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STUDY OF POLYCYCLIC AROMATIC HYDROCARBON-TRANSFORMING MICROBIAL COMMUNITIES FROM CONTAMINATED SITES

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LIST of ORIGINAL PAPERS

This thesis comprised the following articles, which are referred in the text by the roman numerals:

[I] Zocca C, Di Gregorio S, Visentini F and Vallini G. . 2004. Biodiversity amongst cultivable polycyclic aromatic hydrocarbon-transforming bacteria isolated from an abandoned industrial site. FEMS Microbiol Lett. 238: 375-82;

[II] Di Gregorio S, Zocca C, Sidler S, Toffanin A, Lizzari D and Vallini G. 2004. Identification of two new sets of genes for dibenzothiophene transformation in *Burkholderia* sp. DBT1. Biodegradation. 15: 111-23

RIASSUNTO

INTRODUZIONE

Gli idrocarburi policiclici aromatici (IPA) sono sostanze altamente inquinanti, rilasciate nell'ambiente a seguito della incompleta combustione di matrici di origine fossile (petrolio e carbone) e come emissione di numerosi processi produttivi.

Gli IPA possiedono uno scheletro molecolare costituito da due o più strutture cicliche condensate rappresentate sia da anelli benzenici che da anelli furanici. Gli IPA sono composti organici costituiti essenzialmente da carbonio ed idrogeno, tuttavia possono recare gruppi sostituenti ovvero eteroatomi di zolfo, ossigeno ed azoto inseriti nello scheletro carbonioso. Gli IPA, a temperatura ambiente, si presentano allo stato solido ed hanno punti di fusione ed ebollizione elevati; la loro tensione di vapore è generalmente bassa, ed è inversamente proporzionale al numero di anelli presenti nella struttura molecolare. I composti appartenenti a questa classe sono sostanze poco solubili o del tutto insolubili in acqua, con solubilità decrescente in funzione dell'aumento del peso molecolare. La dispersione degli IPA nell'ambiente dipende principalmente dalla solubilità in acqua e dalla pressione di vapore: composti più solubili presentano maggiore capacità di migrazione mentre quelli ad alto peso molecolare hanno una maggiore tendenza ad essere trattenuti dalle diverse matrici ambientali.

Gli IPA in generale possiedono proprietà tossiche, mutagene e cancerogene. Gli effetti degli IPA sulla crescita, sul metabolismo e sulla formazione di tumori nei mammiferi, ivi compreso l'uomo, sono ampiamente documentati nella letteratura scientifica [Goldman et al., 2001; Mastrangelo et al., 1996]. Sulla base della diffusione e tossicità l'Agenzia per la Protezione dell'Ambiente americana (United States Environmental Protection Agency, US-EPA) ha stilato una lista di 16 composti più rappresentativi (Figura I nel testo).

Naftalene e fenantrene sono le due molecole a basso peso molecolare che vengono generalmente prese a modello degli IPA non sostituiti. D'altra parte il dibenzotiofene, (Figura II nel testo), rappresenta il composto modello della classe degli idrocarburi policiclici aromatici eterosolforati. Il naftalene rappresenta tra gli IPA la molecola con struttura più semplice (scheletro carbonioso formato dall'unione di soli due anelli condensati) e a minor peso molecolare. È un frequente microinquinante delle acque e la sua tossicità, anche nei confronti dell'uomo, è purtroppo ben nota [Mastrangelo et al., 1996]. Il fenantrene possiede una struttura chimica derivante dalla condensazione di tre anelli aromatici. Questo composto può essere considerato il primo della classe degli IPA con dimostrata cancerogenicità nei confronti dell'uomo [Bucker et al., 1979]. Il dibenzothiofene costituisce il modello di riferimento delle

reazioni biodegradative a carico dei composti organici solforati presenti nelle matrici fossili in quanto può rappresentare adeguatamente il nucleo di molti composti organosolforati presenti nelle matrici combustibili.

La diffusione nell'ambiente di sostanze tossiche e potenzialmente cancerogene rappresenta un problema sempre più rilevante per la salute e la qualità della vita delle persone. La necessità di risanare aree contaminate da queste ultime ha portato allo sviluppo di numerose metodologie di bonifica. Nella fattispecie, nell'ultimo decennio, lo sforzo tecnologico in campo ambientale è stato rivolto verso la ricerca di sistemi di bonifica, anche di aree contaminate da IPA, che fossero in grado di preservare le caratteristiche originarie del sito da risanare, risultando di conseguenza non distruttivi. Con questi presupposti ha quindi ricevuto notevole impulso la diffusione di sistemi di risanamento biologico in grado di sfruttare le potenzialità metaboliche di organismi viventi (principalmente microorganismi ma anche piante superiori) per promuove la trasformazione delle sostanze inquinanti in altre non nocive (Tabella II, nel testo). Tra i diversi sistemi di bonifica, di particolare interesse risultano essere i trattamenti di *bioaugmentation* e *phytoremediation*. Il primo approccio prevede l'introduzione massiva di biomasse microbiche selezionate nelle matrici da risanare al fine di incrementare l'attività bio-trasformatrice intrinseca delle stesse. D'altro canto l'approccio di *phytoremediation* sfrutta le capacità di sistemi vegetali di intervenire direttamente nell'attività di bonifica (ad esempio trasformando, volatilizzando o stabilizzando i composti contaminanti) ovvero di fungere da stimolo e supporto all' attività microbica (ad esempio attraverso la produzione di nutrienti attraverso il sistema radicale).

Nel caso specifico degli IPA una grande varietà di microrganismi, come batteri e funghi, sono noti per essere in grado di trasformare queste molecole in composti organici a struttura più semplice o nei prodotti finali della completa mineralizzazione, anidride carbonica ed acqua [Cerniglia, 1992]. In particolare, la degradazione batterica in condizioni aerobiche, inizia in genere mediante l'azione di una diossigenasi su uno degli anelli aromatici con formazione di un *cis*-diidrodiolo. Attraverso ulteriori passaggi enzimatici caratteristici della via degradativa, la molecola di *cis*-diidrodiolo subisce modificazioni sequenziali che portano infine alla formazione di catecolo. Il catecolo è poi facilmente degradato con produzione di composti che entrano nella via metabolica degli acidi tricarbossilici [Cerniglia, 1992]. All'opposto, il contributo dei sistemi vegetali sembra essere essenzialmente di supporto all'azione microbica [Anderson et al., 1994].

Negli ultimi anni, oltre alla caratterizzazione biochimica del processo di trasformazione batterica degli IPA, è risultata sempre più interessante, dal punto di vista applicativo, l'indagine dei geni catabolici che sovrintendono alla trasformazione di questa classe di composti. Informazioni in questo senso possono, di fatto, permettere di sviluppare approcci molecolari per il monitoraggio delle popolazioni batteriche IPA trasformatrici in matrici contaminate [Lloyd-Jones et al., 1999; Widada et al., 2002a]. Infatti, prima di

allestire un intervento di bonifica biologica devono poter essere valutate la presenza e le caratteristiche metaboliche delle comunità batteriche adattate al sito da risanare. Approcci molecolari basati sull'ibridazione o sull'amplificazione di geni catabolici coinvolti nelle trasformazioni di interesse potrebbero quindi rivelarsi utili per ottenere queste informazioni [Widada et al., 2002c].

D'alto canto, l'utilizzo di trattamenti di *bioaugmentation* prevede la necessità di sviluppare metodiche che permettano di seguire il destino delle biomasse microbiche all'interno dell'ambiente nel quale sono state aggiunte. Ad oggi diversi approcci di tipo molecolare sono stati sviluppati in questo senso e diversi sono i geni che sono stati scelti come *target* di reazione. In particolare, il monitoraggio dei ceppi introdotti, attraverso la ricerca dei rispettivi geni catabolici, può fornire informazioni riguardanti la persistenza (analisi basate sul DNA) e l'attività degradatrice (analisi basate sul mRNA) di questi ultimi all'interno dei sistemi nei quali sono stati introdotti [Widada et al., 2002c].

SCOPO E ALLESTIMENTO SPERIMENTALE

Nell'arco dei tre anni di questa ricerca, è stato caratterizzato dal punto di vista microbiologico un campione di suolo proveniente da un sito industriale dismesso, (area ex-Carbochimica, Trento), storicamente contaminato da IPA. Gli obiettivi generali della sperimentazione sono stati:

1) studiare le comunità batteriche adattate e trasformatrici presenti nel sito per conoscere il potenziale di risanamento intrinseco allo stesso e per poter scegliere un protocollo di bonifica biologica coerente con lo scenario microbico della matrice da risanare

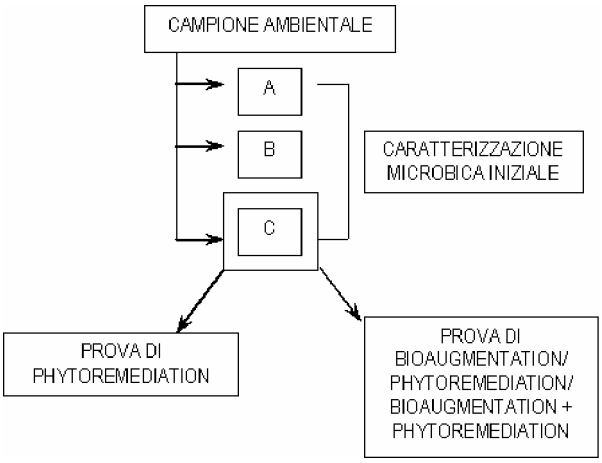
2) investigare il possibile modificarsi delle comunità batteriche in seguito ad alcuni interventi di bonifica messi in atto (su scala di laboratorio) per cercare di dare una spiegazione di tipo microbiologico alle prestazioni ottenute

3) valutare la possibilità di monitorare con metodiche molecolari ceppi batterici degradatori introdotti nella matrice da bonificare.

La sperimentazione condotta si è quindi articolata in due fasi (*Schema 1*):

- *prima fase*: caratterizzazione delle popolazioni IPA degradatici selezionatesi in tre aliquote di suolo (A: 200, B: 400, C: 14000 mg di IPA per kg di suolo) provenienti dal medesimo campionamento;
- *seconda fase*: allestimento di due prove di *bioremediation*, su scala di laboratorio, dell'aliquota maggiormente contaminata. Nel dettaglio sono state realizzate: una prova di bonifica fito-assistita (*Phytoremediation)* e una seconda prova che ha previsto l'utilizzo di approcci di *Bioaugmentation,* di *Phytoremediation* e di un protocollo misto *Phytoremediation-Bioaugmentation.* Questa seconda prova è stata condotta dopo un anno dall'allestimento della prima.

Schema 1



Prima fase

La CARATTERIZZAZIONE MICROBICA INIZIALE del campione di suolo in esame ha riguardato:

- la caratterizzazione delle comunità batteriche adattate presenti nel suolo e la valutazione del loro modificarsi in base al variare della concentrazione dei contaminanti attraverso la metodica molecolare 16SrDNA PCR-*Denaturing Gradient Gel Electrophoresis* (DGGE);

- l'isolamento attraverso colture selettive di arricchimento di ceppi batterici che possiedono le capacità cataboliche necessarie alla trasformazione degli IPA;

- l'identificazione dei geni funzionali che sovrintendono ai processi di trasformazione di interesse mediante reazioni di PCR condotte sia a partire da DNA genomico dei ceppi isolati sia direttamente dal DNA totale estratto dalla matrice in studio.

L'obbiettivo di questa prima fase di sperimentazione è stato quello di valutare il potenziale di biorisanamento intrinseco alla matrice da trattare, di individuare nuove classi di geni responsabili per la trasformazione degli IPA, di isolare ceppi batterici da utilizzare in successivi protocolli di *bioaugmentation* (Figura VIII nel testo).

Seconda fase

Sulla scorta dei risultati ottenuti durante la prima fase della sperimentazione, sono state condotte due prove di *bioremediation*.

L'esperimento di *Phytoremediation* (PROVA di PHYTOREMEDIATION) ha previsto l'allestimento di microcosmi di suolo vegetati con *Zea mays* var. Matisse oppure con *Lupinus albus* var. Multitalia. Nei sistemi così ottenuti sono stati valutati:

- l'abbattimento dei contaminanti in presenza e in assenza delle specie vegetali utilizzate;

- il modificarsi, dal punto di vista tassonomico, della comunità batterica degradatrice coltivabile al passaggio dalla condizione di non-vegetazione a quella di vegetazione (sia *L. albus* che *Z. mays*);

- l'eventuale capacità dei sistemi vegetali di selezionare specie batteriche depositarie di classi specifiche di geni codificanti gli enzimi coinvolti nella attività di trasformazione (genotipi degradatori).

L'indagine è stata condotta utilizzando gli stessi approcci adottati nella prima prova per poter confrontare i risultati ottenuti (Figura IX nel testo).

L' ultima prova (PROVA di BIOAUGMENTATION, PHYTOREMEDIATION e BIOAUGMENTATION + PHYTOREMEDIATION) ha invece previsto l'allestimento di microcosmi vegetati o non vegetati con *Lupinus albus*, inoculati con:

- una coltura mista costituita da tre tra i ceppi batterici (NDP003; 5N-C; L8A-C) trasformatori isolati dalla stessa matrice in esame (*bioaugmentation* con microflora autoctona) (INOCULO BATTERICO I) (Figura X nel testo);
- una coltura pura di un ceppo batterico (*Burkholderia* sp. DBT1) precedentemente selezionato per la capacità di trasformare IPA [I] (*bioaugmentation* con microflora alloctona) (INOCULO BATTERICO II) (Figura X nel testo).

Per ogni tipo di microcosmo è stata valutata:

- la capacità di abbattimento degli IPA in base alla strategia di bonifica adottata;

- la possibilità di formulare una possibile spiegazione microbiologica dei risultati di abbattimento registrati nei diversi sistemi, mediante l'applicazione dell'approccio molecolare 16SrDNA PCR-DGGE (Figura X nel testo);

- la possibilità di quantificare e monitorare la presenza dell' INOCULO BATTERICO II e del suo caratteristico genotipo degradatore sia nei microcosmi inoculati sia in quelli non inoculati (valutazione del contenuto intrinseco alla matrice), attraverso un protocollo TaqMan Real Time PCR (Figura X nel testo).

RISULTATI

CARATTERIZZAZIONE MICROBICA INIZIALE

- Lo studio delle comunità batteriche adattate e trasformatrici coltivabili condotto attraverso l'analisi *Most Probable Number* (MPN), sulle tre aliquote di suolo (A, 200 mg IPA/kg suolo; B, 400 mg IPA/kg suolo; C, 14000 mg IPA/kg suolo), ha permesso di stabilire che nel sito è presente una comunità batterica coltivabile (eterotrofi totali) dell'ordine di 10^5 MPN/g di suolo indipendentemente dalla

concentrazione dei contaminanti. Tuttavia, l'aumentare del livello di contaminazione ha determinato un aumento della frazione di batteri autoctoni in grado di trasformare gli IPA (21% nell'aliquota C, 14000mg IPA/kg suolo, *vs*. 2.07-0.94% nelle aliquote A e B meno contaminate);

- L'analisi PCR-DGGE ha permesso di stabilire che nel sito in esame le comunità batteriche adattate risultano complesse e eterogenee indipendentemente dal livello di contaminazione. Inoltre in tutti e tre i profili relativi alle aliquote analizzate (A, B e C) si e potuta riscontrare la presenza di almeno due specie dominanti riconducibili a ceppi appartenenti ai generi *Cytophaga/Flavobacterium* e *Arthrobacter/Acinetobacterium*. L'analisi dei profili evidenzia inoltre l'aumento della dominanza relativa di queste due specie nell'aliquota a contaminazione maggiore;

- L'analisi dei geni catabolici che sovrintendono alla trasformazione degli IPA condotta direttamente da DNA estratto da suolo ha permesso di identificare chiaramente la presenza della classe di geni più ampiamente descritta in letteratura (geni *nah* simili) in tutte e tre le aliquote di suolo. Inoltre è stato possibile identificare per la prima volta in suolo la presenza del genotipo non classico *dbt*. Tuttavia questo secondo genotipo è stato rintracciato solo nelle aliquote a minore contaminazione (A e B).

- L'isolamento attraverso colture di arricchimento a permesso di isolare 107 ceppi in grado di trasformare gli IPA che sono stati suddivisi mediante metodica *Amplified Ribosomal DNA Restriction Analisys* (ARDRA) in 26 diverse *Operational Taxonomic Units* (OTU). L' 84.6% delle OTU ottenute ospita ceppi batterici appartenenti al phylum dei Proteobatteri (22/26 OTU).

-- l'analisi dei geni catabolici che sovrintendono alla trasformazione degli IPA condotta a partire dal DNA genomico dei singoli isolati ha permesso di stabilire che il 19% delle OTU, sono in grado di utilizzare gli IPA grazie alla presenza di geni *nah*-simili. La natura dei geni coinvolti nella degradazione delle rimanenti unità tassonomiche rimane ad oggi non caratterizzata.

PROVA DI PHYTOREMEDIATION

- L'applicazione del protocollo di *phytoremediation,* utilizzando le specie vegetali *Lupinus albus* e *Zea mays*, ha permesso di ottenere, dopo 10 settimane di trattamento, una diminuzione del livello iniziale di contaminazione dell' aliquota C (14000 mg IPA/kg) rispettivamente del 31.7% e del 22.2%. Di contro l'abbattimento registrato nel suolo non vegetato si attesta attorno al 18.6% della concentrazione iniziale;

- l'analisi dei geni catabolici coinvolti nella trasformazione degli IPA ,condotta direttamente da DNA estratto da suolo, ha permesso di identificare la presenza dei geni *nah* simili nel DNA estratto da entrambi i sistemi vegetali. Inoltre è stato possibile rintracciare la presenza del genotipo non classico

dbt nel DNA estratto dalla rizosfera di *Lupinus albus*. Questo genotipo rimane comunque non individuabile nel DNA estratto dal suolo non vegetato e da quello derivante dalla rizosfera di *Z. mays*;

- Il protocollo di isolamento adottato ha permesso di isolare 18 e 21 ceppi IPA trasformatori rispettivamente dalla rizosfera di *Z. mays* e *L. albus*. Mediante metodica ARDRA i complessivi 39 isolati sono stati distribuiti in 16 diverse *Operational Taxonomic Units* (OTU) (*Z. mays*, 10 OTU; *L.albus* 11 OTU). L'analisi tassonomica delle OTU ha dimostrato che la presenza dei due sistemi vegetali comporta una preferenziale selezione di ceppi appartenenti ai phyla degli Actinobatteri (50% e 54.5% rispettivamente in *Z. mays* e *L. albus*) e Firmicutes (10% e 36.4% rispettivamente in *Z. mays* e *L. albus*) rispetto al phylum dei Proteobatteri (40% e 9.1% rispettivamente in *Z. mays* e *L. albus*). Questa distribuzione risulta sostanzialmente diversa da quella ottenuta in condizioni di non vegetazione (84.6% Proteobatteri);

- L'analisi dei genotipi IPA trasformatori condotta a partire dal DNA genomico dei singoli isolati ha permesso di caratterizzare il 19% delle unità tassonomiche individuate. In particolare, ancora una volta, ceppi appartenenti a queste ultime sono in grado di utilizzare gli IPA grazie a geni *nah*-simili. Comunque, la natura dei geni coinvolti nella degradazione delle rimanenti unità tassonomiche rimane ad oggi non caratterizzata. Poiché, sia in presenza che in assenza di vegetazione, più del 70% delle OTU individuate rimane non caratterizzata rispetto al genotipo degradatore non è stato possibile investigare l'eventuale effetto di selezione di una specifica classe di geni trasformatori, operato da parte dei sistemi vegetali utilizzati.

PROVA di BIOAUGMENTATION, PHYTOREMEDIATION e BIOAUGMENTATION + PHYTOREMEDIATION

- L'abbattimento del contenuto totale di IPA nel suolo sterilizzato è risultato pari al 5.8% del contenuto iniziale (10000 mg IPA/kg suolo, dopo un anno dall'allestimento della prova di *phytoremediation*). Al contrario, in tutti i sistemi non sterilizzati i valori percentuali di abbattimento sono risultati significativamente più grandi. Tuttavia, l'applicazione dei protocolli di *phytoremediation* (suolo vegetato con *L. albus*), di *bioaugmenation* con INOCULO BATTERICO I (coltura mista) e INOCULO BATTERICO II (coltura pura) e l'approccio combinato *bioaugmenation+phytoremediation* con INOCULO BATTERICO I (coltura mista) e *L. albus,* non hanno sostanzialmente incrementato il complessivo abbattimento registrato nei microcosmi non trattati, ossia non vegetati e non inoculati. Tuttavia l'analisi dettagliata della velocità di trasformazione di alcuni dei 16 composi presi in esame evidenzia che, nella maggior parte dei casi, l'applicazione dei diversi protocolli di bonifica accelera la dissipazione dei contaminanti;

- L'intervento di bonifica biologica condotto secondo il protocollo *bioaugmenation+phytoremediation* con INOCULO BATTERICO II (coltura pura) ha permesso di ottenere un abbattimento complessivo statisticamente maggiore (31,1%) rispetto a quello del suolo non trattato (21.5%). Inoltre questo trattamento è risultato in grado di accelerare la dissipazione di tutti i contaminanti, considerati nel dettaglio, all'interno delle prime due settimane di applicazione;

- L'analisi PCR-DGGE ha permesso di dare una possibile spiegazione dal punto di vista microbiologico ai risultati di abbattimento ottenuti. Infatti ha permesso di correlare le scarse prestazioni ottenute mediante l'applicazione dei protocolli di *bioaugmenation* con INOCULO BATTERICO I (coltura mista) e l'approccio combinato *bioaugmenation+phytoremediation* con INOCULO BATTERICO I (coltura mista) e *L. albus* con la non persistenza durante tutto l'arco della sperimentazione di tutti i ceppi introdotti;

Inoltre l'analisi PCR-DGGE ha permesso di ipotizzare una positiva correlazione tra le buone prestazioni ottenute con l'applicazione del protocollo *bioaugmenation+phytoremediation* con INOCULO BATTERICO II (coltura pura) e la persistenza del INOCULO BATTERICO II per tutto l'arco della sperimentazione;

- Il protocollo TaqMan Real Time adottato ha dimostrato di possedere, ad oggi, limiti di determinazione molto alti e di necessitare di ulteriori fasi di ottimizzazione. Tuttavia, la coppia di primer e sonda disegnati si sono rivelati specifici per il gene usato come target di reazione per monitorare la presenza dell' INOCULO BATTERICO II nel suolo. Inoltre, il protocollo adottato benché non ottimizzato ha permesso di seguire la presenza dell' INOCULO BATTERICO II durante tutto il corso della sperimentazione. Non solo, il protocollo saggiato sembra in grado di contrastare gli effetti negativi delle sostanze co-purificate con il DNA sulla reazione di PCR e sulla accuratezza della quantificazione del gene target nel DNA estratto da suolo. D'alto canto non è stato possibile valutare la presenza naturale del gene target di amplificazione nei sistemi non inoculati probabilmente a causa dei limiti intrinseci alle condizioni di reazione.

CONCLUSIONI

- La CARATTERIZZAZIONE MICROBICA INIZIALE delle popolazioni autoctone al sito in studio ha permesso di evidenziare la presenza, all'interno del sistema, di ceppi batterici in grado di trasformare gli IPA e quindi virtualmente in grado di contribuire alla risanamento della matrice in esame;

- Le indagini molecolari condotte a partire dal DNA estratto da suolo, al fine di caratterizzare la diversità dei genotipi degradatori che sovrintendono alla trasformazione degli IPA, hanno permesso di individuare almeno due classi di geni funzionali, in particolare geni *nah*-simili e *dbt*-simili. La presenza di

specie batteriche recanti il genotipo classico *nah* è stata dimostrata in tutte le aliquote con differente grado di contaminazione. D'altro canto, la presenza del genotipo *dbt* è stata rintracciata solo nelle aliquote meno contaminate. Risulta comunque importante sottolineare che questa sembra essere la prima evidenza sperimentale della presenza del genotipo *dbt* in campioni ambientali;

- Le indagini molecolari condotte a partire dal DNA genomico dei singoli isolati ha permesso di stabilire che la maggior parte dei ceppi batterici ottenuti in questo studio non possiede geni trasformatori ad oggi noti. Tuttavia, aver isolato in colture pura questi microrganismi fornirà la possibilità, attraverso successive indagini molecolari, di ampliare le informazioni disponibili rispetto alla diversità esistente all'interno delle classi di geni coinvolti nelle trasformazioni di interesse;

- La PROVA DI PHYTOREMEDIATION condotta ha permesso di stabilire che l'introduzione delle specie vegetali *Lupinus albus* e *Zea mays* ha comportato una riduzione della concentrazione iniziale degli IPA. Parallelamente, dal punto di vista microbiologico non è stato osservato un significativo aumento del numero dei microrganismi coltivabili in grado di trasformare gli IPA. Tuttavia, la presenza delle due specie vegetali ha comportato un cambiamento nella composizione della popolazione batterica trasformatrice dal punto di vista tassonomico. Inoltre, la presenza di *L. albus* ha determinato un arricchimento nel suolo del genotipo trasformatore non classico *dbt.*

- I protocolli di bonifica adottati nella prova di BIOAUGMENTATION, PHYTOREMEDIATION e BIOAUGMENTATION + PHYTOREMEDIATION sono risultati meno efficienti, in termini di abbattimento rispetto alla PROVA DI PHYTOREMEDIATION. Tuttavia, nella maggior parte dei casi, l'applicazione dei diversi protocolli di bonifica accelera la dissipazione dei contaminanti. In generale, comunque l'intervento che si è rivelato più efficacie è stato quello che ha previsto l'impiego del protocollo *bioaugmenation+phytoremediation* con INOCULO BATTERICO II (coltura pura);

D'altro canto l'uso della tecnica molecolare 16SrDNA-PCR-DGGE ha permesso, in modo efficacie, di seguire il modificarsi della composizione delle comunità microbiche a seguito degli interventi messi in atto e il destino dei ceppi batterici introdotti. L'analisi dei profili DGGE ottenuti ha permesso di fornire alcune possibili spiegazioni dei dati di abbattimento registrati;

-Per quanto concerne il protocollo RealTime adottato, esso dovrà essere ulteriormente ottimizzato al fine di abbassare il limite di determinazione attuale della tecnica. Tuttavia quest'ultima, nelle condizioni saggiate, ha comunque permesso di monitorare e quantificare la presenza dell' INOCULO BATTERICO II (coltura pura) durante tutto l'arco della sperimentazione.

1. INTRODUCTION

1.1 POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous class of hydrophobic organic compounds that are formed and released as a result of the incomplete combustion of organic material. Anthropogenic sources such as road traffic, petroleum sludge, asphalt, wood preservative wastes and combustion of fossil fuels, predominate [Cooke and Dennis, 1983]; anyway also natural sources such as volcanic eruptions and forest fires contribute to the total PAHs burden. PAHs consist of fused benzene rings in linear, angular or clustered arrangements. They contain by definition only carbon and hydrogen atoms. However, sulfur, nitrogen and oxygen atoms may readily substitute in the benzene rings to form heterocyclic aromatic compounds, which are commonly grouped with the PAHs. PAHs have been thoroughly studied due to their toxicity, persistency and environmental impact [Howsam and Jones, 1998; Blumer, 2003]. On the basis of their diffusion and toxicity, 16 PAHs compounds have been identified as priority pollutants by the United States Environmental Protection Agency (US-EPA) (Figure I) [Keith and Telliard, 1979].

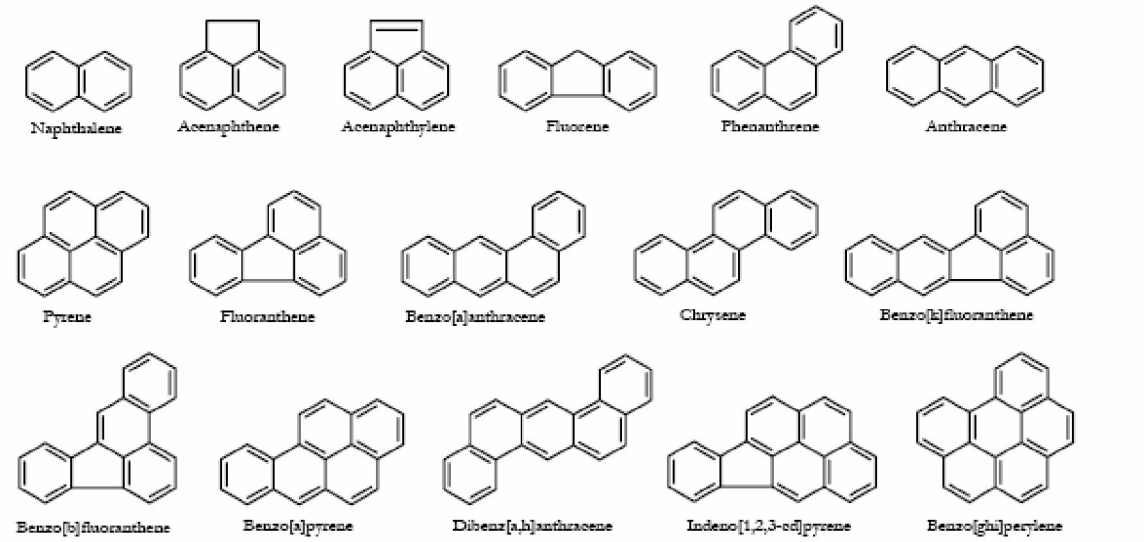


Figure I. Chemical structures of the 16 priority PAH compounds identified by US-EPA [Keith and Telliard, 1979].

1.1.1 PROPERTIES AND ENVIRONMENTAL FATE

Generally, PAHs are lipophilic compounds that show high affinity with organic matter. However, single PAHs differ substantially in their physico-chemical properties. As shown in Table I, properties such as aqueous solubility and vapour pressure range within five and twelve orders of magnitude, respectively, while considering two to six benzene rings in PAH-molecules. Thus, low molecular weight (LMW) PAHs are much more water soluble and volatile than high molecular weight (HMW) relatives, hence HMW

PAHs show higher hydrophobicity than LMW compounds [Mackay et al., 1992a]. The difference in hydrophobicity is also reflected by the octanol-water partitioning coefficient (K_{ow}) shown in Table I. These physico-chemical properties largely determine the environmental behaviour of PAHs and indicate that transfer and turnover will be more rapid for LMW PAHs than for the heavier PAHs [Wild and Jones, 1995]. The semivolatile nature of the LMW PAHs means that they exist in the atmosphere partly as vapours and are therefore highly susceptible to atmospheric degradation processes. Similarly, in aqueous environments, the LMW PAHs are partly dissolved, making them highly available to various degradation processes. On the other hand, HMW PAHs are primarily associated with particles in atmosphere and water and therefore are less available for degradation. Furthermore, PAHs adsorbed to particles may be transported over long distances in the atmosphere, thus becoming ubiquitous in the environment [Howsam and Jones, 1998; Wilson and Jones, 1993].

Table I. Properties of the 16 US-EPA PAHs, from Mackay et al. 1992a

	Number of rings	Molecular weight	Aqueous solubility (mg/l)	Vapor press. (Pa)	Log K_{ow}
Naphthalene	2	128	31	$1.0x10^{2}$	3.37
Acenaphthylene	3	152	16	$9.0x10^{-1}$	4.00
Acenaphthene	3	154	3.8	$3.0x10^{-1}$	3.92
Fluorene	3	166	1.9	$9.0x10^{-2}$	4.18
Phenanthrene	3	178	1.1	$2.0x10^{-2}$	4.57
Anthracene	3	178	0.045	$1.0x10^{-3}$	4.54
Pyrene	4	202	0.13	$6.0x10^{-4}$	5.18
Fluoranthene	4	202	0.26	$1.2x10^{-3}$	5.22
Benzo[*a*]anthracene	4	228	0.011	$2.8x10^{-5}$	5.91
Chrysene	4	228	0.006	$5.7x10^{-7}$	5.91
Benzo[*b*]fluoranthene	5	252	0.0015	-	5.80
Benzo[*k*]fluoranthene	5	252	0.0008	$5.2x10^{-8}$	6.00
Benzo[*a*]pyrene	5	252	0.0038	$7.0x10^{-7}$	5.91
Dibenzo[*a,h*]anthracene	6	278	0.0006	$3.7x10^{-10}$	6.75
Indeno[1,2,3-*cd*]pyrene	6	276	0.00019	-	6.50
Benzo[*ghi*]perylene	6	276	0.00026	$1.4x10^{-8}$	6.50

1.1.2 PAHs IN SOIL

Most PAHs are strongly sorbed to the organic matter in soil. Thus, they are relatively unavailable to degradation processes [Wild and Jones, 1995]. PAHs can therefore remain in soil for many centuries, posing a long-term threat to the environment [Howsam and Jones, 1998], despite partial degradation, volatilization and leaching of LMW PAHs [Wilson and Jones, 1993]. Sorption generally increases with the number of benzene rings in PAH-molecule [Bossert and Bartha, 1986; Sims and Overcash, 1983], since this implies higher lipophilicity. Furthermore, it has been shown that degradability and extractability of organic compounds in soil decrease with the time they have been in contact with soil: a phenomenon

referred to as “aging” or “weathering” [Hatzinger and Alexander, 1995; Loehr and Webster, 1996]. Aging is mainly a result of slow diffusion into the soil organic matter, but other mechanisms include the formation of bound residues and physical entrapment within soil micro pores [Pignatello and Xing, 1996; Alexander, 1995]. Sorption and aging limit, on one hand, the degradability of the contaminants. On the other hand, these processes reduce the toxicity of the soil contaminants, by lowering the fraction available to living organisms [Weissenfels et al., 1992; Alexander, 1995].

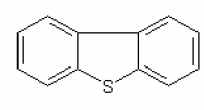
1.1.3 PAHs TOXICITY

Many PAHs have toxic, mutagenic and/or carcinogenic properties [Goldman et al., 2001; Mastrangelo et al., 1996]. Since PAHs are highly lipidsoluble, they are readily absorbed by the gastrointestinal tract of mammals [Cerniglia, 1984]. They rapidly diffuse in a wide variety of tissues with a marked tendency for the localization in body fats. Metabolism of PAHs occurs via the cytochrome P450-mediated mixed function oxydase system with oxidation or hydroxylation as the first step [Stegeman et al., 2001]. The resulting epoxides or phenols might get detoxified in a reaction with the production of glucoronides, sulfates or glutathione conjugates. Some of the epoxides might be metabolized into dihydrodiols which, in turn, could undergo conjugation to form soluble detoxification products or be oxidized to diol-epoxides. Many PAHs contain a ‘bay-region’ as well as ‘K-region’, both of which allow metabolic formation of bay- and K-region epoxides, which are highly reactive. Carcinogenicity has been demonstrated by some of these epoxides [Goldman et al., 2001]. For these reasons, PAHs are considered environmental pollutants that can have a detrimental effect on the flora and fauna of affected habitats, resulting in the uptake and accumulation of toxic chemicals in food chains and, in some instances, in serious health problems and/or genetic defects in humans. Naphthalene, the first member of the PAH group, is a common micropollutant in potable water. The toxicity of naphthalene has been well documented and cataractogenic activity has been reported in laboratory animals [Goldman et al., 2001; Mastrangelo et al., 1996]. Naphthalene binds covalently to molecules in liver, kidney and lung tissues, thereby enhancing its toxicity; it is also an inhibitor of mitochondrial respiration [Falahatpisheh et al., 2001]. Acute naphthalene poisoning in humans can lead to haemolytic anaemia and nephrotoxicity. In addition, dermal and ophthalmological changes have been observed in workers occupationally exposed to naphthalene. Phenanthrene is known to be a photosensitizer of human skin, a mild allergen and mutagenic to bacterial systems under specific conditions [Mastrangelo et al., 1996]. It is a weak inducer of sister chromatid exchanges and a potent inhibitor of gap junctional intercellular communication [Weis et al., 1998]. Equivocal results for tumour initiation have been obtained with skin-painting studies in mice. Interestingly, because phenanthrene is the smallest PAH to have a bay-region and a K-region, it is often used as a model substrate for studies on the metabolism of carcinogenic

PAHs [Bucker et al., 1979]. Little information is available for other PAHs such as acenaphthene, fluranthene and flourene with respect to their toxicity in mammals. However, the toxicity of benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluranthene, dibenz(a,h)anthracene and indeno(1,2,3-c,d)pyrene has been studied and there is sufficient experimental evidence to show that they are carcinogenic [Mastrangelo et al., 1996; Liu et al., 2001; Sram et al., 1999].

1.1.4 DIBENZOTHIOPHENE

Heterocyclic sulfur polynuclear aromatic compounds have received increasing attention in recent years because of their carcinogenic and mutagenic properties combined with their nearly ubiquitous distribution in depositional environments [Kennicutt II et al., 1991, 1992; Domine et al., 1994; Yang, 1996]. Their existence can be explained as a result of chemical and biological processes through the biogeochemical cycle of sulfur as well as a consequence of anthropogenic activities. Dibenzothiophene (DBT) (Figure II) is a major compound of polycyclic aromatic sulfur heterocycles [Joan et al., 1991; Darren et al., 1995] and it has been selected as a model molecule for the description of the microbial capacity to transform organosulphurs [Kodama et al. 1970, 1973]. DBT occurs in petroleum, coal, airborne particulates, storm water runoff, living organisms (Bioaccumulation Factor, BCF: 470) and various depositional environments [Neff et al., 1992; James et al., 1995]. DBT persists mainly in soil (72%) and water (19%); nevertheless its half-life in sediment is greater than in the other two media (140 days *vs.* 30 and 15 in soil and water respectively) [determine by Level III multimedia mass balance model (fugacity model) of Mackay et al., 1992b, on line available: www.pbtprofiler.net]. Concerning DBT toxicity, several studies were conduced on mice [Jacob, 1990] and fishes [Eastmond et al., 1984]. Acute dibenzothiophene poisoning in mice can lead to death caused by gross lesions included pulmonary congestion and edema, mild hydrothorax, intestinal haemorrhage, and mottled livers. The major histological lesions were severe centrilobular hepatic necrosis, necrosis of lymphocytes in thymic cortices, and degenerative changes in the walls of small arteries in the lung [Leighton, 1989]. Moreover, dibenzothiophene–treated zebrafish embryo shows cardiac dysfunction, edema, spinal curvature, and reduction in the size of the jaw and other craniofacial structures [Incardona et al., 2004].



Dibenzothiophene

Number of rings	Molecular weight	Aqueous solubility (mg/l)	Vapor press. (Pa)	Log K_{ow}
3	184.3	0.74	$6.1x10^{-5}$	4.44

Figure II. Physical-chemical properties of Dibenzothiophene

1. 2 MICROBIAL DEGRADATION OF PAHS

Microrganisms can degrade PAHs by either metabolic or cometabolic reactions. Co-metabolism is defined as the oxidation of nongrowth substrates during the growth of an organism on another carbon or energy source. Cometabolis is important for the degradation of both PAHs mixture and HMW-PAHs. On the other hand, PAHs with 2- to 4- benzene rings have been describe for years to act as growth substrate for microbes, mainly bacteria. Although metabolism of PAHs by bacterial axenic cultures in anaerobic condition has been reported [Rockne et al., 2000], attention has been mostly paid to the aerobic metabolism. A variety of bacteria capable of utilising these compounds have been investigated so far. The initial step of the aerobic metabolism of PAHs is biologically slow and metabolically expensive [Huang et al., 2001]. The latter usually occurs *via* incorporation of molecular oxygen into the aromatic nucleus, by a multicomponent dioxygenase enzyme system which gives rise to a *cis*-dihydrodiol. This compound is then aromatised again through a *cis*-diol dehydrogenase to yield a dihydroxylated intermediate. The latter substrate can be cleaved by dioxygenase activity, *via ortho* or *meta*-cleavage, leading to linear compound entering the tricarboxylic acid cycle (TCA).

1.2.1 BACTERIAL METABOLISM OF NAPHTHALENE, PHENANTHRENE AND DIBENZOTHIOPHENE

Naphthalene has been used often as model compound of PAHs degradation. The degradation of naphthalene in soil pseudomonas was first reported by Davies and Evans [Davies and Evans, 1964] (Figure III). In the first catabolic step (in the pathway know as “upper catabolic pathway of naphthalene”) an oxygen molecule is introduced at the 1,2-position of the aromatic nucleus to produce *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene by naphthalene dioxygenase (NDO). This dioxygenase system consists of three components, a ferredoxin reductase, a ferredoxin, and an iron sulfur protein (ISP) composed of two non-identical subunits, α (large) and β (small). The α subunit contains the active site

mononuclear iron and a Rieske [2Fe-2S] center [Kauppi et al., 1998]. On the other hand, β subunit does not appear to be directly involved in the catalysis or substrate specificity, its main role is probably structural [Kauppi et al., 1998]. A single two-electron transfer from NAD(P) H to FAD in a ferredoxin reductase initiates the electron transport, which generate a fully reduced form of FAD. The reduced FAD provides one electron each to the [2Fe-2S] cluster of a ferredoxin. These electrons are finally transferred to an ISP and used in its active site to facilitate the addition of an oxygen molecule to the naphthalene. The substrate specificity of NDO has been analysed in detail [Resnick et al., 1996], and three-dimensional structure of NDO components was also identified [Kauppi et al., 1998]. Parales and co-workers [Parales et al., 1999], found that Asp-205 in the catalytic domain of NDO is essential for its activity, and the crystal structure showed that an alternative NDO substrate, indole, binds near the mononuclear iron, suggesting that the residue is the oxygen-activating site [Carredano et al., 2000]. *cis*-Naphthalene dihydrodiol is then dehydrogenated to 1,2-dihydroxynaphthalene by *cis*-naphthalene dihydrodiol dehydrogenase. 1,2- Dihydroxynaphthalene is *meta*-cleavaged by 1,2-dihydroxynaphthalene dioxygenase, and the resulting ring-cleavage product spontaneously recyclises to form 2-hydroxy-2*H*-chromene-2-carboxylic acid. Enzymatic reactions by an isomerase and hydratase aldolase result in the production of salicylaldehyde, which is then transformed to salicylate by salicylaldehyde dehydrogenase. Salicylate is further metabolised via catechol or gentisate to TCA cycle intermediates.

Phenanthrene has been used also as a model compound in PAHs degradation studies. The enzymes involved in the conversion of naphthalene to salicylate can degrade phenanthrene and dibenzothiophene to 1-hydroxy-2-naphthoate and 3-hydro-2-formilbenzothiophene respectively, through similar catabolic steps [Menn et al., 1993; Kodama et al., 1973] (Figure IV and V). Although it was suggested by Pinyakong and colleagues [Pinyakong et al., 2000] that *Sphingomonas* sp. strain P2 degrades phenanthrene *via* another pathway, by dioxygenation at the 1,2 position in addition to the known one, a pathway *via* dioxygenation at the 3,4 position is the major catabolic pathway of phenanthrene. In general phenanthrene is degraded to 1-hydroxy-2-naphthoate, which is further metabolised through two pathways. One of them produces 1,2-dihydroxynaphthalene, which then enters in the naphthalene degradation pathway. Alternatively, 1-hydroxy-2-naphthoate is directly cleaved by 1-hydroxy-2-naphthoate dioxygenase. Later enzymatic reactions result in production of phthalate that is further metabolised *via* protocatechuate to TCA cycle intermediates.

Through the initial dioxygenolytic attack at the 1,2 position, dibenzothiophene is converted to 1,2-dihydroxy dibenzothiophene. 1,2-Dihydroxy dibenzothiophene is degraded to 3-hydro-2-formilbenzothiophene, which is not further metabolised. This biotransformation mechanism, in which no desulfurisation occurs, was first established by Kodama and colleagues [Kodama et al., 1973].

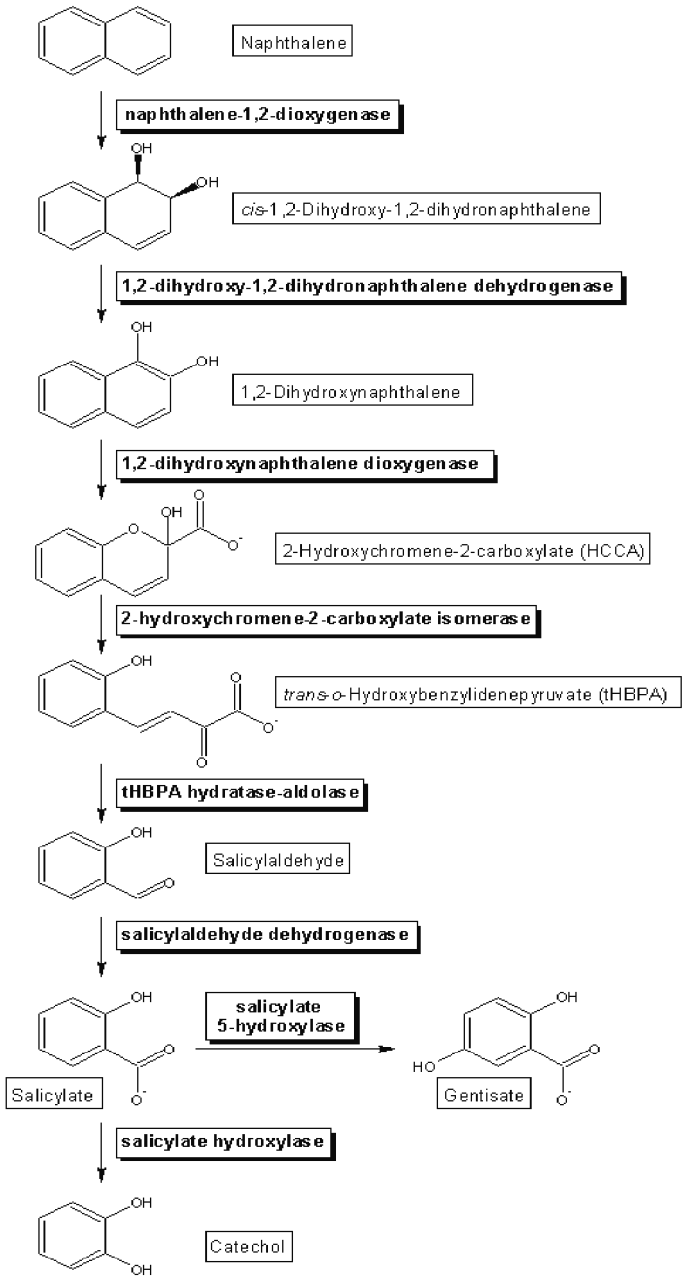


Figure III. Proposed "upper" catabolic pathway of Naphthalene by aerobic bacteria. On line available: http://umbbd.ahc.umn.edu/pha/pha_image_map_1.html

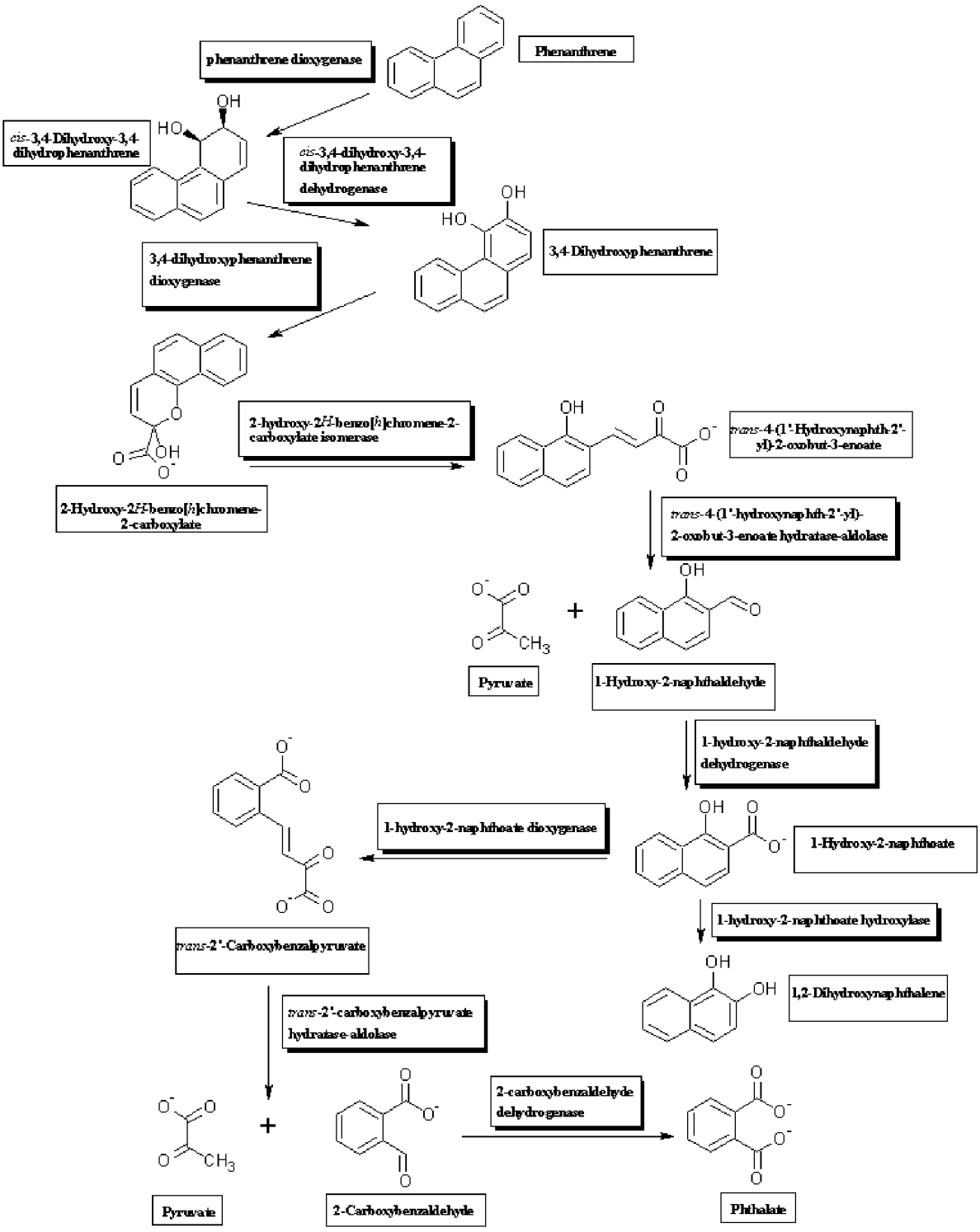


Figure IV. Proposed “upper” catabolic pathway of Phenanthrene by aerobic bacteria. On line available: http://umbbd.ahc.umn.edu/pha/pha_image_map_1.html

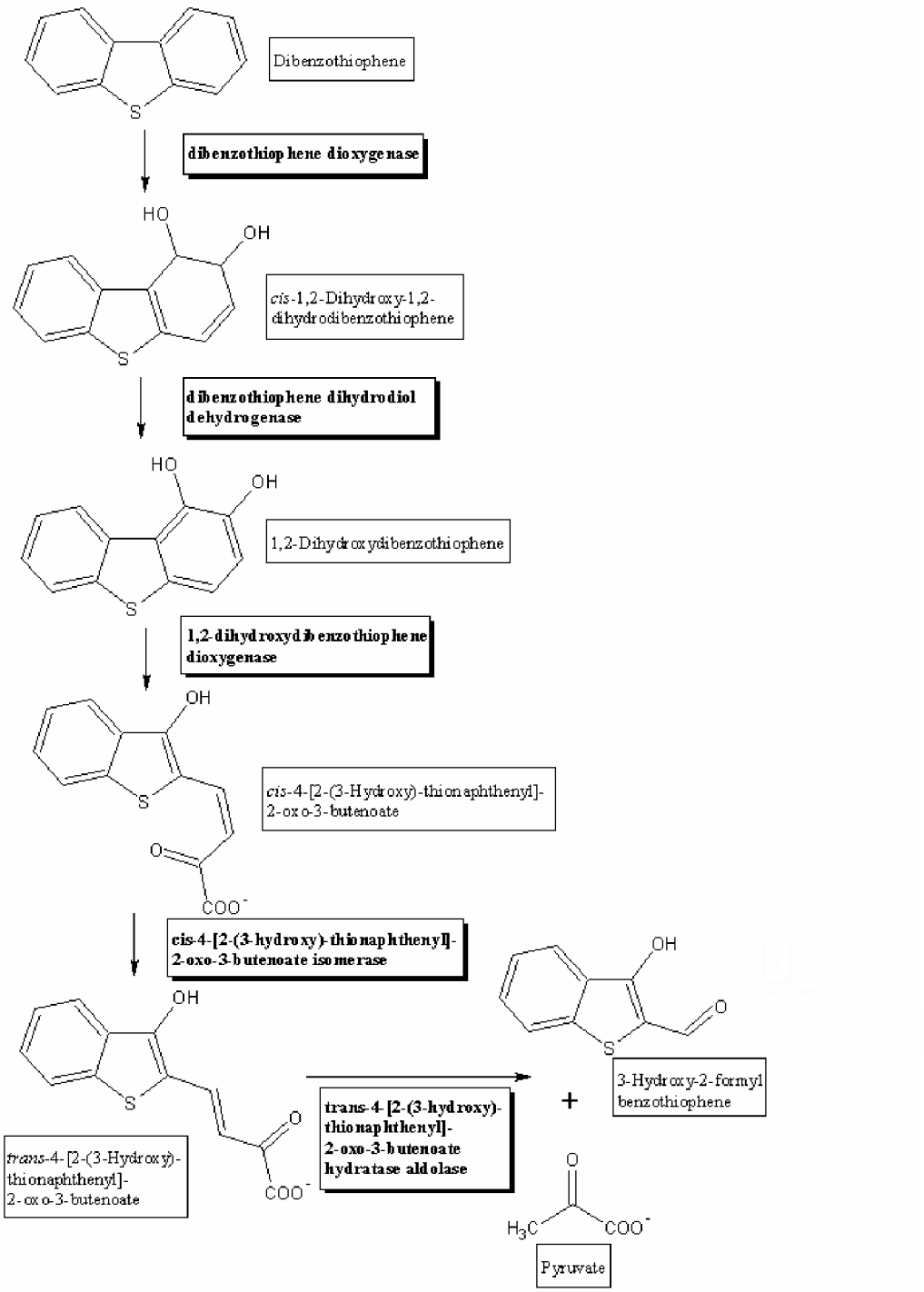


Figure V. Proposed oxidative pathway of Dibenzothiophene, *Kodama pathway*. On line available: http://umbbd.ahc.umn.edu/pha/pha_image_map_1.html

1.2.2 GENETICS OF PAHs IN DIVERSE AEROBIC BACTERIA

Recently, several genes encoding PAHs-catabolic enzymes have been characterised. Analysis of the PAHs-catabolic genes is of interest in applied aspects. Indeed, genetic information will be useful for the monitoring of bacterial populations that degrade PAHs in contaminated soils. In the last two decades, genetic analyses of PAHs degradation by aerobic bacteria have focused on naphthalene catabolic genes (*nah*-genes) from *Pseudomonas* species. However, the analysis of a single, high homologous group of genes may not reflect the true catabolic diversity of PAH-catabolic genes among the bacteria. For this reason, recent studies are focusing on the recovering and characterisation of pollutants catabolic genes from Gram-negative bacteria, other than those in genus *Pseudomonas*, and also from Gram-positive bacteria.

1.2.2.1 PAHs transforming genotypes of Gram-negative bacteria*: nah*-like of *Pseudomonas* strains

The metabolism of naphthalene has been well studied genetically in *Pseudomonas putida* strain G7 because a transmissible plasmid coding for naphthalene catabolism was isolated by Dunn and Gunsalus [Dunn and Gunsalus, 1973]. The catabolic genes are organized in three operons on the 83-kb plasmid NAH7: one encoding the “upper” pathway enzyme involved in the conversion of naphthalene to salicylate (Figure III), the second encoding the lower pathway enzymes involved in conversion of salcylate to TCA cycle intermediate via meta-ring cleavage, and the third encoding a regulatory protein (NahR) [Yen and Gunsalus, 1985]. Nucleic sequences of genes encoding the naphthalene catabolic enzymes from several *Pseudomonas* strains were reported: *ndo* genes from *P. putida* strain NCIB 9816 [Kurkela et al., 1998], *nah* genes from *P. putida* strain G7 [Simon et al., 1993], *P. putida* strain NCIB 9816-4 [Eaton, 1994] and P. stutzeri strain AN10 [Bosch et al., 1999], *dox* genes from *Pseudomonas* sp. strain C18 [Denome et al., 1993], *pah* genes from *P. putida* strain OUS82 [Kiyohara et al., 1994] and *P. aeruginosa* strain PaK1 [Takizawa et al., 1999]. Each name was chosen according to the strain’s feature of substrate use: *nah* for naphthalene degradation, *ndo* naphthalene dioxygenation, *dox* for dibenzothiphene oxidation and *pah* for polycyclic aromatic hydrocarbon. The gene organisation (Figure VI, panel A) and sequence homology (about 90%) among the upper catabolic pathway genes of these strains were similar to those of the *nah* genes from the NAH7 plasmid of strain G7. These genes are usually called “classical *nah*-like genes”.

1.2.2.2 PAHs transforming genotypes of Gram-negative bacteria*: phn* genes of *Burkholderia* sp. strain RP007

Characterisation of novel *phn* operon of *Burkholderia* sp. strain RP007 expanded our knowledge of the genetics of bacterial PAHs catabolism [Laurie and Lloyd-Jones, 1999]. Strain RP007 was isolated from a PAHs-contaminated site in New Zealand on the basis of its ability to degrade phenanthrene as sole carbon and energy source. This strain also utilises LMW-PAHs like naphthalene and anthracene as sole carbon source. The naphthalene and phenanthrene are degraded through a common "upper" pathway via salicylate and 1-hydroxy-2-naphthoic acid, respectively (Figure III and IV). The *phn* locus on an 11.5 kb *Hind*III fragment, which contained nine ORFs, was cloned on the basis of the ability to form indigo from indole [Ensley et al., 1983], and it was found that these "upper" pathway *phn* genes were different in sequence similarity and gene organisation from the isofunctional *nah*-like genes (Figure VI, panel B).

1.2.2.3 PAHs transforming genotypes of Gram-negative bacteria*: dbt* genes of *Burkholderia* sp. strain DBT1

In our laboratory, two novel sets of genes for dibenzothiophene transformation (*dbt* genes) were cloned and characterised [II]. The genotype was discovered by investigating insertional mutants of genes involved in DBT transformation in *Burkholderia* sp. strain DBT1. *B*. sp. DBT1 was isolated from a drain receiving oil refinery wastewater in Italy on the basis of its ability to transform DBT. This strain utilises also naphthalene and phenanthrene as carbon source. The *dbt* genes encoding the enzymes involved in the conversion of dibenzothiophene to 3-hydro-2-formilbenzothiophene by the *Kodama pathway* (Figure V). They constitute two separate operons instead of being clustered in a unique transcript, like previously reported for the *dox*-catabolic genes (*nah*-like transforming genotype) (Figure VI, panel A) [Denome et al., 1993]. The *dbts* were different from *nah*-like genes and also from *phn*-genes both in gene organisation and in sequence similarity [II] (Figure VI, panel B).

1.2.2.4 PAHs transforming genotypes of Gram-negative bacteria*: phd* genes of *Comamonas testosteroni* strain GZ39; *nag* genes of *Ralstonia* sp. strain U2*; phn* genes of *Alcaligenes faecalis* AFK2

Comamonas testosteroni strain GZ39 is capable to utilise phenanthrene as its sole carbon source, and the genes responsible for this catabolic activity is not very similar in sequence and gene organisation from the *nah*-like genes of *P. putida* NCIB 9816-4 [Goyal and Zylstra, 1996]. However, detailed comparison of the *phd* genes with analogous ones is not possible because the sequence of *phd* gene

from strain GZ39 is not available on any database. On the other hand, the naphthalene utilising bacteria *Ralstonia* sp. strain U2 was isolated from oil-contaminated soil in Venezuela [Fuenmayor et al., 1998]. By screening for *E.coli* transformants that formed indigo [Ensley et al., 1983] naphthalene dioxygenase genes (*nag* gene) were cloned and characterised. Recently, Zhou and co-workers [Zhou et al., 2001] reported the whole gene organisation of the *nag* operon. The genes for the conversion of naphthalene to gentisate (Figure III) in strain U2 were in the same order and similar to the genes in the classical *nah*-like operon of *Pseudomonas* strains with the exception of the insertion of two more genes (*nagG* and *nagH*) among ferredoxin reductase and ferrodoxin ones (Figure VI, panel B). Sequences of *nagG* and *nagH* were similar to α and β subunits ones of ISP of other aromatic-ring dioxygenase. A further difference between *nag* and *nah* operons is the location of the regulatory gene (*nagR*).

Concerning *phn* genes of *Alcaligenes faecalis* AFK2, information are available only on databases (Ac No. AB024945). Strain AFK2 can utilise phenanthrene as the sole carbon source through the *o*-phthalate pathway, but not naphthalene [Kiyohara et al., 1982a]. The *phn* genes have a novel structure in comparison with classical *nah*-like operon; however, details have not been reported.

Unlike other Gram-negative bacterial strains, the members of genus *Sphingomonas* and related species can utilise a wide variety of aromatic compounds, including PAHs, as carbon and energy source. Nevertheless, elucidation of the genetics of PAHs-degrading sphingomonads is under way to learn how these bacteria can utilise such a variety of aromatic compounds.

1.2.2.5 PAHs transforming genotypes of Gram-positive bacteria*: phd* genes from *Nocardioides* sp. strain KP7; *nar* genes from *Rhodococcus sp.* strain NCIMB12038

The *phd* genes of *Nocardioides* sp. strain KP7 are the most studied PAH-catabolic genes in Gram-positive bacteria, and belong to a new class of PAH-catabolic genes because of differences in gene organisation (Figure VI, panel B) and sequence similarity. Strain KP7 was isolated on the basis of its ability to grow on phenanthrene at 40°C from marine samples, and it degrades phenanthrene *via* phthalate pathway [Iwabuchi et al., 1998]. Saito and colleagues [Saito et al., 1999] reported the nucleotide sequence of the gene cluster encoding enzyme responsible for the transformation of phenanthrene to 1-hydroxy-2-naphthoate (Figure IV). Interestingly, the genes of this cluster, that encode α and β subunits of ISP of phenanthrene dioxygenase, had less than 60% sequence identity to the corresponding ones of the other aromatic-ring dioxygenase.

On the other hand, Larkin and co-workers [Larkin et al., 1999] reported the purification and the characterisation of a novel ISP of naphthalene dioxygenase from *Rhodococcus* sp. strain NCIMB12038. Protein similarity analyses of the products of α and β subunits, of the *nar* dioxygenase, showed that

they are 31-39% identical to the corresponding isofunctional ones of the other aromatic-ring dioxygenase. Despite the low overall similarity they have conserved key catalytic residues, Rieske [2Fe-2S] center [Larkin et al., 1999].

					1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
A	*ndo* (NCIB9816)					A	B	C						
	nah (G7)				Aa	Ab	Ac	Ad	B	F	C	Q	E	D
	nah (NCIB9816-4)				Aa	Ab	Ac	Ad						
	dox (C18)					A	B	D	E	F	G	H	I	J
	pah (Pak1)				A1	A2	A3	A4	B	F	C	Q	E	D
	pah (OUS82)				Aa	Ab	Ac	Ad	B	F	C	Q	E	D
	nah (AN10)				Aa	Ab	Ac	Ad	B	F	C		E	D
B	*nag* (U2)	Y	R	Aa	G	H	Ab	Ac	Ad	B	F	C	Q	E	D
	phn (RP007)		R		S	F	E	C	D	Ac	Ad	B			
	dbt (DBT1)	*first operon*				R		D	Ac	Ad	B	orf5	X	F	
		second operon				orf10	orf8	orf9	C	Ab	orf6	orf7			
	phd (KP7)		E		F		A	B		G	H	C	D		

Figure VI. Gene organisation of the "upper" pathway of Naphthalene degradation in *Pseudomonas* strains (panel A) and in *Ralstonia sp.* strain U2 (panel B). Gene organisation of the "upper" pathway of Phenanthrene degradation in *Burkholderia* sp. strain RP007 (panel B). Gene organisation of the *Kodama pathway* of Dibenzothiophene in *Burkholderia* sp. strain DBT1 (panel B). Gene organisation of the "upper" pathway of Phenanthrene degradation in *Nocardioides* sp. strain KP7 (panel B). References for the sequences are the follows: *ndo* genes from *P. putida* strain NCIB9816 (M23914); *nah* genes from *P. putida* G7 (M83949, U09057); *nah* genes from *P. putida* strain NCIB9816-4 (AF491307); *dox* genes from *P.* sp. strain C18 (M60405); *pah* genes from *P. aeruginosa* strain Pak1 (D84146); *pah* genes from *P. putida* strain OUS82 (AB004059); *nah* genes from *P. stutzeri* strain AN10 (AF039533); *nag* genes from *Ralstonia* sp. strain U2 (AF036940); *phn* genes from *Burkholderia* sp. strain RP007 (AF061751); *dbt* genes from *Burkholderia* sp. strain DBT1 (AF380367, AF404408); phd genes from in *Nocardioides* sp. strain KP7 (AB017794, AB017795 and AB031319). In Panel A the enzyme designation are the follows: lane *1*, genes coding for NDO ferredoxin reductase; lane *2*, NDO ferredoxin; lane *3*, α subunit of NDO; lane *4*, β subunits of NDO; lane *5*, naphthalene *cis*-dihydrodiol dehydrogenase; lane *6*, salicylaldeide dehydrogenase; lane *7*, 1,2-dihydroxynaphthalene dioxygenase; lane *8*, unknown ORF; lane *9*, *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase; lane *10*, 2-hydroxychromene-2-carboxylate isomerase. In Panel B the enzyme designation are the follows: *nag* genes*:* Y, chemotaxis gene; R, regulatory gene*;* G, α subunit of NDO; H, β subunits of NDO; C, 1,2-dihydroxynaphthalene dioxygenase; D, 2-hydroxychromene-2-carboxylate isomerase; the enzyme designations of the remaining *nag* genes correspond to those with the same letters in the Panel A. *phn* genes: R and S, *regulatory* gene*;* C, 1,2-dihydroxynaphthalene dioxygenase; D, 2-hydroxychromene-2-carboxylate isomerase; the enzyme designations of the remaining *phn* genes correspond to those with the same letters in the Panel A. *dbt* genes: R regulatory gene, orf 5, putative oxidoreductase; X, unknown ORF; orf10, aromatic oxygenase; orf8, isomerase; orf9 and orf6,oxydoreductase; orf7, hydrolase; the enzyme designations of the remaining *dbt* genes correspond to those with the same letters in the Panel A. *phd* genes: A, α subunit of NDO; B, β subunits of NDO; C NDO ferredoxin; D, NDO ferredoxin reductase; E, dihydrodiol dehydrogenase; F, extradiol dioxygenase; G, hydratase-aldolase; H, aldeide dehydrogenase

1.3 SOIL BIOREMEDIATION OF PAHs

Estimated costs for cleaning up contaminated sites by conventional techniques such incineration and landfilling are enormous. In the next decades, billons of dollars will be spent to reclaim quite a vast number of sites polluted with PAHs [Rosenberg, 1993]. Moreover, conventional techniques are limited in their application and are only partially effective [Dixon 1996]. Actually, incineration may cause air pollution; leaches from landfills can reach groundwater and drinking waters, whereas excavation of soil can lead to generation of toxic emission. Therefore, alternative methods are required to restore polluted sites in a less-expensive, less labor-intensive, safe, and environmental friendly way. Such an alternative approach is consistent with bioremediation. By definition, bioremediation is the use of living organisms, primarily microrganisms, to degrade the environmental contaminants into less toxic forms [Caplan, 1993; Dua et al., 2002]. Bioremediation can be applied both *ex situ* and *in situ.* Some of the examples of *ex situ* and *in situ* bioremediation are listened in the Table II. *Ex situ* technologies are the bio-treatments that remove contaminants at a separate treatment facility. On the contrary, i*n situ* bioremediation technologies involve the treatment of the contaminants without the removal and transport of polluted soil and without disturbance of the soil matrix. Since it is possible to clean up a site in its original place these *in situ* treatments result more attractive than the *ex-situ* ones.

In the past two decades, the application of bioremediation has been growing, partly because of the better understanding of the biochemical and molecular microbial processes [Bollag and Bollag 1992; Johri et al. 1996]. Sites polluted with PAHs have been treated with some success, with the Exxon Valdez oil spill as an important example [Pritchard et al., 1992]. In order to design the most effective method of soil treatment, and to best decide which technique will be applied, an elaborated study of the site should be made. Important parameters for bioremediation are 1) the nature of the pollutant, 2) the soil structure and hydrogeology (movement of pollutants through the soil and ground water), and 3) the nutritional state and microbial composition of the site [Blackburn and Hafker 1993; Dua et al., 2002], many cases, the most effective solution combines several treatment techniques.

Table II. Several examples of bioremediation strategies

Treatment	Description	Applicability	Limits
Bioventing In-situ	The oxygen is introduced in the ground with systems of forced airing to increase the aerobiosis and to enhance the biodegradation of the contaminants	Used for enhancing the microbial degradation of hydrocarbons, oil, solvents and pesticides.	- Its effectiveness results to be low in grounds with low moisture. - The gaseous emissions from the surface of the ground have to be monitored
Biostimulation In-situ	The natural degradation activity of the microrganisms is stimulated by the addition of nourishing substances and oxygen to enhance the degradation of the organic	It is effective above all for the reclamation of grounds with low levels of IPA contamination, pesticides and other organic substances. It doesn't	- Ineffective if the nature of the ground prevents the contact between contaminants and microrganisms.

	contaminants and the stabilization of the inorganic ones.	need expensive interventions and it doesn't originate final products that require further treatments.	- The injection of watery solutions in the ground may enhance the mobility of the contaminants. - In grounds of heterogeneous composition there is no uniform distribution of the nutrients and the oxygen - The presence of heavy metals can be toxic for the microrganisms. - Low temperatures may slow down the degradation processes
Bioaugmentation In-situ	Specific microrganisms able to degrade the xenobiotic of interest were seed in he contaminated matrix	Used for enhancing the microbial degradation of hydrocarbons, oil, solvents and pesticides.	- Environmental constraints - Inocula preparation - Inocula Monitoring difficulties
Phytoremediation In-situ	This process exploits the ability of the plants to transfer, to remove, to stabilize and to degrade the organic and inorganic contaminants in a ground.	Plants hyper-accumulator of metals can absorb the inorganic contaminants in the roots. Studies are in progress about the abilities of the plants to absorb and to mineralise organic contaminants.	- High concentrations of contaminants could be toxic for the plants. - It may enhance the transfer of the contaminants from the ground to the air. - Low effectiveness with substances strongly adsorbed to the ground.
Biopile Ex-situ	The contaminated ground is set in covered heaps and a system of forced airing provides oxygen and nutrients for the microrganisms	Used for the reclamation from semi-volatile organic compounds and hydrocarbons derived by the oil	- Preventive studies are required to determine the levels of oxygenation and the nutrients to add. - The stillness of the process does not enhance a uniform treatment of the matrix.
Soil composting Ex-situ	The ground is excavated, set in streaks and added with organic material (hay or fertilizer) as source of carbon and nitrogen with the purpose of the aerobic degradation by the microrganisms.	Effective for the reduction of the level of the toxic substances in grounds contaminated by IPA and by other organic compounds.	- The treatment requires great surfaces for the storage of the heaps - The addition of organic material involves a considerable increase of volume of the matrix to be treated - Ineffective for the reclamation from heavy metals that may result toxic for the microrganisms.
Landfarming Ex-situ	The contaminated matrix is set in long heaps and periodically turned for enhancing the airing	Very used for the degradation of the hydrocarbons with high molecular weight in matrixes already treated with other systems to remove the most volatile fraction of the contaminants.	- Ineffective for the reclamation from inorganic contaminants. - The matrix has to be pre-treated to remove the volatile fraction of the contaminants that could be released in the atmosphere
Bioreactors Ex-situ	The ground is introduced in reactors with the addition of water to maintain in suspension the contaminants and to enhance the contact with the microrganisms.	Used for the reclamation from hydrocarbons, pesticides and solvents, in grounds with low permeability or in the case that a rapid intervention is required.	- The treatment of the ground to be introduced in the reactor may result difficult and expensive, particularly with heterogeneous and very clayey grounds. - The final process of dehydration is very expensive.

However, we have to state that bioremediation is still an immature technology. Although microbes play an essential role in biogeochemical cycles [Wolin and Miller 1987] and they are the primary stimulant in the bioremediation of contaminated environments, current knowledge of changes in microbial communities during bioremediation is limited, and the microbial community is still treated as a “black box”. The reason for this is that many environmental bacteria cannot be cultured yet by conventional

laboratory techniques [Kogure et al., 1979; Olsen and Bakken, 1987]. This has led to two essential questions related to the implementation of bioremediation in the field. These are 1) how to clarify the biological contribution to the effectiveness of bioremediation and 2) how to assess the environmental impact of bioremediation. Because of the technical limitations in monitoring the target bacteria directly related to the degradation of contaminants, bioremediation often faces the difficulty of identifying the cause and developing measures in the case of failure remediation from a microbiological standpoint. Moreover, our limited understanding of the changes in microbial communities during bioremediation makes it difficult to assess the impact of bioremediation on the ecosystem. However, the rapid advancement of molecular biological methods has facilitated the study of microbial community structure without bias introduced by cultivation [Widada et al., 2002c].

1.3.1 in situ PAHs BIOREMEDIATION PROTOCOLS

In order to reclaim PAHs contaminated soil, different bioremediation protocols can be used. In particular, this study focused on bioaugmentation and phytoremediation *in situ* bioremediation approaches.

1.3.1.1 Bioaugmentation treatments

Bioaugmentation is a method to improve the degradation and to enhance the transformation rate of xenobiotics by the addition (seedling) of specific microbes, able to degrade the pollutants of interest. The reinoculation of soil with indigenous microrganisms directly isolated from the same soil is often included in the term bioaugmentation [Phelps et al., 1994, Otte et al., 1994]. Many microbes (bacteria) are described to have genetic tools to utilise recalcitrant contaminants such as PAHs (1.2.2 section) and have been successfully utilised in bioaugmentation protocols [Barathi and Vasudevan, 2003; Widada et al., 2002b].

However, the laboratory scale results of seedling bacteria for degradation of soil are ambiguous. Goldstein and associates [Goldstein et al., 1985] reported five possible reasons for the failure of bacteria inocula. First, the concentration of the contaminants at the site can be too low to support growth of the inoculum. This also includes the problem of low bioavailability of the pollutants [Johnsen et al., 2005]. Uptake of PAHs by bacteria proceeds via the water phase and, therefore, depends upon their solubility in water [Bouwer and Zehnder, 1993]. In addition, the sorption to the soil particles will protect these molecules from degradation by microbes. Consequently, the inoculated PAHs-degrading bacteria (as well as the natural PAHs-transforming populations) may be physically separated from the PAHs-sources, and therefore unable to grow on these carbon sources. One way to improve the availability of xenobiotics may be the use of surfactants. Many bacteria can produce such surface-active agents,

referred to as biosurfactants. Although the effects of biosurfactants can be dualistic [Allen et al., 1999] and their mode of action needs further detailed research, the use of biosurfactants, or better the inoculation of biosurfactants-producing bacteria, can be considered a valuable tool to improve bioremediation. Second, inocula fail because of inability of the microbes to spread through the soil and research the pollutant. Third, the inoculum prefers to use other carbon sources present in the soil, instead of the contaminant. Fourth, the presence of certain compounds in the environment can inhibit the growth or the activity of the inoculum. Fifth, protozoan-grazing rates on the inoculum can be higher than the growth rate of the bacteria. In order to enhance degradation of pollutants by both the indigenous microbial communities and the introduced microbes fertilisation of sites using compost, nitrogen and phosphorus has been reported [Rosenberg, 1993]. However the addition of nutrients yielded inconclusive results, because publications exist in which no influence of addition of limiting nutrients was shown [Heitkamp and Cerniglia, 1989; Manilal and Alexander 1991]. However, this unpredictability of this bioremediation approach often is caused by the lack of knowledge of the persistence of microbial populations in the environment [Head, 1998].

1.3.1.2 Phytoremediation treatments

To be effective, remediation techniques should enhance the rates of PAHs removal and degradation. Experimental evidence suggests that organic contaminants often disappear more quickly from planted soils than from soils without vegetation [Walton et al., 1994]. Such observations have led to propose the use of plants to stimulate bioremediation of contaminated soils. Most studies dealing with xenobiotic degradation in the rhizosphere have been carried out so far through empiric protocols. They have focused mainly on the screening of various plant species to identify those promoting degradation rates or on field trials at contaminated sites. In general, this research has shown that monocot species with fibrous rooting systems appear to be most effective in enhancing degradation rates of organic contaminants in planted soils [Anderson et al., 1993; Ferro et al., 1994; Schwab and Banks, 1994]. However, little mechanistic information regarding accelerated degradation in the rhizosphere is available. Two different strategies for phytoremediation of organic are suggested: direct phytoremediation and phytoremediation *ex-planta* [Anderson et al., 1993]. However, PAHs phytoremediation seems to occur mainly by *ex-planta* strategy. The latter is based on the secretion by plants of their photosynthate in root exudates, which may support the overall growth and metabolic activities of bacterial communities in the rhizosphere [Anderson et al., 1994]. Root exudates may also selectively enrich for specific degraders, or alternatively secrete compounds that induce the necessary degradative pathways [Walton et al., 1994]. On the contrary, direct phytoremediation is primarily limited

by the availability of the target compound and uptake mechanisms [Salt et al., 1998]. The main factors that govern the uptake of pollutants are their physical-chemical characteristics, and in particular their octanol-water partition coefficient (log K_{ow}). Organics that are most likely to be taken up by plants are moderately hydrophobic compounds with octanol-water partition coefficients ranging from 0.5 to 3.0 [Ryan et al., 1988]. Therefore, contaminants, like PAHs, with al log K_{ow} > 3.5 show high sorption to the roots, but slow or not translocation to the stems and leaves [Trapp et al., 2001].

The advantages of phytoremediation compared with other approaches are: 1) it preserves the natural structure and texture of the soil; 2) energy is derived primarily from sunlight; 3) high levels of microbial biomass in the soil can be achieved; 4) it is low in cost; and 5) it has the potential to be rapid. Although using plants for remediation of persistent contaminants may have advantages over other methods, many limitations exist for large-scale application of this technology [US EPA, 2000]. One serious limitation is that many plant species are sensitive to contaminants including PAHs [Huang et al., 2001; Burd et al., 1998]. Therefore, they grow slowly, and it is difficult to establish sufficient biomass for meaningful soil remediation. In addition, in most contaminated soils, the number of microrganisms is depressed so that there are not enough bacteria either to facilitate contaminant degradation or to support plant growth [Glick, 1995; Siciliano and Germida, 1997].

Despite the above-mentioned problems, it may be useful to facilitate phytoremediation through the augmentation of specific contaminant degrading bacteria, in a combined phytoremediation/ bioaugmentation way [Burd et al., 1998; Siciliano and Germida, 1997]. Actually, inoculation of plants with exogenous PAHs degrading bacteria, may improve the microbial degradation process for of PAHs removing. Moreover, the potential of this approach may also be improved by co-injection, in soil, of plant growth promoting rhizobacteria (PGPR), which may provide better plant growth by increasing plant tolerance to contaminants in the soil, and by using combined physical-chemical treatments [Huang et al., 2004]. However, the success of these process is based on the rhizosphere competence of microbes [Lugtemberg and Dekkers, 1999] which is reflected by the ability of the bacteria to survive in the rhizosphere, compete for the exudates nutrients, sustain in sufficient numbers and efficiently colonized the growing roots system [Lugtemberg and Dekkers, 1999]. Moreover, to date, the application of this combined protocol is better described for processes of biocontrol of soilborne plant diseases [Ching-A-Woeng et al., 1998] biofertilisation, phytostimulation [Okon et al., 1998] than to bioremediation applications. Nevertheless, successful organic pollutants degradation using genetically engineered microbes (GEM) have been reported [Yee et al., 1998; Ronchel and Ramos, 2001]

1.3.2 MOLECULAR MICROBIAL ECOLOGICAL METHODS IN BIOREMEDIATION

To implement bioremediation in the field, structure and role of the biological system involved in the degradation of pollutants need to be clarified. In this context, analysis of microbial communities that take part in *in situ* bioremediation is of great importance. To figure out structure and dynamic succession of natural microbial communities in contaminated sites, approaches complementary to the conventional culture-dependent techniques should be applied. The recourse to molecular biological techniques to detect and identify microrganisms by certain molecular markers has been more and more frequently used in microbial ecological studies. Same examples of the molecular microbial ecological methods that can be used for *in situ* bioremediation are given below.

1.3.2.1 Monitoring changes in bacterial diversity

Microbial communities play an essential role in biogeochemical cycles and contribute to the maintenance of the ecosystem. Therefore, investigating the influence of bioremediation on the microbial community is necessary to prove the safety of *in situ* bioremediation. This investigation may be distinguished mainly in DNA-based methods and RNA-based methods. DNA hybridization techniques, using labelled DNA as a specific probe, have been used in the past for identification of specific microrganisms in environmental samples [Atlas, 1992; Sayler and Layton, 1990]. Although these techniques are still useful for monitoring a specific genome in nature, they have some limitations. Colony hybridization can only be used for detection of culturable cells, and slot blot and Southern blot hybridization methods are not adequately sensitive for the detection of low cell numbers. On the other hand, greater sensitivity of detection, without reliance on cultivation, can be obtained using PCR [Jansson, 1995]. One disadvantage of DNA-based methods is that they do not distinguish between living and dead organisms, which limits their use for monitoring purposes. The mRNA level may provide a valuable estimate of gene expression and/or cell viability under different environmental conditions [Fleming et al., 1993]. Retrieved mRNA transcripts can be used to compare the expression level of individual members of gene families in the environment. Thus, when properly applied to field samples, mRNA-based methods may be useful in determining the relationships between the environmental conditions prevailing in a microbial habitat and particular in situ activities of native microrganisms [Wilson et al., 1999]. Moreover, RT-PCR gives us the ability to detect and quantify the expression of individual structural genes. However, nucleic acid isolation from an environmental sample is the most important step in examining the microbial community and catabolic genes diversity. Procedures for DNA isolation from soil and sediment were first developed in the 1980s, and today several methods are

evaluated and improved [Widada et al., 2002c]. In contrast to extraction of DNA, extraction of mRNA from environmental samples is quite difficult and is further hampered by the half-lives of prokaryotic mRNA being very short.

Genetic fingerprinting techniques provide a pattern or profile of the genetic diversity in a microbial community. Recently, several fingerprinting techniques have been developed and used in microbial ecology studies such as bioremediation. These techniques are important in separating and identifying DNA/RNA PCR-amplified products that might have the same size but slightly different nucleotide sequences. Amplified ribosomal DNA restriction analysis (ARDRA) [Vaneechoutte et al., 1992], Terminal restriction fragment length polymorphism analysis (T-RFLP) [Marsh, 1999] and Denaturing gradient gel electrophoresis (DGGE) [Muyzer et al., 1993] are three of the main molecular methods successful utilised in the microbial community analysis.

1.3.2.2 Detection and monitoring of target bacteria

Because different methods for enumeration of microrganisms in environmental samples sometimes provide different results, the method used must be chosen in accordance with the purpose of the study. Not all detection methods provide quantitative data; some only indicate the presence of an organism and others only detect cells in a particular physiological state [Jansson and Prosser, 1997]. Several molecular approaches have been developed to detect and quantify specific microrganisms (Table III).

Table III. Molecular approaches for detection and quantification of specific microrganisms in environmental samples (adapted from Jansson and Prosser, 1997).

Identification method	Detection and quantification method	Cell type monitored
Fluorescent tags on rRNA probes	Microscopy Flow cytometry	Primary active cells
lux or *luc* gene	Luminometry/scintillation counting Cell extract luminescence	Active cells Total cells with translated luciferase protein Culturable luminescent cells
gfp gene	Luminescent colonies Fluorescent colonies Microscopy Flow cytometry	Active cells Total cells with translated luciferase protein Culturable luminescent cells
Specific DNA sequence	cPCR MPN-PCR, RLD-PCR[a]	Total DNA (living and dead cell and free DNA) Culturable cells
Specific mRNA transcript	Slot/dot blot hybridization Colony hybridization Competitive RT-PCR Slot/dot blot hybridization Plate Counts colony hybridization	Catabolic activity of cells

Other marker genes (e.g., *lacZY*, *gusA*, *xylE*, and antibiotic resistance genes)	Quantitative PCR Slot/dot blot hybridization	Culturable marked cells and indigenous cells with marker phenotype Total DNA (living and dead cells and free DNA)
[a]: *cPCR* Competitive PCR; *MPN-PCR* most probable number PCR; *RLD-PCR* replicative limiting dilution PCR		

Quantification by PCR/RT-PCR is now used for sensitive detection of specific DNA in environmental samples. Sensitivity can be enhanced by combining PCR with DNA probes, by running two rounds of amplification using nested primers [Moller et al., 1994], or by using real-time detection systems [Widada et al., 2001]. Detection limits vary for PCR amplification, but usually between 10^2 and 10^3 cells/g soil can be detected by PCR amplification of specific DNA segments [Fleming et al., 1994; Moller et al., 1994]. However, despite its sensitivity, until recently it has been difficult to use PCR quantitatively to calculate the number of organisms (gene copies) present in an environmental sample. While standard PCR technique can be problematic, their complexity pales in comparison to quantitative PCR methods in an environmental context. The relative absence of quantitative PCR methodologies for environmental applications might be due to the chemical complexity of the environmental samples. In fact, the application of PCR techniques in environmental is complicated by innumerable organic and inorganic contaminants and enzyme inhibitors that copurify with the nucleic acids. These complications are compounded in quantitative PCR methods, which are predicated upon subtle yet significant assumptions of amplification efficiency and representativeness of sample with respect to the environment from which it was obtained. However, Competitive PCR (cPCR), Most Probable Number PCR (MPN-PCR) and Replicative Limiting Dilution PCR (RLD-PCR) are three of the recently developed techniques often utilise (with successes and limitations) for environmental application [Chandler, 1998].

1.4 EX-CARBOCHIMICA: THE STUDIED CASE

The soil sample analysed in this research work came from a former industrial area, named “ex Carbochimica” (point D2 of the grid of sampling), located in the northern outskirt of the city of Trento (Italy). The western part of the area is limited by the Brennero railway line while on the east it is limited by the national route n°12 “dell’Abetone e del Brennero” (Figure VII). The site has a surface of about 42700 m^2.

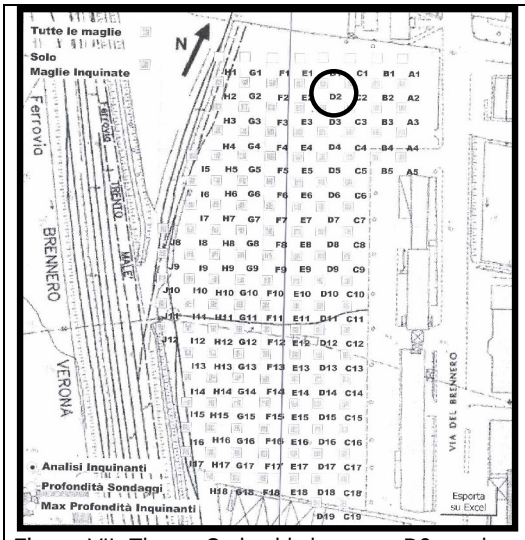


Figure VII. The ex-Carbochimica area. D2, analysed soil sample in the grid of sampling (black circle).

The industrial activity of this site began in 1905. The initial activities concerned the tar distillation of coal through humid treatment, for applications in the building sector (e.g. waterproof materials and wood impregnating solutions). The place in which the activities began was located in the central area of the present site and was extended for all the width till the railway line on the north west. The activity expansion in the sixties brought at the purchasing of the northern area on which material depots were built. The southern area, which was initially occupied by handling and stocking structures, after the year 1960 has been dedicated to the production of phtalic anhydride and fumaric acid. Starting from the middle seventies the productions were progressively brought away of the Trento premises. In the year 1975 the activity of naphthalene purification was transferred to Fidenza, followed, in the year 1977, by the tar distillation, transferred to Porto Marghera. The complete ceasing of the activities was declared in March 1984 and, in the following years, took place the dismantling process of the structures and residues, in cooperation with the local authorities. Studies conducted in the years 1997-1998 permitted to state that the main contaminant substances present in the site belonged to the class of the PAHs, and the contamination sources have been located in the compounds dispersed in the various productive cycles and present in the final products. In particular the total surface contaminated by IPA (16 priority US-EPA) results to be of 31.050 m^2 for a total contaminated volume of 57420 m^3. The contamination levels in the entire area vary between 10 mg and 18830 mg of 16 priority US-EPA per kg of dry soil.

AIMS AND EXPERIMANTAL DESIGN

Polycyclic Aromatic Hydrocarbons (PAHs), which consist of two or more fused aromatic rings, are widespread in the environment and persist over long periods of time. The decontamination of PAH-polluted environment is of importance because some PAHs are health hazards. As part of the efforts to establish remediation process, the use of aerobic bacteria has been extensively investigated, and both biochemical and genetic studies are underway for the purpose of effective biodegradation. In particular, in applied studies, genetic information will be useful for the monitoring of bacterial populations that degrade PAHs in contaminated soils. Most studies of PAHs bioremediation involve either stimulation of indigenous microbial populations by addition of nutrients (biostimulation) and by introduction of plant species (phytoremediation) to the environment, or inoculation of exogenous microbial populations that transform the pollutants (bioaugmentation). Before a stimulation of a contaminated site, the presence in the environment of bacteria that degrade PAHs should be confirmed. Moreover, the impact of the adopted stimulation protocol on the bacterial communities should be also evaluated. Therefore, microbiological as well as molecular approaches are useful for the detection, characterisation and monitoring of transformers. On the other hand, during the application of a bioaugmentation protocol, the fate of augmented degraders in the new environment also should be investigated. Therefore, PCR based protocols could be a way to help the monitoring of degraders in a contaminated environment.
Consistent with the above consideration, the experimental design of this research work was arranged as follows (Figure VIII):

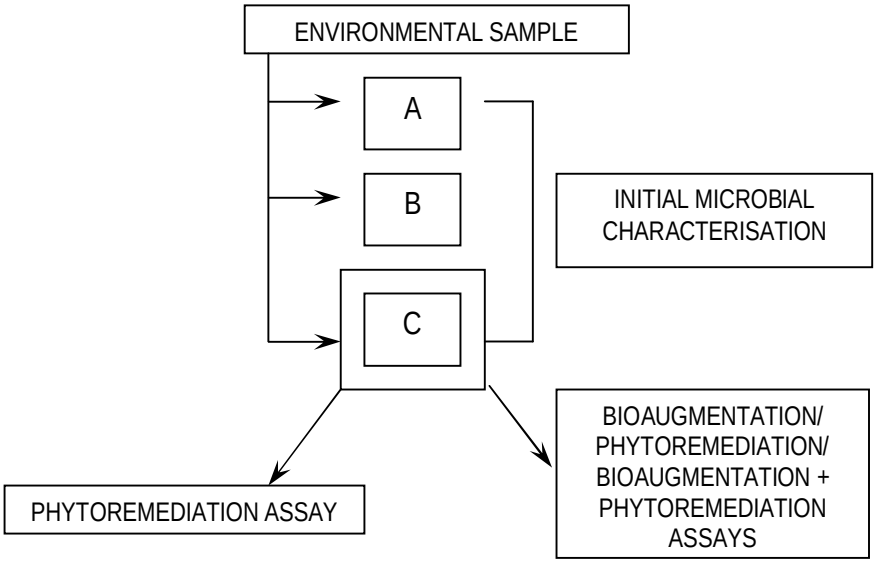


Figure VIII. Graphical representation of the experimental design. ENVIRONMENTAL SAMPLE, PAHs contaminated soil sample collected from the Ex-Carbochimica industrial area. A, B and C: random sub-samples that had different degree of

PAHs contamination (A and B low contamination level; C high contamination level). The bacterial community settled down in all the three sub-samples was subjected to an INITIAL MICROBIAL CHARACTERISATION. Only the soil aliquot C was utilised to arrange two bioremediation protocols (PHYTOREMEDIATION ASSAY and BIOAUGMENTATION/ PHYTOREMEDIATION/ BIOAUGMENTATION + PHYTOREMEDIATION ASSAYS).

In this study were analysed three random sub-samples that had different degree of PAHs contamination (A, B and C). Each sub-sample was characterised with the aim to know the soil adapted-PAHs-transforming bacterial communities and the intrinsic bioremediation potential of the matrix (INITIAL MICROBIAL CHARACTERISATION). Furthermore, the soil aliquot C was utilised to test two bioremediation protocols: PHYTOREMEDIATION ASSAY and BIOAUGMENTATION/ PHYTOREMEDIATION/ BIOAUGMENTATION + PHYTOREMEDIATION ASSAYS. The main aim of PHYTOREMEDIATION ASSAY was to understand the changes in microbial communities during performed bioremediation protocols. Moreover, we were interested in verifying the possible plant-dependent selection of specific classes of transforming genes as a consequence of the adopted treatment. Furthermore, by both INITIAL MICROBIAL CHARACTERISATION and PHYTOREMEDIATION ASSAY we try to improve the current knowledge about the catabolic diversity of PAHs-transforming genes. On the other hand, the aims of BIOAUGMENTATION/ PHYTOREMEDIATION/ BIOAUGMENTATION + PHYTOREMEDIATION ASSAYS was to suggest, from a microbial standpoint, some possible explanations concerning the performances of the adopted treatments and to evaluate the possibility to monitor the fate of augmented PAHs transforming strains in the polluted matrix by using two different DNA PCR-based approaches.

In order to achieve these aims the adopted experimental steps were graphically represented in Figures IX to XI.

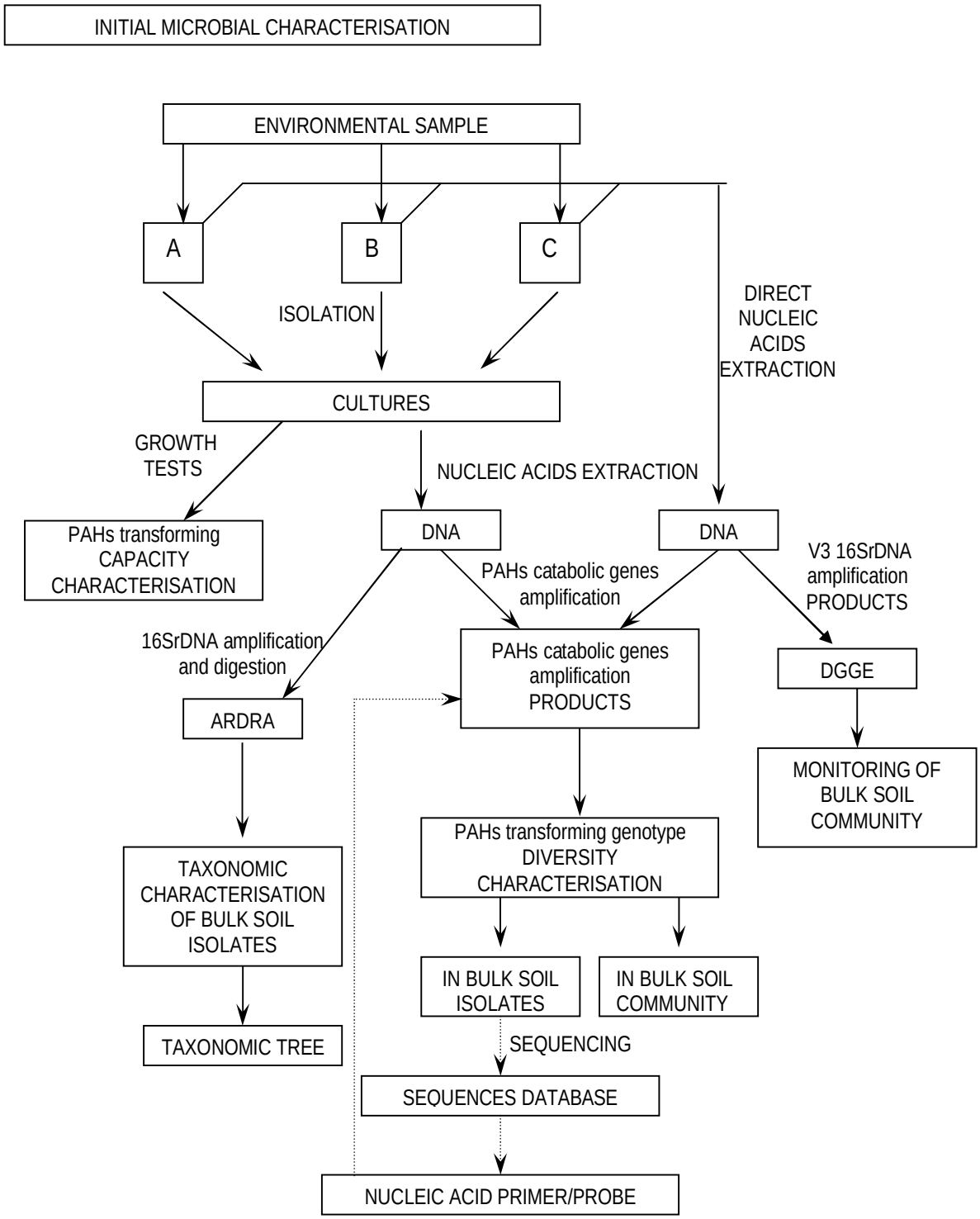


Figure IX. INITIAL MICROBIAL CHARACTERISATION. The bacterial communities of the three soil aliquots were biochemically, taxonomically and genetically characterised, by using culture dependent and independent approaches (solid arrows). The results obtained may improve our knowledge about bacterial composition and bioremediation potential of the matrix. The future cloning and sequencing of the, uncharacterised oxigenase system, involved in the PAHs-transformation, may provide new tool to investigate, through molecular approaches, the PAHs-transforming genetic diversity in the environment (dotted arrows)

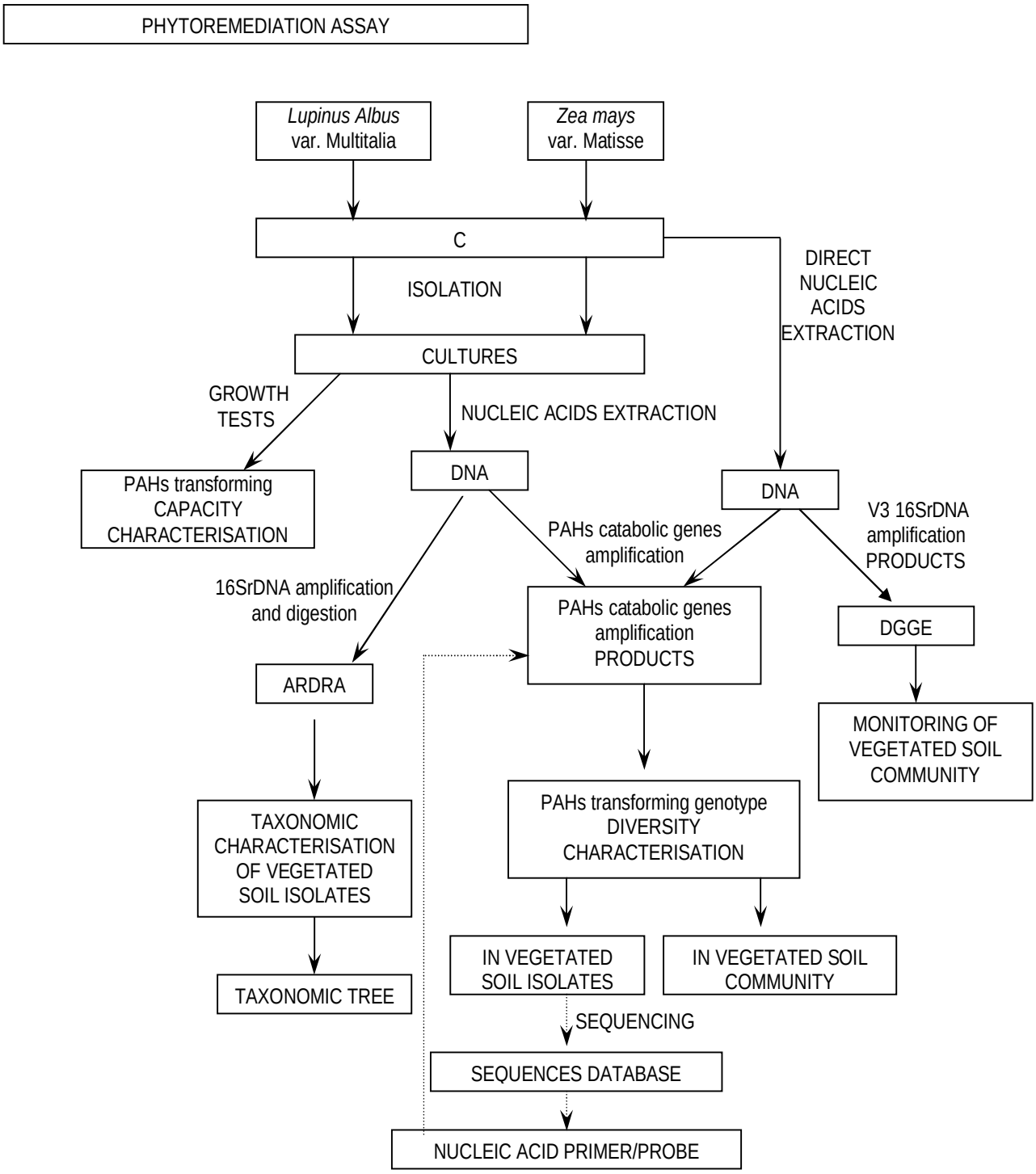


Figure X. PHYTOREMEDIATION ASSAY. The bacterial communities of the soil aliquot C, vegetated with *L. albus* and *Z. mays*, were biochemically, taxonomically and genetically characterised, by using culture dependent and independent approaches (solid arrows). The results obtained may improve our knowledge about the modification occurred in bacterial taxonomic and PAHs-transforming genes class composition as a result of plants introduction in the systems. The future cloning and sequencing of the, uncharacterised oxigenase system, involved in the PAHs-transformation, may provide new tool to investigate, through molecular approaches, the PAHs-transforming genetic diversity in the environment (dotted arrows)

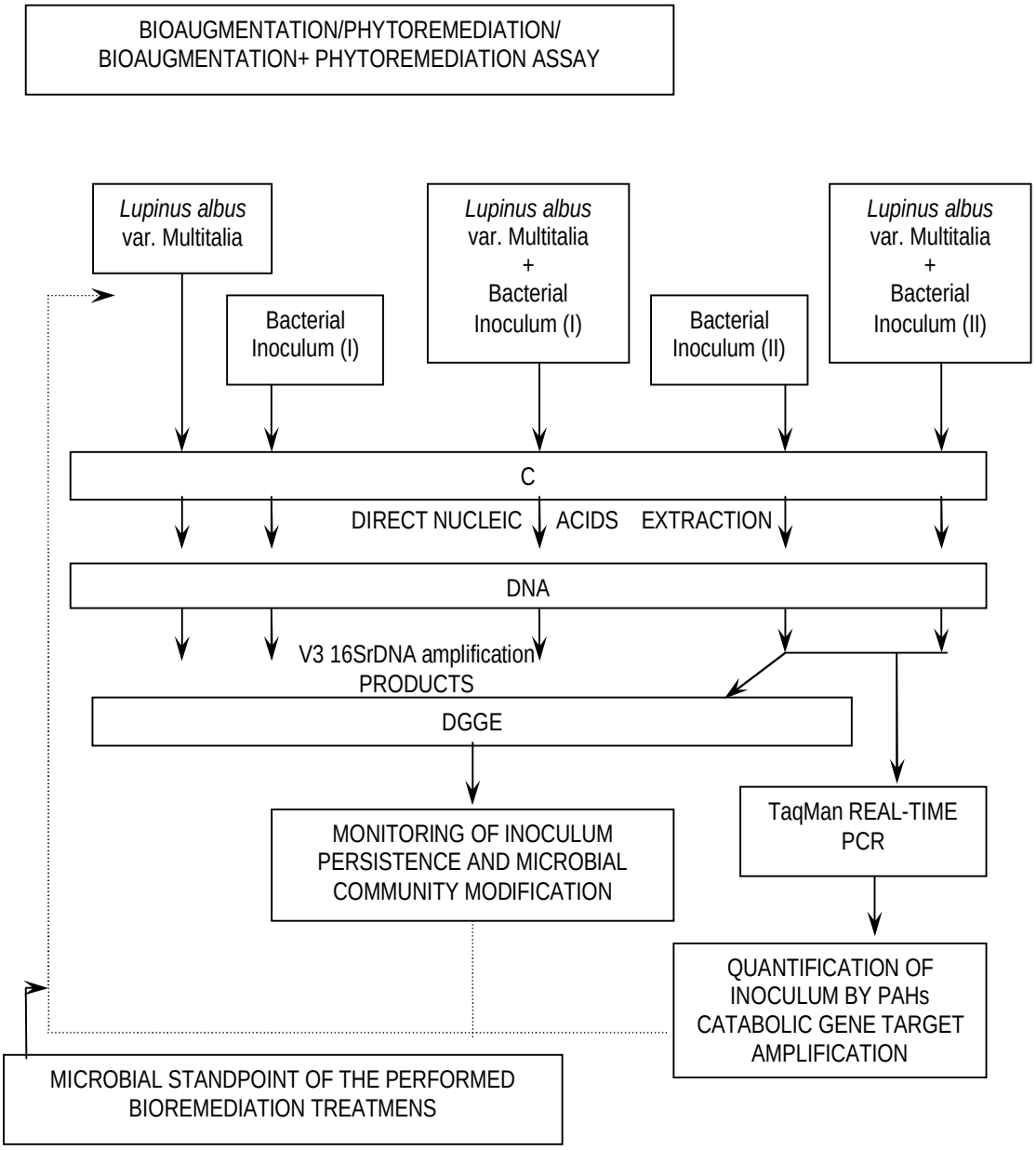


Figure XI. PHYTOREMEDIATION ASSAY and BIOAUGMENTATION/ PHYTOREMEDIATION/ BIOAUGMENTATION + PHYTOREMEDIATION ASSAYS. The soil aliquot C was vegetated with *L. albus* (PHYTOREMEDIATION ASSAY) or inoculated with two different bacteria inocula (Bacterial inoculum I and Bacterial inoculum II) (BIOAUGMENTATION ASSAY). When necessary the BIOAUGMENTATION and PHYTOREMEDIATION approaches were combined (*L. albus* +Bacterial inoculum I and *L. albus*+ Bacterial inoculum II; BIOAUGMENTATION + PHYTOREMEDIATION ASSAY). All the obtained systems were investigated by DGGE to suggest, from a microbial standpoint, some possible explanations concerning the performances of the adopted treatments (dotted arrows) and to monitor the fate of augmented PAHs transforming strains. With the same aim, the systems arranged by using the Bacterial Inoculum II were also studied through an original REAL TIME PCR approach.

2. MATERIALS AND METHODS

2.1 CHEMICALS AND CULTIVATION MEDIA

Chemicals were of analytical grade. Naphthalene, phenanthrene, dibenzothiophene and solvents were purchased from Sigma Aldrich. Bacteriological preparations were purchased from Oxoid. Liquid growth media were supplemented with an excess of the three PAHs as *N-N*-dimethylformamide solutions. Solidified growth medium (Bacteriological agar) was supplemented with dibenzothiophene by spraying the plate surface with an ethanol-free diethyl ether solution (5% [wt vol^{-1}]) [Kiyohara et al., 1982b], with naphthalene by placing crystals within the lids of the plates, and with phenanthrene by the overlay method [Bogardt and Hemmingsen, 1992].

2.2 REFERENCE BACTERIAL STRAINS

Pseudomonas putida ATCC17484 and *Burkholderia* sp. RP007 ICMP 13529 as representatives of *nah* and *phn* transforming genotype were maintained in Nutrient medium supplemented with naphthalene and phenanthrene respectively. *Burkholderia* sp. DBT1 as representative of *dbt* transforming genotype was maintained in YMB medium [Yeast Extract 0.4 g/l; Mannitol 10 g/l; KH_2PO_4 0.5g/l; $MgSO_4$ 0.2 g/l; NaCl 0.1 g/l] supplemented with dibenzothiophene. The growth temperature of all strains was 28°C.

2.3 SOIL SAMPLES

PAHs-contaminated soil samples were collected from a former industrial area placed in Trento, Italy. Three random samples were taken from 0 to 2 m deep in the soil of an area, which had different degrees of PAHs-contamination. In particular two samples, named A and B, had a low contamination level (200 and 400 mg 16 US-EPA PAHs /kg of dry soil) and another, named C, had a high pollution level (14,000 mg 16 US-EPA PAHs /kg of dry soil). All samples showed dark brown colour and medium texture, the pH ranged from neutral to sub-alkaline values and all soil samples evidenced quite high contents of organic matter (Table 1).

Table1. Characteristic of analysed soil samples

Soil samples	16 US-EPA PAHs concentration (mg/kg)	Texture	Moisture (%)	Organic C (%)	Inorganic C (%)	Fulvic acids (%)	Humic Acids (%)	Total N (%)	pH
A	200	Loam	7.48	12.5	3.5	0.249	0.659	0.212	8.2
B	400	Loam	7.61	6.5	2.3	0.284	0.223	0.053	8.1
C	14,000	Sandy Loam	3.12	11.4	3.4	0.257	0.317	0.14	7.8

2.4 SOIL MICROCOSMS EXPERIMENTS

2.4.1 PHYTOREMEDIATION ASSAY

10 microcosms were arranged with the high contaminated soil aliquot C and maintained at controlled light, temperature and moisture conditions (day, 14 h at 24°C; night, 10 h at 19°C; moisture 70%) for 10 weeks. Eight black pots were filled with 200g of polluted soil, four of them (named L) were seeded with *Lupinus albus* var. Multitalia and the other four (named M) with *Zea mays* var. Matisse. Two additional control pots were maintained not seeded at the identical chamber conditions for the same time (Table 2). PAHs transformation and microbial as well as molecular characterisation of autochthonous soil bacteria communities were evaluated at the end of the experimentation.

Table 2. Microcosms design in Phytoremediation assay

Microcosms	Pots number	Soil characteristic	*Lupinus albus*
K	2	Native soil [a]	no
L	4	Native soil [a]	yes
M	4	Native soil [a]	yes

[a]: soil aliquot C, 14,000 16 US-EPA PAHs mg/kg dry soil

2.4.2 BIOAUGMENTATION/ PHYTOREMEDIATION/ BIOAUGMENTATION and PHYTOREMEDIATION ASSAYS

The potential of bioaugmentation, phytoremediation or of the combined bioaugmentation–phytoremediation approaches to reclaim an aged polluted soil was evaluated on microcosm scale. PAHs-concentration decrease was evaluated periodically, i.e. after zero, 2, 4, 6 and 9 weeks. At the same time samples of each soil microcosms were subjected to direct molecular analyses to monitor the bacterial inocula introduced in the systems.

2.4.2.1 Bacteria inocula

Two types of bacteria inocula were utilised in the performed bioaugmentation and bioaugmentation-phytoremediation treatments, which were, a pure culture of the *Burkholderia* sp. DBT1 (BACTERIAL INOCULUM II, Figure XI), a previously described PAHs transforming bacteria [II], and a mixed culture of three selected bacteria (BACTERIAL INOCULUM I, Figure XI), two of them belonging to *Pseudomonas* sp. (NDP003, 5N-C) and one to *Bacillus* sp. (L8A-C) genera. All of them were isolated in this study.

Burkholderia sp. DBT1 was grown in 50ml YMB medium, supplemented with 100mg/l respectively of naphthalene, phenanthrene and dibenzothiophene at 28°C in constant agitation (250 rpm). The *Burkholderia* sp. DBT1 stationary culture phase (53 hours old, Figure 1, panel A) was collected by centrifugation, washed twice and then re-suspended in bi-distilled water [Kastner et al., 1998]. 10ml of this suspension was applied and distributed uniformly throughout the 200g of the soil microcosms by mixing, at an initial concentration of 6 x 10^7 CFU/g of soil.

Three bacterial strains NDP003, 5N-C and L8A-C were isolated and characterised for their capacity to transform PAHs *in vitro* and subsequently selected as mixed bacterial autochthonous inoculum for these bioaugmentation/bioaugmentation-phytoremediation assays. All the isolated were grown in 50 ml Nutrient broth, supplemented with 100mg/l respectively of naphthalene, phenanthrene and dibenzothiophene, at 28°C in constant agitation (250 rpm). At the stationary culture phases (45 hours old, for each isolated, Figure 1, panel B) the bacterial biomasses were collected by centrifugation, washed twice, re-suspended in bi-distilled water and then mixed together in a 1.5:1:2.5 (NDP003: 5N-C: L8A-C) proportion. A 10ml sample of this suspension was applied and distributed uniformly throughout the 200g of the soil microcosms by mixing, at an initial concentration of 2 x 10^7 CFU/g of soil for each bacterial culture.

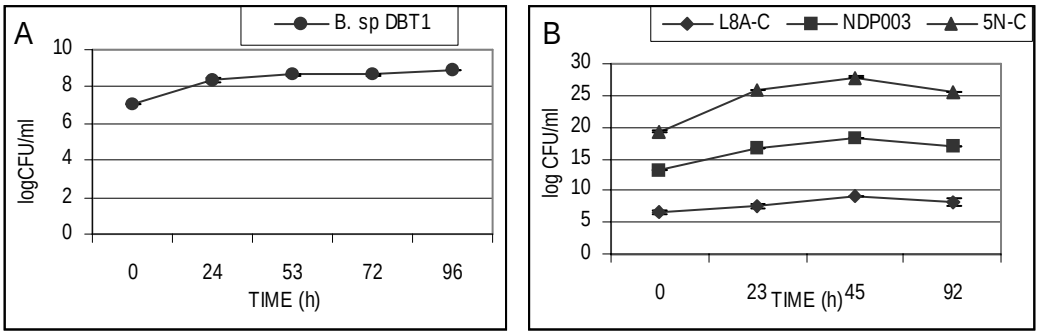


Figure 1. Cell viability of bacteria used as inocula. Panel A: Growth of *Burkholderia* sp. DBT1 in YMB amended with Naphthalene, Phenanthrene and Dibenzothiophene. Panel B: Growth of NDP003, 5N-C and L8A in Nutrient Broth amended with Naphthalene, Phenanthrene and Dibenzothiophene.

2.4.2.2 Microcosms arrangement

The second set of experiments was performed after the ageing of the high-contaminated soil aliquot C for 365 days. During aging, the polluted soil was kept outdoors, in dark condition, at temperature ranging from 5 to 25°C and was maintained a 60% of the water holding capacity. Microcosms were arranged in black pots filled with 200g soil as listening in Table 3. When necessary, the microcosms were seeded with *Lupinus albus* var. Multitalia 24 hours after the bacterial inoculum. The assay was conducted at controlled light, temperature and moisture conditions (day, 14 h at 24°C; night, 10 h at

19°C; moisture 70%) during 9 weeks. PAHs-concentration decrease was evaluated, after zero every 2 weeks as well as the molecular monitoring of the bacterial inocula.

Table 3. Microcosms design in Bioaugmentation/ Phytoremediation/ Bioaugmentation-Phytoremediation assays

Microcosms	Pots number	Soil characteristic	*Lupinus albus* var. Multitalia	Bacterial inocula
A	10	Sterilised soil	no	no
B	10	Native soil	no	no
C	10	Native soil	no	6 x 10^7 CFU/g of soil of mixed culture[a]
D	10	Native soil	no	6 x 10^7 CFU/g of soil of *Burkholderia* sp. DBT1[b]
E	10	Native soil	yes	no
F	10	Native soil	yes	6 x 10^7 CFU/g of soil of mixed culture[a]
G	10	Native soil	yes	6 x 10^7 CFU/g of soil of *Burkholderia* sp. DBT1 [b]

[a]: Mix of three bacterial isolates. 2 x 10^7 CFU/g of soil NDP003, isolated from soil aliquot A; 2 x 10^7 CFU/g of soil 5N-C, isolated from soil aliquot C; 2 x 10^7 CFU/g of soil L8A-C, isolated from the rhizosphere of *Lupinus albus* var. Multitalia, soil aliquot C

[b]: *Burkholderia* sp. DBT1, selected PAHs transforming bacteria [II]

2.4.3 PAHS ANALYSES

1g of soil from each microcosm was dried to the air and drawn out more times with 5 volumes of toluene and, alternatively, of carbon tetrachloride, more proper for breaking up bituminous matrixes. The extraction took place in a bath of ultrasounds at the temperature of 80°C for 20 minutes for each cycle. The repeated extractions on every sample have been from seven to nine, and the process have been interrupted only after the obtainment of a colourless extract. The extracts have been all reunited, brought to volume (50ml) with toluene and diluted in two following steps: at first withdrawing 40 µl of it and bringing it to 5 ml with toluene; then withdrawing 80 µl of the obtained solution and bringing it to 2 ml with hexane. Finally the internal standard has been added, the solution has been injected in GC/MS and further analysed to determine the 16 US-EPA PAHs residual concentration. The gas-chromatography analyses were performed in a TRACE GC couplet to a mass spectrometer TRACE DSQ (ThermoFinnigan). Compounds were separated with He flow on a 30 m DB5 MS column, 0.25 mm internal diameter and 0.25µm thick film. The column oven temperature was: 60-120°C at 30°C/min and 120-300 at 7°C/min. The PTV (Programmed Temperature Vaporization) autoinjector AI 3000 temperature was 55°C for 6 s, and then up to 15°C/s until the temperature of transfer (320 °C) for 2 min. Detection and quantification of 16 USA EPA PAHs were carried out by Single Ion Monitoring. The

data elaboration has been made through the software Xcalibur 1.4. All the analyses were performed twice for each sample of soil.

2.5 CULTURE DEPENDENT APPROACHES

2.5.1 ENUMERATION OF CULTURABLE PAHS TRANSFORMING BACTERIA AND TOTAL MICROFLORA

PAHs-transforming microrganisms were quantified using a Most Probable-Number (MPN) procedure [Stieber et al., 1994] with some modifications. Briefly, 96-well microtiter plates were filled with 20 µl per well of a mixture of naphthalene (100 mg/l), phenanthrene (100 mg/l) and dibenzothiophene (100 mg/l) in hexane. The hexane was evaporated in a sterile laminar flow fumehood prior to adding 180 µl per well of Defined Mineral medium, DM [Na_2HPO_4 2.2 g/l, KH_2PO_4 0.8 g/l, NH_4NO_3 3 g/l, Yeast extract 0.05 g/l, Vitamin solution 1ml/l, Wolfe mineral solution 10ml/l [Frassinetti et al., 1998]. Serial dilutions (10^{-3}-10^{-8} in 0.9% NaCl) of 5 g of soil in 100 ml of 0.9% NaCl, were inoculated into 24 well for each dilution, including un-inoculated controls and controls without PAHs. The plates were incubated for three weeks at room temperature in the dark. The presence of PAHs transforming microrganisms was assessed when the medium turned yellow or brown due to the appearance of PAH metabolites, as quantified by spectrophotometry (OD at 415 nm minus OD 650 nm). The total number of heterotrophs was estimated similarly using Nutrient broth and the same soil suspension-dilution method. The plates were incubated for one week at the same conditions mentioned above and the total microflora was estimated by spectrophotometry at 650 nm. A computer program (MPN calculator 1.0 exe, on line available http://microtitermethods.dmu.dk) was used to calculate MPN value of total heterotrophs and PAHs transforming bacteria for each sample.

2.5.2 ENRICHMENT CULTURES AND ISOLATION OF PAHS-TRANSFORMING BACTERIA

Enrichment cultures were set up with 1 g of each sample soil in 99 ml of DM medium in 250 ml Erlenmeyer flasks aseptically supplemented in excess with naphthalene, phenanthrene, dibenzothiophene respectively or the combination of the three PAHs as carbon sources. After four sub-cultures, an appropriate dilution of the resultant cultures was spread onto solidified DM plates containing naphthalene, phenanthrene or dibenzothiophene. Colonies from this plates that revealed the presence of PAHs transforming capacity (by observing the appearance of coloured intermediates or a clearing zone on solidified media) were purified by streaking on the same medium. Stocks for storage at –80°C

were then prepared from single colonies. Isolates thus obtained were subjected to further characterisations.

2.5.3 BIOCHEMICAL CHARACTERISATION OF PAHS-TRANSFORMING BACTERIA ISOLATES

2.5.3.1 Dioxygenase test and carbon source utilisation

Dioxygenase activity of the bacterial isolates was examined by monitoring the conversion of indole to indigo on solidified plates. The bacterial strains were pre-grown on Nutrient solidified media supplemented with naphthalene, phenantrene or dibenzothiphene. After three days new ones containing indole crystals replaced the lids of the Petri dishes. The plates were maintained at room temperature for two weeks and then evaluated for the emergence of indigo or red colour on the agar surface.

PAHs utilisation was determined in solidified DM medium by observing the appearance of colour intermediates or a clearing zone on solidified media.

2.5.3.2 Biosurfactants production test

PAHs-utilizing bacteria were screened for the ability of producing surfactant, extracellular glicolipids, by cultivation in blue agar plates containing 0.2 g/l cetyltrimethylammonium bromide (CTAB) and methylene blue (5μg/ml) [Siegmund and Wagner, 1991] with 2% dextrose or mannitol as carbon sources. For biosurfactants production on PAHs the isolates were pre-grown onto solidified DM medium supplemented with a naphthalene, phenantrene, dibenzothiphene mixture (100mg/l each), in order to induce their catabolic genes, and then streaked on blue agar plates supplemented with the same PAHs mixture. Biosurfactants production was observed by formation of dark blue halos around the colonies.

2.5.4 TAXONOMIC CHARACTERISATION OF PAHS-TRANSFORMING BACTERIA ISOLATES

2.5.4.1 DNA isolation

Bacterial genomic DNA was extracted using the Nucleospin Tissues Kit (BD Bioscences Clontech) following the manufacturer's instructions.

2.5.4.2 Amplified ribosomal DNA restriction analysis (ARDRA)

The recovered isolates were clustered in different Operational Taxonomic Units (OTUs) by Amplified Ribosomal DNA Restriction Analysis, ARDRA [Vaneechoutte et al., 1992]. The gene encoding for the 16S rRNA of each isolates was first amplified using the fD1/rP2 primer set (Table 4).

Table 4. PCR 16SrDNA gene primers used in this study		
Primer sets	Sequences (5'to3')	References
fD1	GAGTTTGATCCTGGCTCAG	[Weisburg et al., 1991]
rP2	ACGGCTACCTTGTTACGACT	[Weisburg et al., 1991]
p2	(40bp-GC-clamp)CCTACGGGAGGCAGCAG	[Muyzer et al., 1993]
p3	ATTACCGCGGCTGCTGG	[Muyzer et al., 1993]

All PCR amplifications were performed with *Mastercycler® personal* (Eppendorf) in the reaction conditions listed below using 2-5 ng of genomic DNA as template

50µl PCR mix	fD1/rP2 reaction conditions
1x reaction buffer Dynazyme, including 1.5 mM MgCl 0.4 mM of dNTPs mix 20 pmol of both the primers 1.5 U of DyNAzyme™ II DNA polymerase (Finzymes)	5 min at 95°C 30 cycles of 45 s at 95°C, 45 s at 54°C 2 min at 72°C hold 5 min at 72°C

The subsequent digestion of PCR amplification products was performed with *Hae*III, *Hha*I, *Alu*I, and, when necessary, with *Rsa*I restriction enzymes (Amersham Bioscience). All analyses were carried out at least twice for each isolate.

One strain for each OTU was chosen as candidate for the following analyses. The 16S rRNA gene of each candidate was amplified, single strands sequenced and aligned to the sequence databases using BLASTN [Altshul et al., 1997]. Moreover, to infer taxonomy correlation among the OTUs, the 16S rRNA gene sequence of all candidate was on line aligned both to the sequence of closely related bacteria and to the ones of some selected bacteria type species contained in The Ribosomal Database Project (RDP)-Hierarchy Browser (http://rdp.cme.msu.edu). The evolutionary Distance Matrix was determined by using neighbor-joining method [Saitou and Nei, 1987] as implemented in the NEIGHBOR program in PHYLIP software package [Felsenstein, 1982]. Taxonomy tree was finally constructed from the Distance Matrix by neighbor-joining method.

2.5.5 PAHS-TRANSFORMING GENOTYPE CHARACTERISATION OF BACTERIA ISOLATES

2.5.5.1 Amplification by PCR of the genes encoding the α subunit of the initial PAHs-dioxygenase

The primer sets listed in Table 5 were used to amplify the gene encoding the α subunit of the initial PAHs-dioxygenase from genomic DNA of the soil isolates. Primer sets P20/P54 and dbtAcfor/dbtAcrev

were designed on the nucleotide sequence of Burkholderia sp. DBT1 *dbt*Ac gene (AF380367). The P20/P54 primers amplify the entire *dbt*Ac gene (from 1 to 1368 position) and the dbtAcfor/dbtAcrev were designed, as described in the 2.6.4.1 section, to amplify a nucleotide fragment spanned from 823 to 963 positions on *dbt*Ac gene

Table 5. PCR primers for α subunit of initial PAHs dioxygenase used in this study		
nahAcfor	TGGCGATGAAGAACTTTTCC	[Laurie and Lloyd-Jones, 2000]
nahAcrev	AACGTACGCTGAACCGAGTC	[Laurie and Lloyd-Jones, 2000]
P8073	TTCGAGCTGGAATGTGAGC	[Laurie and Lloyd-Jones, 2000]
P9047	AATAACCGGCGATTCCAAAC	[Laurie and Lloyd-Jones, 2000]
P20	ACAGGATCCAGATGGGTGAATATGGTACTGGTG	This study
P54	CGGGATCCTTATTTTCCGATGAGCTCGCGCTT	This study
dbtAcfor GC	(40bp-GC-clamp)GGCAAGCTCTACCGAAAATGG	This study
dbtAcfor	GGCAAGCTCTACCGAAAATGG	[I]
dbtAcrev	GTGTTAGTACCCCAGAGATATGAGTTGT	[I]
COM1for	AAAAGAGTTGTACGGCGATG	[Moser and Stahl, 2000]
COM1rev	ACGGTAGAATCCGCGATAGC	[Moser and Stahl, 2000]
RHO1for	CAAGGATGCGTATGGCAATC	[Moser and Stahl, 2000]
RHO1rev	TTCGAGGAAGTTGCGCTGGT	[Moser and Stahl, 2000]
RieskeF	TGYCGBCAYCGBGGSAWG	[Kasuga et al., 2001]
RieskeR	CCAGCCGTGRTARSTGCA	[Kasuga et al., 2001]
AJ025	TAYATGGGBGARGAYCCVGT	[Armengaud et al., 1998]
AJ026	GCRAAYTTCCARTTRCABGG	[Armengaud et al., 1998]
Do-1s	TGYAGYTWYCAYGGNTGG	[Saito et al., 2000]
Do-1a	TCNRCNGCRAAYTTCCARTT	[Saito et al., 2000]

Genomic DNAs from *Pseudomonas putida* ATCC17484, *Burkholderia* sp. RP007 ICMP 13529, and *Burkholderia* sp. DBT1 were used as positive controls. Reactions were performed in 50 µl final volume with 2-5 ng of genomic DNA, 1x reaction buffer Dynazyme, including 1.5 mM MgCl, 0.4mM dNTPs mix, 2U of DyNAzyme™ II DNA polymerase (Finzymes) and 20 pmol of each specific primer and 100 pmol of each degenerate primer. All PCR amplifications were performed with *Mastercycler® personal* (Eppendorf). The reaction conditions, for the different primer sets, were:

Primers	reaction conditions
nahAcfor/nahAcrev (992bp)[a]; P9047/P8073 (993bp)[a]	5 min at 95°C; 25 cycles of 45 s at 95°C; 45 s at 53°C; 1 min at 72°C; hold 5 min at 72°C
P20/P54 (1368bp)[a]	5 min at 95°C; 35 cycles of 1 min at 95°C; 1 min at 60°C; 1.30 min at 72°C; hold 10 min at 72°C

dbtAcfor/dbtAcrev (140bp) [a]	2 min at 94°C; 30 cycles of 45 s at 94°C; 45 sec at 58°C; 45 sec at 72°C; hold 5 min at 72°C
RHO1for/RHO1rev (906bp) [a]; COM1for/COM1rev (894bp) [a]	5min at 94°C; 35 cycles of 45 s at 94°C; 45 sec at 55°C; 1 min at 72°C; hold 5 min at 72°C
DO-1s/DO-1a (300bp) [a]	5 min at 95°C; 40 cycles of 1 min at 95°C; 1 min at 47; 1 min at 72°C; hold 5 min at 72°C
AJ025/AJ026 (434bp) [a]	5 min at 95°C; 5 cycles of 1 min at 95°C; 1 min at 38°C; 1 min at 72°C and 40 cycles of 1 min at 95°C; 1 min at 48; 1 min at 72°C; hold 5 min at 72°C
RieskeF/RieskeR (78bp) [a]	5min at 95°C; 40 cycles of 45 s at 95°C; 1 min at 58°C; 45 s at 72°C; hold 5 min at 72°C
[a]: expected amplification fragments size	

2.5.5.2 *nahAc* Restriction analysis

The Southern confirmed positive nahAcfor/nahAcrev PCR products were further digested with *Hae*III (Amersham Bioscience) restriction enzyme and separated onto 2% (w/v) agarose gel in order to investigate the α subunit gene encoding polymorphism in the genomes of the isolates [modified from Ferrero et al., 2002].

2.6 CULTURE INDEPENDENT APPROACHES

2.6.1 DNA EXTRACTION FROM SOIL

Total DNA from 500 mg of each soil samples was extracted using the FastDNA® SPIN® Kit (For Soil) (Q-BIOgene, Resnova) following the manufacturer's instructions. To assess DNA yield and quality (average molecular size), the soil DNA was run on 0.8% (w/v) agarose gels [Sambrook et al., 1989] with a molecular size marker (1-kb ladder) as reference. The DNA purity was assessed on the basis of PCR amplifiability.

2.6.2 PCR-DGGE COMMUNITIES ANALYSIS AND MONITORING OF PAHS-TRANSFORMING INOCULA

The PCR-DGGE approach has been adopted in order to evaluate the changes in adapted bacterial community due to the presence of different PAHs-contamination level or plant rhizospheres as well as to monitoring the bacteria inocula bioaugmented into the assessed microcosms.

The gene encoding for the 16S rRNA (1 to 1512 position, *E. coli* numbering) was initially amplified using the fD1 and rP2 primer (Table 4). Total DNA, extract from the bulk soil samples A, B, C as well as from all rhizospheric soil samples (Phytoremediation assay and Bioaugmentation/ Phytoremediation/ Bioaugmentation and Phytoremediation assays) was used as reaction template (5-20ng). 1U of MasterTaq (Eppendorf) was used as reaction enzyme. Subsequently, the universal bacterial primers

targeting 16SrDNA V3 variable region, p3/p2 (Table 4) were used to amplify fragments sized about 200 bp [Muyzer et al., 1993]. The reactions were set up using a suitable dilution of the first PCR products, as template, and 1.5 U of DyNAzyme™ II DNA polymerase (Finzymes) as reaction enzyme. In both conditions the template was substituted with ultrapure water in the negative control samples. The different PCR protocols were performed as follows:

100 µl PCR mix	fD1/rP2 reaction conditions
1x reaction buffer MasterTaq, including 15mM $Mg(OAc)_2$ 1x enhancer buffer MasterTaq 300µg BSA 20 pmol of both the primers 0.5 mM of dNTPs mix 1U of MasterTaq (Eppendorf)	1 min at 70°C 38 cycles of 1 min at 95°C 1 min at 40°C 3 min at 72°C and 1 min at 95°C 1 min at 40°C 10 min at 72°C

50 µl PCR mix	p2/p3 reaction conditions
1x reaction buffer Dynazyme, including 1.5 mM $MgCl_2$ 20 pmol of both the primers 0.4 mM of dNTPs mix 1.5 U of DyNAzyme™ II DNA polymerase (Finzymes)	5 min at 95°C 30 cycles of 45 s at 95°C, 30 sec at 57°C 30 sec at 72°C hold 5 min at 72°C

All PCRs were performed with *Mastercycler® personal* (Eppendorf).

The DGGE was carried out using a Dcode Universal Mutation Detection System instrument (Bio-Rad). The acrylamide concentration in the gel was 8%, and the denaturing gradient was 30% to 60% (the 100% denaturant solution contained 7 M urea, 40% (v/v) formamide, 8% acrylamide/bis-acrylamide (37.5:1) and 0.5x TAE buffer (pH 8) in ultrapure water). 1.5 µg of p2/p3 PCR products were transferred to the bottom of the well and then the gel was run at 60°C for 5 h at 180V. The gel images were acquired using the ChemDoc (Bio-Rad) gel documentation system.

Some selected DGGE bands were first scratched then reamplified, purified, cloned in pBluescript® SK (+/-) Phagemid vector, single strand sequenced and aligned to the sequence databases using BLASTN [Altshul et al., 1997]. DNA sequence data were analysed using PRISM Ready Reaction DNA terminator

cycle sequencing Kit (Perkin-Elmer) running on ABI 377 instrument and then were assembled by using ABI Fractura and Assembler computer packages

2.6.3 PCR-BASED EVALUATION OF PAHS-TRANSFORMING CAPACITY IN THE BACTERIAL COMMUNITIES

2.6.3.1 Amplification by PCR of the genes encoding the α subunit of the initial PAHs-dioxygenase from soil DNA extracts

The nahAcfor/nahAcrev, P9047/P8073, P20/P54 primer sets (Table 5) were used to amplify the gene encoding the α subunit of the initial PAHs-dioxygenase using 50-400ng of total DNA extracted from soil as a template. The reaction conditions were the same described above (2.5.5.1 section) however two different PCR mixture were tested in this occasion:

50 µl PCR mix	100 µl PCR mix
1x reaction buffer Dynazyme, including 1.5 mM $MgCl_2$ 20 pmol of both the primers 0.4 mM of dNTPs mix 1.5 U of DyNAzyme™ II DNA polymerase (Finzymes)	1x reaction buffer MasterTaq, including 15 mM $Mg(OAc)_2$ 1x enhancer buffer MasterTaq 300µg BSA 20 pmol of both the primers 0.5 mM of dNTPs mix 1U of MasterTaq (Eppendorf)

Genomic DNAs from *Pseudomonas putida* ATCC17484, *Burkholderia* sp. RP007 ICMP 13529, and *Burkholderia* sp. DBT1 were used as positive controls. The template was substituted with ultrapure water in the negative control. All the PCRs were performed with *Mastercycler® personal* (Eppendorf).

2.6.3.2 PCR-DGGE analysis of the *dbt*-genotype

A suitable dilution of positive products of P20/P54-PCR (Table 5) has been used as template of a second amplification with dbtAcforGC/dbtAcrev primer set (Table 5) and 1.5 U DyNAzyme™ II DNA polymerase (Finzymes) as reaction enzyme [PCR conditions performed with *Mastercycler® personal* (Eppendorf): 2 min of denaturation at 94°C; followed by 25 cycles of 45 s at 94°C; 45 s at 58°C; 45 sec 72°C; final extention 5min at 72°C]. dbtAcfor GC primer was obtained by adding a 40bp GC clamp [Muyzer et al., 1993] to dbtAcfor primer. The amplified products obtained were separated by DGGE. The acrylamide concentration in the gel was 10%, and the denaturing gradient was 40% to 60% (the 100% denaturant solution contained 7 M urea, 40% (v/v) formamide, 8% acrylamide/bis-acrylamide (37.5:1) and 0.5x TAE buffer (pH 8) in ultrapure water). 900ng and 300ng of dbtAcfor GC/dbtAcrev PCR products from soil DNA and from genomic DNA of *B*. sp DBT1 were transferred to the bottom of the well and then the gel

was run at 60°C for 5 h at 180V. The gel images were acquired using the ChemDoc (Bio-Rad) gel documentation system.

2.6.3.3 Southern hybridisation using PAHs gene probes

Products of PCR amplifications, obtained using the primer sets reported in Table 5 from the total soil DNA, and from genomic DNA of the isolates, were resolved on agarose gel and blotted on a nylon membrane (Hybond N^+, Amersham Bioscience) using standard protocols [Sambrook et al., 1989]. Two DNA fragments were utilised as probe: nahAc probe, a portion of the *nah*Ac gene (AF004284) amplified using the nahAcfor/nahAcrev primer set on genomic DNA from *Pseudomonas putida* ATCC 17484 and dbtAc probe, a portion of *dbt*Ac gene (AF380367) amplified using dbtAcfor/dbtAcrev primer set on genomic DNA from *Burkholderia* sp. DBT1. The labelling of the probes was performed using the Gene Images™ CDP-Star™ Detection Module (Amersham Biosciences) following the manufacturer's instructions. Pre-hybridisation, hybridisation and the final blots washes were performed as follows:

PCR fragments obtained by using	Pre-hybridisation conditions	Hybridisation conditions	Washes
nahAcfor/nahAcrev dbtAcfor/dbtAcrev	10ml Buffer: SSC 5X [1X SSC is 0.15M NaCl plus 0.015 M sodium citrate]; 0.1% SDS; 5% dextran sulfate; 5ml liquid block; 1mg of sonicated and boiled salmon sperm DNA Treatment: 1 hour at 60°C	10ml Buffer: SSC 5X; 0.1% SDS; 5% dextran sulfate; 5ml liquid block; 1mg sonicated and boiled salmon sperm DNA; 300ng of boiled nahAc or dbtAc probe Treatment: overnight at 60°C	1) 50ml of SSC 1x; SDS 0.1%, 15 min at 60°C 2) 50ml of SSC 0.5X; SDS 0.1%, 15 min at 60°C
AJ025/AJ026	10ml Buffer: SSC 6X; 0.5% SDS; 5% dextran sulfate; 2.5ml liquid block; 1mg sonicated and boiled salmon sperm DNA Treatment: 4 hour at 55°C	10ml Buffer: SSC 6X; 0.5% SDS; 5% dextran sulfate; 2.5ml liquid block; 1mg sonicated and boiled salmon sperm DNA 300ng of boiled nahAc probe Treatment: overnight at 55°C	1) 50ml of SSC 1x; SDS 0.1%, 2x 15 min at 37°C 2) 50ml of SSC 0.1X; SDS 0.1%, 2x 15min at 40°C
RieskeF/RieskeR	10ml Buffer: SSC 5X; 0.1% SDS; 5% dextran sulfate; 5ml liquid block; 1mg sonicated and boiled salmon sperm DNA Treatment: 1 hour at 65°C	10ml Buffer: SSC 5X; 0.1% SDS; 5% dextran sulfate; 5ml liquid block; 1mg sonicated and boiled salmon sperm DNA; 300ng of boiled nahAc probe Treatment: overnight at 65°C	1) 50ml of SSC 1x; SDS 1%, 2x 7 min at 37°C 2) 50ml of SSC 0.1X; SDS 1%, 2x7 min at 65°C

2.6.4 MONITORING OF Burkholderia sp. DBT1 BY TaqMan RealTime PCR IN BIOAUGMENTATION/ PHYTOREMEDIATION/ BIOAUGMENTATION and PHYTOREMEDIATION ASSAYS

2.6.4.1 TaqMan primers/probe design and specificity

The primers dbtAcfor and dbtAcrev (Table 5) as well as dbtAcFAM/TAMRA TaqMan probe (TCGACGACATGCGACCCAAGA) were designed with Primer Express 1.0 software (Perkin-Elmer) according to the recommended guidelines for TaqMan probe design. The TaqMan probe 5' labelled with the fluorescent dyes 6-carboxyfluorescein (FAM), and 3' labelled with 6-carboxy-tetramethyl rhodamine (TAMRA) were obtained from Perkin-Elmer. The criterion used for primers and probe design was the absence of adventitious amplification of any non-target sequences. By CLUSTAL-W [Thompson et al., 1994] alignment of the *dbt*Ac gene sequence (AF380367) with analogous ones, available in on-line databases, a suitable region was selected (823-963 in *dbtAc* gene sequence) for the three oligo design. In particular *dbtAc* gene sequence was aligned with:

genes	Bacteria	AC number	genes	Bacteria	AC number
ntdAc	*Pseudomonas* sp. strain JS42	U49504	*nbzAc*	*Comamonas* sp. strain JS765	AF379638
dntAc	*Burkholderia cepacia* strain R34	AF169302	*dntAc*	*Burkholderia* sp. strain RASC	U62430
pahAc	*Comamonas testosteroni* strain H	AF252550	*nagAc*	*Ralstonia* sp. strain U2	AF036940
nahAc	*Pseudomonas stutzeri* strain AN10	AF039533	*pahAc*	*Pseudomonas aeruginosa* strain PaK1	D84146
ndoC2	*Pseudomonas fluorescens* strain ATCC 17483	AF004283	*pahAc*	*Pseudomonas putida* strain OUS82	AB004059
nahAc	*Pseudomonas putida* strain G7	M83949	*ndoB*	*Pseudomonas putida* strain NCIB9816	M23914
doxB	*Pseudomonas* sp. strain C18	M60405	*nahAc*	*Pseudomonas* sp. strain 9816-4	U49496
nahA3	*Pseudomonas putida* strain BS202, plasmid NPL1	AF010471	*narAa*	*Rhodococcus* sp. strain NCIMB12038	AF082663
phnAc	*Burkholderia* sp. RP007	AAD09872	*phnAc*	*Alcaligenes faecalis* strain AFK2	AB024945

2.6.4.2 TaqMan RealTime PCR optimisation

PCR was performed in 25 µl volumes using MicroAmp Optical 96-well reaction plates and MicroAmp Optical Caps (Applied Biosystems). A 140-bp product was amplified using primers dbtAcfor and dbtAcrev and probe dbtAcFAM/TAMRA (Table 5). The PCR DNA targets were extracted from soil microcosms listed below (Table 6):

Table 6. Soil DNA extracts utilised as template in TaqMan PCR reactions		
Microcosms	*Lupinus albus*	Bacterial inocula
L*	yes	no
E0[a] and E9°	yes	no
D0 to D9°	no	6 x 10^7 CFU/g of soil of *Burkholderia* sp. DBT1
G0[a] to G9°	yes	6 x 10^7 CFU/g of soil of *Burkholderia* sp. DBT1
*: Microcosms performed in the Phytoremediation assay;°: Microcosms performed in the Bioaugmentation/ Phytoremediation/ Bioaugmentation and Phytoremediation assays [a]: E0, G0: sampling after 24h from the inoculation and before plant sowing		

5 µl (200 ng) of diluted template was added to 20 µl of PCR master mix, 2.5x RealMasterMix Probe ROX (Eppendof) [12.5mM $Mg(OAc)_2$, 1mM dNTPs with dUTP and 1U/µl HotMaster Taq DNA Polimerase]. The TaqMan reaction mixture was optimised, as described below, in order to minimize the humic acids interferences both on *Taq* polimerase [Tebbe and Vahjen, 1993; Toranzos, 1997; Wilson, 1997] and on the fluorescence detection [Stults et al., 2001].

25µl PCR mix	reaction conditions
5µl of target DNA 1X RealMasterMix Probe ROX (Eppendorf) 1X MasterTaq PCR Enhancer (Eppendorf) 25 µg BovineSerum Albumin (Sigma Aldrich) 0.0075 mM Brilliant Black BN (Sigma Aldrich) 200mM dbtAcFAM/TAMRA probe 900mM of both primers	2 min at 94°C 40 cycles of 15 s at 94°C, 1min at 60°C

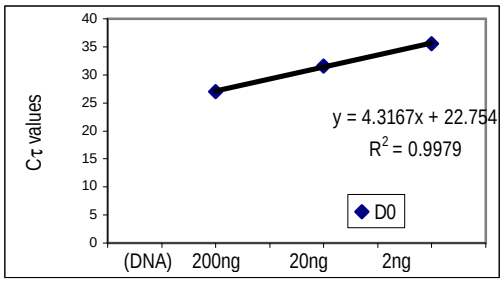
Bovine Serum Albumin (BSA) and Brilliant Black BN (4-acetamido-5-hydroxy-6-[7-sulfonato-4-(4 sulfonatophenylazo)-1-naphtylazo] naphthalene 1,7-disulfonate, E151) were added to the reaction mixture as water DNA-se and RNA-se free solutions. 75, 37.5 and 25 µg BSA /reaction were pre-tested to determine the protein appropriate reaction quantity. On the other hand, 0.5mM, 0.05mM, 0.01mM, 0.0075mM and 0.005mM Brilliant Black BN reaction concentrations were pre-tested to determine the dye suitable reaction amount.

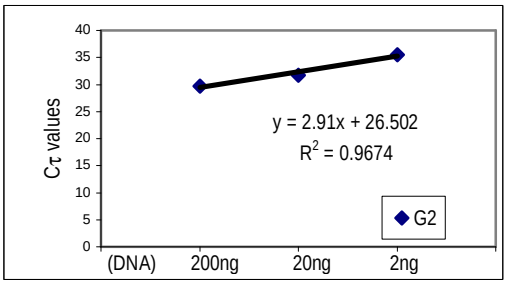
The positive reaction controls were performed using 1ng of *B*. sp. DBT1 genomic DNA, negative reaction controls were carried out without template (NTC) and without reaction enzyme (NAC). Amplification, data acquisition, and data analysis were carried out routinely in an ABI Prism 7700 Sequence Detector (PE Applied Biosystems). Data were analysed with Sequence Detector Software (version 1.7). Threshold determinations were manually performed. The cycle at which a sample crosses

the threshold (a PCR cycle where the fluorescence emission exceeds that of non-template controls) is called the threshold cycle, or Cτ. A high Cτ value corresponds to a small amount of template DNA, and a low Cτ corresponds to a large amount of template initially present. Holland and co-workers [Holland et al., 1991], provide more detailed information on the TaqMan PCR quantification method. The template copy number was determined from Cτ values by using a standard curve.

2.6.4.3 Brilliant Black BN fluorescence normalisation

The normalising fluorescence effect of the selected 0.0075mM Brilliant Black BN concentration was assed by performing PCRs with 10fold-dilution or 2fold-dilution template quantity. The normalising effect of the black dye is achieved because of PCR performed on diluted soil DNA produced amplification curves with linearly related Cτ values. The results obtained from the DNA extracts of D0 (non-vegetated; *B.*sp.DBT1 inoculated, t=0) and G2 (vegetated; *B.*sp.DBT1 inoculated, t=2) microcosms were reported as example. In these cases, PCRs were performed as described above, using 0.0075mM Brilliant Black BN and, as template, 200ng, 20ng and 2ng of soil DNA of each sample.





DNA extracts	Cτ	Standard deviation (Cτ)	DNA extracts	Cτ	Standard deviation (Cτ)
D0 200 ng	26.96	0.078	G2 200 ng	29.72	0.357
D0 20 ng	31.62	0.574	G2 20 ng	31.71	0.061
D0 2 ng	35.59	1.800	G2 2 ng	35.54	1.880

2.6.4.4 TaqMan quantification

A single copy of *dbt*Ac gene, amplified with P20/P54 (Table 5), was cloned into 2958bp pBluescript® SK (+/-) Phagemid cloning vector. Serial dilutions (10-fold) of plasmid DNA obtained were utilised to construct a linear *dbt*Ac standard curve with the lower detection limit. The detection limit optimisation was conducted adjusting BSA-Brilliant Black BN reaction concentrations.

3. RESULTS AND DISCUSSION

3.1 INITIAL CHARACTERISATION OF SOIL BACTERIAL COMMUNITIES

The initial purpose of this research was to characterise the adapted bacterial community of an aged PAHs-contaminated soil (ex-Carbochimica, Trento, Italia), to evaluate the intrinsic bioremediation potential of the site. Therefore, culture-dependent and culture-independent approaches were adopted to study the bacterial communities of three soil aliquots (coming from the same soil sample) sharing different level of PAHs-contamination, in detail 200 (soil aliquot A), 400 (soil aliquot B) and 14,000 (soil aliquot C) mg of 16 US-EPA PAHs per kg of dry soil.

3.1.1 COLTURE DEPENDENT SOIL CHARACTERISATION: ENUMERATION OF CULTURABLE BACTERIAL STRAINS AND PAHs TRANSFORMING BACTERIA

Only 1%-10% of environmental microrganisms can be cultivated [Alexander 1976; Bakken 1985], because of the selective pressure imposed by the requirement of growth on laboratory-made substrates, leading to the isolation of a subpopulation from the communities. Therefore, the microbial numbers obtained with microplates technique are underestimate. However, the evaluation of the culturable total heterotrophic bacteria, in a contaminated soil, can provide useful information on the extent to which the indigenous microbial community has acclimated to the site conditions. On the other hand, the enumeration of PAHs-transforming bacteria can indicate the dimension of the microbial population potentially capable of supporting bioremediation. Moreover, since the total culturable microflora and PAHs degraders were estimated using the same method, these numbers can be compared [Binet et al., 2000]. In this study, three soil aliquots with similar chemical-physical properties (Table 1), but different degree of PAHs contamination were analysed. The MPN estimations of total microflora and of PAHs-transforming bacteria in the three samples were listed in the Table 7.

Table 7. PAHs-transforming bacteria and Total heterotrophic MPN enumerations

Soil aliquots	Total heterotrophs MPN/g of dry soil	95% Confidence limits	PAHs-transforming bacteria MPN/g of dry soil	95% Confidence limits	PAHs-transforming bateria/total heterotrophs (%)
A	$2.85X\ 10^5$	$1.30X\ 10^5$-$5.55X\ 10^5$	$5.90X\ 10^3$	$3.15X\ 10^3$-$1.41X\ 10^4$	2.07
B	$1.46X\ 10^5$	$4.58X\ 10^4$-$2.88X10^5$	$1.37X\ 10^3$	$6.78X\ 10^2$-$2.77X10^3$	0.94
C	$6.60X10^5$	$3.55X\ 10^4$-$1.50X\ 10^6$	$1.43X10^5$	$4.41X\ 10^4$-$2.85X\ 10^5$	21.7
Z-value (MPN_A-MPN_B): 0.61[a]			Z-value (MPN_A-MPN_B): 1.33[a]		
Z-value (MPN_C-MPN_A): 0.78[a]			Z-value (MPN_C-MPN_A): 2.93[a]		
Z-value (MPN_C-MPN_B): 1.39[a]			Z-value (MPN_C-MPN_B): 4.27[a]		

A: 200 mg of 16 US-EPA PAHs per kg of dry soil; B: 400 mg of 16 US-EPA PAHs per kg of dry soil; C: 14,000 mg of 16 US-EPA PAHs per kg of dry soil.

[a]: Critical Z-value at 95% level=1.96. MPN-estimates with calculated Z-values smaller then 1.96 are not significantly different at 95% level

Total heterotrophs values obtained for the studied aliquots, were significantly lower than the previous reported ones for other PAHs polluted soil (averagely 10^7 MPN/g dry soil) [Joner et al., 2002; Binet et al., 2000]. Nevertheless, the number of heterotrophs in all soil aliquots was of the same order of magnitude, independently of the PAHs contamination degree. Therefore, the presence of pollutants seemed to not affect the total microbial community. On the other hand, high contamination level enriched the percentage of total culturable microflora able to transform PAHs. In fact, the percentage of PAHs-utilizing bacteria varied from 0.94-2.07%, in the low polluted aliquots, to 21.7% in the 14,000mg/kg PAHs-contaminated one (Table 7).

3.1.2 CULTURE INDEPENDENT CHARACTERISATION: PCR-BASED EVALUATION OF TOTAL BACTERIAL COMMUNITY AND PAHs TRANSFORMING BACTERIA

Culture-independent methods have been proposed to provide a lesser-biased picture of the richness of bacterial communities than culture-dependent methods [Amann et al., 1995]. However, culture-independent methods are typically dependent on PCR. Several potential biases have been shown because of the required extraction of community DNA, the optimisation of the PCR conditions as well as the subsequent PCR-products manipulations [Wintzingerode et al., 1997]. The implication of poor DNA recovery is the possible reduction of detection limits achievable using PCR. In some situations, particularly when target genes are poor represented in the final extract, this potential detection limit (about 10^6 copies of a gene/g of dry soil [Lloyd-Jones and Hunter 2001]) can prevent PCR amplifications and thus generate false negative results. Moreover, the choice of particular PCR conditions or reagents as well as the intrinsic resolution limit of the PCR-products screening techniques, contribute, once again, to reduce the potential of the culture-independent approach on the characterisation of the total microbial community. Nevertheless, DNA can be isolated from bacterial fraction containing 50 to 80% of the soil bacteria [Holben et al., 1998; Steffan and Atlas, 1988; Torsvik, 1980], and, also due to the power of the PCR, may provide genetic information about culturable as well as nonculturable bacteria. Moreover, the heterogeneity of this DNA is a measure of the total number of genetically different bacteria in soil (means genetic complexity of a soil) [Torsvik et al., 1990]. It is important to underline that the genomic complexity recovered by culturing methods is two and one order of magnitude lower than those gained in pristine and in perturbed soil respectively by culture independent methods [Torsvik and Øvreas, 2002]. These evidences provide that the culture-independent potential is high enough to overcome its own limits. Nevertheless, the culture-independent and culture-dependent views are not necessarily mutually exclusive; indeed investigations performed

combining the two different approaches can provide a more detailed image of the bacterial community of a complex matrix.

In this study, using the FastDNA® SPIN® Kit (For Soil) (Bio101) extraction method it has been possible to obtain DNA from all soil aliquots. All DNA extracts were sufficiently pure to serve as template for direct PCR amplification using the MasterTaq (Eppendorf) as DNA polimerase. The MasterTaq (Eppendorf) polimerase, in the formulated reaction mixture, resulted to be a suitable enzyme to amplify environmental DNA samples. The average DNA quantity recovered from the different soil samples by the utilised extraction kit was 39.4 ± 7.3 μg DNA/g of dry soil. This quantity was comparable with the previous reported one for the DNA extracted by the same extraction kit from soil with analogous texture and composition [soil samples 2 and 3 in Lloyd-Jones and Hunter 2001]. Considering 1 bp as 649 Da [Sambrook et al. 1989], and an average soil bacteria genome of 5 Mb [Lloyd-Jones and Hunter 2001], we should have obtained an average starting microbial biomass investigable by PCR-based approaches valuable as 7.3 x 10^9 genome equivalents/g of dry soil.

3.1.2.1 Analysis of bacterial communities by PCR-Denaturing Gradient Gel Electrophoresis (DGGE)

The electrophoresis separation onto a denaturing gradient gel of V3-16SrRNA PCR-products is a common way to directly evaluate the genetic diversity of complex microbial communities [Muyzer et al. 1993]. It is based on the fact that a DNA strand may denature dependently on its nucleotide sequence composition [Muyzer et al. 1993]. Therefore, PCR products with different compositions, but with the same length, will migrate different distances when exposed to gradients of denaturing substances. This procedure allows to directly identifying the presence of different species within a community (represented by bands within one electrophoresis profile) and their relative abundance. This approach can successfully be adopted to qualitatively determine the modifications occurred in a microbial community through the time or because of the presence of different concentrations of any selective agents [Nakatsu et al. 2000; Torsvik et al., 1998]. Nevertheless, because of the intrinsic resolution limit of the gel separation, up to 40% of all possible 16S rRNA variant sequences present in a natural microbial community could be adequately detected [Myers et al., 1987; Sheffield et al., 1989]. Therefore, the technique has some limitations to quantify the extent of the differences among communities with great diversity [Nakatsu et al. 2000]. However, total DNA extracted from complex microbial communities, such as soil one, contains too many information to be analysed directly by high-resolution methods, like ARDRA or T-RFLP [Torsvik et al., 1998]. On the contrary the lower resolution DGGE

analysis provided more useful information on the overall diversity and changes in the community structure [Torsvik et al., 1998].

In this way, we successfully adopted the V3-16SrRNA-PCR-DGGE analysis for the initial evaluation of the existing differences in the total bacterial communities as result of the different level of PAHs-contamination. With the same approach it has also been possible to identify the more representative bacterial species adapted at the site conditions. The obtained PCR-DGGE profiles showed that the contaminated site was characterised by heterogeneous bacterial communities (Figure 2). Each profile consisted of 9-14 intense bands, in addition to a greater number of weaker ones. No great differences in the patterns of dominating bands were noted among the samples. Nevertheless, a substantial enrichment of major bands (1 and 2) in the higher contaminated soil aliquot C is evident. Moreover some bands that were not present in the others can be observed in this last profile. Both these evidences may be due to the more selective pressure imposed by the higher contamination level.

The scrapping and sequencing of bands 1 and 2 (Table 8) showed high homology with bacterial strains belonging to the group genera *Cytophaga/Flavobacterium* and *Arthrobacter/Acinetobacterium*. It is important to underline that PAHs-transforming strains belonging to these genera have already been characterised [Stach and Burns, 2002; Huang et al., 2004]. It is reasonable to suggest that the recovered dominant species may have a similar transforming activity in the studied soil.

Table 8. Taxonomic characterisation of cloned bands from DGGE profiles

Scrapped bands	Organism showing greatest similarity	% Homology
1	*Cytophaga* sp. type 0092 (X85210)	99%
2	*Acinetobacterium* sp.RG-73 (AY561633)	100%

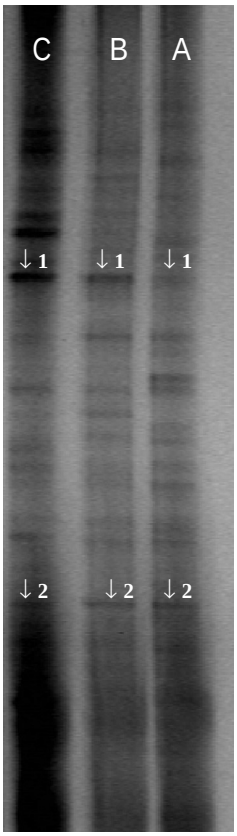


Figure 2. DGGE profiles. Lane C, B, A, 1.5 μg of PCR amplification products (p3/p2) of V3 region 16S rDNA of the bacterial community soil aliquot C (14,000 mg/kg 16 US-EPA PAHs), B (400 mg/kg 16 US-EPA PAHs), and A (200 mg/kg 16 USA EPA PAHs), respectively. Arrows 1and 2, scrapped bands.

3.1.2.2 PCR based evaluation of PAHs-transforming capacity within the bacterial communities

Recently, the characterization of genes involved in bacterial organic pollutant degradation has promoted the development and application of nucleic-acid-based methods to determine the potential for successful bioremediation before setting up a field system [Chandler and Brockman, 1996] and to monitor the performance of *in situ* bioremediation [Burlage et al., 1994]. Therefore, PCR amplifications of specific target sequences, as well as gene probes hybridisations, has been used to detect the presence of catabolic genes within bacterial populations from contaminated soils, and on the basis of those results, it has been possible to assess their biodegradation potential [Fleming et al., 1993; Chandler and Brockman, 1996; Whyte et al., 1997; Milcic-Terzic et al., 2001].

With the same aim, in this study PCRs were performed (using primers listened in Table 5), directly to the soil total DNA, to detect the presence of catabolic genes responsible for the bacterial aerobic PAHs transformation. In particular, PCRs were carried out to detect the presence of three divergent PAHs-transforming genotypes, which were *nah, phn* and *dbt* genotypes [Laurie and Lloyd-Jones, 1999; II]. Therefore, we attempted to evaluate the biodegradation potential of the studied matrix by selective amplification of the α subunit of the genes (*nahAc*, *phnAc* and *dbtAc*) encoding the initial-dioxigenase enzyme in the corresponding catabolic pathways. The results obtained were listened in Table 9.

Table 9. PAHs-transforming genotype soil characterisation using MasterTaq (Eppendorf) as reaction enzyme

Soil aliquots	PCR using[a]		
	nahAcfor/nahAcrev[b] (*nah* genotype)	P9047/P8073 (*phn* genotype)	P20/P54[c] (*dbt* genotype)
A	+	-	+
B	+	-	+
C	+	-	+/-

[a]: +, PCR product of expected length; +/- no reproducible PCR products. All PCRs were performed twice on three replicates of the same environmental DNA

[b]: All products were verified through Southern hybridisation with nahAc probe generated by PCR amplification with *P. putida* ATCC17484

[c]: All products were verified through Southern hybridisation with dbtAc probe generated by PCR amplification with *B.* sp. strain DBT1

As a result, the performed reaction conditions permitted to recover the *nah* genotype within all bacterial communities but not the *phn* genotype. This failure could be due to not optimised reaction conditions or to the absence of this genotype in the investigated site. Nevertheless, it is also possible that the *phnAc* target gene was poorly represented in the final DNA extracts, and its copy number not exceed the PCR detection limits. Finally, the *dbt* genotype has been clearly detected in the two poorly contaminated aliquots, but not in the highly polluted one. In fact, we observed a lack of PCR reproducibility using DNA extracted from aliquot C as template. Probably, a gene target copy number very close to the PCR

detection limit could explain the obtained data. While the *nah* and *phn* genotypes both represent a potential capacity for PAHs transformation in the majority of other studied soils [Lloyd-Jones et al., 1999], to date no information are available concerning *dbt* genotype soil presence. Therefore, we were interested in the further investigation of this genotype. Thus, *dbtAc* fragments obtained by P20/P54 amplifications (from soil aliquots A and B) were re-amplified with dbtAcforGC/dbtAcrev primer set and then separated through DGGE (Figure 3). This assay was performed to characterise the potential *dbt* genotype polymorphism in the studied soil. The DGGE analysis, of the products generated with the dbtAcforGC/dbtAcrev primer set, showed that the fragments amplified from the PAHs-polluted soil samples resembled that generated with *Burkholderia* sp. strain DBT1, since the respective bands migrate to the same position in the denaturing gel. The blotting of the gel and the hybridisation with the dbtAc probe, under stringency conditions, provided the evidence that the melting behaviour of the soil derived products was similar to that generated with *B.* sp. strain DBT1. This result suggests a limited sequence divergence from the *B.* sp. strain DBT1 *dbtAc* gene in the *dbt* soil genes in the region spanned by the primers. The obtained data suggested a conservative nature of the *dbt* genotype. However, to date, *B.* sp. strain DBT1 *dbtAc* sequence (AF380367) is the only one *dbtAc*-like sequence cloned and sequenced, so we cannot confirm this DGGE data with some other sequence information.

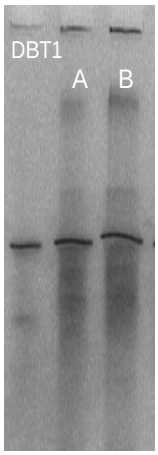


Figure 3. DGGE profiles. DBT1: 300 ng of PCR amplification products (dbtAcforGC/dbtAcrev) of *dbtAc* gene of *Burkholderia* sp. DBT1 genomic DNA; A and B: 900 ng of PCR amplification products (dbtAcforGC/dbtAcrev) of *dbtAc* gene from the bacterial community soil aliquot A (200 mg/kg 16 US-EPA PAHs) and B (400 mg/kg 16 US-EPA PAHs), respectively.

In conclusion, the performed PCR-based approach, permitted to determine, directly from the soil DNA, the presence of the suitable catabolic capacity to reclaim the polluted soil. These data were coherent to those obtained with the selective MPN enumeration. Moreover, the assessed nucleic acid-base method permitted to determine the classes and the heterogeneity of genes potentially involved in the bioremediation of the site.

3.1.3 ISOLATION OF PAHS-TRANSFORMING BACTERIAL STRAINS

The isolation of PAHs-transforming bacteria was performed, in this study, for several reasons. First, to assess the possibility to isolate, among the PAHs-utilizing bacteria, someone corresponding to dominant

species within the bacterial communities, in order to better microbiologically characterise the studied polluted matrix. Moreover, to compare the evidences obtained by PCR-based investigation about the PAHs-tranforming genotypes, on the soil DNA extracts, with those achievable by the characterisation of PAHs-utilizing cultivable strains. Finally, to gain pure culture of bacteria, with suitable biochemical capacity, to use in bioaugmentation protocols.

3.1.3.1 Biochemical characterisation

Therefore, with the purpose of isolating and characterising the culturable PAHs-transforming strains, selective enrichment cultures were set up. Naphthalene phenanthrene, dibenzothiophene and the combination of the three PAHs were used as carbon sources. A total of 21, 43 and 43 PAHs-transforming isolates were recovered from the soil aliquots A, B and C respectively. Carbon utilisation and indole transformation of the isolates were showed in the same tables (Table 10, 11 and 12).

Table 10. Biochemical characterisation of bacteria isolated from soil aliquot A (200 mg/kg PAHs)

Bacterial isolates	Dioxygenase test	Growth on[a]		
		Nah	Phn	DBT
NDP006, NDP007; NDP008;	-	+	-	+
NDP004; NDP005; NDP009; NDP010, NDP011;	-	+	+	+
N001; N002; N003; N004; N005; N006; NDP001; NDP002; NDP003; NDP012; NDP013; NDP014; NDP015;	+	+	+	+

[a]: Nah, naphthalene; Phn, phenanthrene; DBT, dibenzotiophene

Table 11. Biochemical characterisation of bacteria isolated from soil aliquot B (400 mg/kg PAHs)

Bacteria isolates	Dioxygenase test	Growth on[a]		
		Nah	Phn	DBT
1N-B; 2N-B; 3N-B; 4N-B, 5N-B; 6N-B; 7N-B; 8N-B; 9N-B; 10N-B; 12N-B; 13N-B; 14N-B; 1D-B; 3D-B; 4D-B; 6D-B; 7D-B; 8D-B; 1P-B; 2P-B; 5P-B; 1D750-B; 2D750-B; 4D750-B; 5D750-B; 6D750-B; 1P750-B; 4P750-B; 5P750-B	-	+	+	+
4P-B	-	-	+	-
2D-B; 5D-B; 3NDP-B; 1N750-B	+	+	+	+
3D750-B; 2P750-B; 3P750-B	++	+	+	+
11N-B; 1NDP-B; 2NDP-B; 4NDP-B; 5NDP-B	+++	+	+	+

[a]: Nah, Naphthalene; Phn, phenanthrene; DBT, dibenzothiofene

Table 12. Bacterial characterisation of bacteria isolated from soil aliquot C (14,000 mg/kg PAHs)				
Bacterial isolates	Dioxygenase test	Growth on[a]		
		Nah	Phn	DBT
1N-C; 6N-C; 10N-C; 12N-C; 13N-C; 14N-C; 15N-C; 17N-C; 2D-C; 3D-C; 4D-C; 5D-C; 6D-C; 8D-C; 9D-C; 10D-C; 11D-C;12D-C; 15D-C;16D-C; 1P750-C; 2P750-C; 3P750-C; 4P750-C; 1NDP-C; 2NDP-C; 3NDP-C; 1D750-C; 2D750; 3D750-C	-	+	+	+
1P-C; 2P-C; 3P-C; 4P-C	-	-	+	-
14D-C; 5P-C	-	-	+	+
16N-C; 1D-C; 4D750-C	+	+	+	+
4N-C; 5N-C	++	+	+	+
2N-C; 13D-C	+++	+	+	+
[a]: Nah, naphthalene; Phn, phenanthrene; DBT, dibenzotiophene				

Biochemical screening showed that the PAHs-transforming bacteria had multiple phenotypes in terms of PAHs utilisation, suggesting the possibility that there were various PAHs transformation pathways among the strains isolated from the same site. Based on carbon source utilisation, the 107 isolates could be grouped into four catabolic sets, naphthalene/phenanthrene/dibenzothiophene transforming set (90.7%), naphthalene/dibenzothiophene transforming set (2.8%), phenanthrene/dibenzotiophene transforming set (1.9%) and phenanthrene transforming set (4.7%). To date, it is well documented that the enzymes involved in the conversion of naphthalene to salycilate (naphthalene upper pathway) can degrade phenanthrene and dibenzothiophene in 1-hydroxy-2-naphthoate and 3-hydroxy-2-formil-benzothiophene respectively, through similar catabolic steps [Menn et al., 1993; Sanseverino et al., 1993; Kiyohara et al., 1994; Yang et al., 1994; Denome et al., 1993]. As a result, theoretically, bacteria able to grow on naphthalene can grow on phenanthrene and dibenzothiophene. It is not truly realised. Indeed, for instance, regarding phenanthrene, the 1-hydroxy-2-naphthoate is further metabolised through salicylate-catechol or through phthalate-protocatecuate. Some authors have recently reported that phenanthrene-degrading bacteria *via* phthalate-protocatecuate cannot grow on naphthalene as sole carbon source [Takizawa et al., 1999; Saito et al., 2000; Goyal and Zylstra 1996]. Therefore, it may suggest that the strains 1P-C, 2P-C, 3P-C, 4P-C and 4P-B (Table 11 and 12), unable to degrade naphthalene but able to transform phenanthrene, could utilise the latter *via* phthalate-protocatecuate. On the other hand, concerning dibenzothiphene, the previously characterised dibenzothiphene-utilizing bacteria, *via* Kodama pathway [Kodama et al., 1973], were also able to degrade naphthalene [Denome

et al., 1993; Kasai et al., 2002; II]. In our knowledge, this is the first evidence of dibenzothiphene-transforming bacteria (14D-C and 5P-C) able to utilise phenanthrene but not naphthalene as carbon source. Therefore, it may at least suggest that dibenzothiphene-transformation in these strains proceed trough an unknown pathway.

The 107 bacterial isolates were also tested for the capacity to convert indole to indigo. The indigo formation from indole is a property of dioxygenase enzyme system that forms *cis*-dihydrodiols from aromatic hydrocarbons [Ensey et al., 1983]. Of the many aromatic hydrocarbons shown to undergo reaction of this type, examples of a range of mono- and multinuclear substrates can be cited. Thus, benzene, toluene, naphthalene, phenanthrene and biphenyl have all been shown to yield *cis*-dihydrodiols [Eaton and Chapman 1995]. Nevertheless, the investigation of aromatic ring oxygenase activity of the isolates showed that the 73.8% of the PAHs-transforming bacteria recovered were unable to convert indole to indigo. Similar results have just been reported in literature by other authors [Widada et al., 2002a; Lloyd-Jones et al., 1999]. One explanation for this failure may be the existence of oxygenase systems with original enzymatic attack of the molecular oxygen to the aromatic hydrocarbons.

Moreover, we were interested in finding out if PAHs-transforming bacteria isolated might be able to secrete surface-active agents. In fact, biosurfactants production is often associated with the capacity of microrganisms to utilize hydrocarbons as substrates [Itoh and Suzuki, 1972; Oberbremer and Muller-Hurting, 1989] because of the water-immiscible nature of these compounds. Therefore, the 107 bacteria isolates were screened for the biosurfactants, as rhamnolipids, synthesis, onto blue agar plates, while growing on dextrose, mannitol or a mixture of the three selected PAHs (Table 13, 14 and 15).

Table 13. Production of extracellular biosurfactant by bacteria isolated from soil sample A (200 mg/kg PAHs)

Bacterial strains	Biosurfanctant production growing on [a]		
	Dextrose	Mannitol	Nah/Phe/DBT
N001 N002 N003 N004 N005 N006 NDP001 NDP002 NDP004 NDP011 NDP005 NDP009 NDP010	++++	+++	++
NDP012 NDP013 NDP014 NDP015	++	++++	++++
NDP003	+	+	+
NDP006 NDP007 NDP008	+	++	++

[a]: +: dark blue halos production; +/- : weak blue halos production; -: no blue halos production

Table 14. Production of extracellular biosurfactant by bacteria isolated from soil sample B (400 mg/kg PAHs)			
Bacterial strains	Biosurfanctant production growing on[a]		
	Dextrose	Mannitol	Nah/Phe/DBT
3NDP-B	+++	+++	+
11N-B; 12N-B; 13N-B; 14N-B; 2D-B; 4D-B; 5D-B; 7D-B; 3D750-B; 4D750-B; 3P750-B	++	+	++
7N-B; 1NDP-B;2NDP-B; 4NDP-B; 5NDP-B; 1D750-B; 2D750-B; 5D750-B; 1P750-B; 4P750-B; 1N750-B	++++	-	++
2P750-B	-	+++	+
1D-B; 3D-B; 4P-B; 5P-B	+	+	+
6N-B	-	-	-
1N-B	ng	+	+
5N-B; 9N-B; 10N-B	ng	+	++
2N-B; 3N-B; 4N-B; 8N-B; 6D750-B	+	+/-	+/-
6D-B; 8D-B	+++	-	+/-
1P-B; 2P-B	+/-	+	+
5P750-B	++++	+/-	++
[a]: +: dark blue halos production; +/- : weak blue halos production; -: no blue halos production; ng: no growth detected			

Table 15. Production of extracellular biosurfactant by bacteria isolated from soil sample C (14,000 mg/kg PAHs)			
Bacterial isolates	Biosurfanctant production growing on [a]		
	Dextrose	Mannitol	Nah/Phe/DBT
4N-C; 5N-C; 3D750-C; 3P750-C; 3NDP-C	+++	+++	+
2N-C; 12N-C; 14N-C; 16N-C; 1D-C; 10D-C; 11D-C; 13D-C;16D-C; 1P750-C; 2P750-C; 1NDP-C; 2NDP-C	++	+	++
1N-C; 10N-C; 15N-C; 1D750-C; 2D750-C	++++	-	-
2D-C; 3D-C; 4D-C; 5D-C; 6D-C; 8D-C; 9D-C; 12D-C	+++	-	+/-
15D-C; 17N-C	ng	+	+
6N-C	-	-	-
14D-C	+	+	+/-
1P-C; 2P-C; 3P-C; 4P-C	ng	+	-
5P-C	ng	+	++
13N-C; 4D750-C; 4P750-C	+	+/-	+/-
[a]: +: dark blue halos production; +/- : weak blue halos production; -: no blue halos production; ng: no growth detected			

The obtained results suggest that 72% of the isolates were able to produce dark blue halos when grown on the naphthalene, phenanthrene, and dibenzothophene-tested mixture.

Summarising the results obtained, the 36.4% of the 107 isolates have versatile metabolisms; in fact they were able to transform each tested PAHs, as well as to produce surfactants while growing on a PAHs mixture. Moreover, they probably harboured dioxygenase enzymes, to date not characterised, because they were unable to convert indole to indigo in the tested conditions.

3.1.3.2 Taxonomic characterisation

The results obtained by ARDRA of the isolates, permitted to distinguish the 107 isolates in 26 different Operational Taxonomic Units (in detail, 6 OTUs in soil aliquot A, 15 in soil aliquot B and 12 in soil aliquot C). Every OTU was obtained combining three restriction profiles (*Hha*I, *Hae*III and *Alu*I). The initial OTUB was spread in OTUB1, OTUB2, and OTUB3 by using another restriction enzyme, *Rsa*I. The distribution of the strains, isolated from all soil aliquots, in the different OTUs was graphically represented in Figure 4. One isolate for each OTU was selected for further taxonomic analysis. The taxonomic investigation was performed double strand sequencing the 16S rRNA genes of each candidate and aligning the obtained sequences to on line databases.

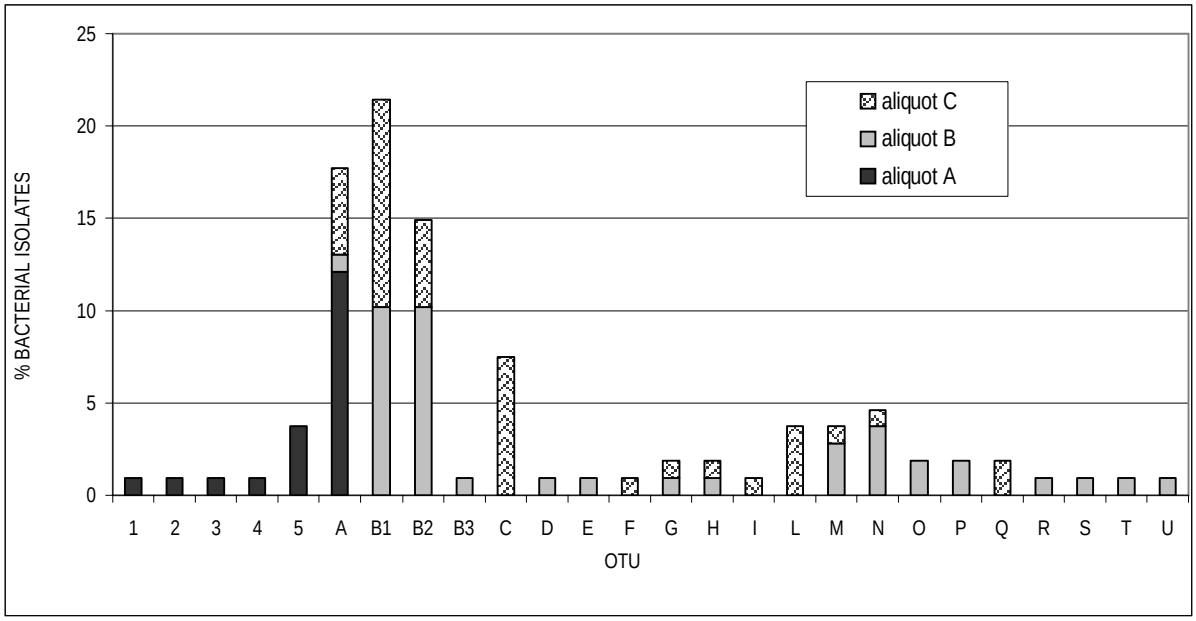


Figure 4. Graphical representation of isolates distribution inside the 26 OTUs. Aliquot A, 200 mg16 USA EPA PAHs per kg dry soil; Aliquot B, 400 mg 16 USA EPA PAHs per kg dry soil; Aliquot C, 14,000 mg 16 USA EPA PAHs per kg dry soil. 100% Bacterial isolates: 107 strains.

By the performed analysis it was possible to share out the 107 isolates in Proteobacteria [α- (7.5 %) β- (8.4%) and γ- (78.5%)], Firmicutes (4.7%) and Actinobacteria (0.9%). Within the Proteobacteria the *Pseudomonas* genus was the more represented one. In particular, the *Pseudomonas* genus

represented the 70.1% of the whole isolates, and the strains belonging to this genus were spread into 9 different OTUs (OTU1, OTUA, OTUB1, OTUB2, OTUB3, OTUC, OTUO, OTUQ, OTUR). It is interesting to note that the OTUA was recovered in all soil aliquots (61.9%, 2.3% and 11.6% respectively) independently of the contaminants content. Moreover, OTUB1 and OTUB2 were well represented both in the middle-polluted soil sample B (25.6% equally) and in the high-polluted soil sample C (30.2% and 11.6% respectively). The preferential recover of *Pseudomonas* genus using selective enrichment cultures as isolations approach was expected [Stach and Burns, 2002]. However it was also possible to recover strains belonging to the genera *Stenotrophomonas* (4.7%, OTU2, OTUM), *Comamonas* (0.9%, OTU3), OTU4), *Klebsiella* (3.7%, OTU5), *Agrobacterium* (1.9%, OTUD, OTUE), *Azospirillum* (3.7%, OTUL), *Brevundimonas* (2.8%, OTUF, OTUS, OTUT), *Achromobacter* (7.5%, OTUH, OTUN, OTUU) as well as *Bacillus* (2.8%, OTUG, OTUI) and *Staphylococcus* (1.9%, OTUP) within the Firmicutes and *Leucobacter* (0.9%) in the Actinobacteria phylum (Table 16). All the genera identified in this study were previously described as involved in PAHs-transformation [Boonchan et al., 1998; Juhasz et al., 2000; Goyal and Zylstra, 1996 and 1997; Widada et al., 2002a; Pospısil et al., 1996; Andreoni et al., 2004; Aitken et al., 1998; Chaineau et al., 1999; Gauthier et al., 2003; Kazunga and Aitken, 2000; Nweke and Okpokwasili, 2003; Zhuang et al., 2003], except *Leucobacter* [first time described in I] and *Azospirillum*. To our knowledge this is the first report on the capacity of these genera to transform PAHs.

Table 16. Taxonomic characterisation of bacteria isolated from the whole industrial soil

OTU	Bacterial Isolates	Organism showing greatest similarity[a]
1	NDP003	*Pseudomonas* sp. HR26 (AY032726) γ-proteobacteria
2	NDP006	*Stenotrophomonas maltophilia* LMG 10877 (AJ131784) γ-proteobacteria
3	NDP007	*Comamonas testosteroni* NCIMB 9872 (AF172067) β-proteobacteria
4	NDP008	*Leucobacter komagatae* (AB007419) Actinobacteria
5	NDP012; NDP013; NDP014; NDP015	*Klebsiella oxytoca* (U78183) γ-proteobacteria
A	N001 to N006; NDP001; NDP002; NDP004; NDP005; NDP009 to NDP011; 3NDP-B; 4N-C; 5N-C; 3D750-C;3P750-C; 3NDP-C	*Pseudomonas* sp. KD (AY456697) γ-proteobacteria
B1	11N-B; 12N-B; 13N-B; 14N-B; 2D-B; 4D-B; 5D-B; 7D-B; 3D750-B; 4D750-B; 3P750-B; 2N-C; 12N-C; 14N-C; 16N-C; 1D-C; 10D-C; 11D-C; 13D-C;16D-C; 1P750-C; 2P750-C; 1NDP-C; 2NDP-C	*Pseudomonas putida* ATCC 17484 (D85993) γ-proteobacteria
B2	7N-B; 1NDP-B;2NDP-B; 4NDP-B; 5NDP-B; 1D750-B; 2D750-B; 5D750-B; 1P750-B; 4P750-B; 1N750-B; 1N-C; 10N-C; 15N-C; 1D750-C; 2D750-C	*Pseudomonas putida* (AY456706) γ-proteobacteria
B3	2P750-B	*Pseudomonas chlororaphis* DSM 6698 (AY509898) γ-proteobacteria
C	2D-C; 3D-C; 4D-C; 5D-C; 6D-C; 8D-C; 9D-C; 12D-C	*Pseudomonas putida* strain KL33 (AY686638) γ-proteobacteria

D	1D-B	*Agrobacterium* sp. PB (AF482682) α-proteobacteria
E	3D-B	*Agrobacterium tumefaciens* TJ6 (AF508094) α-proteobacteria
F	15D-C	*Brevundimonas* sp. FWC43 (AJ227798) α-proteobacteria
G	6N-B; 6N-C	*Bacillus* sp. NK13 (AY654897) Firmicutes
H	1N-B ;17N-C	*Achromobacter xylosoxidans* subsp. *Xylososxidans* (AF225979) β-proteobacteria
I	14D-C	*Bacillus firmus* D1 (AJ491843) Firmicutes
L	1P-C; 2P-C; 3P-C; 4P-C	*Azospirillum* sp. TS6 (AB114139) α-proteobacteria
M	5N-B; 9N-B; 10N-B; 5P-C	*Stenotrophomonas maltophilia* VUN10,035 (AF100735) γ-proteobacteria
N	2N-B; 3N-B; 4N-B; 8N-B; 13N-C	*Achromobacter xylosoxidans* F (AJ491845) β-proteobacteria
O	6D-B; 8D-B	*Pseudomonas putida* (U70977) γ-proteobacteria
P	1P-B; 2P-B	*Staphylococcus* sp. S5-94 (X86648) Firmicutes
Q	4D750-C; 4P750-C	Uncultured *Pseudomonas* sp. AMS1clone (AY635892) γ-proteobacteria
R	5P750-B	*Pseudomonas oryzihabitans* HAMBI2386 (AF501362) γ-proteobacteria
S	4P-B	*Brevundimonas vesicularis* (AY169433) α-proteobacteria
T	5P-B	*Brevundimonas vesicularis* LMG (AJ227781) α-proteobacteria
U	6D750-B	*Achromobacter xylosoxidans* H (AJ4918406) β-proteobacteria
[a]: The percentage of homology between the query and the subject sequences ranged from 98 to 100%		

Although a good number of bacterial strains have been recovered, it was not possible to find out a clear correspondence between any of their V3 16SrRNA-PCR products and the major bands visualised in the total communities DGGE profiles. It was probably due to the poor resolution of the complex bacterial communities onto the denaturing gel. Nevertheless, it was also possible that the dominant bacterial populations selected by polluted soil conditions not included the isolated PAHs-utilising bacteria. However, the dominant bands resolved on DGGE gels may correspond to hard- or un- cultivable bacterial species.

3.1.3.3 PAHs-transforming genotype characterisation

The strains selected for taxonomic analysis were further characterised in relation to the PAHs-transforming genotype. According to this aim, the amplification of the genes encoding the α subunit of the dioxygenases involved in the first step of PAHs aerobic catabolism was attempted and the results were listened in the Table 16. Concerning the *nah* genotype, PCR products of the expected length (992

bp), using the nahAcfor/nahAcrev primer set (Table 5), were gained only within the *Pseudomonas* genus (OTU1, OTUA, OTUB1, OTUB2, OTUB3). Indeed, a 1200bp non-specific amplification fragment was produced with the OTUI (*Bacillus* sp.) reference strain, while no amplifications were obtained with genomic DNA of strains belonging to other genera. Nevertheless, non-specific (850 and 1500 bp) and no amplification products were also obtained from genomic DNA of the remaining *Pseudomonas* reference strains (OTUO and OTUC, OTUQ, OTUR respectively). The presence of *nah*-like catabolic capability in the studied soil bacterial communities was just assessed by direct amplification of soil DNA extracts. Nonetheless, the genetic characterisation of the isolates showed the positive correlation between *nah* genotype and the *Pseudomonas* genus. This evidence was expected since the *nah* genotype was extensively characterised in environmental strains belonging to that genus [Simon et al., 1993; Kiyohara et al., 1994; Takizawa et al., 1994; Denome et al., 1993]. In order to better investigate the diversity inside the *nahAc* amplicons, recovered from the genomic DNA of the OTU1, OTUA, OTUB1, OTUB2, OTUB3 reference strains, a *Hae*III restriction analysis was performed. The obtained digestion profiles were compared to those gained (by nahAcfor/nahAcrev primers amplification and *Hae*III digestion) from *P*. sp. AN10 and *P*. sp. C18 genomic DNA. The analysis of the profiles permitted to distinguish the OTUA from the remaining OTUs. In fact, the first showed an AN10-like restriction profile and the last a C18-like restriction patterns.

On the other hand, no amplification products were obtained using primers for *phn* and *dbt*-genotypes, from any of the selected isolates, even though, at least the *dbt* genotype presence, in the adapted communities, has just been assessed by direct amplification of the soil DNA extracts. A similar result was already been reported in literature [Lloyd-Jones et al., 1999], and it was probably due to the hard-/non- cultivability of bacterial strains harbouring these genotypes or, concerning the *phn*-genotype, to the poverty of this genotype in the matrix.

In order to provide alternative tools to identify the genes encoding the oxygenase system, harboured by the remaining un-characterised isolates, different primer sets, with specific as well as degenerated sequences, were also used (COM1for/COM1rev; RHOfor/RHOrev; DO-1s/DO-1a; RieskeF/RieskeR; AJ025/AJ026) (Table 5). No amplification products were obtained using the COM1for/COM1rev, RHOfor/RHOrev, and DO-1s/DO-1a primer pairs, from all genomic DNA. On the contrary, some results could be achieved by using RieskeF/RieskeR and AJ025/AJ026. Nevertheless, using RieskeF/RieskeR primers, fragments of the expected size (78 bp) were produced exclusively from genomic DNA of the nahAcfor/nahAcrev-positive *Pseudomonas* strains (OTU1, OTUA, OTUB1, OTUB2, OTUB3) (Table 17). Moreover, by using the AJ025/AJ026 primer set, no more than multiple amplification products were obtained from every selected strains, instead of the expected one (434bp). However, even if the OTUC and OTUO reference isolates gave the same non-specific amplification products (700 and 200 bp),

these PCR fragments positively hybridised with the nahAc probe, under non-stringency conditions. The future cloning and sequencing of the OTUC, OTUO AJ025/AJ026-PCR products will allow to confirm the nature of these PCR fragments and to characterise a new potentially transforming genotype. In conclusion, to date, although the presence of *nah*-genotype harbouring bacteria was assessed through both culture independent and dependent approaches, the presence of *dbt*-genotype was assessed by culture independent method and some information will be achieve about the PAHs-transforming genotype of OTUC and OTUO, the 73% of the recovered OTUs host strains with un-characterised PAHs-oxygenase system. The evidence that over 50% of the PAH-degrading isolates remains unclassified has already been reported in literature [Lloyd-Jones et al., 1999]. In fact, these strains may harbour either divergent homologues of the targeted genes, or alternatively, unrelated and uncharacterised genes for PAHs catabolism. Therefore, the failure in the characterisation of the main portion of the functional genes involved in the PAHs utilisation suggests that, at present, we have a poor grasp of the available diversity of PAH catabolic genes. Nevertheless, the future extensive genetic characterisation of the isolated strains (through genomic library construction [Krivobok et al., 2003; Kasai et al., 2003] or by insertional mutagenesis [II]) may permit to increase our knowledge about the diversity within the PAHs-transforming genes. Moreover, the widening of the sequences data available in on-line databases, will allow to develop new primer pairs and probe to use, either in microbiological or in molecular biological approaches, for the further investigations of the intrinsic biodegradation potential of the matrix.

Table 17. PAHs-transforming genotype characterisation

Genera	OTU	PCR using[A]			Hybridisation with nahAc probe[B]		
		nahAcfor/ nahAcrev	RieskeF/ RieskeR	AJ025/AJ026	nahAcfor/ nahAcrev	RieskeF/ RieskeR	AJ025/ AJ026
Pseudomonas	OTU1, OTUA, OTUB1, OTUB2, OTUB3	+	+	m	+	+	m
	OTUO	c	-	c	-	-	+
	OTUC	-	-	c	-	-	+
	OTUR, OTUQ	-	-	m	-	-	m
Other genera	OTUI	c	-	m	-	-	m
	OTU2, OTU3, OTU4, OTU5, OTUD, OTUE, OTUF, OTUG, OTUH, OTUL, OTUM, OTUN, OTUP, OTUS, OTUT, OTUU	-	-	m	-	-	m

[A]: +, PCR product of expected length; -, no PCR product
[B]: +, Hybridisation signal with nahAc probe; -, no hybridisation signal with nahAc probe
c: one/two amplification fragment of incorrect length; m: multiple PCR products or multiple Southern Hybridisation with nahAc probe

In the Figure 5 was graphically summarised the results obtained by the taxonomic and the PAHs-transforming genotype characterisation of the recovered Operational Taxonomic Units (OTUs) from the three studied soil aliquots.

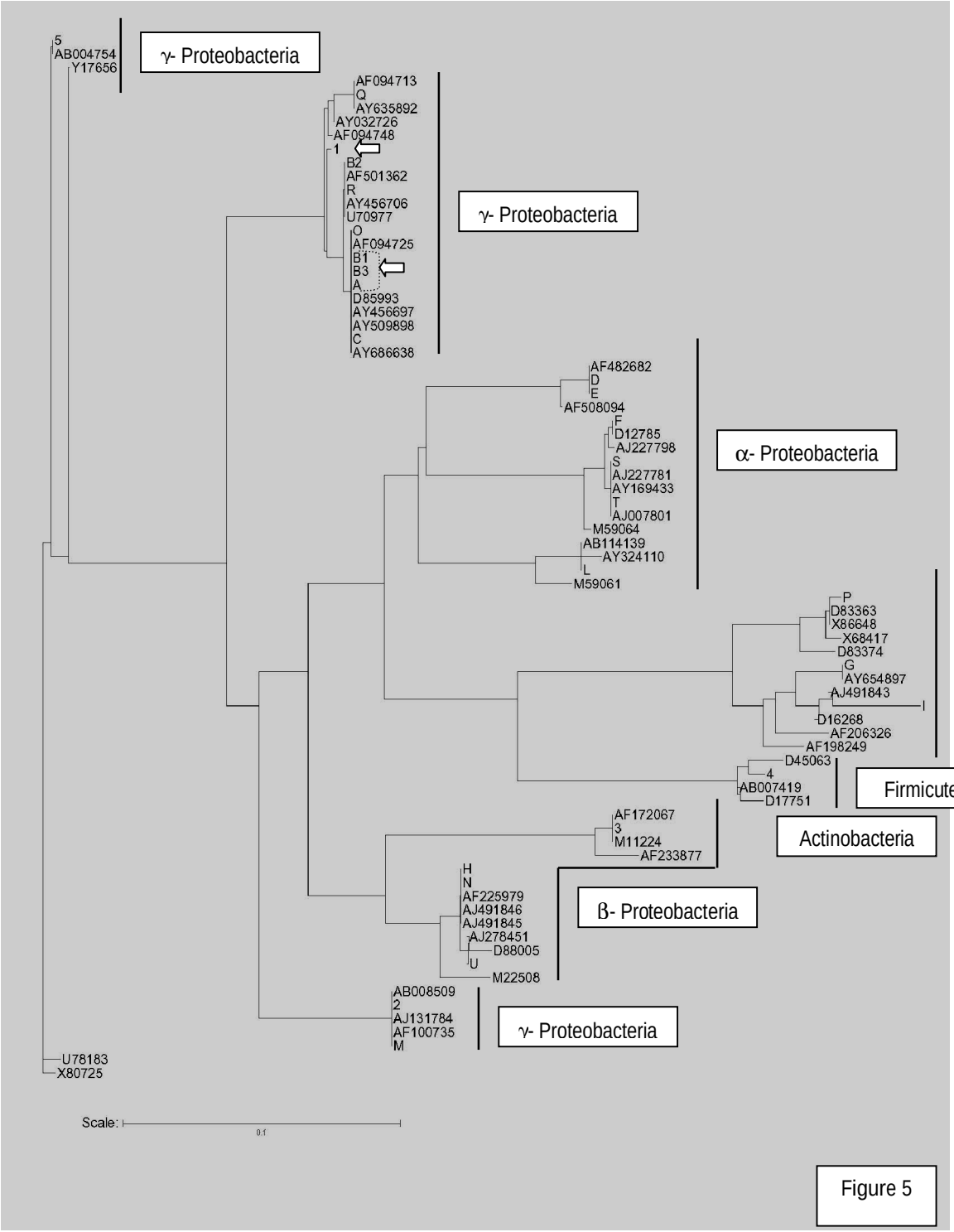


Figure 5. Tree graphical representation of the taxonomic correlation among the 26 OTUs (1, 2, 3, 4, 5, A, B1, B2, B3, C, D, E, F, G, H, I, L, M, N, O, P, Q, R, S, T, U) recovered from the soil aliquots A, 200 mg 16US-EPA PAHs/ kg dry soil; B, 400 mg 16US-EPA PAHs/ kg dry soil; C, 14,000 mg 16US-EPA PAHs/ kg dry soil. The white arrows indicate the OTUs that hosts *nah*-genotype harbouring bacteria. Related and type species reference strains: *Alcaligenes xylosoxidans* strain H (AJ491846), *Pseudomonas aeruginosa* ATCC 10145 (AF094713), *Pseudomonas stutzeri* ATCC 17588 (AF094748), *Pseudomonas fluorescens* ATCC 13525 (AF094725), *Achromobacter xylosoxidans* subsp. *denitrificans* DSM 30026 (AJ278451), *Bacillus firmus* IAM 12464 (D16268), *Bacillus subtilis* LN002 (AF198249), *Bacillus cereus* (AF206326),

Staphylococcus aureus ATCC 12600 (X68417), *Staphylococcus xylosus* ATCC 29971T (D83374), *Staphylococcus epidermidis* ATCC 14990T (D83363), *Brevundimonas diminuta* (M59064), *Brevundimonas vesicularis* ATCC 11426 (AJ007801), *Mycoplana bullata* IAM 13153 (D12785), *Stenotrophomonas maltophilia* ATCC 13637T (AB008509), *Azospirillum brasilense* ATCC 29145 (AY324110), *Azospirillum lipoferum* (M59061), *Alcaligenes faecalis* (M22508), *Achromobacter xylosoxidans* subsp. *xylosoxidans* IAM12684 (D88005), *Comamonas testosteroni* (M11224); *Comamonas denitrificans* (AF233877); *Klebsiella oxytoca* (T) JCM1665 (AB004754); *Klebsiella pneumoniae* (T) ATCC13883T(Y17656); *Leucobacter komagatae* (T) JCM 9414 (D45063); *Leucobacter komagatae* (T) IFO15245T (D17751); *Pseudomonas* sp. HR 26 (Y032726); *Stenotrophomonas maltophilia* LMG 10877 (AJ131784); *Comamonas testosteroni* NCIMB 9872 (AF172067); *Leucobacter komagatae*, (AB007419); *Klebsiella oxytoca* ATCC 13182T (U78183). Sequence used as outgroup: *Escherichia coli* ATCC 11775T (X80725).

3.2 PHYTOREMEDIATION ASSAY

The initial microbial characterisation of the polluted matrix coming from the ex-Carbochimica area has permitted to assess the presence of a selected PAHs-transforming population within the adapted bacterial community. In this second phase of our research, a bioremediation laboratory-scale assay (Phytoremediation assay) was set up (Table 2). Our intents were both to enhance the catabolic potential of the autochthonous transforming bacteria, in order to promote the reclaim of the industrial soil, and above all to characterise the modifications occurred to the soil bacterial community as a result of the performed treatment. Therefore, culture independent and culture dependent approaches were adopted to investigate the impact of two plant species, *Lupinus albus* var. Multitalia and *Zea mays* var. Matisse on the bacterial community settled down in the high contaminated soil aliquot C (14,000 mg 16 US-EPA PAHs/kg of dry soil)

3.2.1 PAHS-TRANSFORMATION IN MICROCOSMS

The phytoremediation treatment, by using *Lupinus albus,* successfully decreased the PAHs concentration in the bulk soil (soil aliquot C) after 10 weeks. In fact, at the end of the experiment, the total extractable PAHs decreased to 68.3% of the initial content while in the non-vegetated microcosms decreased only to 81.6% (Table 18). On the other hand, in the *Zea mays* rhizosphere the pollutants content decrease only to 78.0% of the starting amount.

Table 18. Concentration decrease (mg/kg) of the 16 US-EPA PAHs at the end of Phytoremediation assay.

PAHs	Soil aliquot C (*t0*)	Microcosms		
		K	L	M
naphthalene	163	143	116	101
acenaphthylene	38	36	31	33
acenaphthene	94	75	74	73
fluorene	126	126	85	86
phenanthrene	1459	1004	856	1139
anthracene	359	380	204	200
fluoranthene	2392	1708	1688	1942
pyrene	1845	1646	1293	1556
benzo(a)anthracene	942	875	799	664
chrysene	1574	1211	1064	1402
bezo(b)fluoranthene	1133	1106	988	1119
bezo(k)fluoranthene	1352	626	478	421
bezo(a)pyrene	726	704	498	537
indeno[1,2,3c,d]pyrene	775	728	541	661
Dibenzo[a,h]anthracene	128	173	82	116
benzo[g,h,i]perylene	744	732	669	717
TOTAL	13850	11273	9466	10767

K: soil microcosms non vegetated after 10 weeks; L: soil microcosms vegetated with *Lupinus albus* after 10 weeks; M: soil microcosms vegetated with *Zea mays* after 10 weeks

However, we can observe that the PAHs decrease concerned mainly fluorene (32.5% and 31.7%), anthracene (43.2% and 44.3%), benzo(k)fluoranthene (64.6% and 68.8%) and bezo(a)pyrene (31.4%

and 26.0%) for both *Lupinus albus* and *Zea mays* vegetated microcosms. Nevertheless, phenanthrene (41.3% *vs.* 21.9%), chrysene (32.4% *vs.* 10.9%), pyrene (29.9% *vs.* 15.7%) and indeno[1,2,3c,d]pyrene (30.2% *vs*. 14.7%) decreased more in *Lupinus albus* rhizosphere than in *Zea mays* one (Figure 6). On the other hand, benzo(a)anthracene (29.5%) concentration was reduced more in *Zea mays* root system (Figure 6).

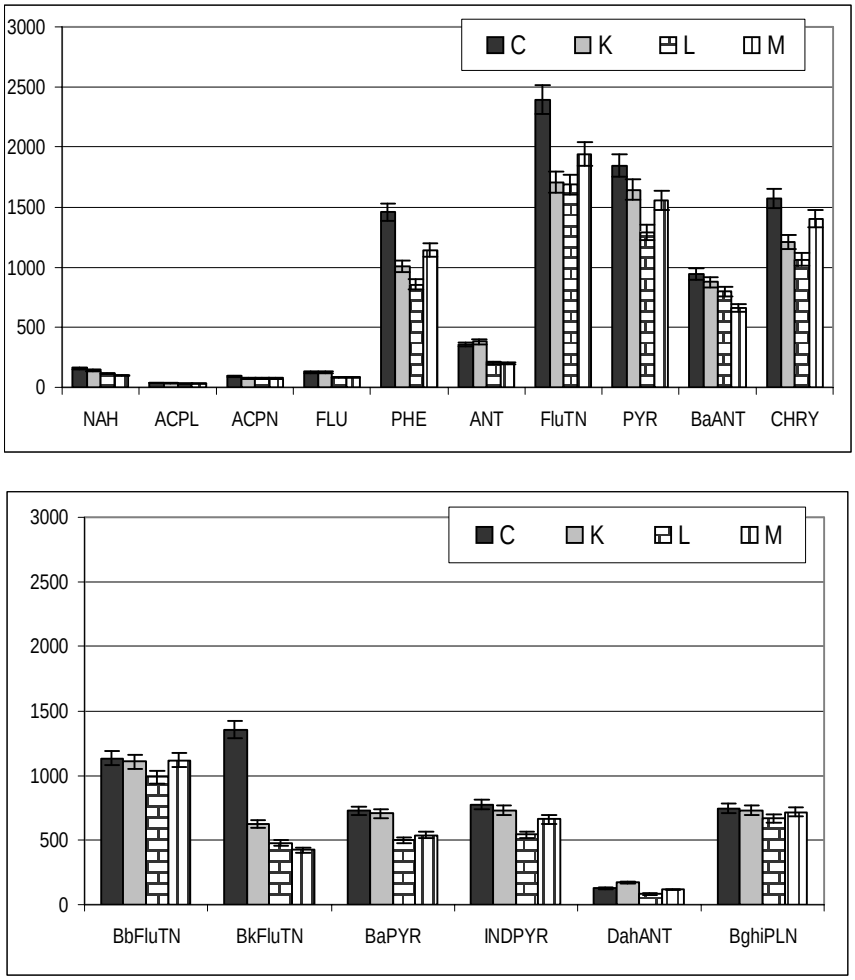


Figure 6. 16-priority PAHs concentration decrease 10 weeks from the microcosms set up. On the x-axis: 16 US-EPA PAHs. On the y-axis: remaining concentration (mg/kg) of PAHs in the soil. C: soil aliquot C non-vegetate at the beginning of the experimentation; K: soil aliquot C non-vegetate at the end of the experimentation; L: soil aliquot C vegetated with *Lupinus albus;* M: soil aliquot C vegetated with *Zea mays*. NAH: naphthalene, ACPL: acenaphtylene; ACPN: acenaphthene; FLU: fluorene; PHE: phenanthrene; ANT: anthracene; FluTN: fluoranthene; PYR: pyrene; BaANT: benzo(a)antracene; CHRY: chrysene; BbFluTN: bezo(b)fluoranthene; BkFluTN: bezo(k)fluoranthene; BaPYR: bezo(a)pyrene; INDPYR: indeno[1,2,3c,d]pyrene; DahANT: Dibenzo[a,h]anthracene; BghiPLN: benzo[g,h,i]perylene

3.2.2 COLTURE DEPENDENT CHARACTERISATION: ENUMERATION OF CULTURABLE BACTERIAL STRAINS AND PAHs-TRANSFORMING BACTERIA IN VEGETATED MICROCOSMS

It has been extensively reported in literature, that the first plant contribution to the organic contaminant removal was the increasing of the microbial biomass and activity [Anderson et al., 1993; Lynch 1990]. Supporting this hypothesis the population levels of contaminant-degrading bacteria and the potential of soil to degrade contaminants typically increases during phytoremediation [Nichols et al., 1997; Radwan et al., 1998]. Therefore, the initial purpose of current investigation was to estimate, through the MPN enumerations, the extent of the impact of *Lupinus albus* and *Zea mays* rhizospheres on the increasing of culturable bacterial biomasses. In particular, we were interested in the evaluation of rizospheric effects on PAHs-utilizing bacteria.

Table 19. PAHs-transforming bacteria and Total heterotrophic MPN enumeration

Soil microcosms [a]	Total heterotrophs MPN/g of dry soil	95% Confidence limits	PAHs-transforming bacteria MPN/g of dry soil	95% Confidence limits	PAHs-transforming bacteria/total heterotrophs (%)
C *(t0)*	$6.60X10^5$	$3.55X\ 10^4$-$1.50X\ 10^6$	$1.43X10^5$	$4.41X\ 10^4$-$2.85X\ 10^5$	21.7
K T10	$1.17x10^5$	$2.98x10^4$-$2.37x10^5$	$4.5x10^4$	$2.29x10^4$-$9.93x10^4$	38.5
L T10	$1.19x10^7$	$6.03x10^6$-$2.60x10^7$	$6.45x10^5$	$3.47x10^5$-$1.47x10^6$	5.4
M T10	$5.76x10^6$	$3.07x10^6$-$1.37x10^7$	$5.26x10^5$	$2.78x10^5$-$1.24x10^6$	9.1
Z-value (MPN_{KT0}-MPN_{KT10}): 1.60 [b]			Z-value (MPN_{KT0}-MPN_{KT10}): 1.06 [b]		
Z-value (MPN_L-MPN_{KT10}): 4.28 [b]			Z-value (MPN_L-MPN_{KT10}): 2.43 [b]		
Z-value (MPN_M-MPN_{KT10}): 3.60 [b]			Z-value (MPN_M-MPN_{KT10}): 2.23 [b]		
Z-value (MPN_L-MPN_M): 0.68 [b]			Z-value (MPN_L-MPN_M): 0.19 [b]		

[a] C *(t0)*: soil aliquot C non vegetated at T0; K T10: control non vegetated at T10; L T10: soil vegetated with *L. albus* at T10; M T10: soil vegetated with *Z.mays* at T10

[b]: Critical Z-value at 95% level=1.96. MPN-estimates with calculated Z-values smaller then 1.96 are not significantly different at 95% level.

The results obtained evidenced that the number of the total microflora and of the PAHs-transforming bacteria in the *Lupinus albus* and *Zea mays* root systems were not significantly different (Table 19). Therefore, the effects of the two different plant species were comparable. Moreover, the presence of the two rhizosphere systems significantly increased, as expected, the cultivable biomasses both of the heterotrophs and of the PAHs-utilising bacteria respect to those estimated in the non vegetate treatments (Table 19). Nevertheless, while the total heterotrophs number increased by 22.6 times (average value) from bulk soil to root systems conditions, on the contrary the PAHs-utilizing bacteria MPN values increased only by 6.2 times (average value). Therefore, although population levels of contaminant-degrading bacteria increase, the fraction of the total heterotrophs able to transform PAHs in rhizospheric soils was lower than that in the non-rhizospheric ones (Table 19). This evidence suggested that the introduced plant species increased the total biomass, but not specifically the PAHs-utilizing bacteria. Moreover, the obtained data, combined with the previous reported ones (3.1.1 section) led to conclude that the PAHs-transforming bacteria were mainly stimulated by the presence of high

PAHs concentrations rather than by the presence of a root system. However, despite the narrow augmentation of the PAHs-utilizing biomass, the presence of the selected plant species positively contributed to the contaminants-transformation activity in the arranged microcosms (3.2.1 section).

3.2.3 CULTURE INDEPENDENT CHARACTERISATION: PCR-BASED EVALUATION OF TOTAL BACTERIAL COMMUNITY AND PAHs TRANSFORMING BACTERIA IN VEGETATED MICROCOSMS

In order to better characterise the modification occurred in bacterial community of the soil aliquot C, because of the introduction of the two selected plant species, nucleic-acids-based approaches were once again adopted. The average DNA quantity recovered from the vegetated soil samples by the utilised extraction kit was 33.7 ± 3.5 μg DNA/g of dry soil. Considering 1 bp as 649 Da [Sambrook et al. 1989], and an average soil bacteria genome of 5 Mb [Lloyd-Jones and Hunter 2001], we should have obtained an average starting microbial biomass investigable by PCR-based approaches valuable as 6.19 x 10^9 genome equivalents/g of dry soil.

3.2.3.1 Analysis of bacterial communities by PCR-Denaturing Gradient Gel Electrophoresis (DGGE)

The results obtained by the performed V3-16SrRNA-PCR-DGGE analysis (Figure 7), confirm the previous finding [Anderson et al., 1993; Shann and Boyle, 1994] that the presence of plant species modify the structure of the acclimatised bulk bacterial community. In particular, the presence of the two root systems seems able to change, in its own way, the relative abundance of the dominant species in the adapted bacterial community. Therefore, in the *Zea mays* corresponding profile (M) two bacterial species seem to prevail (bands 2 and 3) among a great number of weaker ones. On the other hand, the *Lupinus albus* DGGE profile (L) was clearly represented by the bands 2 and 3 and also by the band 1. Moreover, in the L profile can be also recovered a band (band 4) which was not so enriched in the other treatments. The sequences analysis of the bands 1 and 2 in the vegetated profiles confirms the data obtained for the corresponding ones in the bulk profiles (section 3.1.2.1) (Table 20). On the other hand the remainder of the sequences obtained from the bands 3 and 4 showed great homology to two uncultured bacteria (Table 20). No information can be recovered for the band 3 related bacteria species, and only few ones were available for the band 4 corresponding strain. However, concerning to the latter band, it corresponds to an actinobacterium environmental clone recovered from a metal contaminated

soil [Stach et al., 2003]. Actually, only the bands 1 and 2 seem to be clearly associated to an organic contaminated scenario and however they persist in the *L. albus* rhizosphere.

Table 20. Taxonomic chacactrerisation of cloned bands from DGGE profiles

Scrapped bands	Organism showing greatest similarity	% Homology
1	*Cytophaga* sp. type 0092 (X85210)	99%
2	*Acinetobacterium* sp.RG-73 (AY561633)	100%
3	Uncultured bacterium WkB15 (AF257865)	100%
4	Uncultured actinobacterium (AY124390)	100%

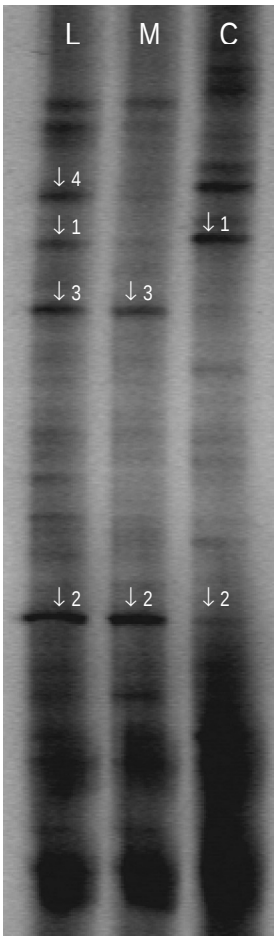


Figure 7. DGGE profiles. Lane C, M, L, 1.5 μg of PCR amplification products (p3/p2) of V3 region 16S rDNA of the bacterial community soil aliquot C (14,000 mg/kg 16 USA EPA PAHs), M (microcosms vegetated with *Z. mays*), and L (microcosms vegetated with *L. albus*), respectively. Arrows 1, 2, 3 and 4, scrapped bands.

3.2.3.2 PCR based evaluation of PAHs-transforming capacity in the bacterial communities

The performed DGGE analysis allows suggesting that the presence of two root systems was able to modify the relative abundance of several bacterial species within the studied polluted matrix. However, we were interested in determining if the phytoremediation treatments stimulated specific functional genotypes harbouring bacteria inside the bacterial community. Therefore, an initial PCR-based evaluation was performed. PCRs designed to amplify the *nah* genotypes gave amplification products of the expected length from bulk soil DNA as well as from both rhizosphere DNA samples (Table 21). PCRs performed to recovered *phn* genotype once again failed to give same products from all soil DNA extracts. On the other hand, reactions designed to amplify *dbt* genotype gave no or not reproducible amplification products from *Z. mays* rhizospheric and bulk soil DNA respectively, nevertheless it produced a weak amplification fragment with the correct length from *L. albus* rhizosphere DNA samples.

Table 21. PAHs-transforming genotype soil characterisation using MasterTaq (Eppendorf) as reaction enzyme			
Soil microcosms[a]	PCR using[b]		
	nahAcfor/nahAcrev [c] (*nah* genotype)	P9047/P8073 (*phn* genotype)	P20/P54 [d] (*dbt* genotype)
K	+	-	+/-
M	+	-	-
L	+	-	+

[a]: K : control non vegetated at T10; L : microcosms soil vegetated with *L. albus* at T10; M : microcosms soil vegetated with *Z.mays* at T10

[b]: +, PCR product of expected length; +/- no reproducible PCR product. All PCRs were performed twice on three replicates of the same environmental DNA

[c]: All products were verified trough Southern hybridisation with nahAc probe generated by PCR amplification with *P. putida* ATCC17484

[d]: All products were verified trough Southern hybridisation with dbtAc probe generated by PCR amplification with *B.* sp. strain DBT1

The reproducibility of the P20/P54 product from the *L. albus* rhizosphere DNA extracts was probably due to the increasing, over the PCR detection limit, of the *dbtAc* gene copy number in the soil DNA samples. This result may suggest that the presence of *Lupinus albus* rhizosphere enriches the *dbt* harbouring bacteria within the bulk community of the high-contaminated C aliquot. The positive P20/P54-PCR products were re-amplified with dbtAcforGC/dbtAcrev and then electrophoretically separated by DGGE (data not shown). The fragment amplified from the vegetated-polluted soil sample resembled that generated with *Burkholderia* sp. strain DBT1, as well as the ones obtained from aliquot A and B (section 3.1.2.2). Once again the nature of the *dbt* genotype in the studied matrix seemed to be substantially conserved.

3.2.4 ISOLATION OF PAHs-TRANSFORMING BACTERIAL STRAINS FROM VEGETATED MICROCOSMS

The isolation of the PAHs-utilizing bacteria from the root systems of the two selected plant species (using the same method adopted for the bacteria isolation from the non vegetate soil) permits to evaluate the impact of the vegetation on the previously assessed taxonomic structure of the culturable PAHs-transforming bacteria (3.1.3 section). Moreover, it is interesting to verify the possibility to isolate *dbt* genotype harbouring bacteria from *L. albus* rhizosphere, because of the suggested enrichment of this functional genotype by this root system (3.2.2.2 section). Finally, PAHs-utilizing bacteria acclimatised in a root system, may be further utilised as appropriate bacterial inoculum in plant assisted bioremediation protocols.

3.2.4.1 Biochemical characterisation of bacterial strains isolated from rhizospheres of *Zea mays* and *Lupinus albus*

Therefore, the isolation of the PAHs-transforming strains from the two rhizospheres was performed by selective enrichment cultures using naphthalene phenanthrene and dibenzothiophene and the combination of the three PAHs as carbon source. A total of 18 and 21 PAHs-transforming isolates were recovered from the soil rhizosphere of *Zea mays* and *Lupinus albus* respectively. Carbon utilisation and indole transformation of the isolates as well as the biosurfatants production were listed in the Table 22, 23, 24 and 25.

Table 22. Biochemical characterisation of bacteria isolated from *Zea mays* rhizosphere

Bacterial isolates	Dioxygenase test	Growth on[a]		
		Nah	Phn	DBT
M3A-C	-	+	-	-
M2A-C	-	+	-	+
M2-C; M6-C; M0A-C; M1A-C; M4A-C; M6A-C; M7A-C; M1ATT-C; M2ATT-C; M4ATT-C	-	+	+	+
M1-C; M4-C; M7-C; M8-C; M5A-C; M8A-C	+	+	+	+

[a]: Nah, naphthalene; Phn, phenanthrene; DBT, dibenzotiophene

Table 23. Biochemical characterisation of bacteria isolated from *Lupinus albus* rhizosphere

Bacterial isolates	Dioxygenase test	Growth on[a]		
		Nah	Phn	DBT
L3A-C; L4ATT-C	-	+	-	-
L1-C; L3-C; L4C; L0A-C; L1A-C; L2A-C; L4A-C; L5A-C; L6A-C; L7A-C; L8A-C; L9A-C; L10A-C; L11A-C; L12A-C; L1ATT-C; L7ATT-C; L11ATT-C	-	+	+	+
L2-C	+	+	+	+

[a]: Nah, naphthalene; Phn, phenanthrene; DBT, dibenzotiophene

Table 24. Production of extracellular biosurfactant by bacteria isolated from *Zea mays* rhizosphere

Bacterial strains	Biosurfanctant production growing on		
	Dextrose	Mannitol	Nah/Phe/DBT
M8A-C; M6A-C; M2-C; M1-C; M4A-C	++++	+++	++
M6-C	+++	++	++
M5A-C	+	+/-	+

M4-C	ng	+	+
M7-C	ng	+	-
M8-C	ng	+	-
M1ATT-C; M2ATT-C; M4ATT-C; M3A-C; M7A-C	ng	ng	-
+: dark blue halos production; +/-: weak blue halos production; -: no blue halos production; ng: no growth detected			

Table 25. Production of extracellular biosurfactant by bacteria isolated from *Lupinus albus* rhizosphere

Bacterial strains	Biosurfanctant production growing on		
	Dextrose	Mannitol	Nah/Phe/DBT
L0A-C; L2-C	++++	+++	++
L8A-C	+	+	+
L7A-C; L9A-C	+	+/-	+
L1-C	+/-	+	+
L4-C; L5A-C; L12A-C	+/-	+/-	+/-
L4ATT-C; L7ATT-C	+/-	ng	+/-
L3-C; L1ATT-C; L11ATT-C	+/-	-	-
L4A-C	-	-	-
L1A-C; L2A-C; L6A-C; L10A-C	ng	-	-
L3A-C; L11A-C	ng	ng	-
+: dark blue halos production; +/-: weak blue halos production; -: no blue halos production; ng: no growth detected			

Based on carbon source utilisation, the 43 strains isolated from high-polluted non-vegetated soil have been grouped into three catabolic sets (naphthalene/ phenanthrene/ dibenzothiophene; phenanthrene/ dibenzotiophene and phenanthrene) (3.1.3.1 section). Conversely, the 18 bacteria isolated from *Z. mays* rhizosphere were grouped into three different catabolic sets and the 21 strains isolated from *L. albus* rhizosphere into other two ones. In particular, in the first case, naphthalene/ phenanthrene/ dibenzothiophene; naphthalene/ dibenzothiophene as well as naphthalene transforming bacteria were recovered. On the other hand, in *Lupinus albus* root system were recruited only naphthalene/ phenanthrene/ dibenzothiophene and naphthalene transforming bacteria. It is interesting to note that, in vegetated conditions, strains harbouring peculiar phenanthrene catabolic capacity (such as phenanthrene/ dibenzothiophene or phenanthrene transforming bacteria) were not isolated, moreover, strains unable to transform both phenanthrene and dibenzothiophene were also recovered. However, the high percentage of the isolates from bulk soil and from the two plant rhizospheres was able to transform all the tested PAHs (86.0%, 88.9%, 90.5% bulk, *Z. mays*, *L. albus* in that order). Concerning the aromatic ring oxygenase activity, the performed tests showed that the greatest portion of the isolates was able to transform PAHs without converting indole to indigo (66.7% *Z. mays* and 95.2% *L. albus*). In

particular, 10/16 (from *Z. mays*) and 18/19 (from *L. albus*) of the strains, able to use all tested PAHs, did not show classical oxygenase activity. The recovered strains were also analysed for the production of biosurfactants. The 41.9% of the bulk soil isolates were able to synthesize surfactants (3.1.3.1 section), *versus* the 61.1% and the 28.6% ones in the *Z. mays* and in the *L. albus* rhizospheres.

3.2.4.2 Taxonomic characterisation of the bacterial strains isolated from rizospheres of *Zea mays* and *Lupinus albus*

The results obtained by ARDRA permitted to distinguish the 39 isolates in 16 Operational Taxonomic Units (in detail, 10 OTUs from *Z. mays*, and 11 OTUs from *L.albus*). Every OTU was obtained combining three restriction profiles (*Hha*I, *Hae*III and *Alu*I). In Figure 8, was graphically represented the distribution of the isolates, recovered from the vegetated microcosms, in the 16 OTUs, compared with the sharing of the 43 isolates, recovered from the bulk aliquot C, in 12 OTUs (3.1.3.2 section). The figure clearly shows that the isolates recovered from the vegetated soil and those recovered from the bulk soil were spread into completely different OTUs (except OTUA).

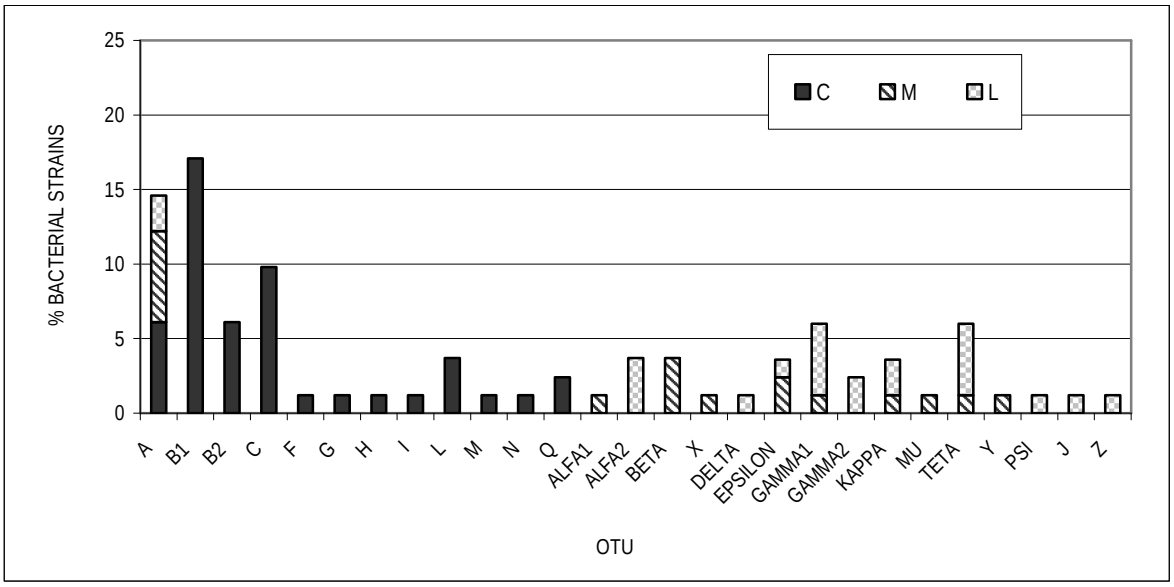


Figure 8. Graphical representation of isolates distribution inside the OTUs. C: Non-vegetated aliquot C, 14,000 mg 16 USA EPA PAHs per kg dry soil; M: microcosms vegetated with *Zea mays*; L: microcosms vegetated with *Lupinus albus*. 100% Bacterial isolates: 82 strains.

Furthermore, one isolate for each OTU was selected for subsequent taxonomic analyses. The sequencing of the corresponding 16S rRNA genes indicated that the bacterial strains, isolated from the two rhizospheres systems, were also distributed into different and more heterogeneous genera than bulk ones (Table 26).

Table 26. Taxonomic characterisation of bacteria isolated from *Zea mays* and *Lupinus albus* rhizospheres		
OTU	Bacterial Isolates	Organism showing greatest similarity[a]
A	L0A-C; L2-C; M8A-C; M6A-C; M2-C; M1-C; M4A-C	*Pseudomonas* sp. KD (AY456697) γ-proteobacteria
ALFA 1	M6-C	*Pseudomonas* sp. SCD-7 (AF448041) γ-proteobacteria
ALFA2	L1ATT-C; L3-C; L1ATT-C	*Bacillus* sp. A4 (AY489119) Firmicutes
BETA	M1ATT-C; M2ATT-C ; M4ATT-C	*Streptomyces* sp. LK1312.4 (AY465295) Actinobacteria
X	M4-C	*Brevundimonas* sp. DW114b, isolate DW114b (AJ534862) α-proteobacteria
DELTA	L11A-C	*Promicromonospora sukumoe* strain type DSM 44121 (AJ272024) Actinobacteria
EPSILON	L3A-C; M3A-C; M7A-C	*Streptomyces setonii* (D63872) Actinobacteria
GAMMA1	L4-C; L12A-C; L5A-C; M1A-C; M2A-C	*Arthrobacter* sp. 108 (AY238501) Actinobacteria
GAMMA2	L7ATT-C; L4ATT-C	*Arthrobacter* sp. pfB10 (AY336539) Actinobacteria
KAPPA	L7A-C; L9A-C; M5A-C	*Rhodococcus* sp. A2Y26 (AY512637) Actinobacteria
MU	M8-C	*Cellulosimicrobium funkei* strain CDC#JB2769ot (AY523788) Actinobacteria
TETA	L6A-C; L10A-C; L2A-C; L8A-C; M0A-C	*Bacillus* sp. PAMU-1.13 (AB118223) Firmicutes
Y	M7-C	*Stenotrophomonas* sp. S1 (AY179327) γ-proteobacteria
PSI	L1A-C	*Staphylococcus* sp. strain AG-30 (X86641) Firmicutes
J	L4A-C	*Staphylococcus* sp. MO28 (AY553115) Firmicutes
Z	L1C-C	*Arthrobacter* sp. Amico3 (AY512634) Actinobacteria
[a]: The percentage of homology between the query and the subject sequences ranged from 98 to 100%		

By the performed analysis, it was possible to share out the 18 strains isolated from the *Z. mays* rhizosphere in Proteobacteria [α- (5.6%) and γ- (38.9%)], Firmicutes (5.6%) and Actinobacteria (50%). In the same way, the 21 strains recovered from the *L. albus* root system can be grouped in γ-Proteobacteria (9.5%), Firmicutes (42.9%) and Actinobacteria (47.6%). On the other hand, in the non-vegetated condition, the isolates have been shared out in Proteobacteria [α- (2.3%) β- (2.3%) and γ- (82.9%)], Firmicutes (4.7%) (3.1.3.2 section). It is clear that the presence of the two root systems determine an enrichment of culturable transforming bacteria belonging to Actinobacteria rather than Proteobacteria, therefore altering the taxonomic structure of the bulk soil PAHs-bacteria populations. Moreover, in presence of *L. albus* rhizosphere, there was also an increasing of bacteria belonging to Firmicutes that caused a more consistent structure modification. Nevertheless, bacteria belonging to the OTUA (*Pseudomonas* sp., γ- Proteobacteria) were recovered in every environmental condition, independently by the contamination level and with or without vegetation (both *L. albus* and *Z. mays*). Moreover, the Proteobacteria still remained widely represented (44.5%) in *Z. mays* rhizosphere. However, *Arthrobacter* sp. (Actinobacteria), *Streptomyces* sp. (Actinobacteria) and *Bacillus sp*. (Firmicutes) were the main represented genera in the vegetated conditions.

Unfortunately, once again, it was not possible to find out a clear correspondence between any of PAHs-transforming isolates V3 16SrRNA-PCR products and the dominant bands visualised in the DGGE profiles of the acclimatised rhizospheres community (data not shown). Nevertheless, it is important to underline that one of the major bands scrapped from both vegetated profiles (band 2), corresponded to a bacterial species belonging to the Actinobacteria phylum and in particular to the *Arthrobacter/Acinetobacterium* group. This species was also represented in the bulk soil profile, but was enriched by the presence of the root systems. This evidence may support the hypothesis of a main involvement of these genera in the PAHs dissipation, in the studied matrix. Nevertheless, while strains belonging to the Actinobacteria phylum were well represent both in *Z. mays* and in *L. albus,* strains fit in Firmicutes seem to be preferentially selected by the *L. albus* rhizosphere presence.

However, strains belonging to the same genera of almost all those isolated in this study were previously characterised in other experiments investigating contaminant degradation [Boonchan et al., 1998; Juhasz et al., 2000; Widada et al., 2002a; Chaineau et al., 1999; Gauthier et al., 2003; Kazunga and Aitken, 2000; Nweke and Okpokwasili, 2003; Zhuang et al., 2003; Kosono et al., 1997; Allen et al., 1997; Di Gennaro et al., 2001; Sutherland et al., 1990; Samanta et al., 1999]. Nevertheless, this was the first report on the capacity of the genera *Promicromonospora* and *Cellulosimicrobium* to transform PAHs. However, these genera were probably preferentially stimulated by the presence of the plant species (*L. albus* for the first and *Z. mays* for the second) rather than the PAHs contamination.

3.2.4.3 PAHs-transforming genotype characterisation

The strains selected for taxonomic analysis were further investigated in relation to the PAHs-transforming genotype. According to this aim, the amplification of the genes encoding the α subunit of the dioxygenase involved in the first step of PAHs aerobic catabolism was attempted (Table 27). Concerning the *nah* genotype, PCR products of the expected length were obtained within the *Pseudomonas* genus (OTUA, OTUALFA1) but also inside the *Brevundimonas* one (OTUX). In this case, the genetic characterisation of the isolates showed the correlation between *nah* genotype and the γ- (*Pseudomonas* genus) and α- (*Brevundimonas* genus) Proteobacteria. This is the first evidence of the presence of a *nah* like functional genotype in the *Brevundimonas* genus. Furthermore, also the other *Brevundimonas* strains isolated from the bulk soil aliquots (3.1.3.3 section) did not harbour a *nah* genotype, on the contrary their oxygenase system remain un-characterised (3.1.3.3 section). A more extensive investigation of the *nah*Ac-PCR products amplified from the genomic DNA of the selected strains was assessed by *Hae*III restriction analysis. The obtained digestion profiles confirmed the presence of an AN10-like restriction pattern in the OTUA representing strain and showed a C18-like

restriction pattern in the OTUALFA1 and OTUX representing strains. On the other hand, no amplification products were obtained using primers for *phn* and *dbt*-genotypes, from any of the selected isolates. The obtained results were analogous to those gained for the isolates recovered from bulk aliquot C. Nevertheless, the direct amplification of the *L. albus* vegetated soil DNA extracts clearly showed, at least, the presence of the *dbt* harbouring bacteria in this root system. Once again, the failure in the recruitment of the *dbt*-genotype carrying strains may be caused by the hard-or non-cultivability of the bacteria that harbour this genotype [Lloyd-Jones et al., 1999]. Furthermore, the performed isolation protocol, by selective enrichment cultures, may not be suitable for the recover of these specific PAHs-utilizing bacteria [Stach and Burns, 2002]. Moreover, in order to extend the characterisation of the oxygenase systems involved in the PAHs utilisation, several other primer sets (RieskeF/RieskeR; AJ025/AJ026, COM1for/COM1rev, RHOfor/RHOrev, DO-1s/DO-1a) were tested. As already evidenced (3.1.3.3 section), the RieskeF/RieskeR primers produced correct amplification fragments only from genomic DNA of nahAcfor/nahAcrev-PCR positive strains, corresponding, in this case, to the OTUA, OUTALFA1 and OTUX representative strains (*Pseudomonas* sp. and Brevundimonas sp.). By using the AJ025/AJ026 primer set multiple amplification products were obtained from every selected strains. Finally, no amplification products were gained using all the other tested primer pairs.

Table 27. PAHs-transforming genotype characterisation

Genera	OTU	PCR using[A]		Hybridisation with nahAc probe[B]	
		nahAcfor/ nahAcrev	RieskeF/ RieskeR	nahAcfor/ nahAcrev	RieskeF/ RieskeR
Pseudomonas	OTUA, OTUALFA1	+	+	+	+
Brevundimonas	OTUX	+	+	+	+
Other Genera	OTU ALFA2, OTU BETA, OUT DELTA, OTU EPSILON, OTU GAMMA1, OTU GAMMA2, OTU KAPPA, OTU MU, OTU TETA, OTU Y, OTU PSI, OTU J, OTU Z	-	-	-	-

[A]: +, PCR product of expected length; -, no PCR product
[B]: +, Hybridisation signal with nahAc probe; -, no hybridisation signal with nahAc probe

In conclusion, the results obtained from the transforming genotype characterisation of the strains isolated in non vegetated condition (aliquot A, B and C) showed that the 19% (5/26) of the recovered OTUs host *nah*-genotype harbouring strains, while the 73% (19/26) of them host strains with un-characterised genotype (3.1.3.3 section). In the same way, in vegetated soil condition the 19% (3/16) of the recovered OTUs host *nah*-genotype harbouring strains, while no data was available concerning the nature of the oxygenase system present in the remaining 75% (13/16) of them. Therefore, by this approach, no information can be achieved in relation to the impact of the two root systems on the possible selection of specific PAHs-transforming genotype, because of the main part of the oxygenase system-encoding genes were not characterised. Indeed, from a microbiological perspective, we are restricted to what we can cultivate, which we have demonstrated includes isolates which host divergent

catabolic genes not represented in the sequence databases. However, molecular approaches are also restricted due to the limited number of characterised sequences available for most phenotypes. Nevertheless, in Figure 9 was graphically summarised the obtained correlation among the taxonomic group and the available PAHs-transforming genotype data about the strains isolated from both of the two root systems and the corresponding bulk soil.

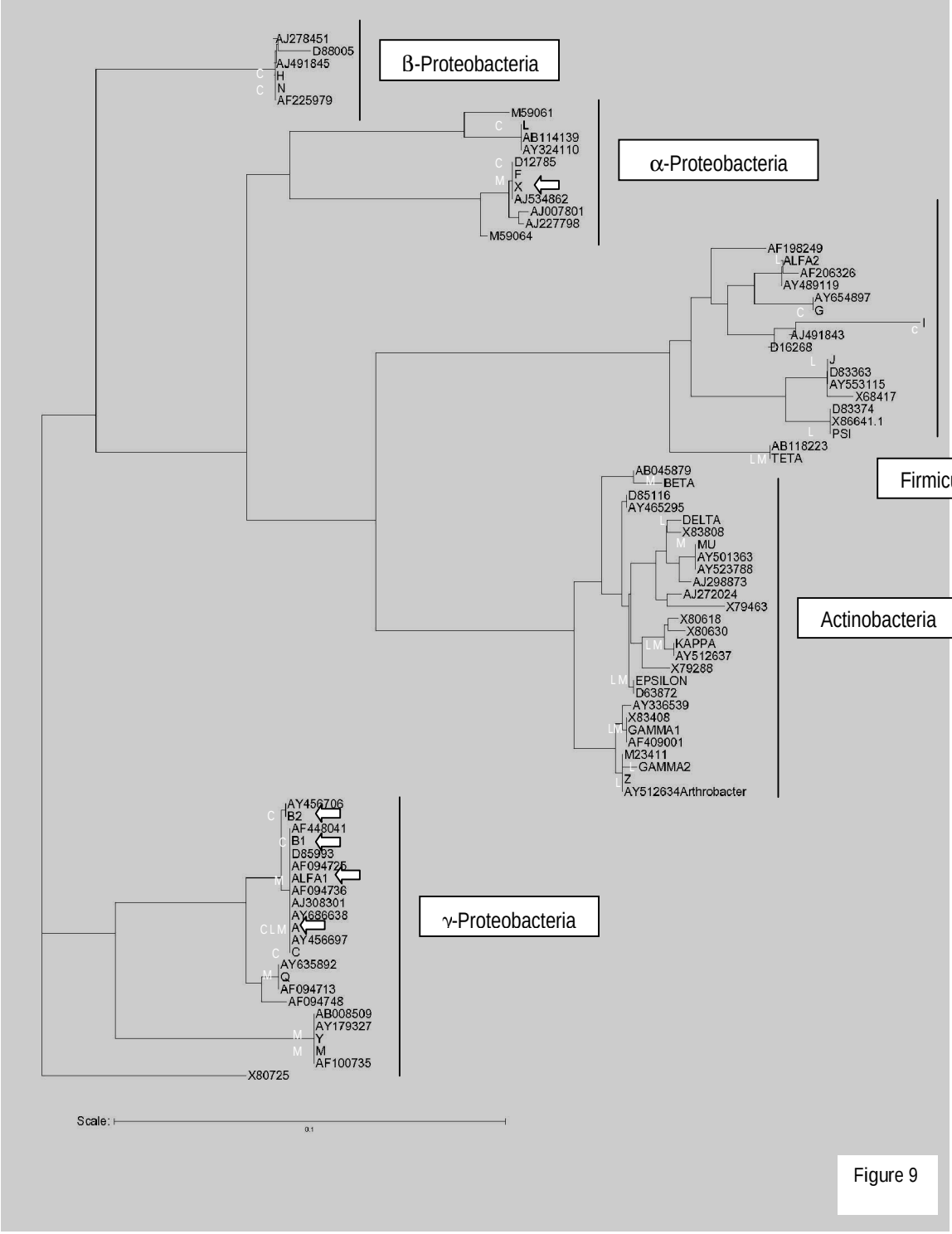


Figure 9. Tree graphical representation of the taxonomic correlation among the 26 OTUs (A, B1, B2, C, F, G, H, I, L, M, N, Q, ALFA1, ALFA2, BETA, GAMMA1, GAMMA2, DELTA, EPLSILON, TETA, KAPPA, MU, X, PSI, J, Y, Z) recovered from the

soil aliquots C, 14,000 mg 16US-EPA PAHs/ kg dry soil (indicated with white C); microcosms vegetated with *Lupinus albus* (indicated with white L); microcosms vegetated with *Zea mays* (indicated with white M). The white arrows indicate the OTUs that host *nah*-genotype harbouring bacteria. Related and type species reference strains: *Pseudomonas* sp. KD (AY456697), *Pseudomonas putida* ATCC 17484 (D85993), *Pseudomonas putida* (AY456706), *Pseudomonas putida* KL33 (AY686638), *Brevundimonas* sp. FWC43 (AJ227798), *Bacillus* sp. NK13 (AY654897), *Achromobacter xylosoxidans* subsp. *xylosoxidans* (AF225979), *Bacillus firmus* D1 (AJ491843), *Azospirillum* sp. TS6 (AB114139), *Stenotrophomonas maltophilia* isolate VUN10,035 (AF100735), *Alcaligenes xylosoxidans* strain F (AJ491845), Uncultured *Pseudomonas* sp. AMS1 (AY635892), *Pseudomonas* sp. SCD-7 (AF448041), *Bacillus* sp. A4 (AY489119), *Streptomyces* sp. LK1312.4 (AY465295), *Arthrobacter* sp. Ellin159 (AF409001), *Arthrobacter* sp. pfB10 (AY336539), *Promicromonospora sukumoe* DSM 44121 (AJ272024), *Streptomyces setonii* (D63872), *Bacillus* sp. PAMU-1.13 (AB118223), *Rhodococcus* sp. A2Y26 (AY512637), *Cellulosimicrobium funkei* strain CDC#JB2769ot (AY523788), *Staphylococcus* sp. MO28 (AY553115), *Brevundimonas* sp., isolate DW114b (AJ534862), *Stenotrophomonas* sp. S1 (AY179327), *Arthrobacter* sp. Amico3 (AY512634), *Staphylococcus* sp. AG-30 (X86641.1), *Pseudomonas aeruginosa* ATCC 10145 (AF094713), *Pseudomonas stutzeri* ATCC 17588 (AF094748), *Pseudomonas fluorescens* ATCC 13525 (AF094725), *Achromobacter xylosoxidans* subsp. *denitrificans* DSM 30026 (AJ278451), *Bacillus firmus* IAM 12464 (D16268), *Bacillus subtilis* LN002 (AF198249), *Bacillus cereus* (AF206326), *Staphylococcus aureus* ATCC 12600 (X68417), *Staphylococcus xylosus* ATCC29971T (D83374), *Staphylococcus epidermidis* ATCC 14990T (D83363), *Brevundimonas diminuta* (M59064), *Brevundimonas vesicularis* ATCC 11426 (AJ007801), *Mycoplana bullata* IAM 13153 (D12785), *Stenotrophomonas maltophilia* ATCC 13637T (AB008509), *Azospirillum brasilense* ATCC 29145 (AY324110), *Azospirillum lipoferum* (M59061), *Achromobacter xylosoxidans* subsp. *xylosoxidans* IAM12684 (D88005), *Pseudomonas putida* (AF094736), *Pseudomonas chlororaphis* (AJ308301), *Promicromonospora citrea* DSM 43110T (X83808), *Cellulomonas flavigena* NCIMB 8073 (X79463), *Rhodococcus rhodochrous* DSM 43241 (X79288), *Rhodococcus opacus* DSM43205 (X80630), *Rhodococcus erythropolis* DSM43188 (X80618), *Cellulosimicrobium cellulans* ATCC 12830 (AY501363), *Cellulosimicrobium variabile* MX5 (AJ298873), *Arthrobacter oxidans* DSM 20119 (X83408), *Arthrobacter globiformis* (M23411), *Streptomyces tauricus* JCM 4837 (AB045879), *Streptomyces lavendulae* IFO 12789 (D85116). Sequence used as outgroup: *Escherichia coli* ATCC 11775T (X80725)

3.3 BIOAUGMENTATION / PHYTOREMEDIATION / BIOAUGMENTATION and PHYTOREMEDIATION ASSAYS

In the previous chapters we have described the modifications occurred to the bulk soil bacteria community as a result of the presence of different level of PAHs contamination or plant root systems. We have assessed that, in the studied aged polluted aliquots, there is a PAHs-transforming population potentially able to reclaim the investigated industrial soil. Moreover, we have verified that the introduction of plant systems can beneficially alter the microbial diversity for the specific soil contamination scenario, producing a sensible reduction of the PAHs content in the analysed soil aliquot (soil aliquot C). On the basis of the obtained information, in the third part of this work, laboratory scale Bioaugmentation (microcosms C and D), Phytoremediation (microcosm E) and combined Bioaugmentation/Phytoremediation (microcosms F and G) assays were performed (Table 3, from Materials and Methods). In these experiments, *Lupinus albus* var. Multitalia and two different types of bacterial inocula (Table 3, from Materials and Methods) were utilised as plant and microbes remediation promoters. The bioremediation treatments were performed using the soil aliquot C, one year after the arrangement of the first experiment (Phytoremediation assay, 3.2 section). After the ageing, the 16 priority PAHs concentration value reached 10,312 mg/ kg dry soil. The aims of the research were both to compare the potential of the three bioremediation approaches on the removal of PAHs from soil, and above all, to evaluate the possibility of monitoring the augmented bacteria inocula with molecular protocols.

Table 3, from Materials and Methods. Microcosms design in Bioaugmentation/ Phytoremediation/ Bioaugmentation-Phytoremediation assays

Microcosms	Pots number	Soil characteristic	*Lupinus albus* var. Multitalia	Bacterial inocula
A	10	Sterilised soil	no	no
B	10	Native soil	no	no
C	10	Native soil	no	6×10^7 CFU/g of soil of mixed culture[a]
D	10	Native soil	no	6×10^7 CFU/g of soil of *Burkholderia* sp. DBT1[b]
E	10	Native soil	yes	no
F	10	Native soil	yes	6×10^7 CFU/g of soil of mixed culture[a]
G	10	Native soil	yes	6×10^7 CFU/g of soil of *Burkholderia* sp. DBT1 [b]

[a]: Mix of three bacterial isolates. 2×10^7 CFU/g of soil NDP003, isolated from soil aliquot A; 2×10^7 CFU/g of soil 5N-C, isolated from soil aliquot C; 2×10^7 CFU/g of soil L8A-C, isolated from the rhizosphere of *Lupinus albus* var. Multitalia, soil aliquot C

[b]: *Burkholderia* sp. DBT1, selected PAHs transforming bacteria [II]

3.3.1 BACTERIA INOCULA

The inoculation of pollutant degrading bacteria can be an important additive to improve the efficiency of intrinsic (natural attenuation) or plant-assisted remediation of a PAHs contaminated soil. These bacterial inocula can be constituted by selected indigenous- as well as foreigner- transforming bacteria. In the performed bioaugmentation and Bioaugmentation/ Phytoremediation treatments both types of inocula were utilised, and their molecular monitoring was attempted by using V3-16S rDNA PCR DGGE approach. The indigenous inoculum was constituted by a mixed culture of three strains (NDP003, 5N-C and L8A-C) isolated during this research work (3.1.3 and 3.2.3 sections). They were selected for their ability to transform PAHs, both as sole carbon source and by means of co-metabolism, and to produce surfactants when grown on the tested PAHs mixture. Two of them, NDP003 and 5N-C are *nah*-genotype transforming bacteria isolated from the bulk soil, and in particular to the aliquots A (low polluted) and C (high polluted) respectively (3.1.3 section). They were affiliated to the same taxon (γ-Proteobacteria, *Pseudomonas* genus), but fitted into two different OTUs (OTU1 and OTUA). Moreover, by using PCR-DGGE technique they can be distinguished and monitored thanks to their different V3-16S-rDNA DGGE profiles. Strains belonging the *Pseudomonas* genus were already utilized in bioaugmentation [Barathi and Vasudevan, 2003; Widada et al., 2002b], as well as in Bioaugmentation/Phytoremediation treatments of various organic polluted environmental samples [Geels and Schippers 1983; Kuiper et al., 2002]. Furthermore, same *Pseudomonas* spp. can also promote plant growth [Lugtenberg and Dekkers, 1999]. Along these lines, NDP003 and 5N-C were chosen as possible suitable candidates to enhance the PAHs dissipation in the performed bioremediation treatments. On the contrary, L8A-C was isolated from rhizosphere of *Lupinus albus* (3.2.3 sections). L8A-C belonged to the Firmicutes phylum (this phylum seems to be positively selected by the plant root system, 3.2.3.2 section) and in detail to the *Bacillus* genus. L8A-C showed original V3-16S-rDNA DGGE profile; therefore it can be distinguished from the other two inoculated strains, and monitored by using PCR-DGGE technique. Concerning the functional genes involve in the PAHs transformation the latter strain host an uncharacterised oxygenase system. The *Bacillus* genus has already been reported in literature as involved in PAHs transformation [Gauthier et al., 2003; Kazunga and Aitken, 2000], even if not in plant-association. Therefore, we were interested in investigating the possible role of this strain in both the arranged bioaugmentation and plant-assisted treatments.

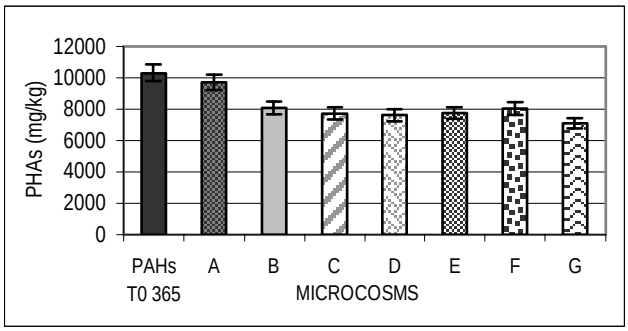
The second type of utilised inoculum was constituted by a pure culture of a previously characterised, foreigner bacterium, *Burkholderia* sp. strain DBT1 [II]. This strain was a versatile PAHs transformer, a *dbt*-genotype harbouring bacteria, and had a specific V3-16S-rDNA DGGE profile, so it can be clearly monitored onto denaturing gels. Strains belonging to this genus were previously recovered in PAHs-

polluted soil and were well known as xenobiotic degraders [Johnson et al., 2000; Suen et al., 1996; Laurie and Lloyd-Jones, 1999]. Moreover, many *Burkholderia* spp. were recognized for their ability to nodulate legumes [Moulin et al., 2001; Chen et al., 2003] and to fix N_2 [Gillis et al., 1995; Estrada-De Los Santos et al., 2001]. In addition, recently, Unno and co-workers reported the isolation of strains belonging to this genus, from *Lupinus albus* rhizosheath and rhizoplane [Unno et al., 2005]. These bacteria, thanks to *L. albus*-released citrate, can utilize soil organic phosphorus (as phytates). By such way, they make P available for plant and therefore may act as plant growth promoters in case of low inorganic P conditions. However, other studies reported the *B.* spp. ability to promote plants yield and growth [Bevivino et al., 1998; Chiarini et al., 1998; Tran Van et al., 2000]. For these considerations, *B.* sp. DBT1 was selected as suitable candidate to be used in the assessed bioremediation operations. Moreover, it has to be remembered that *Lupinus albus* var. Multitalia seems to be able to stimulate the *dbt*-genotype harbouring bacteria (3.2.2.2 section). Therefore, we were interested in investigating the effects of the *L. albus*-*B.* sp. DBT1 interaction on the PAHs transformation. On the other hand, we were fascinated by the possibility of evaluating the both *B*. sp. DBT1 persistence and *dbt*-genotype presence in the soil microcosms using an original TaqMan RealTime PCR approach, targeting the *dbtAc* dioxygenase encoding gene. However, the parameters optimisation of this PCR protocol is underway.

3.3.2 PAHS TRANSFORMATION IN MICROCOSMS

These experimentation were carried out, using the soil aliquot C, after one year from the first experiment setting (*Phytoremediation assay* 3.2 section). The total 16 US-EPA PAHs concentration in the soil aliquot C after the ageing had decreased to 74.5% of the initial amount (13,850 mg 16 US-EPA PAHs/kg dry soil, 3.2.1 section) and in particular the PAHs decrease concerned mainly phenanthrene (34.6%), anthracene (41.0%) and fluoranthene (36.7%).

At the end of the present microcosm experimentations (9 weeks), the total 16 priority PAHs remaining concentration were showed in Figure 10.

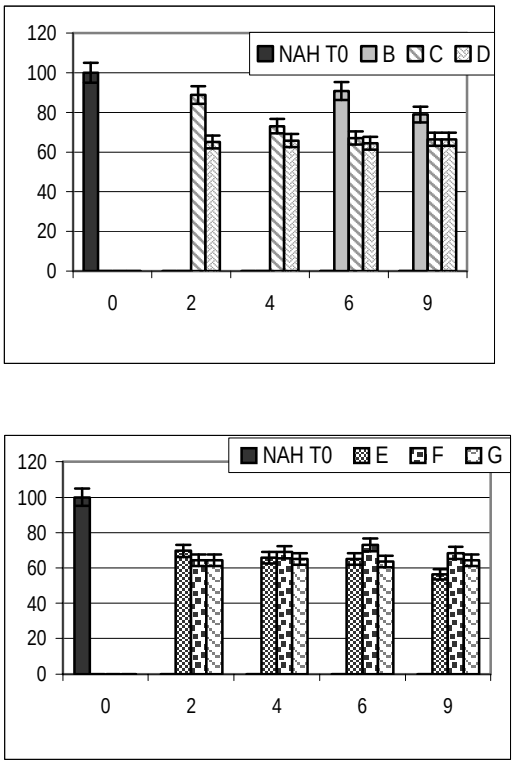


Microcosms	16 US-EPA PAHs concentration (mg/kg)	Percentage of PAHs transformation
PAHs $T0_{365}$	10,312	-
A	9,712	5.8
B	8,090	21.5
C	7,722	25.1
D	7,614	26.2
E	7,922	23.2
F	7.969	22.7
G	7,096	31.2

Figure 10. 16 priority PAHs transformation after 9 weeks from the microcosms set up. PAHs $T0_{365}$: PAHs initial concentration in the soil aliquot $C_{365\text{-days}}$; A: sterilised soil microcosms; B: native soil; C: native soil inoculated with NP003:5N-C:L8A-C; D: native soil inoculated with *B*. sp strain DBT1; E: native soil vegetate with *L. albus*; F: native soil vegetate with *L. albus* and inoculated with NP003:5N-C:L8A-C; G: native soil vegetate with *L. albus* and inoculated with *B*. sp DBT1.

PAHs concentration in the abiotic control (A) decreased during 9 weeks of about 5.8% of the initial content. In contrast, all biotic conditions (B, C, D, E, F and G) showed a more sensible decrease in the PAHs concentration. Best results, concerning the total PAHs transformation, have been achieved through the application of Bioaugmentation/Phytoremediation protocol G (31.2%). On the contrary, no substantial difference can be showed among B, C, D, E and F treatments regarding the whole extractable pollutants concentrations at the end of the experiment. However, more information can be obtained comparing the transformation rate of some selected 16 priority PAHs, in the different microcosm conditions. The figures below (Figures 11-17) graphically represent the decreasing trend of selected 2- 3- 4- 6- rings PAHs.

NAPHTHALENE



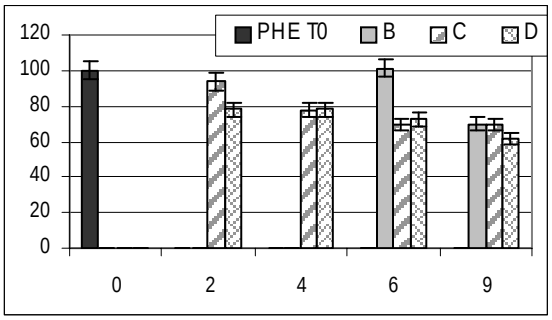
NAH T0 (mg/kg) 152	% Transformation in the arranged Microcosms		
	NON-VEGETATED		
	B	C	D
0-9 weeks	21.1	33.2	34.3
NAH T9 (mg/kg)	120	101	101
	VEGETATED		
	E	F	G
0-9 weeks	42.1	32.6	35.5
NAH T9 (mg/kg)	86	104	98

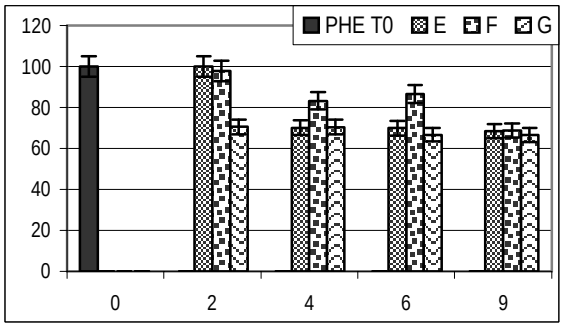
Figure 11. Percentage of Naphthalene transformation in the arranged microcosms during the time. NAH T0: NAH initial concentration in the soil aliquot $C_{365\text{-days}}$; NAH T9: NAH final concentration in the arranged microcosms. B: native soil; C: native soil inoculated with NP003:5N-C:L8A-C; D: native soil inoculated with *B.* sp. strain DBT1; E: native soil vegetate with *L. albus*; F: native soil vegetate with *L. albus* and inoculated with NP003:5N-C:L8A-C; G: native soil vegetate with *L. albus* and inoculated with *B.* sp. DBT1

In the regard of naphthalene (Figure 11), the higher percentage of transformation, at the end of the experimentation, was obtained in the vegetated non–inoculated soil system E (42.1%). The biotransformation performances of the two inoculated microcosms, C and D, were comparable (33.2% and 34.3% respectively) and also higher than that registered in the system B (non-vegetated, non–inoculated) (21.1%). Therefore the bioaugmentation treatments enhance the intrinsic natural bio-dissipation capacity. On the other hand, the vegetated and bioaugmented microcosms F and G showed

the same decreasing percentage (32.6% and 35.5% respectively) but lower than that recorded in the vegetated non-inoculated treatment (E). Consequently the potential of the combined approaches results less pronounced than the one achieved in the Phytoremediation protocol E. However, the maximum percentage of transformation occurred in the first 2 weeks for all vegetated systems (E 30.3%, F 35.5%, and G 35.5%) and also in the bioaugmentation treatment D (34.2%).

PHENANTHRENE



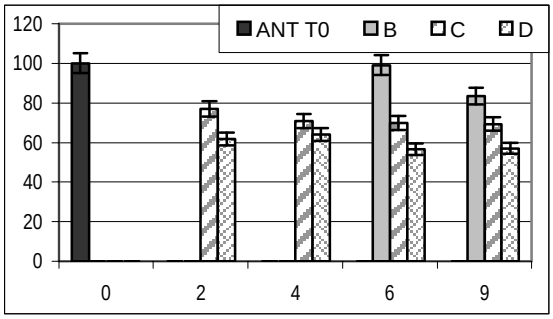


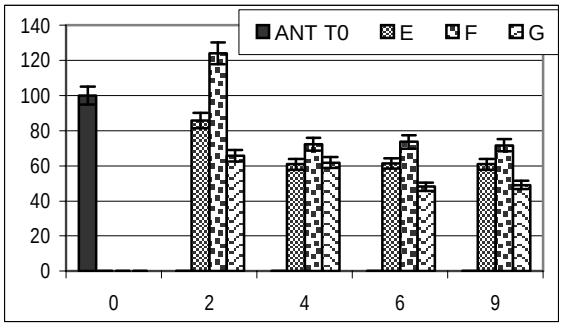
PHE T0 (mg/kg) 952	% Transformation in the arranged Microcosms		
	NON -VEGETATED		
	B	C	D
0-9 weeks	29.9	30.1	38.3
PHE T9 (mg/kg)	667	664	587
	VEGEATED		
	E	F	G
0-9 weeks	30.5	31.1	31.4
PHE T9 (mg/kg)	652	656	634

Figure 12. Percentage of Phenanthrene transformation in the arranged microcosms during the time. PHE T0: PHE initial concentration in the soil aliquot $C_{365\text{-days}}$; PHE T9: PHE final concentration in the arranged microcosms. B: native soil; C: native soil inoculated with NP003:5N-C:L8A-C; D: native soil inoculated with *B*. sp. strain DBT1; E: native soil vegetate with *L. albus*; F: native soil vegetate with *L. albus* and inoculated with NP003:5N-C:L8A-C; G: native soil vegetate with *L. albus* and inoculated with *B*. sp. DBT1

On the other hand, the phenanthrene maximum reduction percentage occurred in the non-vegetated but inoculated soil system D (38.3%) (Figure 12). However, at the end of the experimentation, no great differences in phenanthrene transformation can be detected in the arranged microcosms. Nevertheless, it was to underline that the presence of *B*. sp. DBT1, with (G) or without vegetation (D), permitted to obtain the maximum concentration decrease in the first 2 weeks (29.4% and 21.6% respectively). Moreover, it was interesting to note that the *L.albus* alone (E) enhanced the transformation rate more than the indigenous inolucum introduction (C) or than the concomitant presence of plant and these augmented bacteria (F).

ANTHRACENE



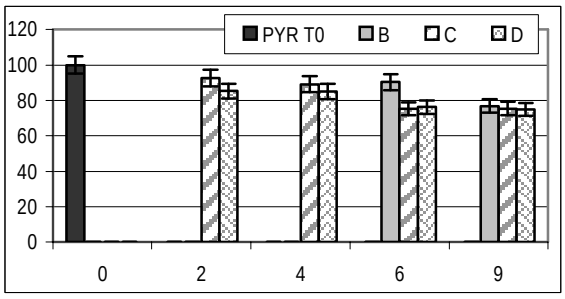


ANT T0 (mg/kg) 212	% Transformation in the arranged Microcosms		
	NON-VEGETATED		
	B	C	D
0-9 weeks	16.5	30.0	43.2
ANT T9 (mg/kg)	177	147	121
	VEGETATED		
	E	F	G
0-9 weeks	39.0	27.5	51.4
ANT T9 (mg/kg)	129	152	104

Figure 13. Percentage of Anthracene transformation in the arranged microcosms during the time. ANT T0: ANT initial concentration in the soil aliquot $C_{365\text{-days}}$; ANT T9: ANT final concentration in the arranged microcosms. B: native soil; C: native soil inoculated with NP003:5N-C:L8A-C; D: native soil inoculated with *B.* sp. strain DBT1; E: native soil vegetate with *L. albus*; F: native soil vegetate with *L. albus* and inoculated with NP003:5N-C:L8A-C; G: native soil vegetate with *L. albus* and inoculated with *B.* sp. DBT1

Concerning anthracene the maximum transformation percentage occurred in the vegetated and *B.* sp. DBT1 inoculated soil system G (51.4%) (Figure 13). The presence of this non-indigenous bacterium greatly enhanced the anthracene transformation also in the arranged D microcosms (*B.* sp. DBT1-inoculated, non-vegetated); however the root system significantly increased the degrading microbial activity, perhaps stimulating the inoculated strain. In both cases the transformation mainly occurred in the first 2 weeks (G 34.4% and D 38.2%).

PYRENE



PYR T0 (mg/kg) 1277	% Transformation in the arranged Microcosms		
	NON-VEGETATED		
	B	C	D
0-9 weeks	23.2	24.7	24.4
PYR T9 (mg/kg)	981	962	957
	VEGETATED		
	E	F	G
0-9 weeks	16.4	18.2	31.1
PYR T9 (mg/kg)	1104	996	880

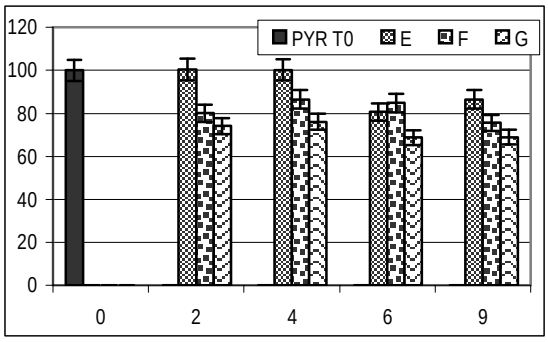
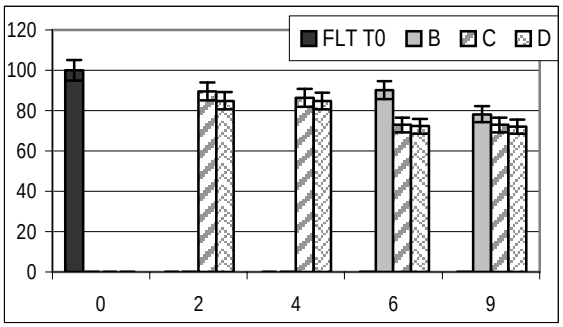


Figure 14. Percentage of Pyrene transformation in the arranged microcosms during the time. PYR T0: PYR initial concentration in the soil aliquot $C_{365\text{-}days}$; PYR T9: PYR final concentration in the arranged microcosms. B: native soil; C: native soil inoculated with NP003:5N-C:L8A-C; D: native soil inoculated with *B*. sp. strain DBT1; E: native soil vegetate with *L. albus*; F: native soil vegetate with *L. albus* and inoculated with NP003:5N-C:L8A-C; G: native soil vegetate with *L. albus* and inoculated with *B*. sp. DBT1

FLUORANTHENE



FLT T0 (mg/kg) 1515	% Transformation in the arranged Microcosms		
	NON-VEGETATED		
	B	C	D
0-9 weeks	21.8	26.7	27.8
FLT T9 (mg/kg)	1184	1104	1091
	VEGEATED		
	E	F	G
0-9 weeks	27.3	26.3	34.1
FLT T9 (mg/kg)	1104	1117	999

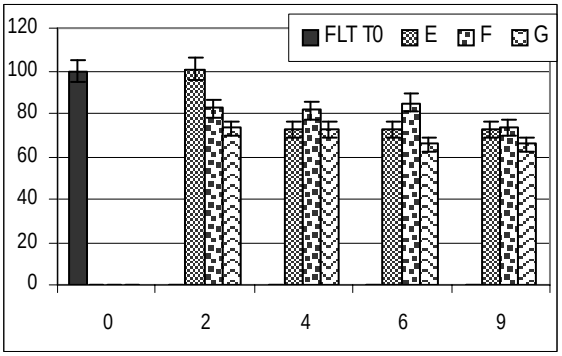
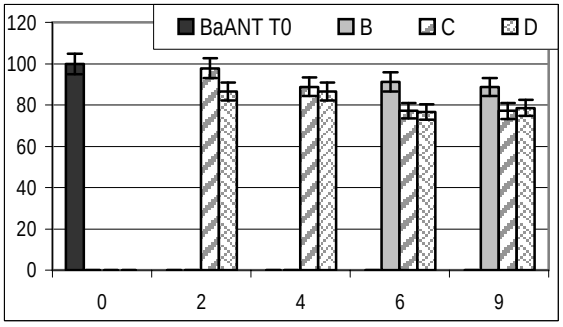


Figure 15. Percentage of Fluoranthene transformation in the arranged microcosms during the time. FLT T0: FLT initial concentration in the soil aliquot $C_{365\text{-}days}$; FLT T9: FLT final concentration in the arranged microcosms. B: native soil; C: native soil inoculated with NP003:5N-C:L8A-C; D: native soil inoculated with *B*. sp. strain DBT1; E: native soil vegetate with *L. albus*; F: native soil vegetate with *L. albus* and inoculated with NP003:5N-C:L8A-C; G: native soil vegetate with *L. albus* and inoculated with *B*. sp. DBT1

BENZO(a)ANTHRACENE



BaANT T0 (mg/kg) 753	% Transformation in the arranged Microcosms		
	NON-VEGETATED		
	B	C	D
0-9 weeks	11.3	22.7	22.4
BaANT T9 (mg/kg)	668	581	592
	VEGETATED		
	E	F	G
0-9 weeks	21.1	18.3	31.0
BaANT T9 (mg/kg)	594	615	519

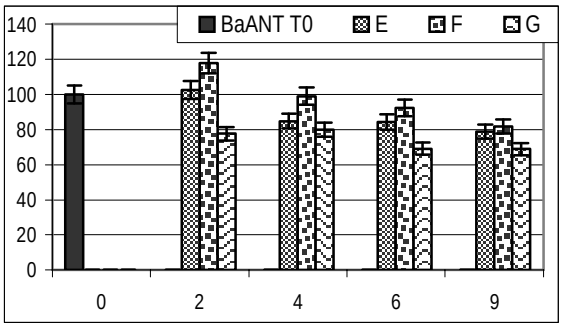
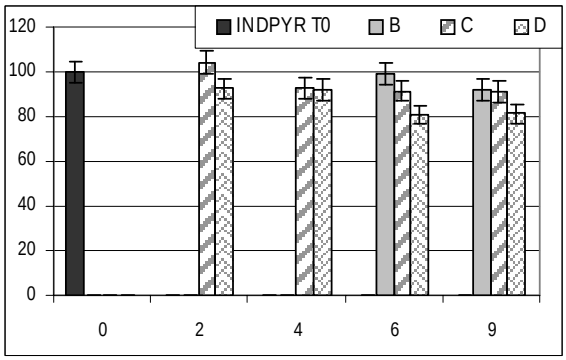
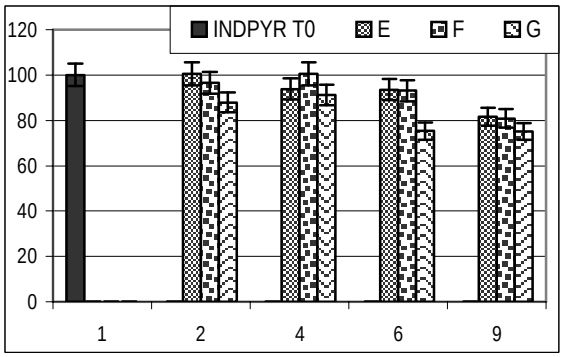


Figure 16. Percentage of Benzo(a)anthracene transformation in the arranged microcosms during the time. BaANT T0: BaANT initial concentration in the soil aliquot $C_{365\text{-}days}$; BaANT T9: BaANT final concentration in the arranged microcosms. B: native soil; C: native soil inoculated with NP003:5N-C:L8A-C; D: native soil inoculated with *B.* sp. strain DBT1; E: native soil vegetate with *L. albus*; F: native soil vegetate with *L. albus* and inoculated with NP003:5N-C:L8A-C; G: native soil vegetate with *L. albus* and inoculated with *B.* sp. DBT1

INDENO[1,2,3 c,d]PYRENE





INDPYR T0 (mg/kg) 559	% Transformation in the arranged Microcosms		
	NON-VEGETATED		
	B	C	D
0-9 weeks	7.9	8.1	18.8
INDPYR T9 (mg/kg)	515	509	454
	VEGETATED		
	E	F	G
0-9 weeks	18.4	19.1	24.8
INDPYR T9 (mg/kg)	456	452	420

Figure 17. Percentage of Indeno[1,2,3 c,d]pyrene transformation in the arranged microcosms during the time INDPYRT0: INDPYR initial concentration in the soil aliquot $C_{365\text{-}days}$; INDPYR T9: INDPYR final concentration in the arranged microcosms. B: native soil; C: native soil inoculated with NP003:5N-C:L8A-C; D: native soil inoculated with *B.* sp. strain DBT1; E: native soil vegetate with *L. albus*; F: native soil vegetate with *L. albus* and inoculated with NP003:5N-C:L8A-C; G: native soil vegetate with *L. albus* and inoculated with *B.* sp. DBT1

Regarding pyrene, fluoranthene and benzo(a)anthracene the maximum percentage of transformation was achieved in the treatment G (vegetated, *B.* sp. DBT1-inoculated) (31.1%, 34.1% and 31.0% respectively). In particular, the dissipation mainly occurred in the first 2 weeks (pyrene 25.8%, fluoranthene 26.9%, benzo(a)anthracene 22.3%) (Figures 14-16). On the contrary, no great differences in the contaminants concentration reduction can be detected in the other microcosms.

Nevertheless, comparing the F (vegetated, inoculated with indigenous inolucum) and C (non-vegetated, inoculated with indigenous inolucum) treatments, the presence of the plant root system (F) enhanced

the transformation rate of the pollutants (except for benzo(a)anthracene) in the first 2 weeks. On the other hand, the Phytoremediation protocol (E) overall improved the intrinsic soil community transformation performances on the regard of fluoranthene and benzo(a)anthracene, but not of pyrene.

As regard indeno[1,2,3 c,d]pyrene (Figure 17), the higher dissipation was once again obtained in the microcosms arranged with *L. albus* and *B.* sp. DBT1 (G), 24.8%. In the latter case the maximum reduction percentage was mainly registered 4-6 weeks after the assessment of the microcosms (24.7%). On the contrary, in the other treatments the indeno[1,2,3 c,d]pyrene concentration decrease mostly occurred after 6 weeks.

Moreover, it was important to note that also the acenaphthylene, acenaphthene, fluorene, benzo(a)pyrene and benzo[g,h,i]perylene concentration reduction was mostly achieved by the application of G treatment (vegetated, *B.* sp. DBT1-inoculated) (17.3% 21.3%, 34.1%, 30.3% and 26.3% respectively). Concerning dibenzo[a,h]anthracene substantial dissipation was registered after the G treatment (28.5%), however best result (35.4%) was obtained with the application of the Phytoremediation approach E (vegetated non–inoculated soil). On the other hand, all the performed bioremediation protocols did not enhance the chrysene, bezo(b)fluoranthene and bezo(k)fluoranthene dissipation over that registered in the B (non-vegetated, non-inoculated) soil system.

In conclusion, our study showed that the best results, concerning the PAHs transformation, have been achieved through the application of Bioaugmentation/Phytoremediation with *Burkholderia* sp. strain DBT1 (G). Indeed, this treatment successfully enhanced the dissipation of several PAHs, accelerating their transformation rate mainly in the first 2 weeks. Concerning the Phytoremediation protocol (E), after the soil ageing, the rhizospheric effect was less pronounced than that obtained after the first experiment (Phytoremediation assay 3.2 section). Nevertheless, analogous results were already reported by Binet and colleagues [Binet et al., 2000], and may be due to the decreased availability of PAHs with the ageing process [Hatzinger and Alexander, 1995]. In fact, an ever greater percentage of these compounds becomes less bio-available and more resistant to biodegradation with time [Hatzinger and Alexander, 1995]. However, naphthalene, anthracene, benzo(a)anthracene and indeno[1,2,3 c,d]pyrene concentration still decreased more significantly in vegetated soil (E), after ageing, than in non-vegetated one (B) (Figures 13, 16 and 17). Regarding the application of F protocol (vegetated, inoculated with indigenous inolucum), actually it did not effectively decrease the contaminants content more than the corresponding non-vegetated soil (C). Nevertheless, the naphthalene, pyrene and fluoranthene transformation was accelerated in the first 2 weeks by F treatment. Moreover, total amount of the indeno[1,2,3 c,d]pyrene dissipation was increased by using the latter approach.

3.3.3 INOCULA PERSISTENCE AND COMMUNITY MODIFICATION MONITORING BY PCR-DGGE

The impossibility to understand and explain the effect of the performed bioremediation treatments often is caused by the lack of knowledge both of persistence of the inoculated bacterial populations in the environment [Head, 1998] and of the modifications occurred in the microbial community as a result of the adopted protocol. In this way, a molecular approach such as the PCR DGGE, able of monitoring, through the time, the presence of the bacterial inocula and the bacterial community modifications became necessary. Therefore, 16S rDNA PCR-DGGE method was successfully utilised in this study to suggest some microbial explanation for the observed PAHs-transformation activity.

In the Figure 18 was shown the relative DGGE profiles of the non-inoculated/non-vegetated systems (B) and of the corresponded vegetated ones (E) during the experimentation (from 0 to 9 weeks). The analysis of the native bulk community profiles (B0 to B9) showed that no modifications occurred in the dominant bands patterns during the research trial. Therefore, the observed PAHs natural dissipation may be due to an increase of the bacterial activity produced by the suitable physical-chemical chamber conditions. On the other hand, the dominant bands pattern in the rhizosphere bacterial community varied during the time (E0 to E9). In particular, the most important modifications occurred in the first 4 weeks, matching to the early stage of plants growth and to acclimation phase of the plant-microbe interaction. After 4 weeks, the bacterial community seemed to substantially set down until the experimentation end. It was interesting to note, that in the vegetated system E, the maximum percentage of PAHs transformation generally occurred between 0 and 4 weeks (Figures 11-12-13-15 and 16).

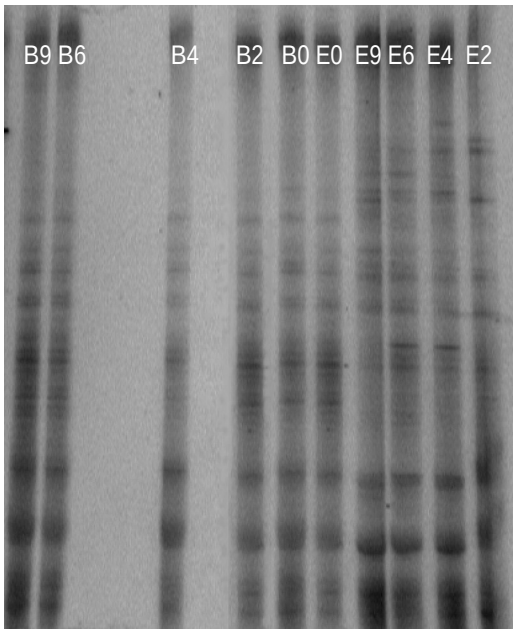


Figure 18. Denaturing gradient gel electrophoresis of bacterial community V3-16S rDNA PCR products, amplified from non-inoculated/ non-vegetated microcosms (Lane B0 to B9) and non-inoculated/ vegetated microcosms (Lane E0 to E9) soil-DNA extracts. Letters correspond to the microcosm type and numbers to the sampling week.
E0: sampling after 24h from the inoculation and before plant sowing

The same PCR-DGGE analysis was performed for the C and F bioremediation treatments as well as for D and G ones, the obtained profiles were shown in the Figure 19 and 20.

The analysis of the DGGE profiles produced by PCR amplification of the DNA extracted from the microcosms C (non-vegetated, inoculated with indigenous inolucum) showed that the introduction of the autochthonous bacterial inoculum does not modify the bulk soil bacterial community structure (Figure

19, panel A). Moreover, the sequencing of the soil bands, from C0 profile, corresponding to the inoculated bacteria strains, NDP003 (lane 1), 5N-C (lane 2) and L8A-C (lane 3), confirms the presence of all three strains, at the beginning of the experimentation. In the same way, the presence of L8A-C was determined until 6 weeks. On the contrary, the presence of NDP003, but above all, of 5N-C, remained unclear through the time. However, none of the inoculated strains can be detected after 6 weeks. The disappearance of the DGGE bands related to the selected strains may be due to the relative decrease of the corresponding DNA amplification targets inside the total nucleic acid extract. This evidence, may suggest a reduced amount (growth) of corresponding strains in the bacterial community. Therefore we can conclude that the bacterial inoculum NDP003:5N-C:L8A-C did not persist in the assessed system C. In the same way, the overall PAHs concentration reduction obtained by the application of the latter treatment (non-vegetated, inoculated with autochthonous inoculum) not substantially overcame that achieved in B soil microcosms (non-inoculated, non-vegetated). Nevertheless, the PAHs transformation rate in the C treatment was greater than that registered in the B microcosms (non-vegetated, non-inoculated) (Figures 11, 12, 14, 15 and 16). Moreover, naphthalene, anthracene and benzo(a)anthracene amount reduction was significantly higher in the bioaugmented soil systems (C). Therefore, these evidences may suggest that at least L8A-C presence enhanced the biotransformation potential of the soil matrix. Consisting with the latter hypothesis, the PAHs dissipation, in this type of treatment, generally occurred until 6 weeks (Figures 11, 12, 14, 15 and 16). Therefore, PAHs content seems to decrease until L8A-C was detectable.

Different consideration could be made for the F treatment (vegetated, inoculated with indigenous inolucum) relative DGGE profiles (Figure 19 panel B). The analysis of the obtained DGGE profiles showed that even if the three bacterial strains were present at the beginning of the experimentation, 4 weeks after the microcosms arrangement only NDP003 (lane 1) still remained. Therefore, we can conclude that the bacterial inoculum NDP003:5N-C:L8A-C did not persist in the system. This evidence was analogous to that obtained by the analysis of the C treatment (non-vegetated, inoculated with autochthonous inoculum) DGGE profiles. Nevertheless, in F soil microcosms (vegetated, inoculated with autochthonous inoculum), NDP003 instead of L8A-C can be detected after the time zero. However, NDP003 persists in soil less than L8A-C (4 weeks *vs.* 6 weeks). The first evidence may suggest that NDP003 has been specifically stimulated by the plant system. This is particularly increasing when we consider that L8A-C, and not NDP003, was isolated from the *L. albus* rhizosphere (3.2.3 section).

The obtained PCR-DGGE picture of the F soil (vegetated, inoculated with autochthonous inoculum) bacterial communities corresponds to an overall not-significant PAHs dissipation of the F Bioaugmentation *plus* Phytoremediation treatment in comparison with that obtained by the application of

C (bioaugmentation with autochthonous inoculum), but above all B (natural attenuation, non-vegetated, non-inoculated), protocols (3.3.2 section).

Nevertheless, the pyrene and fluoranthene transformation rates, in the F treatment, were greater than those registered in the C (non-vegetated, inoculated with autochthonous inoculum) and B (non-vegetated, non-inoculated) microcosms. Indeed, in the soil system F (vegetated and inoculated, with autochthonous inoculum) the reduction of the two pollutants occurred mainly in the first two weeks (Figures 14 and 15). Therefore, it seems to happen concomitantly with the NDP003 presence in the systems. Nevertheless, this reduction may be due to the presence of *L. albus*. However, even if in the vegetated non-inoculated microcosms (E) the pyrene and fluoranthene dissipation values were comparable with those achieved in F condition, the transformation rate of the two molecules was lower in E than that registered in the latter treatment (Figures 14 and 15). This evidence may confirm the significant role of NDP003 in the pyrene and fluoranthene reduction concentration in studied soil sample.

On the other hand, the transformation rate of anthracene and benzo(a)anthracene was enhanced in the C (non-vegetated, inoculated with autochthonous inoculum) but not in the F microcosms (Figures 13 and 16). The latter evidence seems to confirm our hypothesis of a specific role of L8A-C in the dissipation of these two compounds in the studied soil matrix.

However, in conclusion, the indigenous strains inoculation not successfully enhanced the overall biotransformation performances of the adapted community. This result may be caused by a non-suitable selection, size or relative proportion of the bacterial strains in the mixed inoculum. Moreover, the ability of the introduced bacteria to preserve, *in vivo,* the *in vitro* assessed PAHs-transforming catabolic activities were still to verify. On the other hand, concerning the Bioaugmentation *plus* Phytoremediation F treatment the nature of the established interaction among the augmented inoculum and the adopted plant species remain to be extensively investigated. Actually, the study of the capacity of the bioinoculants to compete for the exudates nutrients or to efficiently colonize the growing root system was underway. For these reasons, we are conscious that this study must be considered at the preliminary stage. Indeed, to date, we had focused our attention only on the possibility to monitor the persistence, in the soil, of the introduced bacteria inocula during the experimentation by using molecular approaches.

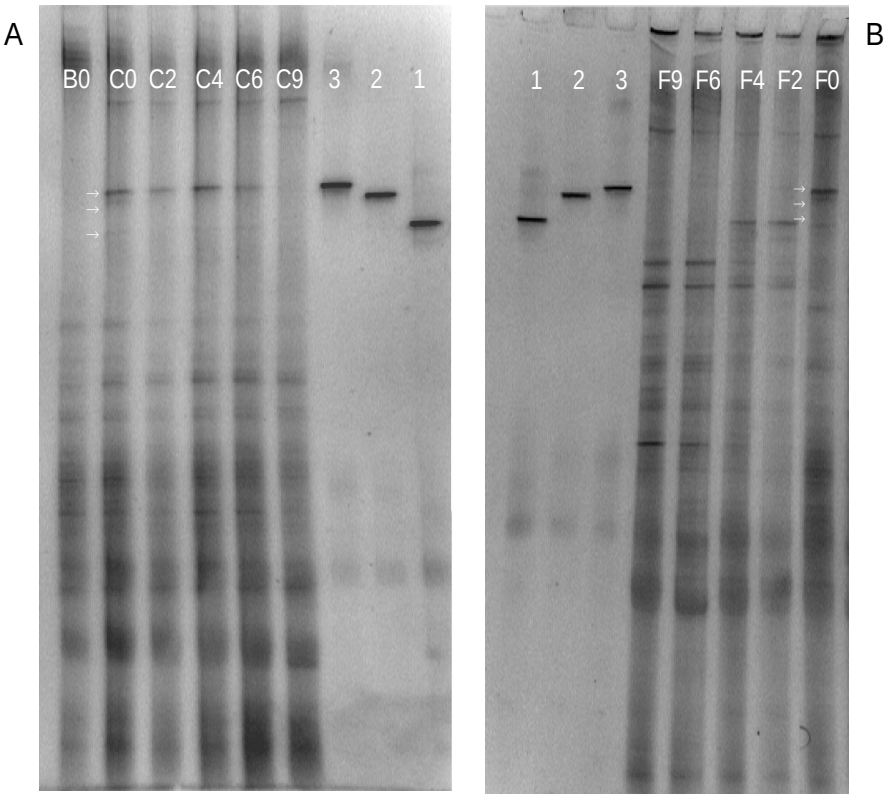


Figure 19. Panel A: Denaturing gradient gel electrophoresis of bacterial community V3-16S rDNA PCR products amplified from inoculated/ non-vegetated microcosms (Lane C0 to C9) soil-DNA extracts; Lane B0 V3-16S rDNA PCR products amplified from non-inoculated/ non-vegetated microcosms soil-DNA extract at the beginning of the experimentation; Lane 1 to 3: V3 region 16S rDNA PCR products amplified from NDP003, 5N-C and L8A-C genomic DNA. Panel B: DGGE of bacterial community V3-16S rDNA PCR products amplified from inoculated/ vegetated microcosms (Lane F0 to F9) soil-DNA extracts. Lane 1 to 3: V3 region 16S rDNA PCR products amplified from NDP003, 5N-C and L8A-C genomic DNA. The bacterial inoculum was constituted by NDP003: 5N-C: L8A-C as 1.5:1:2.5 mixed bacterial culture. Arrows indicate the inoculated bacterial strains in the community profiles. Letters correspond to the microcosm type and numbers to the sampling week.

With reference to D (non-vegetated, inoculated with *B.* sp. DBT1) and G (vegetated, inoculated with *B.* sp. DBT1) bioremediation treatments, the obtained DGGE profiles were showed in the Figure 20. The presence of the inoculated *Burkholderia* sp. strain DBT1 in the D and G soil microcosms was confirmed at all sampled times. In particular, in the non-vegetated microcosms (D), DBT1 seemed to be the dominant species. On the contrary, some other major bands (probably corresponding to bacterial strain stimulated by the plant) can be recovered in the rhizospheric community

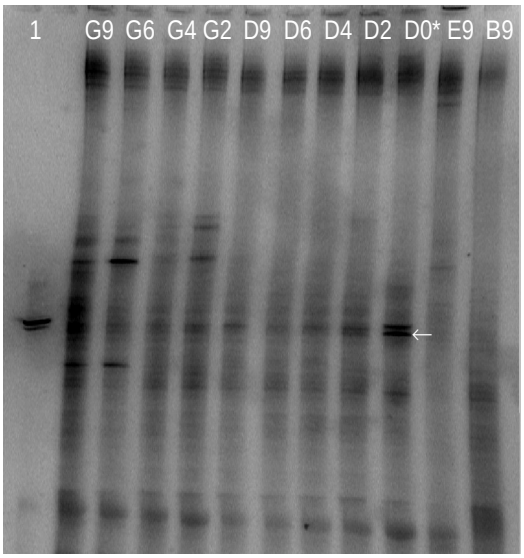


Figure 20. Denaturing gradient gel electrophoresis of bacterial community V3-16S rDNA PCR products amplified from inoculated/ non-vegetated microcosms (Lane D0 to D9) and inoculated/ vegetated microcosms (Lane G2 to G9) soil-DNA extracts; Lane B9: V3-16S rDNA PCR products, amplified from non-inoculated/ non-vegetated microcosms soil-DNA extract at the end of the experimentation; Lane E9: V3-16S rDNA PCR products, amplified from non-inoculated/ vegetated microcosms soil-DNA extract at the end of the experimentation; Lane 1 V3 region 16S rDNA PCR products amplified from *B.* sp. DBT1 genomic DNA.
The bacterial inoculum was constituted by pure culture of *Burkholderia* sp. DBT1. Arrows indicate the inoculated bacterial strain in the community profiles. Letters correspond to the microcosm type and numbers to the sampling week. *: D0=G0 therefore the latter is omitted

profiles (G). Therefore, the higher PAHs dissipation percentage achieved in the G treatment (vegetated, inoculated with *B.* sp. DBT1) may be due to a positive *L. albus*-soil bacterial community- *B.* sp. DBT1 interaction. Anyhow, also in this case, the plant-bacteria interaction remains to be extensively investigated.

Although the strain was persistent, the highest percentage of PAHs transformation in these treatments was reached in the first 2 weeks from the microcosms setting. This evidence may suggest that the metabolic and/or the PAHs transforming activity of *B.* sp. DBT1 could be reduced after this time.

3.3.4 MONITORING OF Burkholderia sp. DBT1 *BY TaqMan-REALTIME PCR*

In the 3.1.2.2 section we emphasized that the characterization of genes involved in bacterial organic pollutant degradation has promoted the development and application of PCR-based methods to monitor the performance of *in situ* bioremediation [Burlage et al., 1994]. One of the forefront PCR methods is quantitative PCR [Jansson and Leser, 1996; Lee et al., 1996], and in particular 5' fluorogenic exonuclease assay (TaqMan) PCR approach, that represents the latest development in real-time qPCR methods [Holland et al., 1991; Lie, and Petropoulos. 1998]. By utilizing an internal probe in addition to standard PCR amplification primers, TaqMan chemistry combines the amplification power of PCR with the specificity and verification of Southern hybridization. Labelling the internal probe with fluorescent dyes provides, in-tube, real-time detection of PCR product accumulation during each amplification cycle and at very early stages in the amplification process.

In this study, we utilised the TaqMan quantitative PCR assay to monitor and quantify the presence of the bioaugmented *Burkholderia* sp. DBT1 strain, in the soil microcosms D and G, (non-vegetated, inoculated; vegetated, inoculated respectively), targeting the *dbt*Ac dioxygenase encoding gene. The presence of only 1 copy of *dbtAc* gene per *B.* sp. DBT1 genome was estimated by Southern hybridisation [II]. By this approach, we also tried to recover and quantified the soil natural *dbt*-genotype presence, to obtain an estimation of intrinsic *dbt*-related bioremediation potential of studied matrix. Nevertheless, environmental samples have level of chemical complexity, which may affect the ability of the TaqMan PCR to quantify RNA and DNA in these matrices [Stults et al., 2001]. In particular, these matrices contain organic contaminants (metals, humic acids or other inhibitory compounds) that can copurify with nucleic acids and complicate the amplification process [Toranzos, 1997; Wilson, 1997]. These inhibitors may also interfere with fluorescence detection independently of their effect on *Taq* polimerase. In fact, compound such as humic acids can either quench or auto-fluorescence at the excitation emission wavelengths of common fluors (FAM, TAMRA). The consequence of fluorescence

quenching is to increase Cτ and underestimated the starting target concentration in the original sample. The consequence of auto-fluorescence is to decrease Cτ and overestimate the starting target concentration, irrespective of assay precision [Stults et al., 2001]. In order to avoid fluorescence detection interferences as well as to limit the DNA contaminants inhibitory effects on the *Taq* polymerase activity, the optimisation of an original Brilliant Black BN-Bovine Serum Albumin TaqMan PCR protocol is underway, in our laboratories.

3.3.4.1 TaqMan PCR optimisation for *dbtAc* quantification from soil-DNA extracts

One primer pair and a probe were developed (2.6.4.1 section) to target a 140 bp region inside *dbtAc* gene (AF380367). Primers specificity was tested against genomic DNA from two bacterial strains harbouring divergent PAHs-transforming genotypes (*Pseudomonas putida* ATCC17484 and *Burkholderia* sp. RP007 ICMP 13529 as representatives of *nah* and *phn* transforming genotype) in a TaqMan PCR assays without BSA and Brilliant Back BN dye. Two different DNA concentrations (1 and 10 ng), extracted from bacterial mixed as well as pure culture of *P. putida* ATCC17484, *B.* sp. RP007 and *B.* sp. DBT1, were tested to determine *B.* sp. DBT1 primer and probe specificity. Unspecific signal associated with non-target bacterial cultures was obtained using dbtAcfor (position 823 to 843), dbtAcrev (position 935 to 963) and dbtAcFAM/TAMRA TaqMan probe (position 845 to 865) in the performed TaqMan Real Time reactions (data not shown). On the other hand, we can successfully estimate that 1ng *B.* sp. DBT1 of genomic DNA harboured 1.17×10^7 ($\pm$ 1.01×10^6) *dbtAc* gene copies. The initial copy number of a gene target was estimated, in the performed RealTime PCR protocol from the exponential PCR phase of the products accumulation and by comparison to an external standard curve. The obtained linearity between TaqMan Cτ values and gene target concentration in the standard samples spanned from 4.95×10^{10} to 4.95 *dbtAc* gene copies with the following correlation curve equation: y= -3.770194x+45.924427. Therefore, the primer pair and probe, designed in this study, could be considered specific for *Burkholderia* sp. DBT1 *dbtAc* gene or better for the *dbt* genotype. The latter consideration validated the use of these oligo for PCR monitoring and/or quantification of *Burkholderia* sp. DBT1 as well as *dbt* genotype presence in total soil DNA extracts.

Therefore, with the purpose to minimise the humic acids effect on the *Taq* polymerase (when PCRs from soil DNA extracts were performed), we adopted RealMasterMix Probe ROX (Eppendof) combined with MasterTaq PCR Enhancer (Eppendorf) and Bovine Serum Albumin. Concerning BSA, the protein concentration was empirically determined (1μg/μl) to achieve both some PCR amplification and the better assay sensitivity. On the other hand, the amount of Brilliant Black BN dye solution (0.0075mM final concentration) was empirically determined both to normalise the humic acids fluorescence

interference (quenching or auto-fluorescence) (2.6.4.3 section), and to permit the reporter dye (FAM) signal detection. This approach was chosen to overcame, and then normalise, the unpredictable contribution of the humic substances in the soil DNA extracts. In fact, in this way an equal and note amount of dye, in the target soil DNA as well as in the standard references DNA samples, was present. This setting might provide accurate target quantification, because the fluorescence interference in the standard samples should be comparable with the one in the soil DNA extracts. The results obtained showed that the tested BSA and Brilliant Back BN concentrations might be used in TaqMan quantitative assay. However, to date, the method must be greatly improved. In fact, the initial copy number of a *dbtAc* gene was estimated from the exponential PCR phase of the products accumulation and by comparison to the external standard curve. Nevertheless, in our laboratory trials, the best linearity between TaqMan Cτ values and *dbtAc* gene concentration in the standard samples was observed only over three orders of magnitude (8.85×10^8 to 8.85×10^5) (Figure 21). Therefore, the obtained data established that quantification of target DNA was possible, by this approach, but that the technique detection limit, up till now, is very high. Moreover, the better observed PCR efficiency (slope = -5.434203) and the linear correlation ($r^2 = 0.965217$) were lower than those usually recommended by Perkin-Elmer, for precise and accurate quantification. Nevertheless, the adopted reaction mixture has never been previously tested; therefore we cannot compare the obtained data with analogous ones.

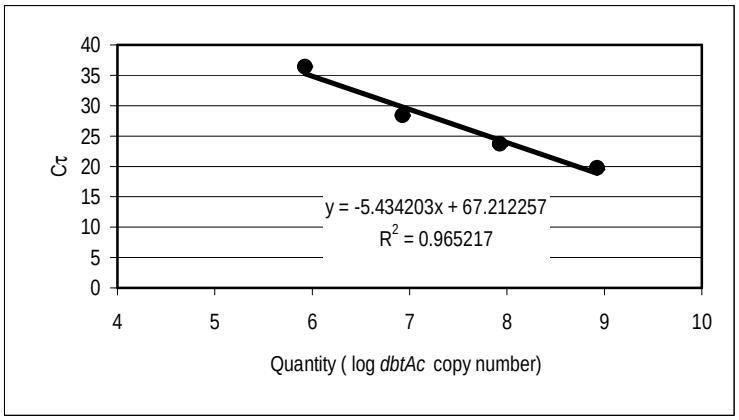


Figure 21. TaqMan standard curve of serially diluted *dbtAc*-containing plasmid pBluescript® SK, using Brilliant Black BN in the reaction mixture. Each dot represents the result of triplicate PCR amplification for each dilution. Standard error of triplicates was smaller than the size of the symbol.

Despite the intrinsic high resolution limits of the protocol, we however tried to quantify and detect the *B.* sp. DBT1 and the *dbt*-genotype presence in the matrix. Therefore, TaqMan Real Time PCRs were

performed on the 200ng of DNA extracts from the soil microcosms as listed below (Table 6, from Material and Methods).

Table 6, from Material and Methods. Soil DNA extracts utilised as template in TaqMan PCR reactions

Microcosms	*Lupinus albus*	Bacterial inocula
L*	yes	no
E0[a] and E9°	yes	no
D0[b] to D9°	no	6 x 10^7 CFU/g of soil of *Burkholderia* sp. DBT1
G0 [a] to G9°	yes	6 x 10^7 CFU/g of soil of *Burkholderia* sp. DBT1

*: Microcosms performed in the Phytoremediation assay; °: Microcosms performed in the Bioaugmentation/ Phytoremediation/ Bioaugmentation and Phytoremediation assays
[a]: E0, G0: sampling after 24h from the inoculation and before plant sowing
[b]: D0: sampling after 24h from the inoculation

The obtained PCR results suggested that this assay may actually provide a measure of *B.* sp. DBT1 abundance in the soil samples, in which it was bioaugmented, at every sampling time (D0 to D9, non-vegetated, inoculated microcosms; G0 to G9 vegetated, inoculated respectively). Moreover, the presence of Brilliant Black BN dye in the reaction mixture seems able to provide a more accurate estimation of the target gene than that gained without the presence of the dye. Indeed, assuming 1.17x10^7 *dbtAc* copies number in 1 ng of *B*. sp. DBT1 genomic DNA (see above in this section), when we used *B*. sp. DBT1 genomic DNA as reaction template, we estimate a *dbtAc* gene copy number of 1.11x10^7 (± 4.11x10^6) and of 3.31x10^6 (± 7.24x10^4) in the "black" and in the "white" conditions respectively (Tables 28 and 29). Therefore, the latter conditions seem to underestimate the amount of *dbtAc* gene in the *B.* sp. DBT1 genomic DNA. It is also evident by using soil DNA extracts as reaction templates, especially with low target gene copies (Tables 28 and 29 and Figure 22).

Nevertheless, we were not able to quantify the intrinsic *dbt*-genotype amount in the non-inoculated microcosms (L, E0 and E9) (Tables 28 and 29). Indeed, soil DNA extracted from these microcosms did not result in fluorescence increase above the threshold value. The latter data, at least concerning L and E9 soil DNA extracts, were probably due to the low sensitivity of the assessed method rather than to the absence of the target gene in the corresponding DNA template. In fact, at least the presence of *dbt*-genotype in the DNA extracted from soil microcosms L was already assessed in this study (3.2.2.2 section).

Table 28. TaqMan estimates of *dbtAc* copy number without Brilliant Black BN in reaction mixture ("white" conditions)				
200 ng soil DNA extracts	Cτ	Standard deviation (Cτ)	Mean Quantity	Standard deviation (Quantity)
D0	22.65	0.468	1.80E+07	3.74E+06
D2	23.93	0.241	7.89E+06	8.60E+05
D4	25.99	0.119	2.11E+06	1.13E+05
D6	26.37	0.181	1.65E+06	1.36E+05
D9	27.43	0.991	8.35E+05	3.63E+05
G0	22.74	0.499	1.69E+07	4.01E+06
G2	26.88	0.315	1.21E+06	2.46E+05
G4	29.07	0.009	2.93E+05	1.64E+03
G6	34.11	0.212	1.15E+04	1.56E+03
G9	34.87	0.340	7.15E+03	1.46E+03
L, E0, E9	-	-	BDL*	-
B. sp. DBT1[a]	28.51	0.048	3.31E+06	7.24E+04

dbtAc quantity was estimated by comparison to "white" external standard curves:
$y = -3.586493x+48.668972$; $r^2 = 0.978691$
Linearity between TaqMan Cτ values and target concentration in the standard samples was observed over four orders of magnitude $6.05x10^7$-$6.05x10^3$
*: below detection limit
[a]: 1 ng of *B*. sp. DBT1 genomic DNA

Table 29. TaqMan estimates of *dbtAc* copy number with Brilliant Black BN reaction mixture, ("black" conditions)				
200 ng soil DNA extracts	Cτ	Standard deviation (Cτ)	Mean Quantity	Standard deviation (Quantity)
D0	26.58	0.772	3.12E+07	1.09E+07
D2	28.39	0.816	1.45E+07	4.88E+06
D4	29.55	0.761	8.82E+06	2.90E+06
D6	30.54	0.856	5.59E+06	1.14E+06
D9	36.22	0.395	5.09E+05	8.86E+04
G0	26.84	0.675	2.69E+07	9.56E+06
G2	31.51	0.141	3.71E+06	2.22E+05
G4	32.52	0.884	2.51E+06	9.17E+05
G6	33.05	0.214	2.40E+06	1.50E+06
G9	38.94	0.047	1.60E+05	3.20E+03
L, E0, E9	-	-	BDL*	-
B. sp. DBT1[a]	29.04	0.882	1.11E+07	4.11E+06

dbtAc quantity was estimated by comparison to "black" external standard curves:
$y = -5.434203x + 67.212257$; $r^2 = 0.965217$
Linearity between TaqMan Cτ values and target concentration in the standard samples was observed over four orders of magnitude $8.85x\ 10^8$ to $8.85x\ 10^5$
*: below detection limit
[a]: 1 ng of *B*. sp. DBT1 genomic DNA

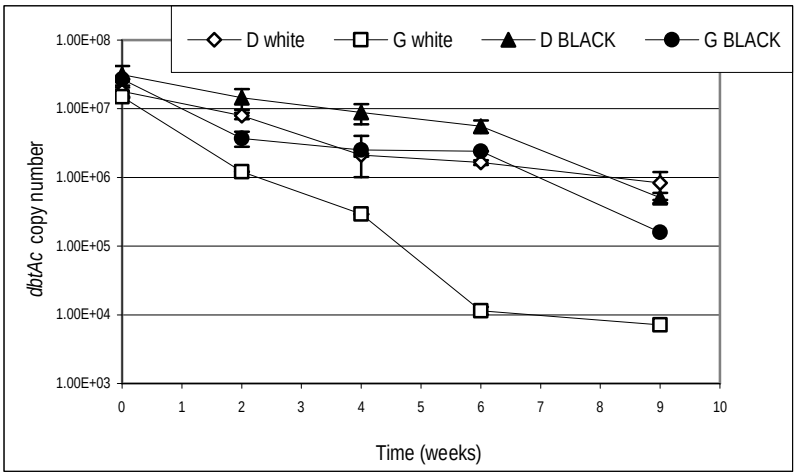


Figure 22. Estimation of *B.* sp. DBT1 *dbtAc* copy number by TaqMan real time PCR at every sampling time during the experimentation (from 0 to 9 weeks). Soil DNA, used as reaction templates, were extracted from D, non-vegetated/*B.* sp. DBT1 inoculated soil microcosms and G, vegetated/*B.* sp. DBT1 inoculated soil microcosms.
D white: *dbtAc* quantity obtained without Brilliant Black BN in the reaction mixture from non-vegetated/*B.* sp. DBT1 inoculated soil DNA extracts; G white: *dbtAc* quantity obtained without Brilliant Black BN in the reaction mixture from vegetated/*B.* sp. DBT1 inoculated soil DNA extracts; D BLACK: *dbtAc* quantity obtained with Brilliant Black BN in the reaction mixture from non-vegetated/*B.* sp. DBT1 inoculated soil DNA extracts; G BLACK: *dbtAc* quantity obtained with Brilliant Black BN in the reaction mixture from vegetated/*B.* sp. DBT1 inoculated soil DNA extracts

Actually the obtained data suggest few considerations. Indeed, the Brilliant Black BN-Bovine Serum Albumin TaqMan PCR protocol actually permits to specifically monitor the presence of the bioaugmented *B*. sp. DBT1 until the experimentation end. Moreover, gained results showed that the *B*. sp. DBT1 quantity, in the soil samples (both in the non-vegetated/inoculated soil D microcosms and in vegetated/inoculated G soil microcosms), decrease during the time, however still remaining over the (high) detection limit of the adopted PCR protocol. This data may be due to the relative decrease of the corresponding DNA amplification targets inside the total nucleic acid extracts and may suggest a reduced amount (growth) of *B.* sp. strain DBT1 in the bacterial communities. On the other hand, by using this approach we can confirm and quantify the presence of the *dbt*-related PAHs transforming capacity also 9 weeks after the bacterial culture inoculation. Nevertheless, no information can be achieved, by using this DNA based approach, concerning the DBT1 PAHs-transforming activity in soil. However, further extensive optimisations of the protocol may provide a more sensible and accurate quantification of the DNA target gene and perhaps may permit to applied this amplification condition to a mRNA-PCR based approach.

CONCLUDING REMARKS

Polycyclic aromatic hydrocarbons (PAHs) are a recalcitrant group of contaminants and are known to be highly persistent in the environment [Cooke and Dennis, 1983]. There are many sources for PAH contamination in soils. These include creosote, petroleum products, and coke products [Cooke and Dennis, 1983]. It is expensive and time consuming to remediate persistent contaminants such as PAHs from soils. At the present time, the techniques used to reclaim contaminated soils tend to be inefficient. Physical removal of contaminated soil and washing of those soils with solvents are expensive, and have met with mixed results [Dixon 1996]. Alternatively, *in situ* microbial remediation (bioremediation) has been attempted. However, in most contaminated soils, the number of microrganisms is depressed so that there are not enough bacteria to facilitate contaminant degradation. Moreover, PAHs are very stable compounds, and the initial oxidation step is biologically slow and metabolically expensive [Huang et al., 2001]. Thus, microbial remediation alone is probably too slow to be a realistic approach to this problem. More recently, there have been some improvements in the strategies for bacterial remediation of contaminated soil, including the stimulation of indigenous bacterial community, or inoculation with bacteria that were selected from PAHs contaminated sites [US EPA, 2000]. The stimulation of autochthonous bacteria, to facilitate PAHs transformation, may be due by the addition of nutrients or by using higher plants (phytoremediation). Indeed, the rhizosphere effect (physical-chemical modification of soil, production of exudates) is well known to increase microbial density and activity, compared to those for bulk soil, and/or to select specific microrganisms [Lynch, 1990]. However, for bioremediation to be effective, the overall rate of PAHs transformation and degradation must be accelerated above natural microbial processes.

The preliminary focus of this thesis was characterised the indigenous bacterial community which can survive in an industrial PAHs contaminated soil in order to recover the presence of the catabolic capacity that may be responsible for the pollutants biodegradation. The results achieved during this research work indicate that, in the studied soil aliquots, a heterogeneous bacterial community is present that actually host PAHs-transforming populations. The further PCR-based characterisation permits to clearly ascribe the intrinsic bioremediation potential of the soil only to the classical *nah*-like transforming genotype harbouring bacteria, irrespectively of the contamination level, and to *dbt*-transforming genotype carrying bacteria in association with low pollution concentration. To our knowledge, this is the first report on the *dbt*-genes recruitment in soil. Moreover, the performed isolation protocol permits to recover several strains able to utilise the pollutants. The further taxonomic analysis allows inferring a taxonomic tree, in which the Proteobacteria phylum constitutes the main branches (94.4% of the isolates belonging to this phylum). The application of the lab-scale phytoremediation treatment

(PHYTOREMEDIATION ASSAY) has reached its goal permitting to positively stimulate the PAHs-transforming populations in the high polluted soil aliquot C (14,000 mg PAHs/kg). Indeed, the presence of *Lupinus albus* determines an increase of the microbial catabolic activity that produced a significant (31.7%) overall reduction of the 16 priority US-EPA PAHs concentration, in the investigated soil aliquot, while the natural microbial process removes only the 18.4% of the initial PAHs amount. On the other hand, although the presence of *Zea mays* root system results to be partially effective (22.0%), it enhanced the transformation of several compound such as benzo(a)antracene, benzo(a)pyrene and benzo(k)fluorantene over the natural attenuation. The transformation activity increase is probably due to an enhancement of general microbial activity. Really, root systems do not seem specifically stimulate the PAHs-transforming population. However, the transformation increase seems to be correlated with a plant-dependent taxonomic modification of the pollutants transforming bacterial population. Indeed, the results obtained, by culture dependent approach, reveal that the taxonomic structure of the isolates, recovered from both rhizosphere systems, is altered in comparison with that assessed in the bulk soil, by using the same isolation protocols. In particular, from *Z. mays* rhizosphere, we can recover strains belonging to Proteobacteria (44.5%) but also to Actinobacteria (50%) phylum. On the other hand, from *Lupinus albus* root system a high proportion of the recovered isolates belonged to the Actinobacteria (47.6%) and to Firmicutes (42.9%) instead of Proteobacteria (9.5%) phylum. Moreover, by direct *Lupinus albus*-rhizospheric DNA amplification we can assess that the presence of the latter root system is able to enrich, over the detection limit of the utilised technique, the presence of the *dbt*-transforming genotype harbouring bacteria. Therefore we can conclude that the adopted treatment successful enhances the decrease of the contaminants and that the best results were obtained by using *Lupinus albus* as plant remediation promoter. Moreover, from a microbiologic point of view, tanks to the information gained by culture dependent and independent adopted protocols, we can suggest that this success positively correlate whit the specific plant selection of bacteria belonging to the Firmicutes phylum and with the plant dependent stimulation of the *dbt*-genotype harbouring bacteria.

When, after one year from the first experimentation, we try to improve the PAHs microbial reclaim of the site by using separated and combined bioaugmentation and phytoremediation approaches (BIOAUGMENTATION/PHYTOREMEDIATION/ BIOAUGMENTATION-PHYTOREMEDIATION ASSAYS), less pronounced results were obtained. However, gained evidences were expected because of the reduction, during the time, of the more bioavailable compounds [Wilson and Jones, 1993] and the increasing of the soil particles sorption of PAH-molecules [Hatzinger and Alexander, 1995]. Along this way, the overall contaminants decrease obtained by the application of bioaugmentation treatment both with indigenous (*Pseudomonas* spp. and *Bacillus* sp.) and foreigner (*Burkholderia* sp. DBT1) inoculum and by using both *Lupinus albus* and indigenous inoculum-*Lupinus albus* combined approach do not

substantial exceed the potential of natural microbial process. However, our results confirm that these reclaim operations successful permit to accelerate the transformation rate of several selected PAHs like naphthalene, anthracene, fluoranthene and benzo(a)anthracene. On the other hand, the combined introduction of *Burkholderia* sp DBT1 and *Lupinus albus* significant overcame the reduction efficiency of other treatment, above all, enhance the transformation rate of many analysed PAHs within the first two weeks of experimentation. Therefore, it is possible to suggest a positive plant-soil bacteria-inoculum interaction even if the nature of the established relation among the augmented bacteria and *Lupinus albus* remains to be extensively investigated.

However, the mainly focus of this study was to understand the bacterial contribution to the effectiveness of the bioremediation. Therefore we have successfully adopted culture dependent and independent approaches. The utilised isolation protocol permits to recover bacterial strains, which are able to transform tested PAHs and that are shared in different genera. Further genomic DNA investigation of these strains may permit to improve the overall knowledge of the nature of the class of genes involved in the environmental PAHs-transformation. In particular, it may allow enlarging the picture of the true diversity of PAHs-catabolic genes in the analysed soil aliquots also as consequence of the imposed selection of the performed bioremediation treatments.

Moreover, we have optimised a PCR protocol that permits to directly amplify soil DNA extracts and therefore to clarify the modification occurred to the bacterial community during bioremediation. Along this line, 16SrDNA PCR-DGGE was effectively adopted. 16SrDNA PCR-DGGE is also successfully adopted to monitor the fate of augmented PAHs transforming strains in the polluted matrix during performed bioremediation protocols. The latter approach permits also to suggest some possible explanations, from a microbial point of view, concerning the performances of the adopted treatments. In fact, it allows correlating the persistence of the introduced bacteria to the performance of the bioremediation treatments.

Regarding TaqMan Real Time protocol, we actually tested original reaction conditions that seem to overcome the negative effects generated by contaminant compounds copurify with soil DNA. Indeed, it permits the quantification of the inoculated strain (*B.* sp DBT1) even if, to date, the PCR methods must to be extensively improved.

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CHAPTER I

Biodiversity amongst cultivable polycyclic aromatic hydrocarbon-transforming bacteria isolated from an abandoned industrial site.

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Biodiversity amongst cultivable polycyclic aromatic hydrocarbon-transforming bacteria isolated from an abandoned industrial site

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Abstract

The characterisation of a microbial community of a polycyclic aromatic hydrocarbons (PAHs) contaminated site (formerly Carbochimica, Trento, Italy) was carried out. A preliminary evaluation of the heterogeneity and the metabolic activity of the microbial community were attempted by denaturing gradient gel electrophoresis (DGGE) and reverse transcriptase-denaturing gel electrophoresis (RT-DGGE). The presence of a heterogeneous and metabolically active microbial community was found. To evaluate the PAH-transforming potential of the soil bacterial community, enrichment cultures were set up. Taxonomically diverse bacteria, showing different biochemical PAH-transforming pathways were obtained. Some of the isolates showed not *nah*-homologous PAH-transforming genotypes.

Keywords: Bioremediation; Denaturing gradient gel electrophoresis; Microbial cenosis; Polycyclic aromatic hydrocarbons; PAH-transforming genotypes; Soil bacteria

1. Introduction

Many naturally occurring microorganisms are able to degrade polycyclic aromatic hydrocarbons (PAHs) [1–6]. Most of these belong to the genus *Pseudomonas* and possess genes involved in the PAH-biodegradative pathway that are highly homologous to the cluster of naphthalene genes (*nah* genes) cloned from the NAH7 plasmid harboured by *Pseudomonas putida* G7 [7]. However, the nucleotide sequence homology and the organisation of isofunctional clusters of genes cloned from genera *Burkholderia* [8,9], *Comamonas* [10], *Ralstonia* [2], and *Sphingomonas* [11,12] are not conserved in respect with the *nah* genes.

By utilization of a bioremediation protocol to reclaim a PAH-contaminated soil, preliminary characterisation of the autochthonous microbial community is important to evaluate the intrinsic biodegradation potential of the contaminated site. However, PAH-transformation may involve genes that are not highly homologous to the *nah*-like class of genes. In this context, the detection of PAH-transforming microorganisms using *nah*-like gene probes and specific primers for the *nah*-like genotype has sometimes failed [1,13,14], confirming that the PAH-transforming genotypes are actually diverse. An alternative approach to detect PAH-transforming

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microorganisms consists in designing primers for the detection of an increasing number of diverse PAH-transforming genes [15–17]. However, this approach is limited by the availability of databases of appropriate gene sequences that are still lacking PAH-transforming genes.

The aim of this study was the characterisation of a PAH-contaminated industrial soil (formerly Carbochimica, Trento, Italy) by studying the taxonomic diversity of the autochthonous PAH-transforming bacteria and the diversity of their PAH-transforming genes. Both cultivation-based and non-cultivation-based protocols were adopted in order to gain information on the PAH-biodegradation potential of the site.

2. Materials and methods

2.1. Chemicals

Chemicals were of analytical grade. PAHs and solvents were purchased from Sigma–Aldrich, (Milan, Italy). Bacteriological preparations were purchased from Oxoid (Milan, Italy).

2.2. Cultivation media

Enrichment cultures were set up in minimal Defined Medium [18] supplemented in excess either with naphthalene (500 mg l^{-1}) or with a combination of phenanthrene, naphthalene, and dibenzothiophene (250 mg l^{-1} each) provided as *N*-*N*-dimethylformamide solutions.

Substrate utilization was performed in Defined Medium supplemented with phenanthrene, naphthalene, dibenzothiophene or salicylate (1 g l^{-1} ethanol solution); gentisate or phthalate (1 g l^{-1} aqueous solutions). Solidified medium (Bacteriological agar) was supplemented with dibenzothiophene by spraying the plate surface with an ethanol-free diethyl ether solution (5% [wt vol^{-1}]) [19], with naphthalene by placing crystals within the lids of the plates, and with phenanthrene by the overlay method [20].

2.3. Molecular techniques

Standard procedures were used for DNA manipulation and agarose gel electrophoresis [21]. Bacterial genomic DNA was extracted using the Nucleospin Tissues Kit (BD Bioscences Clontech, Milan, Italy) following the manufacturer's instructions. Total DNA and RNA from soil were extracted using the FastDNA® SPIN® Kit (For Soil) (Q-BIOgene, Resnova, Italy). For RNA extraction, the modifications reported in [22] were adopted. DNA was manipulated using enzymes purchased from Amersham Bioscience (Milan, Italy) and sequenced using a PRISM Ready Reaction DNA terminator cycle sequencing Kit (Perkin–Elmer, Milan, Italy) running on an ABI 377 instrument. Nucleotide sequence data were assembled using the ABI Fractura and Assembler computer packages and analyzed using ClustalW [23] and Omiga (version 1.1) (Oxford Molecular Group, UK). Polymerase chain reactions (PCR) were performed using DyNAzyme II DNA Polymerase (Finnzymes, Celbio, Milan, Italy), repetitive sequence element polymerase chain reaction (REP-PCR) were performed using Eurobiotaq® DNA Polymerase (Eurobio, France).

2.4. Isolation of PAH-transforming bacteria

Enrichment cultures were set up with 1 g of soil in 99 ml of Defined Medium in 250 ml Erlenmeyer flasks aseptically supplemented with naphthalene or the combination of naphthalene, phenanthrene and dibenzothiophene as carbon sources. After four sub-cultures, an appropriate dilution of the resultant culture was spread onto solidified Defined Medium plates containing naphthalene, phenanthrene or dibenzothiophene.

2.5. Isolate taxonomic characterisation by amplified ribosomal DNA restriction analysis and REP-PCR

The recovered isolates were clustered in different Operational Taxonomic Units (OTUs) by amplified ribosomal DNA restriction analysis (ARDRA) [24] and REP-PCR [25,26]. The ARDRA was performed digesting the amplification products with *Hae*III and *Hha*I. All analyses were performed at least twice for each isolate. The gene encoding for the 16S rRNA of one microorganism for each OTU was amplified, sequenced on both strands, and aligned to the sequence databases using BLASTN [27].

2.6. Substrate utilisation and dioxygenase tests

PAH utilisation was determined in Defined Medium by observing the appearance of coloured intermediates or a clearing zone on solidified media. Utilisation of salicylate, gentisate and phthalate was evaluated by observing the growth media turbidity increase. Dioxygenase activity was examined by monitoring the conversion of indole to indigo on solidified plates as previously described [28]. All the analyses were performed at least twice for each isolate.

2.7. Denaturing gradient gel electrophoresis and reverse-transcriptase denaturing gradient gel electrophoresis analyses

The V-3 region (position 341–534, *E. coli* numbering) of bacterial 16S rDNA was amplified by PCR using primers p3/p2 [29]. Soil 16S ribosomal copy (rcDNA) was obtained by reverse-transcriptase PCR (RT-PCR) with the M-MLV reverse transcriptase, RNA H Minus, Point Mutant (Promega, Milan, Italy), from 70 ng of to-

tal soil RNA by using the p2 primer (primer annealing at 42 °C for 10 min, extension at 50 °C for 1 h). An appropriate dilution of the obtained product was used as template for PCR reactions with the p3/p2 primer set.

The PCR products were separated on polyacrylamide gels (8% [wt vol^{-1}], 37.5:1 acrylamide-bisacrylamide) with a 30–60% linear gradient of urea. Denaturing gels were run using the Dcode Universal Mutation Detection System (Bio-Rad, USA). The gels images were acquired using the ChemDoc (Bio-Rad) gel documentation system.

2.8. Amplification by PCR of the genes encoding the α subunit of the initial PAH-dioxygenase

The primer sets nahAcfor/nahAcrev, P9047/P8073, dbtAcfor/dbtAcrev (Table 1) were used to amplify the gene encoding the α subunit of the initial PAH-dioxygenase using total DNA extracted from soil as a template. Genomic DNAs from *Pseudomonas putida* ATCC17484, *Burkholderia* sp. RP007 ICMP 13529, and *Burkholderia* sp. DBT1 [8] were used as positive controls. The reaction conditions for the dbtAcfor/dbtAcrev primer set were as follows: 94 °C for 2 min, at 58 °C for 45 s, 72 °C for 45 s, 25 cycles, 72 °C for 5 min. All the primer sets described in Table 1 were used to amplify genomic DNA extracted from the PAH-transforming isolates.

2.9. Southern hybridisation

Products of PCR amplifications, obtained by using the primers sets reported in Table 1 on genomic DNA of the isolates, were resolved on agarose gel and blotted on a nylon membrane (Hybond N^+, Amersham Bioscience) using standard protocols [21]. A portion of the *nah* Ac gene (AF004284) was amplified using the nahAcfor/nahAcrev primer set on genomic DNA from *P. putida* ATCC17484 and used as a probe (nahAc probe). The labelling of the probe and hybridisation of the membrane were performed using the Gene Images™ CDP-Star™ Detection Module (Amersham Biosciences) following the manufacturer's instructions.

3. Results

3.1. PCR-based analyses of the soil bacterial community

Analysis of soil 16S rDNA and 16S rcDNA by Denaturing gradient gel electrophoresis (DGGE) and reverse-transcriptase denaturing gradient gel electrophoresis (RT-DGGE), respectively, showed that a metabolically active bacterial community characterised the contaminated site. The predominance of a restricted bacterial population was not observed (Fig. 1).

PCRs designed to amplify the *nah*, *phn* and *dbt* PAH-transforming genotypes with primer sets nahAcfor/nahAcrev, P9047/P8073, dbtAcfor/dbtAcrev, respectively (Table 1), gave no amplification products on DNA extracted from soil.

3.2. Isolation and biochemical characterisation of PAH-transforming bacterial strains

In order to isolate PAH-transforming strains, selective enrichment cultures were set up. Twenty-one PAH-transforming isolates were recovered, 6 from enrichment culture with naphthalene (N001–N006), and 15 from enrichment culture with the three PAHs (NDP001–NDP015) (Table 2).

Biochemical analyses of the 21 isolates are shown in Table 2. Investigation of aromatic ring oxygenase activity showed that most strains were able to convert indole to indigo. Based on carbon source utilisation, the isolates can be grouped into two catabolic sets, a set transforming naphthalene, phenanthrene and dibenzothiophene, and a set transforming naphthalene and dibenzothiophene. Among the naphthalene-transforming isolates, some of

Table 1
PCR primers for the α subunit of the initial PAH-dioxygenases used in this study

Primers	Sequences (5′–3′)	References
nahAcfor	TGGCGATGAAGAACTTTTCC	[13]
nahAcrev	AACGTACGCTGAACCGAGTC	[13]
P8073	TTCGAGCTGGAATGTGAGC	[13]
P9047	AATAACCGGCGATTCCAAAC	[13]
dbtAcfor	GGCAAGCTCTACCGAAAATGG	This study
dbtAcrev	GTGTTAGTACCCCAGAGATATGAGTTGT	This study
COM1for	AAAAGAGTTGTACGGCGATG	[32]
COM1rev	ACGGTAGAATCCGCGATAGC	[32]
RieskeF	TGYCGBCAYCGBGGSAWG	[33]
RieskeR	CCAGCCGTGRTARSTGCA	[33]
AJ025	TAYATGGGBGARGAYCCVGT	[34]
AJ026	GCRAAYTTCCARTTRCABGG	[34]
Do-1s	TGYAGYTWYCAYGGNTGG	[35]
Do-1a	TCNRCNGCRAAYTTCCARTT	[35]

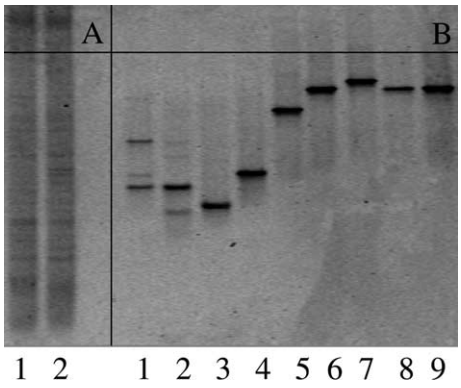

Fig. 1. DGGE profiles. *Panel A*: lane 1, 1 μg of PCR amplification product (p3/p2) of V3 region of rcDNA of the bacterial community; lane 2, 1 μg of PCR amplification product (p3/p2) of V3 region of 16S rDNA of the bacterial community. *Panel B*: 200 ng of PCR amplification products (p3/p2) of V3 region of 16S rDNA of the nine isolates; lane 1, NDP012 (OTU9); lane 2, NDP008 (OTU8); lane 3, NDP007 (OTU7); lane 4, NDP006 (OTU6); lane 5, NDP003 (OTU5); lane 6, NDP005 (OTU4); lane 7, NDP004 (OTU3); lane 8, (OTU2); lane 9, N001 (OTU1).

these took place through the salicylate-catechol pathway [7], while others were incapable of metabolising salicylate and gentisate, both intermediates of the naphthalene-transforming pathway [2]. Actually any of the strains utilised gentisate. In relation to phenanthrene, some isolates went through salicylate-catechol and through phthalate-protocatecuate, while the others were incapable of metabolising both intermediates of the phenanthrene-transforming pathway [30]. All the isolates transformed dibenzothiophene producing orange coloured metabolites, as reported by Kodama et al. [31].

3.3. Taxonomic characterisation of the bacterial strains isolated

Combining the results obtained by ARDRA and REP-PCR analyses of the 21 isolates, nine distinct OTUs were obtained (Table 2). One isolate for each OTU was selected for further taxonomic analysis (N001, NDP001, NDP003, NDP004, NDP005, NDP006, NDP007, NDP008, NDP012). The sequencing of the corresponding 16S rRNA genes indicated that the isolates belonged to different genera (Table 2). The genus *Pseudomonas* was represented by two different ARDRA and five different REP-PCR profiles. All the other genera were represented by a single ARDRA and REP-PCR profile.

The DGGE pattern of the partially amplified 16S rDNAs of the nine isolates selected for taxonomic analysis were compared to the DGGE and RT-DGGE profiles of the entire bacterial community (Fig. 1). Any of the major bands in the DGGE profile migrated in the gel at the same position of the nine isolates, indicating that these latter were not numerically more represented than others.

3.4. Characterisation of PAH-transforming genotypes

The nine strains selected for taxonomic analysis were further analysed in relation to the PAH-transforming genotype. Amplification of the α subunit of the initial PAH-dioxygenases was attempted using the primers sets shown in Table 1. Using the nahAcfor/nahAcrev set of primers, products of the expected size (992 bp) were obtained for all the *Pseudomonas* sp. strains (Table 3). The identity of the amplification products was verified by hybridisation and Southern analysis with the nahAc probe (Table 3). The nahAcfor/nahAcrev set of primers gave no amplification products for the isolates belonging to *Stenothrophomonas*, *Klebsiella*, *Comamonas*, and *Leucobacter* sp. By using primers specific for *phn* and *dbt*-genotypes, no amplification products were obtained for any of the nine isolates selected; no amplification products were obtained using the COM1for/COM1rev primers set, specific for the α subunit characterising the genus *Comamonas*.

Table 2
Taxonomic and biochemical characterization of PAH-transforming isolates

Bacterial isolates	OTU	Organism showing greatest similarity[a]	Dioxygenase test	Growth on[b]			
				Nah/DBT	Phn	SA	PA
N001 N002 N003 N004 N005 N006	OTU1	*Pseudomonas* sp. Fa27	+	+	+	+	–
NDP001 NDP002	OTU2	*Pseudomonas* sp. Fa24	+	+	+	+	–
NDP004 NDP011	OTU3	*Pseudomonas* sp. HR13	–	+	+	+	–
NDP005 NDP009 NDP010	OTU4	*Pseudomonas* sp. dcm7B	–	+	+	+	+
NDP003	OTU5	*Pseudomonas* sp. HR26	+	+	+	+	+
NDP006	OTU6	*S. maltophilia* LMG 10877	–	+	–	–	–
NDP007	OTU7	*C. testosteroni* MBIC 3840	–	+	–	–	–
NDP008	OTU8	*L. komagatae*	–	+	–	–	–
NDP012 NDP013 NDP014 NDP015	OTU9	*K. oxytoca*	+	+	+	–	–

[a] *Pseudomonas* sp. Fa27 (AY131221); *Pseudomonas* sp. Fa24 (AY131219); *Pseudomonas* sp. HR13 (AY032725); *Pseudomonas* sp. dcm7B (AF430125); *Pseudomonas* sp. HR26 (AY032726); *S. maltophilia* LMG 10877 (AJ131784); *C. testosteroni* MBIC 3840 (AF172067); *L. komagatae* (AB007419); *K. oxytoca* (U78183).

[b] Nah, naphthalene; DBT, dibenzothiophene; Phn, phenanthrene; SA, salicylic acid; PA, phthalic acid.

Table 3
Genotypic characterization of PAH-transforming isolates

Bacterial isolates[a]	PCR using[b]						Hybridization with nahAc probe[c]	
	nahAcfor/nahAcrev	P8073/P9047	dbtAcfor/dbtAcrev	COM1for/COM1rev	RieskeF/RieskeR		nahAcfor/nahAcrev	RieskeF/RieskeR
N001	+	–	–	–	(+)[d]		+	+
NDP001 NDP003 NDP004 NDP005	+	–	–	–	+		+	+
NDP006 NDP007 NDP008 NDP012	–	–	–	–	–		–	–
ATCC17484	+	–	–	nd	+		+	+
RP007	–	+	+	nd	nd		–	nd
DBT1	–	–	+	nd	nd		–	nd

[a] ATCC17484, *P. putida* ATCC17484; RP007, *Burkholderia* sp. RP007 ICMP 13529; DBT1, *Burkholderia* sp. DBT1 [8].
[b] +, PCR product of expected length; (+), weak PCR product of expected length; –, no PCR product; nd, not determined.
[c] +, Hybridization signal with nahAc probe; –, no hybridization signal with nahAc probe; nd, not determined.
[d] Several PCR products of different length.

Degenerate primer sets (RieskeF/RieskeR; DO-1s/DO-1a; AJ025/AJ026) were also used. By using RieskeF/RieskeR (Table 2), amplification products of the expected size were obtained only for *Pseudomonas* sp. isolates. Strain N001 showed a multiple profile with a faint band of the expected size (78 bp). In this case, the identity of the amplification products was also verified by hybridisation and Southern analysis using the nahAc probe.

By using the DO-1s/DO-1a and AJ025/AJ026 primer sets, non-specific amplification products were obtained. The possibility that these might also include the amplification product of the expected size (300 and 434 bp, respectively) was verified hybridising the blotted amplicons with the nahAc probe. No hybridisation of the expected size was obtained.

4. Discussion

The intrinsic capacity of contaminated soils to degrade PAHs is related to the presence of a possibly heterogeneous but metabolically active microbial community. Microbial counts have been reported to provide information about the presence of viable microorganisms in a contaminated site; however, the protocol suffers from a number of problems related to the fact that only a small fraction of microorganisms (1–10%) can be isolated and cultivated on laboratory media [36,37]. In this study, DGGE and RT-DGGE analysis of the soil bacterial community provided a more valid protocol to initial evaluation of the complexity of the latter. In fact, the number of bands observed by DGGE analysis is interpreted as an indication of the number of predominant microbial members in the bacteria community. The number of bands in RT-DGGE profile approximately represents the number of metabolically active bacteria strains. Our results indicate that in the site of interest, PAH contamination is associated with the presence of a heterogeneous bacterial community that is numerically rich in metabolically active candidates, with any strong evidence for the presence of a dominant, restricted microbial population. Different approaches were attempted in order to characterize the PAH-transforming capacity of this heterogeneous bacterial community.

Non-cultivation-based molecular techniques for detecting microbial genes in environmental samples are considered a powerful tool to study the presence of a PAH-transforming bacterial community. However, in this study, previously described primers specifically designed to recover the *nah* and *phn* genotypes (nahAcfor/nahAcrev, P9047/P8073), failed to give amplification products on DNA directly purified from soil. Moreover, the same result was obtained by using the dbtAcfor/dbtAcrev primers, here designed to recover the *dbt* genotype. One set (nahAcfor/nahAcrev), among the three

used, gave amplification products only on isolates recovered from enrichment cultures. An impediment to PCR-based protocols is the initial concentration of the template that, in this case, is directly related to the numerical representation of the target genotype. Thus, the result of PCR amplification obtained may be affected by the dilution factor imposed by the heterogeneity of the bacterial community and eventually by the heterogeneity of the related PAH-transforming genotypes.

The failure to evaluate the PAH-transforming capacity of the bacterial community of interest by non-cultivation PCR-based protocols suggested the use of a cultivation-based approach in order to eventually characterise the genotypes of interest in a less complex context as axenic cultures. Thus, cultivable PAH-transforming bacteria were recovered and affiliated to different taxa by ARDRA and REP-PCR analyses. Almost all the microbes could be identified at species level. Most of the isolates were Gram-negative (95.2%), belonging to the γ (90.4%), and β (4.8%) Proteobacteria. The members of the genus *Pseudomonas* formed a remarkably high proportion of the total (66.6%). The Gram-positive isolates belonged to the Actinobacteria represented by the genus *Leucobacter*.

The genera *Comamonas* [5,10,17], *Klebsiella* [38] and *Stenotrophomonas* [39,40] have been previously reported to be capable of transforming PAHs. To our knowledge, this is the first report on the capacity of the genus *Leucobacter* to transform PAHs.

A reduction in recovered bacterial species diversity from enrichment cultures has been reported [41], the same as the dominance of a single species [42]. However, the results here obtained showed that the enrichment protocols adopted led to the isolation of taxonomically different bacteria. Comparing the DGGE profile of the entire bacterial community to the DGGE pattern of the PAH-transforming isolates, these latter microbes were not found numerically more represented than others. In fact, any of the major bands in the DGGE profile migrated in the gel at the same position of the nine isolates. However, their involvement in the PAH-transforming potential of the site is confirmed by the biochemical characterisation, demonstrating their capacity to transform PAHs.

In relation to their PAH-transforming potential, even though many authors observed that isolates from the same environmental sample seemed to be similar to each other regarding specific catabolic activity [14], the isolates analysed here utilised different PAH-transforming pathways. Naphthalene was transformed through salicylate-catechol, as described in *P. putida* G7, and through an unknown pathway related to the strains incapability of metabolising either salicylate or gentisate. Phenanthrene was metabolised as previously reported through salicylate-catechol and through phthalate-protocatecuate. However, it was also metabolised through an unknown pathway related to the incapacity of the strains to metabolise either salicylate or phthalate. Although the novelty of the unknown transforming pathways has to be verified in the present context for both naphthalene and phenanthrene, it is not without precedence [17].

The characterisation of the genotypes responsible for the observed diverse PAH-transformation pathways was further attempted by using degenerate primers for PAH-transforming dioxygenases.

PCR analyses using degenerate primers may provide tools for the detection of PAH-transforming genes, and successful results were reported for isolates derived from enrichment cultures [15,17]. In this study, one primer set (RieskeF/RieskeR) among the three used (RieskeF/RieskeR, DO-1s/DO-1a; AJ025/AJ026) gave specific amplification products only on the *Pseudomonas* sp. isolates, which were previously determined to possess a *nah*-like genotype. In conclusion, the results obtained herein showed that 44% of the taxonomically characterised PAH-transforming isolates did not harbour genes homologous to the classical *nah* genes.

As previously stated, the protocols based on the use of degenerate primers suffer from the limitation of databases related to gene nucleotide sequences of interest. Consequently, the cloning of new PAH-transforming genes will be crucial in order to make a substantial progress.

The integration of non-cultivation and cultivation-based protocols adopted herein was the only way to monitor the PAH-transforming potential of the bacterial community of interest. However, our approach provided the possibility to isolate microbes harbouring new PAH-transforming genotypes. The cloning of the latter may also allow the development of new primer sets and/or probes for the detection of unidentified PAH-degrading isolates in environmental samples. Such analyses are underway by using an insertional mutagenesis approach previously described [8].

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CHAPTER II

Identification of two new sets of genes for dibenzothiophene transformation in Burkholderia sp. DBT1.

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Identification of two new sets of genes for dibenzothiophene transformation in *Burkholderia* sp. DBT1

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Abstract

A novel genotype for the initial steps of the oxidative degradation of dibenzothiophene (DBT) is described in a *Burkholderia* sp. strain isolated from a drain receiving oil refinery wastewater. The strain is capable of transforming DBT with significant efficiency when compared to other microorganisms. Its genotype was discovered by investigating insertional mutants of genes involved in DBT degradation by the Kodama pathway. The cloned *dbt* genes show a novel genomic organization when compared to previously described genes capable of DBT catabolism in that they constitute two distinct operons and are not clustered in a single transcript. Sequence analysis suggests the presence of a σ^{54}-dependent positive transcriptional regulator that may be involved in the control of the transcription of the two operons, both activated by DBT. The achieved results suggest the possibility of novel features of DBT biotransformation in nature.

Introduction

Crude oils contain between 0.04 and 5% (wt/wt) sulfur (Speight 1980), which is mainly represented by organosulfurs including thiols, sulfides, and thiophenes. Condensed thiophenes such as dibenzothiophenes are among the most commonly found organosulfurs in fossil fuels (Bence et al. 1996) and represent the predominant portion of the so-called heavy fraction of crude oils. Moreover, they are more recalcitrant to biodegradation than the non-sulfur containing polycyclic aromatic hydrocarbons (PAH). Dibenzothiophene (DBT) serves as a model molecule for the description of the microbial capacity to transform organosulfurs (Kodama et al. 1970, 1973; Crawford & Gupta 1990; Kropp & Fedorak 1998). One of the described microbial DBT oxidative pathways, the Kodama pathway (Figure 1), transforms the molecule to the final product 3-hydroxy-2-formylbenzothiophene (HFBT). The intermediates of the Kodama pathway are colored compounds whose occurrence in suspension culture is an indication of the microbial activation of this specific oxidative process (Kodama et al. 1970, 1973). Denome et al. (1993) cloned the genes responsible for the Kodama pathway from *Pseudomonas* sp.. These genes are organized in a single operon, encode enzymes of the upper naphthalene catabolic pathway, and belong to a group of genes showing a high degree of homology to the *nah* genes from *Pseudomonas putida* G7 (Simon et al. 1993). The *nah*-like class of genes, cloned from different microorganisms, are highly conserved and are involved in the transformation of molecules that constitute the low molecular weight fraction of PAHs, including DBT (Denome et al. 1993; Menn et al. 1993; Sanseverino et al. 1993; Kiyohara et al. 1994; Geiselbrecht et al. 1998). They

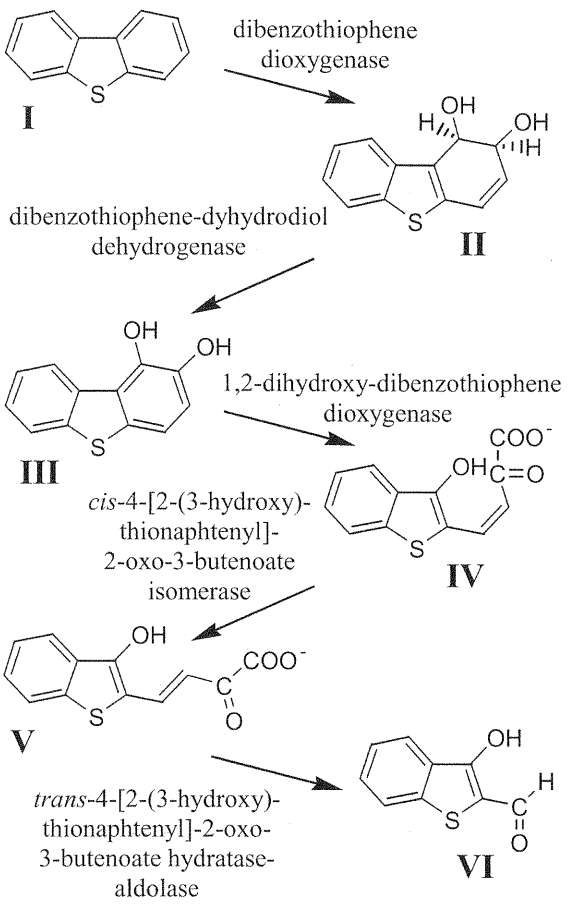

Figure 1. Kodama pathway showing the degradation of DBT to HFBT. (I) dibenzothiophene (DBT); (II) *cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene; (III) 1,2-dihydroxy-dibenzothiophene; (IV) *cis*-4[2-(3-hydroxy)-thionaphthenyl]-2-oxo-3-butenoate; (V) *trans*-4[2-(3-hydroxy)-thionaphthenyl]-2-oxo-3-butenoate; (VI) 3-hydroxy-2-formyl benzothiophene (HFBT).

are normally clustered in a single operon under the control of a single promoter.

The scope of the present work was the isolation and molecular characterization of microbial strains capable of transforming DBT. By analysis of enrichment cultures of an inoculum derived from a drain receiving oil refinery wastewater and grown in presence of DBT, we found a microbial candidate capable of transforming the organosulfur via the Kodama pathway. The microorganism belongs to the *Burkholderia* genus and transforms DBT with significant efficiency compared to other microorganisms. Detailed characterization of the isolate showed the presence of a novel set of genes responsible for the early part of the Kodama pathway. The genes are organized in two operons instead of one, and show only low similarity to the *nah*-like class of genes. The unusual gene organisation suggests the possibility of novel features of DBT transformation in natural context.

Materials and methods

Nucleotide sequences accession numbers

The nucleotide sequences of the DBT oxidizing genes (*dbts*) in *Burkholderia* sp. DBT1 are available in GenBank with accession numbers AF380367 and AF404408.

Chemicals

DBT and solvents were purchased from Sigma Aldrich. Bacteriological preparations were purchased from Difco. Other chemicals and solvents were analytical grade.

Cultivation media

Enrichment cultures were set up in minimal defined medium DM (Frassinetti et al. 1998) supplemented with DBT that was added in excess to DM medium: 500 mg l^{-1} DBT as *N-N*-dimethylformamide (Amersham Pharmacia Biotech) solutions. The solidified medium (noble agar) was supplemented with DBT spraying the surface of the plates with an ethanol free diethyl ether solution (5%) (Kiyohara et al. 1982).

Yeast Mannitol Broth (YMB): 0.5 g l^{-1} K_2HPO_4; 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$; 0.1 g l^{-1} NaCl; 1.0 g l^{-1} yeast extract; 10 g l^{-1} mannitol and Yeast Mannitol Agar (YMA): YMB solidified with 15 g l^{-1} of noble agar were utilized.

Molecular techniques

Standard procedures were used for plasmid DNA preparation, manipulation and agarose gel electrophoresis (Sambrook et al. 1989). Plasmid DNA was extracted using the Qiaprep Spin Plasmid Kit (Qiagen), bacterial genomic DNA using the Nucleospin Tissues Kit (Clontech), and total RNA using the TRIzol Reagent (Invitrogen), following the manufacturer's instructions. Genomic DNA was manipulated using enzymes purchased from Amersham Pharmacia Biotech. Cloned DNA was sequenced using a PRISM Ready Reaction DNA terminator cycle sequencing Kit (Perkin-Elmer) and run on an Applied Biosystems Inc. (ABI) 377 instrument. Nucleotide sequence data

were assembled using the ABI Fractura and Assembler computer packages and analyzed using ClustalW (Thompson et al. 1994) and Omiga (version 1.1) (Oxford Molecular Group, Oxford, UK).

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* DH5α was the host strain for replicative units pH1A, p46 and p51, derived from the insertional mutants, and for pMB393Ac and pMB393C plasmids used for the complementation of the insertional mutants. *E. coli* S17-1 (λ*pir*) was the host and mobilizing strain for the suicide vector pSS240, which harbors a self-cloning promoter-probe minitransposon m*Tn*5Ss*gus*AoriR (see below). *E. coli* harboring the recombinant constructs were routinely grown and maintained on either solidified or liquid Luria Bertani (LB). The antibiotics used were either 100 μg ml^{-1} ampicillin or 50 μg ml^{-1} streptomycin-spectinomycin (Sigma Aldrich). *Burkholderia* sp. DBT1 and its selected insertional mutants were routinely grown at 30 °C; *E. coli* strains at 37 °C.

Isolation of DBT1

Enrichment cultures were set up as described in Frassinetti et al. (1998). The enrichment inoculum was collected from a drain receiving oil refinery wastewater. Twenty-one isolates were recovered and clustered in different operational taxonomic units (OTUs) by ARDRA analysis using primers fD1 and rP2 (Weisburg et al. 1991) to amplify the 16S rRNA genes and digesting the amplification products with *Hae*III and *Hha*I. All the isolates were screened for their capacity to transform DBT based on the capacity to develop an orange color on agar plates supplemented with the molecule. One isolate showed the orange color indicating that the Kodama pathway was active. The corresponding microorganism was sub-cultured for further analysis and the 16S rRNA gene was amplified, sequenced on both strands, and aligned to the database sequences using BLAST (Altschul et al. 1997).

Testing of the isolate

The strain DBT1 was inoculated in tightly closed 125 ml flasks containing 25 ml of DM supplemented with DBT. The growth of the isolate was evaluated by means of CFU counts on YMA plates. The transformation of DBT was monitored spectrophotometrically at 325 nm analyzing the hexane extracts of microbial cultures filtrates, (pore size 0.2 μm), as described by Setti et al. (1995). The DBT concentration was measured using the content of the entire flask. The TLC analyses was performed as described in Frassinetti et al. (1998). The purification and the identification of HFBT were performed by ^{1}H-NMR and GC-MS analysis as previously described by Frassinetti et al. (1998).

Molecular characterization of DBT1

PCR reactions were set up in order to partially clone the genes involved in DBT transformation. The primers used were nahAcfor and nahAcrev, which amplify a 992 bp region encompassing nucleotides 63 to 1055 of *Pseudomonas putida* G7, *nah*Ac (GenBank accession no. M83949) (Simon et al. 1993) and P8073, P9047, which amplify a 993 bp region encompassing nucleotides 82 to 1075 of *Burkholderia* sp. RP007, *phn*Ac (GenBank accession no. AF061751) (Laurie & Lloyd-Jones 2000).

Transposon mutagenesis

Insertional mutants of the strain DBT1 were generated using the protocol by Ditta (1986). In order to obtain a self-cloning promoter-probe minitransposon, the origin of replication pMB1 (oriR), was amplified from plasmid pRL278 (GenBank accession no. L05083) with PCR primers GTCGACCAAAATCCCTTAACGTGA and GTCGACATACGCGTAAAACAGCCAGC. A 1.1 kb amplification product was obtained and cloned into plasmid pCAM140 (Wilson et al. 1995) partially digested with *Sma*I, to form plasmid pSS240. The resulting minitranposon construct harboured the pMB1 (oriR) origin of replication between the promoterless β-glucoronidase (*gus*A) gene and the antibiotic spectinomycin-streptomycin resistance cassette (Sm/sp). Putative insertional mutants (>10,000 total) selected on 50 μg ml^{-1} spectinomycin-streptomycin YMA plates, sprayed with DBT, were tested for the loss of the capacity to form orange colonies. Genomic DNA of putative transposon recipients was extracted and digested with the restriction endonuclease *Pst*I that does not cut inside the m*Tn*5Ss*gus*AoriR minitransposon. The digested DNA was circularized using T4 ligase and electroporated into competent *E. coli* DH5α, successively streaked on solidified LB with 50 μg ml^{-1} spectinomycin-streptomycin. Different replicative units were obtained as plasmid

Table 1. Bacterial strains and plasmids

Strains and plasmids	Description	Sources
Strains		
Burkholderia sp. DBT1	Transforms DBT	Present study
E. coli DH5α	F-, *phi*80d *lac*Z Delta M15, Delta (*lac*ZYA-*arg*F), U169, *deo*R, *rec*A1, *had*R17 (rk−, mk+), *gal*-, *pho*A, *sup*E44, Lambda-, thi-1, *gyr*A96m *rel*A1	Invitrogen
E. coli S17-1 (λ-*pir*)	λ-pir lysogen of S17-1 [*thi pro hsd*R$^-$ *hsd*M$^+$ recA RP4 2-Tc::Mu-Km::*Tn*7(Tp^r/Sm^r)]	Victor de Lorenzo, Centro de Investigaciones Biologicas Madrid, Spain
Plasmids		
pMB393	Spectinomycin-resistant derivative of pBBR1MCS	(Gage et al. 1996)
pMB393Ac	Spectinomycin-resistant derivative of pMB393 complementing MH1A	Present study
pMB393C	Spectinomycin-resistant derivative of pMB393 complementing M51	Present study
pCAM140	Sm/sp, Ap, m*Tn*5S*gus*A40 in pUT/mini-*Tn*5 Sm/sp	(Wilson et al. 1995)
pSS240	Spectinomycin-resistant derivative of pCAM140 harbouring a pMB1 origin of replication	Present study

preparations from several insertional mutants and successively sequenced by inverse PCR on both strands to gain information on the nucleotide sequence of the transposon flanking regions. For this purpose, the primers gus55-TTTGATTTCACGGGTTGG and aadA1846-GCTGGCTTTTTCTTGTTATCG were designed, which anneal to the end regions of the m*Tn*5S*gus*AoriR minitransposon. Three replicative units, pH1A, p46 and p51, were completely sequenced by inverse PCR.

RT-PCR reactions

The strain DBT1 was grown to exponential phase in DM in the presence of DBT or 250 mg l^{-1} citrate (Sigma Aldrich). A total of 10^8 cells were pelleted from which RNA was isolated and successively treated with DNAse I. RNasin (Promega) was added to maintain the integrity of the mRNA. RT-PCR was carried out with M-MLV Reverse Transcriptase, RNA H Minus, Point Mutant (Promega), using respectively, primers p3-CGAATTTCCCGAAGTCCCAATT, p5-GTCGGATCCCAATGCAGAAACTCC, p7-CCGACAATGATAATATGGTCACCGC for the genes cloned on the pH1A replicative unit and p9-CTGATAATCGAGGCGCAAATAGA, p11-TTGTTCCGGTTTACATCGTAGCTC, p13-ATACTGCATGGGCGATATGTTTAAC for the genes cloned on the p51 replicative unit (Figure 2).

The reaction conditions were as follows: annealing at 42 °C for 10 min and extension at 50 °C for 1 h. The PCR reactions were carried out using respectively primers p1-GGGACGAGAAAACGTTATCAATT GCG, p2-GGTCGGAAACATGGGGTAATGGA, p4-GCTCCCATTTCAATGGCACCGTTCTT, p6-GGAATGAAAACGGAGCTGTCCGGA for the genes cloned on the pH1A replicative unit, (Figure 2) and p8-TAGGCTGGCCTATTCCACCTTCA, p10-AGCGGCCAAGCGAATCAATCATTT, p12-GTCTTCCCGGTTATGTGGACATTA or the genes cloned on the p51 replicative unit (Figure 2).

Appropriate positive and negative PCR controls were included. The cycling conditions for the RT-PCR amplification were as follows: 94 °C for 1 min, at 64 °C for 1 min, 72 °C for 2 min, followed by 25 cycles, 72 °C for 5 min. The amplification products were then sequenced.

Complementation of the characterized mutants

MH1A and M51 insertional mutants were complemented. The complementing genomic fragment was obtained by PCR reactions and cloning of the amplified fragments in the broad host range cloning vector pMB393 (Gage et al. 1996). The primers PAcF-CGG*CTCGAG*GCCGTCTCCTCTCGGGAATTTTG-C and PAcR-GGG*GGGCCC*TTATTTTCCGATGAG-CTCGCGGTTCC were designed for DBT1 genomic DNA (Figure 2) to complement the MH1A mutant.

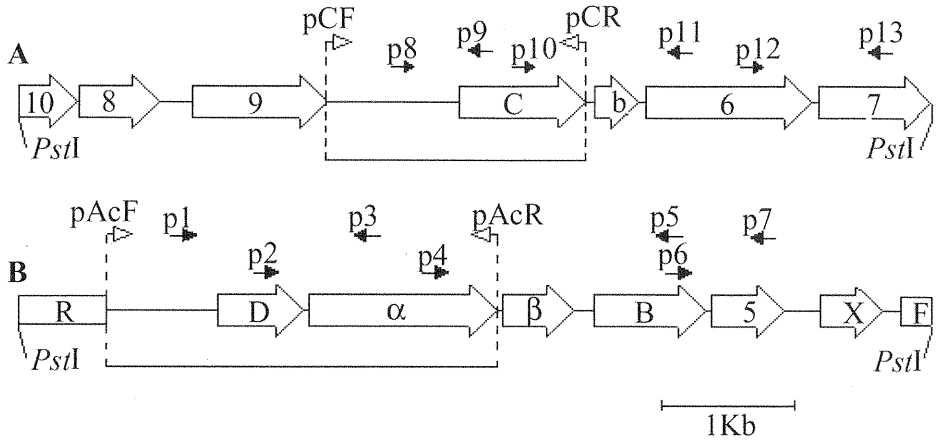

Figure 2. Map of the two DBT oxidizing operons in DBT1. (A) Map of the DBT1 *Pst*I genomic fragment mutagenized in MH1A and M46 insertional mutants. (B) Map of the DBT1 *Pst*I genomic fragment mutagenized in M51. The primers used for RT-PCR experiments (p1–p13) and complementation tests (pAcF/pAcR, pCF/pCR) are reported.

They amplified product from nucleotide 643 to 3486 on the genomic fragment deposited with the accession number AF380367. The cycling conditions were as follows: 94°C for 1 min, at 69 °C for 1 min, 72 °C for 2 min, followed by 25 cycles, 72 °C for 5 min. Both primers had a clamp: *Xho*I clamp was present in pAcF and *Sma*I clamp was present in the pAcR primer.

The primers pCF-CGG*GGTACC*CAATTGGAT TCCCGGATATCTGTCC and pCR-GGG*GGGCC C*TTATTCTTTCAATTTGACGTCAAGGCCG were designed for DBT1 genomic DNA (Figure 2) to complement the M51 mutant. They amplified from nucleotides 2351 to 4162 on the genomic fragment with the accession number AF404408. The cycling conditions were as described above. The pCF primer had a *Kpn*I clamp and the pCR primer had a *Sma*I clamp. The expected products were amplified using the Herculase DNA Polymerase (Stratagene). The PCR products were sequenced and successively cloned in pMB393 as *Xho*I *Sma*I and *Kpn*I *Sma*I fragments respectively, obtaining the pMB393Ac and the pMB393C plasmids complementing MH1A and M51 mutants. The two plasmids were transformed into competent MH1A and M51 mutant cells by electroporation (Dennis & Sokol 1995).

Fluorimetric GUS assay

The assay was performed growing *Burkholderia* sp. DBT1, MH1A, M46 and M51 mutants in YMB and YMB containing DBT. The bacterial cells were collected by centrifugation at different times, resuspended in 50 mM sodium phosphate buffer (pH 7.0), 10 mM β-mercaptoethanol, 10 mM disodium EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100, sonicated, and centrifuged at 16,000 g for 10 min at 4 °C. GUS activity was assayed in the supernatant. Fluorimetric GUS assays were performed as described by Jefferson (1987). Protein concentration in bacterial extracts was determined using the Bradford reagent (BioRad) according to the manufacturer's instructions.

Results

Isolation and biochemical characterization of DBT1

With the scope of isolating microorganisms capable of transforming organosulfurs, 21 candidates were recovered by enrichment cultures of a inoculum derived from a drain receiving oil refinery wastewater grown in the presence of DBT. A single microorganism capable of transforming DBT via the Kodama pathway was selected. The gene encoding for 16S rRNA was amplified, sequenced and analyzed, tentatively assigning the microorganism to the *Burkholderia* genus (99% homology to *Burkholderia* sp. isolate N2P5). The strain was designated *Burkholderia* sp. DBT1 due to its capacity to transform DBT.

The DBT transformation capacity of the strain, initially indicated by the release of colored compounds in the growth medium, was confirmed by the removal of DBT from the growth minimal medium when the molecule was used as a sole carbon and energy source (Figure 3). DBT removal was associated with corresponding microbial growth. The DBT content of the

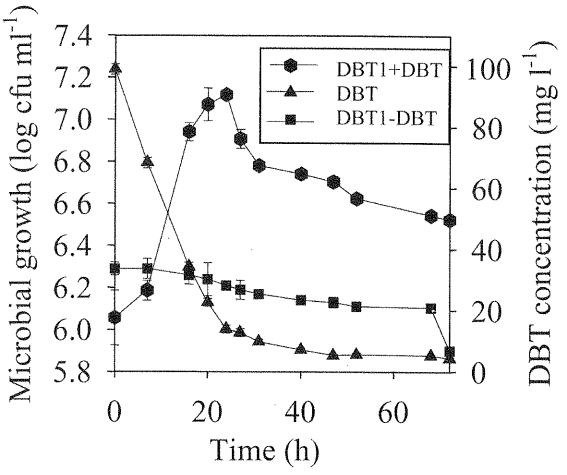

Figure 3. Growth of *Burkholderia* sp. DBT1 in DM supplemented with DBT. Growth of DBT1 cells is recorded only in presence of DBT and is concomitant with a decrease of DBT content in the growth medium. DBT1 does not growth in absence of DBT.

culture decrease by 93% after 72 h of incubation. Separation of the reaction intermediates at the end of the bacterial growth was performed by TLC and six colored spots characterizing the Kodama pathway were recovered (Kodama et al. 1973) (data not shown). A pale yellow compound with an absorption peak at 394 nm (Rf = 0.85) was purified by a column chromatography procedure (Frassinetti et al. 1998) and further analyzed by ^{1}H-NMR. The spectrum indicated four non-equivalent aromatic protons at δ 7.45 (triplet, J = 9 Hz, where J is the coupling constant value) that collapse to doublet upon irradiation at δ 7.96, 7.60 (triplet, J = 9 Hz), 7.80 (doublet, J = 9 Hz), and 8.05 (doublet, J = 9 Hz). The collapsing is ascribable to an aldehydic proton. The mass spectrum of the molecule showed the molecular ion at m/z 178 (100% relative intensity; $C_9H_6O_2S$ requires M^+178) and important fragment ions at m/z 177 (M^+–H), 121, 77 that are expected from an aldehyde. These data are consistent with the final product HFBT reported by Kodama et al. (1973) confirming that DBT1 transform DBT via the Kodama pathway (Figure 1), as expected.

Cloning of dbt genes

To clone the genes involved in the DBT transformation pathway, primers designed to amplify the *nah*-like class of genes were used. In the genus *Burkholderia*, an isofunctional cluster of genes (*phn* operon) has been cloned from a strain capable to transform phenanthrene (Laurie & Lloyd-Jones 1999). Primers designed from *phn* genotype were also used. However, using both the combination of primers, no amplification products were detected using the DBT1 genomic DNA as template.

To characterize the DBT1 genes involved in the transformation of DBT, insertional mutants of the isolate were generated and screened for the loss of the capacity to produce orange-colored intermediates on solidified medium sprayed with DBT. Three stable insertional mutants were obtained (Table 2). As expected, the selected insertional mutants lost the capacity to remove DBT from liquid culture (data not shown).

A set of inverse PCR reactions was programmed on the transposon flanking regions of the three insertional mutants. The results indicated that in two of these (MH1A, M46), the transposon was inserted in the iron sulfur protein (ISP) large (α) subunit of the initial dioxygenase with an opposite orientation. In the third mutant (M51) the transposon was inserted in the PAH extradiol dioxygenase (Table 2). Three replicative units, pH1A, p46 and p51 deriving, respectively, from the three insertional mutants (Figure 4), were completely sequenced.

Based on nucleotide and amino acid sequence similarities, putative gene identifications were assigned to the resulting sequences (Tables 3 and 4). The information obtained from sequence data indicated the occurrence of different ORFs corresponding to (i) genes encoding for enzymes catalysing the different steps of the Kodama pathway and (ii) genes encoding for protein not involved in the same pathway. Thus, the former ORFs were named *dbt* and the latter were not given names. Eight putative ORFs were recovered internal to the p46 and pH1A replicative units (GeneBank accession no. AF380367) (Figure 4). Seven of these ORFs initiated with the expected ATG start codon, while the truncated ORF R (*dbt*R) started with a GTG start codon. A putative ribosomal binding site preceded each start codon. ORF D, encoded for DbtD, a 2-hydroxychromene-2-carboxylate isomerase. ORF α and β encoded DbtAc and DbtAd, respectively the large (α) and small (β) subunit of the initial dioxygenase (ISP). ORF B encoded DbtB, a dihydrodiol dehydrogenase. Downstream the four genes, ORF 5 encoded for a protein similar to a NADH:FMN oxidoreductase, the first of these genes that are not involved in the Kodama pathway. The close arrangement of the described ORFs suggested that the related genes constitute a transcriptional unit (*dbt*D,Ac,Ad,B,ORF5). No ORF was detected 271 bp downstream ORF 5 and an inverted repeat was

Table 2. Burkholderia sp. DBT1 replicative units derived from insertional mutants

Replicative units – Insertional mutants	Sequencing results: primer *gus55*	Sequencing results: primer *aada1846*
pH1A–MH1A *dbt*Ac::m*Tn*5Ss*gus*AoriR	ISP α subunit IC	ISP α subunit
p46 - M46 *dbt*Ac::m*Tn*5Ss*gus*AoriR	ISP α subunit	ISP α subunit IC
p51–M51 *dbt*C::m*Tn*5Ss*gus*AoriR	PAH extradiol diox IC	PAH extradiol diox

ISP, iron sulfur protein; IC, inverse complement; diox, dioxygenase.

Table 3. dbt genes cloned on pH1A replicative unit

ORF	Gene	Nucleotide	Protein feature	% homology isofunctional genes	
R	*dbt*R	642-1	transcriptional activator	*phlR* 60% ***phnR*** 65%	(X91145) (AF061751)
D	*dbt*D	1443-2066	isomerase	***nahD*** 64% ***phnD*** 60%	(U09057) (AF061751)
α	*dbt*Ac	2119-3486	ISP α subunit	***ndoAc*** 68% ***phnAc*** 68%	(M23914) (AF061751)
β	*dbt*Ad	3516-4034	ISP β subunit	***pahAd*** 54% ***phnAd*** 59%	(AB004059) (AF061751)
B	*dbt*B	4182-5000	dihydrodiol dehydrogenase	***bphB*** 65% ***phnB*** 76%	(D17319) (AF061751)
5	ORF5	5029-5562	putative oxydoreductase	*ntaB* 62%	(I40751)
X	ORFX	5833-6282	unknown protein	***nahX*** 69% ***phnX*** 83%	(AF100302) (AF112137)
F	ORFF	6418-6643	salicylaldeyde dehydrogenase	***bphA1C*** 83%	(AF079317)

The cloned ORFs, where possible, were compared either to the highly conserved *nah*-like class of genes or to the *phn* sequences (indicated in boldface). The GeneBank accession numbers are reported.

detected 91 bp downstream the ORF 5 stop codon. The inverted repeat is capable of forming a stem-loop structure that may be a transcriptional terminator. Downstream the above-mentioned 271 bp, two putative ORFs, ORF X and the truncated ORF F, were detected. They encoded, respectively, for a protein homologous to PhnX cloned in *Burkholderia* sp. RP007 and for the N-terminal of a salicylaldehyde dehydrogenase. Neither protein is involved in the Kodama pathway.

Upstream all the described structural ORFs, separated by 800 bp, ORF R encoded for the N-terminal of DbtR, a putative σ^{54}-dependent transcriptional regulator. The above-mentioned regulative ORF R was divergently transcribed with respect to the structural ORFs described. The putative partial coding region showed 65% of homology to the amino acid sequences of the positive transcriptional regulators of the NtrC family (Buikema et al. 1995).

In summary, the described organization of the different ORFs cloned from the p46 and pH1A replicative units suggests that they contain two transcriptional units, the *dbt*D,Ac,Ab,B,ORF5 and the truncated ORFX,F both preceded by the regulative truncated gene *dbt*R.

With regards to p46 and pH1A, the complete nucleotide sequence of p51 replicative unit was determined, and seven putative ORFs were cloned (Figure 4) (GeneBank accession AF404408). The cloned ORFs initiated with a ATG start codon and a putative ribosomal binding site preceded each of the ORFs. ORF 10 encoded the C-terminal of a protein homologous to

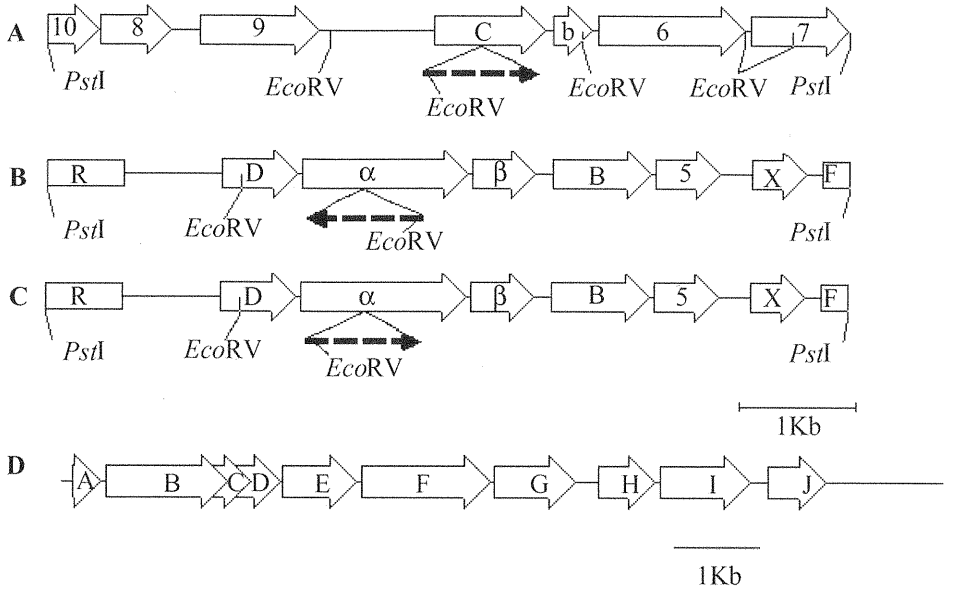

Figure 4. Diagram of the p51 (A), p46 (B), pH1A (C) replicative unit structures. The dotted arrow represents the transposon and the orientation of the reporter gene *gus*A with respect to the coding orientation of the mutagenized transcripts. The DBT1 genes responsible for oxidation of DBT via the Kodama pathway are designated as R for *dbt*R, D for *dbt*D, α for *dbt*Ac, β for *dbt*Ad, B for *dbt*B, C for *dbt*C, b for *dbt*Ab. The other ORFs were indicated as follows: 5, ORF5; 6, ORF6; 7, ORF7; 8, ORF8, 9, ORF9, X, ORFX; F, ORFF. (D) Diagram of the cluster of *dox* genes responsible for oxidation of DBT in *Pseudomonas* sp. (Denome et al. 1993). The genes are designated as A for *dox*A, B for *dox*B, C for *dox*C, D for *dox*D, E for *dox*E, F for *dox*F, G for *dox*G, H for *dox*H, I for *dox*I, J for *dox*J.

the large subunit of an aromatic oxygenase. ORF 8 encoded for a protein analogous to 2-hydroxychromene-2-carboxylate isomerase from the PAH catabolic pathway, showing 57% of homology to the isofunctional DbtD cloned on p46 replicative unit. ORF 9 encoded for a protein homologous to an oxidoreductase. No ORFs were observed in the 974 bp region downstream ORF 9. Downstream the untranscribed region, four ORF were recovered. ORF C and ORF b encoded, respectively, for DbtC, an extradiol dioxygenase, and for DbtAb, a ferrodoxin subunit of the initial dioxygenase (ISP). ORF 6 and ORF 7 encoded for proteins homologous to an oxidoreductase and for the C-terminal of an hydrolase, respectively, which are not involved in the Kodama pathway. The described organization of the different ORFs suggests that the p51 unit harboured two truncated transcriptional units, ORF10,9,8 and *dbt*C,Ab,ORF6,ORF7.

In summary, these results indicated that two independent insertional events in two genes encoding for dioxygenases determines the loss of the capacity of DBT1 to transform DBT via the Kodama pathway. The results of sequence analysis indicated that knocking out two genes putatively determines also the loss of two distinct transcriptional units, resulting from the close arrangement of the cloned genes on the flanking regions. Thus, the co-transcription of the genes constituting the putative transcriptional units involved in DBT transformation was verified by RT-PCR. RNA was extracted from DBT1 grown under inducing (DM, DBT) and uninducing (DM, citrate) conditions and used as target for RT-PCR amplification of partial gene sequences (Figure 2). The cDNAs derived from induced cells showed amplification products of the size expected by co-transcription (data not shown). No cDNAs were obtained from uninduced cells. All positive and negative PCR controls gave the expected results. The identity of the RT-PCR products was confirmed by sequencing the amplified fragments and indicated that DBT induces the transcription of the transcriptional units *dbt*D,Ac,Ad,B,ORF5 and *dbt*C,Ab,ORF6,ORF7. No transcription was observed upstream *dbt*D and *dbt*C and downstream ORF5.

The two transcriptional units might constitute two separate operons, and, by examination of the orientation of transcription, the expected transcription initiation sites and the promoter regions should be located upstream *dbt*D and *dbt*C. Thus, the untranscribed regions upstream the two clusters of oxidative genes, respectively 800 bp on pH1A and 974 bp

Table 4. *dbt* genes cloned on p51 replicative unit

ORF	Gene	Nucleotide	Protein feature	% homology isofunctional genes	
10	ORF10	1-435	aromatic oxygenase	*bphA1c* 83%	(AF079317)
8	ORF8	449-1039	isomerase	***pahD*** 60%	(AB004059)
				phnD 66%	(AF061751)
9	ORF9	1275-2264	oxydoreductase	*mll7162* 64%	(AP003011)
C	*dbt*C	3239-4162	PAH extradiol	***nahC*** 74%	(AF039533)
			dioxygenase	***phnC*** 21%	(AF061751)
b	*dbt*Ab	4236-4547	ISP ferrodoxin	***doxA*** 53%	(M60405)
			subunit	***phnAb*** 72%	(AF061751)
6	ORF6	4602-5822	oxydoreductase	*spr0246* 44%	(AE008406)
7	ORF7	5877-6707	hydrolase	*todF* 47%	(D90906)

The cloned ORFs, where possible, were compared either to the highly conserved *nah*-like class of genes or to the *phn* sequences (indicated in boldface). The GeneBank accession numbers are reported.

on p51 replicative unit, might have the consensus sequences necessary to function as promoters. In order to verify this hypothesis, the insertional mutants were tentatively complemented by constructing two expression vectors harboring the two untranscribed regions as inducible promoters (Figure 2). The complementing genomic fragments were obtained by PCR reactions on DBT1 genomic DNA using primers pAcF and pAcR to complement MH1A mutant, and pCF and pCR to complement M51 mutant (Figure 2). The two genomic regions amplified by PCR were cloned into pMB393, obtaining the pMB393Ac and the pMB393C plasmids complementing, respectively, the MH1A and M51 insertional mutants. The complemented insertional mutants recovered the capacity to produce orange-colored colonies on solid medium sprayed with DBT, indicating the occurrence of DBT transformation. The results indicated the presence of two distinct promoters upstream the two transcriptional units induced by DBT, confirming the hypothesis that these transcriptional units constitute two distinct operons.

GUS assay

In order to further analyze the induction of the two operons, a GUS activity fluorimetric assay was performed on MH1A, M51, M46 insertional mutants and on DBT1 as a control, in presence (inducing conditions) and absence (non-inducing conditions) of DBT (Figure 5). GUS activity was not detected in uninduced bacteria grown in YMB. Addition of DBT to the growth medium rapidly induced GUS activity in the MH1A and M51 mutants. GUS activity was not induced in either the M46 mutant (internal control) or in DBT1. The specific activity of GUS at the end of the exponential growth phases of the two mutants is 8 to 10 times higher than that observed at the two initial growth phases (Figure 5).

Discussion

The objective of the present study was the isolation and characterization of microorganisms capable of transforming DBT. By a culture-based approach, *Burkholderia* sp. DBT1, capable of transforming DBT via the Kodama pathway, was isolated from a drain receiving oil refinery wastewater. The assumption that DBT1 is able to transform DBT was initially based on the observation of the microbial release of colored intermediates in presence of DBT. This initial assumption was confirmed by the recovery, in the microbial growth medium, of the final product of the Kodama pathway (HFBT). Moreover, amending DBT as the sole carbon and energy source, DBT1 in liquid culture shows a clear growth, concomitantly to decreased levels of the compound in growth medium. In fact, after three days DBT is nearly completely removed. The DBT transformation time course may also be compared to that reported for other DBT transforming microorganisms. *A Rhizobium* strain, under similar growth conditions and following the same oxidative pathway, has been reported to remove the same DBT content in a significantly longer time (5 days) (Frassinetti et al. 1998). In our opinion, DBT1 may be

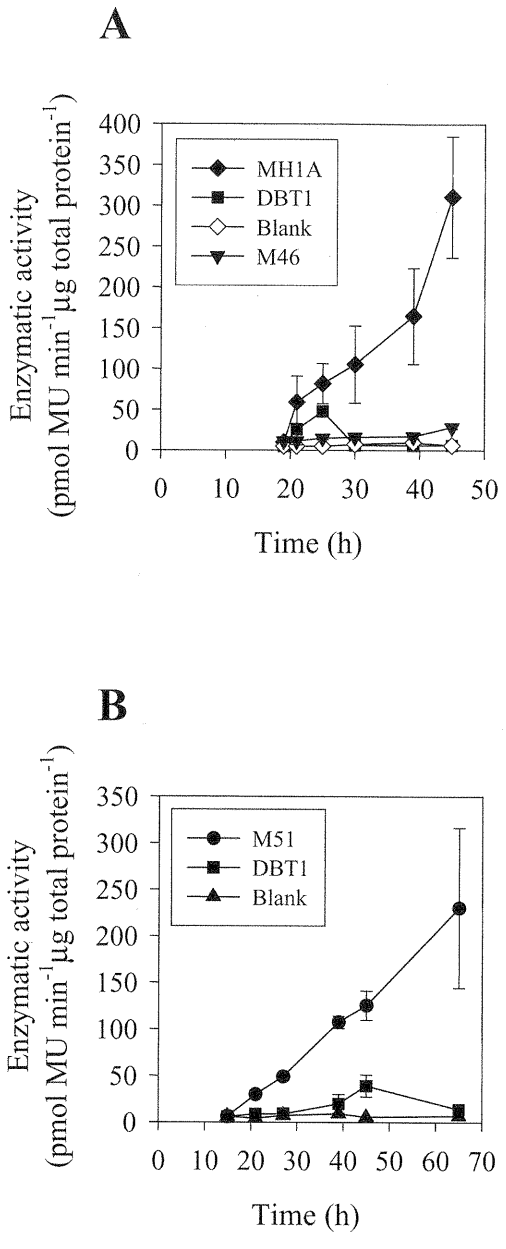

Figure 5. GUS activity in MH1A and M51 mutants harboring the reporter gene *gus*A with the same transcription orientation with respect to the one of the mutagenized operons. As controls, the GUS activity of DBT1 and M46 mutant (internal control because *gus*A in the reverse orientation with respect to the mutagenized operon) is reported.

considered an efficient bio-catalyst of DBT conversion to HFBT. Recently, mixed cultures capable to mineralize HFBT to CO_2 has been described (Bressler & Fedorak 2001). Thus, the isolation of a microorganism that rapidly transforms DBT to molecules that are easily mineralized, may prefigure new potentialities in the exploitation of microbial DBT biodegradation.

The transforming capacity of DBT1 is attributable to two novel sets of genes encoding for the initial steps of the oxidative Kodama pathway that were cloned by insertional mutagenesis.

The genes responsible for the Kodama pathway were initially cloned from *Pseudomonas* sp. by Denome et al. (1993) and were clustered into a single operon, responsible for the oxidation of naphthalene to catechol. They belong to the *nah*-like class of genes that, as previously mentioned, encode for enzymes catalyzing the transformation of the low molecular fraction of PAHs, and are usually involved in the oxidation of DBT via the Kodama pathway. These genes have been reported to be as highly conserved, clustered in one operon, and under the control of a single promoter (Zylstra et al. 1997).

The failure to recover an amplification product using primers designed to amplify the *nah*-like class of genes was the first evidence that DBT1 genes involved in DBT transformation were not highly similar to the conserved isofunctional oxidative genes. At the same time, the primers designed to amplify the isofunctional *phn* genes cloned from *Burkholderia* sp. RP007, also failed to yield a product, showing low similarity in the nucleotide sequences of isofunctional genes, even within the same microbial genus.

In order to clone the DBT1 genes involved in the Kodama pathway an insertional mutagenesis approach was adopted. The capacity of DBT1 to transform DBT was lost by knocking out the genes encoding for the initial dioxygenease (ISP) and the dihydrodiol dioxygenase (1,2-dihydroxy-dibenzothiophene dioxygenase). Sequence analysis of the replicative units derived from the insertional mutants suggested that in DBT1, the DBT transforming genes are organized in two separate transcriptional units. The results with RT-PCR corroborated this hypothesis, as co-transcription of the genes harbored by the two putative transcriptional units was observed and co-transcription of the latter with their upstream and downstream flanking regions was not recovered. As predicted, the cloned genes showed only 60% similarity to the highly conserved *nah*-like class of genes, instead of 90% reported

for the latter, as well as also 60% homology with the isofunctional *phn* genes.

The complementation of the insertional mutants indicated that the genomic regions cloned upstream of the two functional transcriptional units functioned as promoters, indicating that the knocking out of the genes encoding for the two dioxygenases also knocked out two separate operons. The two operons encode for the enzymes involved in the initial steps of the Kodama pathway (e.g. the large (α) and small (β) subunit of the initial dioxygenase (ISP), the corresponding ferrodoxin subunit, the dibenzothiophene-dihydrodiol dehydrogenase, the 1,2-dihydroxy-dibenzothiophene dioxygenase, and the isomerase). However, the enzyme catalyzing the last step of the HFBT formation (the *cis*-4-[2-(3-hydroxy)-thionaphtenyl]-2-oxo-3-butenoate hydratase-aldolase) (Figure1) has not been recovered by insertional mutagenesis, suggesting the presence of a third operon harboring the missing enzyme. In conclusion, even though as previously mentioned, isofunctional genes have been described as highly clustered, we conclude that DBT1 shows a scattered genomic organization of the genes involved in the catabolism of DBT.

An additional result of RT-PCR reaction consisted in the evidence that the two cloned operons were both transcribed in presence of DBT. Moreover, the GUS-assays showed that DBT strongly induces the translation of the two operons, as indicated by the significant increment in β-glucoronidase activity observed in presence of the molecule.

As a consequence, it can be suggested that the strong activation of the two operons by DBT observed may be responsible for the high efficiency of DBT transformation by DBT1. The presence of a gene (*dbt*R) encoding for a σ^{54}-dependent regulator indicates a possible involvement of the *dbt*R in the transcriptional activation of the two DBT1 operons. The location and direction of transcription of *dbt*R with respect to the structural genes harbored by one of the two operons has been reported in different aromatic catabolic operons (Inouye et al. 1988; Shingler et al. 1993; Ng et al. 1995). Therefore, despite the novel structural gene organization, DBT1 appears to have a conserved putative mechanism of transcription activation of catabolic genes by DBT.

The scattered genetic organization described in DBT1 resembles that one observed in *Sphingomonas* genus, where the catabolic genes involved in the oxidation of aromatic compounds to catechol intermediates are distributed on multiple gene clusters (Armengaud et al. 1998; Romine et al. 1999). It is also worth noting that the oxidative genes cloned in DBT1 are not organized in discrete pathway units. They are grouped on the same operon with oxidizing genes not associated with the Kodama pathway. Even though the DBT accumulating metabolite identified in the growth medium of DBT1 was HFBT, as expected, the involvement of genes not associated with the Kodama pathway in oxidative reactions on the DBT accumulating metabolites could not be excluded and must be further investigated. However, the involvement of these genes in the oxidative pathways of different classes of compounds may be also possible. This unusual organization of oxidizing genes in relation to pathway units has been described also in *Sphingomonas* sp. (Zylstra & Kim 1997), and, although speculative, it is possible to suggest that the activation of operons that encode for groups of genes of different oxidizing pathways may increase the capabilities of a microorganism in response to different environmental conditions.

Conclusions

A microbial strain, *Burkholderia* sp. DBT1, capable of efficiently transforming DBT to intermediates that can be mineralized has been isolated. The microorganism joins a high efficiency in DBT transformation to low similarity in nucleotide sequences and genomic organisation of *dbt* genes with isofunctional one. At present, the explanation of the relation between the two evidences may be only speculative. However, the cloning of the *dbt* genes expands current knowledge of the genetics of the microbial capacity to transform DBT. The enlargement of the current information on the genes governing the process is, in our opinion, an important step in understanding the prevalence and distribution of new groups of genes involved in the catabolism of organosulfur compounds. In fact, probes developed for specific transforming genes should eventually provide a useful tool for a detailed characterization of the ecology of organosulfur transforming microorganisms within a contaminated site.

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