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NEW INSIGHTS IN MICROBIAL TRANSFORMATION OF SELENIUM: A BIOTECHNOLOGICAL PERSPECTIVE

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[I] Di Gregorio S., **Lampis S.**, Vallini G. 2005. Selenite precipitation by a rhizospheric strain of *Stenotrophomonas* sp. isolated from the root system of *Astragalus bisulcatus*: a biotechnological perspective. *Environment International*, 31: 233– 241.

[II] Vallini G., Di Gregorio S., **Lampis S**. 2005. Rhizosphere-induced selenium precipitation for possible applications in phytoremediation of Se polluted effluents. *Z. Naturforsch*. 60c, 349-356.

[III] Di Gregorio S., Barbafieri M., **Lampis S.**, Sanangelantoni A.M., Tassi E., Vallini G. 2006. Combined application of Triton X-100 and *Sinorhizobium* sp. Pb002 inoculum for the improvement of lead phytoextraction by *Brassica juncea* in EDTA amended soil. *Chemosphere,* 63(2):293-299.

[IV] Di Gregorio S., **Lampis S.**, Malorgio F., Petruzzelli G., Pezzarossa B., Vallini G. 2006. *Brassica juncea* can improve selenite and selenate abatement in selenium contaminated soils through the aid of its rhizospheric bacterial population. *Plant soil* (in press) [*not attached*].

RIASSUNTO

Introduzione.

Il selenio è un elemento che, in tracce, risulta essenziale per molti organismi, compreso l'uomo. Tuttavia, a dosi di poco eccedenti la soglia ottimale, esso può risultare tossico (Barceloux, 1999).

Pur essendo distribuito sulla crosta terrestre a basse concentrazioni, il selenio risulta essere un contaminante ambientale il cui rilascio nell'ambiente è legato per lo più ad attività di natura antropica. Attività industriali, quali la raffinazione del petrolio e l'impiego in numerosi processi tecnologici, possono infatti contaminare le acque di superficie fino a concentrazioni di Se decisamente elevate (Lawson and Macy, 1995). Inoltre, numerose pratiche agricole, quali l'irrigazione di suoli con elevato contenuto in selenio, possono incrementarne la solubilizzazione e la mobilità, aumentandone la concentrazione nelle acque di drenaggio (Dungan and Frankenberger, 1999).

Le principali forme di selenio che si diffondono in tal modo nell'ambiente sono gli ossianioni selenito (Se^{+4}, SeO_3^{2-}) e selenato (Se^{+6}, SeO_4^{2-}), che costituiscono le specie chimiche del selenio maggiormente tossiche, in quanto altamente solubili e quindi maggiormente biodisponibili (Dungan and Frankenberger, 1999; Barceloux, 1999).

Risulta quindi di fondamentale importanza la loro rimozione dai reflui industriali e dalle acque di drenaggio. I metodi chimico fisici finora applicati, quali la precipitazione chimica e la riduzione catalitica, risultano tuttavia non pienamente efficienti e molto costosi (Kashiwa *et al.*, 2000); si presenta quindi la necessità di sviluppare processi alternativi.

Oggi, la possibilità di rimuovere contaminanti metallici attraverso l'impiego di piante e/o microorganismi capaci, non solo di resistere alle elevate concentrazioni degli inquinanti oggetto dell'intervento, ma, addirittura, di rimuovere il metallo dal sito accumulandolo nelle proprie strutture cellulari, ovvero di condurre reazioni di detossificazione del metallo stesso attraverso processi di trasformazione delle forme più tossiche in specie chimiche meno nocive, apre interessanti prospettive alla bonifica biologica (*bioremediation*).

È noto infatti come i microorganismi siano in grado di attuare reazioni di trasformazione delle forme tossiche del selenio, SeO_3^{2-} e SeO_4^{2-}, nella forma inerte Se^0, che risulta essere quindi non tossica. Dal momento che la forma elementare non tossica del selenio può essere facilmente rimossa dalla fase acquosa sfruttando la sua insolubilità, questo tipo di trasformazioni microbiche può essere sfruttato per la messa a punto di sistemi per il trattamento biologico di reflui contaminati da ossianioni del Se. D'altra parte, la *phytoremediation*, definita come l'impiego di piante per estrarre, sequestrare o

detossificare i contaminati, costituisce anch'essa una promettente alterativa all'impiego dei metodi chimico-fisici, sia in termini di costi (vantaggiosi), sia perché mantiene per lo più inalterate la struttura e le caratteristiche della matrice ambientale trattata.

Quando si parla di *phytoremediation* dei metalli ci si riferisce principalmente a due principali strategie di intervento: fitostabilizzazione e fitoestrazione (Salt *et al.*, 1998).

La fitostabilizzazione, prevede l'immobilizzazione del contaminante attraverso l'assorbimento e l'accumulo all'interno delle radici, l'adsorbimento al di fuori delle radici, ovvero attraverso la precipitazione o l'immobilizzazione all'interno dell'area radicale. D'altra parte, la strategia più efficace, ma anche quella più difficile da attuare, è la fitoestrazione che consiste, invece, nell'assorbimento attraverso l'apparato radicale del contaminante e nel suo accumulo nei tessuti vegetali epigei. Alla fase di accumulo del contaminante nei tessuti vegetali, segue quindi il raccolto della biomassa vegetale, l'essiccamento ovvero l'incenerimento della stessa e il conseguente confinamento in discarica. Tuttavia, nella valutazione di applicabilità di un approccio fito-assistito non si può prescindere dal considerare il contributo apportato al sistema dalla rizosfera della specie vegetale interessata nel processo di *phytoremediation*. È oramai noto che i microorganismi del suolo possiedono meccanismi capaci di alterare la mobilità dei contaminanti metallici presenti nell'ambiente influenzando di conseguenza il loro assorbimento da parte dell'apparato radicale della pianta (Lasat, 2002). Inoltre i microorganismi della rizosfera possono influenzare la crescita della pianta tramite la produzione di fitoormoni, aumentare l'assorbimento di minerali ed acqua e, tramite la produzione di antibiotici, inibire i patogeni del suolo. A sua volta la pianta fornisce ossigeno e, attraverso la produzione di essudati radicali e la decomposizione di composti organici vegetali, nutrienti e fonti di carbonio per la crescita microbica (Azaizeh *et al.*, 2003; Kapulnik, 1996). Tra le specie vegetali capaci di accumulare elevate concentrazioni di selenio, *Brassica juncea* è stata identificata come una delle migliori per la bonifica di matrici contaminate da Se. Essa infatti produce una cospicua biomassa ed è in grado di accumulare Se nell'ordine delle centinaia di ppm (Zayed *et al.*, 1998; Terry *et al.*, 2000).

Obiettivi

Allo scopo di valutare l'applicabilità di processi di bonifica biologica di reflui contaminati da Se, inizialmente la ricerca è stata finalizzata all'ottenimento di ceppi microbici resistenti alla specie ossianioniche tossiche selenito (SeO_3^{2-}) e selenato (SeO_4^{2-}) e capaci di attuare reazioni di detossificazione delle stesse.

Successivamente, l'attenzione è stata posta su alcuni isolati capaci di attuare reazioni di detossificazione della specie ossidata selenito, la più tossica in ambiente ossidativi. Gli obiettivi principali della sperimentazione sono stati quindi, da un lato la caratterizzazione di tali ceppi e lo studio dei meccanismi di trasformazione dell'ossianione selenito da essi attuati, dall'altro la messa a punto di sistemi che prevedessero il loro impiego, in scala di laboratorio, per la bonifica del selenio in fase acquosa. Sempre a tale scopo, è stato inoltre valutato l'impiego della specie vegetale *Brassica juncea* coadiuvata da microorganismi, nella rimozione di SeO_3^{2-} e SeO_4^{2-} da reflui trattati in coltura idroponica. Questo sistema è stato allestito allo scopo di valutare le interazioni pianta-microorganismi della rizosfera, considerando l'eventuale ruolo di tali microorganismi sia nei meccanismi di accumulo del contaminante che nella crescita e sviluppo della pianta stessa.

Schema sperimentale.

La prima fase della sperimentazione ha riguardato l'isolamento di ceppi batterici capaci di crescere in presenza di SeO_3^{2-} e SeO_4^{2-}. L'ottenimento di tali ceppi è stato condotto a partire dalla rizosfera di piante di *Astragalus bisulcatus* (nota iper-accumulatrice di Se) (Neuhierl and Böck, 1996) coltivate su di un suolo storicamente contaminato da Se, mediante arricchimenti selettivi in presenza di 0.2 mM SeO_3^{2-} ovvero SeO_4^{2-}, ammendati come sali di sodio. I ceppi ottenuti in coltura axenica sono stati quindi analizzati per la loro capacità di ridurre le forme ossidate tossiche del selenio (SeO_3^{2-} e SeO_4^{2-}) nella forma elementare inerte, non tossica (Se^0). Degli isolati ottenuti, 3 sono risultati di particolare interesse e quindi sono stati oggetto di successive indagini.

In particolare 2 ceppi batterici , SeITE01 e SeITE02, sono risultati capaci di ridurre SeO_3^{2-} a Se^0 e di crescere ad elevate concentrazioni di questo ossianione, mentre il ceppo SeATE17 è risultato capace di ridurre entrambi gli ossianioni alla forma elementare.

Nella seconda fase della sperimentazione, i due ceppi SeITE01 e SeITE02 sono stati analizzati per loro capacità di ridurre elevate concentrazioni di selenito. Quindi, allo scopo di valutare l'efficienza di riduzione del selenito a selenio elementare, i ceppi SeITE01 e SeITE02 sono stati coltivati in mezzo di crescita ricco (Nutrient Broth) in presenza di concentrazioni crescenti di SeO_3^{2-} (0.2, 0.5, 2 mM Na_2SeO_3). Parallelamente al processo di riduzione del SeO_3^{2-} è stata seguita, per entrambi i ceppi, anche la cinetica di crescita microbica.

Inoltre, ulteriori prove sono state condotte allo scopo di studiare il meccanismo responsabile della riduzione del selenito in questi due isolati. È stata quindi valutata l'efficienza di riduzione dei singoli ceppi a seguito di induzione con concentrazioni subletali

di selenito (0.2 mM) ed è stato inoltre studiato l'effetto causato dall'ammendamento del contaminante in questione a tempi diversi nell'arco della crescita batterica sia sul processo di riduzione, che sulla cinetica di crescita stessa. Infine in colture batteriche dei ceppi SeITE01 e SeITE02 è stata studiata la formazione di precipitati di selenio elementare (nanosfere di Se^0) a seguito dell'avvenuta riduzione di SeO_3^{2-} e la loro localizzazione mediante studi di microscopia elettronica a trasmissione (TEM) e successiva analisi EDX.

Nella terza fase della sperimentazione, si è proceduto con la realizzazione di una simulazione su scala di laboratorio di un processo di rimozione biologica del selenio da un mezzo acquoso mediante l'applicazione di un SBS (Sequencing Batch System) (Kashiwa *et al.*, 2000). Tale sistema ha previsto l'impiego del ceppo SeITE01 mantenuto in condizioni di crescita stazionaria ed alimentato ogni 24 ore con un mezzo di crescita ricco ammendato con selenito alla concentrazione di 0.5 mM.

Infine è stato allestito un esperimento di bonifica biologica fito-assistita utilizzando un sistema in idroponica in cui la specie vegetale (*Brassica juncea*) veniva coltivata su letti di agriperlite (una matrice silicica inerte).

Le prove sono state condotte in vasche di polietilene da 20 L ciascuna. Il selenio è stato fornito al sistema in un unico apporto all'inizio della sperimentazione.

Sono state allestite prove in presenza di selenito (Se^{+4}) e selenato (Se^{+6}) ammendati come sali di sodio a tre diverse concentrazioni: 15 mg Se/kg (0.2 mM), 75 mg Se/kg (1.0 mM) e 150 mg Se/kg (2.0 mM). La durata della prova è stata di 6 settimane dall'ammendamento del contaminante.

Allo scopo di valutare l'influenza esercitata dai microrganismi sul processo di fitoestrazione, è stato fornito alle piante un inoculo microbico che, a seconda della prova, è consistito in:

- Una <u>comunità tellurica</u> mista pre-adattata (I), proveniente dalla rizosfera della stessa pianta precedentemente coltivata su terreno in presenza di selenio;
- <u>Ceppi in coltura pura</u>. I ceppi inoculati sono stati appunto gli isolati SeITE01, SeITE02 e SeATE17.

I ceppi SeITE01 e SeITE02 sono stati utilizzati come inoculo nelle prove effettuate in presenza di selenito, mentre il ceppo SeATE17 è stato utilizzato come inoculo in coltura pura nelle prove effettuate in presenza di selenato. Per le prove allestite in presenza di inoculo in coltura pura è stata testata un'unica concentrazione di selenio: 75 mg Se/kg (1.0 mM). L'entità dell'inoculo è stata, per ogni prova, di 10^7 CFU/g di agriperlite.

Sono state inoltre allestite prove di controllo in presenza di selenio, alle tre concentrazioni di cui sopra, ma in assenza di inoculo. Sono stati effettuati campionamenti sia della

porzione vegetale (3 piantine per ogni campionamento), sia della matrice di supporto (agriperlite) in tre momenti durante l'intero arco della sperimentazione, indicati come:

T0, tempo di inizio della sperimentazione, dopo ammendamento con selenio

T3, dopo 3 settimane dall'inizio della sperimentazione

T6, dopo 6 settimane dall'inizio della sperimentazione

La porzione vegetale prelevata ad ogni campionamento è stata suddivisa nelle porzioni epigea ed ipogea sulle quali è stata effettuata la determinazione del peso secco e del contenuto totale in selenio. Sulla porzione di agriperlite prelevata ad ogni campionamento è stata effettuata la conta microbica, l'estrazione del DNA genomico totale e successiva amplificazione della regione V3 del gene 16S rRNA con conseguente analisi DGGE (Denaturing Gradient Gel Electrophoresis).

L'analisi DGGE aveva lo scopo sia di valutare gli eventuali cambiamenti nella microflora della rizosfera delle piante di *B. juncea* in seguito ai diversi trattamenti, sia di seguire durante l'intero arco della sperimentazione i ceppi in coltura pura ammendati.

I dati ottenuti dalla determinazione nel contenuto totale in selenio sono stati espressi come:

- Fattore di Traslocazione (TF), definito come il rapporto tra la concentrazione di selenio presente nei tessuti vegetali aerei (parte epigea) e quella presente nelle radici (parte ipogea);
- Fattore di Bioconcentrazione (BF), definito come il rapporto tra la concentrazione di selenio presente nel tessuto vegetale e quella nella matrice contaminata (in questo caso, agriperlite);
- Efficienza di fitoestrazione (FE), definita come il rapporto tra il prodotto del contenuto in Se totale nei tessuti vegetali con la biomassa prodotta e la quantità di Se ammendata al sistema al T0.

Risultati e breve discussione.

Gli arricchimenti selettivi, effettuati a partire dalla rizosfera di *Astragalus bisulcatus* coltivata su suolo contaminato da Se, hanno permesso l'isolamento in coltura axenica di 10 ceppi batterici. Tre di questi ceppi (SeITE01, SeITE02 e SeATE17) sono stati oggetto di successive analisi. Gli isolati SeITE01 e SeITE02, ascrivibili rispettivamente alle specie *Bacillus mycoides* e *Stenotrophomonas maltophilia*, sono risultati capaci di ridurre la forma ossidata tossica SeO_3^{2-} a Se^0, forma inerte e non tossica, in coltura liquida, in condizioni di aerobiosi. Dai successivi studi effettuati su questi due ceppi, è stato evidenziato come il ceppo SeITE01 risulti di gran lunga più efficiente di SeITE02 nella rimozione del selenito

dal mezzo di coltura. Infatti, in condizioni di crescita ottimale (mezzo ricco), il ceppo SeITE01 è stato capace di rimuovere completamente il selenito presente nel mezzo in 6, 12 e 24 ore, quando la concentrazione iniziale di SeO_3^{2-} era pari a 0.2, 0.5 e 2.0 mM, rispettivamente, mentre il ceppo SeITE02, nelle stesse condizioni di crescita, è risultato capace invece di rimuovere completamente il contaminante in 24 e 52 ore, quando la concentrazione iniziale era 0.2 e 0.5 mM, rispettivamente. In presenza di 2.0 mM selenito, invece, SeITE02 è risultato capace di rimuovere solamente 87% del contenuto iniziale di SeO_3^{2-} nel mezzo di coltura. Allo scopo di valutare l'effetto di una pre-induzione con selenito sull'efficienza di riduzione dei due ceppi, sono state allestite delle prove in cui i due ceppi venivano pre-trattati con concentrazioni subletali di SeO_3^{2-} (0.2mM). Dai dati ottenuti è risultato evidente come il pre-trattamento con SeO_3^{2-} abbia un effetto positivo sia sulla crescita che sull'efficienza di riduzione del ceppo SeITE02. Questo ceppo, infatti, se pre-indotto, riusciva a ridurre completamente il contenuto in SeO_3^{2-}, presente alla concentrazione iniziale di 2.0 mM, in 48 ore. D'altro canto, il pre-trattamento con selenito sembrava non avere alcun effetto, invece, sull'efficienza di riduzione e sulla crescita del ceppo SeITE01. Dalle analisi di microscopia elettronica effettuate a tempi diversi della crescita microbica su colture liquide dei due ceppi cresciute in presenza di selenito alla concentrazione di 0.5 mM, è stato possibile osservare la formazione di particelle elettron-dense di Se^0. La localizzazione di tali granuli di Se^0, è risultata essere diversa per i due ceppi. Infatti, in colture di SeITE02 è stata osservata la presenza di granuli di selenio elementare sia all'interno della cellula batterica (nel citoplasma), sia all'esterno, nello spazio extracellulare. Tuttavia, è stato osservato che le colture analizzate dopo 48-60 ore di crescita presentavano cellule batteriche morte e la presenza nello spazio extracellulare di grossi granuli di selenio elementare. È stato quindi ipotizzato che il Se^0 venga inizialmente accumulato all'interno della cellula e che, solo in un secondo momento, a seguito di lisi cellulare venga rilasciato nell'ambiente circostante. Per quanto riguarda invece il ceppo SeITE01, non sono state raccolte evidenze della presenza di granuli di Se elementare all'interno della cellula, nemmeno per colture analizzate dopo 12 ore di crescita. Inoltre, le micrografie ottenute dalle analisi al microscopio elettronico con *negative staining* del campione hanno permesso di individuare, in colture di SeITE01, la presenza di granuli di Se^0 sulla parte esterna della parete cellulare. I due ceppi batterici, SeITE01 e SeITE02, sembrano quindi avere meccanismi diversi di riduzione del selenio. Tuttavia, dai risultati ottenuti sulla crescita dei due ceppi in presenza di selenito, risulta evidente come per entrambi si possa ipotizzare un meccanismo di detossificazione. Infatti, la riduzione dell'ossianione selenito non è in grado di supportare la crescita dei due ceppi

batterici in condizioni di microaerofilia o anaerobiosi (dati non mostrati). Inoltre i dati relativi alla cinetica di crescita batterica in presenza di selenito hanno indicato come per entrambi i ceppi la riduzione dell'ossianione avvenga principalmente nella fase esponenziale della crescita. Sebbene molti microrganismi presentino la capacità di ridurre SeO_3^{2-} a Se^0, il meccanismo di questa trasformazione microbica non è ancora stato chiarito. È stato infatti ipotizzato che tale reazione di riduzione sia catalizzata da una nitrito reduttasi periplasmatica (DeMoll-Decker and Macy, 1993), ovvero da una idrogenasi I (Yanke *et al.*, 1995), oppure che sia promossa da reazioni non enzimatiche (Turner *et al.*, 1998). Tuttavia, molto deve ancora essere studiato su questo argomento.

Alla luce dei risultati ottenuti, è stata quindi realizzata la simulazione su scala di laboratorio di un processo di rimozione biologica del selenio da un mezzo acquoso mediante l'applicazione di un SBS (Sequencing Batch System) che ha previsto l'impiego del ceppo SeITE01, di gran lunga più efficiente di SeITE02 nella riduzione del selenito in fase acquosa, mantenuto in condizioni di crescita costante ed alimentato ogni 24 ore con un mezzo di crescita ricco ammendato con selenito alla concentrazione di 0.5 mM. Dai dati ottenuti è risultato evidente come il sistema riesca a rimuovere 0.5 mM di selenito in un ciclo di 24 ore mantenendo costante la biomassa per almeno 10 cicli. L'impiego del ceppo SeITE01 per la rimozione dell'ossianione SeO_3^{2-} da un mezzo acquoso è risultata quindi efficace. Tuttavia, è necessario considerare che tale prova è stata realizzata in condizioni di crescita ottimali (mezzo di crescita ricco) per il ceppo. In futuro, tale sistema verrà quindi testato in condizioni di crescita sub-ottimali, al fine di mimare le caratteristiche di un refluo industriale.

Infine, la prova di bonifica fito-assistita, ha previsto l'allestimento di un sistema in coltura idroponica in cui le piante di *Brassica juncea* sono state cresciute su letto di agriperlite, ammendate con diverse concentrazioni di selenito ovvero di selenato e inoculate con microflora tellurica mista pre-adattata ovvero con i ceppi SeITE01, SeITE02, SeATE17 in coltura pura.

I dati ottenuti indicano come l'efficienza di fitoestrazione dell'intero sistema sia risultata maggiore per la rimozione del selenato che per la rimozione di selenito (vedi Tab. 10 e 12 nella sezione 3.6) alle tre concentrazioni di Se testate. Una maggiore efficienza di *Brassica juncea* nella rimozione del selenato era comunque un risultato atteso e confermato da dati riportati in letteratura (Terry *et al.*, 2000; Ximenez-Embun *et al.*, 2004). Il selenato viene infatti assorbito dalla pianta in modo attivo, mediante il meccanismo di trasporto del solfato, sfruttando la solfato permeasi presente nella membrana plasmatica delle cellule radicali. Per il selenito invece è stato ipotizzato un meccanismo di trasporto passivo (Terry

et al., 2000; Zayed *et al.*, 1998). Inoltre, le piante cresciute in presenza di selenato hanno accumulato selenio maggiormente nella porzione epigea, mentre quelle cresciute in presenza di selenito riportavano un maggior contenuto in selenio nella porzione ipogea. I valori di TF (Fattore di Traslocazione) ottenuti per le piante cresciute in presenza di selenato risultavano infatti > 1. Tuttavia, anche questo dato risulta in accordo con dati di letteratura che riportano TF maggiori per il selenato che per il selenito (Terry *et al.*, 2000; de Souza *et al.*, 1998; Zayed *et al.*, 1998).

Un importane risultato riguarda invece l'effetto positivo esercitato dalla presenza dell'inoculo microbico sulla capacità di bioaccumulo del Se nei tessuti vegetali. Infatti, l'inoculo sia della popolazione mista pre-adattata, sia dei ceppi in coltura pura ha prodotto sia nelle prove trattate con selenato che in quelle ammendate con selenito, un aumento del fattore di bioconcentrazione (BF) nei tessuti vegetali. Tale incremento, in alcuni casi (in presenza di 75 mg Se/kg selenato, e inoculo con la popolazione mista pre-adattata) risulta essere di 3.5 volte rispetto al valore ottenuto in assenza di inoculo. La presenza dell'inoculo batterico, inoltre, provoca anche un aumento nel fattore di traslocazione. Risultati analoghi sono stati riscontrati anche da de Souza e collaboratori (1999) per piante di *Brassica juncea* trattate con selenato e inoculate con microrganismi della rizosfera. Tuttavia, il dato interessante riguarda invece l'incremento nell'accumulo di Se in piante trattate con selenito. Alla luce delle conoscenze fino ad oggi in nostro possesso, non vi sono dati in letteratura che riportino un incremento nell'accumulo di selenito nei tessuti vegetali a seguito d inoculo massivo della pianta con microorganismi.

D'altra parte, in presenza di inoculo misto pre-adattato, sia in presenza di selenito che di selenato, è stata registrata una forte diminuzione della produzione di biomassa. Tale dato, ha inficiato negativamente sulla efficienza di fitoestrazione dell'intero sistema che è risultata maggiore in assenza di inoculo a tutte e tre le concentrazioni testate. La riduzione nella produzione di biomassa vegetale, causata dall'inoculo microbico potrebbe essere dovuta sia all'incremento delle concentrazioni di Se accumulate dalla pianta sia ad una competizione tra la pianta ed i microorganismi per i nutrienti. In quest'ultimo caso, è quindi possibile ipotizzare che in presenza di un eccesso di nutrienti tale fenomeno possa essere contenuto.

Un'importante eccezione rispetto a questo andamento è rappresentata dal risultato ottenuto con l'inoculo del ceppo *S. maltophilia* SeITE02 in presenza di 75 mg Se/kg selenito. In questo caso la presenza del ceppo batterico pur inducendo un incremento nell'accumulo di Se nei tessuti vegetali, non ha causato alcun effetto negativo sulla produzione della biomassa vegetale. La prova allestita in presenza di selenito e inoculata

con il ceppo SeITE02 ha riportato quindi un'efficienza di fitoestrazione maggiore rispetto a quella ottenuta sia in presenza di inoculo tellurico misto pre-adattato che in assenza d inoculo.

Infine, la tecnica PCR-DGGE effettuata sui prodotti di PCR ottenuti mediante amplificazione della regione V3-16S rDNA dal DNA totale estratto dai campioni di agriperlite prelevati a tempi diversi lungo l'intero arco della sperimentazione, ha permesso con successo di monitorare la presenza dei ceppi batterici inoculati.

1. INTRODUCTION

1.1 Selenium in the environment

Selenium is a naturally occurring element that belongs to Group VI A of the Periodic Table. It has been classified as a metalloid, having properties of both a metal and a non-metal.

Selenium is markedly similar to sulphur in its chemistry, with its primary oxidation states being +6, +4, 0 and –2.

Selenate, SeO_4^{2-} (Se^{+6}), and selenite, SeO_3^{2-} (Se^{+4}), are the most common Se oxyanions found in soil solutions and in natural waters. Elemental selenium (Se^0), highly insoluble in water solutions, is stable and exists in several allotropic forms. Three are generally recognized, but as many as six have been claimed. Selenium exists as either an amorphous or crystalline structure. The colour of amorphous selenium is either red, in powder form, or black, in vitreous form. Crystalline monoclinic selenium is a deep red; crystalline hexagonal selenium, the most stable variety, is a metallic grey (Barceloux, 1999; Kessi *et al.*, 1999). The red and black amorphous allotropes are the forms that are most likely to occur in soils. Red amorphous Se^0 originates when Se^0 precipitates in aqueous solution (Geering *et al.*, 1968). At temperature greater than 30°C, red amorphous selenium gradually reverts to the black amorphous form (Gattow and Heinrich, 1964). This form is then slowly transformed into the much more stable grey hexagonal allotrope or is reoxidized, depending on the redox conditions and the pH of the soil. Se is present as Se^{-2} in the inorganic and organic reduced forms. The inorganic reduced Se-forms include mineral selenides and hydrogen selenide (H_2Se). Organic Se-containing compounds include volatile methyl species such as dimethylselenide (DMSe, $[CH_3]_2Se$), dimethyldiselenide (DMDSe, $[CH_3]_2Se_2$), dimethylselenone ($[CH_3]_2SeO_2$), methane selenol (CH_3SeH), and dimethylselenylsulfide (DMSeS, ($[CH_3]_2SeS$), and Se-substituted amino acids such as selenomethionine (Se-met), selenocysteine (Se-cys) and selenocystine. It is important to note that these amino acids, particularly Se-met, are the predominant form of Se in food (Levander, 1983), whereas inorganic selenium (selenate and selenite) is generally only included in the diet through supplements.

As described in the next section, selenocysteine, the 21st amino acid, has a central role as a component of the catalytic site of enzymes with important biological functions (Böck *et al.*, 1991).

1.1.1 Toxicity and deficiency of selenium

Selenium, which is not considered to be an essential element for plants, is essential in

trace for growth of fish, birds, animals, humans, and many microorganisms. However there is a fine threshold between Se toxicity and deficiency.

Selenium, in fact, as selenocysteine, is a component of selenoproteins, some of which have important enzymatic functions, especially in humans and animals (Barceloux, 1999). It has been recognised that all these enzymes are selenium-dependent, generally with selenocysteine at the active site. Differences between the chemistry of Se and S result in the sulfhydryl groups of cysteine being mostly protonated at physiological pH, whereas the analogous groups of Se-cyst are largely dissociated, which facilitates the catalytic role of Se in the selenoproteins (Combs and Combs, 1984). Here, indeed, selenium functions as a redox centre. The best-known example of this redox function is the reduction of hydrogen peroxide, damaging lipid and phospholipid hydroperoxides to harmless products (water and alcohols) by the family of selenium-dependent glutathione peroxidases (GSHPx). This function helps to maintain membrane integrity and reduces the likelihood of propagation of further oxidative damage to biomolecules such as lipids, lipoproteins, and DNA with the associated increased risk of conditions such as atherosclerosis and cancer. Activities similar to activities by glutathione peroxidase have also been measured in yeast, in algae and in higher plants (Leisinger *et al.*, 1999).

In bacteria, three enzymes containing Se as Se-cys have been identified: a formate dehydrogenase in *Escherichia coli* , a hydrogenase finding both in *Methanococcus vannielii* and *Desulfovibrio baculatus* and finally a glycine reductase in *Clostridium sticklandii*. As glutathione peroxidase, all these bacterial enzymes catalyse oxidation/reduction reactions, and it is assumed that the selenocysteine moiety is part of the active centre (Stadtman, 1990).

It has been demonstrated that the incorporation of the active site-SeCys into these essential selenoproteins is a cotranslation process directed by a UGA codon (Böck *et al.*, 1991). UGA normally functions as a universal termination codon; in order for UGA to function as a SeCys codon, both specific secondary structural elements in the mRNA and a unique SeCys-charged $tRNA^{ser/sec}$ that contains the UGA anticodon are required (Stadtman, 1996).

Another beneficial effect of dietary selenium in mammals is to prevent chemicals from inducing tumours, but the mechanism of this prevention is not clearly understood (Ip, 1998; Shanberger, 1985). Selenium has been shown to inhibit the intracellular JNK/SAPK signalling and $p38^{MAPK}$ cascades (Park *et al.*, 2000b) and some transcription factors (Kim and Stadtman, 1997; Spyrou *et al.*, 1995). Some of these inhibitions occur through a thiol redox mechanism (Park *et al.*, 2000a; Park *et al.*, 2000b) but it is not known whether this

mechanism is involved in the anticarcinogenic properties of this element.

1.1.1.1 Selenium deficiency

In animals and humans the symptoms associated with a deficiency of Se are closely related. A severe deficiency of Se is characterized by cardiomyopathy, such as Keshan disease, a juvenile cardiomyopathy associated with low Se status (Chen *et al.*, 1980), while a moderate deficiency produces less severe myodegenerative syndromes such as muscular weakness and pain as well as a variety of Se-associated diseases.

The suggested recommended daily dietary allowance of Se is 55 µg for women and 70 µg for men (Levander, 1991). “White muscle disease”, a commonly known Se deficiency in livestock, is a myodegenerative disorder. Depending on the animal species and the level of vitamin E in the diet, the required Se intake for adequate animal nutrition is between 0.04 and 0.1 mg Se kg^{-1} dry matter.

1.1.1.2 Selenium toxicity

Although Se is an essential micronutrient for humans and animals, at excessive concentrations in the environment, Se bioaccumulates in the food chain and constitutes a serious threat to wildlife.

Selenium toxicity strongly depends on the chemical form of selenium. Regarding selenium salts, it has been demonstrated that, in animals, selenite is slightly more toxic compared with selenate. Although selenocysteine possesses similar toxicity in animals compared with selenite, selenomethionine is somewhat less toxic (McAdam and Levander, 1987). Other methyl-alkyl selenium compounds are much less toxic in animal studies compared with selenite, in fact DMSe is considered 500 to 700 times less toxic to rats than aqueous SeO_3^{2-} and SeO_4^{2-} ions (Table 1) (Spallholz, 1994; Frankenberger and Karlson, 1995). Selenious acid is the most toxic form of selenium and ingestion of this compound often causes fatalities as a result of refractory hypotension from direct myocardial depression and from peripheral vasodilatation.

Compound	Parameter	Value (mg Se/kg)
$(CH_3)_2Se$	ipr-rat LD_{50}	2200
	scu-rat LD_{50}	2180
$(CH_3)_3SeCl$	ipr-rat LD_{50}	100
Na_2SeO_4	ipr-rat LD_{Lo}	9
	scu-rat LD_{Lo}	19
	ipr-rat LD_{75}	13
Na_2SeO_3	ipr-rat LD_{50}	3
	scu-rat LD_{50}	2
	ipr-rat LD_{75}	8
Se-methionine	ipr-rat LD_{50}	11
Se-cystine	ipr-rat LD_{50}	9

Table 1 – Toxicity of some selenium species

[ipr-rat = intraperitoneal injection into rats; scu-rat = subcutaneous injection into rats]

Toxicity studies indicate that birds, fish, and animals suffer from chronic selenium toxicosis (selenosis) when diets contain more than 5 to 25 mg Se kg^{-1} (Mayland, 1994). Drinking water selenium concentrations below 20 $\mu g\ L^{-1}$ are unlikely to cause health impacts in animals (Ayers and Wescot, 1985), while direct human health problems are unlikely to occur when Se concentrations in drinking water are less than the present federal drinking water standard of 50 $\mu g\ L^{-1}$ (U.S. EPA, 1996). Selenosis is responsible for "alkali disease" in livestock and is believed to be the cause of "blind staggers" in ruminants. Alkali disease is a disorder characterised by hair loss, deformed hoofs, poor growth and stiffness of joints. Blind staggers, which has been attributed to excess Se in the diet, may also be the result of ingesting excess S instead of Se (Beke and Hironaka, 1991).

Skorupa *et al.* (1990) have found that embryonic deformities in waterfowl were consistently observed in populations that had a mean egg Se concentration > 20 mg Se kg^{-1}. In the Tulare Lake basin, California, 15 $\mu g\ L^{-1}$ has been associated with substantial bioaccumulation of Se in waterfowl eggs (Skorupa *et al.*, 1990).

Why is selenium toxic?

It has been proposed that selenium (selenite) toxicity was due to its interaction with vicinal thiols in proteins and consequently production of reactive oxygen species (ROS),

such as $O_2^{\bullet-}$ and H_2O_2. In fact, in vitro studies (Seko *et al.*, 1989) have shown that the reduction of selenite involves reactions with sulfhydryl groups of thiol-containing molecules such as glutathione (GSH), leading to the production of intermediate metabolites selenodiglutathione (GS-Se-SG), glutathioselenol (GS-SeH), hydrogen selenide (HSe^-) and finally to elemental selenium (Fig. 1). Certain reactions of this pathway produce hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$), which can cause damage to cell membranes and DNA. Thus, it has been suggested that selenite toxicity is due to oxidative stress, while selenate has toxic effects only after being reduced to selenite or selenol (-SeH).

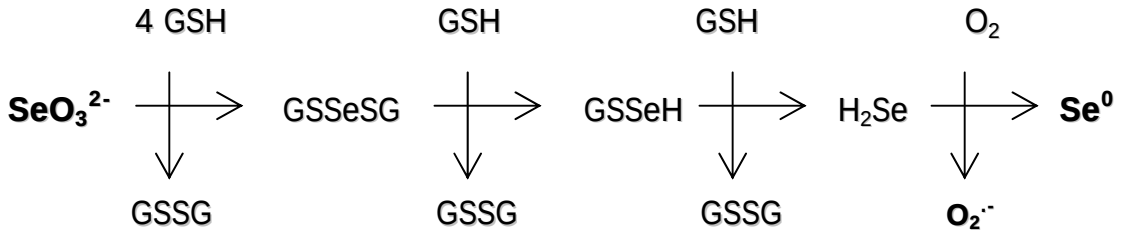


Figure 1 – Reaction proposed by Seko and coworkers (1989) on selenium toxicity mechanism

Morover, Spallhollz and coworkers (1993) have tested a number of different selenium compounds to determine their *in vitro* reaction with GSH to produce superoxide ion. These selenium compounds are given in Table 2.

Se compounds that produce $O_2^{\bullet-}$ (*in vitro*)	Se compounds that don't produce $O_2^{\bullet-}$ (*in vitro*)
Selenite	Elemental Se
Selenium dioxide	Selenate
Selenocystine	Selenomethionine
Selenocystamine	Selenobetaine

Table 2 – Glutathione oxidase activity of selenium compounds

From this list of selenium compounds a pattern emerged that permitted an *a priori* determination whether or not any selenium compound would react with GSH or other thiols to produce ROS.

The hypothesis that evolved from these *in vitro* studies was that:

1. Selenium compounds, i.e. selenite and selenium dioxide, can react with GSH and other thiols to form selenotrisulfides that will ultimately react to produce superoxide and hydrogen peroxide, and are toxic.
2. Diselenides, i.e., selenocystine and selenocystamine, in the presence of GSH and other thiols, are reduced to selenols (RSeH), which are catalytic, produce superoxide and and hydrogen peroxide, and are toxic.
3. Selenium compounds that do not react with thiols, i.e., selenate and all tested selenoethers (RSeR) do not produce superoxide or hydrogen peroxide *in vitro* and are not toxic *per se*.
4. Selenate and selenoethers are toxic in tissue cultures or *in vivo* only after being reduced to selenite or a selenol.
5. Se toxicity manifests itself acutely or chronically when oxidative damages excess antioxidant defences or the ability of either plants or animals to form selenoproteins, selenoethers or elemental selenium.

1.2 Global cycling of selenium

The global cycling of Se in the environment is driven by both biogenic and anthropogenic activities.

Selenium is distributed throughout the environment by processes such as volcanic activity and hot springs, sea salt spray, forest wildfires, combustion of fossil fuels, incineration of municipal waste, weathering of rocks and soils, dust, soil leaching, copper/nichel production, lead and zinc smelting, iron and steel production, crop-fallow and irrigation practices, fertilizers, groundwater transport, plant and animal uptake and release, adsorption and desorption, chemical and biological redox reactions and mineral formation (Mayland *et al.*, 1989; McNeal and Balilestri, 1989; Nriagu, 1989). Estimated Se fluxes indicate that the natural sources of Se emission are as important as anthropogenic emission (Nriagu, 1989).

In general, the Se concentration in soil or ground and fresh water depends upon the parent material, climate, topography, age of the soil, and agricultural or industrial utilization.

Terrestrial systems

Selenium is widely dispersed in the earth's crust at low concentrations. Most soils contain between 0.1 and 2 mg se Kg^{-1} (Elrashidi *et al.*, 1989; Mayland *et al.*, 1989). However, elevated concentrations of this metalloid are associated with various soils, notably those of marine sedimentary parent material and soils impacted by industrial activity such as petroleum refining (Weres *et al.*, 1989; Haygarth, 1994). In fact, anthropogenic activities, such as irrigated agriculture and mining can lead to solubilization and migration of Se, significantly raising Se levels in localized soils and ground or surface waters (Presser *et al.*, 1994; Kauffman *et al.*, 1986).

The concentration and speciation of Se in soil depend on the pH, redox potential, solubility, complexing ability of soluble and solid ligands, biological interactions, and reaction kinetics (Barrow and Whelan, 1989; McNeal and Balilestri, 1989). Of these, redox status is perhaps the major controlling influence (Fig. 2) (Masscheleyn *et al.*, 1990). Under acidic, reducing conditions in soils which may be waterlogged and rich in organic matter, elemental Se (Se^0) and selenides (Se^{-2}) are the predominant species (McNeal and Balilestri, 1989). From pH 4 to 8, stable adsorption complexes or co-precipitates with sesquioxides are prevalent (Ullrey, 1981) and at moderate redox potentials, either $HSeO^{3-}$ or SeO_3^{2-} (Se^{+4}) are the predominant species in soil solution. At high redox, in well-

aerated, alkaline soils the highly soluble SeO_4^{2-} (Se^{+6}) is the predominant species (Elrashidi *et al.*, 1989). Selenate ions do not form stable adsorption complexes or co-precipitates with sesqioxides (Ullrey, 1981). Therefore, under most pH and redox conditions, SeO_3^{2-} and SeO_4^{2-} are the dominant forms of Se found in soils.

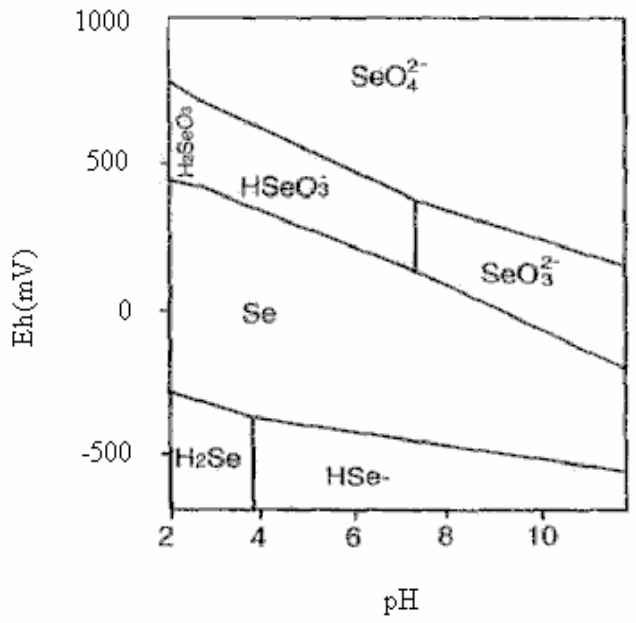


Figure 2 – Redox potential (Eh) and pH effect on selenium speciation

Aquatic systems

Most natural waters have low concentrations of Se, ranging from 0.1 to 100 μg L^{-1} (NAS, 1983). However, some evaporation ponds in the California San Joaquin Region, a major agricultural region in California's Central Valley, have reached levels in excess of 1000 μg L^{-1} (Thompson-Eagle *et al.*, 1989). Soils in this region were derived from seleniferous Cretaceous sedimentary strata and therefore contain high levels of Se (Presser *et al.*, 1994). Se was mobilized by infiltrating irrigation water, which was then discharged to surface water by subsurface drainage or to groundwater by deep percolation. The drainage waters were then disposed of in evaporation ponds, where Se accumulated to toxic levels in the sediments and water.

On the other hand, Se is also found in the form of SeO_3^{2-} and/or SeO_4^{2-} in the effluents from thermal power stations, oil refineries and smelting plants. Nevertheless, as a consequence of the wide employment of this metalloid in various productive activities, industrial effluents can contain considerable amounts of soluble Se. Moreover, while in the agricultural wastewaters Se is present at relatively low concentrations (0.3 – 0.5 mg L^{-1}),

some industrial wastewater can be expected to contain high soluble Se concentrations (up to 250 mg L^{-1}) (Kashiwa *et al.*, 2000).

As with soils, under most pH and redox conditions, SeO_3^{2-} and SeO_4^{2-}, are the dominant forms of Se in water, with several forms of Se^{2-} also being present (Cutter and Bruland, 1984).

1.2.1 Biological reactions in selenium cycling

Selenium is predominantly cycled via biological pathways (Shrift, 1973), similar to that of sulfur (Fig. 3).

Like sulfur, Se undergoes various oxidation and reduction reactions that are biological in nature, supporting by both microorganisms and plants, and that directly affect its oxidation state and hence, its chemical properties and its behaviour in the environment.

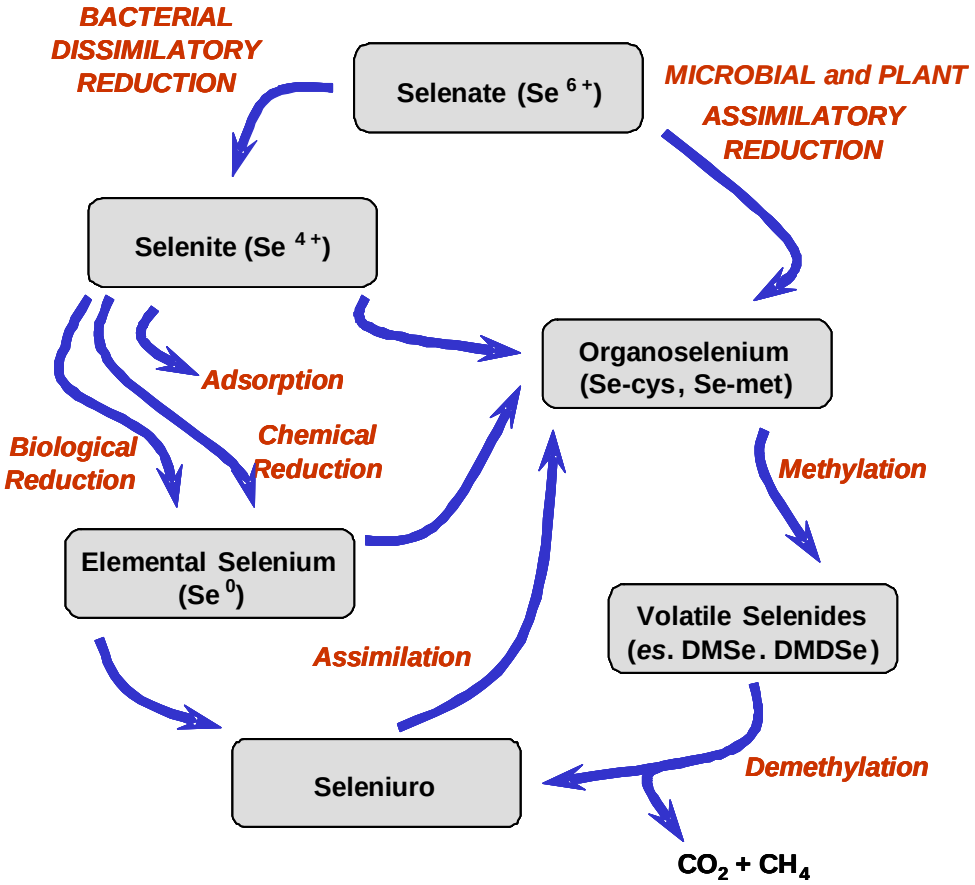


Figure 3 – Biogeochemical cycle of selenium (modified from Oremland, 1994)

1.2.1.1 Microbial transformations of Se

Most of the work to date, on microbial transformation of selenium forms, has focused on the reductive reactions in the Se cycle, whereas much less is known about Se oxidation.

Actually, four biological transformations of Se are known to occur: reduction (assimilatory, dissimilatory, detoxification), oxidation, methylation, and demethylation.

The purpose of discussing each of the biological transformations of Se in detail is to demonstrate the significant role that each mechanism may play in the fate and transport of Se in the environment. Furthermore, great importance will be given to the dissimilatory and aerobically reduction and methylation reactions because these mechanisms are considered to be the most significant with respect to bioremediation schemes.

(a) Selenium oxyanions reduction

SeO_4^{2-} and SeO_3^{2-} oxyanions do not readily undergo chemical reduction under physiological conditions of pH and temperature (Oremland *et al.*, 1989; Jajaweera and Biggar, 1996). Thus, it is highly unlikely that abiotic reduction of selenate or selenite, such as by green rust (Mynemi *et al.*, 1997), plays an important role in natural environments.

In soil, sediment and water, SeO_4^{2-} and SeO_3^{2-} ions undergo microbial reduction to produce Se^0. Microorganisms can reduce selenium oxyanions mainly through a dissimilatory or assimilatory reduction mechanism. Dissimilatory reduction generally refers to reduction of compounds as terminal electron acceptors in energy metabolism, whereas assimilatory reduction refers to reduction and incorporation of Se into organic compounds. Another mechanism of SeO_4^{2-} and SeO_3^{2-} reduction in anaerobic growth conditions is the one carried out by phototrophic bacteria for the regulation of reducing equivalents (Moore and Kaplan, 1992). However, other mechanisms, apparently not belonging to these categories, have been noted. For example, some microorganisms are capable of transforming the toxic selenium forms (SeO_4^{2-} and SeO_3^{2-}) into less toxic ones (elemental or methylated forms) as a means of detoxifying their immediate environment (Lovely, 1993). These phenomena are generally referred to as 'detoxification mechanisms' and selenium oxyanions aerobically reduction to Se^0 and methylation of Se are believed to fall into this category (Lortie *et al.*, 1992).

To date, most research has focused on the reduction of Se oxyanions (especially dissimilatory reduction) because of its possible application in remediating seleniferous environments, both soils and aqueous streams, by producing insoluble Se^0, which is biologically unavailable and can easily be removed from an aqueous phase.

Assimilatory reduction

Selenate is thought to enter the cell through the sulphate permease system (*cysA*, *cysU*, *cysW*) (Heider and Böck, 1993). Mutations in these genes give rise to selenate resistance. Selenite can also enter the cell through this sulphate transporter; however, an alternative yet undefined carrier likely exists because repression of sulfate permease expression does not inhibit selenite uptake completely (Turner *et al.*, 1998).

Once in the cell, both SeO_4^{2-} and SeO_3^{2-} ions undergo assimilatory reduction to Se^{2-}, which can then be incorporated into selenoamino acids (selenomethionine and selenocysteine) and then in proteins. As it has been described in the previous section, Se-cys is present in the catalytic site of seleno enzymes with essential function for microbial metabolism, such as formate dehydrogenase enzyme. The incorporation of this non-standard amino acid takes place co-translationally: biosynthesis occurs on a specific tRNA and insertion into a growing polypeptide is directed by a UGA codon in the mRNA (Böck *et al.*, 1991). Although it is recognised that trace amounts of Se are essential for some microbial proteins, when excess amounts of Se are present, the cell begins to indiscriminately substitute Se for its analogous S in cellular components. Because Se compounds are more reactive and less stable than S compounds, the organism begins to experience metabolic problems, which ultimately leads to death.

Nowadays it appears that a treatment system utilizing assimilatory reduction would be of limited use, because microbes will only assimilate enough Se to make all necessary proteins, therefore, a system of this type would provide poor removal efficiency.

Dissimilatory reduction

Many bacteria are responsible for dissimilatory reducing both Se oxyanions to Se^0.

While the reduction of SeO_4^{2-} and SeO_3^{2-} is often observed, it has been reported that only SeO_4^{2-} can support bacterial growth under anaerobic conditions. Oremland *et al.*, (1989) demonstrated that SeO_4^{2-} dissimilatory reduction to Se^0 is a major sink for Se oxyanions in anoxic sediments.

This bacterial transformation is important because of its potential application for remediating Se oxyanions from seleniferous water.

An overview of SeO_4^{2-} and SeO_3^{2-} reducing bacteria in anaerobic growth conditions, including a description of their involvement in Se reduction is provided in Table 3.

Sulfurospirillum barnesii strain SES-3, isolated by Oremland and co-workers (1994), can achieve respiratory growth with a variety of electron acceptors including As(V), NO_3^-, $S_2O_3^{2-}$, S(0) and Fe(III) in addition to SeO_4^{2-} and is also capable of weak microaerobic

growth. Of great significant is that it does not use sulfate as an electron acceptor. It was initially believed, because S and Se are closely related group VI A elements, that selenate reduction could be attributed to sulfate reducing bacteria (Zehr and Oremland, 1987). The characterization of *S. barnesii* and other selenate respiring bacteria has clearly shown that the dissimilatory reduction of SO_4^{2-} to SO_3^{2-} and SeO_4^{2-} to SeO_3^{2-} and Se^0 are achieved by very different microbes using different biochemical pathways and with much different cellular energy yields.

Organism	Description of reaction	Ref.
***Sulfurospirillum barnesii* strain SES-3**	Respires SeO_4^{2-} and can reduce SeO_3^{2-} to Se^0	Oremland *et al*. (1994)
***Enterobacter cloacae* strain SLD1a-1**	Respires SeO_4^{2-} and NO_3^-, and reduces SeO_4^{2-} to Se^0 only in the presence of NO_3^-	Losi and Frankenberger (1997)
Thauera selenatis	Grows anaerobically using SeO_4^{2-}, NO_3^- and NO_2^-. SeO_4^{2-} is completely reduced to Se^0 only when NO_3^- is present	Macy (1994)
***Bacillus* sp. strain SF-1**	Respires SeO_4^{2-} and NO_3^-, and reduces SeO_4^{2-} to Se^0 with transient accumulation of SeO_3^{2-}	Fujita *et al*. (1997)
***Bacillus arsenicoselenatis* strain E-1H**	Grows anaerobically using SeO_4^{2-} and reducing it to SeO_3^{2-}	Switzer-Blum *et al*. (1998)
***Bacillus selenitireducens* strain MLS-10**	Grows anaerobically using SeO_3^{2-} and reducing it to Se^0	Switzer-Blum *et al*. (1998)
Enterobacter taylorae	Respires SeO_4^{2-} following the order: $SeO_4^{2-} \rightarrow SeO_3^{2-} \rightarrow Se^0 \rightarrow Se^{2-}$	Zahir *et al.* (2003)
Wolinella succinogenes	Reduces SeO_4^{2-} and SeO_3^{2-} to Se^0 under anaerobic conditions. Both selenium oxyanions could not support growth	Tomei *et al*. (1992)
Desulfovibrio desulfuricans	Reduces SeO_4^{2-} and SeO_3^{2-} to Se^0 under anaerobic conditions. Both selenium oxyanions could not support growth	Tomei *et al.* (1995)
Rhodobacter sphaeroides	Reduces SeO_4^{2-} and SeO_3^{2-} to Se^0	Moore and Kaplan

	under anoxic phototrophic growth conditions	(1992)
Rhodospirillum rubrum	Reduces SeO_3^{2-} to Se^0 under anoxic phototrophic growth conditions	Kessi *et al.* (1999)

Table 3 – Selenium reducing microorganisms in anaerobic growth conditions

Macy (1994) isolated *Thauera selenatis,* a SeO_4^{2-}, NO_3^-, and NO_2^- respiring bacterium from seleniferous sediments of the San Joaquin Valley (California). The reduction of SeO_4^{2-} to SeO_3^{2-} and NO_3^- to NO_2^- by *T. selenatis* occurs through the use of separate terminal reductase, a SeO_4^{2-} and NO_3^- reductase, respectively. The complete reduction of SeO_4^{2-} to Se^0 only occurred when the organism was grown in the presence of both SeO_4^{2-} and NO_3^- ions. Selenite produced during the concomitant respiration of SeO_4^{2-} and NO_3^- is believed to be reduced via a periplasmic NO_2^- reductase (DeMoll-Decker and Macy, 1993). The selenate reductase from *T. selenatis* was subsequently purificated and characterized. This enzyme resulted to be specific for the reduction of selenate, whereas nitrate, nitrite, and sulfate ions were not reduced at detectable rates. These studies constitute the first description of a selenate reductase, which represents a new class of enzymes (Schröder *et al.*, 1997).

Enterobacter cloacae SLD1a-1, a facultative anaerobe isolated by Losi and Frankenberger (1997), operates under mechanisms very similar to that of *T. selenatis*. *E. cloacae* uses SeO_4^{2-} and NO_3^- as terminal electron acceptors during anaerobic growth and can reduce SeO_4^{2-} to Se^0 in growth experiments and washed-cell suspensions under microaerophilic conditions. Although *E. cloacae* respires SeO_4^{2-} anaerobically, the complete reduction of SeO_4^{2-} to Se^0 did not occur unless NO_3^- was present, suggesting that NO_3^- is necessary for the reduction of SeO_3^{2-} to Se^0.

A gram-positive, facultative anaerobic, rod-shaped bacterium was isolated from selenium-polluted sediment and designated as *Bacillus* sp. strain SF-1 (Fuiita *et al.*, 1997). This bacterium grows with lactate as an electron donor and SeO_4^{2-} as an electron acceptor in the absence of oxygen and presence of yeast extract (SeO_4^{2-} respiration). There is a stoichiometric balance between the cell growth, lactate consumption and selenate reduction. Strain SF-1 can completely reduce up to 1 mM SeO_4^{2-} to amorphous Se^0 with transient accumulation of SeO_3^{2-}. The presence of NO_3^- ion inhibits the SeO_4^{2-} reduction, suggesting a relationship between the nitrate and selenate reduction mechanisms.

Bacillus arsenicoselenatis strain E-1H and *Bacillus selenitireducens* strain MLS-10, are novel species of Gram-positive bacteria that respire selenium oxyanions. They were isolated from Mono Lake, California, an alkaline (pH=9.8) and hypersaline (salinity = 90 g L^{-1}) soda lake (Switzer-Blum *et al.*, 1998). Strain E-1H is a spore former and strict anaerobe that grows by reducing SeO_4^{2-} to SeO_3^{2-}. It can use lactate or malate as electron donors as well as starch. Strain MLS-10 does not form spores and grows by reduction of SeO_3^{2-} to Se^0. Although a number of bacteria are capable of reducing highly toxic SeO_3^{2-} to Se^0 for the purpose of detoxification, strain MLS-10 is the first organism that can respire this substrate to sustain growth and achieves this at elevated SeO_3^{2-} concentrations (i.e. 10 mM). When the two strains are grown in co-culture, SeO_4^{2-} is quantitatively reduced to Se^0 (Switzer-Blum *et al.*, 1998).

More recently, an *Enterobacter taylorae* strain was isolated from a rice straw bioreactor channel system tested to remove SeO_4^{2-} from agricultural drainage water (Zahir *et al.*, 2003). From this study resulted that *E. taylorae* can remove SeO_4^{2-} from drainage water through two pathways: SeO_4^{2-} reduction to Se^0, followed by the precipitation of Se^0 and Se volatilization to the atmosphere. The efficiency of SeO_4^{2-} reduction by *E. taylorae* was related to the amount of yeast extract added, salinity, and NO_3^- levels in the drainage water. The presence of NO_3^- negatively affected the bacterial SeO_4^{2-} reduction efficiency, being NO_3^- a competitive electron acceptor in SeO_4^{2-} reduction to Se^0 in aquatic system (Zhang *et al.*, 2003).

Regulation of reducing equivalents

In some phototrophic bacteria, such as *Rhodobacter sphaeroides* and *Rhodospirillum rubrum,* which are both purple nonsulfur bacteria, the intracellular redox potential is maintained through the continuous dumping of electrons (Table 3) (Moore and Kaplan, 1992, Moore and Kaplan, 1994). During anaerobic growth in the light, a pool of reducing equivalents is built up, which eventually affects photosynthesis. One mechanism by which this pool is diminished is through the reduction of heavy metal oxyanions. Although the exact mechanism has not been worked out, Moore and Kaplan (1992) have identified a membrane-bound, $FADH_2$-dependent metal reductase.

A side effect of this process is that the cells are resistant to high levels of heavy metal oxides ($Cr_2O_7^{2-}$; Rh_2O_3; Eu_2O_5; TeO_4^{2-}; TeO_3^{2-}; AsO_4^{3-}) including SeO_4^{2-} (Moore and Kaplan, 1994).

R. sphaeroides is able to tolerate high concentrations of SeO_4^{2-} and SeO_3^{2-} oxyanions, up

to 10 mM, and to carry out reduction and methylation reactions of these oxidative Se forms to Se organic compounds and Se^0. Although *R. sphaeroides* is capable to metabolize both SeO_3^{2-} and SeO_4^{2-}, it exhibits a profound preference for the former. However, the biochemical basis for the greater uptake of SeO_3^{2-} than of SeO_4^{2-} remains to be clarified. The primary requirement of selenium is for incorporation into amino acids, but the necessary levels are so low that it's difficult to detect. When even the lowest addition of SeO_3^{2-} or SeO_4^{2-} exceeds the requirements of growth, the system initially seems to put the excess into a form very similar or perhaps identical to Se-met. Further excess seems to be detoxified as the red elemental form, which has a very low bioavailability (Van Fleet-Stalder *et al.*, 2000).

As *R. sphaeroides*, also *R. rubrum* is able to tolerate high concentrations of the Se oxyanions. The ability of this phototrophic bacterium to reduce SeO_3^{2-} is greater under anoxic phototrophic growth conditions, under which SeO_3^{2-} concentrations up to 1.5 mM are completely reduced, than under oxic growth conditions (Kessi *et al.*, 1999).

Other reports of SeO_4^{2-} reduction in anaerobic conditions

Other interesting strains were isolated and studied for their capacity to anaerobically grow in presence of SeO_4^{2-} or SeO_3^{2-} ions.

Anaerobic cultures of *Wolinella succinogenes* reportedly reduced SeO_4^{2-} and SeO_3^{2-} to Se^0 in solution culture after reaching the stationary growth phase (Tomei *et al.*, 1992). This bacteria was unable to grow using either SeO_4^{2-} or SeO_3^{2-} as terminal electron acceptors and the mechanism, although not elucidated, was probably enzymatic in nature.

Tomei and co-workers (1995) reported a similar study with a SO_4^{2-} reducing bacterium, *Desulfovibrio desulfuricans*, an organism that was also adapted to grow in the presence of SeO_4^{2-} and SeO_3^{2-}. *D. desulfuricans* was able to reduce SeO_4^{2-} and SeO_3^{2-} to Se^0 under anaerobic conditions, but neither Se oxyanions could be used for respiratory growth, which suggested that a detoxification mechanism was at work.

SeO_3^{2-} reduction in aerobic conditions

Aerobic reduction of Se oxyanions to Se elemental form may also occur. In this case, the reduction of SeO_4^{2-} or SeO_3^{2-} to Se^0 does not occur to support microbial growth, but, rather, to detoxify the microbial immediate environment (Lovely, 1993).

Although microorganisms capable of reducing SeO_4^{2-} ion have been isolated, SeO_3^{2-} is more easily reduced than SeO_4^{2-} (Doran, 1982).

In the last two decades, several bacteria, that are capable of reducing both selenium oxyanions to Se^0 in aerobic growth conditions, have been isolated from seleniferous sediments and waters (Table 4) (Lortie *et al.*, 1992; Roux *et al.*, 2001; Dungan *et al.*, 2003; Rathgeber *et al.*, 2002)

Organism	Description of reaction	Ref.
Pseudomonas stutzeri	Reduces SeO_4^{2-} and SeO_3^{2-} to Se^0 under aerobic growth conditions	Lortie *et al.* (1992)
Stenotrophomonas maltophilia	Reduces SeO_4^{2-} and SeO_3^{2-} to Se^0 under aerobic and microaerophilic growth conditions	Dungan *et al.* (2003)
Ralstonia metallidurans	Reduces SeO_3^{2-} to Se^0 and transforms SeO_4^{2-} to organic selenium compounds under aerobic growth conditions	Roux *et al.* (2001) Sarret *et al.* (2005)

Table 4 – Selenium reducing bacteria in aerobic growth conditions

A *Pseudomonas stutzeri* strain, isolated by Lortie and co-workers (1992), was capable of reducing up to 6.3 mM of selenate and up to 5.2 mM of selenite in 24h. The deep red colour of the medium during the growth confirms the reduction of SeO_4^{2-} or SeO_3^{2-} to Se^0. Moreover aeration was found to be necessary for both selenite and selenate reduction.

Dungan and co-workers (2003) isolated a strain of *Stenotrophomonas maltophilia* from Se-contaminated evaporation pond sediment in the Tulare Lake Drainage District, California. This organism is capable of rapidly reducing SeO_4^{2-} or SeO_3^{2-} to Se^0 and to volatilizing Se and for this reason it may be useful in a low-cost remediation scheme designed to treat seleniferous agricultural wastewater.

Ralstonia metallidurans has been found in the metal-rich sediments of a zinc factory in Belgium and has been demonstrated to prevail in industrial anthropogenic biotypes such as metallurgic wastes (Mergeay *et al.*, 1985). It harbors plasmide-borne multiple resistance to several heavy metals and oxyanions and it is able to resist up to 5 to 6 mM SeO_3^{2-} when it is cultured in solid minimal mineral medium. Moreover, this strain is capable to reduce SeO_3^{2-} oxyanion to elemental selenium form in aerobic growth conditions (Roux *et al.*, 2001). Recent studies have demonstrated that this strain is also capable to transform SeO_4^{2-} oxyanion (Sarret *et al.*, 2005). Furthermore, kinetic reduction

studies demonstrated that the reduction reaction to Se^0 occurs preferentially when selenite was present in the medium. On the contrary, when selenate is present in the medium, an assimilative reduction process takes place, leading to the formation of Se-alkyl compounds rather then to elemental selenium forms (Sarret *et al.*, 2005).

Se^0 nanospheres: their nature and mechanisms of biotic formation.

Multiple detoxification processes may occur during selenite and selenate reduction by microorganisms since elemental selenium has been described as being deposited in the cytoplasm (Tomei *et al.*, 1992; Tomei *et al.*, 1995), in the periplasmic space (Gerrard *et al.*, 1974), and outside the cell (Losi and Frankenberger, 1997; Yamada *et al.*, 1997; Dungan *et al.*, 2003; Zahir *et al.*, 2003). According to Tomei et al. (1995), the particles containing elemental selenium found outside cells are released by cell lysis, while Losi and Frankenberger (1997) suggested that the reduction reaction occurs close to the membrane, possibly as a result of a membrane-associated reductase(s), and that the precipitate is rapidly expelled by a membrane efflux pump. They observed spherical protrusions on the surfaces of *Enterobacter cloacae* cells grown in the presence of selenite, as well as selenium-containing particles in the culture medium, but no intracellular Se was present.

Dungan *et al.* (2003) tested *Stenotrophomonas maltophilia* cells in the presence of SeO_4^{2-} and SeO_3^{2-} ions. As Losi and Frankenberger (1997), they observed Se deposits located near the periphery of the cell wall. These deposits were also found free in the medium. Thus, they suggested that Se oxyanions are reduced to Se^0 on the surface of the cell wall or alternatively in the inner membrane space, followed by expulsion of Se particles from the cells.

In agreement with Losi and Frankenberger (1997), also Zahir *et al.* (2003) observed Se^0 particles free in the growth medium and on the surface of *Enterobacter taylorae* cells, grown in the presence of SeO_4^{2-} and SeO_3^{2-} ions.

On the contrary, Kessi and co-workers (1999) observed the presence of selenium-containing particles both inside (in the citoplasm) and outside *Rhosospirillum rubrum* cells, grown in the presence of selenite. Nevertheless, they suggested that a vesicular mechanism of excretion occurs in this strain. Indeed, they observed intact cells after selenite reduction. This evidence, led the authors to exclude a cell lyses process for releasing Se particles in the growth medium (Kessi *et al.*, 1999).

Interestingly, in *Escherichia coli* elemental selenium deposition has been observed both in the periplasmic space (Gerrard *et al.*, 1974) and in the cytoplasm (Silverberger *et al.*,

1976).

Moreover, elemental selenium deposited inside or outside cells has been described as being in spherical or spherical to oval-shaped structures (Losi and Frankenberger, 1997; Tomei *et al.*, 1995), fibrillar and granular structures (Silverberg *et al.*, 1976), or amorphous aggregates (Yamada *et al.*, 1997).

(b) Selenium oxidation

The oxidation of reduced forms of Se to Se oxyanions represents an important reaction in the environment, not only because SeO_4^{2-} and SeO_3^{2-} are soluble and toxic, but because the biomethylation of Se relies largely on these oxidized selenium forms.

The oxidation of Se^0 has been studied in several soil and sediment experiments (Masscheleyn *et al.*, 1990; Tokunaga *et al.*, 1996; Dowdle and Oremland, 1998; Losi and Frankenberger, 1998), but very little information is available on the oxidation of other reduced Se compounds and organisms that may carry out these transformations.

Losi and Frankenberger (1998) demonstrated that the oxidation of Se^0 to SeO_4^{2-} and SeO_3^{2-} was largely biotic in nature and occurs at relatively slow rates. The oxidation of Se^0 was reportedly carried out by both heterotrophic and autotrophic organisms.

Dowdle and Oremland (1998) studied the oxidation of Se^0 in oxic soil slurries and found that Se^{4+} was the main product, with lesser quantity of Se^{6+} being produced.

Moreover, it was speculated that Se^0 oxidation was carried out by chemoheterotrophs and chemoautotrophic thiobacilli. The microbial oxidation of Se^0 to Se^{4+}, by a group of unidentified autotrophic bacteria, was first reported by Lipman and Waksman (1923).

Torma and Habashi (1972) described the oxidation of copper (II) selenide to Se^0 by *Thiobacillus ferroxidans*.

Saratchandra and Watkinson (1981) reported the oxidation of Se^0 to SeO_3^{2-} and trace amounts of SeO_4^{2-} by a heterotrophic bacterium, *Bacillus megaterium*.

The oxidation of Se^0 and other Se reduced forms has not been fully addressed and is clearly an area of vast research opportunities. Furthermore, little information is available on the mechanisms used and the diversity of organisms that carry out such reactions.

(c) Selenium methylation

The formation of methylated Se compounds from Se oxyanions and organo-Se compounds is known to occur in seleniferous soil, sediment and water (Doran, 1982). The methylation of Se has been shown to be mainly a biotic process and is primarily thought to be a protective mechanism to detoxify their surrounding environment.

The predominant groups of Se-methylating organisms isolated from soils and sediments are bacteria and fungi (Doran, 1982; Karlson and Frankenberger, 1988) while bacteria are thought to be the major Se methylating organisms in water (Thomson-Eagle and Frankenberger, 1991).

The volatile Se compound product by most organisms is DMSe (Doran, 1982; Karlson and Frankenberger, 1988; Thomson-Eagle and Frankenberger, 1991). Other volatile Se compounds produced in much smaller amounts are DMSe, DMSeS, methaneselenone, and methane selenol. Although the biological significance of Se methylation is not clearly understood, once volatile Se compounds are released to the atmosphere and diluted, Se loses its hazardous potential.

Selenium methylation pathway

In general, methylation of inorganic Se involves a reduction and a methylation step, but the exact order in which these reactions occur is still highly debated. Initially it was thought that DMSe (dimethylselenide) was the only product of selenium methylation reactions (Challanger, 1945). Subsequently Reamer and Zoller (1980) identified DMDSe and dimethylselenone in addition to DMSe as products from soil and sewage sludge amended with either SeO_3^{2-} or Se^0 and proposed a methylation pathway (Fig. 4). From this study, it appears that the production of DMDSe is dependent on the Se concentration, where high Se concentrations may inhibit specific microbial communities responsible for producing DMSe. Alternatively it may be more energetically favorable to produce DMDSe rather than DMSe.

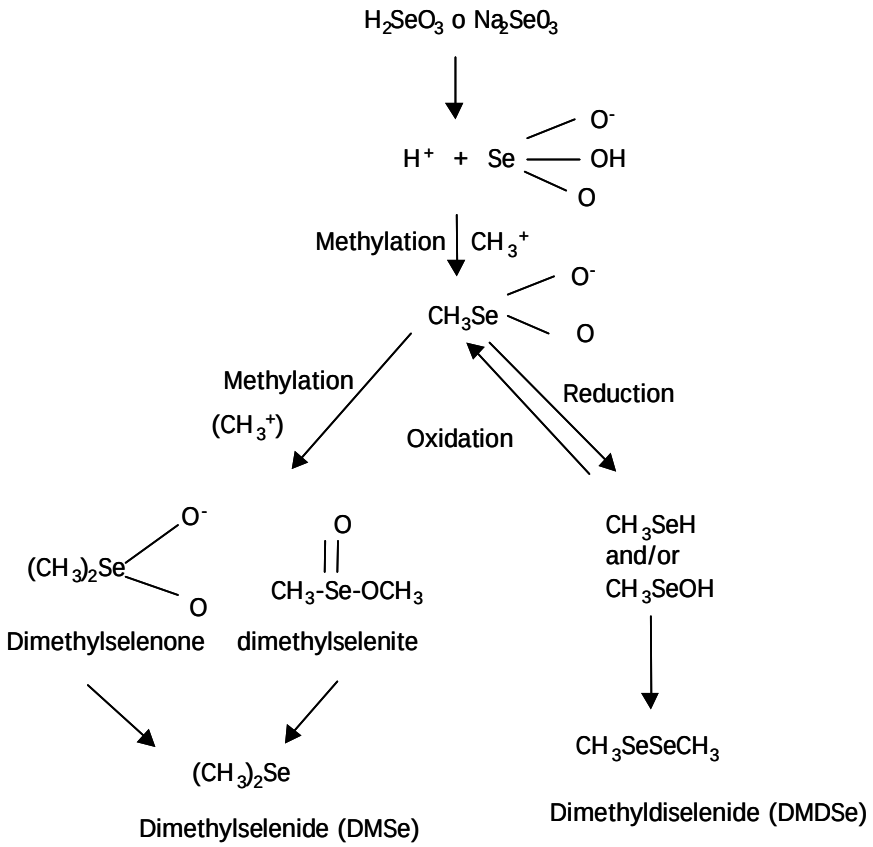


Figure 4 – Selenium methylation pathway (proposed by Reamer and Zoller, 1980)

(d) Selenium demethylation

In soil and water systems, DMSe and its analogue dimethylsulfide (DMS), undergo biological demethylation reactions. Demethylation can be defined as the removal of a methyl group form the central atom of a methylated compound. Several soil microorganisms have been isolated that are capable of demethylating volatile Se compounds. Doran and Alexander (1977) isolated from seleniferous clay, a *Pseudomonas* strain able to use TMSe, two DMSe demethylating pseudomonads, and strains of *Xanthomonas* and *Corynebacterium* that were able to grow on DMDSe as the sole C source.

Oremland and co-workers (1989) hypotised that methylotrophic bacteria carry out demethylation, while certain hydrogen-oxidizing methanogenes may be involved in reductive methylation. The magnitude of demethylation reactions in seleniferous environments has yet to be determined and may be of significant interest if Se volatilization is to be implemented as a remediation technique.

1.2.1.2 Selenium in higher plants

Plants differ in their ability to accumulate Se in their tissues. Pioneering research conducted in the 1930s led to the discovery that several plant species originating on seleniferous soils in the western US had the capacity to accumulate high levels of Se (Beath *et al.*, 1934).

Actually, certain native plants are able to hyperaccumulate Se in their shoots when they grow on seleniferous soil. These species are called Se accumulators and include a number of species of *Astragalus*, *Stanleya*, *Morinda*, *Nepturia*, *Oonopsis*, and *Xylorhiza* (Brown and Shrift, 1982; Trelease and Trelease, 1939). On the other hand, most forage and crop plants, as well as grasses, contain less than 25 mg Se kg^{-1} dry weight and do not accumulate Se much above a ceiling of 100 mg Se kg^{-1} dry weight when grown on seleniferous soils. These plants are referred to as Se nonaccumulators (Brown and Shrift, 1982; White *et al.*, 2004).

A third category of plants, known as secondary Se accumulators (Brown and Shrift, 1982), grow on soil of low-to-medium Se content and accumulate up to 1000 mg Se kg^{-1} dry weight. Examples of plants in this group are species of *Aster*, *Astragalus*, *Atriplex*, *Castilleja*, *Comandra*, *Grayia*, *Grindelia*, *Gutierrezia* and *Machaeranthera* (Parker and Page, 1994).

Most recently, research by Banuelos and co-workers has identified the fast growing *Brassica* species, Indian mustard (*Brassica juncea*) and canola (*Brassica napus*), as new secondary Se-accumulator plant species with a typical Se concentration of several hundred micrograms of Se g^{-1} dry weight in their shoot tissues when grown on soils contaminated with moderate levels of Se (Banuelos *et al.*, 1997).

Is Se an essential element for plants?

Although there are evidences that Se is required for the growth of algae (Price *et al.*, 1987; Yokota *et al.*, 1988), the question of the essentiality of Se as a micronutrient in higher plants is unresolved and remains controversial. While there are indications that Se may be required for Se-accumulating plants (Broyer *et al.*, 1972a,b), which are endemic on seleniferous soils, there are no evidences for a Se requirement in non accumulators (Shrift, 1969).

In order to investigate the essentiality of Se in higher plants, attempts have been made to establish whether plants contain essential selenoproteins, such as those discovered for bacteria and animals. However, on the basis of all the available information published, to

date no essential higher plant selenoprotein has been clearly identified by either protein or DNA sequences analysis (Terry *et al.*, 2000).

Selenium uptake and transport in plants

The rate and form of Se uptake depend on the concentration and chemical form of Se in the soil solution, as well as rhizosphere conditions such as pH and the presence of sulfate and phosphate, which compete with Se uptake (Bell *et al.*, 1992; Blaylock and James, 1994; Dhillon and Dhillon, 2003).

Se is taken up into plant roots from the soil solution predominantly as SeO_4^{2-}, yet SeO_3^{2-} and organic Se compounds are also taken up readily (Shrift and Ulrich, 1969; Martin *et al.*, 1971; Asher *et al.*, 1977; White *et al.*, 2004).

There are considerable differences between the mechanisms involved in the uptake and transport of SeO_4^{2-} and SeO_3^{2-} and organic Se compounds like SeMet.

Both SeO_4^{2-} and organic Se compounds absorption in plants from the soil solution are active processes, whereas SeO_3^{2-} seems to be accumulated through passive diffusion and can be inhibited by phosphate (Shrift and Ulrich, 1969; Abrams *et al.*, 1990).

Asher and co-workers (1977) demonstrated that when SeO_4^{2-} was supplied to severed tomato roots, the concentration of SeO_4^{2-} in the xylem exudates was 6-13 times higher than in the external medium. On the other hand, SeO_3^{2-} concentrations in the exudates were always lower than in the external solution.

SeO_4^{2-} readily competes with the uptake of SO_4^{2-} and it has been proposed that both anions are taken up via a sulphate transporter in the root plasma membrane (Abrams *et al.*, 1990; Arvy, 1993). The absorption of SO_4^{2-} from the soil occurs through the use of high affinity (K_m = 9 μM) and low affinity (K_m = 100 μM) transporters that are localized in root epidermal and cortical cells (Leustek and Saito, 1999). Yet, the high affinity transporter is expressed exclusively in roots and is believed to be the primary transporter of SO_4^{2-} from the soil (Smith *et al.*, 1997; Shibagaki *et al.*, 2002). On the other hand, the low affinity transporters, which are expressed in leaves and roots, are postulated to be involved in both uptake of SO_4^{2-} from the soil solution to the roots and intracellularly from the apoplast into the symplast.

On the other hand, also the translocation of Se from root to shoot is dependent on the form of Se supplied. SeO_4^{2-} is transported much more easily than SeO_3^{2-}, or organic Se, such as SeMet. Zayed *et al.* (1998) showed that the shoot Se/root Se ratio ranged from 1.4 to 17.2 when SeO_4^{2-} was supplied but was only 0.6 to 1 for plants supplied with SeMet and less than 0.5 for plants supplied with SeO_3^{2-}. Arvy (1993) demonstrated that within 3

hours, 50% of the SeO_4^{2-} taken up by bean plant roots moved to shoots, whereas in the case of SeO_3^{2-}, most of the Se remained in the root and only a small fraction was found in the shoot. Time-dependent kinetics of Se uptake by *Brassica juncea* showed that only 10% of the SeO_3^{2-} taken up was transported from root to shoot, whereas SeO_4^{2-} (which was taken up twofold faster than SeO_3^{2-}) was rapidly transported into shoots (de Souza *et al.*, 1998). Thus, plants transport and accumulate substantial amounts of SeO_4^{2-} in leaves but much less SeO_3^{2-} or SeMet. The reason why SeO_3^{2-} is poorly translocated to shoots may be because it is rapidly converted to organic forms of Se such as SeMet (Zayed *et al.*, 1998), which are retained in the roots.

Selenium biochemistry in plants

(a) Pathway in Se Nonaccumulators

Nowadays, it has been demonstrated that higher plants metabolize Se via the S-assimilation pathway (Zayed *et al.*, 2000). This involves the nonspecific incorporation of Se into selenoamino acids and their proteins, as well as volatilization, which occurs when Se is supplied to plants in excess of any potential Se requirement.

After SeO_4^{2-} is absorbed into the root via the SO_4^{2-} transporter, it is translocated without chemical modification through the xylem to leaves (de Souza *et al.*, 1998; Zayed *et al.*, 1998). Once inside the leaf, SeO_4^{2-} enters chloroplasts where it is metabolized by the enzymes of SO_4^{2-} assimilation. The first step in the reduction of SeO_4^{2-} is its activation by ATP sulfurylase to APSe (adenosine phosphoselenate), an activated form of SeO_4^{2-}. Molecular studies provided the evidence that this enzyme is rate limiting for both SeO_4^{2-} reduction and Se accumulation (Pilon-Smits *et al.*, 1999).

Subsequently, APSe can be reduced either nonenzymatically to GSH-conjugated selenide ($GS\text{-}Se^-$) (Fig. 5, Panel A) or enzymatically to selenide (Se^{2-}) via APS reductase and sulfite reductase (Fig. 5, Panel B). SeO_3^{2-} can enter the pathway via a nonenzymatic reaction to selenodiglutathione (GS-Se-SG), which is reduced to the selenol (GS-SeH).

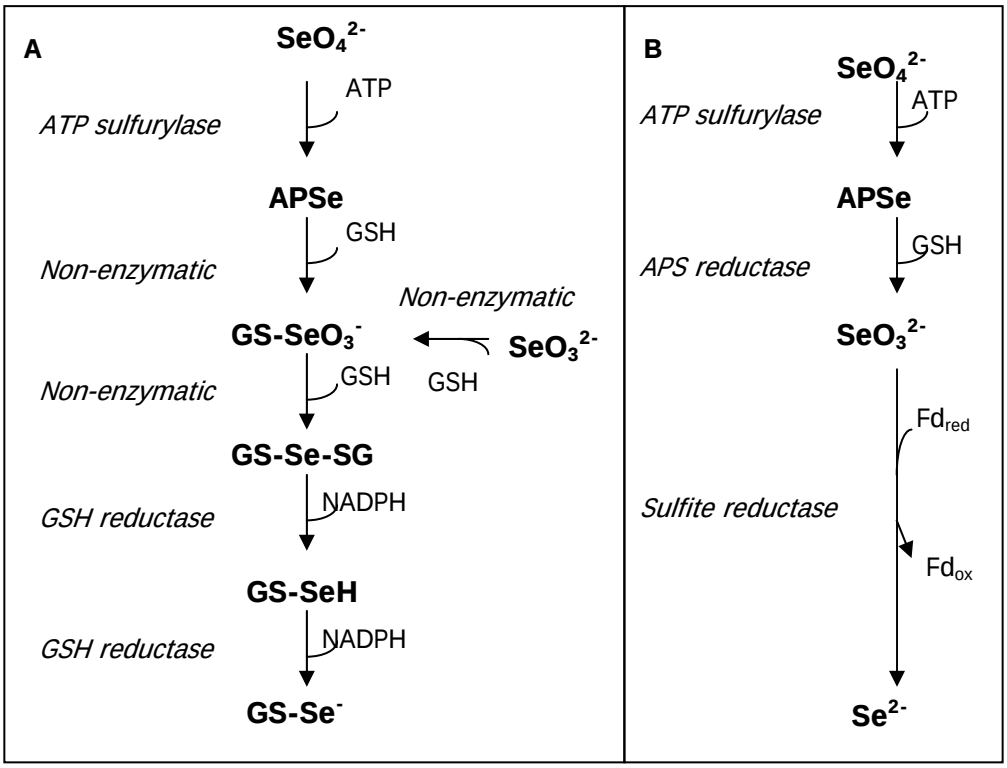


Figure 5 – Pathways of SeO_4^{2-} reduction to GS-Se$^-$ via nonenzymatic reactions (panel A) or to Se^{2-} via APS reductase and sulfite reductase (panel B)

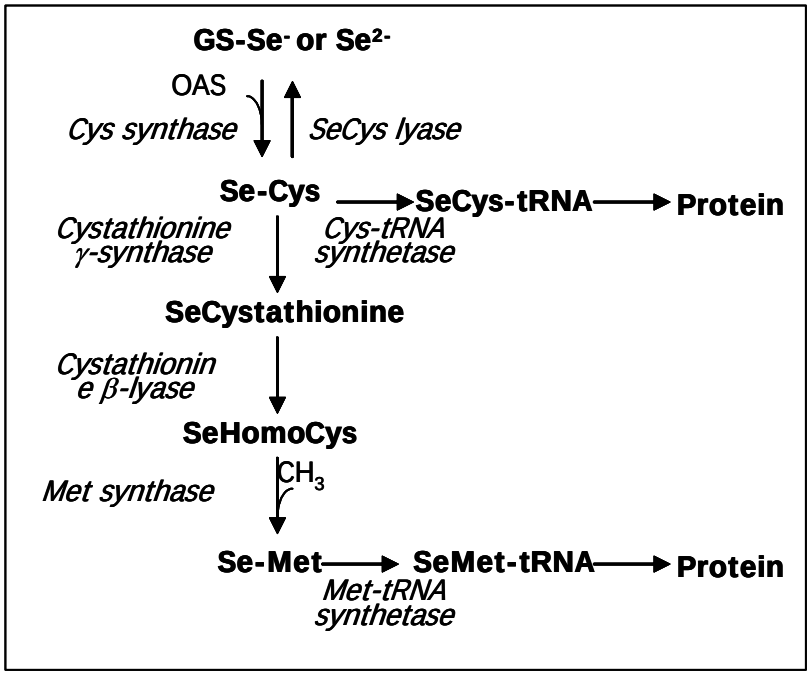


Figure 6 – Pathway of selenide incorporation into SeCys, SeMet, and proteins

GSH-conjugated selenide or selenide can be incorporated in SeCys or SeMet ammino acids following the pathways schematized in Figure 6.

SeCys is formed by the action of Cys synthase, which couples selenide with O-acetylserine (Ng and Anderson, 1978), whereas SeMet may be produced from SeCys via SeCystathionine and SeHomoCys (Droux *et al.*, 2000). The most likely enzyme for the synthesis of SeMet from SeHomoCys is the cytosolic enzyme Met synthase (Fig. 6).

Thus, selenium is readily incorporated into proteins in nonaccumulator plants treated with Se (Brown and Shrift, 1981). The incorporation into proteins occurs through the nonspecific substitution of SeCys and SeMet in place of Cys and Met, respectively (Brown and Shrift, 1982) (Fig. 6).

SeMet may be also methylated to Se-methylSeMet and subsequently originate DMSe, a volatile Se organic compound (Fig. 7). The most likely enzyme responsible for this reaction is S-methylMet hydrolase, which produces DMS from S-methylMet in higher plants (Gessler and Bezzubov, 1988). Another possible pathway for DMSe production is via the intermediate dimethylselenoniopropionate (DMSeP) (Ansede and Yoch, 1997) (Fig. 7).

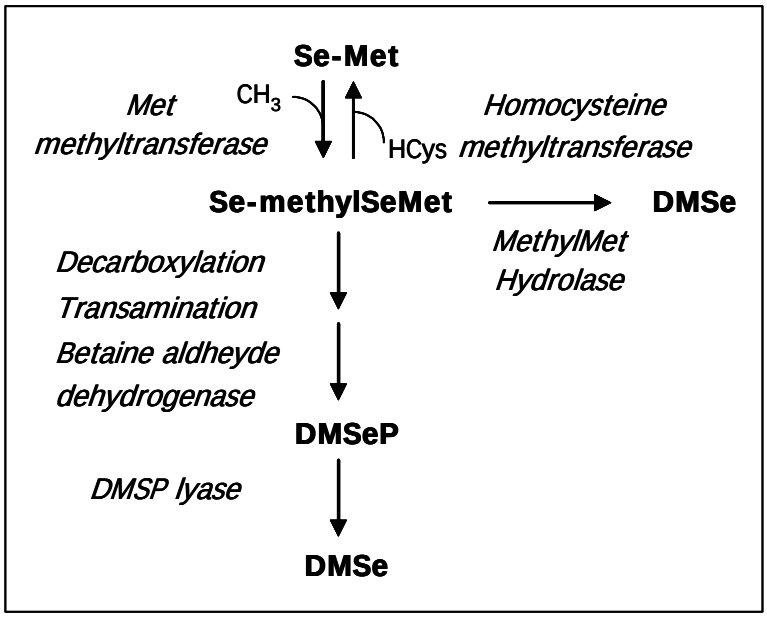


Figure 7 – Possible pathways for DMSe production from SeMet

(b) Pathway in Se Accumulators

The pathway for the assimilation of inorganic forms of Se to SeCys in Se accumulators is believed to be the same as for Se nonaccumulators (Brown and Shrift, 1982; Terry and Zayed, 1994) (Fig. 8). However, Se accumulators differ from nonaccumulators because they metabolize SeCys primarily into various nonprotein selenoamino acids. The synthesis

of these nonprotein selenoamino acids probably occurs along pathways associated with S metabolism (Brown and Shrift, 1982). SeCys can be converted to:

(I) Se-methylSeCys, which has been found in many Se-accumulators (Brown and Shrift, 1982);

(II) Se-cystathionine (Peterson and Robinson, 1972);

(III) γ-glutamyl-Se-methylSeCys, which has been observed in two *Astragalus* Se-accumulators species (Nigam *et al.*, 1969)

Se-methylSeCys is the product of the enzyme SeCys methyltransferase, which has recently been purified and cloned from *Astragalus bisulcatus* (Neuhierl and Böck, 1996; Neuhierl *et al.*, 1999). SeCys methyltransferase methylates SeCys specifically into methylSeCys (Neuhierl and Böck, 1996). It may be further methylated to produce DMDSe, which is volatilized (Fig. 8).

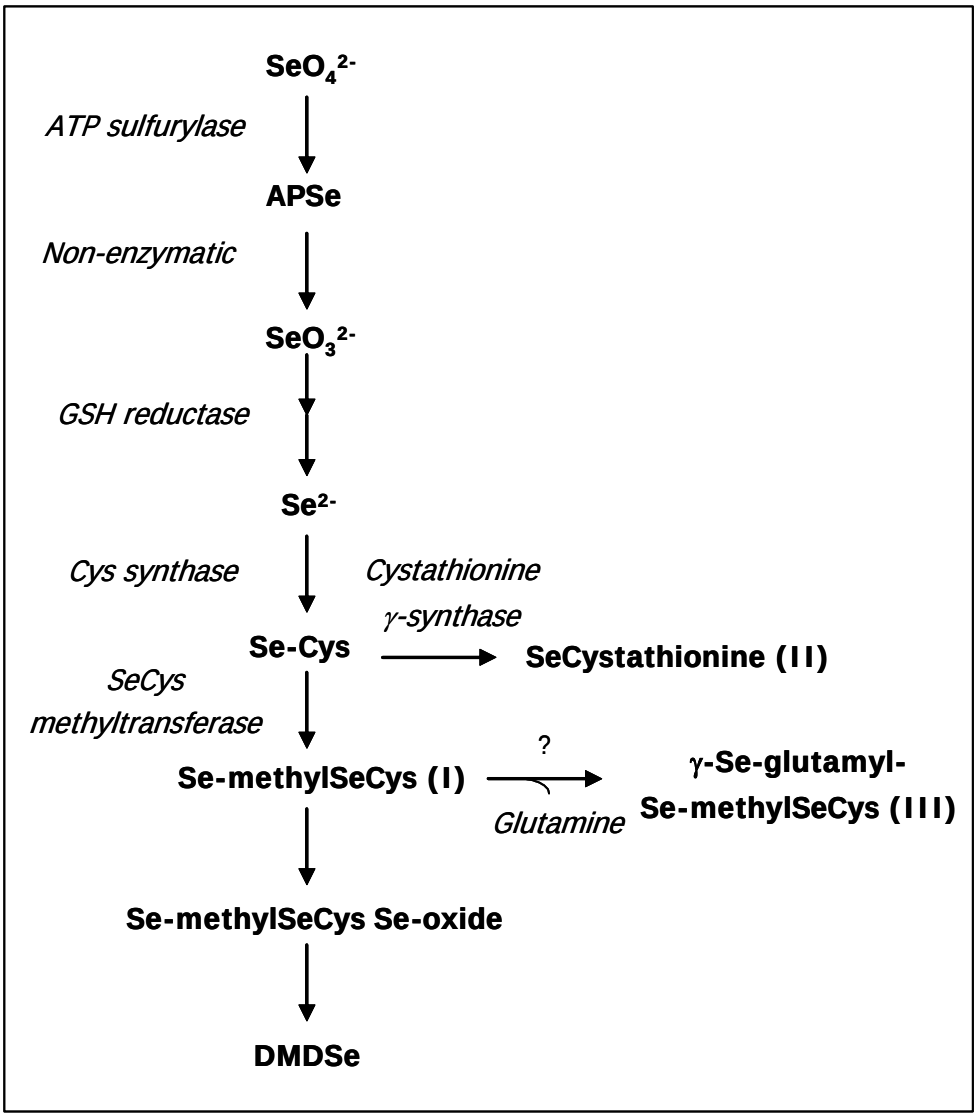


Figure 8 – Pathway of SeO_4^{2-} assimilation by Se accumulators

In nonaccumulators plants, the non-specific integration of the selenoamino acids SeCys and SeMet into proteins is believed to be the major contributor of Se toxicity in plants (Brown and Shrift, 1981). Thus, the metabolic capacity of Se accumulator plants to incorporate SeCys into non-protein amino acids, makes them able to accumulate and tolerate high concentrations of Se.

1.3 Possible exploitation of biological processes for the treatment of Se polluted matrices

Over the last two decades, attention has been given to the development of an effective remediation technology for the permanent removal of Se oxyanions from seleniferous soil and water. A majority of the focus has been applied to contaminated agricultural drainage water, which has been responsible for a number of well-documented ecotoxicological problems. One approach to alleviating this problem is treating the water before disposal.
Because Se undergoes microbial and plant transformations, their application may be potentially useful as bioremediation strategies.
Several different bioremedial approaches have or are being developed, which include a variety of bioreactors utilizing bacteria with the ability to reduce the toxic, soluble Se oxyanions to insoluble Se^0. These systems are designed to remove Se from contaminated wastewater (industrial or agricultural) before release into the environment. Another means to remove Se from contaminated soil and water involves stimulation of the indigenous microorganisms that volatilize Se. This process has proven effective as an *in situ* treatment for seleniferous soils in the San Joaquin Valley, California (Frankenberger and Karlson, 1995; Flury *et al.*, 1997).
On the other hand, also plants have been shown to be highly effective in the remediation of Se contaminated soils and waters (phytoremediation process).
With their root systems, plants can scavenge large areas and volumes of soils, removing Se as selenate, selenite, and organic forms of selenium such as SeMet. Once absorbed by plant roots, as described in section 1.2.1, Se is translocated to the shoot, where it may be harvested and removed from the site; alternatively, once in the shoots, Se may be volatilized.
Concerning Se polluted streams, another effective solution is to use constructed wetlands, a technique for treating wastewater that is based on the auto depurative capacity of

complex ecosystems like natural wetlands, reproducing them in an artificial environment in an engineered way: up to 90% of the Se from oil refinery effluents was shown to be removed by this means (Hansen *et al.*, 1998).

1.3.1 Microbe-induced bioremediation

Several systems have been investigated that utilize Se bioreduction to remove Se oxyanions from solution with the goal of treating agricultural and industrial wastewater (Mattison, 1992).

Oremland (1991) holds a patent describing a method that involves a 2-stage reduction process that uses algae in the aerobic first stage to deplete NO_3^- concentrations in contaminated water to < 1 mM. The water is then fed to an anoxic bioreactor containing SeO_4^{2-} respiring bacteria where SeO_4^{2-} is reduced to insoluble Se^0 and deposited within the biomass. Influent concentration of approximately 56 mg Se L^{-1} was reduced by 99%.

Another technique, the Oswald Process (Gerhardt *et al.*, 1991), is similar to Oremland's technology in that the system uses aerobic algal growth to take up NO_3^-, lowering its concentration to <10 mg L^{-1}. The biomass suspension is then transferred to an anoxic unit where denitrifying and SeO_4^{2-} respiring bacteria carry out reduction of SeO_4^{2-} to SeO_3^{2-} rather than to Se^0. After the anoxic reduction step, $FeCl_3$ is added to precipitate SeO_3^{2-}, thereby reducing soluble selenium levels from 200-400 μg L^{-1} to 7-12 μg L^{-1}.

The Owens Process (Owens, 1998) also uses a technology based on an anaerobic mechanism to reduce SeO_4^{2-} to Se^0. The carbon source used in the system was methanol. Most of the C added to the system was used during denitrification, thus enough methanol must be added to support both denitrification and Se reduction. Denitrification is important to the process because Se reduction will not occur until NO_3^- is removed. It was reported that the reactor was able to remove 94 % of the soluble Se.

The use of *Thauera selenatis*, a SeO_4^{2-} respiring bacterium, in a biological reactor system to remediate both selenium oxyanions from contaminated water has been described by Macy *et al.* (1993), Lawson and Macy (1995) and Cantafio *et al.* (1996). The latest pilot scale system, which consisted of a series of four medium-packed tanks, was used to treat seleniferous agricultural drainage water (Cantafio *et al.*, 1996). Using acetate as the electron donor, Se oxyanions and NO_3^- concentrations were reduced by 98%. An earlier system included the use of two bioreactors in series; the first being an aerobic sludge blanket reactor and the second a fluidized bed reactor (Macy *et al.*, 1993). Once again acetate was used as the electron donor and the SeO_4^{2-}, SeO_3^{2-} and NO_3^- levels were all reduced by 98% in the influent. A similar system was successively used to remediate

SeO_3^{2-} from oil refinery wastewater, which reduced the Se oxyanion concentration by 95%. Although Macy (1994) has shown that *T. selenatis* can reduce both SeO_4^{2-} and NO_3^- simultaneously, NO_3^- must be present in the system for SeO_4^{2-} to be completely reduced to Se^0, because the nitrite reductase will only catalyze the reduction of selenite when denetrification is occurring.

Finally, Adams and coworkers (1993) conducted a pilot study in which *E. coli* was used to treat a weak acid effluent from a base metal smelter, containing 30 mg Se L^{-1}. This system was able to remove 97% of the Se within 4 h. A bench-scale system was also tested on mining process waters. Using *Pseudomonas stutzeri*, with molasses (1 g L^{-1}) as the C source, 97% of the Se was removed in 6-h retention time.

Volatilization of Se is also a potentially important microbial process in relation to bioremediation. The process removes Se from soil and water permanently. Microbial volatilization of Se has been tested as a bioremediation approach to remove toxic levels of Se in soils at Kesterson Reservoir, California. Field investigations were conducted with the goal of identifying the most effective practices for accelerating volatilization and to obtain information necessary for the determination of time and factors affecting this technology (Frankenberger and Karlson, 1995; Flury *et al.*, 1997).

At Kesterson Reservoir, 68 to 88 % of the total Se inventory (0-15 cm) was dissipated over 100 months (Flury *et al.*, 1997). The highest Se removal rates were observed in soils amended with casein. At the Sumner Peck Ranch (California), 32% of the Se was removed over 22 months from the dewatered seleniferous sediment with the application of moisture and tillage, and this was increased to 59 % with the addition of a carbon source (Frankenberger and Karlson, 1995).

1.3.2 Plant-assisted bioremediation (Phytoremediation)

Phytoremediation is defined as the use of green plants to remove pollutants from the environment or to render them harmless (Kramer, 2005; Salt *et al.*, 1998).

There are two distinct strategies in soil phytoremediation: phytostabilization and phytoextraction (Salt *et al.*, 1998). The former is used to provide a cover of vegetation for a moderately to heavily contaminated site, thus preventing wind and water erosion. Plants suitable for phytostabilization develop an extensive root system, provide good soil cover, posses' tolerance to the contaminant metals, and ideally immobilize the contaminants in the rhizosphere. Phytostabilization is often performed using species from plant communities occurring on local contaminated sites.

The most effective but also technically the most difficult phytoremediation strategy is phytoextraction. It involves the cultivation of tolerant plants that concentrate soil contaminants in their above-ground tissue. At the end of the growth period, plant biomass is harvested, dried or incinerated, and the contaminant-enriched material is deposited in a special dump or added into smelter. For phytoextraction to be worthwhile, the dry biomass or the ash derived from above ground tissues of a phytoremediator crop should contain substantially higher concentrations of the contaminant than the polluted site.

Many species have been evaluated for their efficacy in selenium phytoremediation (Banuelos and Meek, 1990; Bell *et al.*, 1992; Mayland *et al.*, 1989; Wu *et al.*, 1988). As described in section 1.2.1.2, Se hyperaccumulator plants are naturally capable of accumulating Se in their above-ground tissues, without developing any toxicity symptoms (Backer and Brooks, 1989). The concentration of Se in dry leaf biomass is usually up to 100-fold higher than the concentration in the polluted matrix (McGrath and Zhao, 2003) and the shoot:root ratio of concentrations of the hyperaccumulated Se is above unity (Zayed *et al.*, 1998).

Certain species of *Astragalus* were found to accumulate high Se levels in their tissues (e.g. *A. bisulcatus*) which led some researchers to suggest the use of the Se-accumulators, *A. bisulcatus* and *A. racemosus* (Parker *et al.*, 1991) for Se phytoremdiation. However, these are slow-growing plants and Se accumulated in *Astragalus* shoots is mostly soluble and can easily be leached from plant tissue back to the soil by rainfall (Cowgill, 1990).

Actually, the ideal plant species for phytoremediation of Se is one that can accumulate and volatilise large amounts of Se, grow rapidly and produce a large biomass, tolerate salinity and other toxic conditions, and, if possible, provide a safe source of forage for Se-deficient livestock. *Brassica juncea*, which tipically contains 350 mg Se kg^{-1} dry weight, has most of these attributes (Banuelos and Meek, 1990; Zayed *et al.*, 2000) and can find promising application in Se phytoremediation.

1.3.2.1 Plant-microbe interaction in Se phytoremediation processes

Rhizosphere microorganisms have been shown to have an important role in phytoremediation processes. It is worth noting that these microbes can positively affect both plant growth and Se uptake or Se volatilization by plant. On the other hand, plant may increase the growth and the metabolic activity of the microorganisms in the rhizosphere.

Actually, the exudation of nutrients by plant roots creates a nutrient-rich environment in which microbial activity is stimulated. Plant root exudates contain sugars, organic acids and amino acids as main component (Vancura and Hovadik, 1965). In addition, the mucigel secreted by root cells, lost root cap cells, the starvation of root cells, or the decay of complete roots provide nutrients for microbes (Lugtenberg and de Weger, 1992; Lynch and Whipps, 1990). In turn, rhizosphere organisms also have a large impact on plants, because many microbes isolated from the rhizosphere are described to have root growth-stimulating or growth-inhibiting properties (Campbell and Greaves, 1990). In fact, rhizosphere bacteria can stimulate plant growth by producing phytohormones (Fallik *et al.*, 1994), enhancing mineral and water uptake (Lin *et al.*, 1983), producing antibiotics to inhibit pathogenes (Lesinger and Margraff, 1979) and altering root morphology (Kapulnik, 1996). Moreover several lines of evidence suggest that soil microorganisms possess mechanisms capable of altering environmental mobility of metal contaminants with subsequent effects on the potential for root uptake (Lasat *et al.*, 2000). For example, chemolithotrophic bacteria have been shown to enhance environmental mobility of metal contaminants via soil acidification, or, in contrast, to decrease their solubility due to precipitation as sulfides (Kelley and Tuovinen, 1988). In addition, soil microorganisms have been shown to exude organic compounds which stimulate bioavailability and facilitate root absorption of a variety of metal ions including Fe^{2+}, Mn^{2+} and Cd^{2+} (Bural *et al.*, 2000; Salt *et al.*, 1995)

Concerning Se phytoremediation process, de Souza and co-workers (1999) demonstrated that bacteria increase the plant's potential for Se phytoremediation because they facilitate Se accumulation and volatilization. Their experiments with antibiotic ampicillin showed that bacteria facilitated 35% of plant Se volatilization and 70% of plant tissue accumulation. By comparing Se phytoremediation assays carried out with axenic plants and with plants inoculated with rhizosphere bacteria, they found that plants with bacteria contained a heat-labile compound in their root exudates. When this compound was added to the rhizosphere of axenic plants, they observed an increment of Se accumulation in plant tissues (de Souza *et al.*, 1999). However no more information is available for this heat-labile compound.

1.3.2.2 Selenium removal by constructed wetlands

Constructed wetlands constitute a complex ecosystem, the biological and physical components of which interact to provide a mechanical and biogeochemical filter capable of removing many different types of contaminants from water. They have been used as a

low-cost treatment to remove a wide-range of waterborne contaminants from polluted waters such as municipal wastewater and effluents from electricity generating facilities and oil refineries in the United States (Brown and Reed, 1994) and Europe (Haberl *et al.*, 1995). The anoxic environment and organic matter production in wetlands promote biological and chemical processes that transform contaminants to immobile or less toxic forms (Gao *et al.*, 2003). Plants support microbially mediated transformations of contaminants by supplying fixed-carbon as an energy source for bacteria and by altering the chemical environment in their rhizosphere (Oremland *et al.*, 1990; Terry *et al.*, 1992). Plants also take up and accumulate metals and metalloids in their tissues (Zhu *et al.*, 1999). Once entering the plant tissue, some metals and metalloids can be metabolized to non-toxic and/or volatile forms, which may escape the local ground ecosystem by release to the atmosphere (Hansen *et al.*, 1998).

The first indication that wetlands might be useful in the removal of Se from wastewaters came from a study of 36-ha constructed wetland located adjacent to San Francisco Bay, California. Analysis of the wetland inlet and outlet waters showed that the constructed wetland was successful in removing at least 70% of the Se from the wastewater passing through it (Duda, 1992).

In 1996, an experimental wetland was constructed at the Tulare Lake Drainage District (TLDD) in the San Joaquin Valley (Calif.). Its purpose was to evaluate the potential of constructed wetlands for the removal of Se from agricultural irrigation drainage water. Ten individual cells were tested, either unvegetated or vegetated singly or with a combination of sturdy bulrush [*Schoenoplectus robustus* (Pursh) M.T. Strong], Baltic rush (*Juncus balticus* Willd.), smooth cordgrass (*Spartina alterniflora* Loisel.), rabbitfoot grass [*Polypogon monspeliensis* (L.) Desf.], saltgrass [*Distichlis spicata* (L.) Greene], cattail (*Typha latifolia* L.), tule [*Schoenoplectus acutus* (Muhl. ex Bigelow)], and widgeon grass (*Ruppia maritima* L.) (Gao *et al.*, 2003). On average, the wetland cells removed 69% of the total Se mass from the inflow. Vegetated wetland cells removed Se more efficiently than the unvegetated cell, without significant differences among vegetated cells (Lin and Terry, 2003).

Microcosm experiments provide an initial means of evaluating the remediation potential of a constructed wetland with a greater degree of experimental control, less cost, and substantially reduced environmental risk than a study of the wetland itself. Such a microcosm study was used to evaluate the potential of constructed wetlands to remediate effluent containing highly toxic selenocyanate (SeCN), As, and boron (B) generated by a coal gasification plant (Ye *et al.*, 2003). The concentrations of these contaminants were

several orders of magnitude higher than those normally treated by constructed wetlands. The microcosms removed 79% Se, 67% As, 57% B, and 54% CN mass, significantly reducing the toxicity of the effluent. Because cattail (*Typhia latifolia* L.), *Thalia dealbata* Fraser ex Roscoe, and rabbitfoot grass (*P. monspeliensis* L. Desf.) showed no growth retardation when supplied with the contaminated wastewater, constructed wetlands planted with these species show particular promise for remediating this highly toxic effluent.

Although constructed wetlands offer a less expensive alternative to other water-treatment methods, the approach needs to be optimized to enhance efficiency and reproducibility, and reduce ecotoxic risk. Most of the contaminants removed from the waste-stream are immobilized in the sediment. For example, in the microcosm experiment discussed above, the sediment contained 63% of the Se, 51% of As, and 36% of B, while only 2–4% was accumulated in plant tissue (Ye *et al.*, 2003). In the TLDD wetland, 41% of the supplied Se left the wetland; the remaining 59% was retained in the wetland cell, partitioned between the surface sediment (0–20 cm; 33%), organic detrital layer (18%), fallen litter (2%), standing plants (<1%), and standing water (<1%) (Gao *et al.*, 2003). The Se in the agricultural drainage water entering the TLDD wetland was predominantly in the form of selenate (95%); it was reduced in sediment to a mixture of elemental Se (45%), organic Se (40%), and selenite (15%, Lin and Terry, 2003). As described in the section 1.1.1, although elemental Se is essentially non-toxic, some selenite and some species of organic Se are more toxic than selenate. There is concern that, since Se concentrations in the organically rich surface sediments increased over time, Se could eventually enter the aquatic food chain and exert ecotoxic effects.

1.4 AIM OF THE WORK

Naturally occurring selenium is essential for biological system as trace element (Shamberger, 1983). However, at high concentrations, this metalloid is toxic even to humans (Barceloux, 1999). In particular, the oxyanions SeO_3^{2-} and SeO_4^{2-} are the dominant Se species in aerobic environments and at the same time they also represent the Se forms with the highest toxicity level. These toxic oxidized forms can be found in high concentrations in some habitats as a consequence of agricultural practices or industrial discharges (Losi and Frankenberger, 1997). Severe Se pollution in agriculture is restricted to seleniferous soils where repeated irrigation leads to the accumulation of this metalloid into the drainage water, eventually entering the food chain. On the other hand, selenium-laden effluents and wastes are principally associated to oil refining and mining activities, to the production of pigments, metallurgical additives and pharmaceutical preparates or to the use in electronics and glass manufacturing. Therefore, increasing selenium contents in soils and waters may become troublesome to the environment. Thus, it's becoming even more necessary to set up effective systems for the remediation of polluted effluents. Microbial reduction of bioavailable selenium oxyanions into elemental selenium or to relatively non-toxic forms, is of great interest for bioremediation, expecially for the treatment of Se-laden effluents and industrial outlets. Thus, an interesting alternative for a cost-effective abatement of Se oxyanions may be represented by biological treatments relying on the exploitation of either microbes or plants capable to reduce, volatilise or accumulate toxic selenium forms (Cantafio *et al.*,1996; Fujita *et al.*, 2002; Azaizeh *et al.*, 2003).

With the main purpose of set up bioremediation protocols for the remediation of selenium laden effluents, the aims of the present work were:

- to isolate Se-resistant strains capable to carry out transformation reactions (mainly Se oxyanions reduction) of toxic selenium forms;
- to characterize the isolated strains in order to investigate the mechanisms responsible for their reduction capability;
- to test these strains for the biological removal of soluble selenium forms on a lab scale. Two different protocols were tested: a Sequencing Batch System for the removal of selenite from aqueous solution and a hydroponic system for a plant-assisted phytoremediation process.

2. MATERIALS AND METHODS

2.1 Chemicals, culture media and solutions

Chemicals purchased from Sigma-Aldrich (Milan, Italy) were all analytical grade. Nutrient Broth, Yeast Extract and Bacteriological Agar were furnished by Oxoid Italia Spa (Garbagnate Milanese, Italy). Mineral medium (DM) was prepared as described by Frassinetti *et al.* (1998) with small modifications (addition of 0.1% Yeast Extract).

Na_2SeO_3 and Na_2SeO_4 were prepared as a 100 mM stock solution in deionised water and sterilized by filtration.

Half-strength Hoagland solution was prepared as described in Azaizeh *et al.*, 2003 and sterilized by filtration.

2.2 Isolation of Se-resistant bacterial strains

Enrichment cultures for bacterial isolation were inoculated with soil samples from the rhizosphere of *A. bisulcatus*, a leguminous plant grown throughout six months in seleniferous soil collected at a mine site in Sardinia, Italy (Campostrini *et al.*, 1999).

A. bisulcatus seeds were obtained from the Western Regional PI Station, Washington State University, Pullman, USA.

Enrichment cultures were carried out in 250-ml Erlenmeyer flasks containing 100 ml Nutrient Broth amended with 0.2 mM Na_2SeO_3 or 0.2 mM Na_2SeO_4 (15 mg Se/kg). Flasks were incubated at 28 °C on an orbital shaker (250 rev/min) in the dark. After 1-week incubation in aerobic growth conditions, serial dilutions of the culture medium were plated on agarised Nutrient Broth plates added respectively with 0.2 mM Na_2SeO_3 or 0.2 mM Na_2SeO_4. These plates were incubated at 28 °C for 5 days. Appearance of red-coloured colonies was interpreted as an indication for Se oxyanions reduction to Se^0 (Moore and Kaplan, 1992; Sabaty *et al.*, 2001). Single colonies were then isolated and streaked on fresh agarised Nutrient Broth plates containing either Na_2SeO_3 or Na_2SeO_4 as a tool of selective pressure, in order to obtain axenic cultures. Pure cultures of ten morphologically different bacteria were obtained.

2.2.1 MIC (Minimum inhibitory concentration) determination

Minimum inhibitory concentration (MIC) for SeO_3^{2-} and SeO_4^{2-} oxyanions was determined either on Nutrient Broth or DM agarised plates. Microbial cells were checked for their viability after 5 days of incubation on plates containing increasing concentrations of Na_2SeO_3 and Na_2SeO_4.

2.3 Characterization of strains SeITE01 and SeITE02

Three different strains were chosen for further characterization from those obtained in axenic culture. Strains SeITE01 and SeITE02 were both able to reduce SeO_3^{2-} to Se^0 and revealed the highest value of MIC for Na_2SeO_3. Strain SeATE17 resulted to be the one, from isolated strains, capable to transform both se oxyanions to elemental Se form. SeATE17 characterization is now in progress, thus no date regarding its characterization is reported in this document.

2.3.1 Phylogenetic analysis

Microbial genomic DNA of the selected strains was extracted with the NucleoSpin Tissue Kit (Clontech) following the manufacturer's instructions. Amplification of the gene encoding for the 16*S* rRNA (rDNA) was performed with the primers F8 and R11 (Weisburg *et al.*, 1991).

The product of amplification was directly double-strand sequenced, aligned to the database sequences using BLASTN (Altshul *et al.*, 1997), and analysed using the ARB database (release June 2002) (Strunk O. and Ludwig W., 1993–2002, ARB—a software environment for sequence data, http://www.arb-home.de).

2.3.2 Biochemical and physiological properties

Physiological and biochemical assays were carried out as described in Gerhardt *et al.* (1981). Growth tests at different temperatures and pH values were performed in Nutrient Broth. Assimilation capability was evaluated in DM amended with different carbon sources (0.1% wt./vol.).

2.3.3 Morphological and cytological features

2.3.3.1 Optical microscopy

Cell size and shape were determined by observation at the phase-contrast microscope (Leitz/Leica DMR-HC Series) starting from samples of microbial cultures grown either in Nutrient Broth or in Nutrient Broth amended with 0.2, 0.5, and 2.0 mM Na_2SeO_3, respectively. Samples derived from microbial cultures carried out in presence of 2 mM of Na_2SeO_4 were also analyzed.

2.3.3.2 Electron microscopy

Cell size and shape were also determined through transmission electron microscopy (TEM) or scanning electron microscopy (SEM) analysis starting from samples of microbial cultures grown either in Nutrient Broth or in Nutrient Broth amended with 0.5 mM Na_2SeO_3, respectively. Negative staining analyses were also carried out for the same samples.

TEM analyses. In order to obtain thin sections for electron microscopy analysis, bacterial cells were embedded in Epon-araldite resin after fixation with 2.5% paraformaldehyde + 2.5% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, pH 7.2) and post-fixation with 1% OsO_4 + 0.15% ruthenium red in cacodylate buffer as described by Hess (1966). Sections were prepared by means of a Reichert Ultracut S ultramicrotome (Leica) equipped with a diamond knife. Uranyl acetate and lead citrate were used as contrast agents.

SEM analyses. Bacterial cells analysed through scanning electron microscopy underwent the same fixation and post-fixation processes as it has been described for TEM sample preparation. After fixation, cells were dehydrated with increasing acetone concentrations and dried through the critical point method by using liquid CO_2. Cells were then directly observed through the electron microscope.

Negative staining. Bacterial cells in suspension were fixed on a copper support and contrasted with 2% uranyl-acetate. Cells were then directly observed through the electron microscope.

All observations were carried out with a high resolution electron microscope Jeol JSM 5200. Whereas Energy-dispersive X-ray (EDX) analyses were performed with a high resolution electron microscope (JEOL JEM 2010) operated at high accelerating voltage (200 kV) and equipped with an Inca 100 Link analysis system.

2.3.4 Selenite reduction tests

2.3.4.1 Evaluation of SeITE01 and SeITE02 reduction efficiency at increasing SeO_3^{2-} concentrations

Selenite reduction capability was determined for strains SeITE01 e SeITE02 in rich (Nutrient Broth) growth medium (100ml flasks). Tests were carried out in presence of 3

different Na_2SeO_3 concentrations: 0.2, 0.5 or 2.0 mM. Both microbial growth and SeO_3^{2-} content were measured.

(a) Microbial growth estimation

SeITE02 and SeITE01 cell growth was evaluated by counting the colony forming units (CFU) on agarised Nutrient Broth plates seeded with aliquots of bacterial cultures. All analyses were performed in triplicate.

Bacterial growth in presence of SeO_3^{2-} was checked vs. control cultures incubated in Nutrient Broth with no Na_2SeO_3 added.

(b) SeO_3^{2-} content determination

SeO_3^{2-} concentration in culture medium was determined spectrophotometrically by using the method described by Kessi *et al.* (1999). This method was carried out as follow: first 10 ml of 0.1M HCl, 0.5 ml of 0.1M EDTA, 0.5 ml of 0.1M NaF, and 0.5 ml of 0.1M of disodium oxalate were mixed in a 50 ml glass bottle. A 50- to 250-µl sample containing 100 to 200 nmol of selenite was added, and then 2.5 ml of 0.1% 2,3-diaminonaphthalene in 0.1M HCl was amended. The bottles were incubated at 40°C for 40 min and then cooled to room temperature. The selenium-2,3-diaminonaphthalene complex was extracted with 6 ml of cyclohexane by shaking the bottles vigorously for about 1 min. The absorbance at 377 nm of the organic phase was determined by using a spectrophotometer Heλios β, Unicam. Sterile cultures were also tested for SeIV concentration as negative controls. All manipulations were done in the dark.

Calibration curves were effectuated by using 0, 50, 100, 150, 200 nmol of selenite and were obtained for the growth medium tested (i. e. Nutrient broth).

2.3.4.2 Evaluation of SeITE01 and SeITE02 capability to reduce SeO_3^{2-} added at different times of culture growth

Selenite reduction capacity was determined for both strains in Nutrient Broth in presence of 0.5 mM Na_2SeO_3 added at different times of culture growth:

(a) at the beginning;

(b) in the LAG growth phase;

(c) in the early EXPONENTIAL growth phase;

(d) in the late EXPONENTIAL growth phase;

(e) in the STATIONARY growth phase.

Both microbial growth and SeO_3^{2-} content were evaluated as previously described.

2.3.4.3 Evaluation of SeITE01 and SeITE02 capability to reduce SeO_3^{2-} in adaptive growth conditions

A preculture obtained by growing bacterial cells until stationary phase in presence of either 0.2 mM Na_2SeO_3, 2 mM Na_2SeO_4 and 0.2 mM H_2O_2 were used to inoculate either 100 ml of Nutrient Broth or 100 ml of Nutrient Broth amended with 0.5 and 2.0 mM Na_2SeO_3. Both microbial growth and SeO_3^{2-} content in the culture medium were evaluated as previously described.

2.4 Selenite removal tests using a sequencing batch system (SBS)

A model biological selenite removal process using strain SeITE01 was simulated by aerobic culture in a sequencing batch mode as follows. 250-ml Erlenmeyer flasks, containing 100 ml Nutrient Broth amended with 0.5 mM selenite were inoculated with strain SeITE01. Flasks were incubated under aerobic condition on a rotary shaker at 200 rpm, 28°C. After cultivation for 24 hours, a specific amount of the culture broth (2 ml) was withdrawn and used for routine analysis (cell growth and selenite content determination) and the cells that remained were recovered by centrifugation (8000 x *g*, 10 min, 4°C). The whole amount of recovered cells was transferred into the fresh medium and the selenite reduction tests were carried out again. The experimentation was carried out for 10 cycles. SeO_3^{2-} content in the culture medium was evaluated as previously described. Microbial growth was measured following two different procedures:

- count of colony forming units (CFU) on agarised Nutrient Broth plates seeded with aliquots of bacterial suspensions;
- quantification of total protein content of microbial biomass from different cell suspensions.

All analyses were performed in triplicate.

Protein concentration in bacterial cell extracts was determined by using the Bradford reagent (BioRad) according to the manufacturer's instructions. An aliquot of cells corresponding to 1 ml of bacterial culture was centrifuged at 5000×*g* for 10 min. The pellet was resuspended in 100 µl of extraction buffer (50 mM Na_2 HPO_4, pH 7; 10 mM β-mercaptoethanol; 10 mM Na_2 -EDTA; 0.1% Sodium Lauryl Sarcosine; 0.1% Triton X-100). The resulting suspension was sonicated (Ultrasonic Cleaner, Soltec) for 5 min, stored on ice for 5 min and centrifuged at 18,000×*g* for 10 min at 4°C. The supernatant was collected and measured for protein content. Protein extraction was carried out in

triplicate, whereas all spectrophotometric measurements (Hελios β, Unicam) were done in duplicate.

2.5 Phytoremediation assay

2.5.1 Experimental design

Seeds of *Brassica juncea L.*, accession number n. 173874, were obtained from the North Central Regional Plant Introduction Station, Iowa State University.

Phytoremediation assays were carried out on a perlite hydroponics system. Tests were set up in 20 L polyethylene pods on perlite bed. Before being settled down, perlite was wet with the sterile half strength Hoagland solution until it reached the maximum water content (72.5%).

Seeds, pre-treated with a mixture of hydrogen peroxide and ethanol, were sown directly on perlite beds in number of 50 per pod. Water and nutrients were given to plants once a day through the amendment of a proper quantitative of the half-strength Hoagland solution.

Three different concentrations of soluble selenium, both as selenite and selenate sodium salts, were tested: 15 mg Se/kg (0.2 mM), 75 mg Se/kg (1.0 mM) and 150 mg Se/kg (2.0 mM).

In order to evaluate the influence of Se-resistant bacteria on the phytoremediation process, rhizospheres of *Brassica* plants were inoculated with two different types of bacteria inocula:

1) Inoculum of soil microbial community, collected from the rhizosphere of *Brassica juncea* plants previously grown on soil amended with 30 μM Se (2.5 mg Se/kg), either as Na_2SeO_3 or Na_2SeO_4.
2) Inoculum of strains in axenic culture, isolated from a selenium polluted soil and capable of growing in presence of high selenium oxyanions concentrations: *B. mycoides* strain SeITE01, *S. maltophilia* strain SeITE02 and strain SeATE17.

The bacteria inocula were given after 4 weeks from seeds sowing and then, after 24 hours, soluble selenium was added to the pods as unique amendment.

Each pod was incubated in a glasshouse at temperature ≥ 24°C and with a photoperiod of 12 hours. Each experiment was carried out for 10 weeks.

For each experiment, control pond was set up. In this way a total of 16 different experimental trial were arranged and summarize as follow (Table 5).

	[Se oxyanion]	Type of inoculum
Se^{+4} 0.2 mM + I	15 mg Se/kg SeO_3^{2-}	soil microbial community (I)
Se^{+4} 0.2 mM CTRL	15 mg Se/kg SeO_3^{2-}	NO
Se^{+6} 0.2 mM + I	15 mg Se/kg SeO_4^{2-}	soil microbial community (I)
Se^{+6} 0.2 mM CTRL	15 mg Se/kg SeO_4^{2-}	NO
Se^{+4} 1.0 mM + I	75 mg Se/kg SeO_3^{2-}	soil microbial community (I)
Se^{+4} 1.0 mM + B	75 mg Se/kg SeO_3^{2-}	*B. mycoides* SeITE01 (B)
Se^{+4} 1.0 mM + S	75 mg Se/kg SeO_3^{2-}	*S. maltophilia* SeITE02 (S)
Se^{+4} 1.0 mM + B+S	75 mg Se/kg SeO_3^{2-}	*B. mycoides* SeITE01 and *S. maltophilia* SeITE02 (B+S)
Se^{+4} 1.0 mM CTRL	75 mg Se/kg SeO_3^{2-}	NO
Se^{+6} 1.0 mM + I	75 mg Se/kg SeO_4^{2-}	soil microbial community (I)
Se^{+6} 1.0 mM + 017	75 mg Se/kg SeO_4^{2-}	SeATE17 (017)
Se^{+6} 1.0 mM CTRL	75 mg Se/kg SeO_4^{2-}	NO
Se^{+4} 2.0 mM + I	150 mg Se/kg SeO_3^{2-}	soil microbial community (I)
Se^{+4} 2.0 mM CTRL	150 mg Se/kg SeO_3^{2-}	NO
Se^{+6} 2.0 mM + I	150 mg Se/kg SeO_4^{2-}	soil microbial community (I)
Se^{+6} 2.0 mM CTRL	150 mg Se/kg SeO_4^{2-}	NO

Table 5 – Test set up in the 'perlite hydroponic system'

3 plants and 1 aliquot of perlite from the bulk were collected per pod at three different times:

T0 = immediately after selenium oxyanion amendment

T3 = after 3 weeks of incubation in the glasshouse

T6 = after 6 weeks of incubation in the glasshouse, i.e. at the end of the experimentation

At the end of the experimentation, i. e. at T=6, all plants from each pod were collected and analysed.

Plant tissues were analysed both for selenium content determination and for biomass production, whereas perlite aliquots from the plant rhizosphere were investigated through microbial total counts and DGGE analysis. Total count were also carried out on bulk perlite samples.

2.5.2 Determination of selenium in plant tissues

Plant collected at different times were separated into shoot and root portions. Fresh (FW) and dry weight (DW) of plant samples were recorded. Total selenium content in 50°C oven dried plant tissues was determined as described below.

Samples digestion and Se content determination were performed following the EPA 3052/1996 and the EPA 200.8/1994 methods, respectively.

A representative sample of up to 0.5 g of plant tissue was digested in 10 ml of concentrated nitric acid for 15 minutes using microwave heating with a suitable laboratory microwave system. The temperature profile is specified to permit specific reactions and incorporates reaching 180 ± 5 ºC in approximately less than 5.5 minutes and remaining at 180 ± 5 ºC for 9.5 minutes for the completion of specific reactions. After cooling, the vessel contents were diluted to a volume of 100 ml, and analyzed by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS).

2.5.3 Microbial counts

Microbial total counts were evaluated both in bulk and rhizosphere perlite samples. A total of 5gr of perlite were incubated in 45 ml of 0.9 % (wt/vol) NaCl solution for two hours at 28°C on an orbital shaker. Serial dilutions were plated on Nutrient plates.

2.5.4 Bacterial inocula

Both the two different types of bacteria inocula (i.e. the soil microbial community and the axenic cultures of strains SEITE01, SeITE02 and SeATE17) were bioaugmented to the rizosphere of *B. juncea* plants grown on perlite beds at an initial concentration of 2 x 10^7 CFU/g of perlite. The inocula of soil microbial community were obtained by resuspended a proper aliquot of soil in 0.9 % (wt/vol) NaCl solution for two hours at 28°C on an orbital shaker. After a filtration with Watman paper, the soil suspension was directly utilized for the inoculum. The inocula of axenic cultures were obtained by growing the three different strains separately in 250-ml Erlenmeyer flasks containing 100 ml Nutrient Broth amended with 0.2 mM Na_2SeO_3 (strains SeITE01 and SeITE02) or 0.2 mM Na_2SeO_4 (strain SeATE17). Flasks were incubated on an orbital shaker at 200 rpm at 28°C for 24 hours. Cell cultures were then centrifuge at 8000 x *g* for 10 min, at 4°C. Subsequently, a proper aliquot of cell was resuspended in 200 mL of 0.9 % (wt/vol) NaCl solution and, as for soil suspension, was distributed uniformly throughout the perlite bed.

2.5.5 Molecular analyses

2.5.5.1 DNA extraction from perlite samples

Total DNA extraction from perlite samples was carried out by following the direct DNA extraction from soil protocol through the cooling-heating method (Chao *et al.*, 1996) with some modifications. 1 g of perlite was resuspended in 5 ml of lyses solution (0.15 M NaCl, 0.1 M Na-EDTA, 1% CTAB). 100 μl of lysozime (15 mg/ml) was added and sample was incubated on an orbital shaker at 37°C for 1h. Successively, 5 ml of SDS solution (0.1 M NaCl, 0.5 M Tris-HCl [pH 8.01], 10% SDS) was added. The sample was then incubated at -80°C for 30 min and immediately after transferred to hot bath at +65°C for 30 min. This latter operation was repeated 2 times. The aqueous phase was then extracted twice with chloroform/isoamyl alcohol (25:24:1) (Sambrook *et al.*, 1987) and DNA was precipitated by adding 1 volume isopropanol. After 2 hrs at – 80°C, DNA was pelleted by centrifugation (12000 x *g* for 30 min, at 4 °C) and resuspended in H_2O (200 μl).

2.5.5.2 PCR-DGGE analysis

In order to evaluate the changes in the adapted microbial community due to the presence of different selenite and selenate concentrations in the rhizosphere of *Brassica jucea* plants as well as to monitoring the bacteria inocula bioaugmented as axenic cultures, a PCR-DGGE approach has been adopted.

The gene encoding for the 16S rRNA (1 to 1512 position, *E. coli* numbering) was initially amplified using the F8 and R11 primers. Total DNA extracted from rhizosphere perlite samples was used as template for PCR reaction (5-20 ng). Subsequently, the universal bacterial primers targeting 16SrDNA V-3 variable region (position 341 to 534, *E. coli* numbering), p3 and p2 were used to amplify fragments sized 233 bp (Muyzer *et al.*, 1993). The reactions were set up using a suitable dilution of the PCR products obtained from the amplification of the entire 16S gene.

The two different PCR protocols were performed as follows:

50 µl PCR mix	**F8/R11 reaction conditions**
1X reaction buffer Dynazyme, including 1.5mM $MgCl_2$	4 min at 95°C
20 pmol of each primer	30 cycles of:
0.4 mM of dNTPs mix	45 s at 95°C
1.5 U of DyNAzyme™ II DNA polymerase (Finzymes)	45 s at 42°C
	2 min at 72°C
	and finally 5 min at 72°C

50 µl PCR mix	**p2/p3 reaction conditions**
1X reaction buffer Dynazyme, including 1.5mM $MgCl_2$	4 min at 95°C
20 pmol of each primer	30 cycles of:
0.4 mM of dNTPs mix	45 s at 95°C
1.5 U of DyNAzyme™ II DNA polymerase (Finzymes)	30 s at 42°C
	30 s at 72°C
	and finally 5 min at 72°C

The PCR products were separated on polyacrylamide gels (8% [wt vol^{-1}], 37.5:1 acrylamide-*bis*-acrylamide) with a 30% to 60% linear gradient of denaturant (100% denaturant: 40% [vol vol^{-1}] formamide plus 42% [wt vol^{-1}] urea). Gels were run for 16 hrs at 35 V in 1 X Tris-Acetate EDTA buffer at 60°C. Denaturing gels were run using the Dcode Universal Mutation Detection System (Bio-Rad, USA). Gels were stained for 30 min in ethidium bromide (1 mg l^{-1}) in 1 X Tris-Acetate EDTA buffer and visualised by UV illumination. The gels images were acquired using the ChemDoc (Bio-Rad) gel documentation system.

Some selected DGGE bands were scratched from the gel and successively reamplified using the same set of primers (p2/p3). The PCR products obtained were purified, cloned in pBluescript® SK (+/-) phagemid vector, single strand sequenced and aligned to the sequence databases using BLASTN (Altshuld *et al.*, 1997).

3. RESULTS AND DISCUSSION

3.1 Isolation of bacterial strains from a selenium polluted soil

Ten different bacterial strains were isolated as axenic cultures from the rhizosphere of the selenium hyperaccumulator legume *Astragalus bisulcatus* grown on a selenium polluted soil collected in Sardinia (Italy).

Of these ten strains, three were isolated from microbial enrichment cultures carried out in presence of 0.2 mM Na_2SeO_3 as selective agent, whereas seven were obtained from cultures set up with 0.2 mM Na_2SeO_4. Nevertheless, these isolates are all capable of growing in presence of both SeO_3^{2-} and SeO_4^{2-} oxyanions when they were inoculated in liquid growth medium and incubated at 28°C in aerobic growth conditions. However, they showed different MIC values (Table 6) towards SeO_3^{2-} or SeO_4^{2-} and different capabilities to reduce selenium oxyanions to Se^0 (Table 6), as it was marked from the appearance of red coloured colonies on agarized plates amended with either Na_2SeO_3 or Na_2SeO_4.

strain	SeO_3^{2-}		SeO_4^{2-}	
	Reduction to Se^0	MIC value (mM)	Reduction to Se^0	MIC value (mM)
SeITE01	+	25	-	>200
SeITE02	+	50	-	>200
SeITE03	-	10	-	>200
SeATE02	+	5	-	50
SeATE04	+	35	-	>200
SeATE05	+	35	-	>200
SeATE11	-	15	-	>200
SeATE17	+	10	+	>200
SeATE18	+	5	-	>200
SeATE21	+	20	-	>200

Table 6 - MIC values and reduction capability to Se^0 for Na_2SeO_3 and Na_2SeO_4 referring to the bacterial isolates from SeO_3^{2-} and SeO_4^{2-} enrichment cultures.

It's worth noting that all but two isolates produced red colonies in the presence of Na_2SeO_3, whereas only one strain produced red colonies when exposed to Na_2SeO_4.

This evidence seems to be in agreement with other reports regarding previous studies on soil bacteria (Doran, 1982). These studies have demonstrated that only a small

percentage of isolates reduce selenate to metallic selenium; however the identities of these bacteria have not been reported. Moreover this evidence is consistent with the idea that selenium in the +6 oxidation state (SeO_4^{2-}) is relatively non toxic to aerobic facultative bacteria (Doran, 1982; La Page and Bascomb, 1968) and MIC values obtained for Na_2SeO_4 also supported this evidence.

Actually, the MIC values of Na_2SeO_3 ranged from 5 to 50 mM, whereas MICs of Na_2SeO_4 ranged from 50 to >200 mM. In particular, strain SeATE02 exhibited the lowest MIC values towards both selenite and selenate, whereas strain SeATE18, which also exhibited the lowest MIC value for selenite, showed a high MIC value for selenate. Thus, the data obtained indicated that the relative toxicity of SeO_3^{2-} versus SeO_4^{2-} varied from strain to strain and that SeO_3^{2-} and SeO_4^{2-} resistances seem to be separate phenotypes.

Three strains resulted to be of particular interest both for their SeO_3^{2-} and SeO_4^{2-} reduction capacities and MIC values: SeITE01, SeITE02 and SeATE17.

Strain SeITE01 and SeITE02, obtained from SeO_3^{2-} enrichment cultures, were both capable of reducing SeO_3^{2-} but not SeO_4^{2-} to Se^0. Moreover strain SeITE02 showed the highest tolerance to selenite (up to 50 mM). Strain SeATE17, isolated from SeO_4^{2-} enrichment cultures, was the one, from all the isolates, able to transform both Se oxyanions to Se^0.

Strains SeITE01 and SeITE02 were further characterised, and are the subject of this section, whereas microbial characterization of strain SeATE17 is now in progress. However, all these three strains were used as inoculums in the phytoremediation assay (section 3.6).

3.2 Characterization of strain SeITE01

3.2.1 Taxonomic and biochemical characterization

Molecular taxonomy results obtained from sequencing of 16S rRNA gene, evidenced that strain SeITE01, which showed a 99% identity with *Bacillus cereus* G9667 strain, resulted to be very close to the *Bacillus cereus* group. The *Bacillus cereus* group includes Gram-positive bacteria belonging to four species: *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides*. Actually, phylogenetic analysis, based on 16S ribosomal DNA (rDNA), has shown that these four *Bacillus* species are very close to each other, due to the similarities in 16S rDNA sequences, that differ by only zero to nine nucleotides (Ash *et al.*, 1991; Mantynen and Lindstrom, 1998).

Nevertheless, further biochemical characterization suggested the possible attribution of strain SeITE01 to the species *B. mycoides* (Table 7). In fact, strain SeITE01, when grown on Nutrient agarized pates, showed the typical rhizoid growth which is characteristic of *B. mycoides* species. The elaborated chiral colony pattern belongs to *Bacillus mycoides*, was first described by Flügge in 1886. The author called the species "mycoides" just due to fungal-like growth on agar plates of these rod bacilli as chains of cells forming filaments projecting radially and curving to the left or to the right (Di Franco *et al.*, 2002).

On the other hand, *B. mycoides* species has recently been recognized as a plant growth-promoting bacterium associated to the roots of different conifer species (Petersen *et al.*, 1995). Moreover, the genus *Bacillus* is presently studied for a possible exploitation in bioremediation processes of industrial waste waters polluted with heavy metals, such as nickel, zinc and cadmium (Zaidi and Musarrat, 2004; Yilmaz, 2003; Jezequel *et al.* 2004) and metalloids, such as arsenic and selenium (Fujita *et al.*, 2002; Yamamura *et al.*, 2005; Soda *et al.*, 2006).

As reported in chapter 3.1, this isolate was demonstrated to reduce selenite to Se^0 and to tolerate up to 25 mM SeO_3^{2-} in both rich and mineral agarised medium. This MIC value for SeO_3^{2-} resulted to be comparable to that accounted for other naturally occurring, selenite resistant bacteria (Roux *et al.*, 2001; Van Fleet-Stalder *et al.*, 2000; Kessi *et al.*, 1999; Garbisu *et al.*, 1999) and much higher respect to some selenite reducing bacteria of biotechnological interest (Rathgeber *et al.*, 2002).

CHARACTERISTIC	SeITE01
Cell morphology	Short, ovoid rods
(size [μm])	(0.7 × 2.0)
Colour and aspect of colonies:	
without SeO_3^{2-} addition	Opaque, creamy, rhizoid growth
with SeO_3^{2-} addition	Bright red, rhizoid growth
Fluorescent pigment	-
Growth at 4 °C	-
Growth at 18 °C	-
Growth at 28 °C	+
Growth at 37 °C	+
Growth at 42 °C	-
Growth at pH 4.5	-
Growth at pH 7.2	+
Growth at pH 9	-
Oxidase activity	-
Catalase activity	+
Lipase activity	+
Urease activity	-
Metil red test	-
V/P test	+
Nitrate reduction	+
Nitrite reduction	-
Utilisation of:	
Acetate	-
Caprilate	-
Citrate	-
Gluconate	+
Malate	+
Ossalate	-
Succinate	+
Arabinose	-
Fructose	+
Galactose	+
Glucose	+
Lactose	+
Maltose	+
Mannitole	+
Mannose	+
N-acetyl-glucosamine	-
Raffinose	+
Rhamnose	+
Saccarose	+
D-xylose	+
Hydrolysis of:	
Starch	+
Gelatin	+
Esculine	+

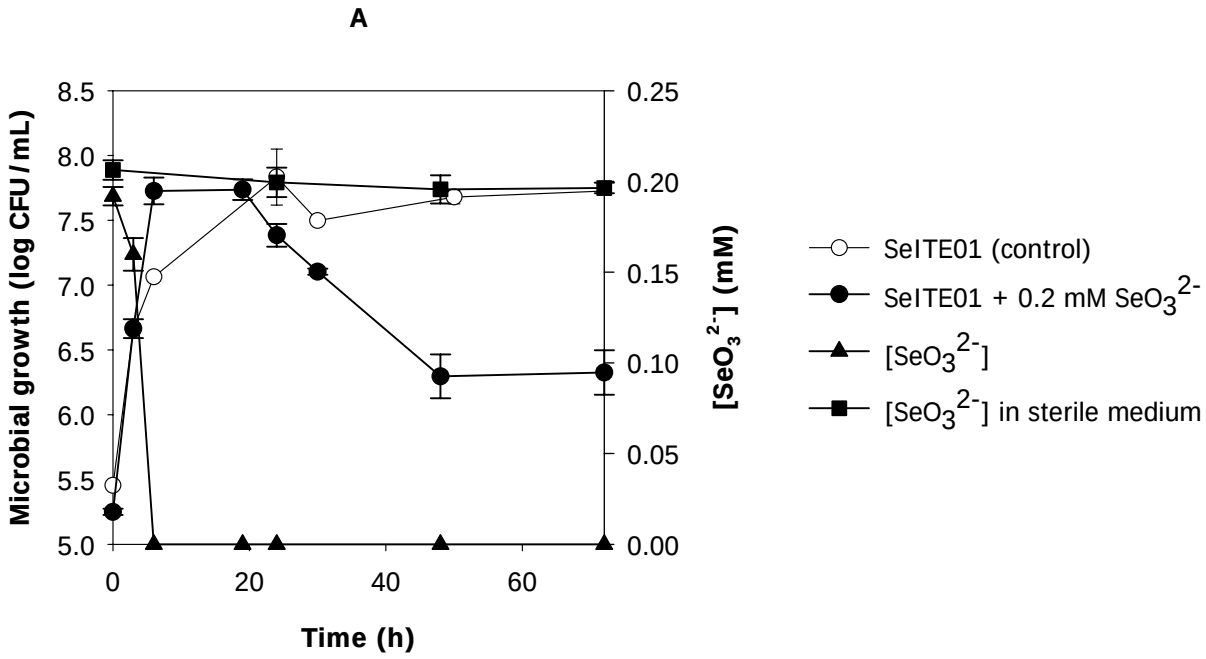
Table 7 - Morphological, biochemical, and growth characteristics of *Bacillus* sp. strain SeITE01 (modified from [**II**])

3.2.2 Selenite reduction tests

3.2.2.1 Evaluation of selenite reduction efficiency and microbial growth at increasing SeO_3^{2-} concentrations

SeO_3^{2-} reduction efficiency of *Bacillus* strain SeITE01 was evaluated in a liquid rich medium (Nutrient Broth) at increasing Na_2SeO_3 concentrations: 0.2 mM, 0.5 mM and 2.0 mM (Fig. 9). Both selenite content and microbial growth were measured.

Because of selenite reduction to the red allotropic form Se^0 (Moore and Kaplan, 1992; Sabaty *et al.*, 2001), strain SeITE01 induced a progressive decrease of SeO_3^{2-} concentrations initially added to the culture medium (Fig. 9). Meanwhile, SeO_3^{2-} was totally recovered in sterile control cultures suggesting for a biologically mediated selenite reduction. Thus, it's possible to evaluate the selenite reduction efficiency of this strain by measuring the depletion of SeO_3^{2-} content in the growth medium.



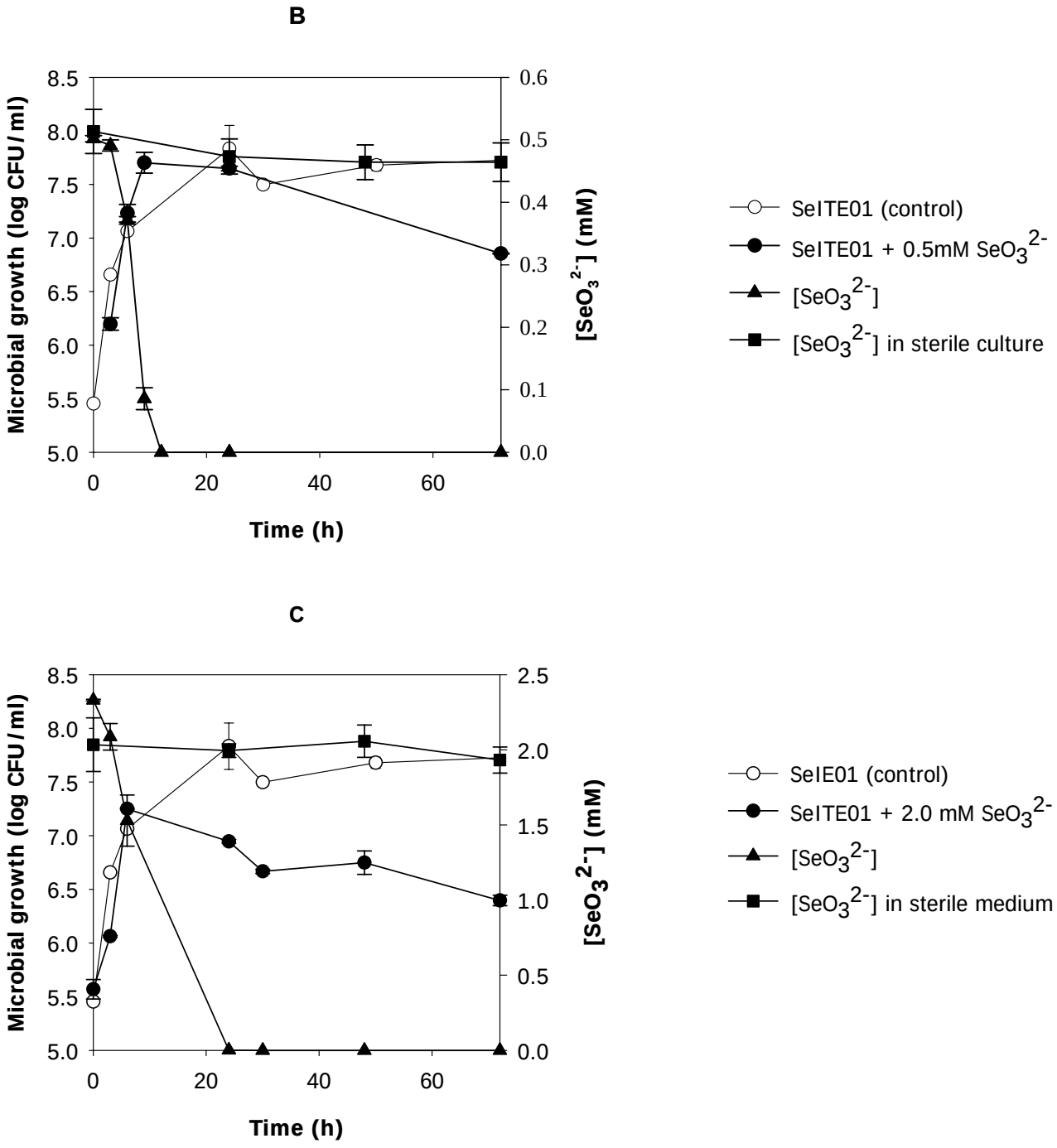


Figure 9 - Time courses of microbial growth and SeO_3^{2-} reduction by SeITE01, in presence of **(A)** 0.2 mM SeO_3^{2-}, **(B)** 0.5 mM SeO_3^{2-}, **(C)** 2.0 mM SeO_3^{2-}. Each curve shows means based on the results of three experiments.

Strain SeITE01 completely reduced selenite within 6, 12 and 24 hours, when SeO_3^{2-} concentration in the growth medium was respectively 0.2, 0.5 or 2.0 mM SeO_3^{2-}. Thus, the selenite reduction efficiency resulted to be higher for lower SeO_3^{2-} concentrations.

At all selenite concentrations tested, reduction process began concomitantly with the

starting of microbial growth. However, in the presence of 0.2 and 0.5 mM SeO_3^{2-}, the whole amount of selenite initially present in the medium was completely depleted during the exponential growth phase, whereas, in the presence of 2.0 mM SeO_3^{2-}, only the 25% of the initial selenite content was reduced during the exponential phase, the remaining being depleted in the stationary phase, between the 6th and 24th hour.

It's worth noting that all SeO_3^{2-} concentrations tested negatively affected both the SeITE01 growth rate and the final cell yield (Fig. 9). As a general rule, at all SeO_3^{2-} concentrations tested we observed that strain SeITE01 reached the stationary phase earlier than in the control tests. In fact, while in cultures set up in absence of selenite the stationary phase was reached after about 24 hours of cell growth, in cultures amended with SeO_3^{2-} the stationary phase was reached after only 6-10 hours of growth. Moreover, it's important to note that when selenium was added at 0.2 and 0.5 mM SeO_3^{2-}, strain SeITE01 resulted to be capable to maintain the stationary phase within the 24 hours of growth, reaching values comparable with those registered for control cultures. Nevertheless after the 24th hour, we observed a decrease in cell growth that caused a reduction in the final cell yield comparing to the control test (-20% for 0.2 mM and -10% for 0.5 mM SeO_3^{2-} tests). On the other hand, for cultures set up in presence of 2.0 mM SeO_3^{2-} , we observed that strain SeITE01 resulted to be unable to maintain the stationary phase after the first 6 hours of incubation. In fact we registered a slight decrease in cell growth just after the 6th hour and, also in this case, the strain reached a lower final cell yield (- 15%) than that obtained for control test.

Concomitantly with the reduction and consequent depletion of SeO_3^{2-} , we observed the appearance, in the growth medium, of a bright red color. Despite the reduction process took place in parallel with microbial growth, the appearance of the red color in cell suspensions occurred later. In particular the cultures turned red after 3, 6 and 9 hours from the beginning of the experimentation when they were amended with 0.2, 0.5, and 2.0 mM SeO_3^{2-} respectively. Thus, it may be suggested that selenite, before being reduced to Se^0, was transformed to another reduced selenium form.

3.2.2.2 Effect of SeO_3^{2-} induction on microbial growth and SeO_3^{2-} reduction efficiency

In order to evaluate the effect of selenite induction on the growth and SeO_3^{2-} reduction efficiency, cultures of strain SeITE01 were set up in presence of 0.5 and 2.0 mM Na_2SeO_3 after a pre-treatment with 0.2 mM SeO_3^{2-} (Fig. 10).

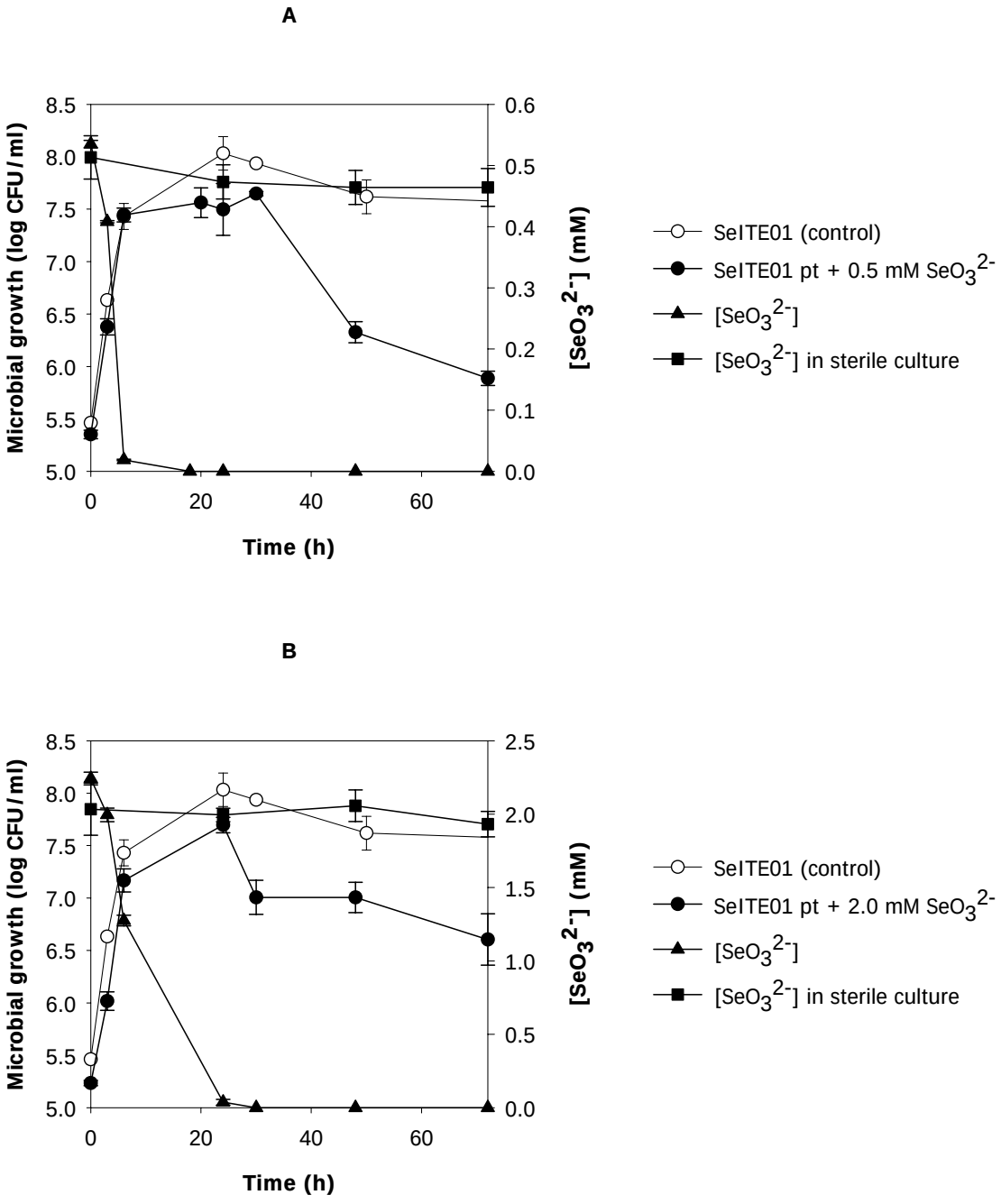


Figure 10 - Time courses of microbial growth and SeO_3^{2-} reduction by SeITE01 pre-tretated with 0.2 mM Na_2SeO_3 (pt, pre-treated), in presence of **(A)** 0.5 mM, **(B)** 2 mM SeO_3^{2-}. Each curve shows means based on the results of three experiments.

Results obtained indicated that the selenite reduction efficiency of strain SeITE01 seems to be not greatly affected by the pre-treatment with 0.2 mM Na_2SeO_3 (Fig. 10). Actually, the time courses of SeO_3^{2-} reduction registered after pre-induction resulted to be very similar to that obtained without pre-induction (Fig. 9). However in presence of 0.5 mM SeO_3^{2-} , pre-treated SeITE01 cultures reduced selenite in the medium within 6th hour

instead of 12th hour of growth. Concerning the microbial growth, in presence of 2.0 mM selenite, pre-treated cultures reached higher values than those registered for the not pre-treated test. Moreover, at this selenite concentration, strain SeITE01 reached the stationary phase after 24 hours of growth, i.e. later than when it was tested without pre-induction.

3.2.2.3 Evaluation of SeITE01 capacity of reducing SeO_3^{2-} added at different time of culture growth

In order to investigate more in detail the selenite reduction process, 0.5 mM Na_2SeO_3 was added at different time of culture growth. Thus, selenite was amended (a) at the beginning (t=0); (b) after 2 hours, during the lag growth phase; (c) after 4 hours, at the early exponential growth phase; (d) after 16 hours, at the late exponential growth phase; (e) after 24 h, during the stationary growth phase (Fig. 11).
Once again, both SeO_3^{2-} content and microbial cell growth were determined (Fig. 12).

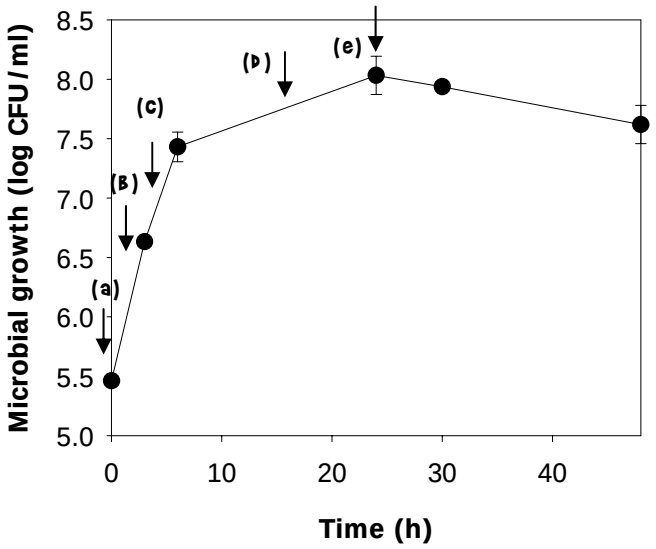


Figure 11 – Representation of Na_2SeO_3 amendments at different time of SeITE01 growth

The addition of SeO_3^{2-} at different time during SeITE01 growth seems not to affect microbial growth rate, except when Se oxyanion was added after 16 hours of microbial growth (during the late exponential growth phase) (Fig. 12A)

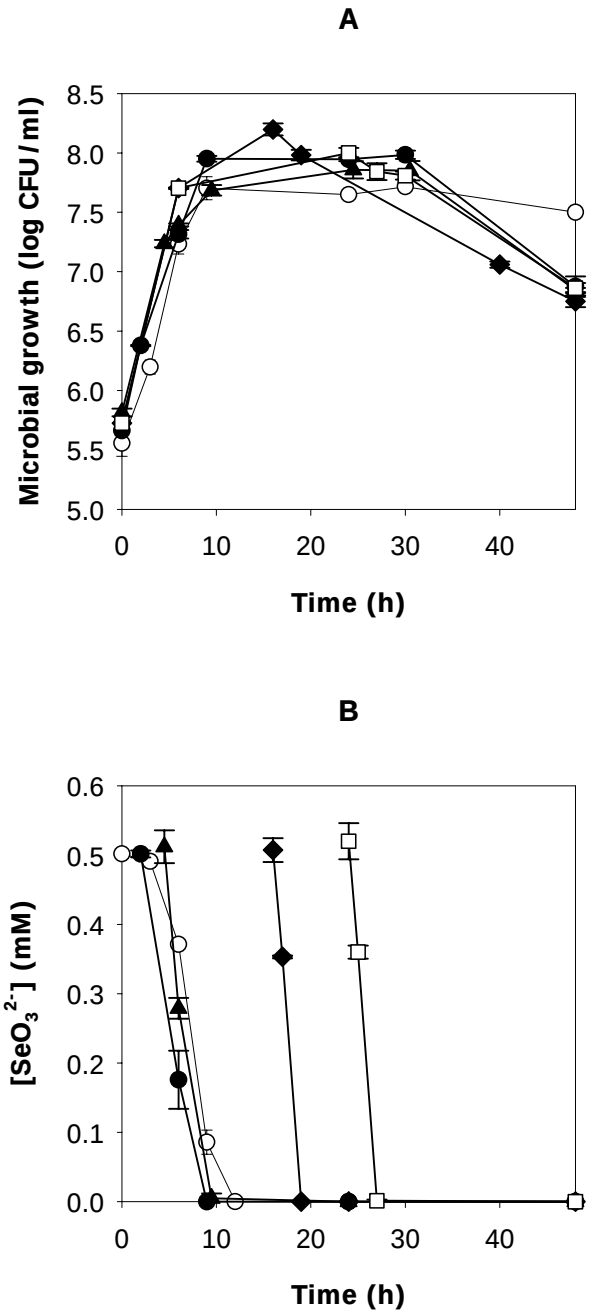


Figure 12 - Time courses of microbial growth **(A)** and SeO_3^{2-} reduction **(B)** by SeITE01 when 0.5 mM Na_2SeO_3 was added at different time of cell growth. Each curve shows means based on the results of three experiments.

In fact, in this case we observed a rapid decrease in cell growth immediately after selenite addition to the medium. However in all tests set up we observed a reduction in the final cell yield, which attested to – 15% comparing to that registered for control cultures. Nevertheless when selenite was added after the beginning of cell growth, strain SeITE01 seemed to be more efficient in selenite reduction (Fig. 12B). In fact, in comparison with a

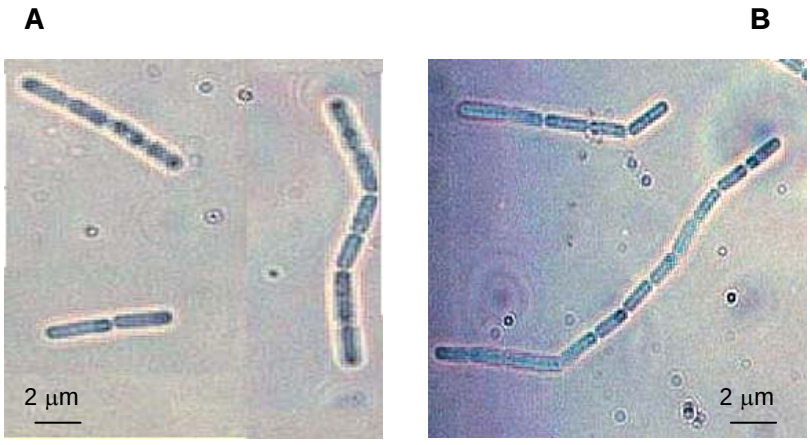
12 hours-time for a complete selenite depletion from the medium registered when SeO_3^{2-} was added at the beginning of microbial growth, we observed that a faster reduction process took place when SeO_3^{2-} was amended after 2 hours [lag phase (b)] and after 4 hours [early exponential phase (c)] of cell growth.

Furthermore when selenite was added in the late exponential growth phase or during the stationary phase we had a complete SeO_3^{2-} reduction in 3 hours. Thus strain SeITE01 seems to be much more efficient in selenite reduction when selenite was added after the beginning of the growth

3.2.3 Phase-contrast microscopy analyses.

Effects of SeO_3^{2-} added to the growth medium on cell morphology of strain SeITE01 was evidenced by phase-contrast microscopy analyses (Figure 13).

Samples collected from microbial cultures grown in absence of selenite evidenced that this isolate normally replicates as oblong rods jointed in short chains (Fig. 13A). On the other hand, when selenite was present in growth medium, SeITE01 cells generated chains much longer than that observed in the control cultures (Fig. 13C, 13D). Moreover these chains of cells evolved progressively in a sort of cell aggregates, entrapping light-refractive selenium granules. These clumps of bacterial cells and selenium granules never occurred in control cultures. At all SeO_3^{2-} concentration tested, clumps were recorded starting from early steps of growth and increased in size along with the progressive reduction of SeO_3^{2-}, as it was shown in micrographs registered at different time of cell growth.



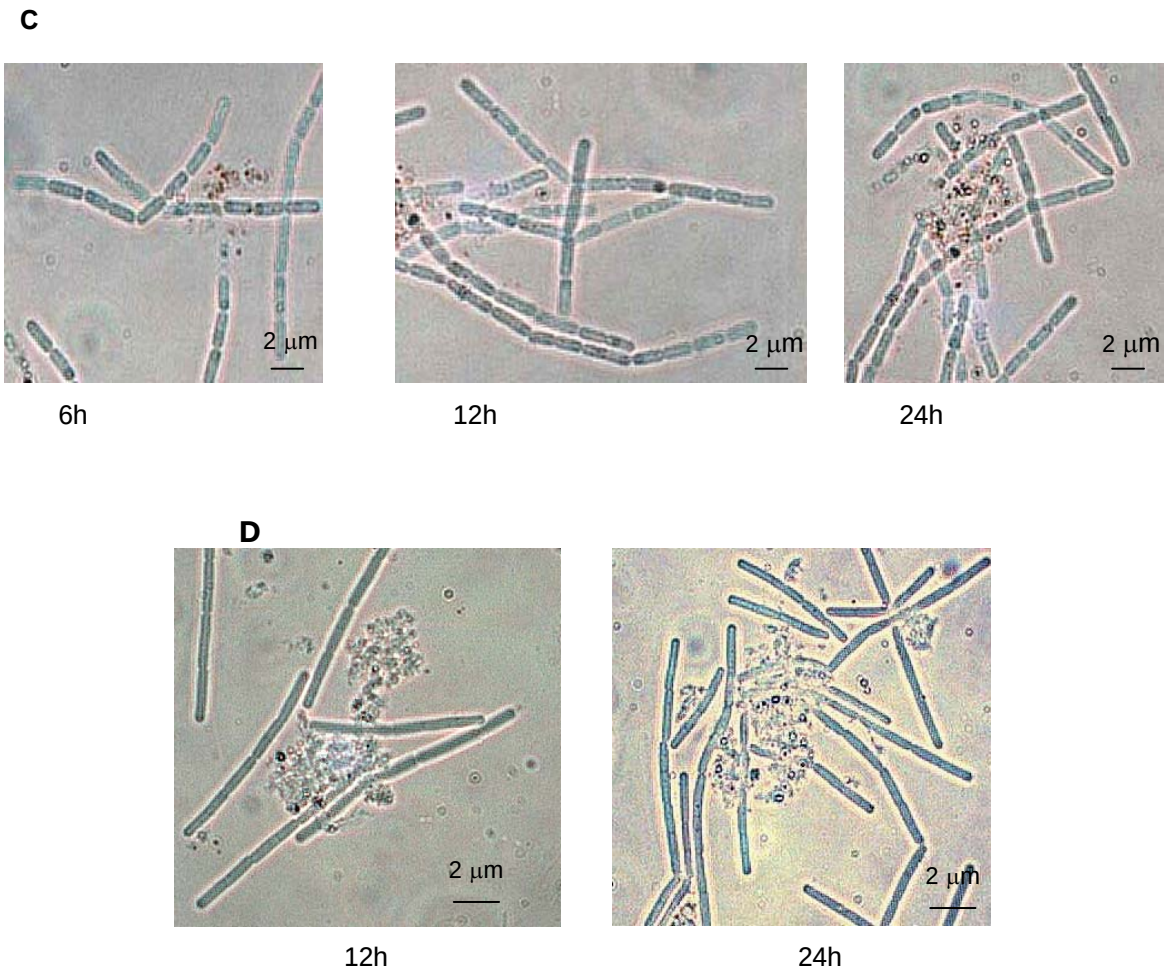


Figure 13 - Phase contrast micrographs of SeITE01 suspension culture grown in absence of Se oxyanions (A), in presence of 2.0 mM SeO_4^{2-} (B),and in presence of 0.5 mM (C) and 2.0 mM (D) of SeO_3^{2-}.

Furthermore, it's important to note that both the size of cell aggregates and the number of light refractive Se granules seem to increase with the increasing of SeO_3^{2-} concentrations tested.

3.2.4 Electronic microscopy analyses

Electron microscopy analyses were carried out on SeITE01 bacterial cultures grown either in absence of selenium or in presence of 0.5 mM SeO_3^{2-}.

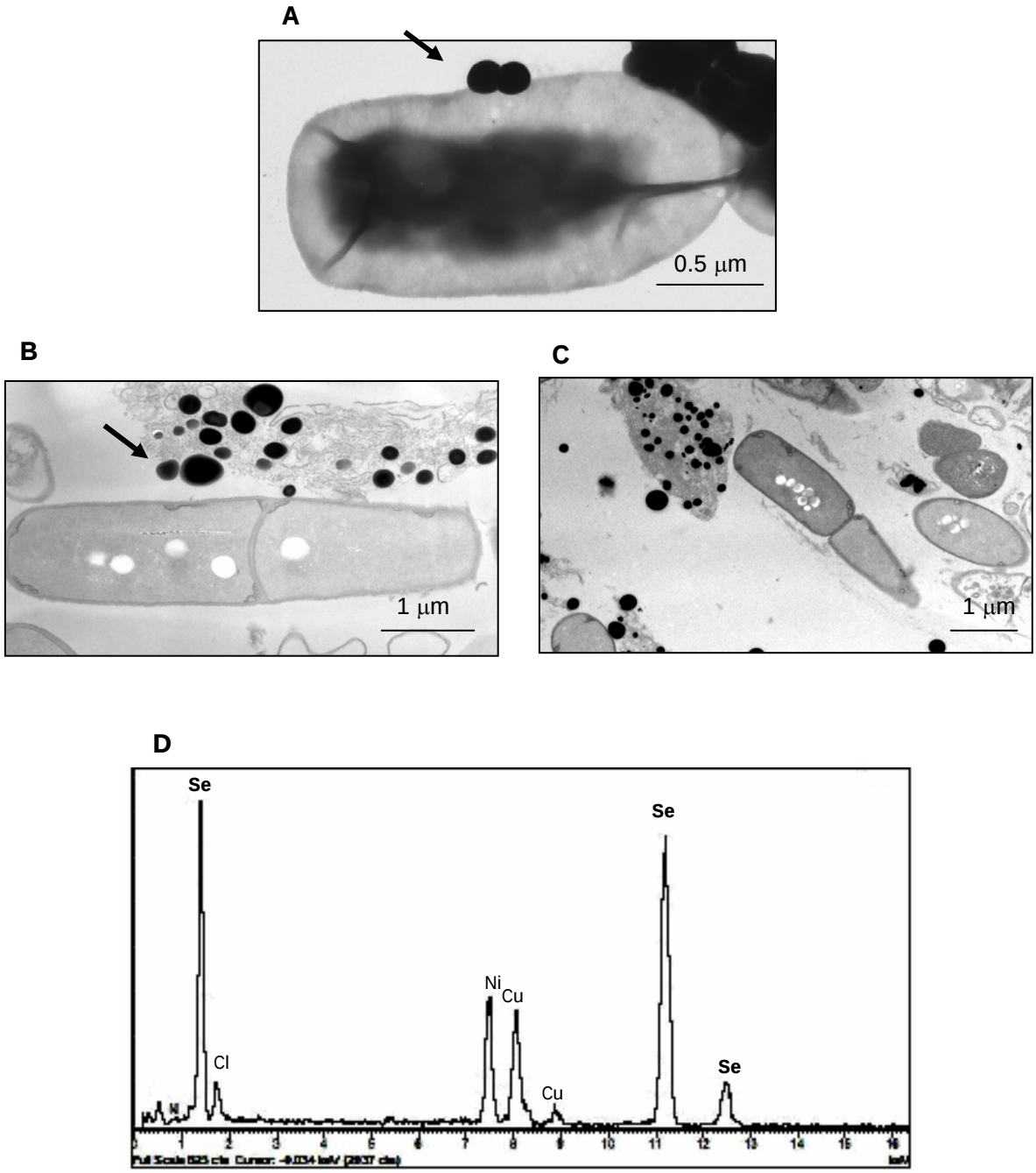


Figure 14 – Electron micrographs of SeITE01 suspension culture grown in presence of 0.5 mM SeO_3^{2-}, obtained through negative staining analysis (**A**) and TEM analysis (**B**, **C**). In Panel **D** was reported the EDX spectrum of Se granules indicated by the arrows.

Results obtained both from Negative Staining (Fig. 14A) and TEM analysis (Fig. 14B, 14C) revealed the presence of electron-dense particles in the extra-cellular space both after 12 (Fig. 14A, 14B) and 24 (Fig. 14C) hours of cell growth. On the other hand, the same electron-dense granules were never detected in cell cultures which had received no SeO_3^{2-} addition in the growth medium (data not shown).

EDX spectra derived from these nanospheres clearly indicated that they were composed entirely of selenium (Fig. 14D)

The Cu and Ni peaks were associated with the TEM grid, whereas the O and C peaks most likely were associated with cellular exudates. The lack of any other metal peaks in the spectrum indicated that the selenium occurred in the elemental state, Se^0, rather than as a metal selenide.

3.3 Characterization of strain SeITE02

3.3.1 Taxonomic and biochemical characterization

After sequencing of 16S rRNA gene, molecular taxonomy results obtained suggested for the attribution of strain SeITE02 to the genus *Stenotrophomonas*. Actually, close clustering with the species *maltophilia* (99% identity with *Stenotrophomonas maltophilia* VUN10-075, AF100734) was recognised. A list of biochemical and physiological features of strain SeITE02 are summarised in Table 8.

Stenotrophomonas maltophilia, previously designed as *Pseudomonas maltophilia* or *Xanthomonas maltophilia* (Palleroni and Bradbury, 1993), is a Gram negative, aerobic, non-fermentative, ubiquitous bacterium widely diffused within different environmental niches. It can be found in aquatic environments, in soils, and on vegetation and even on some animals (Berg *et al.*, 1999). *S. maltophilia* is often associated with the rhizosphere of a variety of plants such as wheat, oat, cucumber, maize, oilseed rape and potato (Berg *et al.*, 1996; Debette and Blondeau, 1980; Heuer and Smalla, 1999; Lambert and Joos, 1989).

Moreover, the genus *Stenotrophomonas* is currently studied for its potential application in bioremediation (Dungan *et al.*, 2003; Juhasz *et al.*, 2000; Rousseaux *et al.*, 2001; Song *et al.*, 2002) as well as in biological control of plant pathogens (Yuen *et al.*, 2001; Zhang and Yuen, 2000).

As reported in the previous section, this isolate was demonstrated to reduce selenite to Se^0 and to tolerate up to 50 mM SeO_3^{2-} in both rich and mineral agarised medium. This MIC value for SeO_3^{2-} is much higher than those reported for other naturally occurring, selenite resistant bacteria such as *Ralstonia metallidurans* (Roux *et al.*, 2001), *Rhodobacter sphaeroides* (Van Fleet-Stalder *et al.*, 2000), *Rhodospirillum rubrum* (Kessi *et al.*, 1999), and *Bacillus subtilis* (Garbisu *et al.*, 1999). For these species, MIC values ranging from about 2.0 mM to 6.0 mM SeO_3^{2-} have been determined in similar growth conditions. Nevertheless, other authors reported MIC values for SeO_3^{2-} up to 40 mM, in aerated cultures (Rathgeber *et al.*, 2002). Unfortunately, at the best of our knowledge, those microorganisms were not further identified.

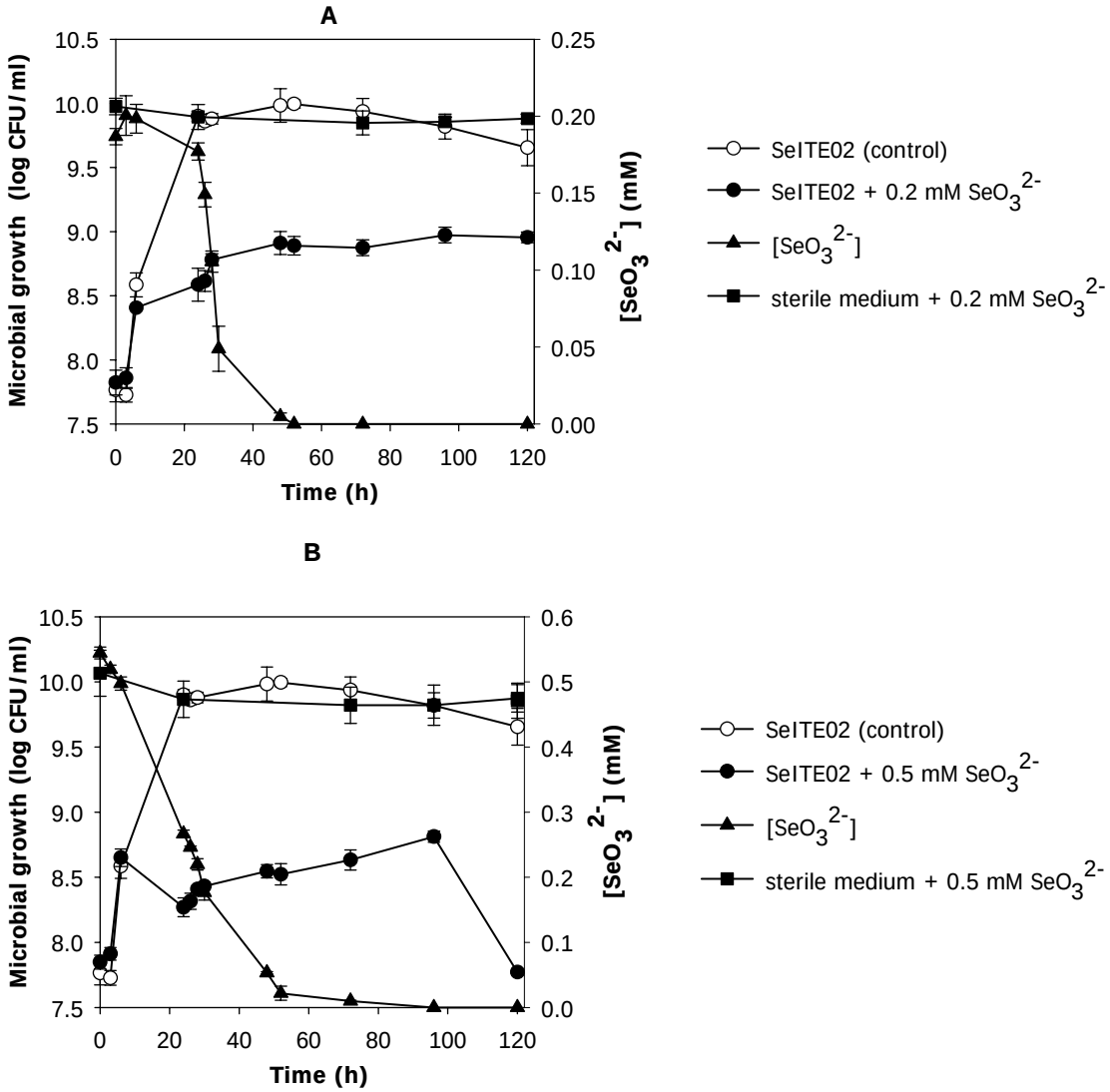
CHARACTERISTIC	SeITE02
Cell morfology (size [μm])	Short ovoid rod, (0.5 × 1.0)
Color and type of colonies:	
without metal addition	Opaque, creamy
with SeO_3^{2-} addition	Bright red
Fluorescent pigment	-
Growth at 4°C	-
Growth at 18°C	+
Growth at 28°C	+
Growth at 37°C	+
Growth at 42°C	-
Growth at pH 4.5	-
Growth at pH 7.2	+
Growth at pH 9	-
Oxidase activity	-
Catalase activity	+
Lipase activity	+
Urease activity	+
Methyl red test	+
V/P test	+
Nitrate reduction	+
Nitrite reduction	-
Utilization of:	
Acetate	-
Caprilate	-
Citrate	-
Gluconate	+
Malate	+
Oxalate	-
Succinate	+
Arabinose	-
Fructose	+
Galactose	+
Glucose	+
Lactose	+
Maltose	+
Mannitole	+
Mannose	+
N-acetyl-glucosammine	-
Raffinose	+
Rhamnose	+
Saccharose	-
D-xylose	+
Hydrolysis of:	
Starch	+
Gelatin	+
Esculine	-

Table 8 - Morphological, biochemical, and growth characteristics of *Stenotrophoonas maltophilia* strain SeITE02 (modified from [**I**])

3.3.2 Selenite reduction tests

3.3.2.1 Evaluation of selenite reduction efficiency and microbial growth at increasing SeO_3^{2-} concentrations

SeO_3^{2-} reduction efficiency of *Stenotrophomonas* strain was evaluated in a liquid rich medium (Nutrient Broth) at increasing Na_2SeO_3 concentrations: 0.2 mM, 0.5 mM and 2.0 mM (Fig. 15). Both selenite content and microbial growth were measured.



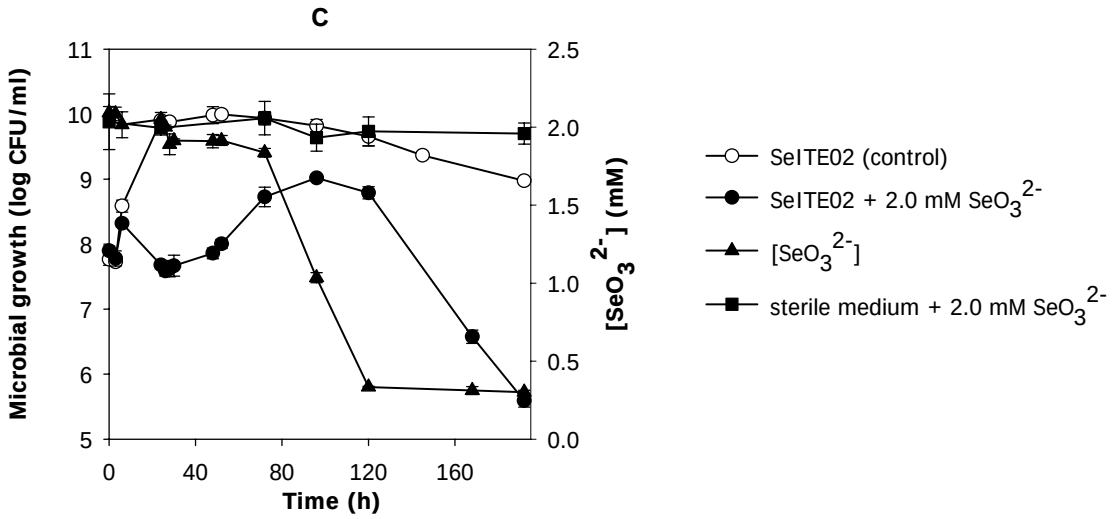


Figure 15 - Time courses of microbial growth and SeO_3^{2-} reduction by SeITE02, in presence of **(A)** 0.2 mM SeO_3^{2-}, **(B)** 0.5 mM SeO_3^{2-}, **(C)** 2.0 mM SeO_3^{2-}. Each curve shows means based on the results of three experiments (modified from [**I**]).

Strain SeITE02 completely reduced selenite within 52 hours, when SeO_3^{2-} concentration in the growth medium was 0.2 or 0.5 mM. In presence of 0.2 mM SeO_3^{2-}, reduction started at the early exponential phase, lasting until the initial stationary phase was reached. With 0.5 mM SeO_3^{2-} in the culture medium, reduction occurred during the exponential growth phase, throughout the interval of depressed cell growth observed between h 6 and h 24. Within this span, 50% of the initial SeO_3^{2-} was reduced, the remaining being reduced during the recovery of cell active growth from h 24 to h 48. At 2.0 mM Na_2SeO_3, reduction of the oxyanion was slower than what verified at 0.2 and 0.5 mM. In this case, selenite concentration drastically dropped between h 72 and h 120. Reduction proceeded slowly during the early exponential phase (first 6 hours), although this metabolic activity was not significantly delayed by low rate growth of the strain SeITE02 between h 6 and h 24. During this interval, 10% of initial SeO_3^{2-} was reduced while a further 77% was transformed throughout the stationary phase. Any reductive activity on selenite stopped after 120 hours of cultivation. At this time, 87% of the initial SeO_3^{2-} added had been reduced. However, the bacterial culture was no longer able to grow and to sustain the reduction of the oxyanion.

Regarding microbial growth, it resulted to be negatively affected at all SeO_3^{2-} concentrations tested (Fig. 15). As a general rule, SeITE02 cell replication decreased with the increase of SeO_3^{2-} concentration in the culture medium. Cultures added with 0.2 mM Na_2SeO_3, evidenced a dynamics of cell growth similar to that of SeITE02 cultured in absence of the oxyanion; however, in stationary phase, lower values were reached. With

0.5 and 2.0 mM Na_2SeO_3 in the culture medium, a marked falling of bacterial cell replication was registered between the 6th and the 24th hour. Afterwards, a partial recovery took place, with microbial growth reaching values comparable to those observed in presence of 0.2 mM SeO_3^{2-}. Nevertheless, a recovery of cell replication was observed with 0.5 and 2.0 mM SeO_3^{2-} in the culture medium, after 96 and 120 hours of incubation, respectively.

Strain SeITE02 formed reddish cell suspensions revealing the capacity to reduce selenite to Se^0, as a consequence of the growth under aerated conditions in liquid medium added with selenite (Moore and Kaplan, 1992; Sabaty *et al.*, 2001). It is worth noting that this color was maintained throughout a long span. Actually, cell suspensions, after three or four weeks of incubation, still manifested the bright red aspect as that already observed after the first 120 hours of cultivation.

3.3.2.2 Evaluation of SeITE02 capacity of reducing SeO_3^{2-} added at different time of culture growth

In order to investigate more in detail the selenite reduction process, 0.5 mM Na_2SeO_3 was added at different time of culture growth. Thus, selenite was amended (a) at the beginning; (b) after 3 hours, during the lag growth phase; (c) after 5 hours, at the early exponential growth phase; (d) after 16 hours, at the late exponential growth phase; (e) after 24 hours, during the stationary growth phase (Fig. 16).

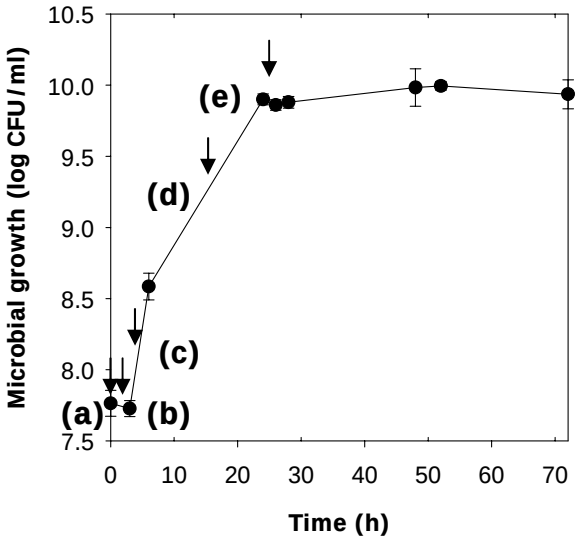


Figure 16 – Representation of Na_2SeO_3 amendments at different time of SeITE01 growth

Once again, both selenite content and microbial cell growth were determined (Fig. 17).

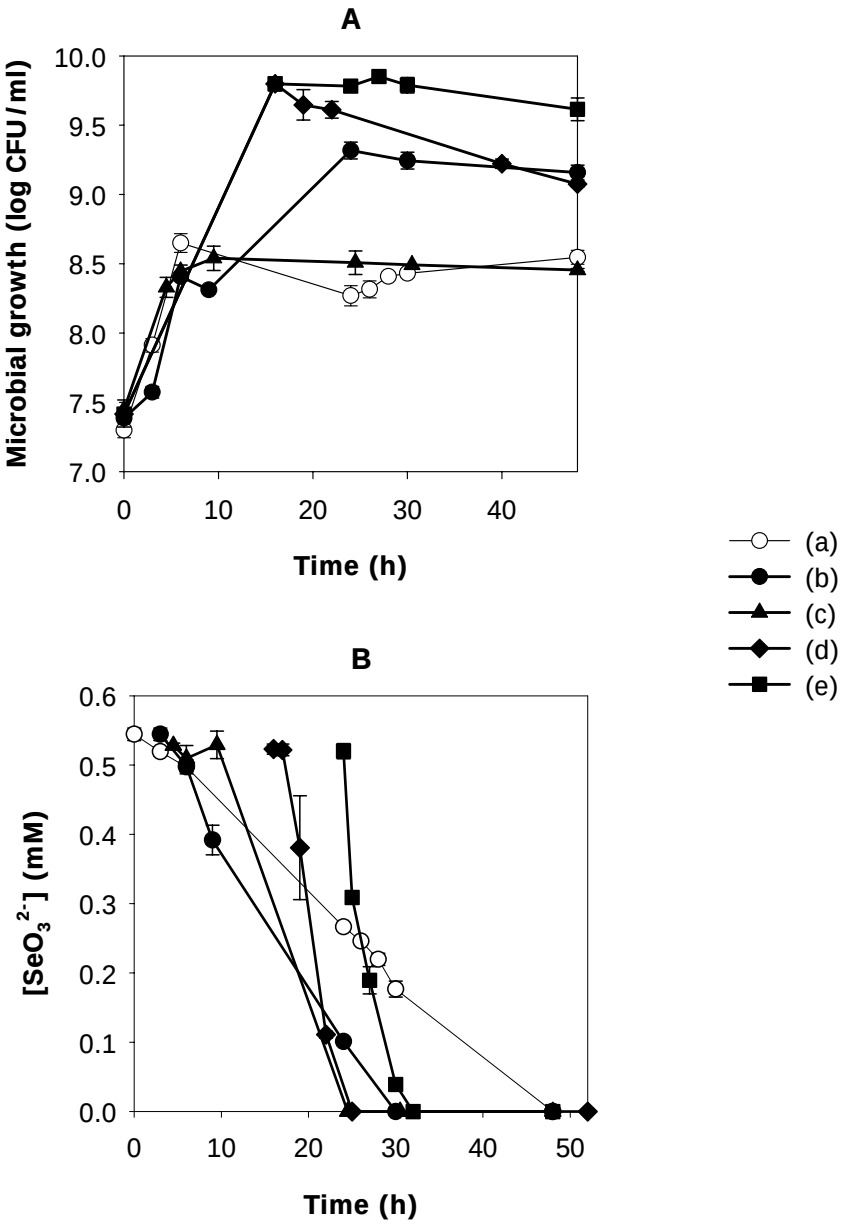


Figure 17 - Time courses of microbial growth **(A)** and SeO_3^{2-} reduction **(B)** by SeITE02 when 0.5 mM Na_2SeO_3 was added at different time of cell growth. Each curve shows means based on the results of three experiments.

Regarding selenite reduction process, as a general rule, we observed an increase in the selenite reduction efficiency when SeO_3^{2-} was added after the beginning of the microbial growth cycle (Fig. 17). More in detail, when selenite was added after 3h from the beginning of culture growth (during the lag phase, (b)), we had a complete selenite depletion in the medium within 30 hours of cells growth. On the other hand, *S. maltophilia* engaged 52 h for completely remove selenite from microbial growth medium when it was added at the beginning of microbial growth (a). We also observed that in this

case, selenite reduction process took place immediately after the oxyanion addition to the medium. Comparing to the results obtained for SeO_3^{2-} addition during lag phase, the selenite reduction process resulted to be faster when SeO_3^{2-} was added at the early exponential growth phase (t=4.5). In fact, in this case we had a complete selenite removal from the medium within 24 hours. Nevertheless, selenite reduction process started after 6 hours from the addition of the Se^{+4} oxyanion. However, the highest selenite reduction efficiencies were observed when SeO_3^{2-} was added after 16 and 24 hours of cells growth. In both of these conditions we had a complete selenite removal from growth medium within 9 hours from the SeO_3^{2-} addition.

Concerning microbial growth, we observed that the addition of SeO_3^{2-} during the lag growth phase (b) gave rise to an increase of the final microbial cell yield, being 1 log higher than that obtained when selenite was added from the beginning (a) (Fig.17). On the other and, when SeO_3^{2-} was added during the early exponential growth phase (c), microbial cell yield was the same that we observed when selenite was added from the beginning. Moreover, after Se^{+4} oxyanion addition in the late exponential growth phase (d), a slight falling of bacterial cell replication was registered. The addition of SeO_3^{2-} after 24 hours of cell growth (e), didn't affect either the rate of microbial growth or the microbial cell yield, which in this case reached the same values achieved for cultures set up in absence of selenite (Fig. 15). Thus, strain SeITE02 appeared to be more sensitive to SeO_3^{2-} when it was added in the lag or exponential growth phases, whereas no influence on microbial growth was registered when SeO_3^{2-} was added in the stationary growth phase.

3.3.2.3 Induction of adaptive response to SeO_3^{2-} in strain SeITE02

Efficiency of strain SeITE02 in reducing selenite to Se^0 and bacterial growth in presence of 0.5 and 2.0 mM Na_2SeO_3 were monitored after pre-induction of microbial cultures with 0.2 mM SeO_3^{2-} (Fig. 18).

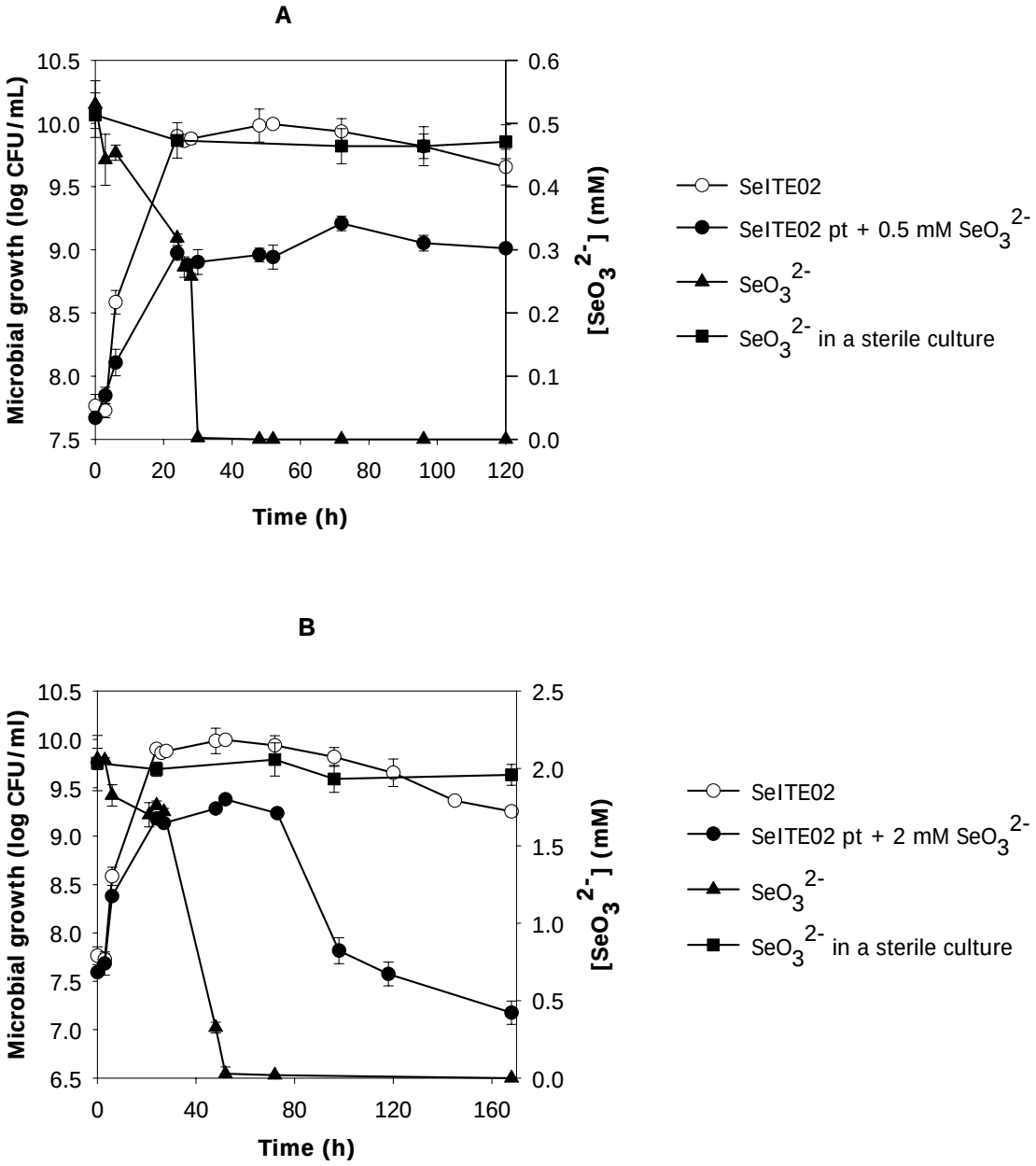


Figure 18 - Time courses of microbial growth and SeO_3^{2-} reduction by SeITE02 pre-tretated with 0.2 mM Na_2SeO_3 (pt, pre-treated), in presence of **(A)** 0.5 mM, **(B)** 2 mM SeO_3^{2-}. Each curve shows means based on the results of three experiments (modified from [**I**]).

As shown in Figure 18, pre-treated SeITE02 cultures did not fully recover the replication rate of cells grown with no selenite added to the growth medium. Nevertheless, despite the decrease of bacterial growth between 6 and 24 hours in not pre-induced cultures, pre-incubation with 0.2 mM SeO_3^{2-} allowed strain SeITE02 to reach the stationary phase after 24 hours, whether it was cultured in presence of 0.5 mM or 2.0 mM SeO_3^{2-}. With 0.5

mM selenite added to the growth medium, the rate of SeO_3^{2-} reduction was not significantly affected by pre-treatment of SeITE02 cells. In these growth conditions, selenite reduction started during the exponential phase lasting until the early stationary phase, according to what observed in not pre-treated cultures. On the other hand, with 2.0 mM SeO_3^{2-} in the culture medium, pre-induction of SeITE02 cells resulted in a significant increase of selenite reduction rate while total depletion of selenite in the substrate occurred in only 52 hours. SeO_3^{2-} reduction was observed starting from the exponential growth phase throughout the initial stationary phase. Thus, SeO_3^{2-} pre-treatment positively affect both SeITE02 growth and selenite reduction process.

Efficiency of strain SeITE02 in reducing selenite to Se^0 and bacterial growth in presence of 2.0 mM Na_2SeO_3 were tested also after pre-induction with either 2.0 mM Na_2SeO_4 and 0.2 mM H_2O_2. SeITE02 pre-treatment with either Na_2SeO_4 or H_2O_2 resulted to positively affect both bacterial growth rate and final cell yield.

Furthermore cells pre-induced showed an increase in the SeO_3^{2-} reduction rate, especially during the stationary phase.

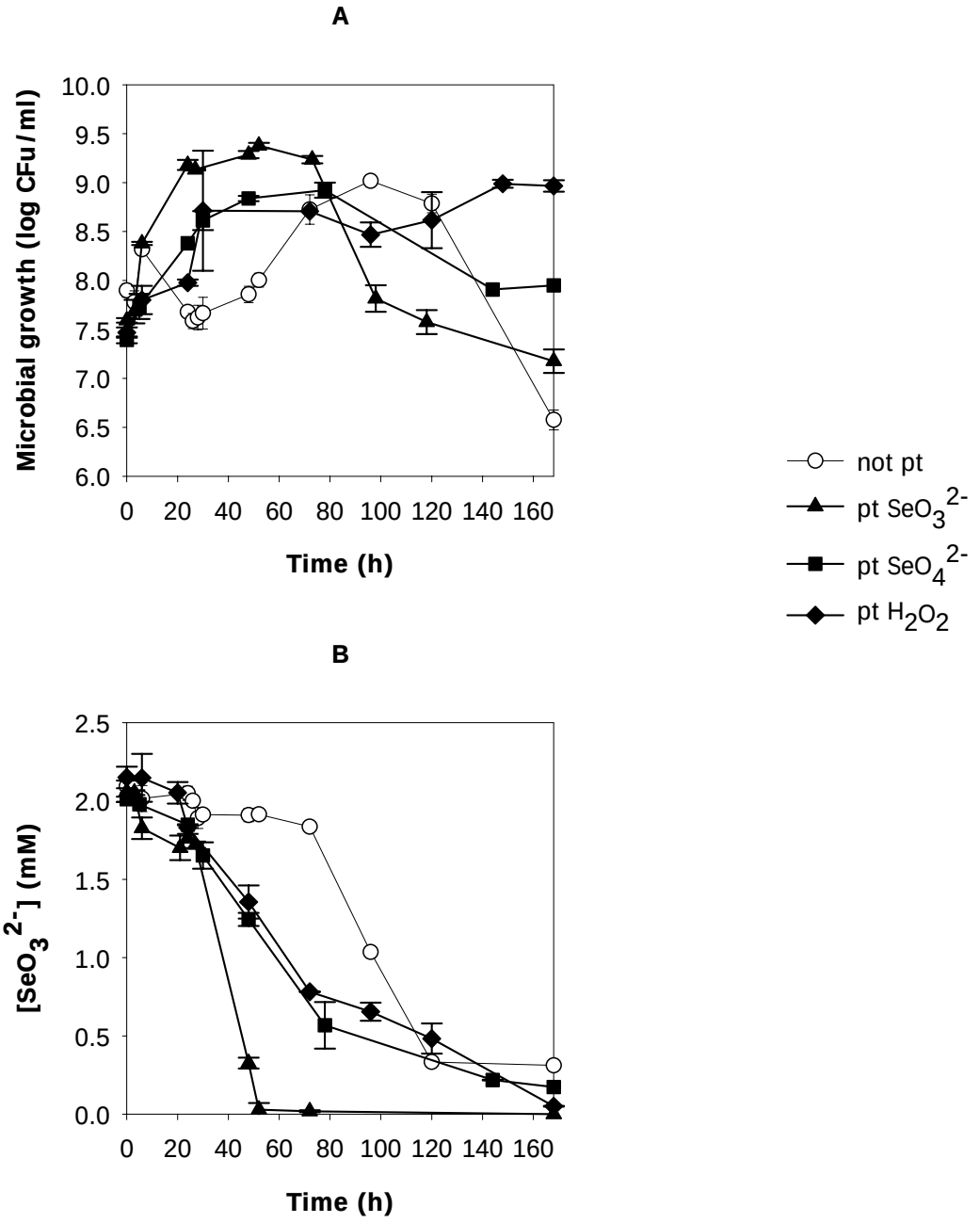


Figure 19 - Time courses of microbial growth **(A)** and SeO_3^{2-} reduction **(B)** by SeITE02 pre-tretated (pt) with either 0.2 mM Na_2SeO_3, 2.0 mM Na_2SeO_4, or 0.2 mM H_2O_2 in presence of 2.0 mM SeO_3^{2-}. Each curve shows means based on the results of three experiments.

3.3.3 Phase-contrast microscopy analyses.

Effects of SeO_3^{2-} added to the growth medium on cell morphology and clustering behavior of strain SeITE02 was evidenced by phase-contrast microscopy analyses (Fig. 20). Actually, the isolate which normally replicates as separate oblong rods, generated more elongated cells, jointed in chains and evolving progressively in a complex aggregates

entrapping light-refractive selenium granules.

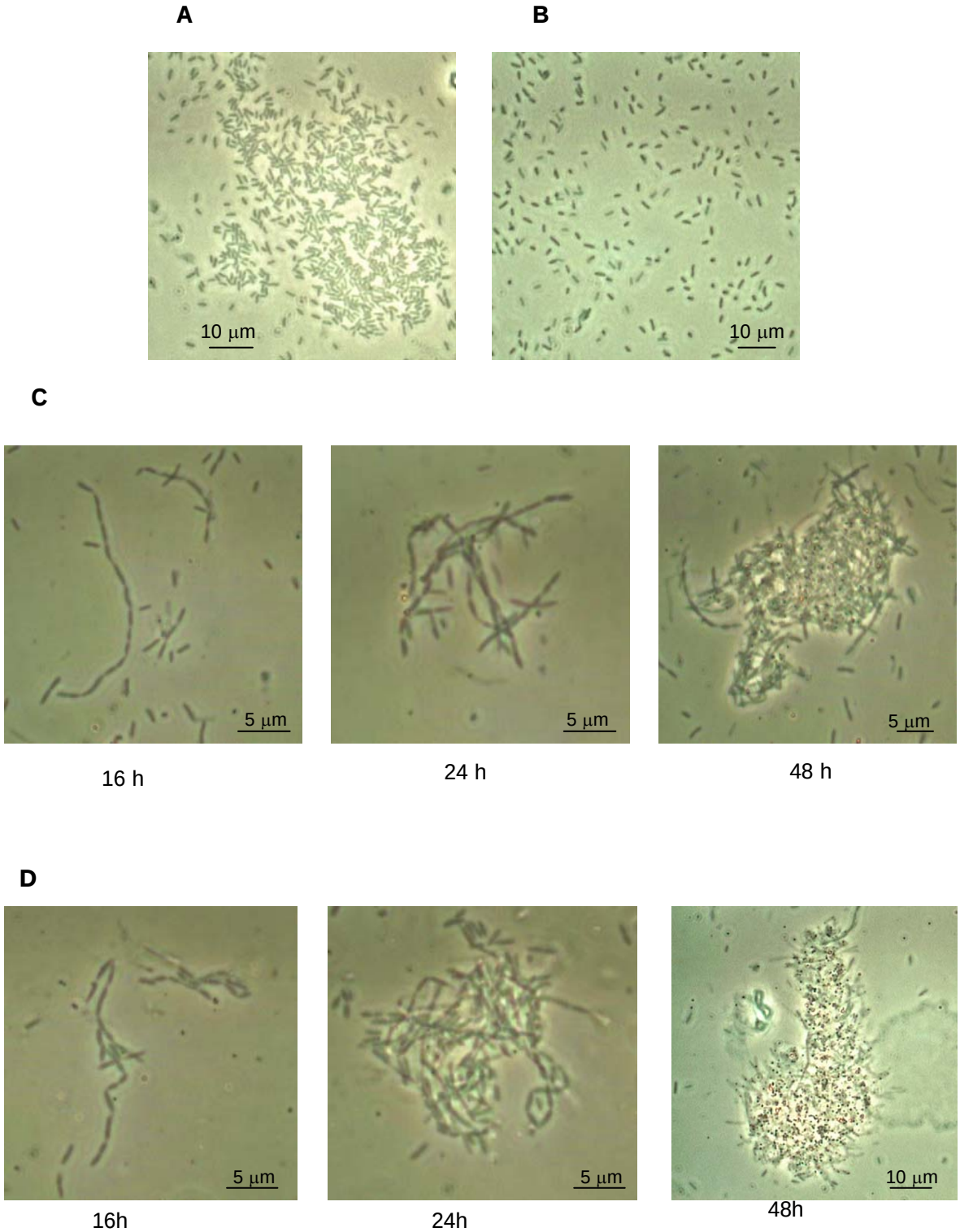


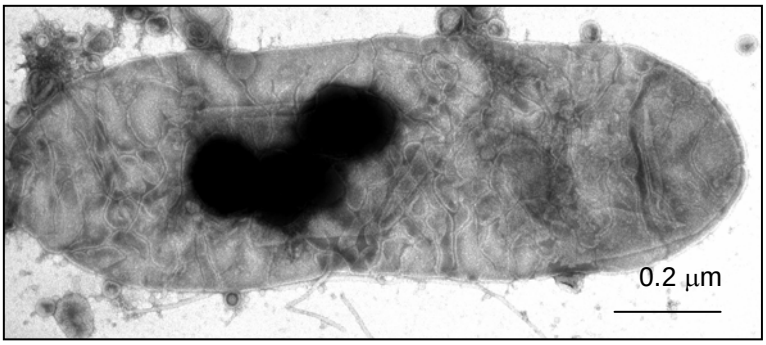
Figure 20 - Phase contrast micrographs of SeITE02 suspension culture grown in absence of Se oxyanions (**A**), in presence of 2.0 mM SeO_4^{2-} (**B**),and in presence of 0.5 mM (**C**) and 2.0 mM (**D**) of SeO_3^{2-} at different time of cell growth

These clumps of bacterial cells and selenium granules never occurred in control cultures. At 0.2 mM and 0.5 mM SeO_3^{2-} in the medium, clumps were recorded starting from early

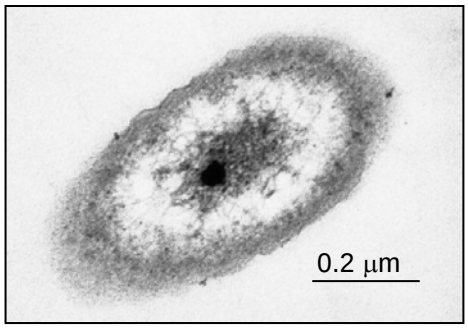
steps of growth. Conversely, at 2.0 mM SeO_3^{2-} in the substrate, those peculiar formations were observed after 48 hours of cultivation. Clumps increased in size along with the progressive reduction of SeO_3^{2-}.

3.3.4 Electronic microscopy analyses

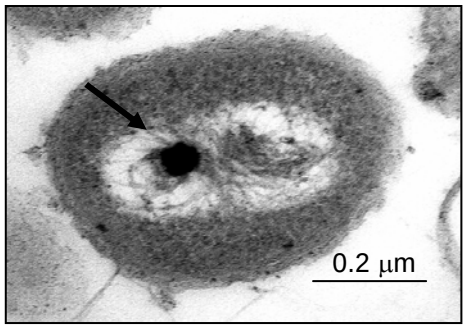
A



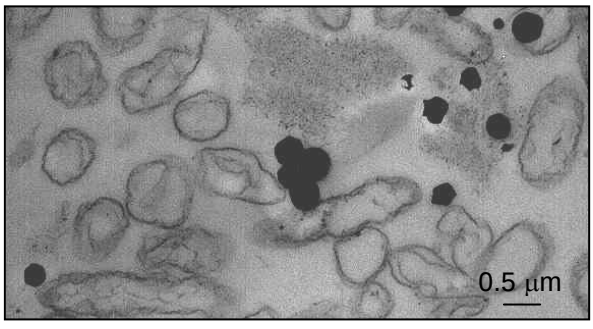
B – 24 h



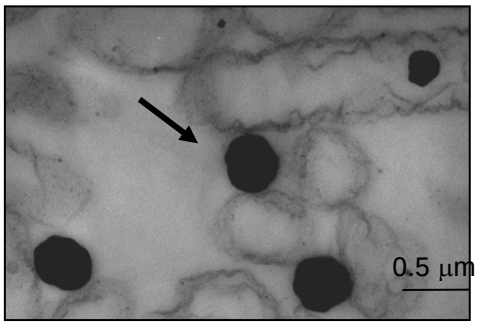
C – 48 h



D – 72 h



E – 120 h



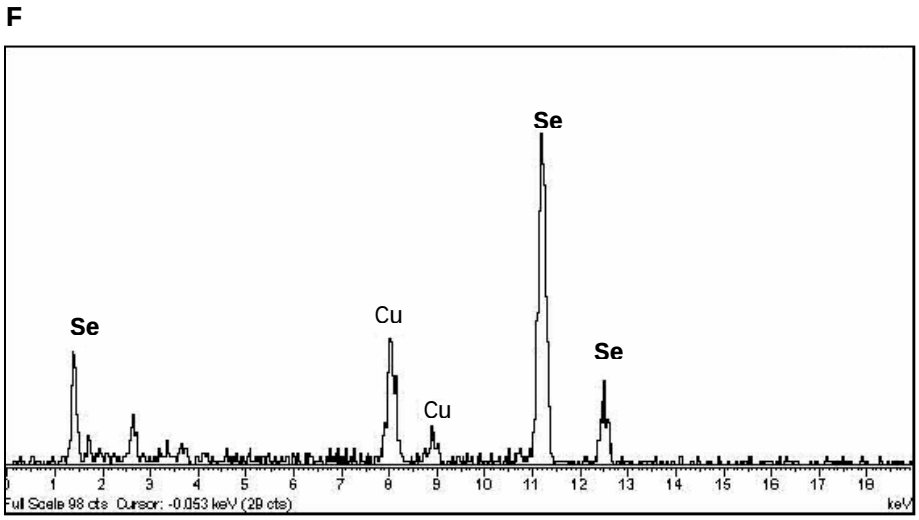


Figure 21 – Electron micrographs of SeITE02 suspension culture grown in presence of 0.5 mM SeO_3^{2-}, and obtained through negative staining analysis (**A**) and TEM analysis (panel **B-E**). TEM analysis were carried out at different time of cell growth, as indicated on each panel. Panel **F** reported the EDX spectrum of Se granules indicated by the arrows.

Electron microscopy analysis of bacterial cells grown in presence of SeO_3^{2-} (Figure 21) carried out both through negative staining (Fig. 21A) and TEM approach (Fig. 21B, 21C) revealed the presence of electron-dense particles inside the cell cytoplasm, at 24 and 48 hours of microbial growth. Identical granules could be observed also in the extra-cellular space after 72 (Fig. 21D) and 120 (Fig. 21E) hours of microbial growth. On the other hand, the same electron-dense granules were never detected in cell cultures which had received no SeO_3^{2-} addition in the growth medium (data not shown).

EDX analyses were carried out on granules which were both inside or outside the cell. Spectra derived from these nanospheres clearly indicated that they were composed entirely of selenium (Fig. 21F)

The Cu and Ni peaks were associated with the TEM grid, whereas the O and C peaks most likely were associated with cellular exudates. The lack of any other metal peaks in the spectrum indicated that the selenium occurred in the elemental state, Se^0, rather than as a metal selenide.

3.4 Comparative considerations on SeITE01 and SeITE02 interactions with Se oxyanions

Tolerance of selenite in strains SeITE01 and SeITE02 can be ascribed to the capacity that these isolates have to reduce soluble and harmful SeO_3^{2-} to the non-toxic and unavailable Se^0, as already reported for other SeO_3^{2-} resistant bacteria (Dungan *et al.*, 2003; Kessi *et al.*, 1999; Rathgeber *et al.*, 2002; Roux *et al.*, 2001; Tomei *et al.*, 1992; Van Fleet-Stalder *et al.*, 2000; Yamada *et al.*, 1997). However these two strains have shown very different behaviors to each others towards selenite, in terms of SeO_3^{2-} reduction efficiency, cell growth, cell morphology, and finally Se nanospheres formation.

Actually selenite reduction process results to be much more efficient in SeITE01 cultures rather than in SeITE02 ones. In fact, at the higher selenite concentration tested (2.0 mM), strain SeITE01 reduces the whole amount of selenium in the medium in 24 hours, whereas SeITE02, after 120 hours of incubation, is able to reduce only the 87% of selenite present at the beginning of cell growth. Moreover, although selenite reduction efficiency of both strains increases when selenite is amended to the cell cultures at times different from the beginning, SeITE01 reduction efficiency registers the highest increment. In fact, when selenite is added in the late exponential growth phase, SeITE01 takes only 3 hours for completely deplete selenite content in the medium.

In both strains (SeITE01 and SeITE02) selenite reduction process accompanies microbial growth, mostly during the exponential phase. The strict correlation between microbial exponential growth and SeO_3^{2-} reduction to Se^0 is consistent with a possible activation of proper detoxifying mechanisms by these two strains (Lortie *et al.*, 1992). Meanwhile, it is worth noting that both SeITE01 and SeITE02 cells maintain their capability to reduce SeO_3^{2-} even under suboptimal growth conditions. Actually the process is not significantly affected by the decrease in cell replication. However cell growth rate of strains SeITE01 and SeITE02 seems to be affected from selenite in different way. Although it has been shown that SeITE01 final cell yield decreases in presence of selenite comparing to that registered in the control tests, the amendment of increasing selenite concentrations in the growth medium seems not to greatly affect microbial growth rate. On the other hand, strain SeITE02 results to be highly affected by the presence of 0.5 and 2.0 mM of selenite in growth medium. In fact, in presence of these two SeO_3^{2-} concentrations, SeITE02 growth showed a depression between 6^{th} and 24^{th} and only after the recovery of cells growth the selenite content was completely reduced. Reaction mechanisms of SeO_3^{2-} reduction by both SeITE01 and SeITE02 need to be further analysed. Although occurring

as a common feature among several microorganisms, reductive reactions for SeO_3^{2-} transformation to Se^0 are not completely clear so far. This reduction has been reported as catalysed by a periplasmic nitrite reductase (DeMoll-Decker and Macy, 1993) or by a hydrogenase I (Yanke *et al.*, 1995), or even promoted by non-enzymatic reactions (Tomei *et al.*, 1992). *In vitro* studies (Turner *et al.*, 1998) have shown that SeO_3^{2-} reduction involves reactions with sulphydryl groups of thiol-containing compounds such as glutathione (see section 1.1.1.2). According to this mechanism, some authors suggested that microbial SeO_3^{2-} toxicity may be due to the oxidative stress experienced by microbial cells during glutathione-mediated reduction of selenite to Se^0 (Kramer and Ames, 1988; Seko and Imura, 1997; Touati, 2000; Kessi and Hanselmann, 2004). However, in the bacterial domain only the cyanobacteria and representatives of the α, β and γ groups of the proteobacteria are able to synthesize glutathione (Newton and Fahey, 1989). Thus, a different mechanism of selenite reduction may take place in Gram-positive bacteria, which are not able to synthesize glutathione but can tolerate high levels of selenite. As for proteobacteria, however, a significant induction of thioredoxin and thioredoxin reductase has been observed in *B. subtils* exposed to millimolar concentration of selenite (Garbisu *et al.*, 1999) and, generally, Gram-positive bacteria accumulate coenzyme A and other organic compounds containing disulfide groups at millimolar levels (Newton and Fahey, 1989). Additionally, a disulfide reductase capable of reducing coenzyme A disulfide and other related disulfides is produced by bacilli (Swerdlow and Setlow, 1983). This suggests that the mechanism of selenite reduction in these bacteria may be similar to that proposed for the proteobacteria with a high glutathione level.

According with this suggestion, we have found that in strain SeITE02, cell pre-treatment with 0.2 mM SeO_3^{2-} seems to stimulate an adaptive response of this bacterium to the toxic effects of high SeO_3^{2-} concentrations (0.5 and 2.0 mM). Such an adaptive response allows the increase of cell growth in the presence of SeO_3^{2-}; in turn, the capability of strain SeITE02 to reduce SeO_3^{2-} is improved. Furthermore these evidences are also supported by tests carried out after pre-treatment with SeO_4^{2-} and H_2O_2. Activation of a sort of adaptive response to selenite toxicity lending support to the hypothesis that in strain SeITE02 reduction of SeO_3^{2-} to Se^0 consists in a detoxification mechanism. On the other hand, in strain SeITE01, cell pretreatment with SeO_3^{2-} seems not to greatly affect selenite reduction process. In fact, the selenite reduction efficiency obtained from the pre-treated inoculum is the same registered in not pretreated cultures. However a slight increase in cell growth was observed. Thus these evidences lead us to suppose that these two strains have different selenite reduction mechanisms.

Another support to this hypotesis is given from electron microscopy analyses. Results obtained indicates that reduction of SeO_3^{2-} to Se^0 by strain SeITE02 leads to accumulation of the metalloid inside bacterial cells. This is in agreement with evidences reported for other SeO_3^{2-} resistant microbial strains (Kessi *et al.*, 1999; Roux *et al.*, 2001; Rathgeber *et al.*, 2002; Bebien *et al.*, 2001). Furthermore, selenium granules were never observed in the periplasm, although they were found extra-cellularly. The presence of selenium granules in extra-cellular locations cannot be explained relying on a transport across membrane that is unlikely for such large particles. Yamada *et al.* (1997) claimed for an extra-cellular reduction of SeO_3^{2-}. Again, several authors suggested a vesicular mechanism of selenium excretion (Kessi *et al.*, 1999) even though vesicular excretion in bacteria is still controversial. With reference to strain SeITE02, a process of cell lysis may be invoked. This should determine release of selenium granules outside the cells. This hypothesis is based on the evidence that, with 2.0 mM SeO_3^{2-} added to the substrate, an increase in culture turbidity can be revealed which appears completely independent from microbial growth (data not shown). Cell lysis, as a consequence of the release of selenium granules into the medium, does increase turbidity of bacterial cultures irrespective of the actual microbial growth. A tentative explanation similar to that here advanced has been formulated also by Tomei *et al.* (1992). Cell lysis may be looked at as a direct consequence of selenium accumulation in form of granules inside microbial cells. This accumulation may even cause the decrease of viability in SeITE02 cultures amended with SeO_3^{2-}. On the other hand, there aren't evidences for strain SeITE01 of an accumulation of the metalloid inside the cell. TEM analyses rather led to suppose that strain SeITE01 reduces SeO_3^{2-} extra-cellularly. In fact there aren't evidence of Se^0 accumulation inside the cell, neither at earlier time in cell growth.

Despite these two strain seem to have different selenite reduction mechanisms, a lot remains to do in order to clarified which specific mechanisms are responsible for such a reduction process.

Finally, it is interesting to point out that the allotropic form of Se^0 produced by both SeITE01 and SeITE02 seems to be highly stable. On the other hand, red amorphous Se^0 is unstable in soil and generally reverts to the black amorphous form (Gattow and Heinrich, 1964). This latter is slowly transformed to the more stable grey hexagonal allotrope or even re-oxidised through abiotic or biotic processes (Geering *et al.*, 1968). The high stability of the red allotropic Se^0 form accumulated by strain SeITE02 and SeITE01, whose cultures remain reddish for at least one month (data not shown), suggests that this selenium species may occur as tightly bound to extra-cellular stabilizing

compounds. Extra-cellular compounds, possibly produced by microbial cells, may prevent the red allotropic form of selenium from transformation in the black form, so avoiding Se^0 re-oxidation. Sequestration of metals in extra-cellular matrices has been recently described in *Klebsiella oxytoca* (Baldi *et al.*, 2001).

Consistently with this scenario, phase-contrast microscopy analyses on strain SeITE02 showed that selenium granules released into the growth medium appeared associated to an extra-cellular matrix, forming a sort of clumps with microbial cells. Also in the case of strain SeITE01 clumps of microbial cells and Se^0 particles were observed in the culture medium. Nevertheless they resulted to be much smaller in size than those observed for SeITE02.

Eventually it's important to underline the potential biotechnological perspectives for the exploitation of these two strains in bioremediation processes. In fact, although SeO_3^{2-} exerts a toxic effect on cellular metabolism, the ability of strains SeITE01 and SeITE02 to reduce selenite to Se^0 offers a possibility to biologically precipitate this metalloid from selenium-laden effluents. Furthermore, it's worth noting that the selenite concentrations tested in this study are comparable to those reported for soluble Se in agricultural drainage wastewaters (~ 0.65 mg/l selenium) (Cantafio *et al.*, 1996) or in industrial wastewater (4 - 42 mg/l selenium) (Lawson and Marcy, 1995; Rege *et al.*, 1999). Consequently, the bacterial isolates SeITE01 and SeITE02 can be considered suitable of application, at either laboratory or pilot scale, in continuously fed wastewater treatment systems in order to optimise the abatement of selenite.

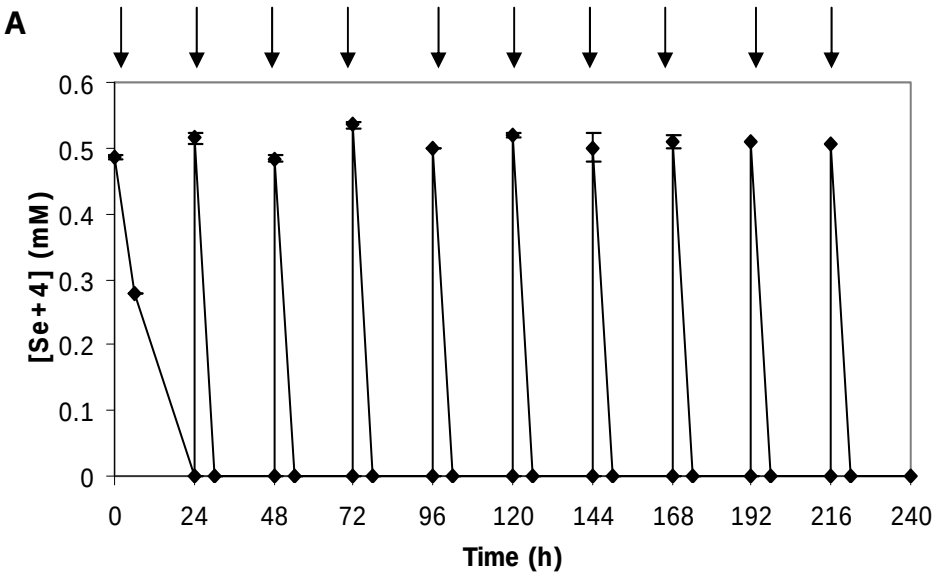
3.5 Selenite removal tests using a SBS (sequencing batch system)

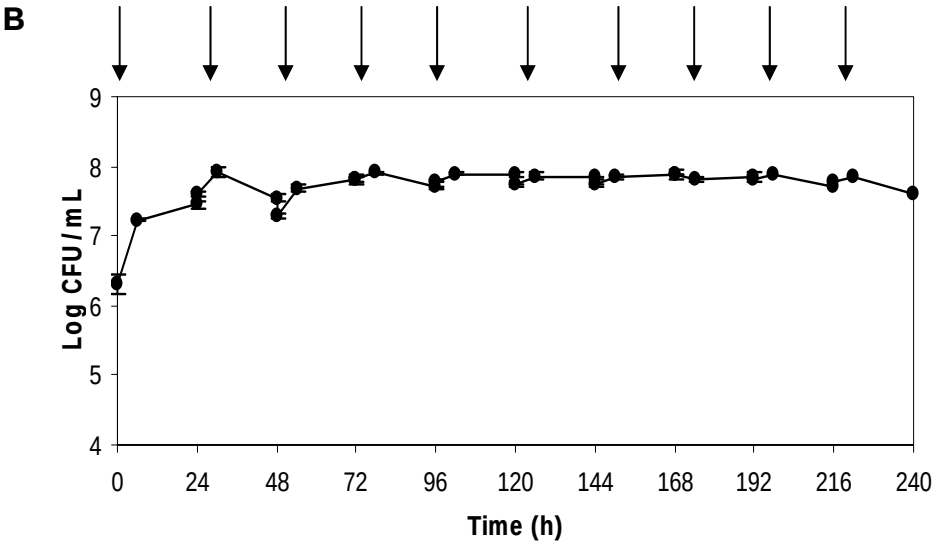
Because of its higher selenite reduction efficiency, comparing to that of strain SeITE02, *Bacillus mycoides* SeITE01 was chosen for further sperimentations in order to test its applicability for SeO_3^{2-} removal processes from aqueous solutions. Since Se^0 is non toxic and can also be easily removed from the aqueous phase on account of its insoluble characteristic, this feature can be applied to develop an economical process for the depletion of soluble Se. As reported in section 1.3.1, some biological treatment systems have already been developed (Gerhardt *et al.*, 1991; Cantafio *et al.*, 1996). However, these systems were set up to treat mainly agricultural drainage water containing low concentration of soluble Se (0.3-0.5 mg/L). Actually, some industrial waste water can be expected to contain higher concentration of selenium oxyanions, that in same case can reach high values, like 250 mg/L (about 3.2 mM). Thus, it is important to develop a system capable of treating high Se concentrations. As we have gained from the results obtained and showed in the previous chapters of this section, strain SeITE01 resulted to be capable to reduce high selenite concentrations whit elevated efficiency (section 3.1). Since *Bacillus mycoides* SeITE01 can tolerate and efficiently reduce high concentrations of selenite, it results to be a promising candidate for application in biological reaction systems for the treatment of waste water with high Se content.

Thus, the possibility of constructing a selenite removal process utilizing strain SeITE01 was investigated using an aerobic culture operated in a sequencing batch mode.

It's important to underline that the process we have set up in this experimental phase, represents only a preliminary step in the development of a system for selenium removal from aqueous solution. In fact SeITE01 was tested in optimal growth condition, i.e. rich growth medium (Nutrient broth). Moreover, the reduction efficiency of strain SeITE01 was evaluated only in presence of selenite.

Both selenite content in the medium and biomass production were measured. Typical results are shown in Fig. 22.


A
[Se+4] (mM)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
24
48
72
96
120
144
168
192
216
240
Time (h)


B
Log CFU/mL
9
8
7
6
5
4
0
24
48
72
96
120
144
168
192
216
240
Time (h)

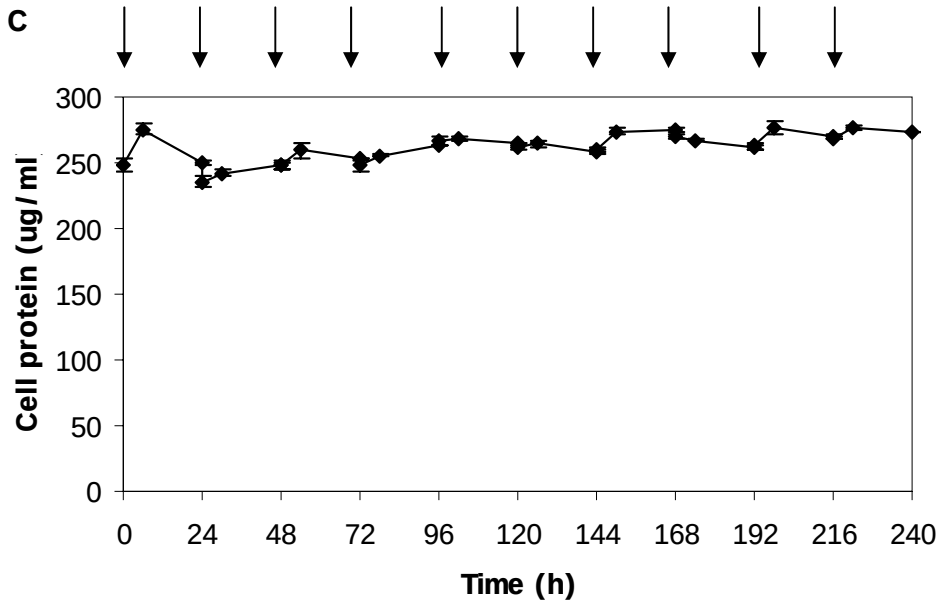


Figure 22 – Sequencing batch tests for selenite removal. Monitoring of selenite reduction efficiency **(A)** and cell biomass production [by counting CFU **(B)** and by measuring cell total protein **(C)**] by strain SeITE01 in presence of 0.5 mM Na_2SeO_3. Arrows indicate the transfer of cells into new medium.

Strain SeITE01 effectively transformed 0.5 mM of selenite into elemental Se within a 24-h cycle over at least 10 cycles (Fig. 22, panel A). The cell density remained relatively stable at 1.0 x 10^8/ml. This evidence was confirmed also by results obtained from the measuring of total protein. During these experiments, a specified amount of the culture broth was withdrawn each day for Se content determination, cell counting an total protein quantification. Since the cell density remained relatively stable throughout the experimental period, the amount of cells withdrawn was estimated to balance the cell grown.

These results clearly indicate that it will be possible to use strain SeITE01 cells repeatedly for removing selenite at a load of approximately 40 g- Se (as selenite)/m^3/d in an aerobic sequencing batch mode.

However, these experiments were performed using a pure culture under sterilized conditions. To realize a practical Se-removal system, further experiments should be carried out under non-sterilized conditions. Furthermore, strain SeITE01 should be tested in sub-optimal growth conditions in order to mime the typical growth conditions in a sewage sludge.

3.6 Phytoremediation assay

In order to evaluate the possibility to utilise an integrate plant-microorganisms system for the remediation of polluted selenium streams, a plant-assisted bioremediation assay was set up using *Brassia juncea* plants as phytoextracting agents.

The choice of *Brassica juncea* for this phytoremediation assay, was due to the fact that this plant species, classified as secondary Se accumulator, can accumulate large amounts of Se, grow rapidly and produce a large biomass, tolerate salinity and other toxic conditions, and, if necessesary, provide a safe source of forage for Se-deficient livestock (Terry *et al.*, 2000).

The phytoremediation assay was set up in a 'perlite hydroponic system', which consisted in plants grown on perlite beds, settle down in ponds and bioaugmented with two different types of bacteria *inocula*, and was arranged in statically conditions. In fact, Hoagland solutions containing selenium oxyanions (either SeO_3^{2-} or SeO_4^{2-}, at three different concentrations) were added as sole amendment at the beginning of the experimentation.

The choice of employing a perlite bed for supporting plants growth, was due to the fact that perlite is an inert silica matrix that, due to its high porosity, allows a good oxygenation of plant root system and thus a good vegetative growth. Moreover, this silica matrix didn't contain humic acids or other clayey compounds which could negatively affect the selenium oxyanions bioavailability.

As described in section 2.5.1, 16 different test were arranged and two different types of bacteria *inocula* were bioaugmented to the rhizosphere of *B. juncea* plants, in order to assess the microbial contribute to the phytoremediation process of a putative selenium polluted stream.

With the purpose of investigate the feasibility of such a bioremediation system, 3 different parameters were monitored:

1) Phytoextraction Efficiency (PE)

$$\mathbf{PE} = \frac{[\text{Se}] \text{ in tissues} \times \text{dried biomass}}{\text{initial Se content}}$$

This parameter is of fundamental importance because it gives the fraction of selenium effectively removed from the contaminated matrix.

2) Bioconcentration Factor (BF)

$$\mathbf{BF} = \frac{\text{[Se] in tissues}}{\text{initial [Se]}}$$

The BF factor is also a very important parameter to consider. It gives a measure of the plant capability to bioconcentrate the toxic element (selenium) into its tissues.

3) Translocation Factor (TF)

$$\mathbf{TF} = \frac{\text{[Se] in shoots}}{\text{[Se] in roots}}$$

Finally, the TF factor gives a quantification of the plant capability of translocate selenium taken up from root to shoot.

Thus, biomass production, as dry weight, and total selenium concentration both in shoots and roots (Se bioaccumulation) were determined for each pond arranged.

3.6.1 Selenate treatment

As described in section 2.5.1, selenate amended Hoagland solution was added to the plant-assisted phytoremediation system at three different SeO_4^{2-} concentrations: 15 mg Se/kg (0.2 mM), 75 mg Se/kg (1.0 mM), and 150 mg Se/kg (2.0 mM).

At all these three concentrations tested, plants were bioaugmented with an adapted soil microbial community, collected from the rhizosphere of *Brassica juncea* plants previously grown on soil amended with 30 μM Se, as Na_2SeO_4. Only at the intermediate concentration, 75 mg Se/kg (1.0 mM), perlite beds were bioaugmented also with an axenic culture of strain SeATE17 which was previously isolated from a selenium polluted soil (section 3.1).

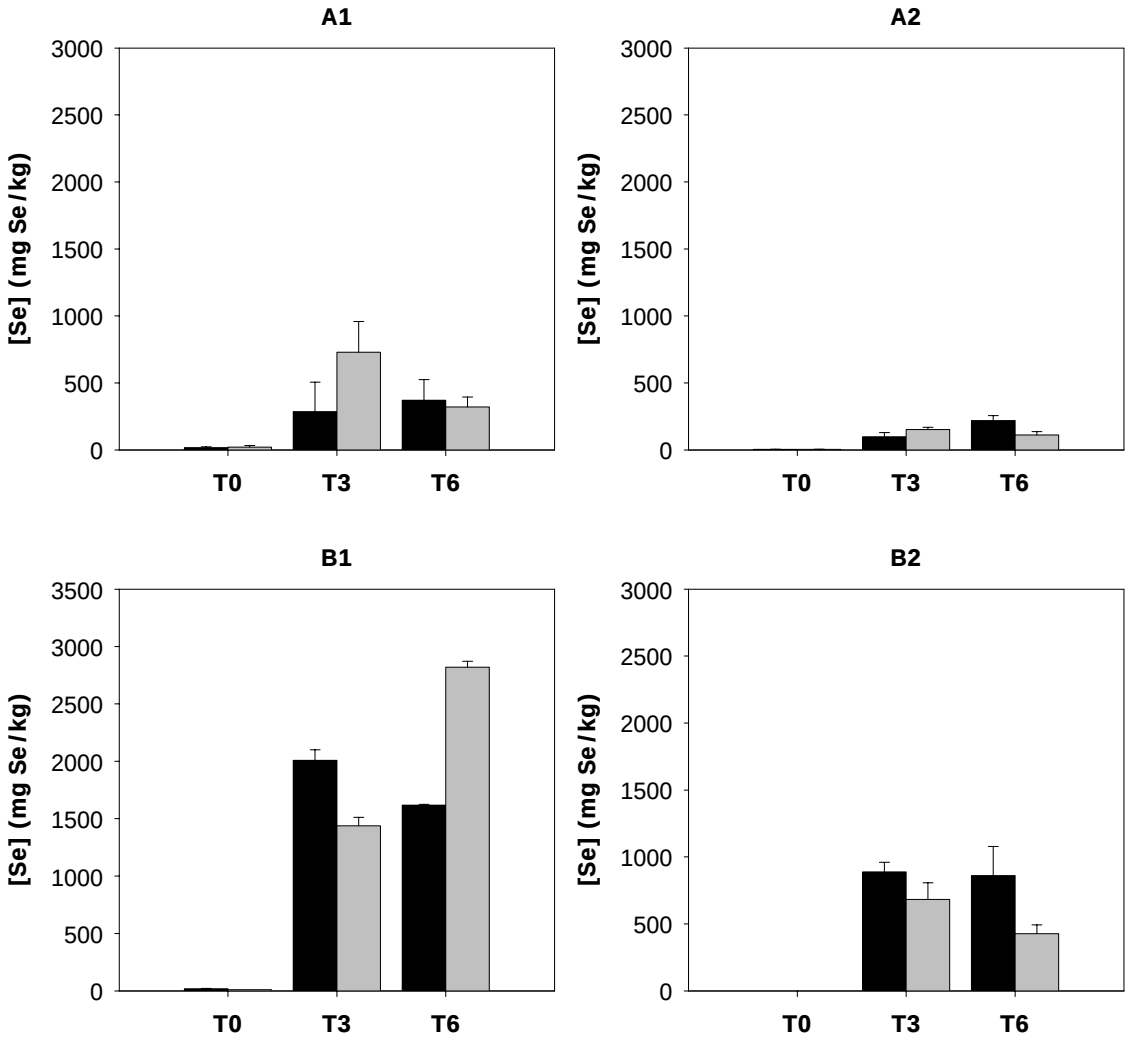
3.6.1.1 Bioaugmentation with the adapted soil microbial community (I)

(a) Se bioaccumulation in plant tissues

Total selenium content determination was carried out on both roots and shoots, at three different sampling times: T0 (at the beginning of the experimentation), T3 (after 3 weeks of incubation), T6 (after 6 weeks of incubation = at the end of the experimentation). The results obtained were shown in Fig. 23.

The time courses of the total selenium contents in plant tissues indicated that selenium accumulation, both in shoots and roots, increased with the increasing of selenate concentration amended.

As a general rule we observed that when selenate was amended, selenium was preferentially accumulated in the shoot portion rather then in the root ones, either with or without soil microbial community addition, at all selenate concentrations tested.



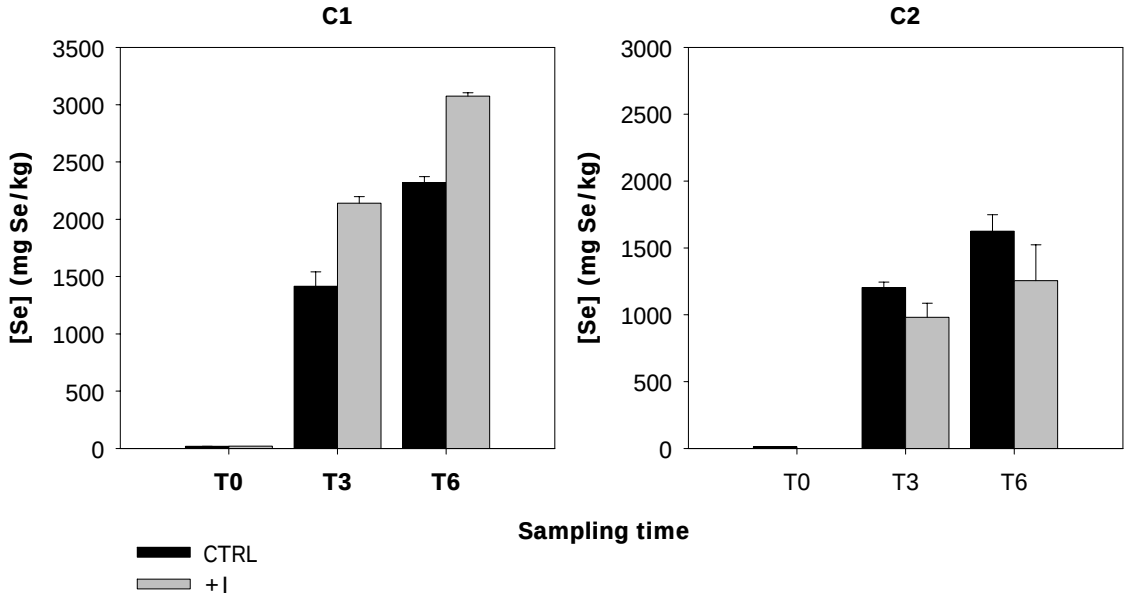
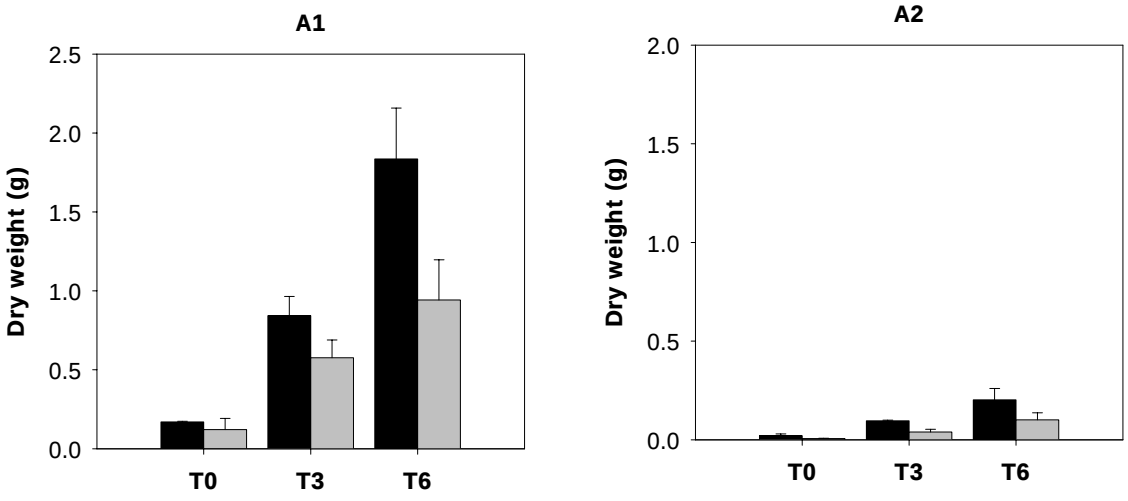


Figure 23– Time courses of selenium contents in the shoot (**1**) and root (**2**) portions, at 15 mg Se/kg (**A**), 75 mg Se/kg (**B**) and 150 mg Se/kg (**C**) of selenate. Each value was calculated as the means of three determinations. [CTRL= plants not bioaugmented; +I=plants bioaugmeted with the adapted soil microbial community]

On the other hand, the bioaugmentation with the soil microbial community seemed to positively affect the plant capability to accumulate selenium in the shoots. Concomitantly we also registered a decrease in selenium accumulation in the root portion.

(b) Biomass production

Dry weight of plant tissues (roots and shoots) was recorded at the three sampling times and reported in Fig. 24.



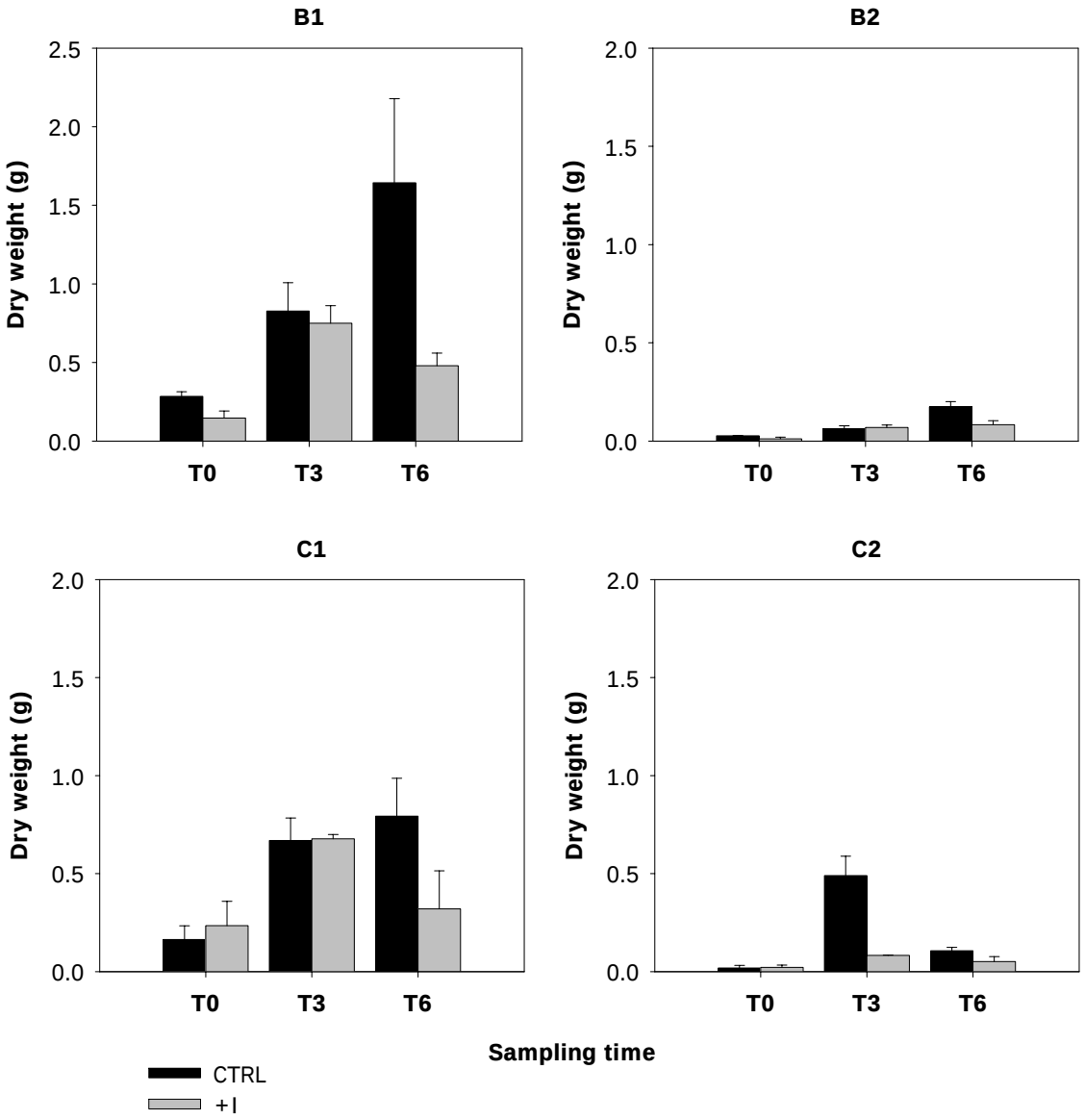


Figure 24 – Time courses of biomass production (dry weight) for shoot (**1**) and root (**2**) portions, at 15 mg Se/kg (**A**), 75 mg Se/kg (**B**) and 150 mg Se/kg (**C**) of selenate. Each value was calculated as the means of three determinations. [CTRL= plants not bioaugmented; +I=plants bioaugmeted with the adapted soil microbial community]

The data obtained, showed a general decrease in biomass production with the increasing of the selenate concentrations tested.

It's worth noting that the presence of the soil microbial community inoculum (I) negatively affected the plant biomass production both in shoot and root portions. Actually, regarding shoot portion, we observed a decrease in the plant biomass production of 48.7%, 70.8% and 59.7% in presence of 15, 75, 150 mg Se/kg respectively, comparing to the values obtained for the not bioaugmented plants (referring to T6). On the other hand, we registered a diminishing in roots biomass of about 50% at all selenate

concentrations tested, in comparison with the values observed without soil microbial community amendment.

Furthermore, plants grew at the highest selenate concentration (150 mg Se/kg), showed phenotypes such as the appearance of violet colour on the edge of leaves (Fig. 25), and a shorter distance in the internodes, comparing with that recordered for plants grown in absence of selenium or in presence of lower selenate concentrations (Fig. 26), that seem to be related to a toxicity effect exerted by high selenate levels. Plants grown in absence of selenium or in presence of selenite never showed such a toxicity symptoms (data not shown).

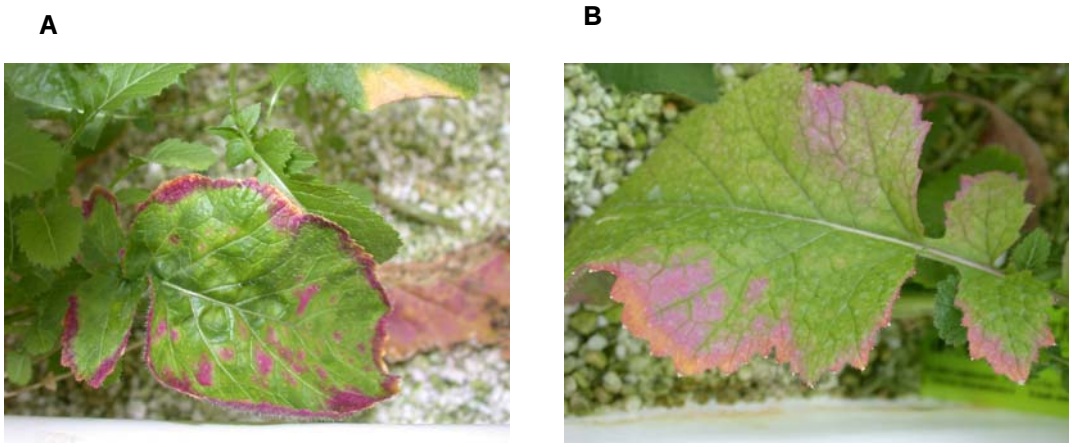


Figure 25 – Photographs of *Brassica juncea* plants grown in presence of 150 mg Se/kg selenate after 3 (**A**) and 6 (**B**) weeks of incubation.

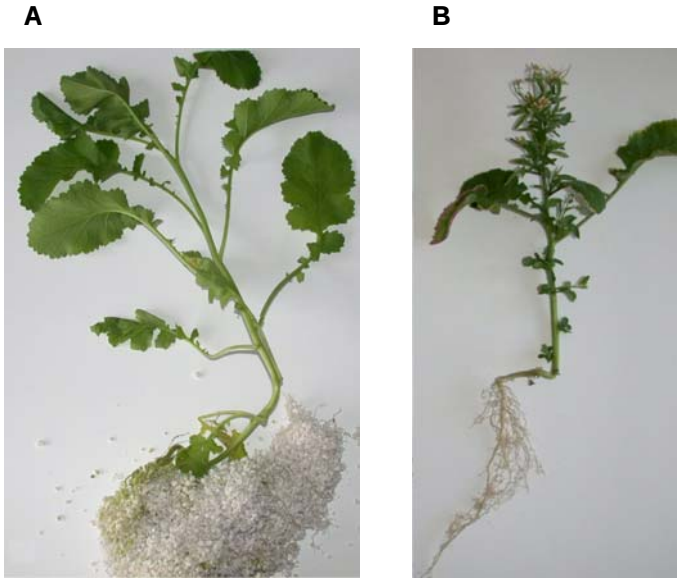


Figure 26 - Photographs of *Brassica juncea* plants grown either in absence of Se (**A**) or in presence of 150 mg Se/kg selenate (**B**)

3.6.1.2 Bioaugmentation with strain SeATE17 as axenic culture

With the purpose of investigate the effect of bacterial strain capable to resist to high selenate concentration on phytoremediation process of such a system, strain SeATE17 was amended as axenic culture into perlite bed. This experiment was set up only at 75 mg Se/kg (1.0 mM) selenate concentration. Data concerning both Se accumulation in plant tissues and biomass production were registered for each sampling time and reported in Fig. 27.

No significant differences were observed in selenium accumulation for plants bioaugmented with strain SeATE17 in comparison with control tests.

On the other hand, the presence of strain SeATE17 negatively affected the biomass production of both shoot and root portions. Actually, for samples collected at the end of experimentation (T6), a decrease of 38.7% as dry weight for shoot portion was registered.

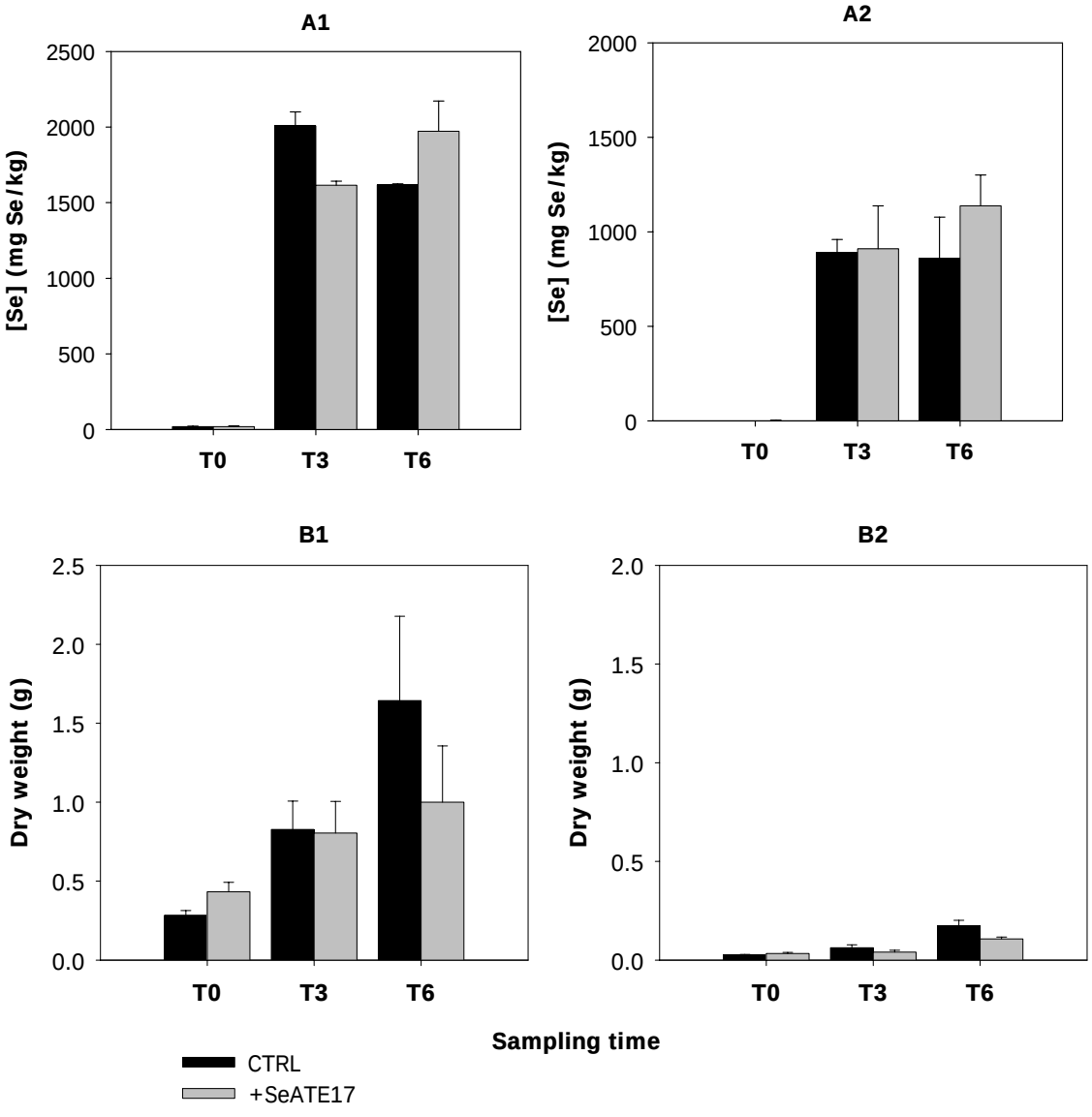


Figure 27 – Time courses of total Se contents (**A**) and biomass production (dry weight) (**B**) in shoot (**1**) and root (**2**) portions, at 75 mg Se/kg of selenate. Each value was calculated as the means of three determinations. [CTRL= plants not bioaugmented; +SeATE17=plants bioaugmeted with strain SeATE17 as axenic culture]

3.6.1.3 Evaluation of BF, TF and PE factors for selenate treatment

BF and TF factors were calculated as described in section 3.6 and are displayed in Table 9.

Concerning Bioaccumulation Factor (BF), we observed that, comparing with the values registered for control tests, the presence of soil microbial community inoculum induced an increase in BF values for the shoot portions at the concentrations of 75 and 150 mg Se/kg selenate. On the other hand a decrease in BF values for the root portions at the same selenate concentrations tested was registered. Furthermore, when strain SeATE17 was bioaugmented at the concentration of 75 mg Se/kg of selenate, we obtained an increase in BF values both for shoot and root portion, in comparison with data obtained for control tests. Nevertheless, the increase in BF values observed in shoot portion for strain SeATE17 was lower than that registered when the soil microbial community was bioaugmented.

	[Se]	BF		TF
		Shoot	Root	
CTRL (15)	15 mg/kg	24.66	14.63	1.69
CTRL (75)	75 mg/kg	21.57	11.48	1.88
CTRL (150)	150 mg/kg	15.46	10.83	1.43
+I (15)	15 mg/kg	21.36	7.4	2.89
+I (75)	75 mg/kg	37.59	5.70	6.59
+I (150)	150 mg/kg	20.50	8.37	2.45
+ SeATE17 (75)	75 mg/kg	26.29	15.17	1.73

Table 9 – Bioconcentration Factor (BF) and Translocation Factor (TF) values for tests carried out in presence of selenate

On the subject of Translocation Factor (TF), values of TF > 1 indicate a preferential accumulation of Se in shoot rather than in root portion. This is a desirable occurrence in a phytoexraction process. Actually, plants capable to concentrate high selenium quantitative in the above ground tissues result to be promising for phytoremediation strategies. In this

case, indeed, it is possible to harvest the above ground plant tissue and thus to remove the pollutant from the contaminated matrix.

Bioaugmentation with the soil microbial community produced an increase in TF values at all selenate concentrations tested, in comparison with those obtained for control experiments. In particular, at the concentration of 75 mg Se/kg, the TF value was 3.5 folds higher than that registered for control test. On the other hand, the strain SeATE17 *inoculum* didn't exert the same positive effect on TF than that achieved from soil microbial community bioaugmentation. Actually, the TF values for SeATE17 bioaugmented test resulted to be slight lower than that observed for control experiment.

However, concerning the phytoextraction efficiency of the whole system, we observed that the highest PE values were reached in the not bioaugmented tests, at all selenate concentrations tested (Table 10). In particular, it has been observed that the system works better at the lowest selenate concentration tested, reaching a PE of 95.65%. Interestingly, the experiment set up with the inoculum of strain SeATE17 showed a phytoremediation efficiency higher than that obtained with the inoculum of the adapted soil microbial community. However, also in this case, the PE value resulted to be lower than that registered for control test, at the same selenate concentration.

	[Se]	**Shoot (μg)**	**Root (μg)**	**Total Se (μg)**	**PE**
CTRL (15)	15 mg/kg	18009.66(94.14%)	1120.71 (5.86%)	19130.37	95.65 %
CTRL (75)	75 mg/kg	83072.45(94.74%)	4606.43 (5.26%)	87678.88	87.68 %
CTRL (150)	150 mg/kg	49654.41(89.37%)	5906.71(10.63%)	55561.11	27.78%
+I (15)	15 mg/kg	11416.18(96.62%)	399.36 (3.38 %)	11815.54	59.07%
+I (75)	75 mg/kg	48153.38(97.37%)	1300.59 (2.63 %)	49453.97	49.45 %
+I (150)	150 mg/kg	34360.47(93.95%)	2211.82 (6.05 %)	36572.29	18.28%
+ SeATE17 (75)	75 mg/kg	61761.76(92.29%)	3738.50 (5.71%)	65500.26	65.50%

Table 10 – Phytoextraction Efficiency (PE) values for tests carried out in presence of selenate

3.6.1.4 PCR-DGGE analyses

In order to evaluate the modifications occurred in the microbial community structure of plant rhizosphere during the experimental time, PCR-DGGE analyses were carried out on V3-16S rDNA PCR products, amplified from total DNA extracted from perlite samples collected from root systems of plant at the three sampling time: T0, T3 and T6.

The DGGE profiles, obtained for selenate treatment tests bioaugmented with the adapted soil microbial community (+I), showed that no great modifications occurred in the dominant bands pattern during the experimental time at all selenate concentration tested (15, 75, 150 mg Se/kg) (Fig. 28). Thus, the presence of selenate in the system seems not to greatly affect the community structure of plant rhizosphere. In fact, we didn't observed a reduction in the number of dominant bands, but rather a redistribution of band intensity in the DGGE profile. Thus, selective pressure exerted by selenate wasn't high enough to cause a speciation in the microbial community. This evidence could be explained by the fact that selenate doesn't exert high toxicity effect on microorganisms under aerobic conditions (Burton *et al.*, 1987). On he other hand, the DGGE profiles clearly showed that the adapted soil microbial community *inocula* persisted during the whole experimental phase. Actually, the adapted soil microbial community *inocula* exerted its effect both on plant Se accumulation and biomass production until the end of experimentation.

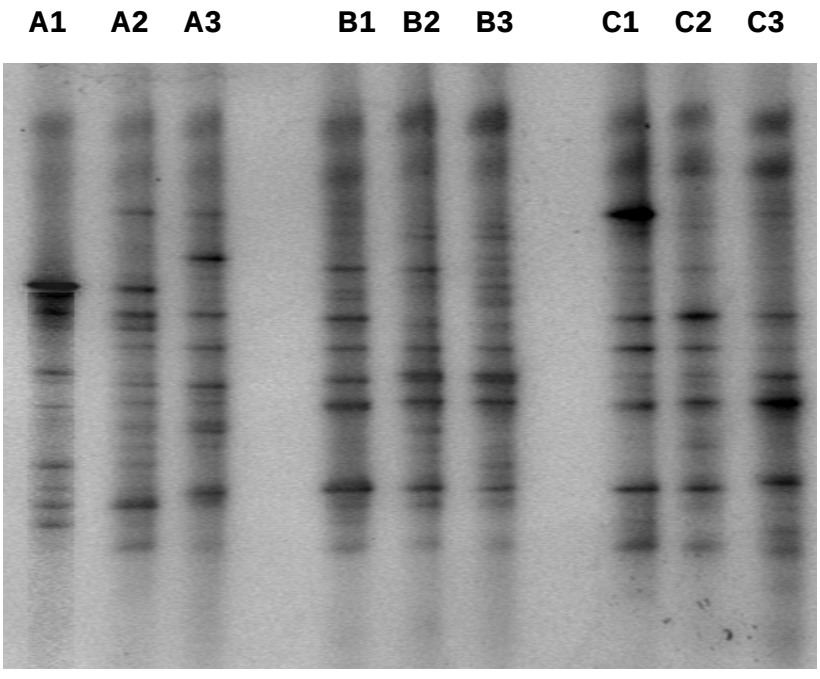


Figure 28 – DGGE profiles of samples collected from the rhizosphere of plants grown at 15 (from **A1** to **A3**), 75 (from **B1** to **B3**), and 150 (from **C1 to C3**) mg Se/kg of selenate and bioaugmented with soil microbial community. Numbers correspond to the sampling time: T0 (**1**), T3 (**2**) and T6 (**3**).

PCR-DGGE analysis was also successfully carried out with the purpose of monitoring the presence of the bacterial strain, augmented as axenic cultures, throughout the whole experimental period.

The presence of strain SeATE17 was checked by comparing the DGGE profile of V3-16S rDNA PCR products amplified from genomic DNA of strain SeATE17 with that resulted from the V3-16S rDNA PCR products amplified from total DNA of perlite samples collected from the rhizosphere of inoculated plants (Fig. 29). In this way, it was possible to verify the presence of strain SeATE17 throughout the whole experimental period. DGGE profiles, obtained for SeATE17-boaugmented test at three different sampling times (lane A1-A3), showed that strain SeATE17 persisted in the plant rhizosphere until the end of the experiment.

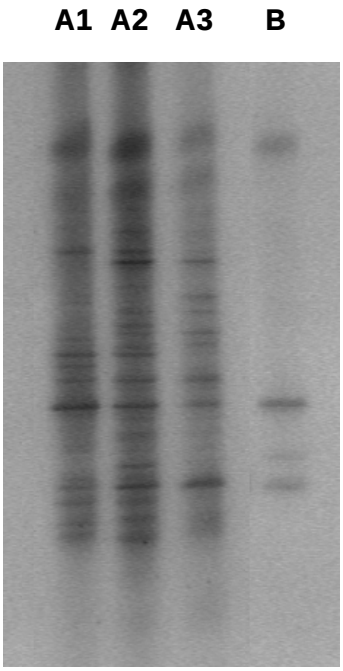


Figure 29 - DGGE profiles of microbial community V3-16S rDNA PCR products, amplified from the rhizosphere of plants grown at 75 mg Se/kg of selenate and bioaugmented with strain SeATE17 (from **A1** to **A3**). Numbers correspond to the sampling time: T0 (**1**), T3 (**2**) and T6 (**3**). Lane **B**: V3-16S rDNA PCR products amplified from strain SeATE17 genomic DNA.

3.6.2 Selenite treatment

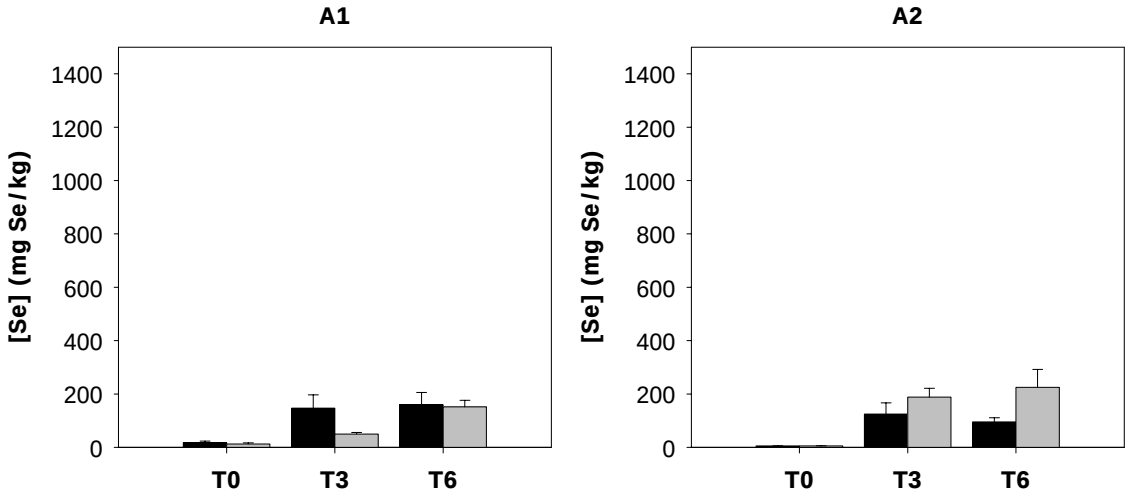
As described in section 2.5.1, selenite amended Hoagland solution was added to the plant-assisted phytoremediation system at three different SeO_3^{2-} concentrations: 0.2 mM (15 mg Se/kg), 1.0 mM (75 mg Se/kg), and 2.0 mM (150 mg Se/kg).

As for selenate treatment, at all these three selenite concentrations, the systems were bioaugmented with an adapted soil microbial community, collected from the rhizosphere of *Brassica juncea* plants previously grown on soil amended with 30 μM Se, as Na_2SeO_3. Only at the intermediate concentration, 1.0 mM (75 mg Se/kg), perlite hydroponics systems bioaugmented with axenic cultures of strains *B. mycoides* SeITE01 and *S. maltophila* SeITE02 as sole *inoculum* and as mixed culture were arranged. These two strains were previously isolated from a selenium polluted soil, and are the subject of the section 3.2 and 3.3 of the present manuscript.

3.6.2.1 Bioaugmentation with the adapted soil microbial community (I)

(a) Se bioaccumulation in plant tissues

Total selenium content determination was carried out on both roots and shoots, at the three different sampling times: T0 (at the beginning of the experimentation), T3 (after 3 weeks of incubation), T6 (after 6 weeks of incubation = at the end of the experimentation). The results obtained were shown in Fig. 30.



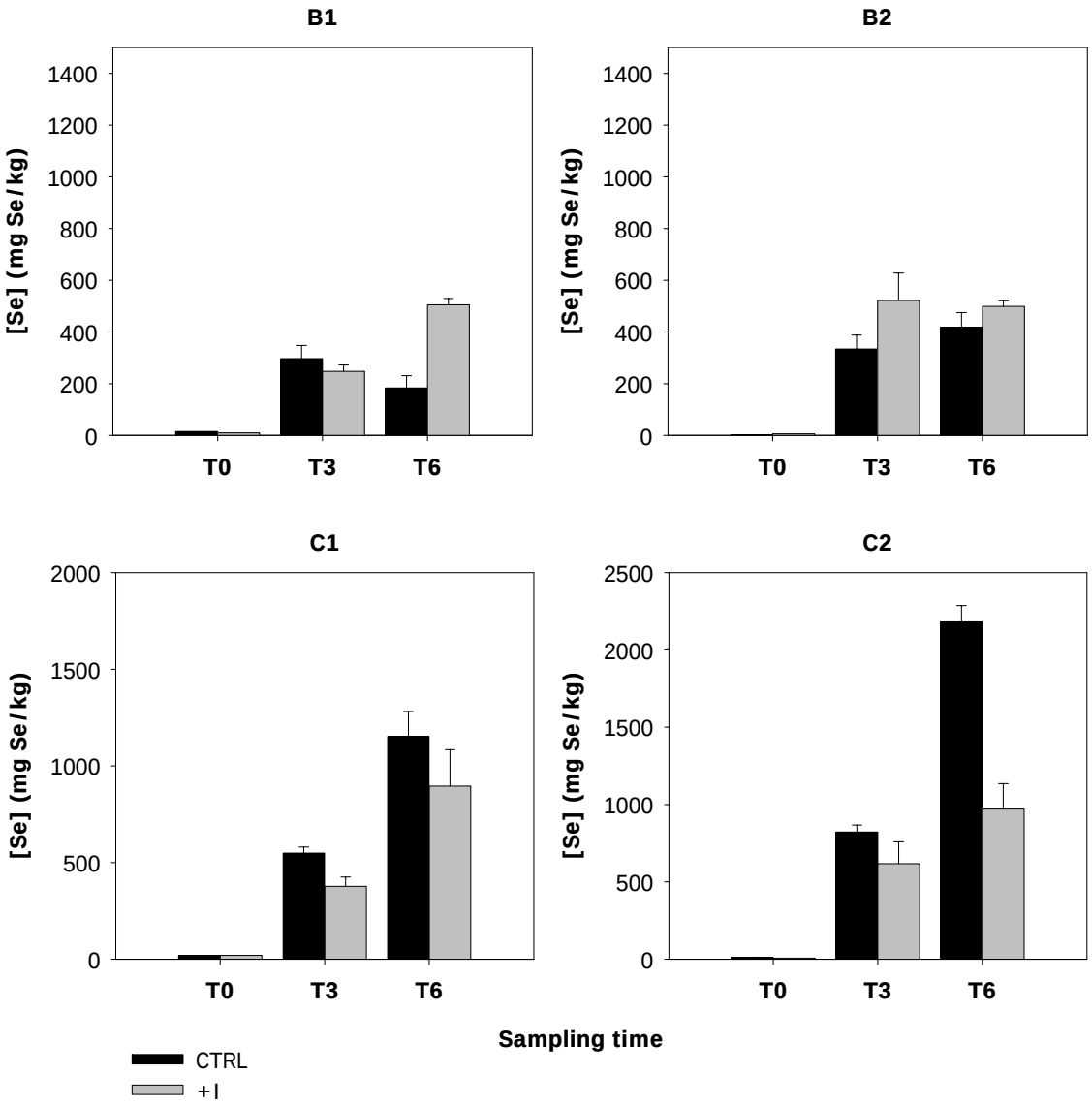


Figure 30 – Time courses of selenium contents in the shoot (**1**) and root (**2**) portions, at 15 mg Se/kg (**A**), 75 mg Se/kg (**B**) and 150 mg Se/kg (**C**) of selenite. Each value was calculated as the means of three determinations. [CTRL= plants not bioaugmented; +I=plants bioaugmeted with the adapted soil microbial community]

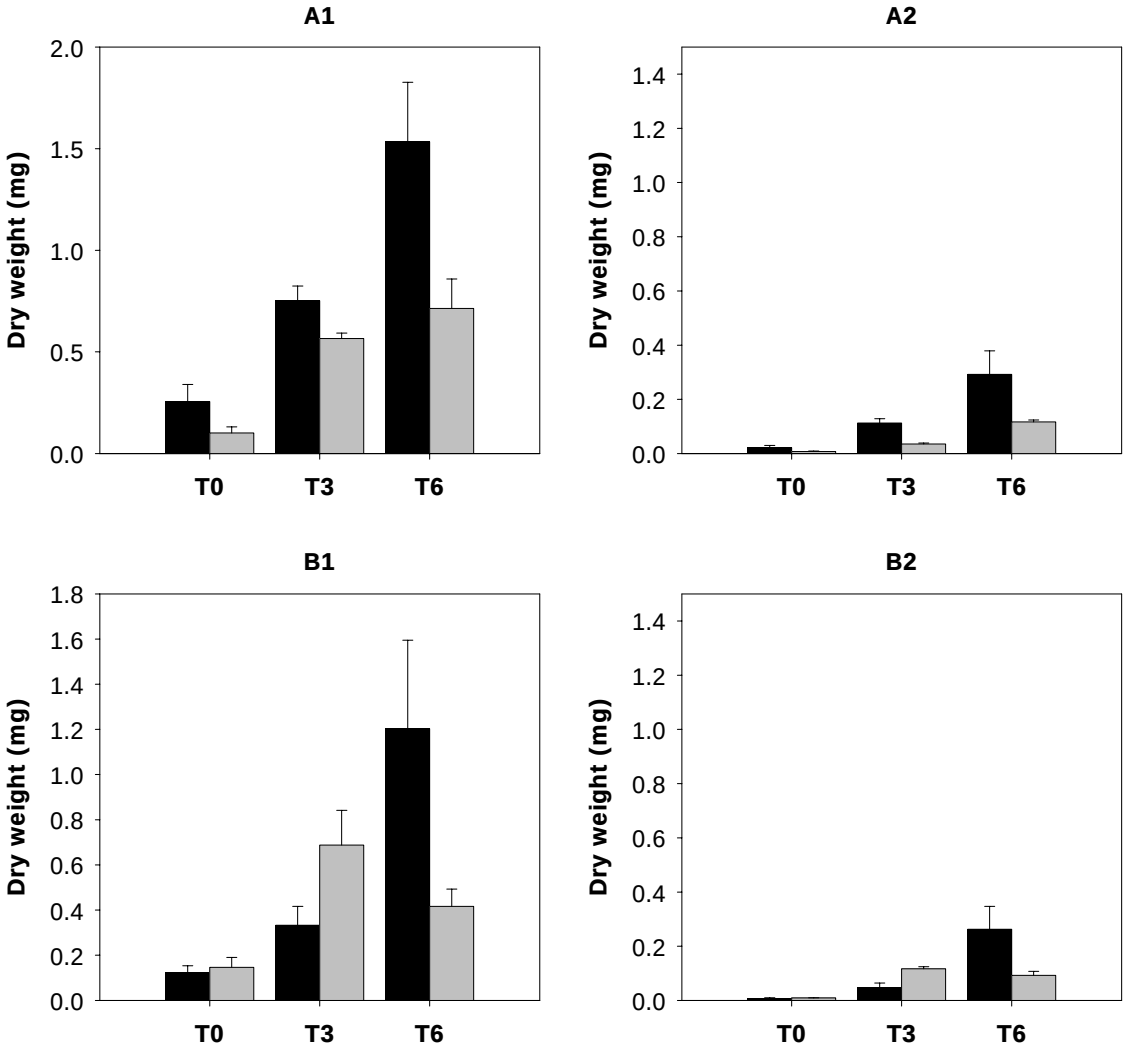
As previously observed for selenate treatment, the time courses of the total selenium contents in plant tissues indicated that selenium accumulation, both in shoots and roots, increased with the increasing of selenite concentration amended.

On the other hand, conversely to what observed for selenate tests, in presence of selenite we registered a preferential bioaccumulation of selenium in the root portion rather then in the shoot ones, either with or without soil microbial community addition, at all concentrations tested.

Moreover, the bioaugmentation with the adapted soil microbial community seemed to positively affect the plant capability to accumulate selenium both in shoots and roots at the concentrations of 15 and 75 mg Se/kg. Nevertheless at the highest selenite concentration tested (150 mg Se/kg) we obtained a decrease in plant tissues Se accumulation in the presence of soil microbial community *inoculum*, comparing to the values observed for control tests.

(b) Biomass production

Dry weight of plant tissues (roots and shoots) was recorded at the three sampling times and reported in Fig. 31.



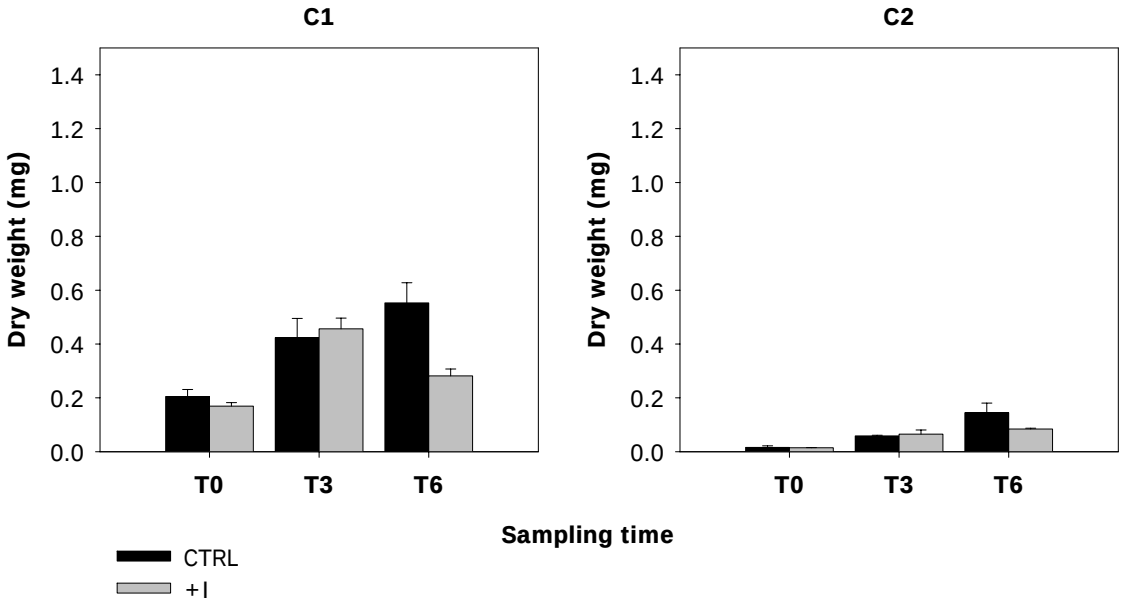


Figure 31 – Time courses of biomass production (dry weight) for shoot (**1**) and root (**2**) portions, at 15 mg Se/kg (**A**), 75 mg Se/kg (**B**) and 150 mg Se/kg (**C**) of selenite. Each value was calculated as the means of three determinations. [CTRL= plants not bioaugmented; +I=plants bioaugmented with the adapted soil microbial community]

Once again, the results obtained, showed a general decrease in biomass production with the increasing of the selenite concentrations tested both in presence and in absence of soil microbial community *inoculum*.

Furthermore, as observed for selenate tests at T6, the presence of the adapted soil microbial community *inoculum* (I) negatively affected the final plant biomass production both in shoot and root portions. Actually, regarding shoot portion, we observed a decrease in the final plant biomass production of 53.46%, 65.33% and 49.20% in presence of 15, 75, 150 mg Se/kg respectively, comparing to the values obtained for the not bioaugmented tests. On the other hand, we registered a diminishing in roots biomass of 60.30 %, 64.64 %, and 48.67% in presence of 15, 75, 150 mg Se/kg respectively, in comparison with the values observed without soil microbial community amendment.

Conversely to what observed for selenate treatment, although plants grown in presence of 150 mg Se/kg of selenite showed a strong decrease in plant biomass production, no toxicity symptoms were registered.

3.6.2.2 Bioaugmentation with strains SeITE01 and SeITE02

In order to survey the effect of Se-resistant bacterial strains, capable to transform selenium oxyanion to Se-compounds with a lower toxicity level, on phytoremediation

process of such a system, strains *B. mycoides* SeITE01 and *S. maltophilia* SeITE02 were amended as axenic cultures into perlite beds. The two strains were bioaugmented both as sole *inoculum* and as dual *consortium*. This experiment was set up only at concentration of 75 mg Se/kg (1.0 mM) selenite. Data concerning both Se accumulation in plant tissues and biomass production were registered for each sampling time and are reported in Fig. 32 and Fig. 33 respectively.

(a) Se bioaccumulation in plant tissues

Bioaugmentation with strain SeITE01 as sole *inoculum* negatively affected the accumulation of Se in plant tissues, especially in root portion. In fact we obtain a reduction of Se concentration in roots of about 50%, comparing with the value obtained for control tests. On the other hand, if we compare this result with Se accumulation in root portion registered with the bioaugmentation of the adapted soil microbial community, we observe that the *inoculum* with strain SeITE01 produced a diminishing of about 70% in Se accumulation in roots.

On the contrary, strain SeITE02 amendment as exclusive *inoculum* achieved an increase in Se accumulation in shoot portion, which resulted to be 2 folds higher than that obtained in control experiments. Nevertheless, the value registered for Se concentration in shoot portion with SeITE02 *inoculum*, resulted to be slightly lower than that obtained with the bioaugmentation of the adapted soil microbial community. On the other hand strain SeITE02 amendment seemed not to affect the Se accumulation in root portion, comparing with control tests.

Furthermore, the bioaugmentation with strain SeITE01 and SeITE02 as dual *consortium* produced Se accumulation values similar to that obtained with the inoculum of strain SeITE02 alone, both in root and shoot portions.

However, the high values obtained for Se accumulation with the amendment of the strains as axenic cultures, both as SeITE02 alone and as dual *consortium* with SeITE01, resulted to be lower than that achieved with the inoculum of the adapted soil microbial community.

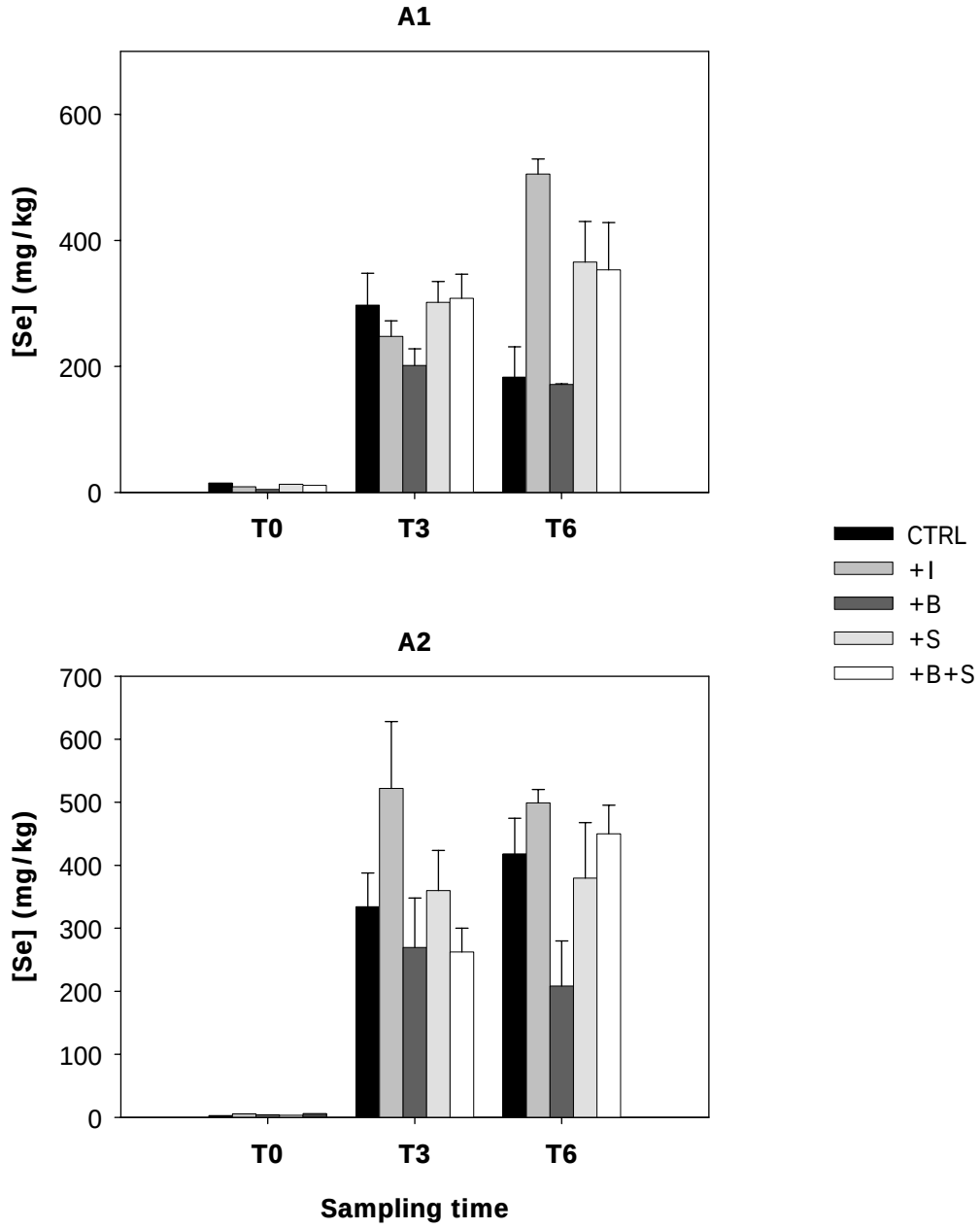


Figure 32 – Time courses of total Se contents in shoot (**A1**) and root (**A2**) portions, at 75 mg Se/kg of selenite. Each value was calculated as the means of three determinations. [CTRL=plants not bioaugmented; +I=bioaugmentation with the adapted soil microbial community; +B=bioaugmentation with strain SeITE01; +S=bioaugmentation with strain SeITE02; +B+S=bioaugmentation with the dual consortium of strains SeITE01 and SeITE02]

(b) Biomass production

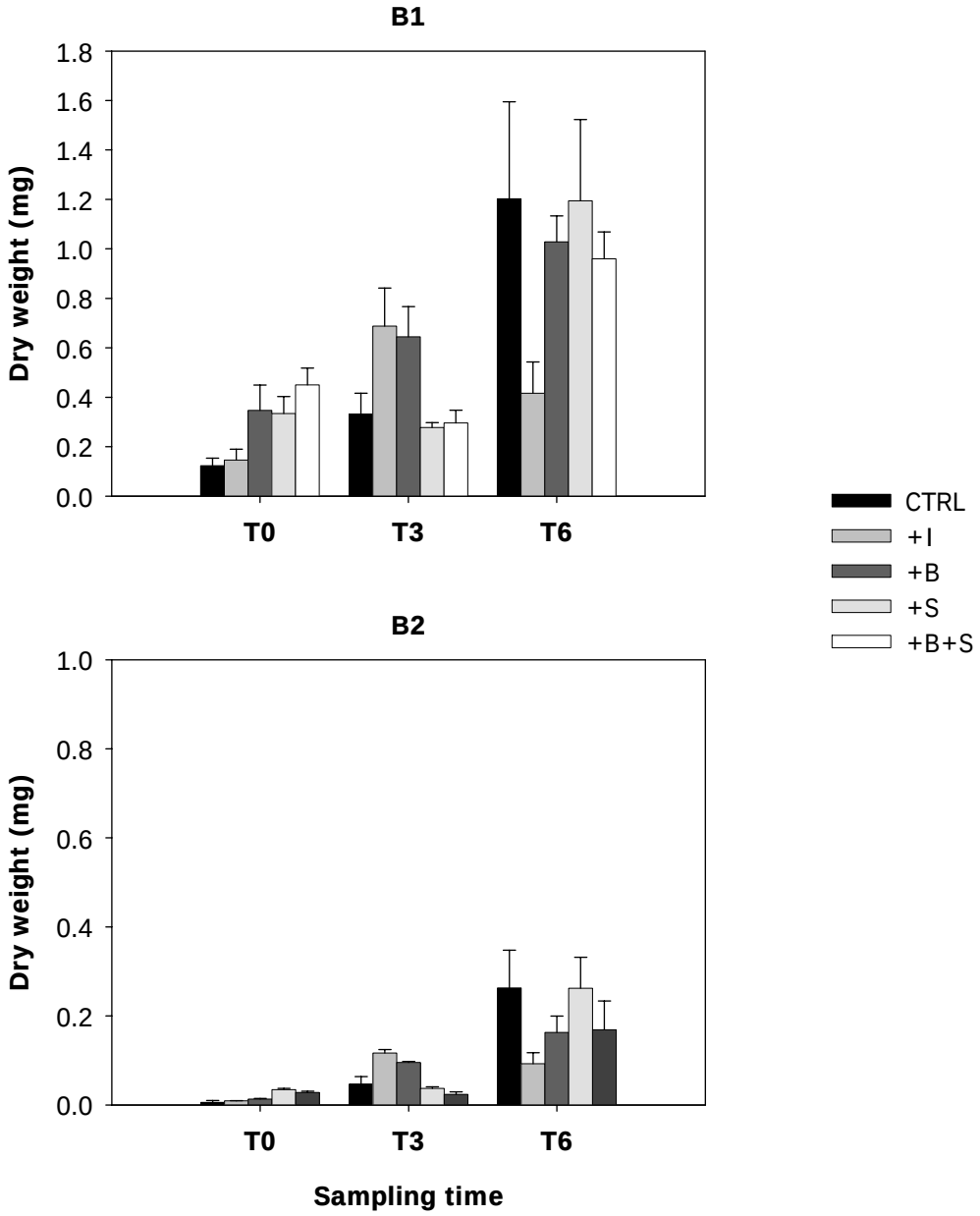


Figure 33 – Time courses of biomass production for shoot (**B1**) and root (**B2**) portions, at 75 mg Se/kg of selenite. Each value was calculated as the means of three determinations. [CTRL=plants not bioaugmented; +I=bioaugmentation with the adapted soil microbial community; +B=bioaugmentation with strain SeITE01; +S=bioaugmentation with strain SeITE02; +B+S=bioaugmentation with the dual consortium of strains SeITE01 and SeITE02]

Interestingly, the amendment with strains as axenic cultures, either as single *inoculum* or as dual *consortium*, didn't negatively affect the final biomass production both in shoot and root portions, comparing with control tests. However, better results were obtained with the bioaugmentation of strain SeITE02 as sole *inoculum*. These results are clearly in

contrast with those obtained with the amendment of the adapted soil microbial community. In that case, indeed, we registered a strong decrease in plant biomass production.

3.6.2.3 Evaluation of BF, TF and PE factors for selenite treatment

BF and TF factors were calculated as described in 3.6 and are displayed in Table 11.

Concerning Bioaccumulation Factor (BF), we observed that, as a general rule, we obtained higher values in root portions than in shoot ones at all selenite concentration tested in control tests. However, comparing with the values registered for control tests, the presence of soil microbial community *inoculum* induced an increase in BF values both for shoot and root portions at the concentrations of 15 and 75 mg Se/kg selenite. The increase resulted to be higher in shoots than in roots. In particular, the BF value for shoots at 75 mg Se/kg selenite was 2.75 folds higher than that obtained in control experiments. On the other hand, bioaugmentation with the adapted soil microbial community produced a decrease in BF values in plant tissues at 150 mg Se/kg selenite.

The amendment of strain *B. mycoides* SeITE01 achieved a strong abating in BF values comparing with those registered both in control tests and in tests inoculated with soil microbial community. Nevertheless, when strain *S. maltophilia* SeITE02 was bioaugmented both as single *inoculum* and as dual *consortium* with strain SeITE01, we obtained an increase in BF values for shoot portion. However, his increase resulted to be lower than that obtained with the augmentation of the adapted soil microbial community.

	[Se]	BF		TF
		Shoot	**Root**	
CTRL (15)	15 mg/kg	6.33	10.6	0.59
CTRL (75)	75 mg/kg	2.45	5.57	0.43
CTRL (150)	150 mg/kg	7.68	14.55	0.53
+I (15)	15 mg/kg	10.10	14.96	0.67
+I (75)	75 mg/kg	6.73	6.65	1.01
+I (150)	150 mg/kg	5.97	6.47	0.92
+B (75)	75 mg/kg	2.29	2.79	0.82
+S (75)	75 mg/kg	4.88	5.05	0.97
+B+S (75)	75 mg/kg	4.70	6.00	0.78

Table 11 - Bioconcentration Factor (BF) and Translocation Factor (TF) values for tests carried out in presence of selenite

Regarding the Translocation Factor (TF), the values registered, for control experiments, at all selenite concentrations tested, resulted to be < 1. Actually, *Brassica juncea* plants treated with selenite, accumulated Se preferentially in root portion. However, bioaugmentation of both the adapted soil microbial community and the bacterial strains as axenic cultures, gave rise to an increase of TF values, comparing to those registered for control tests. In particular, at 75 mg Se/kg selenite, with the amendment of either soil microbial community or strain SeITE02, TF values reached 1.

	[Se]	Shoot (μg)	Root (μg)	Total Se (μg)	PE
CTRL (15)	15 mg/kg	4595.09 (79.34%)	1196.67 (20.66%)	5791.76	28.95%
CTRL (75)	75 mg/kg	7064.02 (66.56%)	3549.28 (33.44%)	10613.30	10.61%
CTRL (150)	150 mg/kg	22224.89 (69.20%)	9892.53 (30.80%)	32117.42	16.06%
+I (15)	15 mg/kg	3840.26 (80.63%)	922.60 (19.37%)	4762.86	23.81%
+I (75)	75 mg/kg	7273.2 (81.47%)	1654.08 (18.53%)	8927.28	8.93%
+I (150)	150 mg/kg	7696.34 (75.07%)	2556.39 (24.93)	10252.73	5.13%
+B (75)	75 mg/kg	6283.62 (84.09%)	1188.60 (15.91%)	7472.22	7.47%
+S (75)	75 mg/kg	13288.46 (81.55%)	3005.65 (18.45%)	16294.11	16.29%
+B+S (75)	75 mg/kg	10687.63 (66.09%)	2369.65 (33.91%)	10687.62	10.68%

Table 12 - Phytoextraction Efficiency (PE) values for tests carried out in presence of selenite

However, concerning the Phytoextraction Efficiency (PE) of the whole system, we observed that tests amended with the adapted soil microbial community reported lower PE values than those obtained for control experiments, at all selenite concentrations tested (Tab. 12). This fact was mainly due to the strong decrease in biomass production caused by soil microbial community augmentation. However, it's worth noting that the best phytoextraction efficiency for selenite treatment at 75 mg Se/kg, was obtained for the test carried out in presence of strain SeITE02 as single *inoculum*. In this case, indeed, we obtained a PE value of 16.29%, which resulted to be about 1.5 and 2 folds higher than those registered for control tests and soil microbial community amended tests, respectively.

3.6.2.4 PCR-DGGE analyses

PCR-DGGE analyses were carried out on V3-16S rDNA PCR products, amplified from total DNA extracted from perlite samples collected from root systems of plant treated with selenite and inoculated either with the adapted soil microbial community or with strain SeITE01 and SeITE02, both as single *inoculum* and dual *consortium*, at the three sampling time: T0, T3 and T6.

The DGGE profiles, obtained from tests bioaugmented with the adapted soil microbial community, showed that the presence of selenite oxyanion produced interesting effects on the microbial community structure during the experimental period. These effects seem to depend on the selenite concentration applied. In particular, at the concentration of 75 mg Se/kg (Fig. 34) we observed a reduction in the number of dominant bands in the profile B2 and B3, obtained from samples collected after 3 (T3) and 6 (T6) weeks of incubations respectively, comparing with that obtained at the beginning of the experiment (lane B1). On the other hand, at 150 mg Se/kg of selenite (lanes from C1 to C3), we observed both a reduction in the number of dominant bands among the profiles obtained at different sampling times, and a redistribution of band intensity in the DGGE profile itself. Finally, at 15 mg Se/kg of selenite (lanes from A1 to A3), that is the lowest selenite concentration applied, no differences were observed among the DGGE profiles obtained at the different sampling times. Actually, conversely to what described for selenate oxyanion, selenite exerts high toxicity effects towards microorganism in aerobic conditions (Burton *et al.*, 1987).

DGGE profiles of V3-16S rDNA PCR products obtained from rhizosphere of plants bioaugmented with bacterial strains as axenic cultures, successfully permitted to monitoring the inoculated strains persistence throughout the experimental time. Once again, the presence of strain SeITE01 and SeITE02 was checked by comparing the DGGE profiles of V3-16S rDNA PCR products amplified from genomic DNA of the two strains with those resulted from the rhizosphere of inoculated plants (Fig. 35).

DGGE profiles of tests set up either with SeITE01 or SeITE02 as sole *inocula*, clearly showed the persistence of the inoculated strains during the whole experimental time (lanes D1-D3 for SeITE01 *inoculum*, lanes E1-E3 for SeITE02 *inoculum*). On the other hand, when the two strains were bioaugmented as dual *consortium* (lanes F1-F3), SeITE02-band resulted to persist until the end (T6) of the experimentation, whereas strain SeITE01-band clearly disappeared at the end of the experimental time, although it was present at the beginning and after 3 weeks of incubation.

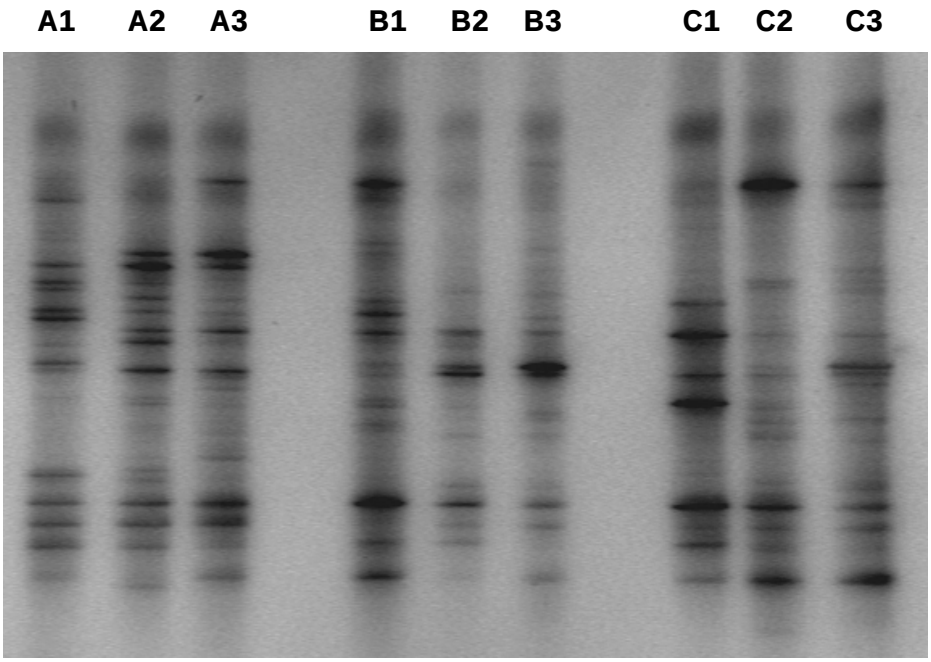


Figure 34 – DGGE profiles of samples collected from the rhizosphere of plants grown at 15 (from **A1** to **A3**), 75 (from **B1** to **B3**), and 150 (from **C1 to C3**) mg Se/kg of selenite and bioaugmented with the soil microbial community (I). Numbers correspond to the sampling time: T0 (**1**), T3 (**2**) and T6 (**3**).

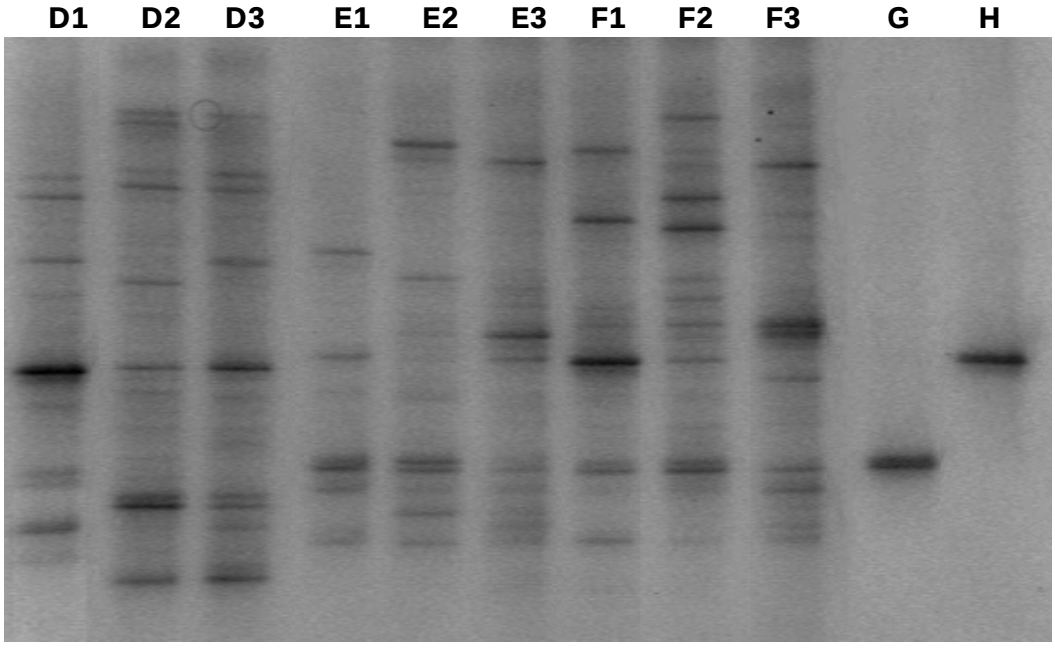


Figure 35 - DGGE profiles of microbial community V3-16S rDNA PCR products, amplified from the rhizosphere of plants grown at 75 mg Se/kg of selenite and bioaugmented with strain SeITE01 (lanes **D1- D3**), SeITE02 (lanes **E1-E3**) and SeITE01 + SeITE02 as dual *consortium* (lanes **F1-F3**). Numbers correspond to the sampling time: T0 (**1**), T3 (**2**) and T6 (**3**). Lane **H** and **G**: V3-16S rDNA PCR products amplified from strain SeITE01 and SeITE02 genomic DNA respectively.

These evidences seem to be in agreement with phytoextraction efficiencies results and with the effects exerted by the two strains on both Se bioconcentration in plant tissues and biomass production. In fact, when SeITE01 was inoculated as single culture, negatively affected both Se accumulation and plant biomass production, whereas when it was augmented as dual consortium with SeITE02, plants accumulated Se at higher concentrations in their tissues than those registered when SeITE01 was inoculated as sole *inoculum*. Moreover, the negative effect on plant biomass production achieved by SeITE01 inoculum decreased when it was inoculated as dual consortium with SeITE02. This was probably due by the fact that SeITE01 didn't persisted during the whole experimental phase. Thus, one possible explanation is that strain SeITE02 exerted an inhibitory effect on SeITE01 growth.

3.6.3 Comparative considerations on selenite and selenate treatments

Phytoremediation assays carried out by growing *Brassica juncea* plants on a 'perlite hydroponic system' resulted to be very efficient in the phytoextraction of Se oxyanions, especially of SeO_4^{2-}. Actually, plants presented higher PE values for selenate than for selenite. Moreover, plant preferentially accumulated selenate in the shoot portion, whereas selenite was bioconcentrated especially in the root ones. However, a higher efficiency in *Brassica juncea* plants phytoextraction of selenate than selenite, was expected and confirms by other authors (Terry *et al.*, 2000; Ximenez-Embun *et al.*, 2004). In fact, it has been demonstrated that selenate uptake by plant is an active process, which is carried out through sulfate uptake system, whereas selenite plant uptake occurs mainly through a passive transport process (Terry *et al.*, 2000; Zayed *et al.*, 1998). Moreover, plants treated with selenate, accumulated higher Se concentrations in shoot than in root portion, whereas plants treated with selenite concentrated Se preferentially in roots. Nevertheless, also these results were expected by other works present in literature (Terry *et al.*, 2000; de Souza *et al.*, 1998; Zayed et al., 1998).

It's worth noting that the *inoculum* either with the adapted soil microbial community or with the bacterial strains as axenic cultures, increased both BF and TF values for plants treated both with selenate and selenite, with some differences. Actually, in presence of selenate, Se accumulation increase was higher in shoot portion than in shoot ones, whereas selenite was amended, plants increased Se accumulation both in shoos and roots. Thus plants treated with selenate showed higher TF values that those grown in presence of selenite. Similar results were reported by de Souza and coworkers (1999) for

Brassica juncea plants grown in presence of selenate and augmented with a rhizosphere microbial community. Nevertheless, the most interesting result is represented by the increase in Se uptake and translocation registered for plants treated with selenite and bioaugmented with the bacterial *inocula*.

The higher Se contents accumulated by plant bioaugmented either with the adapted soil microbial community or with bacterial strains, could be explained by the fact that these microorganisms probably detoxified the system, by transforming toxic Se oxyanions into Se compounds with lower toxicity level. Otherwise microbial *inocula* may have transformed selenium oxyanions into organic Se compounds more easily absorbed and accumulated by plants, such as Se-methionine (Terry *et al.*, 2000).

On the other hand, bioaugmentation with the adapted soil microbial community induced a strong decrease in plant biomass production in presence of both selenate and selenite. This fact could be due to a sort of competitive effect for nutrients exerted by microbial *inoculum* towards the plant (Nehl *et al.*, 1996). On the other hand, the decrease in biomass production could be also due to the higher Se content, which in turn could produce a toxicity effect on plant growth. Actually, at the highest Se concentrations we obtained the lowest plant biomass production. Furthermore at the highest selenate concentration (150 mg Se/kg) we observed the appearance of toxicity symptoms such as the appearance of violet colour on the edge of leaves, and a shorter distance in the internodes.

However, best results were obtained with bioaugmentation of strain SeITE02. actually, when SeITE02 was inoculated in presence of selenite we observed an increase in selenite uptake and translocation in plant tissues without registering a decrease in plant biomass production.

4. CONCLUDING REMARKS

Se forms, such as SeO_3^{2-} (selenite) and SeO_4^{2-} (selenate) oxyanions, are widespread in the environment due to the great Se utilization in industrial processes and in several human activityes, such as combustion of fossil fuels, incineration of municipal waste, copper/nichel production, lead and zinc smelting, iron and steel production, crop-fallow and irrigation practices, and fertilizers production. Moreover, SeO_3^{2-} (selenite) and SeO_4^{2-} (selenate) result to be the most bioavailable Se forms in the environment, and they have been considered to be the most toxic Se species. Thus far, physicochemical metods such as chemical precipitation, catalytic reduction, and ion-exchange have been mainly used for removing soluble Se from industrial wastewater. While these methods are suitable for the removal of selenite, they are not effective in the removal of selenate (Kashiwa *et al.*, 2000). Furthermore, physicochemical methods are costly. SeO_3^{2-} (selenite) and SeO_4^{2-} (selenate) can be transform, through biotic processes, in the elemental Se form or can be removed from contaminated matrices through the employment of an extracting system, like a plant species capable of accumulate them in its tissues.

Since Se^0 is non-toxic and can also be easily removed from the aqueous phase on account of its insoluble characteristic, this process can be applied to develop an economical process for the removal of soluble Se. Actually in this study, 3 different Se-resistant strains have been isolated from the rhizosphere of a Se-accumulating plant grown on a Se polluted soil and two of them, namely *Bacillus mycoides* SeITE01 and *Stenotrophomonas maltophilia* SeITE02 were further characterized for their capability to reduce selenite oxyanion to the elemental selenium form.

Although selenium exerted a toxic effect on cellular metabolism, the ability of these strains to reduce selenite to Se^0 offers a possibility to biologically precipitate this metalloid from selenium laden effluents. On the other hand, the important role of the rhizosphere microorganisms in phytoremediation processes in terms of both plant growth and Se plant uptake, suggests the possibility of employing these strains in an integrated plant-microbe system for the remediation of Se polluted waste waters.

Thus, these strains were effectively employed in two systems for the biological removal of soluble selenium forms on a lab scale: a Sequencing Batch System for the removal of selenite from aqueous solution and a ‘perlite hydroponic system’ for a plant-assisted phytoremediation process.

In particular, *B. mycoides* SeITE01, which resulted to be more efficient in SeO_3^{2-} reduction than *S. maltophilia* SeITE02, was successfully utilized for a selenite removal process set

up in a sequencing batch mode. Actually, *Bacillus mycoides* SeITE01 was grown in a rich medium supplemented with 0.5 mM SeO_3^{2-} and effectively transformed 0.5 mM of selenite into elemental Se within a 24-h cycle over at least 10 cycles, maintaining the cell density relatively stable. Thus, *Bacillus mycoides* strain SeITE01 has revealed to be a promising candidate for application in biological reaction systems for the treatment of waste water with high Se content. However, further sperimentations are necessary in order to optimize the selenium removal process. In fact, new experiments in which different growth media and selenite concentrations are tested, are now in progress.

On the other hand, the plant-assisted phytoremediation process built up on a 'perlite hydroponic system' utilizing *Brassica juncea* as Se extracting agent, was successfully carried out for the remediation of Se oxyanions from aqueous solutions. Promising results were obtained when the system was bioaugmented with selenium-resistant bacteria. In particular, best results in terms of selenite phytoextraction efficiency were obtained when the system was bioaugmented with *Stenotrophomonas maltophilia* strain SeITE02. On the basis of preliminary evidences from the present study, it appears worth of attention the hypothesis to apply selenite/selenate resistant and reducing bacteria in hydroponic systems for treating selenium-laden water streams. In fact, the results obtained open interesting perspective for the application of a continuous bioreactor in which the synergic action of Se-accumulating plants, such as *B. juncea*, which absorb and concentrate the metalloid in their roots and shoots and rhizosphere colonising bacteria can be successfully exploited.

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CHAPTER I

Selenite precipitation by a rhizospheric strain of *Stenotrophomonas* sp. isolated from the root system of *Astragalus bisulcatus*: a biotechnological perspective

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Selenite precipitation by a rhizospheric strain of *Stenotrophomonas* sp. isolated from the root system of *Astragalus bisulcatus*: a biotechnological perspective

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Abstract

A bacterial strain (SeITE02), related to the species *Stenotrophomonas maltophilia* and resistant to selenite (SeIV) up to 50 mM in the growth medium, was isolated from rhizospheric soil of a selenium hyperaccumulator plant, the legume *Astragalus bisulcatus*. The influence of SeIV on the active growth of this Se-tolerant bacterial strain has been investigated in oxic conditions, along with the isolate's ability to reduce selenite to elemental selenium (Se^0). Interestingly, concentrations of 0.5 mM SeIV were wholly reduced by strain SeITE02 in liquid culture within 52 h. Moreover, 87% of SeIV added to the growth medium at the initial concentration of 2.0 mM underwent again reduction in 120 h. Actually, a selenite-mediated induction of a sort of adaptive response to detrimental SeIV effects magnified the efficiency of SeITE02 in reducing this toxic oxyanion. Furthermore, the SeIV influence on cell morphology of strain SeITE02 was evidenced by phase-contrast and electron microscopy analyses. In particular, transmission electron microscopy (TEM)–energy-dispersive X-ray (EDX) analysis of *S. maltophilia* strain SeITE02, grown in presence of SeIV, showed electron-dense Se^0 granules either in the cell cytoplasm or in the extracellular space. Therefore, the capability of strain SeITE02 to quickly reduce soluble and harmful SeIV to insoluble and unavailable Se^0 may be looked at as a promising exploitable option for the setup of low-cost biological treatments tailored to manage contamination in selenium-laden effluents.

Keywords: *Astragalus bisulcatus*; Rhizosphere bacteria; Selenite reduction; Selenite-resistant bacteria; Selenite toxicity; *Stenotrophomonas* sp.

1. Introduction

Selenium is a naturally occurring metalloid which exerts beneficial effects on biological systems as trace element (Shamberger, 1983). Nevertheless, even apparently low concentrations of selenium in the order of few ppm can provoke health disturbances in animals, livestock, and humans (Vinceti et al., 2001). Therefore, increasing levels of selenium in environmental matrices such as soils, freshwater and groundwater, sediments, and wastes may become troublesome to living organisms. Not to mention natural release mechanisms such as erosion or leaching of seleniferous minerals, selenium enters the environment mostly as a consequence of coal mining, oil refining, combustion of fossil fuels, and several industrial activities, namely glass manufacturing, a variety of chemical syntheses as well as varnish and pigment formulation.

Selenium toxicity is strictly related to the intrinsic bioavailability of its different chemical species. In oxic habitats, selenium is present mainly in form of water-soluble and toxic oxyanions represented by selenite (SeO_3^{2-}, SeIV) and selenate (SeO_4^{2-}, SeVI). On the other hand, elemental selenium (Se^0), which is insoluble and harmless, occurs as prevalent species in anaerobic conditions (e.g., anoxic sediments; Barceloux, 1999).

SeIV derived from refining processes of sulphur-containing crude oils (Lawson and Macy, 1995) represents a relevant environmental pollutant in seawater, agricultural

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soils, and groundwaters (Nriagu and Pacyna, 1988). In natural habitats, reduction of SeIV to Se^0 largely occurs through biotic mechanisms. In particular, bacteria use this reaction to overcome toxic effects of selenite (Moore and Kaplan, 1992). Different species of microorganisms have been described as capable of reducing toxic SeIV to nontoxic Se^0 (Dungan et al., 2003; Kessi et al., 1999; Losi and Frankenberger, 1997; Rathgeber et al., 2002; Roux et al., 2001; Tomei et al., 1992; Van Fleet-Stalder et al., 2000; Yamada et al., 1997).

The mechanisms through which SeIV exerts its toxicity in microbes are far from being well understood. Nevertheless, different authors relate selenite toxicity to the strong oxidising effect generated by SeIV reduction to Se^0 which ends to negatively influence the microbial cell metabolism (Bebien et al., 2002; Touati, 2000).

The present study focuses on the capability of reducing SeIV to Se^0 in oxic conditions, shown by a microbial isolate belonging to the γ-proteobacteria. This bacterial strain (SeITE02), related to the genus *Stenotrophomonas* sp., has been obtained in pure culture through enrichment of soil samples from the rhizosphere of the selenium hyperaccumulator *Astragalus bisulcatus* (Neuhierl and Boek, 1996). The capacity of SeITE02 to rapidly reduce SeIV to Se^0 opens possible scenarios for the exploitation of such a bacterial strain in remediation protocols for the treatment of selenium-bearing wastewaters.

2. Materials and methods

2.1. Chemicals

Chemicals purchased from Sigma-Aldrich (Milan, Italy) were all analytical grade.

2.2. Culture media

Nutrient Broth and Bacteriological Agar were furnished by Oxoid Italia Spa (Garbagnate Milanese, Italy). Mineral medium (DM) was prepared as described by Frassinetti et al. (1998).

2.3. Isolation of bacterial strain SeITE02

Enrichment cultures for bacterial isolation were inoculated with soil samples from the rhizosphere of *A. bisulcatus*, a leguminous plant grown throughout six months in seleniferous soil collected at a mine site in Sardinia, Italy (Campostrini et al., 1999). *A. bisulcatus* seeds were obtained from the Western Regional PI Station, Washington State University, Pullman, USA. Enrichment cultures were carried out in 250-ml Erlenmeyer flasks containing 100 ml Nutrient Broth amended with 0.2 mM SeIV sodium salt. Flasks were incubated at 28 °C on an orbital shaker (250 rev/min). After 1-week incubation, serial dilutions of the culture medium were plated on agarised Nutrient Broth plates added with 0.2 mM SeIV sodium salt. These plates were incubated at 28 °C for 5 days. Appearance of red-coloured colonies was interpreted as an indication for SeIV reduction to Se^0 (Moore and Kaplan, 1992; Sabaty et al., 2001). Red colonies were then isolated and streaked on fresh agarised Nutrient Broth plates containing SeIV sodium salt as a tool of selective pressure, in order to get cultures axenic. Pure cultures of three morphologically different bacteria were obtained. Minimum inhibitory concentration (MIC) for SeIV was determined either on DM or on agarised Nutrient Broth plates. Once exposed to SeIV, microbial cells were checked for their viability after 5 days of incubation on plates containing increasing concentrations of selenite. The strain then referred to as SeITE02 revealed the highest value of MIC for SeIV and was further characterised.

2.4. Phylogenetic analysis of strain SeITE02

Microbial genomic DNA of SeITE02 was extracted with the NucleoSpin Tissue Kit (Clontech) following the manufacturer's instructions. Amplification of the gene encoding for the 16*S* rRNA (rDNA) was performed with the primers F8 and R11 (Weisburg et al., 1991).

The product of amplification was directly double-strand sequenced, aligned to the database sequences using BLASTN (Altshul et al., 1997), and analysed using the ARB database (release June 2002) (Strunk O. and Ludwig W., 1993–2002), ARB—a software environment for sequence data, http://www.arb-home.de).

2.5. Biochemical and physiological properties of strain SeITE02

Physiological and biochemical assays were carried out as described in Gerhardt et al. (1981). Growth tests at different temperatures and pH values were performed in Nutrient Broth. Assimilation capability was evaluated in DM amended with different carbon sources (0.1% wt./vol.).

2.6. Morphological and cytological features of strain SeITE02

Gram staining was performed as described in Gerhardt et al. (1981). Cell size and shape were determined by observation at the phase-contrast microscope (Leitz/Leica DMR-HC Series) and through transmission electron microscopy (TEM) starting from samples of microbial cultures grown either in Nutrient Broth or in Nutrient Broth amended with 0.2, 0.5, and 2.0 mM SeIV sodium salt, respectively. In order to obtain thin sections for electron microscopy analysis, bacterial cells were embedded in Epon-araldite resin after fixation with 2.5% glutaraldehyde and 1% osmium tetroxide, as described by Hess (1966). Sections

were prepared by means of a Reichert Ultracut S ultramicrotome (Leica) equipped with a diamond knife. Uranyl acetate and lead citrate were used as contrast agents. Observations and energy-dispersive X-ray (EDX) analyses were performed with a high resolution electron microscope (JEOL JEM 2010) operated at high accelerating voltage (200 kV) and equipped with an Inca 100 Link analysis system.

2.7. Microbial growth estimation

SeITE02 cell growth was evaluated in Nutrient Broth (100 ml) amended with 0.2, 0.5 or 2.0 mM selenite following three different procedures, namely: (a) count of colony forming units (CFU) on agarised Nutrient Broth plates seeded with aliquots of bacterial cultures; (b) measure of culture turbidity by reading absorbance at 600 nm; and (c) quantification of total protein content of microbial biomass from different cell suspensions. All analyses were performed in triplicate.

Protein concentration in bacterial cell extracts was determined by using the Bradford reagent (BioRad) according to the manufacturer's instructions. An aliquot of cells corresponding to 1 ml of bacterial culture was centrifuged at 5000×*g* for 10 min. The pellet was resuspended in 100 μl of extraction buffer (50 mM Na_2HPO_4, pH 7; 10 mM β-mercaptoethanol; 10 mM Na_2-EDTA; 0.1% Sodium Lauryl Sarcosine; 0.1% Triton X-100). The resulting suspension was sonicated (Ultrasonic Cleaner, Soltec) for 5 min, stored on ice for 5 min and centrifuged at 18,000×*g* for 10 min at 4°C. The supernatant was collected and measured for protein content. Protein extraction was carried out in triplicate, whereas all spectrophotometric measurements (Heλios β, Unicam) were done in duplicate.

SeITE02 growth in presence of selenite was checked vs. control cultures incubated in Nutrient Broth with no SeIV sodium salt added.

2.8. SeIV content determination

SeIV concentration in culture medium was determined by reading absorbance at 377 nm of the selenium-2,3-diaminonaphthalene complex in cyclohexane as described by Kessi et al. (1999). Sterile cultures were also tested for SeIV concentration as negative controls.

2.9. Activation of adaptive response in strain SeITE02

Bacterial cultures in stationary phase grown in presence of 0.2 mM SeIV sodium salt were used to inoculate either 100 ml of Nutrient Broth or 100 ml of Nutrient Broth amended with 0.5 and 2.0 mM SeIV sodium salt. Microbial growth of the adapted inoculum and SeIV content in the culture medium were evaluated as previously described.

3. Results

3.1. Isolation and identification of SeITE02 and evaluation of SeIV reducing capability

Three different bacterial strains were isolated from the rhizosphere of the selenium hyperaccumulator legume *A. bisulcatus*. Strain SeITE02 showed the highest tolerance to selenite (up to 50 mM) in both Nutrient and DM plates; it was thus further characterised. Molecular taxonomy results suggested for the attribution of strain SeITE02 to the genus *Stenotrophomonas* sp. Actually, close clustering with the species maltophilia (99% identity with *Stenotrophomonas maltophilia* VUN10-075, AF100734) was recognised. A list of biochemical and physiological features of strain SeITE02 are summarised in Table 1.

Strain SeITE02 formed reddish cell suspensions revealing the capacity to reduce selenite to Se^0, as a consequence of the growth under aerated conditions in liquid medium added with SeIV (Moore and Kaplan, 1992; Sabaty et al., 2001). It is worth noting that this colour was maintained throughout a long span. Actually, cell suspensions, after 3 or 4 weeks of incubation, still manifested the bright red aspect as that already observed after the first 120 h of cultivation.

Selenite toxicity to strain SeITE02 was evaluated in liquid culture (Nutrient Broth) by checking for bacterial cell replication in presence of increasing concentrations of SeIV (Fig. 1). At all concentrations tested (0.2, 0.5, and 2.0 mM), SeIV negatively affected microbial growth. As a general rule, SeITE02 cell replication decreased with the increase of SeIV concentration in the culture medium. Cultures added with 0.2 mM SeIV evidenced a dynamic cell growth similar to that of SeITE02 cultured in absence of the oxyanion; however, in stationary phase, lower values were reached. With 0.5 and 2.0 mM SeIV in the culture medium, a marked falling of bacterial cell replication was registered between the 6th and 24th hours. Afterwards, a partial recovery took place, with microbial growth reaching values comparable to those observed in presence of 0.2 mM SeIV. Nevertheless, a retrieved loss of cell replication was observed with 0.5 and 2.0 mM SeIV in the culture medium, after 96 and 120 h of incubation, respectively.

Because of selenite reduction to the red allotropic form Se^0 (Moore and Kaplan, 1992; Sabaty et al., 2001), strain SeITE02 induced a progressive decrease of SeIV concentrations initially added to the culture medium (Fig. 1). Meanwhile, SeIV was totally recovered in sterile control cultures suggesting for a biologically mediated selenite reduction.

Strain SeITE02 completely reduced selenite within 52 h, when SeIV concentration in the growth medium was 0.2 or 0.5 mM. In the presence of 0.2 mM SeIV, reduction started at the early exponential phase, lasting until the initial stationary phase was reached. With 0.5 mM SeIV in the culture medium, reduction occurred during the exponential growth phase, throughout the interval of depressed cell

Table 1
Morphological and physiological characteristics of *Stenotrophomonas* sp. strain SeITE02

Characteristic	SeITE02
Cell morphology (size [μm])	Short ovoid rod, (0.5×1.0)
Color and type of colonies:	
without metal addition	Opaque, creamy
with SeIV addition	Bright red
Fluorescent pigment	–
Growth at 4 °C	–
Growth at 18 °C	+
Growth at 28 °C	+
Growth at 37 °C	+
Growth at 42 °C	–
Growth at pH 4.5	–
Growth at pH 7.2	+
Growth at pH 9	–
Oxidase activity	–
Catalase activity	+
Lipase activity	+
Urease activity	+
Methyl red test	+
V/P test	+
Nitrate reduction	+
Utilization of:	
Acetate	–
Caprylate	–
Citrate	–
Gluconate	+
Malate	+
Oxalate	–
Succinate	+
Arabinose	–
Fructose	+
Galactose	+
Glucose	+
Lactose	+
Maltose	+
Mannitol	+
Mannose	+
N-acetyl-glucosammine	–
Raffinose	+
Rhamnose	+
Saccharose	–
D-xylose	+
Hydrolysis of:	
Starch	+
Gelatin	+
Esculin	–

growth observed between the 6th and 24th hours. Within this span, 50% of the initial SeIV was reduced, the remaining being reduced during the recovery of cell active growth from the 24th to 48th hours. At SeIV concentration of 2.0 mM, reduction of the oxyanion was slower than what verified at 0.2 and 0.5 mM SeIV. In this case, selenite concentration drastically dropped between the 72nd and 120th hours. Reduction proceeded slowly during the early exponential phase (first 6 h), although this metabolic activity was not significantly delayed by low rate growth of the strain SeITE02 between the 6th and 24th hours. During this interval, 10% of initial SeIV was reduced while a further 77% of SeIV was transformed throughout the stationary phase. Any reductive activity on selenite stopped after 120 h of cultivation. At this time, 87% of the initial SeIV added had been reduced. However, the bacterial

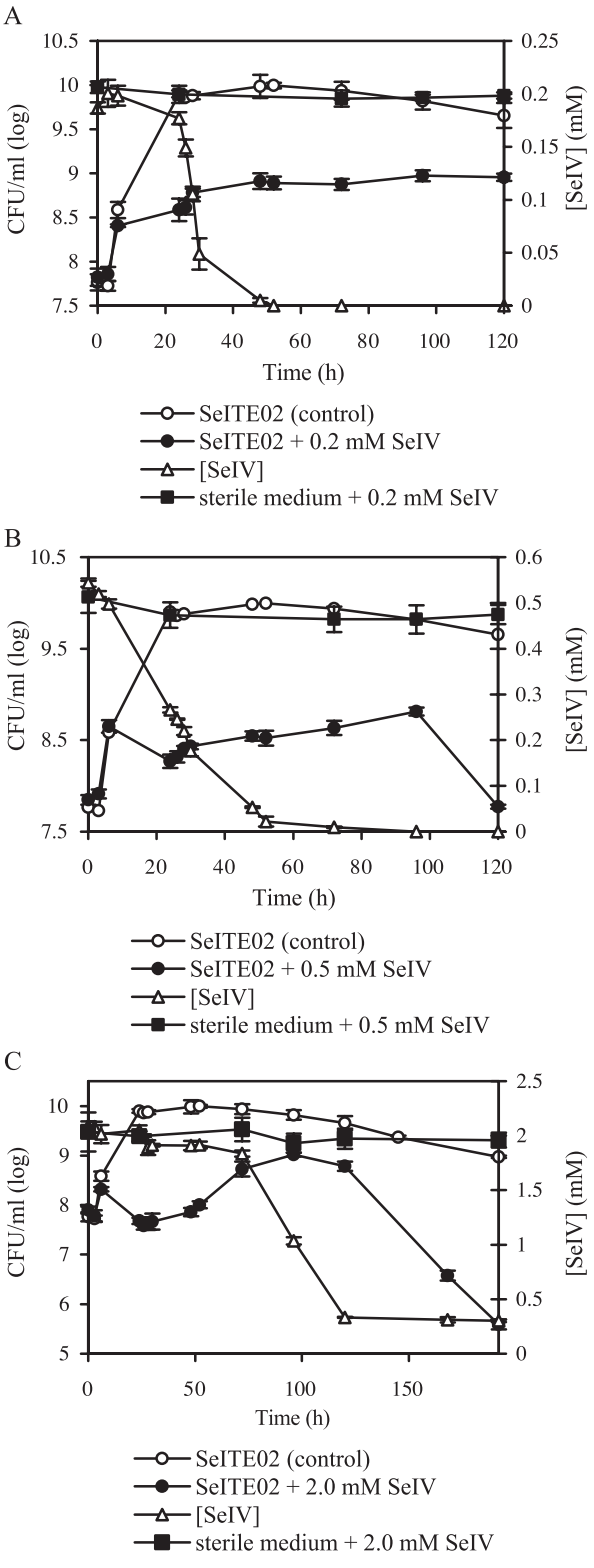

Fig. 1. Time courses of microbial growth and SeIV reduction by SeITE02, in presence of (A) 0.2, (B) 0.5, and (C) 2 mM SeIV. Each curve shows means based on the results of three experiments.

culture was no longer able to grow and to sustain the reduction of the oxyanion.

3.2. Induction of adaptive response to SeIV toxicity in strain SeITE02

Efficiency of strain SeITE02 in reducing selenite to Se^0 and bacterial growth in the presence of 0.5 and 2.0 mM SeIV were monitored after preinduction of microbial cultures with 0.2 mM SeIV. As shown in Fig. 2, pretreated SeITE02 cultures did not fully recover the replication rate of cells grown with no selenite added to the growth medium. Nevertheless, despite the decrease of bacterial growth between the 6th and 24th hours in not preinduced cultures, preincubation with 0.2 mM SeIV allowed strain SeITE02 to reach the stationary phase after 24 h, whether it was cultured in presence of 0.5 or 2.0 mM SeIV. With 0.5 mM SeIV added to the growth medium, the rate of selenite reduction was not significantly affected by pretreatment of SeITE02 cells. In these growth conditions, selenite reduction started during the exponential phase lasting until the early stationary phase, according to what was observed in not pretreated cultures. On the other hand, with 2.0 mM SeIV in the culture medium, preinduction of SeITE02 cells resulted in a significant increase of SeIV reduction rate while total depletion of selenite in the substrate occurred in only 52 h. SeIV reduction was observed starting from the exponential growth phase throughout the initial stationary phase.

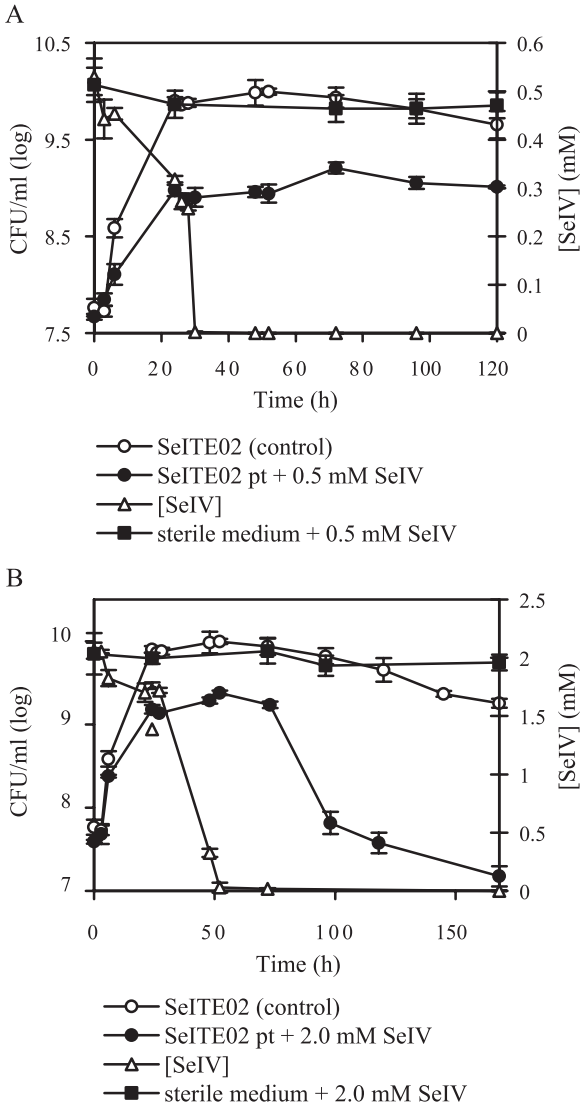

Fig. 2. Time courses of microbial growth and SeIV reduction by SeITE02 pretreated with 0.2 mM SeIV (pt, pretreated), in presence of (A) 0.5 and (B) 2 mM SeIV. Each curve shows means based on the results of three experiments.

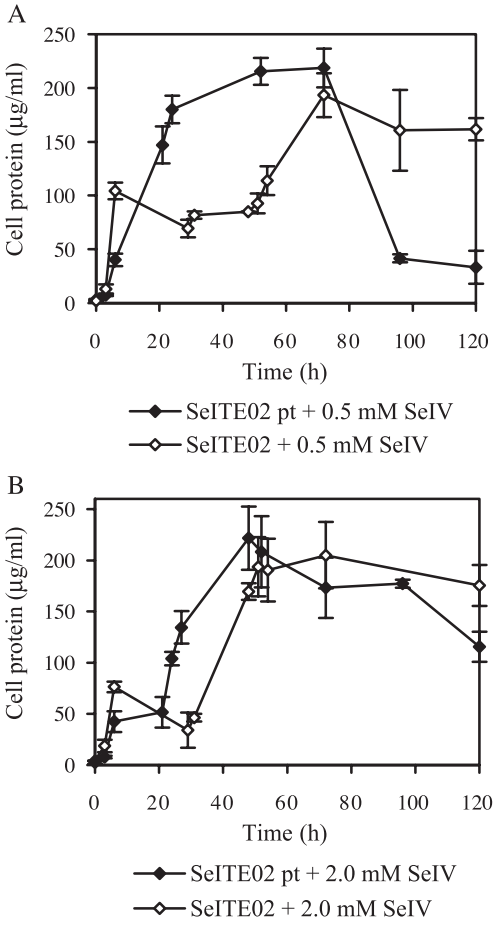

Fig. 3. Time courses of total protein content of SeITE02 suspension culture pretreated and not pretreated with 0.2 mM SeIV (pt, pretreated), in presence of (A) 0.5 and (B) 2 mM SeIV. Each curve shows means based on the results of three experiments.

The influence of cell pretreatment on bacterial cell replication at 0.5 and 2.0 mM SeIV in the growth medium was further evaluated by measuring total protein concentration of strain SeITE02 suspensions. Evidence exists that preinduction of bacterial cultures avoided the decrease of active growth as observed in not pretreated cells between the 6th and 24th hours (Fig. 3).

Quantification of turbidity of strain SeITE02 cultures amended with 0.5 and 2.0 mM SeIV was also carried out (Fig. 4). At 0.5 mM SeIV, culture turbidity was lower than that recorded with no SeIV added to the growth medium.

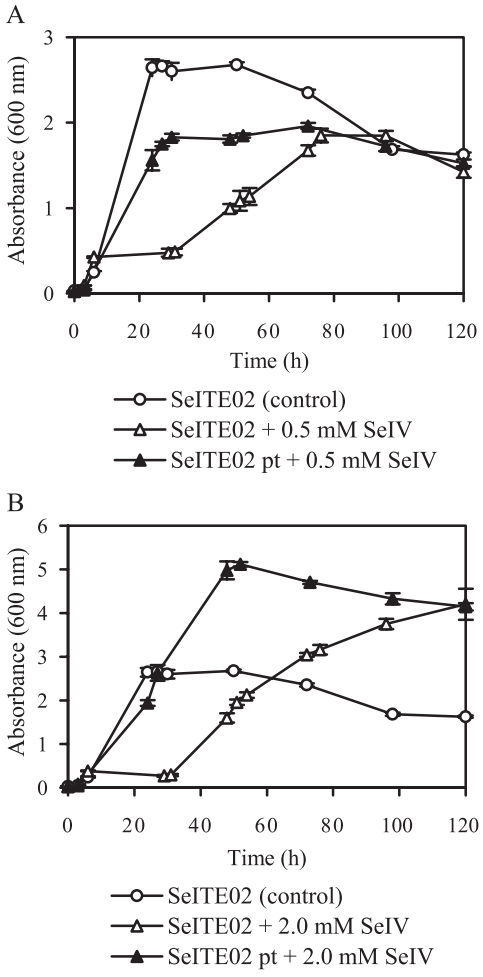

Fig. 4. Time courses of the turbidity increment of SeITE02 suspension culture pretreated and not pretreated with 0.2 mM SeIV (pt, pretreated), in presence of (A) 0.5 and (B) 2 mM SeIV. Each curve shows means based on the results of three experiments.

Nevertheless, a slow increment in turbidity was observed with a maximum after 78 h. In cultures added with 2.0 mM SeIV, turbidity rapidly increased, after a lag phase of 30 h, reaching values higher than those observed in cell suspensions grown with no SeIV in the medium. Pretreatment of bacterial cultures in presence of 0.2 mM SeIV caused a prompt increment in turbidity. In the case of recultivation with 0.5 mM SeIV in the substrate, values comparable to those of nontreated cell suspensions were recorded. At 2.0 mM SeIV in the medium, turbidity of cultures increased without any lag phase. In this case, absorbance rapidly reached values higher than those observed in not preinduced cells. Actually, it is worth noting that, within 48 h, turbidity of pretreated cultures was nearly twice that observed during the stationary phase of SeITE02 cells grown with no SeIV in the growth medium.

3.3. Phase-contrast and transmission electron microscopy/ EDX analyses

Effects of SeIV added to the growth medium on cell morphology and clustering behaviour of strain SeITE02 were evidenced by phase-contrast microscopy analyses (Fig. 5). Actually, the isolate which normally replicates as separate oblong rods generated more elongated cells, jointed in chains and evolving progressively in a complex aggregate entrapping light-refractive selenium granules. These clumps of bacterial cells and selenium granules never occurred in control cultures. At 0.2 and 0.5 mM SeIV in the medium, clumps were recorded starting from early steps of growth. Conversely, at 2.0 mM SeIV in the substrate, those peculiar formations were observed after 48 h of cultivation. Clumps increased in size along with the progressive reduction of SeIV.

EDX analysis of bacterial cells grown in presence of SeIV (Fig. 6) revealed electron-dense granules of Se^0 inside the cell cytoplasm (after 48 h). Identical globules could be observed also in the extracellular space (after 120 h). On the other hand, the same electron-dense granules were never detected in cell cultures that had received no SeIV addition in the growth medium.

4. Discussion

The bacterial isolate here reported as strain SeITE02 has been attributed to the species *S. maltophilia*. *S. maltophilia*,

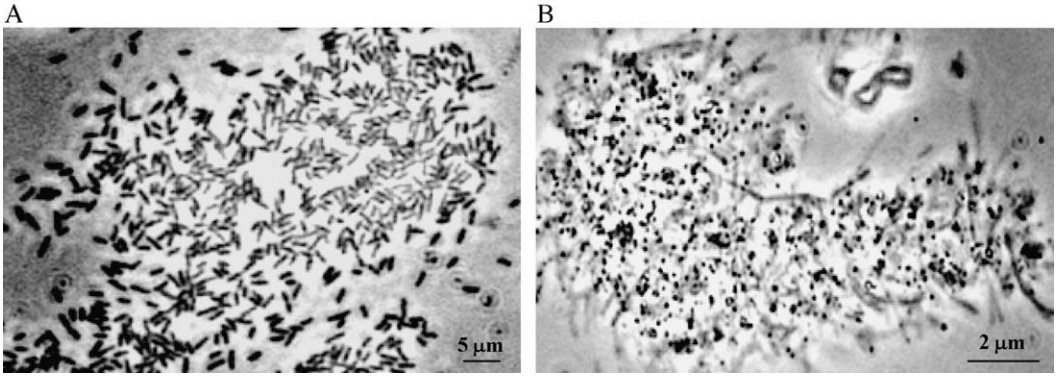

Fig. 5. Phase contrast micrographs of SeITE02 suspension culture grown in absence (A) and presence (B) of 2 mM SeIV. In panel (B), elemental selenium globules (dark bodies) are present as extracellular particles.

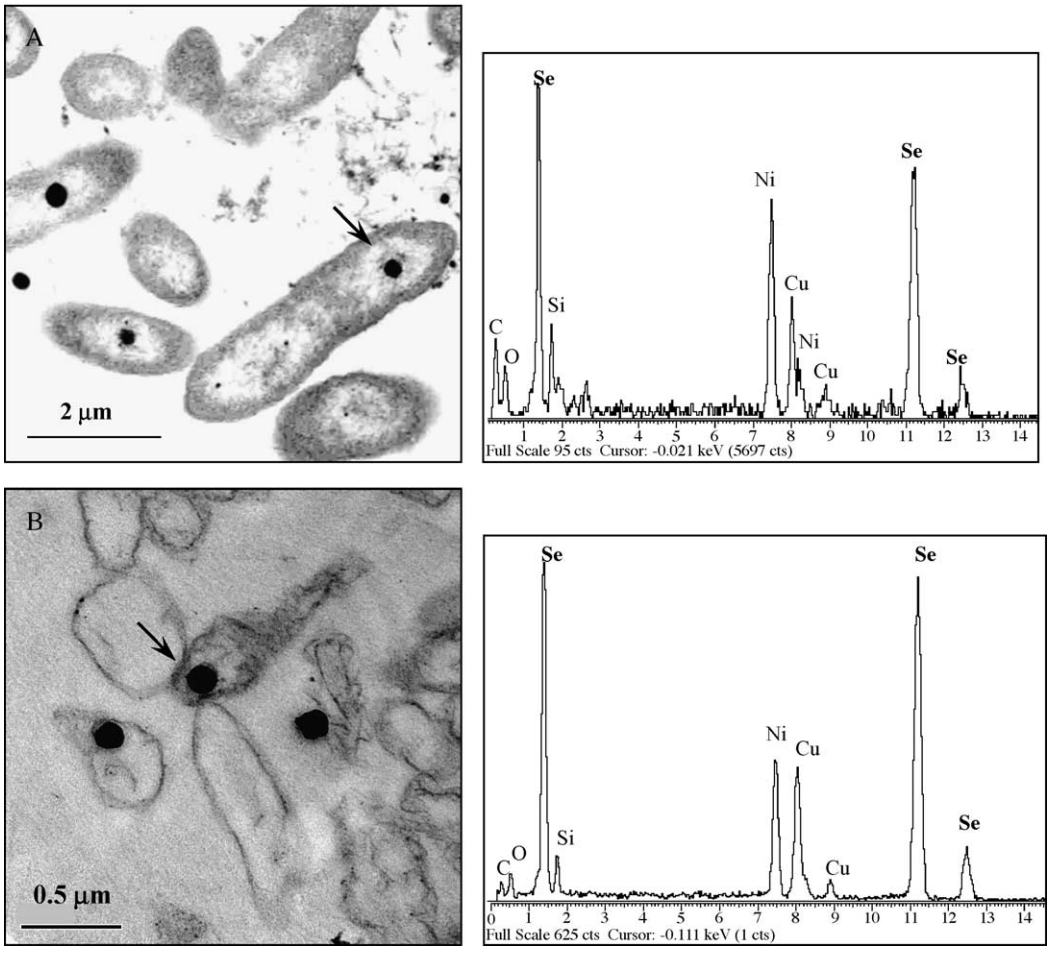

Fig. 6. TEM micrographs and EDX spectra of a SeITE02 culture grown in presence of 2 mM SeIV. (A) Electron-dense granules of elemental selenium located inside the cells. (B) Extracellular occurrence of elemental selenium globules embedded in cell ghosts. Arrows point electron-dense particles whose corresponding EDX spectra are given on the right side of each micrograph.

previously designed as *Pseudomonas maltophilia* or *Xanthomonas maltophilia* (Palleroni and Bradbury, 1993), is a Gram-negative, aerobic, nonfermentative, ubiquitous bacillus widely diffused within different environmental niches. It can be found in aquatic environments, in soils, on vegetation, and even in some animals (Berg et al., 1999). *S. maltophilia* is often associated with the rhizosphere of a variety of plants such as wheat, oat, cucumber, maize, oilseed rape, and potato (Berg et al., 1996; Debette and Blondeau, 1980; Heuer and Smalla, 1999; Lambert and Joos, 1989). The genus *Stenotrophomonas* is currently studied for its potential application in bioremediation (Dungan et al., 2003; Juhasz et al., 2000; Rousseaux et al., 2001; Song et al., 2002) as well as in biological control of plant pathogens (Yuen et al., 2001; Zhang and Yuen, 2000).

Strain SeITE02 has been isolated from the root system of the legume *A. bisulcatus* which is a hyperaccumulator of selenium. This isolate was demonstrated to tolerate up to 50 mM SeIV in both rich and mineral-agarised medium. This MIC value for SeIV is much higher than those reported for other naturally occurring, selenite-resistant bacteria such as *Ralstonia metallidurans* (Roux et al., 2001), *Rhodobacter spheroides* (Van Fleet-Stalder et al., 2000), *Rhodospirillum rubrum* (Kessi et al., 1999), and *Bacillus subtilis* (Garbisu et al., 1999). For these species, MIC values ranging from about 2.0 to 6.0 mM SeIV have been determined in similar growth conditions. Nevertheless, other authors reported MIC values for SeIV up to 40 mM, in aerated cultures (Rathgeber et al., 2002). Unfortunately, at the best of our knowledge, those microorganisms were not further identified.

Tolerance of selenite in strain SeITE02 can be ascribed to the capacity that this isolate has to reduce soluble and harmful SeIV to the nontoxic and unavailable Se^0, as already reported for other SeIV-resistant microbes (Dungan et al., 2003; Kessi et al., 1999; Rathgeber et al., 2002; Roux et al., 2001; Tomei et al., 1992; Van Fleet-Stalder et al., 2000; Yamada et al., 1997). In strain SeITE02, selenite reduction accompanies microbial growth, mostly during the exponential phase. When cultured in the presence of 0.5 or 2.0 mM SeIV, SeITE02 actively reduced selenite once cell viability had been recovered after the transient decrease observed between the 6th and 24th hours. The strict correlation between microbial exponential growth and SeIV reduction to Se^0 is consistent with a possible activation of

proper detoxifying mechanisms by strain SeITE02. Meanwhile, it is worth noting that SeITE02 cells maintain their capability to reduce SeIV even under suboptimal growth conditions. Actually, the process is not significantly affected by the decrease in cell replication. Reaction mechanisms of SeIV reduction by strain SeITE02 need to be further analysed. Although occurring as a common feature among several microorganisms, reductive reactions for SeIV transformation to Se^0 are not completely clear so far. This reduction has been reported as catalysed by a periplasmic nitrite reductase (DeMoll-Decker and Macy, 1993) or by a hydrogenase I (Yanke et al., 1995), or even promoted by nonenzymatic reactions (Tomei et al., 1992). In vitro studies (Turner et al., 1998) have shown that SeIV reduction involves reactions with sulphydryl groups of thiol-containing compounds such as glutathione. According to this mechanism, some authors suggested that microbial SeIV toxicity may be due to the oxidative stress experienced by microbial cells during glutathione-mediated reduction of selenite to Se^0 (Kramer and Ames, 1988; Seko and Imura, 1997; Touati, 2000).

In strain SeITE02, cell pretreatment with 0.2 mM SeIV seems to stimulate an adaptive response of this microbe to the toxic effects of high SeIV concentrations (0.5 and 2.0 mM). Such an adaptive response allows the increase of cell growth in the presence of SeIV; in turn, the capability of strain SeITE02 to reduce SeIV is improved. Activation of a sort of adaptive response to selenite toxicity reinforces the hypothesis that, in strain SeITE02, reduction of SeIV to Se^0 consists in a detoxification mechanism.

Reduction of SeIV to Se^0 by strain SeITE02 leads to accumulation of the metalloid inside bacterial cells. This is in agreement with evidences reported for other SeIV-resistant microbial strains (Kessi et al., 1999; Roux et al., 2001; Rathgeber et al., 2002; Bebien et al., 2001). Furthermore, selenium granules were never observed in the periplasm, although they were found extracellularly. The presence of selenium granules in extracellular locations cannot be explained relying on a transport across membrane that is unlikely for such large particles. Yamada et al. (1997) claimed for an extracellular reduction of SeIV. Again, several authors suggested a vesicular mechanism of selenium excretion (Kessi et al., 1999) although vesicular excretion in bacteria is still controversial.

With reference to strain SeITE02, a process of cell lysis may be invoked. This should determine release of selenium granules outside the cells. This hypothesis is based on the evidence that, with 2.0 mM SeIV added to the substrate, an increase in culture turbidity can be revealed which appears completely independent from microbial growth.

Cell lysis, as a consequence of the release of selenium granules into the medium, does increase turbidity of bacterial cultures irrespective of the actual microbial growth. A tentative explanation similar to that here advanced has been formulated also by Tomei et al. (1992). Cell lysis may be looked at as a direct consequence of selenium accumulation in form of granules inside microbial cells. This accumulation may even cause the decrease of viability in SeITE02 cultures amended with SeIV.

Finally, it is interesting to point out that the allotropic form of Se^0 produced by strain SeITE02 seems to be highly stable. On the other hand, red amorphous Se^0 is unstable in soil and generally reverts to the black amorphous form (Gattow and Heinrich, 1964). This latter is slowly transformed to the more stable grey hexagonal allotrope or even reoxidised through abiotic or biotic processes (Geering et al., 1968). The high stability of the red allotropic Se^0 form accumulated by strain SeITE02, whose cultures remain reddish for at least one month, suggests that this selenium species may occur as tightly bound to extracellular stabilising compounds. Extracellular compounds, possibly produced by microbial cells, may prevent the red allotropic form of selenium from transformation in the black form, so avoiding Se^0 reoxidation. Sequestration of metals in extracellular matrices has been recently described in *Klebsiella oxytoca* (Baldi et al., 2001).

Consistent with this scenario, phase-contrast microscopy analyses on strain SeITE02 showed that selenium granules released into the growth medium appeared associated to an extracellular matrix, forming a sort of clumps with microbial cells.

5. Conclusions

Although SeIV exerts a toxic effect on cellular metabolism, the ability of strain SeITE02 to reduce selenite to Se^0 offers a possibility to biologically precipitate this metalloid from selenium-laden effluents. Thus, *Stenotrophomonas* sp. strain SeITE02 should be exploited for the application in bioremediation of polluted wastewaters deriving from different industrial activities. In particular, strain SeITE02 appears of high interest for its versatile capacity to activate an adaptive response to SeIV that allows the microbe to significantly increment the capacity to precipitate the metalloid. Finally, provided that sequestration of metals by exopolymers of microbial origin represents an important process for removing heavy metals from industrial wastes (Harris and Birch, 1989; Williamson and Johnson, 1991), the nature of the extracellular aggregates, formed during SeIV reduction by strain SeITE02, is worth further investigation. Eventually, the isolate SeITE02 might become of concern for the production of biopolymers of biotechnological relevance.

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CHAPTER II

Rhizosphere-induced selenium precipitation for possible applications in phytoremediation of Se polluted effluents

Rhizosphere-induced Selenium Precipitation for Possible Applications in Phytoremediation of Se Polluted Effluents

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Two bacterial isolates were obtained in axenic culture from the rhizosphere soil of *Astragalus bisulcatus*, a legume able to hyperaccumulate selenium. Both strains resulted of particular interest for their high resistance to the toxic oxyanion SeO_3^{2-} (selenite, Se^{IV}). On the basis of molecular and biochemical analyses, these two isolates were attributed to the species *Bacillus mycoides* and *Stenotrophomonas maltophilia*, respectively. Their capability in axenic culture to precipitate the soluble, bioavailable and highly toxic selenium form selenite to insoluble and relatively non-toxic Se^0 (elemental selenium) was evaluated in defined medium added with 0.2 or 0.5 mM Se^{IV}. Both strains showed to completely reduce 0.2 mM selenite in 120 h, while 0.5 mM Se^{IV} was reduced up to 67% of the initial concentration by *B. mycoides* and to about 50% by *S. maltophilia* in 48 h. Together in a dual consortium, *B. mycoides* and *S. maltophilia* increased the kinetics of selenite reduction, thus improving the efficiency of the process. A model system for selenium rhizofiltration based on plant-rhizobacteria interactions has been proposed.

Key words: Bacterial Reduction, Selenite, Wastewater Rhizofiltration

Introduction

Selenium can be considered as an essential trace element for many living organisms from bacteria to mammals (Shamberger, 1983). Nevertheless, the behaviour of selenium with particular respect to the health of humans and animals is dichotomous. While, as an essential trace nutrient, selenium has a recommended dietary requirement of 70 μg per day, conversely, at intake doses above 350 μg per day, it starts to exert toxic (Rayman, 2000) or even mutagenic effects (Shamberger, 1985).

In oxic environments, selenium is predominantly occurring as Se^{IV} in selenites (SeO_3^{2-}) and as Se^{VI} in selenates (SeO_4^{2-}). These inorganic oxidized forms can be found in high concentrations in some habitats as a consequence of agricultural practices or industrial discharges (Losi and Frankenberger, 1997). Severe Se pollution in agriculture is restricted to seleniferous soils where repeated irrigation leads to the accumulation of this metalloid into the drainage water, eventually entering the food chain. This phenomenon is apparently widespread in the western United States (Weres *et al.*, 1989; Stephens and Waddell, 1998). However, cases of elevated Se concentrations in soils have been reported in several countries world-wide, including Australia, Canada, China, Columbia, India, Ireland, Israel and Mexico (Dhillon and Dhillon, 2001). On the other hand, selenium-laden effluents and wastes are principally associated to oil refining and mining activities, to the production of pigments, metallurgical additives and pharmaceutical preparates or to the use in electronics and glass manufacturing (Bocovay, 1995).

The toxicity of selenium is related to its chemical form. Although the oxyanions selenite and selenate are both soluble and bioavailable, selenium in the form of Se^{IV} is more toxic to most organisms than selenium in the form of Se^{VI}. Contrarily, elemental selenium (Se^0) is insoluble; that is, it can not be absorbed by the biological systems (Barceloux, 1999)

Selenium undergoes various redox reactions within its biogeochemical cycle and microbes play a pivotal role in this context. A number of bacteria have been described as specifically able to reduce Se oxyanions to elemental selenium. *Pseudomonas fluorescens* K27 is a selenium-resistant, facultative anaerobe, that reduces selenite and selenate to Se^0 and volatile organo-selenium methylated com-

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pounds in microaerophilic/anaerobic conditions (Zhang and Chasteen, 1994). Also *Escherichia coli* can transform both oxyanions to Se^0 (Turner *et al.*, 1998). Selenate-reducing bacteria were isolated from extreme environments such as hypersaline ponds (de Souza *et al.*, 2001). On the other hand, certain species, namely *Rhodospirillum rubrum* (Kessi *et al.*, 1999) and *Ralstonia metallidurans* (Roux *et al.*, 2001), have been shown to reduce only selenite. *Rhodobacter sphaeroides*, which reduces mostly selenite, can reduce SeO_4^{2-} to some extent (Van Fleet-Stalder *et al.*, 2000). Selenite is also respired anaerobically by other bacterial species including *Bacillus arsenicoselenatis*, *B. selenitireducens* (Switzer-Blum *et al.*, 1998), *Sulfurospirillum barnesii* (Stolz *et al.*, 1999), and *Thauera selenatis* (Macy *et al.*, 1993), while reduction of selenite in aerobic conditions has been reported in several strains of *Pseudoalteromonas* sp. (Rathgeber *et al.*, 2002) as well as in *Stenotrophomonas maltophilia* (Di Gregorio *et al.*, 2005). This latter was found able to reduce aerobically even selenate (Dungan *et al.*, 2003).

Microbial reduction of bioavailable selenium oxyanions into elemental selenium or to relatively non-toxic gaseous forms is of great interest for bioremediation, expecially for the treatment of Se-laden effluents and industrial outlets. At present, physico-chemical technologies such as chemical reduction and precipitation, adsorption, ion exchange, and membrane processes are mainly applied for remediating wastewater (Twidwell *et al.*, 2000). However, these methods are commonly very costly. Thus, an interesting alternative for a cost-effective abatement of Se oxyanions may be represented by biological treatments relying on the exploitation of either microbes or plants capable to reduce, volatilise or accumulate toxic selenium forms (Cantafio *et al.*,1996; Fujita *et al.*, 2002; Azaizeh *et al.*, 2003).

In the present work, two bacterial isolates from the rhizosphere of the Se hyperaccumulator legume *Astragalus bisulcatus* have been compared for their efficiency in reducing selenite to red amorphous metallic selenium (Se^0) *in vitro*. The main objective of this study was to test such a dual consortium of bacteria in terms of both Se tolerance and SeO_3^{2-} reduction rate in the perspective of possible application in hydroponic rhizofiltration reactors with bacteria entrapped in the root system of *A. bisulcatus*.

Materials and Methods

Chemicals

Chemicals purchased from Sigma-Aldrich were all analytical grade.

Culture media

Nutrient Broth (rich medium) and Bacteriological Agar were provided by Oxoid. Defined medium (DM) was prepared according to Di Gregorio *et al.* (2005), with minor modifications (addition of 0.1% glucose wt/vol. and 0.1% yeast extract wt/vol.).

Isolation of bacterial strains

Enrichment cultures for bacterial isolation were inoculated with rhizosphere soil from plants of *Astragalus bisulcatus* grown for six months in greenhouse conditions at 21 ± 1 °C on seleniferous soil collected at a mine site in Sardinia, Italy (Campostrini *et al.*, 1999). *A. bisulcatus* seeds were obtained from the Western Regional PI Station, Washington State University, Pullman, USA. Enrichment cultures were carried out in 250 ml Erlenmeyer flasks containing 100 ml Nutrient Broth added with 0.2 mM Se^{IV} sodium salt. Flasks were incubated in the dark at 28 °C on an orbital shaker (250 rev/min). After one week of incubation, serial dilutions of the culture medium were plated on agarised Nutrient Broth containing 0.2 mM Se^{IV} sodium salt. Plates were incubated in the dark at 28 °C for 5 d. Appearance of red colored colonies was interpreted as an indication for selenite reduction to elemental selenium (Se^0) (Sabaty *et al.*, 2001). Red colonies were isolated and streaked on fresh agarised Nutrient Broth plates containing Se^{IV} sodium salt. Pure cultures of three morphologically different bacteria were obtained. Minimum inhibitory concentration (MIC) for Se^{IV} was determined on agarised plates prepared with either DM or Nutrient Broth containing increasing concentrations of selenite. Microbial cells were checked for viability after 5 d of incubation. The strains, successively referred to as SeITE01 and SeITE02, revealed the highest value of MIC for Se^{IV} and were further characterized.

Phylogenetic analysis of SeITE01 and SeITE02 strains

Bacterial genomic DNAs of strains SeITE01 and SeITE02 were extracted with the NucleoSpin Tis-

sue Kit (Clontech) following the manufacturer's instructions. Amplification of the genes encoding for the 16S rRNAs (rDNAs) was performed with the primers F8 and R11 (Weisburg *et al.*, 1991).

The products of amplification were directly double-strand sequenced, aligned to the database sequences using BLASTN (Altshul *et al.*, 1997), and analysed by means of ARB (Strunk and Ludwig, 1993–2002).

Se^{IV} reducing activity induction

Stationary phase bacterial cultures grown in presence of 0.2 mM Se^{IV} sodium salt were used to inoculate either 100 ml DM or 100 ml DM added with, respectively, 0.2 and 0.5 mM Se^{IV} sodium salt (initial turbidity: 0.01).

Microbial growth

Microbial growth was evaluated in DM amended with 0.2 or 0.5 mM Se^{IV} by quantifying the turbidity of cell suspensions with a Helios β spectrophotometer, Unicam. All analyses were performed in triplicate. Microbial growth in presence of Se^{IV} was compared to control cultures incubated in DM added with no Se^{IV} sodium salt.

Se^{IV} content determination

Se^{IV} concentration in cultures amended with 0.2 or 0.5 mM Se^{IV} was determined by reading absorbance at 377 nm of the selenium-2,3-diaminonaphthalene complex in cyclohexane as described by Kessi *et al.* (1999). Sterile cultures were also tested for Se^{IV} concentration as negative controls.

Results

Three different bacterial strains (SeITE01, SeITE02 and SeITE03) were isolated from the rhizosphere of the selenium hyperaccumulator legume *Astragalus bisulcatus* (Neuhierl and Boek, 1996). Values of MIC for Se^{IV} were determined. The strains SeITE01 and SeITE02, showing the highest resistance to Se^{IV} (up to 15 and 50 mM, respectively), were further characterized. The corresponding 16S rRNA genes were amplified by PCR. The 1.5 kb fragments obtained were double-strand sequenced and successively analyzed by using BLASTN (Altshul *et al.*, 1997) and ARB (Strunk and Ludwig, 1993–2002). Strain SeITE02 was previously characterized (Di Gregorio *et al.*, 2005) and recognized as belonging to the genus *Stenotrophomonas*, species *maltophilia* (99% identity with *Stenotrophomonas maltophilia* VUN10-075, AF100734). On the other hand, strain SeITE01 resulted very close to the *Bacillus cereus* group (99% identity with *Bacillus cereus* G9667). Nevertheless, further biochemical characterization suggested the possible attribution of SeITE01 to the species *B. mycoides* (Table I). Both strains formed

Table I. Morphological, biochemical, and growth characteristics of *Bacillus* sp. strain SeITE01 isolated from the rhizosphere of the selenium hyperaccumulator *Astragalus bisulcatus*.

Cell morphology (size [μm])	Short, ovoid rods (0.7×1.5)
Colour and aspect of colonies:	
without Se^{IV} addition	Opaque, creamy, rhizoid growth
with Se^{IV} addition	Bright red, rhizoid growth
Fluorescent pigment	–
Growth at 4 °C	–
Growth at 18 °C	–
Growth at 28 °C	+
Growth at 37 °C	+
Growth at 42 °C	–
Growth at pH 4.5	–
Growth at pH 7.2	+
Growth at pH 9	–
Oxidase activity	–
Catalase activity	+
Lipase activity	+
Urease activity	–
Methyl red test	–
V/P test	+
Nitrate reduction	+
Utilisation of:	
Acetate	–
Caprylate	–
Citrate	–
Gluconate	+
Malate	+
Oxalate	–
Succinate	+
Arabinose	–
Fructose	+
Galactose	+
Glucose	+
Lactose	+
Maltose	+
Mannitol	+
Mannose	+
N-Acetyl-glucosamine	–
Raffinose	+
Rhamnose	+
Saccharose	+
D-Xylose	+
Hydrolysis of:	
Starch	+
Gelatin	+
Esculin	+

red cell suspensions, in aerated DM liquid medium added with Se^{IV}, revealing the capacity to reduce Se^{IV} to Se^0 (Sabaty *et al.*, 2001). Conversely, Se^{IV} was totally recovered in sterile control cultures suggesting for a biologically mediated Se^{IV} reduction.

As reported elsewhere (Di Gregorio *et al.*, 2005), the incubation of *S. maltophilia* strain SeITE02 with 0.2 mM Se^{IV} resulted in an increase of either the capability to reduce Se^{IV} to Se^0 or the cell viability in presence of increasing concentration of selenite. Thus, in the present study, both *B. mycoides* SeITE01 and *S. maltophilia* SeITE02 were pre-induced with 0.2 mM Se^{IV} before evaluating selenite resistance and capability to reduce Se^{IV} to Se^0 in both strains.

Se^{IV} toxicity towards strains SeITE01 and SeITE02 was evaluated in liquid culture by checking for bacterial cell growth in presence of increasing concentrations of Se^{IV} (Fig. 1). At the two concentrations tested (0.2, 0.5 mM), Se^{IV} slightly affected the microbial growth of *B. mycoides* strain SeITE01, determining a modest decrease in turbidity compared to the control. On the other hand, neither significant effects by Se^{IV} on cell growth of *S. maltophilia* SeITE02 were revealed nor Se^{IV} negatively influenced the growth of the dual consortium consisting in strain SeITE01 and strain SeITE02 together.

With reference to the capacity of both bacterial strains to reduce Se^{IV} to Se^0, this activity was evaluated for either each strain and for the dual consortium of them (Fig. 2). At 0.2 mM Se^{IV}, *S. maltophilia* strain SeITE02 completely reduced the oxyanion within 120 h. Se^{IV} reduction started at the early exponential phase of growth, lasting throughout the stationary phase (Fig. 1). At 0.5 mM Se^{IV}, strain SeITE02 could reduce only 50% of the initial oxyanion concentration, in 120 h (Fig. 2). Actually, this Se^{IV} reduction was recorded

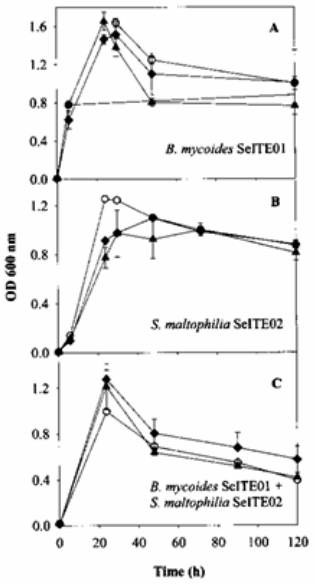

Fig. 1. Time courses of turbidity in presence of 0.2 mM (–▲–), 0.5 mM Se^{IV} (–◆–), or with no selenium (control) (–○–) corresponding to cell suspensions of: (A) *Bacillus mycoides* strain SeITE01, (B) *Stenotrophomonas maltophilia* strain SeITE02, and (C) the dual bacterial consortium consisting of strains SeITE01 and SeITE02 together. Each curve is the mean of three separate experiments.

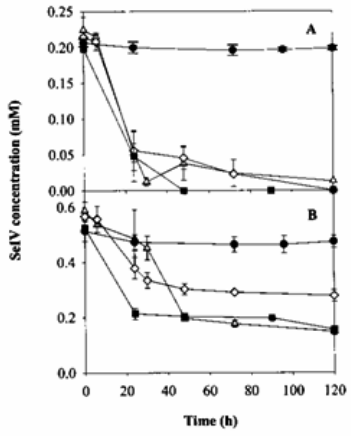

Fig. 2. Time courses of Se^{IV} reduction to Se^0 carried out by *Bacillus mycoides* strain SeITE01 (–△–), *Stenotrophomonas maltophilia* strain SeITE02 (–◇–), and the dual bacterial consortium consisting of strains SeITE01 and SeITE02 together (–■–), in presence of 0.2 mM (A) or 0.5 mM (B) Se^{IV}. Sterile cultures (not inoculated) were also tested for Se^{IV} concentration as negative control (–●–). Each curve is the mean of three separate experiments.

within the first 48 h of incubation. Afterwards, *S. maltophilia* was not capable to reduce the oxyanion any further.

At 0.2 mM Se^{IV}, also *B. mycoides* SeITE01 completely reduced selenite in 120 h (Fig. 2). Conversely, strain SeITE01 did not reduce completely selenite in presence of 0.5 mM Se^{IV}. In fact, *B. mycoides* reduced only 67% of the initial Se^{IV} concentration, mostly within the first 48 h of incubation.

With the dual bacterial consortium consisting of strain SeITE01 and strain SeITE02, the process of selenite reduction was accelerated in comparison with axenic cultures of each single strain. This result was observed at both Se^{IV} concentrations tested. Interestingly, at 0.2 mM Se^{IV}, complete oxyanion reduction occurred in only 48 h vs to 120 h needed with cultures of single strains (Fig. 2). Moreover, at 0.5 mM Se^{IV}, the higher percentage of reduction (67%) previously observed in *B. mycoides* SeITE01 was reached in only 24 h instead of 48 h (Fig. 2). Selenite reduction was recorded in correspondence to an increase of turbidity (*i.e.* cell growth) in cultures of the dual bacterial consortium (Fig. 1).

Discussion

Two bacterial species isolated from the rhizosphere of *Astragalus bisulcatus*, a leguminous plant able to hyperaccumulate selenium, resulted of particular interest for their high resistance to the toxic oxyanion SeO_3^{2-} associated to the capability of reducing selenite to non-bioavailable Se^0. Actually, while the strain SeITE01 assigned to the species *Bacillus mycoides* showed a MIC of 15 mM, either in rich or defined medium, on the other hand, *Stenotrophomonas maltophilia* strain SeITE02 resisted up to 50 mM Se^{IV}. These MIC values for SeO_3^{2-} are much higher than those reported for other naturally occurring, selenite-resistant bacteria such as *Bacillus subtilis* (Garbisu *et al.*, 1999), *Rhodospirillum rubrum* (Kessi *et al.*, 1999), *Rhodobacter spheroides* (Van Fleet-Stalder *et al.*, 2000), and *Ralstonia metallidurans* (Roux *et al.*, 2001). For these species, MIC values ranging from about 2.0 mM to 6.0 mM Se^{IV} have been ascertained in similar growth conditions. Some authors reported MIC values for Se^{IV} up to 40 mM in aerated bacterial cultures, however these isolates were not identified (Rathgeber *et al.*, 2002). The high resistance to selenite and the capability to detoxify SeO_3^{2-} through the reduction to metallic Se^0 make the bacterial isolates of this study attractive for possible application in the remediation of seleniferous effluents.

The *Bacillus cereus* group, which the isolate SeITE01 can be attributed to, includes Gram-positive bacteria belonging to four species: *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides*. In particular, *B. mycoides* has recently been recognized as a plant growth-promoting bacterium associated to the roots of different tree species (Petersen *et al.*, 1995). The genus *Bacillus* is presently studied for a possible exploitation in bioremediation of heavy metals (Fujita *et al.*, 2002; Zaidi and Musarrat, 2004). On the other hand, the species *Stenotrophomonas maltophilia*, which the strain SeITE02 belongs to, is a Gram-negative, aerobic, non-fermentative bacterium widespread in different environmental niches. It has been isolated either from soils or aquatic environments (Berg *et al.*, 1999). Moreover, it occurs in the rhizosphere of a variety of plants such as wheat, oat, cucumber, maize, oilseed rape and potato (Lambert and Joos, 1989; Berg *et al.*, 1996). Even the genus *Stenotrophomonas* is nowadays considered for its potential application in bioremediation (Juhasz *et al.*, 2000; Dungan *et al.*, 2003).

As already reported for other Se^{IV} resistant microbes (Tomei *et al.*, 1992; Yamada *et al.*, 1997; Kessi *et al.*, 1999; Van Fleet-Stalder *et al.*, 2000; Roux *et al.*, 2001; Rathgeber *et al.*, 2002; Dungan *et al.*, 2003), selenite resistance of strains SeITE01 and SeITE02 depends on their capacity to reduce the highly toxic SeO_3^{2-} to the non-bioavailable and relatively non-toxic elemental selenium. Under experimental growth conditions, selenite reduction occurred mainly during the microbial exponential growth, lasting for only a part of the stationary phase. At the lower selenite concentration tested (0.2 mM Se^{IV} corresponding to 15 mg/l selenium), the efficiency of both bacterial isolates to reduce SeO_3^{2-} was similar. Meanwhile, *B. mycoides* strain SeITE01 showed a higher efficiency in Se^{IV} reduction at the higher content of selenite in the medium (0.5 mM Se^{IV} corresponding to 45 mg/l selenium), by reducing up to the 67% of the initial Se^{IV} compared to only the 50% reduced by *S. maltophilia* strain SeITE02. When the dual consortium of SeITE01 and SeITE02 was grown in presence of selenite, SeO_3^{2-} reduction proceeded faster. In fact, the maximum of selenite reduction

was reached in a shorter time, although the percent value of this reduction remained unchanged.

The concentrations of selenite tested in the study are comparable to those reported for soluble Se in agricultural drainage wastewaters (~ 0.65 mg/l selenium) (Cantafio *et al.*, 1996) or in industrial wastewater (4–60 mg/l selenium) (Fujita *et al.*, 2002). Consequently, the bacterial isolates SeITE01 and SeITE02 can be considered suitable for application, at either laboratory or pilot scale, in continuously fed wastewater treatment systems in order to optimise the abatement of selenite. Actually, it is interesting to note that, under the growth conditions adopted in this study, the combination of both bacterial isolates in a dual consortium resulted in a decrease of turbidity of the relative cell cultures, at least, when compared to the suspensions of *B. mycoides* strain SeITE01 alone. However, the dual consortium apparently accelerated selenite reduction with respect to what was observed in axenic cultures of each single bacterial isolate. Growth of the two strains within the dual consortium was likely sub-optimal because of a possible reciprocal inhibition. Nevertheless, strain SeITE02 has been shown to maintain its capability in reducing SeO_3^{2-} even under sub-optimal growth conditions (Di Gregorio *et al.*, 2005). Hence, although the mechanisms of selenite reduction by the strains SeITE01 and SeITE02 need to be further analyzed, the capacity to maintain Se^{IV} reducing activity in sub-optimal growth conditions encourages the application of these strains to treat seleniferous effluents.

Conclusions

Metal pollutants in industrial-process waters or in groundwaters are most commonly removed by precipitation or flocculation, followed by sedimentation and disposal of the resulting sludge. A promising alternative to this conventional clean-up method is rhizofiltration, a phytoremediative technique designed for the removal of metals in aquatic environments (Dushenkov *et al.*, 1995; Zhu *et al.*, 1999). On the basis of preliminary evidences from the present study, it appears worth of attention the hypothesis to apply selenite/selenate-resistant and reducing bacteria in hydroponic systems for treating selenium-laden water streams. In these continuous open bioreactors (Fig. 3), the synergic action of hyperaccumulating plants, such as *A. bisulcatus*, which absorb and concentrate the metalloid in their roots and shoots and rhizosphere colonising bacteria can be exploited. While the plants extract the contaminant in its soluble forms from the effluent flowing through sequencing hydroponic ponds, selenite/selenate-reducing bacteria may cause selenium to precipitate onto

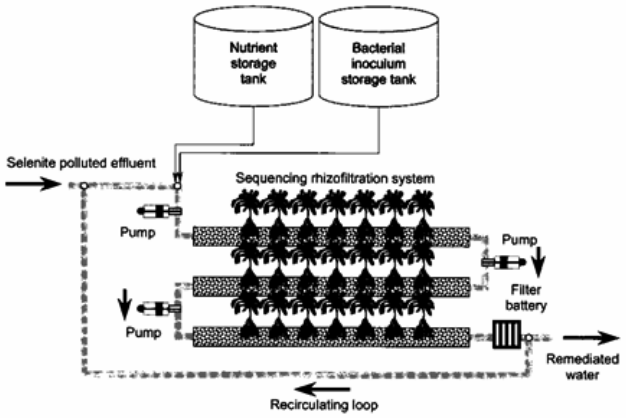

Fig. 3. Schematic representation of a hydroponic rhizofiltration system for the treatment of selenium-laden water streams by means of the synergic activity of a Se hyperaccumulator plant and selenite/selenate reducing bacteria.

the root surfaces or to settle to the bottom. As the plants become saturated with the contaminant, roots or whole plants are harvested for disposal. On the other hand, the remaining suspended Se^0 may be removed from the water by filtering the stream before its final release. Moreover, the possibility to rely on bacterial consortia, whose members exert different capabilities in terms of either selenite/selenate-resistance or reduction kinetics of Se oxyanions, makes the bioprocess more flexible and able to face pollutant fluctuations. For instance, the strain of *Stenotrophomonas* sp. isolated in this study might resist to possible peaking of wastewater selenite concentration, then allowing the *Bacillus* isolate to more efficiently reduce the contaminant in normal steady-state conditions.

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CHAPTER III

Combined application of Triton X-100 and *Sinorhizobium* sp. Pb002 inoculum for the improvement of lead phytoextraction by *Brassica juncea* in EDTA amended soil.

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Combined application of Triton X-100 and *Sinorhizobium* sp. Pb002 inoculum for the improvement of lead phytoextraction by *Brassica juncea* in EDTA amended soil

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Abstract

The process of EDTA-assisted lead phytoextraction from the Bovisa (Milan, Italy) brownfield soil was optimized in microcosms vegetated with *Brassica juncea*. An autochthonous plant growth-promoting rhizobacterium (PGPR), *Sinorhizobium* sp. Pb002, was isolated from the rhizosphere of *B. juncea* grown on the Pb-contaminated soil in presence of 2 mM EDTA. The strain was augmented (10^8 CFU g^{-1} soil) in vegetated microcosms to stimulate *B. juncea* biomass production and, hence, its phytoextraction potential. Triton X-100 was also added to microcosms at 5 and 10 times the critical micelle concentration (cmc) to increase the permeability of root barriers to the EDTA–Pb complexes. Triton X-100 amendment determined an increase in Pb concentration within plant tissues. However it contextually exerted a phytotoxic effect. *Sinorhizobium* sp. Pb002 augmentation was crucial to plant survival in presence of both bioavailable lead and Triton X-100. The combination of the two treatments produced up to 56% increase in the efficiency of lead phytoextraction by *B. juncea*. The effects of these treatments on the structure of the soil bacterial community were evaluated by 16S rDNA denaturing gradient gel electrophoresis (DGGE).

Keywords: Bacterial community structure; Microcosms; Phytoremediation; Soil lead contamination

1. Introduction

Lead is highly toxic to plants, wildlife, and humans (Jin et al., 1997; Bunzl et al., 2001). Soil Pb contamination is mostly due to mining activities, combustion of leaded gasoline, sewage sludge application, and battery disposal (Jarup, 2003). Reclamation of Pb-contaminated soils is mandatory for both environmental restoration and protection of human health. The exploitation of plants for the removal of metals from soil (phytoextraction) is widely considered as a cost-effective approach (Chaney et al., 1997). As an example, *Brassica juncea* has been applied for EDTA-assisted Pb-phytoextraction (Salido et al., 2003; Crist et al., 2004), with EDTA forming soluble Pb-complexes eventually mobilizing the

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metal, otherwise not bioavailable, from soil particles (Kroschwitz, 1995). Moreover, EDTA enhances the translocation of Pb from roots to shoots and leaves (Vassil et al., 1998), and produces a decrease in specificity of transmembrane solute transport into root cells. In fact, at threshold concentration (Vassil et al., 1998), EDTA destroys root barriers (Pasternak, 1987, 1988; Kaszuba and Hunt, 1990). As a result plant permeability to the EDTA–Pb complexes increases.

In the present work, EDTA-assisted Pb-phytoextraction from a historically contaminated soil was monitored in microcosms vegetated with *B. juncea*. Soil was from the dismissed industrial area of the Officina Gas Bovisa, which has been producing for many years (1908–1994) gas from coal for the city of Milan (Italy). As a consequence of this activity, Pb has been released into the environment, in addition to many other pollutants. Two different soil treatments, first applied independently and then in combination, were designed to optimize the remediation process. One treatment consisted in the use of an autochthonous plant growth-promoting rhizobacterium (PGPR), *Sinorhizobium* sp. Pb002, which was massively re-inoculated within the rhizosphere of *B. juncea* to possibly increase plant biomass production (Glick, 1995). The second treatment consisted in the soil addition of the nonionic surfactant Triton X-100, at increasing concentrations. This second approach was adopted to damage the plasma cell membranes of root cells (Cserhati, 1995), making the root barriers more permeable to the EDTA–Pb complexes. The effects of these different amendments on the structure of the soil bacterial community were evaluated by 16S rDNA denaturing gradient gel electrophoresis (DGGE) analyses.

2. Materials and methods

2.1. Chemicals and microbiological growth substrates

Analytical grade chemicals were purchased from Sigma-Aldrich. Bacteriological products were furnished by Oxoid. Luria Bertani (LB)-modified medium was prepared as described by Levison et al. (1996). Minimal defined medium was prepared as described by Frassinetti et al. (1998).

2.2. Plant growth experiments

Plants of *B. juncea* were grown in a temperature-controlled greenhouse (24–28 °C). Seeds of *B. juncea* were obtained from the USDA-ARS North Central Regional Plant Introduction Station, Iowa State University, Ames, IA, USA. Microcosms were prepared with 200 g soil (d.w.) per pot. The main physical and chemical characteristics of soil were: pH, 7.8; conductivity, 1.6 mMhos; CEC, 11.87 meq 100 g^{-1}; clay, 7.1%; sand, 80.7%; silt, 12.3%; total N, 2000 mg kg^{-1}; total P, 700 mg kg^{-1}; total K, 4200 mg kg^{-1}; total Pb, 465 mg kg^{-1}. Seeds were sown directly in soil pots. Five seedlings per pot were grown for six weeks. After four weeks (plant blooming), microcosms were amended with, respectively: (i) 2 mM EDTA, (ii) 5 critical micellar concentration (cmc) Triton X-100, (iii) 10 cmc Triton X-100, (iv) 2 mM EDTA plus 5 cmc Triton X-100, and (v) 2 mM EDTA plus 10 cmc Triton X-100. Six pots were prepared for each treatment. Unvegetated microcosms for a total of six were also set up. Three of them were supplemented with 2 mM EDTA. A similar set of microcosms was arranged with soil previously inoculated with a bacterial culture (10^8 CFU g^{-1} soil) of the PGPR isolated as described below. Inoculated pots were then vegetated.

2.3. Lead content measurement

Lead quantification was performed by atomic absorption spectrophotometry (Perkin Elmer 3030). Total lead content in plant tissues was determined after extraction with a nitric/perchloric mixture, as described by Barbafieri (2000). Plant materials were first washed and dried in a fan oven for 3 d at 60 °C before total lead extraction. Due to a poor roots development, plant shoots and roots were combined prior to the treatment with the acid mixture. Sequential extraction of soil lead was performed with H_2O, KNO_3 and DTPA, as reported by Barbafieri (2000).

2.4. Isolation of rhizobacterial strains

Enrichment cultures for rhizobacterial isolation were inoculated with soil aliquots from the rhizosphere of *B. juncea* plants grown for six weeks in the presence of 2 mM EDTA. One gram of soil was incubated in 250 ml Erlenmeyer flasks containing 100 ml (LB)-modified liquid medium added with 2 mM $Pb(NO_3)_2$. Flasks were maintained at 28 °C on an orbital shaker (250 rpm). After one-week incubation, serial dilutions of the culture medium were plated on solidified (LB)-modified medium supplemented with 2 mM $Pb(NO_3)_2$. Plates were finally incubated for 5 d at 28 °C in aerobic conditions.

2.5. Characterization of rhizobacteria

Bacterial isolates capable of growing on solidified (LB)-modified medium containing 2 mM $Pb(NO_3)_2$ were streaked on fresh plates containing increasing amounts of $Pb(NO_3)_2$ to determine minimum inhibitory concentrations (MIC) for lead. Once exposed to increasing $Pb(NO_3)_2$ levels, microbial cells were checked for viability after 5 d of incubation on selective plates. Each iso-

late was further analyzed for ACC deaminase activity (Honma and Shimomura, 1978). The gene encoding for 16S rRNA of the bacterial strain showing ACC deaminase activity and 3 mM MIC for lead was amplified by fD1 and rP2 primers (Weisburg et al., 1991), sequenced on both strands, and searched for homology using the BLASTN database (Altshul et al., 1997).

2.6. Denaturing gradient gel electrophoresis (DGGE) analyses

Total DNA extraction from soil, bacterial genomic DNA purification, and DGGE analysis were performed as described in Zocca et al. (2004). DGGE bands containing DNA to be sequenced were excised and incubated for 4 h in 100 µl of sterile water. Afterwards, PCR amplification followed, as described in Zocca et al. (2004), except for the use of nonGC-clamped primers. PCR products were transformed into *Escherichia coli* DH5α using the pGEM-T vector system according to the manufacturer's instructions (Promega), sequenced on both strands, and searched for homology using the BLASTN database (Altshul et al., 1997).

3. Results

3.1. Effects of EDTA and Triton X-100 amendments to microcosms

Six sets of experiments were simultaneously assayed: untreated (no chemicals added) vegetated pots, vegetated pots supplemented with (i) 2 mM EDTA, (ii) two different concentrations of Triton X-100, (iii) 2 mM EDTA plus Triton X-100 at two different concentrations. EDTA and Triton X-100 were added at plant blooming. Two weeks later, plant biomass production and lead content in plant tissues were determined (Fig. 1). The sequential extraction of total soil lead was also performed (Fig. 2).

The highest vegetative growth recorded, along with any lead accumulation in plant tissues, was observed in untreated microcosms (Fig. 1). This result was in agreement with the lack of water-soluble lead in soil (Fig. 2). In order to increase soil Pb bioavailability, microcosms were added with 2 mM EDTA. EDTA concentration was chosen, out of an increasing range from 1 to 7 mM, as the threshold concentration corresponding to the highest Pb plant uptake at the lowest symptoms of phytotoxicity (Vassil et al., 1998) (data not shown).

In order to increase plant root permeability to the EDTA–Pb complexes, microcosms were supplemented with increasing concentrations of Triton X-100, because of the surfactant capacity to damage phospholipid membranes (Cserhati, 1995). The surfactant and EDTA were also added in combination. Results indicated that a water-soluble fraction of lead was recovered only in the presence of the latter (Fig. 2). EDTA amendment caused a 37% reduction in plant biomass production, along with a significant accumulation of Pb in plant tissues, (i.e. 146 mg Pb kg^{-1} plant d.w.) (Fig. 1). On the other hand, addition of Triton X-100 alone induced a 72% decrease in plant biomass production and a negligible Pb accumulation in plant tissues (Fig. 1). The soil treatment with both Triton X-100 and EDTA determined a premature plant death (after one day) and minor Pb accumulation in plant tissues (Fig. 1).

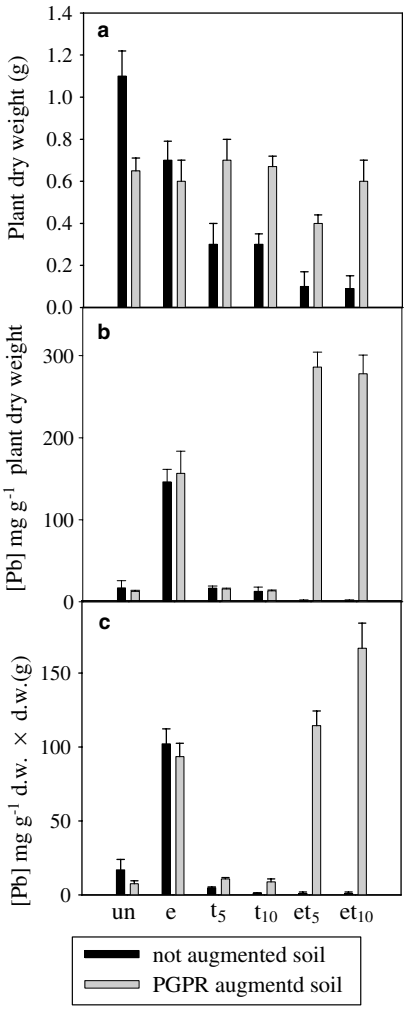

Fig. 1. Biomass (panel a), Pb content (panel b) and phytoextraction efficiency (panel c) of plants grown on untretad soil (un), 2 mM EDTA (e), 5 cmc Triton X-100 (t_5), 10 cmc Triton X-100 (t_{10}), 5 cmc Triton X-100 plus 2 mM EDTA (et_5), 10 cmc Triton X-100 plus 2 mM EDTA (et_{10}) amended soil; d.w., dry weight. Gray boxes represent microcosms augmented with *Sinorhizobium* sp. Pb002.

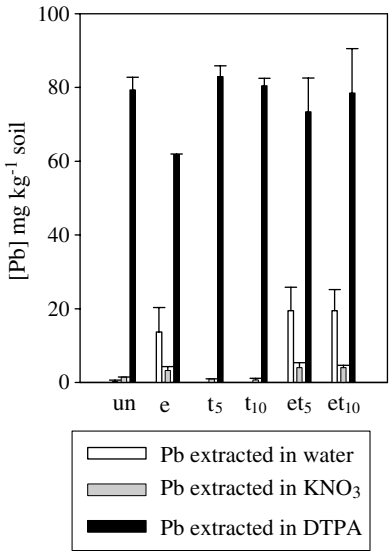

Fig. 2. Lead sequential extraction with H_2O, KNO_3 and DTPA from untretad soil (un), soil added with 2 mM EDTA (e), 5 cmc Triton X-100 (t_5), 10 cmc Triton X-100 (t_{10}), 5 cmc Triton X-100 plus 2 mM EDTA (et_5), 10 cmc Triton X-100 plus 2 mM EDTA (et_{10}).

3.2. Isolation and characterization of rhizobacteria

The cultivable fraction of rhizobacteria colonizing the rhizosphere of plants grown on soil amended with 2 mM EDTA was enriched on selective (LB)-modified medium containing 2 mM $Pb(NO_3)_2$. A total of 26 isolates capable of growth in presence of the metal were obtained. All isolates were analyzed for 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. MICs for lead were also determined. The strain combining a quite high MIC for lead (3 mM) with ACC deaminase activity was taxonomically characterized (*Sinorhizobium* sp. Pb002, 97% homology to *Sinorhizobium* sp. BK1, Accession Number AJ012210). The microbe was then augmented into microcosms as described above.

3.3. Effects of Sinorhizobium sp. Pb002 augmentation into microcosms

The sets of pots previously described were augmented with *Sinorhizobium* sp. Pb002, then vegetated, and analyzed after six weeks for plant biomass production and lead accumulation in plant tissues (Fig. 1). Augmentation of *Sinorhizobium* sp. Pb002 in untreated microcosms was associated with a significant decrease (42%) in plant biomass. Less severe effects (14% decrease) were observed in EDTA-supplemented microcosms. Conversely, when Triton X-100 was present, plant biomass was nearly twice that recorded in the absence of microbial inoculum (Fig. 1). However, positive effects of *Sinorhizobium* sp. Pb002 on plant growth was more pronounced in the case of combined soil amendment with EDTA and Triton X-100. Actually, the presence of the bacterial strain was pivotal for plant survival. The plant biomass produced was comparable to that observed with EDTA amendment alone. At the same time, at either Triton X-100 concentrations, Pb content in plant tissues was twice that recorded after addition of only 2 mM EDTA (about 280 mg instead of 146 mg Pb kg^{-1} plant d.w.) (Fig. 1). Sequential extraction of lead from soil augmented with *Sinorhizobium* sp. Pb002 was also performed. Lead water-soluble fraction was recovered only in presence of EDTA, according to the results obtained in microcosms not augmented with *Sinorhizobium* sp. Pb002 (data not shown).

3.4. Microbial ecology of microcosms

Modifications of the soil bacterial community structure as a consequence of different soil treatments were investigated by 16S rDNA DGGE analysis (Fig. 3). Changes in the DGGE profiles in terms of fluctuation of bands were interpreted as the regression of dominant bacterial species contextual to the rising of different ones. The effect of EDTA addition on soil microbial ecology was initially evaluated by comparing the DGGE profiles of unvegetated microcosms. EDTA amendment did not drastically alter the DGGE profile of not amended soil, indicating only weak effects of the chelant on the soil microbial ecology. One of the most signifi-

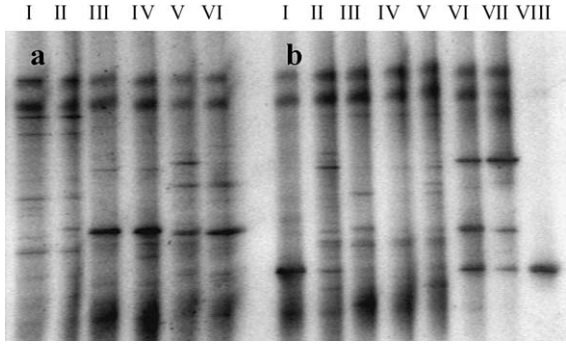

Fig. 3. Panel a: DGGE profile of unvegetated and untreated microcosms (lane I), unvegetated and added with 2 mM EDTA soil (II), vegetated and untreated (III), vegetated and supplemented with 2 mM EDTA (IV), with 5 cmc Triton X-100 (V), with 10 cmc Triton X-100 (VI). Panel b: DGGE profile of microcosms augmented with *Sinorhizobium* sp. Pb002: vegetated microcosms at the time of *Sinorhizobium* sp. Pb002 augmentation (lane I), vegetated and untreated microcosms (II), vegetated and supplemented with 2 mM EDTA (III), with 5 cmc Triton X-100 (IV), with 10 cmc Triton X-100 (V), with 5 cmc Triton X-100 plus 2 mM EDTA (VI), with 10 cmc Triton X-100 plus 2 mM EDTA (VII), amplified fragment of the 16S rDNA of *Sinorhizobium* sp. Pb002 (VIII).

cant modifications of the soil microbial community structure was related to the introduction of *B. juncea* that determined the comparison of a restricted dominant population definitely not resembling that of unvegetated soil. EDTA had only a slight influence on it, while Triton X-100 addition was accompanied by the occurrence of new profiles, corresponding to the incidence of novel dominant bacterial populations.

The presence of *Sinorhizobium* sp. Pb002 into differently treated soil, starting from augmentation throughout six weeks after inoculation, was also monitored. The amplification product of the cloned 16S rDNA of *Sinorhizobium* sp. Pb002 was the molecular marker to monitor the presence of bands corresponding to the microbial inoculum in the different profiles. The bands of interest were gel-excised and sequenced in order to verify their relationship to the 16S rDNA of *Sinorhizobium* sp. Pb002.

After six weeks, in vegetated and untreated soil, the amplification product of interest was above the detection limits of DGGE analysis. On the contrary, in the case of vegetated soil treated with EDTA or Triton X-100, the amplification product was below the detection limits. Moreover, the microbial community structure was significantly different from that observed in inoculated, vegetated but untreated microcosms. At the same time, *Sinorhizobium* sp. Pb002 was one of the dominant species when EDTA and Triton X-100 were simultaneously added. Amendment with these chemicals, along with the PGPR augmentation, determined an evident shift in the bacterial community structure respect to what observed in inoculated, vegetated but untreated soil.

4. Discussion

One of the main strategies for phytoremediation of lead contaminated soils is the EDTA-assisted Pb-phytoextraction. In this study, the process carried out by *B. juncea* has been tentatively optimized. As reported elsewhere (Blaylock et al., 1997; Huang et al., 1997), also in this study the addition of EDTA to soil has been required to make lead bioavailable. However, Pb bioavailability caused toxic effects on *B. juncea*, which resulted in a decrease of plant biomass production. Since the plant phytoextraction efficiency can be defined as the product between plant biomass and Pb concentration in plant tissues, an autochthonous PGPR was augmented to *B. juncea* rhizosphere in order to face the observed biomass loss. The PGPR was isolated from the EDTA-added plant rhizosphere. This allowed the use of a strain capable of synergistic interaction with *B. juncea*, resistant to soil contamination, particularly to lead, and possibly to EDTA as well. This approach was in agreement with previous observation that inoculation of rhizosphere with PGPRs increased plant biomass in *B. juncea* grown on contaminated soils (Belimov et al., 2001). Moreover, PGPRs have been reported to influence plant biomass production (Hall et al., 1996) by lowering plant ethylene synthesis through 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity (Glick, 1995; Glick et al., 1998).

The PGPR strain herein isolated showed a quite high MIC for lead and ACC deaminase activity. The strain was attributed to the genus *Sinorhizobium*, previously reported as PGPR (Gallegiullos et al., 2000). Nevertheless, once the strain was augmented to the rhizosphere of origin, a decrease in plant biomass was observed, with stronger effects in untreated vegetated soil. According to these observations, some authors reported that plant response to rhizosphere inoculation with PGPRs, showing ACC deaminase activity, is affected by the plant trophic conditions. Actually, massive inoculation of the plant rhizosphere with such bacterial strains can decrease plant nutrient uptake (Belimov et al., 2002).

As a matter of fact, the observed *B. juncea* biomass loss was not associated to an increase of Pb accumulation in plant tissues. Thus, because of the failure in optimizing *B. juncea* phytoextraction efficiency by solely increasing biomass, an alternative approach, focusing on the increase of lead concentration in plant tissues, was adopted. The goal may be reached by exploiting the capacity of EDTA to widen root permeability to the EDTA–Pb complexes. However, increasing EDTA concentrations may also cause biomass loss (Vassil et al., 1998; Chen and Cutright, 2001). Furthermore, the amount of EDTA in soil, at a field scale, should not exceed mandatory levels in order to limit the risks of production of bioavailable and toxic chelant–metal complexes, impacting groundwater quality (Schmidt, 2003). Therefore, Triton X-100, a nonionic surfactant, was supplemented to soil. In fact, nonionic surface-active agents, at concentrations above the cmc, damage phospholipid membranes (Cserhati, 1995), putatively increasing their permeability to solutes. Furthermore, as confirmed in this study, synthetic surfactants alone do not solubilize metals in soil, avoiding negative effects of their application on metal groundwater contamination. Actually, Triton X-100 showed toxic effects on *B. juncea*. Since this surfactant did not cause either an increment in Pb bioavailable fraction in soil or accumulation of lead in plant tissues, it is reasonable to suppose a direct phytotoxic effect of the chemical, probably ascribable to the damage of root cell plasma membranes. This hypothesis was confirmed by the combined addition of Triton X-100 and EDTA in presence of *Sinorhizobium* sp. Pb002. Despite a similar biomass production, a significant increase in the concentration of lead in plant tissues was obtained respect to that reached in presence of EDTA alone. Such evidences may be explained

with the increase of plant permeability to the EDTA–Pb complexes in presence of Triton X-100, which ended up in a 48% increment of *B. juncea* phytoextraction efficiency. However, the combined soil supplementation with EDTA and Triton X-100 was lethal to plants, and rhizosphere inoculation with *Sinorhizobium* sp. Pb002 was essential for plant survival. Thus, an improvement in the efficiency of plant phytoextraction occurred only in the presence of *Sinorhizobium* sp. Pb002.

Taking into consideration the bacterial ecology of microcosms after the different treatments, it is interesting to note that, a severe modification of the bacterial community structure of the soil from Officina Gas Bovisa was related to the vegetation with *B. juncea*. This latter caused the disappearance of the dominant bacterial population originally present in unvegetated soil along with the appearance of a new one. EDTA slightly affected the bacterial community structure, with the exception of the concomitant presence of *B. juncea* and *Sinorhizobium* sp. Pb002. On the contrary, the strain augmentation was always accompanied by relevant changes in DGGE profiles, associated to increased bacterial diversity of vegetated microcosms, either in presence or absence of EDTA. The same was observed in the case of Triton X-100 amendment that always determined the occurrence of new dominant bacterial populations, characterized by the massive presence of new bacterial species, when compared to the DGGE profile of vegetated and untreated soil. Noteworthy, although strongly modifying soil microbial ecology, surfactant amendment as well as *Sinorhizobium* sp. Pb002 augmentation did not reduce soil bacterial diversity.

Triton X-100 and EDTA had a negative effect on the growth of *Sinorhizobium* sp. Pb002. This result is apparently in contrast with (i) the observation that PGPR augmentation of Triton X-100-treated soil greatly improved vegetative growth, which, otherwise, was affected by Triton X-100 phytotoxicity, and with (ii) the observation that *Sinorhizobium* sp. Pb002 was present after six weeks as a dominant species within a restricted bacterial population when EDTA and Triton X-100 were concomitantly amended to soil. Here, it should be reminded that the metabolic activity of a strain cannot be unequivocally revealed by 16S rDNA DGGE analysis. The application of the RT-DGGE analysis may be the appropriate tool to monitor the metabolic activity of strains rather than their numerical abundance in a microbial community (Duineveld et al., 2001). In fact, the capacity of *Sinorhizobium* sp. Pb002 to decrease plant ethylene production, rather than the numerical incidence of this strain in the soil bacterial community, may be reasonably involved in decreasing phytotoxicity of Triton X-100 and bioavailable lead. Thus, discrepancies between DGGE profiles, putative presence of *Sinorhizobium* sp. Pb002 in the rhizosphere, and effect of the bacterial strain on plant biomass can be related to the limits of the analytical protocol herein adopted.

5. Conclusions

In heavily contaminated soils where pollutants exceed the limit of plant tolerance, it can be possible to treat plants with PGPRs, in order to stimulate biomass production, and more in general to stimulate plant defenses against stress. In fact, it has been previously observed that genes and/or gene classes associated with plant defenses against abiotic and biotic stress may be co-regulated, and induced by PGPR (Timmusk and Wagner, 1999).

The isolation of the bacterial strain *Sinorhizobium* sp. Pb002 along with the augmentation of *B. juncea* rhizosphere with this microbe allowed to maintain a quite efficient phytoextracting plant biomass under severe stress conditions. *Sinorhizobium* sp. Pb002 overcame plant stress due to possible surfactant-induced disruption of root mechanical barriers. Probably, the strain depressed plant ethylene production. However, regardless of the true mechanism exploited to protect *B. juncea*, *Sinorhizobium* sp. Pb002 can definitely find applications in phytoremediation strategies when plants should be helped in facing toxic effects. Moreover, the combined application of Triton X-100 and *Sinorhizobium* sp. Pb002 certainly improved *B. juncea* phytoextraction efficiency. However, the effectiveness of the integrated approach herein described requires to be verified at field scale, even though it might be critical in terms of application of synthetic surfactants to soils. In fact, nonionic surfactants are used to improve soil bioavailability of organic and inorganic compounds (Shin et al., 2005; Zhao et al., 2005). Nevertheless, albeit not depressing soil bacterial diversity, this study shows that nonionic surfactants can affect the growth of a wide variety of microorganisms. This influence could have a great impact on the environment. Thus, further information on surfactant effects on soil fertility would be of considerable interest for a safe application of these chemicals in the context of bioremediation. At the same time, the use of biosurfactantants and biodegradable chelating agents should be considered in order to develop more environmentally sound treatments exploiting assisted phytoextraction of toxic metals from soil.

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