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Digestate valorization for bioremediation of petroleum hydrocarbons contaminated soils

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Abstract

Petroleum contaminated soils constitute an environmental issue which may be solved with the help of bioremediation. Soil bioaugmentation with petroleum degrading microorganisms is an efficient clean-up strategy. Currently scientific interest is focused on searching new sources of microorganisms able to degrade hydrocarbons which serve as species pools for enrichments during inoculum preparation. Bioaugmentation strategy are especially important in soils with low level of organic matter and low microbial counts (*e.g.* after intensive chemical treatments). No studies were performed up to date while considering the potential of organic fertilizers and amendments as a microbial seeding source for bioremediation. In this thesis, for the first time, digestate as an example of organic amendment, was studied in terms of indigenous microbial community which can be involved in degradation of linear hydrocarbons. Digestate is an organic by-product of biogas production via anaerobic digestion processes and has a great potential as soil fertilizer due to concentrated nutrients and low content of easily biodegradable compounds (which could be used by bacteria as a preferential carbon source over hydrocarbons). However, the potential of microbial community of digestate was never studied in terms of petroleum hydrocarbons (PHCs) degradation.

In this thesis, digestate was examined as microbial seeding for bioremediation of weathered petroleum hydrocarbon contaminated soils. The goals were : I) to check the presence of alkanes degrading bacteria in digestate enrichments and compare alkanes degradation potential with enrichments from petroleum contaminated soils, ii) verify the effect of digestate application on soil microbial community and microbial activity, iii) study the presence of functional genes responsible for alkanes degradation (*alkB* genes) in digestate and amended soils.

During the first experiment, 7 microbial enrichments were developed from various digestates (including composted digestate), a petroleum contaminated soil and from mix of soil with digestate. After 3 weeks of incubation the highest diesel fuel removal was observed for enrichments originating from composted digestate and from the petroleum contaminated soil (78 and 77 % diesel fuel removal, respectively). Enrichments obtained from digestate mixed with soil displayed lower performance than single source enrichments. In all enrichments, presence of *alkB* genes was promoted during the incubation. The experiment revealed the presence of *alkB* genes in bacteria from digestate and confirmed their ability to degrade diesel fuel.

In a second experiment, 6 different treatments were performed in microcosm using two industrial petroleum contaminated soils having different textures: a clay rich soil and a sandy soil. After 30 days of incubation, the highest petroleum hydrocarbons removal was observed in microcosms containing: digestate together with bulking agent (17.8 % and 12.7 % higher than control in clay rich soil and sandy soil, respectively) or; digestate together with immobilized bacteria (13.4 % and 9 % higher than control in clay rich soil and sandy soil, respectively). Distinct microbial groups were formed in amended and non-amended soils. Genera containing species able to degrade hydrocarbons like *Acinetobacter* and *Mycobacterium* were abundant in digestate and soil amended with digestate. The study proved that digestate contains high concentration of *alkB* genes, significantly higher than contaminated soils. Application of digestate significantly increased the level of *alkB* genes in soils which remained high during the treatment.

In a third experiment, a contaminated soil was incubated with digestate and bulking agent (used to increase porosity of soil mixture and facilitate air transfer) in bioreactors with active aeration. Initial *alkB* concentration was 1.5 % in contaminated soil and 4.5 % in digestate. During incubation of soil with digestate, *alkB* percentage increased up to 11.5 % and after additional inoculation with immobilized bacteria this value increased up to 60 % (*alkB* percentage for treatment with mineral nutrients reached 0.4 %). Addition of digestate positively affected soil respiration and bacterial density, which was concomitant with enhanced hydrocarbons degradation. Incubation of soil with digestate for 2 months resulted in 74 % of hydrocarbons removal, while extra addition of immobilized bacteria increased this value to 95 %. Digestate increased soil bacterial density and diversity of hydrocarbons degrading taxa. The experiment clearly revealed the advantage of digestate over mineral fertilizer due to soil enrichment in TPH degrading taxa and thus a more efficient bioremediation.

This thesis for the first time analysed the potential of indigenous bacteria from organic nutrient source in bioremediation. The obtained results proved that digestate is a good source of bacteria caring *alkB* genes curtail in alkanes metabolism. Moreover, observed population of bacterial caring *alkB* genes was significantly greater in digestate comparing to contaminated soils. Application of digestate allowed to increase microbial activity and maintain high content of *alkB* genes in the soil which enhanced PHCs degradation.

Experiments performed during the thesis are contributing for better understanding of bioremediation process with the use of organic amendment as a nutrient source. Presented

advantages of digestate over mineral fertilizers were evaluated and confirmed. This thesis for the first time proposes organic amendment, like digestate to be considered not only as a nutrient source but also as a valuable source of microorganisms for soil bioaugmentation/biostimulation. Developed experimental treatments are a good starting point for further assessment of digestate during field scale treatments, however detailed risk assessment analysis including effect of potential pathogens contained in digestate on human health and studies analysing the effect of digestate leachates on groundwater quality need to be performed.

Résumé

Les sols contaminés par les hydrocarbures pétroliers constituent un problème environnemental qui peut être résolu par l'application de traitements biologiques. Le traitement des sols contaminés par les hydrocarbures pétroliers par bioaugmentation constitue une voie à explorer dans le cadre de la recherche d'une optimisation de traitement. L'intérêt scientifique actuel est axé sur la recherche d'amendements susceptible de contenir des populations microbiennes capables de dégrader les hydrocarbures pétroliers. Ces amendements sont généralement utilisés comme réserve d'espèces pour l'enrichissement lors de la préparation de l'inoculum. Les traitements par bioaugmentation sont particulièrement importants dans les sols à faible teneur en matière organique et à faible numération bactérienne (par exemple après des traitements chimiques intensifs). Le digestat est un sous-produit organique de la production de biogaz via des processus de digestion anaérobie et présente un grand potentiel en tant qu'engrais pour le sol en raison de leur concentration en éléments nutritifs. Cependant, le potentiel de la communauté microbienne de digestat n'a jamais été exploré en termes de dégradation des hydrocarbures pétroliers.

Dans cette thèse, nous avons étudié si les digestats pouvaient être utilisés comme source d'inoculum microbien pour la bioremédiation de sols contaminés par des hydrocarbures pétroliers. Les objectifs de ce travail de thèse étaient les suivants: i) vérifier la présence de bactéries dégradant des hydrocarbures pétroliers dans les enrichissements en digestat et comparer le potentiel de dégradation du diesel aux enrichissements provenant de sols contaminés par le pétrole, ii) vérifier l'effet de l'application de digestat sur la diversité de la communauté microbienne et l'activité microbienne du sol, iii) étudier la présence de gènes fonctionnels responsables de la dégradation des hydrocarbures (gènes *alkB*) dans le digestat et les sols modifiés.

Dans un premier temps, sept enrichissements microbiens ont été obtenus à partir de digestats d'origines différentes, de sols contaminés par des hydrocarbures pétroliers et du mélange de sols contaminés et de digestats. Après 3 semaines d'incubation, il a été observé une élimination très importante d'hydrocarbures pétroliers (e.g. diesel) pour les enrichissements microbiens provenant de digestat composté et de sols contaminés par des hydrocarbures pétroliers (78 et 77% d'élimination du gasoil,

respectivement). Les enrichissements microbiens obtenus à partir de digestat mélangé à du sol contaminé présentent des performances inférieures à celles des enrichissements à source unique. Dans tous les enrichissements, la présence de gènes *alkB* a été favorisée pendant l'incubation.

Dans un deuxième temps, 6 traitements différents ont été effectués dans un microcosme en utilisant deux sols contaminés par des hydrocarbures pétroliers, ayant des textures différentes : un sol argileux et un sol sableux. Après 30 jours d'incubation, on a observé une élimination maximale des hydrocarbures pétroliers dans des microcosmes contenant : un digestat associé à un matériau structurant (17,8% et 12,7% supérieurs aux témoins dans les sols argileux et sableux, respectivement) ou; digestat avec un enrichissement microbien immobilisés sur du biochar (13,4% et 9% de plus que le contrôle dans les sols argileux et sableux, respectivement). Des groupes microbiens distincts ont été retrouvés dans les sols modifiés et non modifiés. Les genres constitués d'espèces capables de dégrader des hydrocarbures pétroliers tels qu'*Acinetobacter* et *Mycobacterium* ont été retrouvé en abondance dans le digestat et dans le sol amendé avec du digestat. Des concentrations élevées de gènes *alkB* ont été mesurées dans le digestat et après l'application au sol, le niveau de gènes *alkB* a augmenté de manière significative dans les sols et est resté élevé pendant toute la durée du traitement (un mois).

Dans un troisième temps, un sol contaminé par des hydrocarbures pétroliers a été incubé avec du digestat et un agent structurant dans des bioréacteurs aérobies. La concentration initiale de gènes *alkB* était de 1,5% dans le sol contaminé et de 4,5% dans le digestat. Au cours de l'incubation du sol avec le digestat, le pourcentage de gènes *alkB* a augmenté jusqu'à 11,5% et, après inoculation supplémentaire avec des bactéries immobilisées sur biochar, cette valeur a augmenté jusqu'à 60% (le pourcentage de gènes *alkB* pour le traitement avec des nutriments minéraux a atteint 0,4%). L'ajout de digestat a eu un effet positif sur la respirométrie du sol et la densité bactérienne, ce qui était concomitant à une dégradation accrue des hydrocarbures pétroliers. L'incubation du sol avec le digestat pendant 2 mois a permis d'éliminer 74% des hydrocarbures pétroliers, tandis qu'une addition supplémentaire d'un inoculum microbien immobilisé sur du biochar a augmenté cette valeur à 95%. Le digestat a augmenté la densité bactérienne du sol et la diversité des taxons dégradant

les hydrocarbures.

Les résultats obtenus au cours de cette thèse confirment que le digestat est une bonne source de bactéries dégradant les hydrocarbures et révèlent son grand potentiel pour augmenter l'abondance de gènes *alkB* responsables de la dégradation des hydrocarbures pétroliers des sols contaminés pour des opérations de bioremédiation.

Tiivistelmä

Maaöljyllä saastuneet maaperät muodostavat ympäristöongelman, joka voidaan ratkaista biologisen kunnostamisen avulla. Maaperän bioaugmentatio öljyä hajottavien bakteerien kanssa on tehokas puhdistusstrategia. Nykyisin tieteellinen kiinnostus kohdistuu sellaisiin bakteerilähteisiin, jotka kykenevät hajottamaan hiilivetyjä, jotka toimivat lajien yhdistelminä rikastukseen siirrostuksen valmistuksen aikana. Bioaugmentointikäsittelyt ovat erityisen tärkeitä maaperässä, jossa orgaanisen aineen pitoisuus on alhainen ja bakteerien määrä alhainen (esim. Intensiivisen kemiallisen käsittelyn jälkeen). Digestaatti on orgaanisen sivutuotteena biokaasun tuotannossa anaerobisten pilkkomisprosessien kautta, ja sillä on suuri mahdollisuus maaperän lannoitteena tiivistettyjen ravintoaineiden takia. Digestaatin mikrobiyhteisön potentiaalia ei kuitenkaan koskaan tutkittu hiilivetyjen hajoamisen suhteen.

Tässä opinnäytetyössä tutkittiin digestaattia mikrobisena kylvönä kosteiden öljyhiilivetyjen saastuneiden maaperien bioremediaatiossa. Tavoitteemme olivat: i) tarkistaa dieselöljyä hajottavien bakteerien esiintyminen mädätysrikasteissa ja vertailla dieselöljyn hajoamispotentiaalia öljysaastuneiden maaperäisten rikastusteiden kanssa, ii) tarkistaa digestaatin levityksen vaikutus maaperän mikrobiyhteisöön ja mikrobitoimintaan, iii) tutkia läsnäoloa funktionaalisten geenien, jotka vastaavat hiilivetyjen hajoamisesta (*alkB*-geenit) mädätteessä ja muunnellussa maaperässä.

Ensimmäisen kokeen aikana kehitettiin seitsemän mikrobirikastetta erilaisista ruuansulatuksista, maaöljyllä saastuneesta maaperästä ja maaperän sekoituksesta digestaatin kanssa. Kolmen viikon inkubaation jälkeen korkein dieselöljypoisto havaittiin rikastuksista, jotka olivat peräisin kompostoidusta digestaatista ja öljysaastuneesta maaperästä (vastaavasti 78% ja 77% dieselöljystä). Rikasteilla, jotka on saatu maaperästä sekoitetusta mäntäaineesta, on huonompi suorituskyky kuin yhden lähteen rikastuksilla. Kaikissa rikastuksissa *alkB*-geenien läsnäoloa edistettiin inkubaation aikana.

Toisessa kokeessa suoritettiin 6 erilaista käsittelyä mikrokosmossa käyttämällä kahta teollisuusöljysaastuttamaa maaperää, jolla oli erilaiset rakenteet: savirikas maa ja

hiekkamaa. 30 päivän inkubaation jälkeen havaittiin suurin öljyhiilivetyjen poistuminen mikrokosmoissa, jotka sisälsivät: digestaattia yhdessä täyteaineen kanssa (vastaavasti 17,8% ja 12,7% korkeampi kuin kontrolli savenrikkaassa ja hiekkaisessa maaperässä) tai; mädätys yhdessä immobilisoitujen bakteerien kanssa (vastaavasti 13,4% ja 9% korkeampi kuin kontrolli savelirikkaassa ja hiekkaisessa maaperässä). Erilliset mikrobiryhmät muodostuivat muutetussa ja muuntamattomassa maaperässä. Sukuja, jotka sisälsivät lajeja, jotka kykenevät hajottamaan hiilivetyjä, kuten Acinetobacter ja Mycobacterium, oli runsaasti ruuansulatuksessa ja maaperässä, joka muutettiin digestaatilla. *AlkB*-geenien korkea konsentraatio havaittiin mädätysastiassa, maaperän levityksen jälkeen *alkB*-geenien taso nousi merkittävästi maaperässä ja pysyi korkeana yhden kuukauden käsittelyn aikana.

Kolmannessa kokeessa saastunutta maata inkuboitiin digestaatin ja täyteaineen kanssa bioreaktorissa aktiivisella ilmastolla. Alkuperäinen *alkB*-konsentraatio oli 1,5% saastuneessa maaperässä ja 4,5% digestaatissa. Maaperän inkubaation aikana mädätteen kanssa *alkB*-prosenttimäärä nousi 11,5%: iin ja lisäinokulaation jälkeen immobilisoiduilla bakteereilla tämä arvo nousi jopa 60%: iin (*alkB*-prosenttiosuus mineraaliravinteiden käsittelyssä oli 0,4%). Digestaatin lisääminen vaikutti positiivisesti maaperän hengitykseen ja bakteerien tiheyteen, mikä oli samanaikainen hiilivetyjen lisääntyneen hajoamisen kanssa. Maaperän inkubaatio digestaatilla 2 kuukautta johti 74% hiilivetyjen poistoon, kun taas immobilisoitujen bakteerien lisäys lisäsi tämän arvon 95%: iin. Digestaatti lisäsi maaperän bakteeritiheyttä ja hiilivetyjen monimuotoisuutta hajottavien taksonien suhteen.

Tämän opinnäytetyön aikana saadut tulokset vahvistavat, että digestaatti on hyvä hiilivetyä hajottavien bakteerien lähde ja paljastaa sen suuren potentiaalin lisätä *alkB*-geenien määrää, jotka vastaavat hiilivetyjen hajoamisesta maaperässä bioremedikaation aikana.

Sommario

I suoli contaminati da petrolio costituiscono un problema ambientale che può essere risolto con l'aiuto del biorisanamento. La bioaugmentazione del suolo con batteri che degradano il petrolio è un'efficace strategia di trattamento. Attualmente l'interesse scientifico è focalizzato su batteri in grado di degradare gli idrocarburi da utilizzare per l'arricchimento microbico durante la preparazione dell'inoculo. I trattamenti di bioaugmentazione sono particolarmente importanti in terreni con basso livello di materia organica e bassa conta batterica (ad es. dopo trattamenti chimici intensivi).
Il digestato è un sottoprodotto organico del processo di digestione anaerobica finalizzato alla produzione di biogas e ha un grande potenziale come fertilizzante del suolo a causa dell'elevato contenuto di nutrienti. Tuttavia, il potenziale della comunità microbica del digestato non è mai stato studiato in termini di degradazione degli idrocarburi.

In questa tesi abbiamo esaminato il digestato come fonte microbica per il biorisanamento di terreni contaminati da idrocarburi. I nostri obiettivi erano: i) verificare la presenza di batteri che degradano il diesel nel digestato e confrontare il potenziale di degradazione del diesel aggiungendo il digestato nei suoli contaminati dal petrolio, ii) verificare l'effetto dell'applicazione del digestato sulla comunità microbica del suolo e l'attività microbica, iii) studiare la presenza di geni funzionali responsabili della degradazione degli idrocarburi (geni *alkB*) nel digestato e nei terreni modificati.

Durante il primo esperimento, sono stati sviluppati sette arricchimenti microbici da vari digestati, terreno contaminato da petrolio e dalla miscela di terreno con digestato. Dopo 3 settimane di incubazione è stata osservata la massima rimozione di diesel per gli arricchimenti originati dal digestato compostato e dal suolo contaminato da petrolio (rispettivamente rimozione del 78 e 77% di diesel). Gli arricchimenti ottenuti dal digestato mescolato con il suolo hanno prestazioni inferiori rispetto agli arricchimenti a fonte singola. In tutti gli arricchimenti, durante l'incubazione, è stata promossa la presenza di geni *alkB.*

In un secondo esperimento, sono stati eseguiti 6 diversi trattamenti nel microcosmo

usando due terreni industriali contaminati da petrolio con trame diverse: un terreno ricco di argilla e un terreno sabbioso. Dopo 30 giorni di incubazione, la più alta rimozione di idrocarburi di petrolio è stata osservata in microcosmi contenenti: digestato insieme ad agente di carica (17,8% e 12,7% superiore al controllo nel terreno ricco di argilla e nel terreno sabbioso, rispettivamente) o digestato insieme a batteri immobilizzati (rispettivamente 13,4% e 9% in più rispetto al controllo nei terreni ricchi di argilla e sabbiosi). Gruppi microbici distinti si sono formati in terreni modificati e non modificati. Le specie contenenti generi in grado di degradare gli idrocarburi come Acinetobacter e Mycobacterium erano abbondanti nel digestato e nel suolo modificato con il digestato. Nel digestato sono state riscontrate alte concentrazioni di geni *alkB*, dopo l'applicazione del suolo, il livello di geni *alkB* è aumentato significativamente nei suoli ed è rimasto elevato durante un mese di trattamento.

In un terzo esperimento, un terreno contaminato è stato incubato con digestato e agente di carica in bioreattori con aerazione attiva. La concentrazione iniziale di *alkB* era dell'1,5% nel suolo contaminato e del 4,5% nel digestato. Durante l'incubazione del terreno con digestato, la percentuale di alkB è aumentata fino all'11,5% e dopo un'ulteriore inoculazione con batteri immobilizzati questo valore è aumentato fino al 60% (la percentuale di alkB per il trattamento con nutrienti minerali ha raggiunto lo 0,4%). L'aggiunta di digestato ha influenzato positivamente la respirazione del suolo e la densità batterica, con una maggiore degradazione degli idrocarburi. L'incubazione del terreno con digestato per 2 mesi ha comportato la rimozione del 74% degli idrocarburi, mentre l'aggiunta di batteri immobilizzati ha aumentato questo valore al 95%. Il digestato ha aumentato la densità batterica del suolo e la diversità degli idrocarburi che hanno degradato i taxa.

I risultati ottenuti durante questa tesi confermano che il digestato è una buona fonte di batteri che degradano gli idrocarburi e rivela il suo grande potenziale per aumentare l'abbondanza di geni *alkB* responsabili della degradazione degli idrocarburi nei suoli durante il biorisanamento.

Samenvatting

Met petroleum verontreinigde bodems vormen een milieuprobleem dat kan worden opgelost met behulp van bioremediatie. Bioaugmentatie van een bodem met aardolie afbrekende micro-organismen is een efficiënte saneringsstrategie. Momenteel is de wetenschappelijke interesse gericht op het zoeken naar nieuwe bronnen van micro-organismen die in staat zijn koolwaterstoffen af te breken om die te gebruiken tijdens de inoculumbereiding. De bioaugmentatiestrategie is vooral belangrijk in bodems met weinig organische stof en lage microbiële diversiteit (bijv. na intensieve chemische behandelingen). Er zijn tot op heden geen studies uitgevoerd waarbij rekening is gehouden met het potentieel van organische meststoffen als bron voor de microbiële enting voor bioremediatie. In dit proefschrift werd voor het eerst digestaat als een voorbeeld van organische stof bestudeerd in termen van inheemse microbiële gemeenschappen die betrokken kunnen zijn bij de afbraak van lineaire koolwaterstoffen. Digestaat is een organisch bijproduct van de productie van biogas via anaërobe vergistingsprocessen en heeft een groot potentiëel als bodemmeststof vanwege de geconcentreerde voedingsstoffen en een laag gehalte aan gemakkelijk biologisch afbreekbare verbindingen (die door bacteriën zouden kunnen worden gebruikt als een preferentiële koolstofbron boven koolwaterstoffen). Het potentieel van de microbiële gemeenschappen van digestaat werd echter nooit onderzocht in termen van afbraak van petroleum-koolwaterstoffen (PHC's).

In dit proefschrift werd digestaat onderzocht als microbiëel inoculum voor de bioremediatie van door verweerde aardolie en koolwaterstoffen verontreinigde bodems. De doelen waren: i) om de aanwezigheid van alkanen afbrekende bacteriën in verrijkingen van digestaat te controleren en hun alkanenafbraakpotentieel te vergelijken met verrijkingen van met aardolie verontreinigde bodems, ii) het effect van toepassing van digestaat op de microbiële gemeenschappen in een bodem en de microbiële activiteit te verifiëren, iii) de aanwezigheid te bestuderen van functionele genen die verantwoordelijk zijn voor de afbraak van alkanen (alkB-genen) in digestaat en gemodificeerde bodems.

Tijdens het eerste experiment werden 7 microbiële aanrijkingen ontwikkeld uit

verschillende digestaten (waaronder gecomposteerd digestaat), één met aardolie verontreinigde grond en uit een mengsel van grond met digestaat. Na 3 weken incuberen werd de hoogste verwijdering van dieselbrandstof waargenomen voor verrijkingen afkomstig van gecomposteerd digestaat en uit de met aardolie verontreinigde grond (respectievelijk 78 en 77% diesel brandstof verwijdering). Verrijkingen verkregen uit digestaat gemengd met grond vertoonden lagere prestaties dan verrijkingen met één bron. In alle verrijkingen werd de aanwezigheid van alkB-genen bevorderd tijdens de incubatie. Het experiment onthulde de aanwezigheid van alkB-genen in bacteriën uit digestaat en bevestigde hun vermogen om diesel brandstof af te breken.

In een tweede experiment werden 6 verschillende behandelingen uitgevoerd in een microkosmos met behulp van twee industriële, met aardolie vervuilde bodems met verschillende structuur: een kleirijke bodem en een zandbodem. Na 30 dagen incubatie werd de hoogste verwijdering van petroleumkoolwaterstoffen waargenomen in de microkosmos bevattende: digestaat samen met vulstof (respectievelijk 17,8% en 12,7% hoger dan controle in kleirijke grond en zandgrond) of digestaat samen met geïmmobiliseerde bacteriën (respectievelijk 13,4% en 9% hoger dan controle in kleirijke grond en zandgrond). Verschillende microbiële groepen werden gevormd in gewijzigde en niet-gewijzigde bodems. Genera die in staat zijn om koolwaterstoffen af te breken, zoals *Acinetobacter* en *Mycobacterium*, waren overvloedig aanwezig in digestaat en bodem gewijzigd met digestaat. De studie heeft aangetoond dat digestaat een hoge concentratie alkB-genen bevat, aanzienlijk hoger dan vervuilde bodems. Toedienen van digestaat verhoogde het niveau van alkB-genen in bodems die tijdens de behandeling hoog bleven.

In een derde experiment werd een verontreinigde grond geïncubeerd met digestaat en vulstof (gebruikt om de porositeit van het grondmengsel te verhogen en luchtoverdracht te vergemakkelijken) in bioreactoren met actieve beluchting. De initiële alkB-concentratie was 1,5% in verontreinigde grond en 4,5% in digestaat. Tijdens incubatie van grond met digestaat steeg het alkB-percentage tot 11,5% en na extra inoculatie met geïmmobiliseerde bacteriën nam deze waarde toe tot 60% (alkB-percentage voor behandeling met minerale voedingsstoffen bereikte 0,4%). Toevoeging van digestaat had een positieve invloed op de ademhaling van de bodem en de bacteriedichtheid, wat

gepaard ging met een verhoogde afbraak van koolwaterstoffen. Incubatie van grond met digestaat gedurende 2 maanden resulteerde in 74% verwijdering van koolwaterstoffen, terwijl extra toevoeging van geïmmobiliseerde bacteriën deze waarde verhoogde tot 95%. Digestaat verhoogde de bodem bacterie dichtheid en diversiteit van taxa die koolwaterstoffen afbreken. Uit het experiment bleek duidelijk het voordeel van digestaat ten opzichte van minerale meststoffen, als gevolg van bodemverrijking in TPH-degraderende taxa en dus een efficiëntere bioremediatie.

Dit proefschrift analyseerde voor het eerst het potentieel van inheemse bacteriën uit organische voedingsstoffen bij bioremediatie. De verkregen resultaten bewezen dat digestaat een goede bron van bacteriën is die alkB-genen beperken in het alkanen metabolisme. Bovendien was de waargenomen populatie van bacteriële alkB-genen aanzienlijk groter in digestaat vergeleken met verontreinigde bodems. Toepassing van digestaat maakte het mogelijk om de microbiële activiteit te verhogen en een hoog gehalte aan alkB-genen in de bodem te handhaven, waardoor de afbraak van PHC's werd verbeterd.

De experimenten die tijdens dit proefschrift zijn uitgevoerd dragen bij aan een beter begrip van het bioremediatieproces met behulp van organische wijzigingen als voedingsbron. De gepresenteerde voordelen van digestaat ten opzichte van minerale meststoffen werden geëvalueerd en bevestigd. Dit proefschrift stelt voor het eerst wijziging van de organische stof in een bodem voor, zoals digestaat, dat niet alleen als een voedingsbron moet worden beschouwd, maar ook als een waardevolle bron van micro-organismen voor bioaumentatie / biostimulatie van de bodem. De ontwikkelde experimentele behandelingen zijn een goed uitgangspunt voor verdere beoordeling van digestaat tijdens veldschaal behandelingen, maar er moet een gedetailleerde risico-analyse worden uitgevoerd, waaronder het effect van potentiële pathogenen in digestaat op de menselijke gezondheid en studies die het effect van digestaat-percolaten op de grondwaterkwaliteit analyseren.

Table of content

List of Symbols and Abbreviations

acyl-CoA	Acetyl coenzyme A
ANOVA	Analysis of variance
BA	Bulking agent
BH	Bushnell Haas
BOD	Biological oxygen demand
BTEX	Benzene, toluene, ethylbenzene and xylenes
DDT	Dichlorodiphenyltrichloroethane
DRI	Dynamic respiration index
GC-FID	Gas chromatography flame ionization detector
GHGs	Greenhouse gases
H	Henry's law constant
HAs	Humic acids
HCH	Hexachlorocyclohexane
HGT	Horizontal gene transfer
HNF	Heterotrophic nanoflagellates
K_{oc}	Organic carbon sorption coefficient
K_{ow}	Octanol/water partition coefficient
LAMAs	Long-chain alkylated monoaromatic hydrocarbons
NADH	Reduced form of nicotinamide adenine dinucleotide
OFMSW	Organic fraction of municipal solid waste
OM	Organic matter
pAHs	Particulate alkane hydroxylases
PAHs	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
PHC	Petroleum hydrocarbons
qPCR	Quantitative polymerase chain reaction (Real Time RCR)
SOM	Soil organic matter
SOUR test	Specific oxygen uptake rate test
SR	Soil respiration
$T_{1/2}$	Half-life of chemical compounds
TN	Total nitrogen
TOC	Total organic carbon
TPH	Total petroleum hydrocarbons
TVS	Total volatile solids
UPW	Ultra-pure water
VOCs	Volatile organic compounds
WHC	Water holding capacity

List of Publications

I. Gielnik A., Pechaud Y., Huguenot D., Esposito G., van Hullebusch E.D., 2019. Bacterial seeding potential of digestate in bioremediation of diesel contaminated soil, Int. Biodeterior. Biodegradation. 143, 104715.

II. Gielnik A., Pechaud Y., Huguenot D., Riom J.-M., Guibaud G., Esposito G., van Hullebusch E.D., 2019. Effect of digestate application on microbial respiration and bacterial communities' diversity during bioremediation of weathered petroleum hydrocarbons contaminated weathered soils, Sci. Total Environ. 670, 271-281.

III. Gielnik A., Pechaud Y., Huguenot D., Esposito G., van Hullebusch E.D. Digestate is a fertilizer rich in *alkB* genes: Microbial population changes during bioremediation of weathered petroleum hydrocarbons in soils, (Submitted to Environmental Pollution on 02.10.2019)

Author's Contribution

Paper I

Anna Gielnik performed the experiments and all the related analysis, wrote the manuscript and is the corresponding author. Yoan Pechaud participated in design and planning of the experiment. Eric D. van Hullebusch, Yoan Pechaud, Giovanni Esposito, David Huguenot and Aurélie Cébron helped in data interpretation and thoroughly revised the manuscript. Aurélie Cébron has participated in all the experimental steps regarding analysis of microbial communities and has provided all necessary equipment.

Paper II

Anna Gielnik performed the experiments and all the related analysis, wrote the manuscript and is the corresponding author. Eric D. van Hullebusch, Yoan Pechaud, Giovanni Esposito and David Huguenot participated in design and planning of the experiment as well as helped in data interpretation and thoroughly revised the manuscript. Aurélie Cébron has participated in all the experimental steps regarding analysis of microbial communities and has provided all necessary equipment. Aurélie Cébron also helped to improve the manuscript. Jean-Michel Riom has participated in the development of the TPH quantification method. Gilles Guibaud helped to develop the conception of the manuscript and revised it.

Paper III

Anna Gielnik performed the experiments and all the related analysis and wrote the manuscript. Eric D. van Hullebusch, Yoan Pechaud, Giovanni Esposito and David Huguenot participated in design and planning of the experiment. Yoan Pechaud has helped in bioreactors setup. Eric D. van Hullebusch, Yoan Pechaud, Giovanni Esposito, David Huguenot and Aurélie Cébron helped in data interpretation and thoroughly revised the manuscript. Aurélie Cébron has participated in all the experimental steps regarding analysis of microbial communities and has provided all necessary equipment.

1. Introduction

Petroleum industry generates substances, which may affect human health as well as soil and water ecosystems, including toxicity towards microorganisms, plants and animals. According to the report of European Commission (European Commission, Progress in the Management of Contaminated Sites in Europe, 2014) up to 45% of total soil pollution in Europe is caused by petroleum hydrocarbons originating from industrial and commercial activities, storage, military activities, transport and land spills. Petroleum products are a diverse group of soil pollutants, which include light distillates e.g. gasoline and naphtha; middle distillates e.g. kerosene, jet fuel, diesel and; heavy distillates e.g. heavy fuel oil, lubricating oils. All of these products have a complex chemical composition which may differ depending on the production process. To illustrate the complexity of a petroleum product the main components encountered in diesel fuel are presented in the Table 1.1.

Crude oil is one of the most economically important organic substances, ensuring proper operation of various industrial sectors, especially transport and energy (Atlas and Hazen 2011). Crude oil is composed of hundreds of diverse hydrocarbons (Atlas and Hazen 2011). Petroleum hydrocarbons spills onto soil can be composed of hundreds different chemical compounds which individual quantification would be challenging. Thus, the term total petroleum hydrocarbon (TPH) was introduced to describe a variety of petroleum hydrocarbon compounds (PHC) which can be extracted from contaminated soils. There are three main fractions of crude oil: paraffin, naphthenes and the aromatic fraction. These fractions are characterized by specific physicochemical properties, and thus can be selectively recovered by distillation (Speight, 2002). Paraffin is usually the largest volume fraction composed of aliphatic hydrocarbons; naphthenes consists of alicyclic hydrocarbons; the aromatic fraction includes hydrocarbons with one or more aromatic rings like monoaromatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) (Fuentes et al. 2014).

Table 1.1 Main chemical components of diesel fuel. Simple alkanes constitute the biggest percentage fraction, with the highest concentration of alkanes ranging from nC11 to nC20.

Compound	Number of carbon atoms	Diesel (wt. %)	References
Simple alkanes			
n-Octane	8	0.1	(ATSDR 1999)
n-Nonane	9	0.19-0.49	(ATSDR 1999)
		0.1	(Heath et al. 1993)
n-Decane	10	0.28-1.2	(ATSDR 1999)
		0.5-2	(Heath et al. 1993)
n-Undecane	11	0.57-2.3	(ATSDR 1999)
		0.98-9	(Heath et al. 1993)
n-Dodecane	12	1-2.5	(ATSDR 1999)
		0.96-11	(Heath et al. 1993)
n-Tridecane	13	1.5-2.8	(ATSDR 1999)
		1.1-10	(Heath et al. 1993)
n-Tetradecane	14	0.61-2.7	(ATSDR 1999)
		1.1-9	(Heath et al. 1993)
n-Pentadecane	15	1.9-3.1	(ATSDR 1999)
		1-7	(Heath et al. 1993)
n-Hexadecane	16	1.5-2.8	(ATSDR 1999)
		1.2-6	(Heath et al. 1993)
n-Heptadecane	17	1.4-2.9	(ATSDR 1999)
		1.2-6	(Heath et al. 1993)
n-Octadecane	18	1.2-2	(ATSDR 1999)
		0.82-5	(Heath et al. 1993)
n-Nonadecane	19	0.7-1.5	(ATSDR 1999)
		0.53-4	(Heath et al. 1993)
n-Eicosane	20	0.4-1	(ATSDR 1999)
		0.23-3	(Heath et al. 1993)
n-Heneicosane	21	0.26-0.83	(ATSDR 1999)
		1	(Heath et al. 1993)
n-Docosane	22	0.14-0.44	(ATSDR 1999)
n-Tetracosane	24	0.35	(ATSDR 1999)
Branched chain alkanes			
3-Methylundecane	12	0.09-0.28	(ATSDR 1999)
2-Methyldodecane	13	0.15-0.52	(ATSDR 1999)
3-Methyltridecane	14	0.13-0.3	(ATSDR 1999)
2-Methyltetradecane	15	0.34-0.63	(ATSDR 1999)
2,6,10-Trimethyldodecane		0.45	(Heath et al. 1993)
Alkyl Benzenes			
Benzene	6	0.003-0.1	(ATSDR 1999)
Toluene	7	0.007-0.7	(ATSDR 1999)
Ethylbenzene	8	0.007-0.2	(ATSDR 1999)
Xylenes	8	0.037-1.11	(ATSDR 1999)
Biphenyl	12	0.01-0.12	(ATSDR 1999)
Polycyclic aromatic hydrocarbons (PAHs)			
Benzo[a]pyrene		0.000007	(Heath et al. 1993)
Naphthalene	10	0.01-0.8	(ATSDR 1999)
		0.13	(Heath et al. 1993)
Methylnaphtalene	11	0.57-0.91	(Heath et al. 1993)
1-Methylnaphtalene	11	0.001-0.81	(ATSDR 1999)
2-Methylnaphtalene	11	0.001-1.49	(ATSDR 1999)
1,3-Dimethylnaphtalene	12	0.55-1.28	(ATSDR 1999)
Fluorene	13	0.034-0.15	(ATSDR 1999)

In a contaminated soil, several physical, chemical and biological processes influence contaminants behaviour (Lim et al., 2016). The relative importance of each process depends on the local physico-chemical properties but also on the physico-chemical properties of the contaminants. Some of the physico-chemical properties of hydrocarbons from different groups are presented in Table 1.2. Diffusivity of a compound is the magnitude of the molar flux through a surface per unit concentration gradient. Specific gravity is the ratio of the mass of a substance to the mass of a reference substance for the same given volume. Both parameters affect the contaminant spreading velocity in soil and in soil solution. Water solubility impacts partially the mobility of contaminants in soil and water bodies. Vapour pressure value affects mobility into gas phase (*i.e.* air) and persistence of the compounds. Henry's law constant (H) indicates the tendency of a compound at the equilibrium to evaporate from aqueous phase and is determined by vapour pressure and solubility. If the H value is higher than 10^{-3} atm.m^3.mol^{-1}, the compound tends to volatilize from aqueous phase; values lower than 10^{-3} atm.m^3.mol^{-1} may support occurrence of different processes like sorption to soil particles (Heath et al. 1993). Hydrocarbons with high volatility value may evaporate from soil before biodegradation takes place. The octanol/water partition coefficient, expressed as K_{ow} or P used in $\log_{10}$ form, describes the hydrophobic or hydrophilic preference and may help to predict affinity for water column. K_{oc} is the organic carbon sorption coefficient which directly indicates sorption of a contaminant to soil organic matter (SOM). The higher is K_{oc} value, the higher sorption will be observed which affects concentration of bioavailable fraction of the contaminant. Half-life ($T_{1/2}$) of chemical compounds represents the time required for 50% total concentration loss, assuming a first order degradation kinetic.

Several studies on hydrocarbons bioremediation reported that n-alkanes and monoaromatic compounds are more biodegradable than iso-alkanes and cycloalkanes (Salanitro 2001; Mao et al. 2009). However, biodegradation efficiency, which means the extent to which contaminants are biodegraded, of individual compounds depends on conditions present on site or applied treatment approach. Different decomposition time between hydrocarbons groups is also related to their bioavailability (Salanitro 2001; Lim et al., 2016). Water soluble hydrocarbons are more accessible for microorganisms and thus easily biodegradable. Water solubility of aromatic compounds is higher in comparison with alkanes with the same number of carbon atoms (Table 1.2). Biodegradability of hydrocarbons is strongly related to their chemical structure (Gargouri et al. 2014). The long-chain alkylated monoaromatic hydrocarbons (LAMAs) which are present in diesel fuel, are less water-soluble then short-chain alkylated PAHs and thus less

biodegradable. This finding was reported in the experiment performed by Mao et al. (2009), in which long-chain alkylated monoaromatic hydrocarbons were a dominant fraction after 20 weeks of bioremediation of a diesel fuel contaminated soil.

Table 1.2 Chemical and physical properties of selected hydrocarbons. Increase of molecular weight among different compounds is connected with decrease of water solubility and often with increased hydrophobicity manifested by high logP or $logK_{ow}$ values. Half-life time of a compound is positively correlated with molecular weight while vapor pressure is negatively correlated (Modified from: Heath et al. 1993; Pasteris et al. 2002; Mackay el al. 2006; Paraíba et al. 2011; Toxnet 2016).

Compound	Molecular wt.	Water solubility (mg/L 25 °C)	Specific gravity	Vapor pressure (mmHg 25 °C)	Henry's law constant (atm-m^3/mol 25 °C)	Diffusivity (cm^2/s)	logP / $logK_{ow}$	Log K_{OC}	$T_{1/2}$ (days)
Benzene	78	1780	0.88	95	$5.5\ 10^{-3}$	$9.30\ 10^{-2}$	1.56-2.15		3.5*
Toluene	92	490-627	0.87	28	$6.7\ 10^{-3}$	$7.80\ 10^{-2}$	2.11- 2.8		4*
Xylenes	106	162-200	0.87	6.6-8.8	$6.3\ 10^{-3}$	$7.20\ 10^{-2}$	2.77-3.2		7*
Benzo[a] pyrene	252	$3.8\ 10^{-3}$ - $4\ 10^{-3}$	1.35	$5.5\ 10^{-9}$	$<2.4\ 10^{-6}$	$4.70\ 10^{-2}$	5.81-6.50	5.00	530**
Fluorene	166	1.66-1.98	1.2	$1\ 10^{-3}$	$2.1\ 10^{-4}$	$5.70\ 10^{-2}$	4.12-4.38	3.24	60**
2-Methylnaphthalene	142	25	1.001	$4.5\ 10^{-2}$	$3.4\ 10^{-4}$	$6.20\ 10^{-2}$	3.86-4.11		
Naphthalene	128	30-34	1.16	$2.3\ 10^{-1}$	$4.6\ 10^{-4}$	$8.20\ 10^{-2}$	3.2-4.7	2.44	48**
Phenanthrene	178	$7.1\ 10^{-1}$ -1.29	1.18	$6.8\ 10^{-4}$	$2.6\ 10^{-5}$	$5.40\ 10^{-2}$	4.2-4.6	3.49	200**
Pyrene	202	$1.3\ 10^{-2}$- $1.71\ 10^{-1}$	1.27	$6.85\ 10^{-7}$ - $2.5\ 10^{-5}$	$1.1\ 10^{-5}$	$5.00\ 10^{-2}$	4.88-5.32	3.87	1870**
n-Hexane	86	18 (20 °C)	0.66	$1.2\ 10^{-2}$ (20 °C)	$7.7\ 10^{-1}$	$7.50\ 10^{-2}$	2.77		2**
n-Heptane	100.21	3		$6.87\ 10^{-2}$	$2.3\ 10^{2}$		3.63		4*
n-Octane	114.23	$6.6\ 10^{-1}$		1.88	$3.0\ 10^{2}$		4.91		4*
n-Nonane	128.26	$7\ 10^{-2}$		$5.71\ 10^{-1}$	$5.0\ 10^{2}$		5.65		4.5*
n-Decane	148.28	$5.2\ 10^{-2}$		$1.75\ 10^{-1}$	$7.0\ 10^{2}$		5.01-6.69		4.7*
n-Undecane	156.32						6.94		5*
n-Dodecane	170.33						6.10-7.24		5*
n-Tetradecane	190.38	$6.96\ 10^{-3}$		$1.27\ 10^{-3}$	$1.1\ 10^{2}$		6.49-8.10		< 15**, 5.6*
n-Hexadecane	226.44	$6.28\ 10^{-3}$		$9.17\ 10^{-4}$	$2.3\ 10^{-1}$		8.86		6*
n-Octadecane	254.4	$2.1\ 10^{-3}$		$2.50\ 10^{-5}$	2.9		8.92-10.39	5.90	6.3*
n-Eicosane	282.6	$1.9\ 10^{-3}$		$2.67\ 10^{-6}$	$2.9\ 10^{-1}$		10.39		7.1*

* in water; ** in soil

Remediation of soils polluted with PHCs may occur naturally, this process is called “natural attenuation” (Tang et al. 2012). However, this process is slow and thus, application of additional treatment strategy is needed to enhance the remediation efficiency. Within soil treatment strategies, bioremediation represents an environmentally friendly solution in comparison with conventional physical treatments such as thermal extraction, solvent extraction, steam stripping, hot-air stripping, immobilization and chemical treatments such as chemical oxidation (Gan et al. 2009; Tang et al. 2012). In contrast to bioremediation, conventional remediation methods are strongly invasive for the treated soils, which may result in environmental perturbations. In most cases, bioremediation has also an economic advantage over physical and chemical treatments. However, the total costs depend on numerous factors like the area of contaminated site, the soil characteristics, the physico-chemical properties of the contaminants as well as depth and the age of the contamination (Saterbak et al. 1999).

Time needed for soil clean-up vary significantly between treatments and soil properties. Solvent extraction methods can be completed within few hours, chemical oxidation last from one day to few weeks, thermal extraction treatments last around a month while immobilization strategies depend on sorption and desorption kinetics and last from few days to several months (Gan et al. 2009). For bioremediation, time needed for soil treatment vary from few months to few years depending on the characteristics of contaminated sites (Table 3.2 in chapter 3 illustrates the time required for an efficient bioremediation treatment at field scale). Application of physical and chemical treatments can be fast and in great extent decrease soil contamination level, however, residual concentrations may remain in soil causing toxicity. For this low remaining concentration of contaminants, physical and chemical treatments may not be longer profitable, thus bioremediation may be selected as an additional treatment (Lin et al. 2017).

Bioremediation is based on the natural capabilities of microorganisms to breakdown hydrocarbons combined with application of methods that allow the enhancement of microbial activity and consequently of the degradation efficiency. Biodegradation of organic contaminants may occur in aerobic and anaerobic conditions. Aerobic methods are more widespread and well-studied (Zappi et al. 1996; Robles-González et al. 2008; Baboshin and Golovleva 2012). The main bioremediation technologies include *in situ* and *ex situ* treatments. *In situ* aerobic techniques including biostimulation, bioaugmentation or bioventing are simple processes based on nutrient application and air or oxygen injection to soil which are performed directly on contaminated sites. Biostimulation involves addition of nutrients to soil in form of

mineral or organic fertilizers, bioventing represents bioremediation treatment in which are is air is actively supplied to soil for example by the system of pipelines. Bioaugmentation is a treatment strategy involving addition of contaminant degrading microorganisms to soil in concentrated form for example as liquid cultures or microbial cells immobilized on a porous carrier (*e.g.* biochar, activated carbon)

In contrast to *ex situ* treatments, *in situ* methods do not include excavation or transport of contaminated soil. The most important *ex situ* technologies include composting in biopiles and treatment in slurry bioreactors (Robles-González et al. 2008). Soil treatment in biopiles represents a composting strategy, in which soil is organized into covered piles and actively aerated by the pipelines system. In slurry bioreactors soil is incubated in constantly or semi constantly mixed reactors in which treatment is conducted in water phase (around 5 – 30 % of solids). *Ex situ* treatments may also combine application of biostimulation, aeration or bioaugmentation.

The main challenge in the design of a bioremediation strategy is to determine the main factors which limit microbial activity and select appropriate treatment conditions (Fuentes et al. 2014). The limiting factors likely to occur on a contaminated site can refer to the physical, chemical or biological aspects. The biological factors that limit biodegradation may include low activity and density of indigenous bacteria as well as interactions between hydrocarbons degrading agents and other organisms *e.g.* prokaryotic predation by viruses or heterotrophic nanoflagellates (HNF) (Sauret et al. 2015). Among the physicochemical aspects, insufficient level of soil aeration, water content and inadequate level of nutrients in soil are the most often listed (Fuentes et al. 2014). Also, low bioavailability and bioaccessibility of the contaminants are common phenomena (Fuentes et al. 2014). In environmental context, bioavailability describes compounds which are freely available to cross the cellular membrane of microorganisms at given time and place (Semple et al., 2004). Bioaccessibility describes the possibility of physical contact with the contaminant at given time and place (Aemig et al. 2016). A non-bioaccessible compound may be for example occluded in soil organic matter and thus being out of reach for microbes at given time (Semple et al., 2004).

The design of a bioremediation strategy should be preceded by proper analysis and adjusted to each case, as each contaminated soil is unique. The analysis of soilshould include texture characterization, since granulometry may influence the bioavailability of the contaminant and thus, the biodegradation efficiency. It has been observed that soils characterized by high clay

and silt content are more problematic for remediation purposes in comparison with a sandy soil due to stronger sorption phenomena as well as low permeability which limits fluid flow and affects mass transfer (Yeh and Young 2003). Petroleum hydrocarbons may also be adsorbed or trapped into soil organic matter and the degree of sorption is correlated with the total content of SOM in the soil (Nishiwaki et al. 2012). The age of the contamination is also an important factor. Over time, organic contaminants are more strongly bound to the soil matrix, resulting in a lower bioavailability and therefore in a lower microbial up-take. In this case, the use of surface-active substances may enhance the mass transfer of the contaminant and thus increase its bioavailability (Baboshin and Golovleva 2012). To minimize the side effects of chemical additions during bioremediation process, the use of biosurfactants is recommended due to low toxicity and better biodegradability (Baboshin and Golovleva 2012). However, even with the application of biosurfactants, in many cases, low bioavailability still constitutes significant limitation for bioremediation efficiency.

If the concentration of contaminants is high, degrading agents may suffer from increased soil toxicity. To temporary decrease the available concentrations of the contaminants, organic matter or porpus amendment can be added. Due to sorption phenomena, contaminants will be bound to the organic amendment, which will decrease bioavailability. During the degradation process, absorbed compounds will be slowly released from organic matter. Another factor, which may cause ecotoxicity, is the accumulation of intermediate metabolites, as a result of incomplete contaminant transformation. Metabolites may be more toxic than initial compounds *e.g.* due to lower hydrophobicity and thus higher bioavailability and intrinsic toxicity (Xu and Lu 2010).

Soil analysis should also include characterization of basic chemical properties such as quantity of organic carbon and nutrients. Nutrient deficiency is often a limiting factor for hydrocarbon degradation. Disturbed nutrient ratio in soils contaminated with organic pollutants may be manifested by elevated total organic carbon (TOC) content and high C:N (up to 58) and C:P ratios (up to 184) (Wang et al. 2010). According to Wang et al. (2010) in hydrocarbons contaminated aged soils, the values of total nitrogen were lower in comparison with uncontaminated soil, while total phosphorus content did not differ significantly. High C:N ratio is not favourable for bioremediation efficiency because microorganisms will deplete nitrogen stock before breaking down organic molecules. Also, high mineral nitrogen application ranging from 200-300 N mg/kg may be toxic for soil biota and inhibit microbial activity (Carmichael

and Pfaender 1997; Braddock et al. 1997). Usually, the optimal C:N:P ratio for bioremediation of hydrocarbons contaminated soils ranges from 100:5:1 to 100:15:1 (Shahi et al. 2016). To balance the C:N:P ratio, inorganic and organic fertilizers are in general used as it will be discussed in chapter 3.

2. Fundamentals of petroleum hydrocarbons metabolism

2.1 Characteristics of petroleum degrading microorganisms

To conduct a successful bioremediation, a wide range of information about the indigenous degrading microbial population present on the site is necessary, including abundance and diversity as well as the optimal conditions of growth such as: energy and carbon sources, amount of dissolved oxygen, pH, salinity and moisture content (Ramirez-Fuentes et al. 2002; Zhang et al. 2005; Masciandaro et al. 2013).

Highly acidic as well as strongly alkaline pH values showed inhibitive impact on the process (Ramirez-Fuentes et al. 2002), nevertheless, slightly alkaline conditions can lead to improved hydrocarbon degradation (Masciandaro et al. 2013). Poor water content, lower than 45% of the soil water holding capacity (WHC), may result in decreased activity and mobility of microorganisms, while high water content, higher than 85%, generally leads to the rise of anaerobic conditions (Masciandaro et al. 2013). Understanding of factors limiting the microbial growth on contaminated site and proper management are the key points in bioremediation planning.

Petroleum degrading microorganisms can be found among bacteria, filamentous fungi and yeasts. The ecological analysis of soil microbiota, based on culture dependent and culture independent studies have revealed a dominance of three bacterial phyla able to degrade hydrocarbons: Proteobacteria, Actinobacteria and Firmicutes (Fuentes et al. 2014). Among Fungi petroleum-degrading agents were found within genera like *Rhodotorula, Sporobolomyces*, *Aspergillus*, *Penicillium* and *Lentinus* (Masciandaro et al. 2013).

Presently more than 79 bacterial genera are classified as petroleum-degrading (Xu et al., 2018). Among them, the most curtail are *Achromobacter, Acinetobacter, Aeromonas, Alcanivorax, Alkanindiges, Alteromonas, Arthrobacter, Bacilli, Burkholderia, Dietzia, Enterobacter, Flavobacteria, Gordonia, Kocuria, Marinobacter, Micrococcus, Mycobacterium, Nocardia, Pandoraea, Pseudomonas, Sphingomonas, Staphylococcus, Streptobacillus, Streptococcus, Streptomyces and Rhodococcus* (Wentzel et al. 2007; Masciandaro et al. 2013; Xu et al., 2018).

Examples of bacterial species able to degrade PHCs are presented in Table 2.1. In last years, popularization of culture independent methods for bacteria identification allowed a deeper understanding of bacterial variety within contaminated soil. However, many hydrocarbon degrading bacteria species still remains undiscovered. Majority of PHCs degraders were obtained from contaminated lands including refinery soils or marine and terrestrial oils spills. It was confirmed, that due to natural presence of hydrocarbons in environment, bacterial species able to degrade crude oil compartments can be found also in uncontaminated habitats, which constitutes an interesting area of studies (Wang and Shao, 2013).

Some bacteria evolved specific mechanism enhancing adaptability. As accessibility to the contaminants often constitutes a major factor limiting the biodegradation rate, some microorganisms have developed a strategy to overcome it. *Mycobacterium* species has developed unique ability to reach the surface of highly hydrophobic contaminants by adhesion caused probably by the presence of high amounts of mycolic acids in the cell walls (Pagnout et al. 2006). Other bacteria species like *Pseudomonas aeruginosa* produce biosurfactants like rhamnolipids to increase bioavailability of hydrophobic compounds (Rahman et al. 2002).

The ability to metabolize wide spectrum of organic compounds including potentially toxic substances is favourable in the evolutionary process. The wider the metabolic capacities of the strain are, the better the fitness of the microbial colony during depletion of preferable carbon source is. Microorganisms evolved many systems enabling survival in diverse and unstable environments. For example, wide substrate range of enzymes allows binding of many compounds with similar structure in an active centre. Wide metabolic range can be crucial in bioremediation considering the diverse composition of petroleum contaminants. Another strategy to metabolize different hydrocarbons is the use of many enzymes with different substrate range, which expression is induced by the current type of available carbon sources (Fuentes et al. 2014). Additionally, the ability to metabolize toxic compounds provides an opportunity to function while other sources of carbon and energy will be depleted (Fuentes et al. 2014).

Table 2.1 Bacterial species able to growth on specific petroleum hydrocarbons. (Adapted from Lumactud et al., 2016; Xu et al., 2018)

Bacterial species	Petroleum hydrocarbons as carbon and energy source	Isolation source	Reference
Dietzia sp.	n-alkanes (C6-C40)	production water of a subterranean oil-reservoir	(Wang et al., 2011)
Pseudomonas sp.	n-alkanes (C14-C30)	n.d.	(Sugiura et al., 1997)
Oleispira antarctica	n-alkanes (C10–C18)	Antarctic coastal sea water	(Yakimov et al., 2003)
Rhodococcus ruber	n-alkanes (C13–C17)	n.d.	(Zhukov et al., 2007)
Geobacillus thermodenitrifican	n-alkanes (C15–C36)	n.d.	(Abbasian et al., 2015)
Rhodococcus sp.	Cyclohexane	oil-contaminated soil	(Lee and Cho, 2008)
Alcanivorax sp.	n-alkanes and branched alkanes	oil-contaminated seawater	(Hara et al., 2003)
Gordonia sihwensis	n-alkanes and branched alkanes	raw sludge from a wastewater treatment facility	(Brown et al., 2016)
Achromobacter xylosoxidans	Mono- /polyaromatics	petroleum-contaminated soil	(Ma et al., 2015)
Aeribacillus pallidus	Mono- /polyaromatics	geothermal oil fiel	(Mnif et al., 2014)
Mycobacterium cosmeticum	Monoaromatics	n.d.	(Zhang et al., 2013)
Pseudomonas aeruginosa	Monoaromatics	crude oil	(Mukherjee et al., 2010)
Cycloclasticus	Polyaromatics	seawater	(Kasai et al., 2002)
Neptunomonas naphthovoran	Polyaromatics	creosote-contaminated sediment	(Hedlund et al., 1999)
Bacillus licheniformis *Bacillus mojavensis*	Polyaromatics	oil-contaminated soil	(Eskandari et al., 2017)
Sphingomonas *Sphingobium* *Novosphingobium*	Polyaromatics	n.d.	(Ghosal et al., 2016)
Pseudomonas sp.	Resins	Sediment from Japanese coasts	(Venkateswaran et al., 1995)
Pseudomonas spp. *Bacillus sp.*	Asphaltenes	oil	(Tavassoli et al., 2012)
Citrobacter sp. *Enterobacter sp.* *Staphylococcus sp.* *Lysinibacillus sp.* *Bacillus sp.*	Asphaltenes	oil contaminated soils and sludge	(Jahromi et al., 2014)

Pseudomonas sp.			
Microbacterium foliorum, Plantibacter flavus Stenotrophomonas rhizophila, Arthrobacter pascens, Curtobacterium, Pseudomonas, Microbacterium oxydans, Xanthomonas gardner	Motor oil	oil contaminated soil	(Lumactud et al., 2016)
Microbacterium foliorum, Plantibacter flavus, Stenotrophomonas rhizophila, Clavibacter michiganensis, Microbacterium oxydans, Xanthomonas gardner	Polyaromatics	oil contaminated soil	(Lumactud et al., 2016)
Microbacterium foliorum, Plantibacter flavus, Stenotrophomonas rhizophila, Microbacterium oxydans, Xanthomonas gardner	Kerosene	oil contaminated soil	(Lumactud et al., 2016)

2.2 Biodegradation of aliphatic hydrocarbons

Metabolic pathway of hydrocarbon degradation is a specific process among different bacterial and fungi species; nevertheless, some similarities can be observed over aerobic catabolism. In case of alkanes (Figure 2.1), hydroxylation reaction is catalysed by alkane oxygenases, which

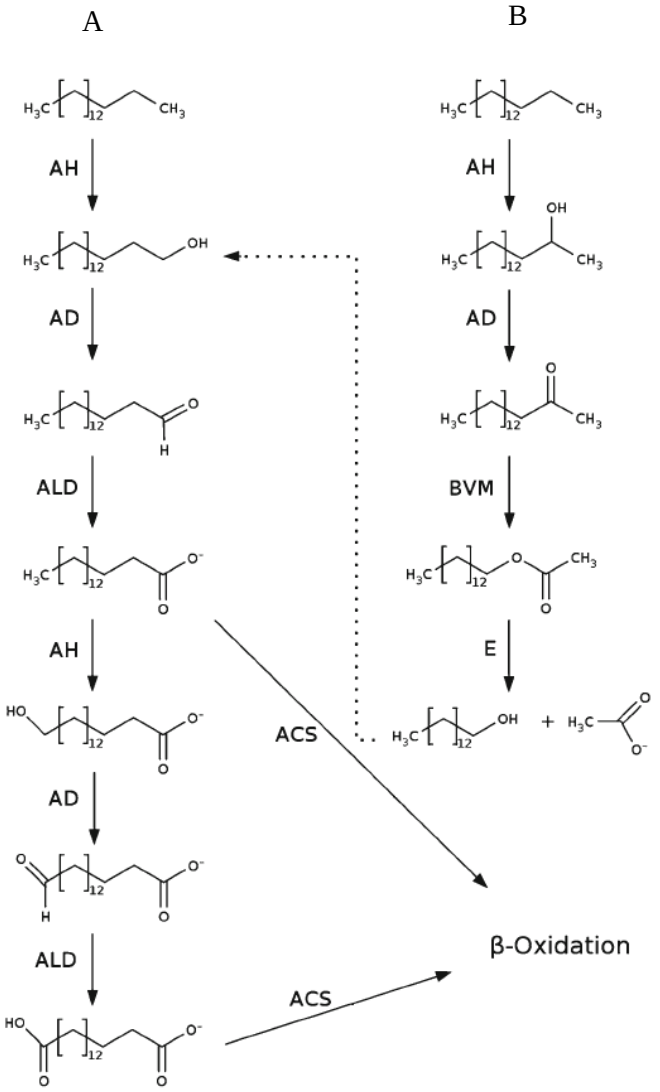

Figure 2.1 Bacterial n-alkane aerobic degradation pathways. A - Alk-like degradation pathway, the reaction product can be carboxylic acid or dicarboxylic acid, if ω-hydroxylation occurred. B - Sub-terminal oxidation of n-alkanes conducted by *Rhodococcus*, *Mycobacterium*, and *Pseudonocardia* strains. Abbreviations: *AH* alkane hydroxylase, *AD* alcohol dehydrogenase, *ALD* aldehyde dehydrogenase, *ACS* acyl-CoA synthetase, *BVM* Baeyer–Villiger monooxygenase, *E* esterase (Adapted from: Fuentes et al. 2014).

is a diverse group of enzymes incorporating bacterial particulate alkane hydroxylases (pAHs) or a cytochrome P450 alkane hydroxylases (van Beilen and Funhoff 2007). The studies on pAH of a *Pseudomonas putida* isolate GPo1 revealed that the enzyme is integral membrane non-hemediiron monooxygenases of the *AlkB*-type (van Beilen and Funhoff 2007). These enzymes cooperate with mononuclear iron rubredoxin reductase and dinuclear iron rubredoxin to transfer electrons from NADH to the active site (Wentzel et al. 2007). *AlkB*-type enzymes were found

within genera like *Acinetobacter*, *Alcanivorax*, *Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus* and were identified with the biodegradation of C5 to C16 alkanes, fatty acids, alkylbenzenes and cycloalkanes. Although some of *alkB* monooxygenases oxidize short-chain alkanes, most of them prefer long chained hydrocarbons with carbon chain longer than C10 (van Beilen and Funhoff 2007). P450 alkane hydroxylases are more frequently reported for yeasts than bacteria. In the study of Maier et al. (2001) the sequence of cytochrome P450 was found in isolates grown on C5 to C10 alkanes. Further analysis has confirmed that the enzyme was responsible for hydrocarbon degradation (van Beilen et al. 2006).

Alkane oxygenases exhibit structural differences, related for example to the active site of the enzyme, which can contain iron–sulphur, di-iron, heme or copper (Fuentes et al. 2014). Monooxygenases are responsible for the oxidation of linear or branched alkanes. The hydroxylation of alkanes leads to the creation of alcohol, which is further oxidized to aldehyde by alcohol dehydrogenase. Aldehyde is subsequently oxidized by aldehyde dehydrogenase to carboxylic acid, which serves a substrate for acetyl coenzyme A (acyl-CoA) synthesis which is further funnelled into β-oxidation pathway (Fuentes et al. 2014). Substrate range of monooxygenases is one of the factors determining the variety of alkanes, which can be degraded by a particular strain.

Microorganisms degrading alkanes can be found within eubacteria, yeasts, fungi and even algae (van Beilen and Funhoff 2007). Bacteria, which possess the ability to degrade alkanes, occur within phyla such as Proteobacteria, Actinobacteria, Actinomycetales, Firmicutes (*Bacillus*, *Geobacillus*), Deinococcus-Thermus (*Thermus*) and Bacteroidetes-Chlorobi (Flavobacteriia, Sphingobacteria) (van Beilen and Funhoff 2007). Alkane degraders occur not only in petroleum-contaminated sites, their presence has also been noted in non-polluted ecosystems (van Beilen and Funhoff 2007).

Aliphatic hydrocarbons are often mineralized into H_2O and CO_2, however production of intermediate metabolites may affect the biodegradation process. Aldehydes are one of the intermediate products on the degradation pathway of alkanes. Water solubility of aldehydes is higher than alkanes, thus temporary accumulation may lead to elevated soil toxicity. This phenomenon may be observed often in the initial phase of bioremediation treatment as microbial activity is stimulated by nutrient addition (Xu and Lu 2010; Qin et al. 2013).

3 The use of organic matter in bioremediation and its environmental implications

3.1 Nutrient source for bioremediation

Solid organic waste originating from agricultural or municipal sources constitutes a valuable amendment for hydrocarbons biodegradation. The use of organic waste in soil bioremediation is cost efficient and resource saving alternative to inorganic fertilizers and it can provide additional benefits to soil in a long term (Steiner et al. 2007). Inorganic amendments are soluble in water and quickly consumed by microorganisms and plants. Mature organic amendments are more stable because of low solubility and low biodegradability. Moreover, addition of organic matter into soil contributes to the turnover of carbon and nutrients in SOM. Addition of organic nutrient has also a positive impact on the improvement of soil physical and chemical characteristics such as aeration, pH neutralization and total organic carbon maintenance (Nardi et al. 2004). Improved soil parameters contribute to creation and maintenance of suitable condition for well-functioning of microbial societies (Masciandaro et al. 2013).

In many studies regarding soil treatments performed at laboratory scale (Table 3.1), addition of organic matter to contaminated soil was shown to significantly improve the rate of hydrocarbon biodegradation (Namkoong et al. 2002; Juteau et al. 2003; Kriipsalu et al. 2007; Sayara et al. 2010c). In order to compare the effectiveness of different nutrient sources, Table 3.1 also presents details regarding studies reporting on the use of mineral fertilizers (Lamy et al. 2013; Sutton et al. 2013; Mihial et al. 2006). To obtain optimal conditions for microbial development, the use of nutrient in bioremediation treatments was combined with the application of bulking agent *e.g.* straws, wood chips, gravel grass clippings (Mihial et al. 2006; Sayara et al. 2010c). This additional amendment allows improving oxygen transfer into the soil mixture, thus enhancing the activity of aerobic bacteria.

Different benefits may be obtained depending on the bulking agent selected. For example, it was observed that peanut hull powder added to hydrocarbon contaminated soil acted as a bio-carrier, improving the mass transfer of water, oxygen, nutrients and contaminants, and constituted an additional nutrient source for microflora (Xu and Lu 2016). The effect of bulking

agents such as pine shavings, chopped grass and wheat straw on C:N ratio during treatment with liquid swine manure was studied by Barrington et al. (2002). In this study nitrogen losses by volatilization were correlated with CO_2 emission while CO_2 emission was correlated with biological oxygen demand (BOD) of the bulking agent.

Studies performed at laboratory scale enable strict control of experimental conditions, and simplification and distinction of specific process parameters, which can allow understanding the complex factors (*e.g.* the effect of microbial cell hydrophobicity on the adhesion to oil droplets). During full-scale experiments a strict control of the operating conditions is rarely possible. However, some processes relate to the scale of experiment cannot be observed during batch or microcosm tests. For example, the temperature rises up to 50°C during composting may only occur during large scale treatments due to higher number of active bacteria and better heat isolation in the middle of the reactors or biopiles. Table 3.2 displays a set of operating conditions for large-scale operations.

In the study of Rojas-Avelizapa et al. (2007), soil originating from oil-based drill muds, with high content of recalcitrant hydrocarbons, was mixed with urea, mineral nutrients and straw as a bulking agent (Table 3.2). The authors have observed almost complete depletion of the contaminant after 180 d of treatment. In another bioremediation experiment, soil polluted with fuel oil was fertilized with mineral nutrient and amended with soft wood sawdust and river sand as a bulking agent (Beškoski et al. 2011). The final 94% decrease of TPH concentration after 150 d of bioremediation was evidenced by 96, 97 and 83% reductions of the aliphatic, aromatic, and NSO-asphaltene fraction, respectively (Beškoski et al. 2011). In the study of Coulon et al. (2010), bunker C fuel oil contaminated soil fertilized with mineral nutrient and installed active air pumping system revealed 80% TPH removal efficiency after 196 d, however, human risk criteria were not fulfilled. Jørgensen et al. (2000) composted lubricating oil-contaminated soil as well as diesel fuel-contaminated soil with mineral nutrients and bark chips as a bulking agent. Over the 150 d of bioremediation, for both soils, the highest degradation intensity was observed within the two first months of treatment and followed a first-order degradation pattern. The authors highlighted that aged TPH at concentrations below 1800 mg kg^{-1} are more difficult to remediate in comparison with freshly contaminated soil due to contaminant ageing and bioavailability decrease, thus final low end-point concentration was not achieved. Li et al. (2002) observed low decrease of TPH content, reaching maximally 57% after 53 d of treatment of oil-contaminated soil fertilized with organic pellet and amended with rice hulk as a bulking

agent. Degradation of resins and asphaltenes was much slower in comparison to aromatic and saturated fractions, affecting the global process efficiency.

Table 3.1 Comparison of the impacts of different nutrient sources and operating conditions on TPH degradation during bioremediation experiments on a laboratory scale. Mineral nutrients as well as organic matter addition to soil result in high bioremediation efficiency. Bioremediation of artificially spiked soil proceed faster in comparison with contaminated soil due to lack of weathering process which decreases contaminant bioavailability and in consequence degradation. Abbreviations: *BA* bulking agent, *TPH* total petroleum hydrocarbons, *PAHs* polycyclic aromatic hydrocarbons, *WHC* water holding capacity.

Contaminant	mperature/ Humidity	Time (d)	Nutrient	Bulking agent	Process efficiency	References
TPH (contaminated soil)	30 °C 1 g soil:2.5 mL	100	Mineral nutrient	Not added	64-100 %	(Sutton et al. 2013)
Hexadecane (spiked soil)	20 °C 1 g soil: 0.15-0.35 mL	14	Mineral nutrient C:N:P ratio of 100:10:1	Not added	40-69%.	(Lamy et al. 2013)
Pyrene (spiked soil)	40–60%	30	OFMSW compost* 1:0.5-1:2 w/w (optimal 1:1.3-1:1.4)	Soil mixture:BA 1:1 v/v	86-100%	(Sayara et al. 2010 c)
PAHs, Alkanes (contaminated soil)	20-22 °C saturated	30	Activated sludge 0.7 and 0.07%w/w	Not added	Alkanes (80%) PAHs (77%)	(Juteau et al. 2003)
PAHs, Alkanes (contaminated soil)	20-22 °C 66% WHC	60	Activated sludge 0.18% w/w	Not added	Alkanes 99% PAHs 80%	(Juteau et al. 2003)
TPH (contaminated soil)	30%	182	Mineral nutrient	BA 25% w/w of gravel grass clippings mixed with sheep manure	96.7%	(Mihial et al. 2006)
Diesel fuel (spiked soil)	20 °C 70%	30	Sewage sludge/compost 1:0.1, 1:0.3, 1:0.5, 1:1 w/w	Not added	Sludge - 88-99.5 %, Compost - 69-99.6 %	(Namkoong et al. 2002)
TPH (contaminated soil)**	-	373	Matured oil compost, kitchen waste compost,	Sand, shredded waste wood	TPH 74% PAHs 97%	(Kriipsalu et al. 2007)

*Compost with different levels of biological stability; **Refinery sludge remediation.

Table 3.2 Operating condition for full-scale hydrocarbon contaminated soils treatments in biopiles. Treatment efficiency depends on contamination and soil type. Period of 5 months or longer is needed for efficient full-scale soil bioremediation. Abbreviations: *TPH* total petroleum hydrocarbons, *WHC* water holding capacity.

Operating time (d)	Contaminant concentration (g kg^{-1} soil)	Soil type	Aeration	Nutrient	Bulking agent	Max. temp.	pH	Efficiency of TPH removal	Moisture content	C :N :P	Reference
180	TPH 99.3 ± 23	Clay loam	Mixing	Urea and mineral nutrient	Soil/straw 97/3 (w/w)	45 °C	7.5-8	94%	30-35%	100:3:0.5	(Rojas-Avelizapa et al. 2007)
150	TPH 5.2*	Sand + clay (%) 61 + 35	Mixing (every15 d)	Mineral nutrient	Soil/softwood sawdust/ river sand 4.2/1/4.8 (v/v)	55 °C	7.1-7.6	94%	13-15%	100:10:1	(Beškoski et al. 2011)
53	TPH 25.8 - 77.2*	nd	Passive aeration	Organic pellet	Soil/rice husk 10/1 (v/v)	52 °C	6.8-8	38-57%	14-18%	23-10:1	(Li et al. 2002)
150	Lubricating oil 2.4* Diesel fuel 0.7	nd	Mixing (every 15/30d)	Commercial fertilizer	Soil/Spruce bark 1/3 (v /v)	42 °C	7.1-7.4	70%, 71%	nd	nd	(Jørgensen et al. 2000)
196	TPH 13*	Coarser textured soil	Air pumping in 48h cycle	Mineral nutrient	Not added	nd	6-7	77-80%	70-80% WHC	100: 20: 2	(Coulon et al. 2010)

* Additional application of inoculation/bioaugmentation; nd – no data.

3.2 Digestate: a valuable resource

Energy production from renewable sources and reduction of greenhouse gases (GHGs) emission are currently strongly encouraged in many countries all over the world. Anaerobic digestion is a process aimed at biogas, mainly methane, production from organic residues (Weiland 2010). Nevertheless, the production of biogas from organic matter may generate large quantities of organic by-products, which are digestates in solid and liquid forms. The physico-chemical characteristics of the final digestate depends on properties of applied feedstock and operating parameters of the process (Appels et al. 2008; Kataki et al. 2017). Digestate production is estimated to range between 5 and 80% of input feedstock (Möller and Müller 2012).

Sustainable practice of organic waste management includes the reduction of the amount of wastes deposited on landfills and the facilitation of organic carbon return into the environment. It can be achieved by field application of the digestate solid fraction *e.g.* obtained through soil-liquid separation, as a fertilizer or an amendment during agricultural activities or bioremediation treatments (Mata-Alvarez and Macé 2004).

Solid digestate has at least two main advantages over fresh feedstocks *e.g.* animal manures, food waste and sewage sludge; i) during the digestion process organic matter becomes stabilized and ii) nutrients such as nitrogen and phosphorus are accumulated in digestate in bioavailable forms (Tambone et al. 2010). However, nutrient status depends on the initial nutrient amount in the feedstock and process parameters and may differ significantly between digestates (Tampio et al. 2016). Microorganisms present in organic matter has diverse metabolic capacity due to presence of plenty diverse organic compounds as potential carbon source. Thus, high microbial density and diversity observed in digestate may potentially contribute to contaminants biodegradation.

3.3 Nutrient status, biological stability and native microflora of digestate

High nutrients status of digestate is ensured by anaerobic conditions, during which mineralization of organic matter leads to preservation and concentration of nutrients (Tambone et al. 2010). Nutrient availability to microorganisms is driven by soil chemical properties like pH or redox potential, which change after application of the digestate. Thus, modified conditions of soil environment will affect not only the soil nutrient status but will also have

direct and indirect influence on the activity and composition of the microbial community (Gómez-Brandón et al. 2016). Studies performed by Tambone et al. (2010) analysed 8 different ingestates and digestates in terms of nutrient content and availability. Results confirmed higher content of nutrients in digestates comparing to raw organic matter used as feedstocks for anaerobic digestion. The same study also compared how fertilizing properties of digestates and composts. In conclusion nutrient status and nutrients availability in digestate were comparable to those in composts. Basing on performed studies, the digestate was classified as good candidate to replace inorganic fertilizers, also contributing, to the short-term soil organic matter turnover (Tambone et al., 2010).

Biological stability can be defined as the extent to which easily biodegradable organic matter has been decomposed (Lasaridi and Stentiford 1998). In other words, biological stable products are rich in recalcitrant organic compounds and have low C:N ratio (Tambone et al. 2009). Biological stability of organic waste influences the quality of fertilizer or amendment. Organic matter stabilization relies on the mineralization of easily biodegradable substrates through anaerobic digestion or composting. Heavy organic fractions such as lignin like compounds as well as lipids (Tambone et al. 2009) and steroids are partially accumulated during this process (Tambone et al. 2010). Further stabilization leads to transformation of heavy fraction into simple compounds like polyphenols, reducing sugars, amino acids or fatty acids, which are mineralized or repolymerized into precursors of humic substances (Qi et al. 2012). Many approaches have been tested to measure the biological stability of organic matter, among which respirometry approaches (Ponsá et al. 2008), like dynamic respiration index (DRI) and specific oxygen uptake rate test (SOUR test), get the most of acceptance (Barrena et al. 2009). Within alternative methods of biological stability measurement, anaerobic biogas production was recognized as the most representative (Ponsá et al. 2008; Barrena et al. 2009).

Composted digestate has higher biological stability due to further reduction of easily biodegradable substances. In the maturation phase of digestate composting, low microbial activity was also observed (Bustamante et al. 2013). Furthermore, composted digestate showed values of pH, total volatile solids (TVS) and C:N ratio similar to those of mature compost (Abdullahi et al. 2008). The complex implications after addition of stabilized organic matter to soil are presented in Figure 3.1.

Stable organic matter has been proved to enhance the level of hydrocarbons degradation in higher degree than low stable matter (Sayara et al. 2010 a, b). The reason relies in the high availability of nutrients impacting microbial activity and low content of bioavailable carbon (Tambone et al. 2010; Torres-Climent et al. 2015). In case of non-stabilized organic amendments, the easily biodegradable substrates may constitute the preferential carbon source and limit the degradation rate of the contaminants (Sayara et al. 2010 a). In stable organic matter, the easily biodegradable substances have already been decomposed and microorganisms are forced to use the contaminant as a carbon source. Stable matter has also high nutrient status defined as N, P, K concentrations in bioavailable forms (Scaglia et al. 2014).

Stable amendment is an organic matrix rich in complex molecules like humic acids, which can contribute to SOM turnover and to the maintenance of soil humus balance (Tambone et al. 2009). The fraction of humic acids dissolved in soil-water solution has been found to act as a carrier of organic compounds (Smith et al. 2009). Therefore, addition of composted digestate to the weathered contaminated soils may increase the bioavailability of hydrophobic substances, like most of petroleum hydrocarbons, which enables biodegradation. Furthermore, the study of Sayara et al. (2010b) revealed a positive impact of the presence of humic-like substances from organic matter on the degradation of organic contaminants in soil. The humic acids (HAs) surfactant effect depends on the applied concentration as well as on the present soil conditions. Depending on the dose and pH value of treated soil mixture, humic acids may inhibit, enhance or have no effect on contaminant biodegradation (Liang et al. 2007). The optimal amount of humic acids for enhancing contaminants degradation depends on the type of soil, contaminant and composition of indigenous bacterial populations (Liang et al. 2007).

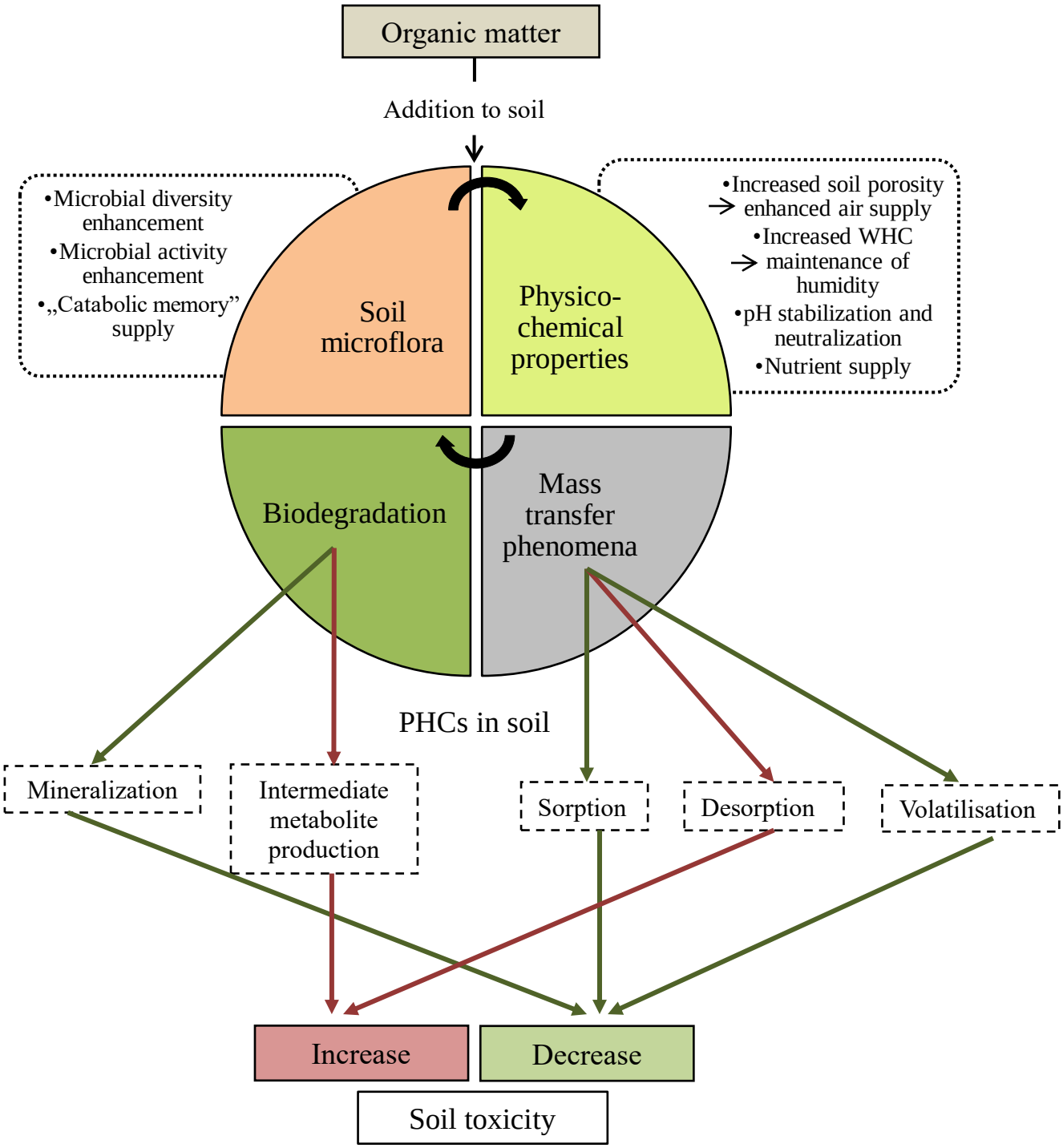

Figure 3.1 Processes occurring after addition of pretreated organic matter to soil. Organic matter improved soil properties and provide wide number of microorganisms including degrading agents. Humic like substances present in organic matter helps to desorb weathered contaminants as well normalize excess concentrations. Abbreviations: *PHCs* petroleum hydrocarbons, *WHC* water holding capacity.

Soil application of organic matter improves total microbial density and diversity (Wang et al. 2016). Organic matter is characterised by rich and diverse native microflora and often contain strains able to degrade complex organic compounds (Liang et al. 2014). In the studies of Liang et al. (2014), sewage sludge addition to soil contaminated with organic compounds (dichlorodiphenyltrichloroethane (DDT) and hexachlorocyclohexane (HCH)) has resulted in soil inoculation with degrading agents including *Pseudomonas sp*., *Bacillus sp*. and *Sphingomonas sp*. and consequently increased biodegradation rate. Moreover, due to horizontal gene transfer (HGT), genes useful in degradation of hydrocarbons may be transferred in soil among bacteria (Shahi et al. 2016). Thus, increase in bacterial diversity and density will positively affect "catabolic memory" of the treated soil (Figure 3.1).

Besides, many studies have suggested that soil humic matter may decrease the bioavailability of organic contaminants through bonding of molecules to the soil matrix (Rivera-Espinoza and Dendooven 2004). Humic acids have a variety of binding sides such as carboxyl, hydroxyl and carbonyl groups which allow for the sorption of diverse trace elements while hydrophobic part is responsible for the sorption of organic compounds (Tejada et al. 2008).

For highly contaminated soils, binding properties of humic matter may be also beneficial in bioremediation process, by decreasing PHCs concentration and thus the toxicity level (Tejada et al. 2008). As a result, organic matter addition can have a stimulating influence on microbial metabolic activity, manifested by increased microbial biomass carbon, enzyme activity and respiration rate (Tejada et al. 2008). Masciandaro et al. (2013) concluded that soil sorption capability is positively correlated with the amount of humic acids in SOM. The final impact of SOM on contaminant mobility depends on (i) the concentration of the contaminant and dissolved humic acids in the soil solution, (ii) nature of interactions between contaminant and other compounds and (iii) the ability of soil matrix to release the contaminant into the aqueous phase (Masciandaro et al. 2013).

4 Hypothesis and aims of the present work

This thesis is aiming to highlight the importance of nutrient source in bioremediation of petroleum hydrocarbons contaminated soils. In the previous chapters the critical view on different types of fertilizers and its potential effect on soil microbiota and contaminant behaviour has been presented. Based on properties favourable for soil bioremediation, digestate was selected for further experimental studies as an example of organic amendment. As soil microorganisms are curtail biological factor in bioremediation, this thesis aimed to focus on the effect of fertilizer (digestate) application on the structure of soil microbial community.

As mentioned previously, digestate contains rich and diverse microbial community which after contact with soil may induce significant changes in soil indigenous microbial structure. Microorganisms native for digestate due to environment providing diverse complex organic compounds (e.g. humic like substances) evolved wide metabolic capacities. Interestingly, the effect of external microbial seeding which is provided by organic fertilizers have never been studied before in terms of microorganisms engaged in PHCs biodegradation. It was hypothesised, that these microorganisms can be also capable of degrading simple petroleum products like linear hydrocarbons. This thesis evaluates potential of digestate as an organic amendment for soil bioremediation, verifies capacity of indigenous microflora of digestate to degrade hydrocarbons and study the effect of soil digestate application on microbial activity and community changes.

Concerning the characteristics of the PHCs contaminated soil used in the study, the special attention was put on bacteria degrading linear hydrocarbons. Usually, saturated hydrocarbons constitute the majority of oil compartments, with aromatic compounds often placed on the second place in significant concentration. In the studied soils, measured concentration of TPH ranged between 5 000 – 32 000 mg kg^{-1} soil. Within measured TPH, concentration of specific PAHs was negligible, and sum of the 16 EPA PAHs did not exceed 20 mg kg^{-1} soil. In this case, special attention was put on the genetic markers indicating degradation of alkanes (*alkB* genes).

The specific objectives of this thesis were as follows:

1. Initial characterisation of digestate microflora:
   - Characterise cultivable petroleum hydrocarbons degrading microorganisms enriched from digestate
   - Study the relation between petroleum hydrocarbons degrading microorganisms enriched from soil and digestate
   - Study the dynamics of petroleum hydrocarbons biodegradation by enrichments originating from soil, digestate or both sources together.
2. Digestate in bioremediation of PHCs contaminated soils:
   - Study how addition of digestate affects petroleum hydrocarbons biodegradation in industrial petroleum contaminated soils under microcosm experimental conditions
   - Study how addition of digestate together with other soil amendments affects microbial respiration in industrial petroleum-contaminated soils with different textures
   - Analyse if digestate addition to industrial petroleum-contaminated soils affects total bacterial abundance in the soils and diversity of PHCs degrading bacterial taxa
   - Study the content of *alkB* genes in digestate and in industrial petroleum-contaminated soils after digestate application.
3. Soil treatment in aerated bioreactors:
   - Valorise digestate as soil bioremediation amendment during treatment in actively aerated bioreactors in comparison to mineral fertilizer
   - Assess the impact of digestate addition on soil remediation through monitoring of PHCs concentration and *alkB* genes dynamics during the treatment together with the diversity of hydrocarbons degrading taxa.

5 Materials and methods

5.1 Overview of the bioremediation experiments

The performed experiments present progressive increase of the complexity of analysed systems and treatment design (Figure 5.1). Initially, to identify the petroleum hydrocarbons degrading capacity of native digestate microflora, a simple batch tests were performed. The enrichment experiment allowed to verify if bacteria originating from digestate are able to utilize petroleum hydrocarbons (added in the batch solution as diesel fuel) as a sole carbon and energy source as well as if these bacteria possess *alkB* genes – curtail in degradation of linear alkanes. Furthermore, the experiment allowed the comparison of hydrocarbon degradation efficiency of bacteria originating from soil and digestate as well as it allowed to study the changes in microbial community structure when both, soil and digestate were enriched together.

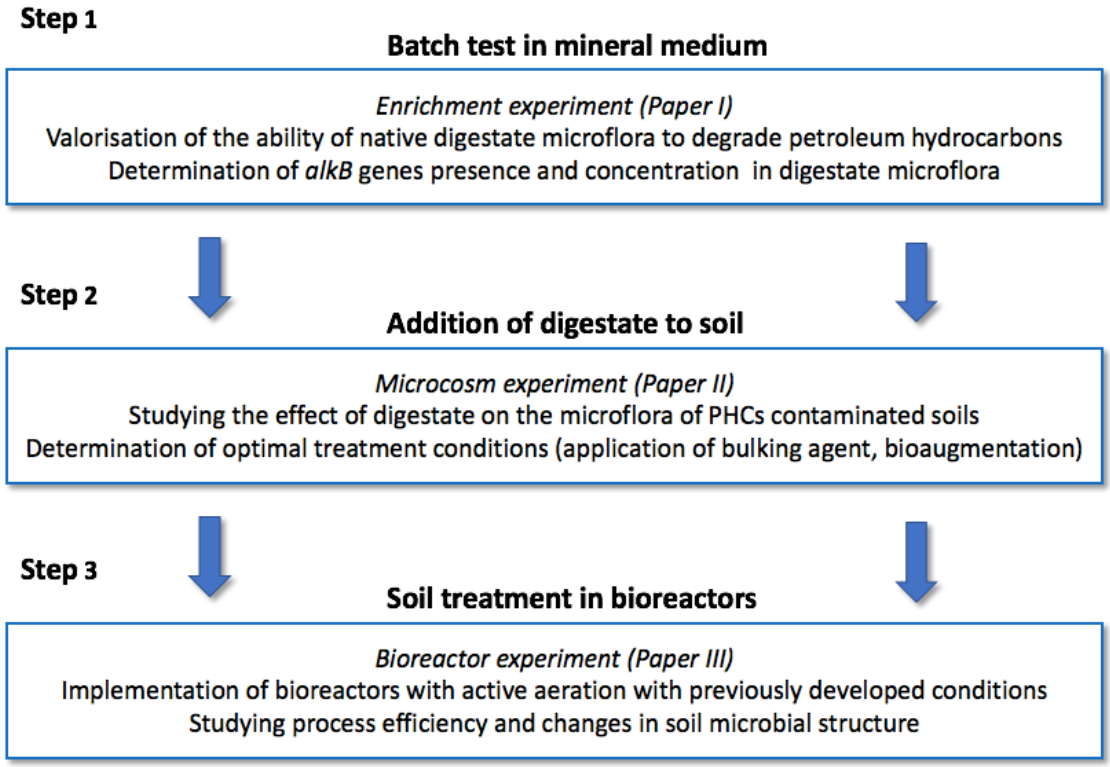

Figure 5.1 Conceptual model of performed experiments.

In the second experiment (Microcosm experiment), PHCs degradation efficiency was studied for industrial refinery soil samples. In this case, the effect of digestate was compared with mineral fertilizer. This simple microcosm experiments allowed to study the microbial abundance and diversity in soil conditions and was not restricted only to cultivable bacterial as in the previous batch tests. Application of digestate alone or together with other soil supplements including bulking agent (saw dust) and inoculum (PHCs degrading bacteria immobilized onto biochar) allowed to recognize the most suitable conditions for soil incubation. The two different types of contaminated soils used in this experiment (clay rich soil and sandy soil) allowed to verify how digestate affects microbial activity represented by soil respiration as a function of soil texture.

The last experiment (Bioreactor experiment) was performed in glass bioreactors with active aeration system. Soil treatment in bioreactors allowed more frequent soil sampling compared to microcosm experiments which enabled detailed studies of changes of the microbial community structure according to available concentration of PHCs. The overview of performed experiments and analytical methods is reported in Table 5.1.

Table 5.1 Overview of the performed experiments and analysed parameters

Analysed parameter	Paper I	Paper II	Paper III
Microbial respiration	OxiTop® system	OxiTop® system	Titration of CO_2 traps
TPH quantification	Final quantification GC-FID	Final quantification GC-FID	Weekly quantification GC-FID
Ecotoxicity assessment	n.a.	Microtox®	Microtox® Germination tests
Real-Time PCR			
16S	Initial and final quantification	Initial and final quantification	Periodical quantification
18S	Initial and final quantification	Initial and final quantification	Periodical quantification
alkB	Initial and final quantification	Initial and final quantification	Periodical quantification
Sequencing	Performed at the end of the experiment	Performed at the end of the experiment	Periodical analysis during the experiment

5.2 Experimental design of performed experiments and materials used

5.2.1 Contaminated soil and digestates used in the batch enrichment experiment (Paper I)

Petroleum hydrocarbons contaminated soil of industrial origin was obtained from a petroleum refinery site located in northern-east of France. The soil was classified as sandy loam. Table 5.2 summarizes detailed characteristics of soils used for all experiments performed during this thesis.

Three types of digestates were used for this experiment. Dry (powder) digestate and fresh digestate were collected from a biogas plant located in Limoges, France. The feedstock used for anaerobic digestion was sewage sludge. The biogas plant in Limoges performs dewatering of digestate and dry digestate was produced on site from fresh digestate. In order to concentrate bacterial cells, fresh digestate was centrifuged and excess of water was removed.
The water content in digestate was reduced from 96 to 81 %. The composted digestate (dry) was obtained from biogas plant located in southern Italy. The feedstock for anaerobic digestion in the plant was organic fraction of municipal solid waste (OFMSW). Composting and drying of digestate were performed by the biogas plant. Characteristics of all digestates used in the thesis are presented in Table 5.3.

Table 5.2 Characteristic of the soils used in the experiments for papers I-III.

Parameter	Paper I	Paper II		Paper III	Method
Texture	Sandy loam	Loam	Fine sand	Sandy loam	NEN 5753
pH (H_2O)	7.4	8.3	5.9	6.2	NF ISO 10693
Water content (%)	0.7	1.3	0.8	0.4	NEN-ISO 11465
WHC (mL cm^{-3})	n.a.	0.44	0.18	0.2	OECD test No. 222
OM (% DW)	n.a.	3.6	2.8	2.2	NF ISO 14235
TOC (g kg^{-1} DW)	16	31	14	15	NEN-EN 13137
TN (g kg^{-1} DW)	0.3	0.9	0.1	0.3	NEN-EN-ISO 11732
P (g kg^{-1} DW)	0.3	0.6	0.1	0.3	NEN 6961, CEN/TS 16171, NF-EN 16179
C:N:P	100:1.9:1.9	100:2.8:1.8	100:0.7:0.6	100:2.3:2	-
TPH (mg kg^{-1} DW)	3200	6100	32600	13200	Internal method
EPA 16 PAHs (mg kg^{-1} DW)	< 1	12	20	25	Internal method (SynLab)
	Elements content (mg kg^{-1} DW)				NEN 6961, NEN-EN-ISO17294-2
Fe	7500	5400	7600	7600	
Cu	n.a.	150	<1	57	
Cd	0.3	0.4	<0.2	0.25	
Cr	8.1	15.1	1.2	8.7	
Co	n.a.	150	<1	n.a.	
Hg	6.3	2.4	<0.1	6.5	
Pb	120.1	210	<10	120	
Ni	5.1	13.0	1.6	7.0	
Zn	84.2	180	<10	88	

Table 5.3 Characteristics of the digestates used in the experiments for papers I-III.

	Paper I and Paper II (FD)			Paper III	
Parameter	**Fresh digestate (FD)**	**Dry (powder) digestate (PD)**	**Composted digestate (CD)**	**Fresh digestate (FD)**	**Method**
Water content (%)	81.1	7.4	8.1	95.6	NEN-ISO 11465
TOC (g kg^{-1} DW)	273	316	256	120	NEN-EN 13137
TN (g kg^{-1} DW)	61	56	21	20.8	NEN-EN-ISO 11732
P (g kg^{-1} DW)	33	32	8	10.0	NEN 6961, CEN/TS 16171, NF-EN 16179
C:N:P	100:22.3:12.1	100:17.7:10.1	100:8.2:3.1	100:17.3:8.3	-
TPH (g kg^{-1} DW)	1.8	1.6	1.2	1.8	Internal method
Elements content (mg kg^{-1} DW)					NEN 6961, NEN-EN-ISO17294-2
Fe	54000	57000	10933	54000	
Cd	2.2	2.1	0.8	0.71	
Cr	135.1	42.6	24.7	34.0	
Hg	2.9	1.8	0.1	0.7	
Pb	115.2	81.1	67.3	27	
Ni	29.2	26.7	10.9	8.9	
Zn	895.1	803.3	1.1	270	

5.2.2 Batch scale tests: preparation of soil and digestate enrichments (Paper I)

The experiments were performed to evaluate PHCs degradation efficiency of native digestate microflora, microflora of industrial contaminated soils and both communities together. The origin of digestate as well as its processing may significantly affect microbial community structure. Thus, digestates of different origins and forms (fresh or dried) were used in the study.

The experiments were performed in 150 mL glass bottles containing soil and digestate microbial enrichments which were cultivated in the presence of diesel fuel. For the preparation of enrichments, the bacterial sources (soil and/or digestate) were placed in sterile glass bottles containing 50 mL of modified Bushnell-Haas medium broth ($MgSO_4$ (0.2), $CaCl_2$ (0.02), KH_2PO_4 (1.0), $(NH_4)_2HPO_4$ (1.0), NH_4Cl (3.5) and $FeCl_3$ (0.05) g L^{-1}) and sterile diesel fuel which was used as a sole carbon source (1% *v*/*v*). Prepared flasks were incubated on rotary shaker at 160 rpm and 21 °C for one week and sub-cultivated to enrich diesel degrading microorganisms. Sub-cultivation procedure was repeated 3 times. The list of 7 developed enrichments is presented in Table 5.4.

Table 5.4 Treatment strategies studied during experiments reported in paper I.

N°	Consortia development conditions
I	BH broth (50 mL) + dry digestate (0.4 g) + diesel fuel (0.5 mL)
II	BH broth (50 mL) + soil (5 g) + dry digestate (0.4 g) + diesel fuel (0.5 mL)
III	BH broth (50 mL) + composted digestate (0.4 g) + diesel fuel (0.5 mL)
IV	BH broth (50 mL) + soil (5 g) + composted digestate (0.4 g) + diesel fuel (0.5 mL)
V	BH broth (50 mL) + fresh digestate (2 g) + diesel fuel (0.5 mL)
VI	BH broth (50 mL) + soil (5 g) + fresh digestate (2 g) + diesel fuel (0.5 mL)
VII	BH broth (50 mL) + soil (5 g) + diesel fuel (0.5 mL)
C	Abiotic control = BH broth (50 mL) + diesel fuel (0.5 mL) + NaN_3 (0.05 g)

Enriched microorganisms were harvested, washed with saline phosphate buffer and adjusted to fit an optical density of 1.5 at 600 nm. Afterwards, 1 mL of inoculum was added to 50 mL of broth containing diesel fuel and incubated for 21 days. The experiment was performed in triplicate. More details regarding the experimental procedure can be found in Paper I at the end of the thesis.

5.2.3 Contaminated soils and digestates used in the microcosm experiment (Paper II)

As in previous experiment, the soil was obtained from a petroleum refinery site located in northern-east of France. However, in this case, soil collection point differed, and the soil was classified as loam with high content of clay. Another soil used in this experiment was collected from an industrial site, located near Lyon, France and was contaminated with motor oil (Table 5.2). The soil was classified as fine sand. In this experiment fresh sewage sludge digestate described before was used.

5.2.4 Microcosm treatment of soil with digestate (Paper II)

To study the effect of digestate on soil bioremediation, soil was mixed with different additives to the total weight of 1 kg and placed in glass bottles (1 L). Composition of the 7 treatments is presented in Table 5.5. For treatment with mineral nutrients, $(NH_4)_2SO_4$ and K_2HPO_4 were applied as N and P nutrient sources (Xu and Lu 2010). The bottles were incubated for 30 days at 21 °C and humidity was set to fit 70% of the water holding capacity (WHC) for each treatment. To maintain oxygen conditions the bottles were mixed every 6 days during sampling. Experiment was performed in triplicate. More details regarding the experimental procedure can be found in Paper II at the end of the thesis.

Table 5.5 Treatment strategies studied during experiments reported in paper II.

Setup	Setup composition	Treatment
C	Soil	Natural attenuation
CF	Soil + mineral fertilizer (C:N:P 100:10:2)	Biostimulation
CD	Soil + digestate (25 % w/w ratio)	Biostimulation
CDA	Soil + digestate (25 % w/w ratio) + bulking agent (25 % w/v ratio)	Biostimulation
CB	Soil + biochar (5 % w/w ratio)	Bioaugmentation
CBIF	Soil + mineral fertilizer (C:N:P 100:10:2) + bacteria* immobilized on biochar (5 % w/w ratio)	Bioaugmentation + Biostimulation
CDBI	Soil + digestate (25 % w/w ratio) + bacteria immobilized on biochar (5 % w/w ratio)	Bioaugmentation + Biostimulation

* Bacteria enriched from clay rich soil or sandy soil, respectively.

5.2.5 Preparation of biochar and bacteria immobilization (Papers II and III)

In microcosm and bioreactor experiments (papers II and III), an additional treatment was performed which aims to assess the effect of digestate addition together with microorganisms immobilized onto biochar on the overall PHCs bioremediation efficiency.

Biochar used for the thesis was produced from sewage sludge digestate by pyrolysis at 350°C using the Biogreen® technology as described by Wongrod et al. (2018). To remove impurities biochar was washed with ultra-pure water (UPW) several times until pH of leaching water was stabilized. Preparation of biochar for immobilization of bacteria, including sterilization and drying was performed according to the previous studies (Xu and Lu, 2010).

Microbial enrichment procedure was performed on Bushnell Haas medium in the presence of diesel fuel as described earlier for enrichments in paper I. Microbial source for enrichment was soil S1 and S2 (paper II) and soil mixed with sawdust and digestate (paper III).

Microbial immobilization procedure was performed according to previous studies (Zhang et al. 2016). In brief, enrichments were incubated with biochar on rotary shaker at 150 rpm for 4 days, drained and dried at room temperature under a sterile hood. At the end, to verify the amount of immobilized bacteria, colony forming unit (CFU) was calculated for immobilized samples (Labana et al. 2005). Until use, inoculated biochar was stored at 4 °C no longer than 3 days (Xu and Lu, 2010).

5.2.6 Contaminated soils and digestates used in the microcosm experiment (Paper II)

As in previous experiments, the soil was obtained from a petroleum refinery site located in northern-east of France. Due to different sampling point, obtained soil differed from previously presented in the concentration of PHCs. Details are presented in the Table 5.2. Fresh sewage sludge digestate with water content of 95.6 %. The water content of digestate differs compared to previous experiment. In this experiment water holding capacity of the soil mixture was higher compering to previous microcosm experiment and digestate did not require concentration. Details about used digestate are presented in Table 5.3.

5.2.7 Bioremediation in aerated bioreactors (Paper III)

In this experiment 12 L glass bioreactors were designed according to a previous study (Namkoong et al. 2002). Bioreactors were aerated from the bottom with the air flow ranging from 190 to 200 L h^{-1} m^{-3} soil. The bioreactors scheme is presented on Figure 5.2.

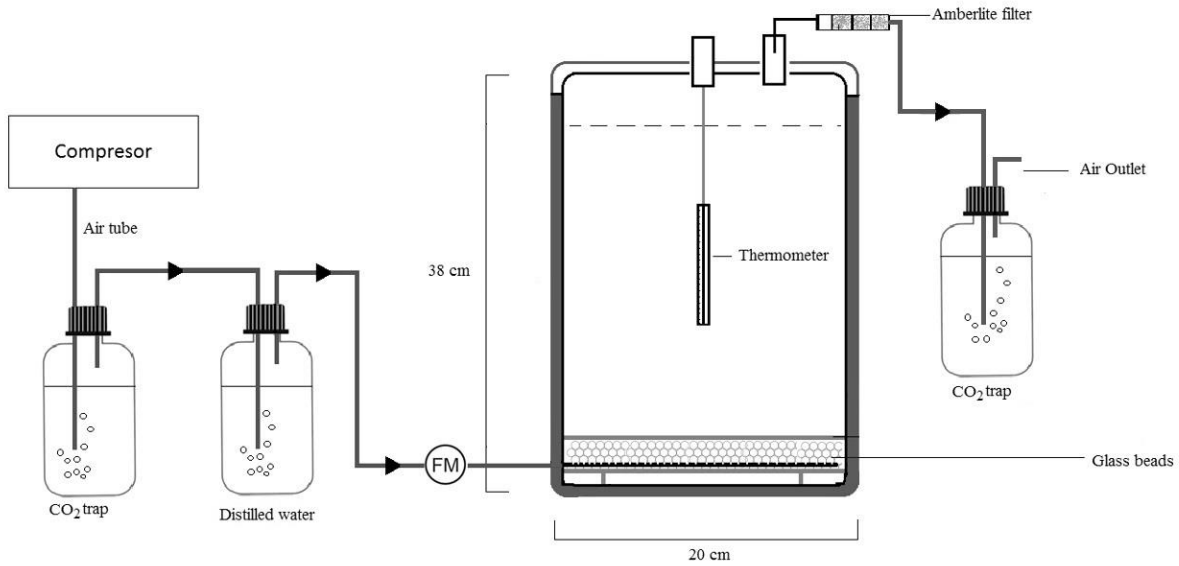

Figure 5.2 Scheme of the bioreactors used for the experiments for paper III.

To study the microbial respiration, represented by the amount of CO_2 evolved during the treatment, the inlet air was passed through a bottle containing NaOH solution acting as CO_2 trap before entering into the reactors. Another NaOH trap was installed in the reactors outlet for titration of CO_2 evolved over time. In order to quantify volatile hydrocarbons or other volatile organic compounds (VOC) released to the air phase, a column with Amberlite® sorbent was

installed in the air outlet for further VOC extraction and quantification. To ensure appropriate porosity of the soil mixture, bulking agent (sawdust) was added in all treatments. In treatment with mineral nutrients (B2) NH_4Cl was used. The list of performed treatments is presented in Table 5.6. The bioreactors were incubated though 63 days at 20 °C (±2 °C). Sampling, mixing of the bioreactors content and humidity adjustment (60 % of the WHC) were performed once a week. Temperature inside the reactors was monitored by thermocouple system. Experiment was performed in duplicate; analyses of each bioreactor were performed in triplicate on three independent samples. More details regarding the experimental procedure can be found in Paper III at the end of the thesis.

Table 5.6 Treatment strategies studied during experiments reported in paper III.

Setup	Description
B1	Control: soil + sawdust (50 % w/v ratio) + water
B2	Soil + sawdust (50 % w/v ratio) + mineral nutrients (C:N:P ratio 100:10:2)
B3	Soil + sawdust (50 % w/v ratio) + digestate (25 % w/w ratio) (D)
B4	Soil + sawdust (50 % w/v ratio) + digestate (25 % w/w ratio) (D) + bacteria immobilized on biochar* (5 % w/w ratio)

5.3 Analytical methods and statistical analysis

5.3.1 Statistical analysis

Statistical analyses were performed using XLStat statistical software for Excel. Significant differences of parameters among the treatments were detected with one-way ANOVA ($p < 0.05$) followed by Tukey test.

5.3.2 TPH and diesel fuel extraction and quantification

The extraction of TPH from the soil samples and quantification with the use of gas chromatography with flame ionization detector (GC-FID) were developed during the thesis. Before each TPH extraction, soil samples were air dried and homogenized by grinding in a mortar. Extraction procedure was developed basing on USEPA 8015B and 3550s methods (USEPA, 1996, 2007). Hexane (main solvent) with methanol (solvent use to break clay aggregates) were used as solvents, extraction was performed by mechanical shaking of samples on the vortex for 1 min and followed by ultrasonic treatment.

For TPH quantification GC-FID (Shimadzu) equipped with capillary column 30 m × 0.25 mm × 0.25 µm (ZB5HT Inferno, Phenomenex) was used. Hydrogen was used as a carrier gas. GC oven and column temperature were adjusted to achieve separation of particular alkanes contained in the standard solution (C6-C40 alkane standard, Sigma). The initial oven temperature was set for 70 °C, kept for 3 minutes and increased by 20°C/minute until 325°C. Injection was done with 1 µL of sample in the split mode with split ratio 20 and temperature 285°C. Column flow was set at 3.0 mL/min with pressure 113.5 kPa. Diesel fuel obtained from a local gas station was used as TPH quantification standard (standard dilutions: 0.0845, 0.845, 2.56, 8.45, 16.90, 42.25, 84.5 mg/mL). To adjust retention time of analysis and identify single alkanes, standard solution containing alkanes ranging from nC7 to nC40 was applied. Androstane was used as internal standard. Terphenyl was used to assess extraction recovery.

5.3.3 Microbial community analysis: Real-Time PCR and Sequencing

For Real-Time PCR and sequencing analysis, meta-genomic DNA was extracted from 500 mg of freeze soil samples (Paper II and III) or 200 µL of microbial enrichments (Paper I) using Fast DNA Spin Kit for Soils (MP Biomedicals) according to manufacturer protocol. Extracted DNA was next eluted in 100 µL of DNA free UPW and testes in terms of purity using spectrophotometer UV-1800 (Shimadzu) equipped with a TrayCell adaptor for micro-volumes (Hellma) (Biache et al. 2017). Such prepared DNA was stored at – 20 ºC until analysis.

Real-time PCR analysis were performed according to existing protocols (Cébron et al. 2008, 2015). The genomic DNA was used to quantify total bacterial (16S) and fungal (18S) populations as well as to quantify *alkB* genes. List of used primers is presented in Table 5.7. Real-time PCR quantifications were performed with the use of CFX96 C1000TM Real Time system (Bio-Rad).

Table 5.7 List of primers used for genes targeting during Real-Time PCR analysis.

Target	Primers	Papers	Reference
16S rRNA gene (DNA)	968F/1401R	I-III	(Felske et al. 1998)
18S rRNA gene (DNA)	Fung5F/FF390R	I-III	(Smit et al. 1999; Vainio et Hantula 2000)
***alkB* rRNA gene (DNA)**	(alkBFd) 50-AAC TAC MTC GAR CAY TAC GG-30 (alkBRd); 50-TGA MGA TGT GGT YRC TGT TCC-30	I-III	(Powell et al. 2006)

Next Generation Sequencing Ilumina MiSeq v3 run (2 x 300 bp) of the V3-4 region of the 16S rDNA was performed by MicroSynth (Switzerland) on extracted meta-genomic DNA. The company is ISO certified according to 9001:2008 and ISO / IEC 17025. Library preparation included sample quality control and Nextera two step PCR amplification using primer set 341f_ill/802r_ill, PCR product purification, quantification and equimolar pooling. Bioinformatic analysis included demultiplexing, merging of forward and reverse reads, quality filtering, trimming, chimera removal, OTU clustering (97 % identity threshold) and subsampling for even sample size (rarefaction to the lower number of reads per sample). Alpha diversity calculation and comparative statistics were done with the use of Phyloseq and DeSeq2 (R packages). Heat map was constructed using Heatmapper software.

5.3.4 Oxygen uptake

Oxygen uptake was monitored during diesel fuel degradation in the liquid broth (I paper) and during microcosm experiment (II paper) with the use of Oxitop® system. During respiration measurement in liquid cultures, 20 mL BH broth and 0.4 mL of appropriate inoculum was sealed inside Oxitop® jars with installed CO_2 trap (in form of NaOH pellet) and incubated on rotary shaker at 160 rpm and 21 °C for 21 days in parallel with experimental batch. In OxiTop system oxygen up-take is measured every 4 hours and registered as a pressure drop (hPa). The oxygen consumption in the flasks was calculated according to equation 1.

$$BR = \frac{\mathrm{M\,(O2)}}{\mathrm{R} \cdot T_m} \cdot \left(\frac{V_b - V_s}{V_s} + \alpha \frac{T_m}{T_0}\right) \cdot \Delta\rho(O_2) \quad (1)$$

where BR: basal respiration (mg O_2 L^{-1}); M(O_2): molecular mass of O_2 (mg mol^{-1}); V_b: bottle volume (L); V_s: sample volume (L); Δρ(O_2): pressure difference (mbar); R: perfect gas constant (L mbar mol^{-1} K^{-1}); T_0: reference temperature (273.15 K); T_m: measuring temperature (K).
Oxygen uptake (soil respiration) was also monitored during microcosm experiment (Paper II). Here, 15 grams of fresh sample was placed in Oxitop® jars and incubated at 21°C, every 6 days samples were replaced. Soil respiration was calculated according to the equation 2.

$$\mathrm{SR} = \frac{\mathrm{M(O_2)}}{\mathrm{R \cdot T}} \cdot \frac{\mathrm{V_{fr}}}{\mathrm{M_s}} \cdot \Delta\rho \quad (2)$$

where SR: soil respiration (mg O_2 g^{-1} DW); M(O_2): molar mass of oxygen (mg mol^{-1}); V_{fr}: free gas volume (L); Δp: pressure difference (mbar); R: general gas constant (L mbar mol^{-1} K^{-1}); T: measuring temperature (K); M_S: soil dry mass (g).

5.3.5 Microtox® ecotoxicity assay

The toxicity of soil elutriates was determined using the Microtox® bioassay according to previous studies (Khudur et al. 2015). Briefly, 1 g of air-dried soil was mixed with 9 mL of UPW and incubated overnight in the dark at 150 rpm. After incubation each sample was centrifuged at 4500 rpm for 10 min. The luminescence was measured on the dilutions of soil leaches at 15 °C after 5 and 15 min of exposure, using a Microtox M500 Analyzer and with *Aliivibrio fischeri* (Hach, France) as a biological reagent. Phenol (20 mg L^{-1}) and zinc sulphate (10 mg L^{-1}) standard as well as reagent control were run with each bath. Delta EC50 represents toxicity loss between samples a day 1 and day 63.

5.3.6 Phytotoxicity assay

Germination tests were performed at the beginning and at the end of the experiments with the use of germination plates and seeds of garden cress (*Lepidium sativum*) and mustard (*Sinapis alba*) purchased from R-Biopharm (France). Germination plates were filled with 80 g of fresh homogenised sample from each reactor. Control consisted of uncontaminated soil (obtained from the same industrial area as contaminated soil) mixed with bulking agent and digestate in the same ratio as soil from bioreactors. Ten seeds were placed equally on each plate. Germination plates were incubated in a growth chamber for 7 days, at 21 °C, with a photoperiod of 12/12 and photosynthetic photon flux density at 100 μmol m^{-2} s^{-1}. After 7 days, germinated plants were counted, and radicle length was measured. Based on obtained data the germination

index (GI) was calculated (Graj et al., 2013) according to the equation:

$$GI = \frac{Gs}{Gc} \times \frac{Ls}{Lc} \times 100[\%] \quad (1)$$

where: Gs and Gc are numbers of seeds germinated in the sample and control, respectively, whereas Ls and Lc are the radicle lengths in the sample and control [mm], respectively.

6 Results and discussion

6.1 Hydrocarbon removal efficiency

6.1.1 Digestate vs. soil - microbial communities potential to remove petroleum products (Paper I)

The potential of native digestate microflora to degrade diesel fuel was analysed during the batch studies (paper I). The results did not reveal significant differences in diesel fueal degradation among microbial inoculum enriched from composted OFMSW digestate and inoculum enriched from petroleum refinery soil. Degradation of diesel fuel for both enrichments was respectively 78 and 77 %, after 21 days (Figure 6.1). Other tested inocula, including enrichments from fresh and dried sewage sludge digestates also showed a great potential to degrade diesel fuel by removing 71 and 74 % of diesel respectively during the incubation time. In the performed study potential of mixed inocula was also analysed, including 3 tested digestates enriched in the presence of soil. Composted digestate, dried digestate and fresh digestate enriched with soil revealed 73, 68 and 64 % of diesel removal after 21 days, respectively. Figure 6.2 presents a summary of the experiment.

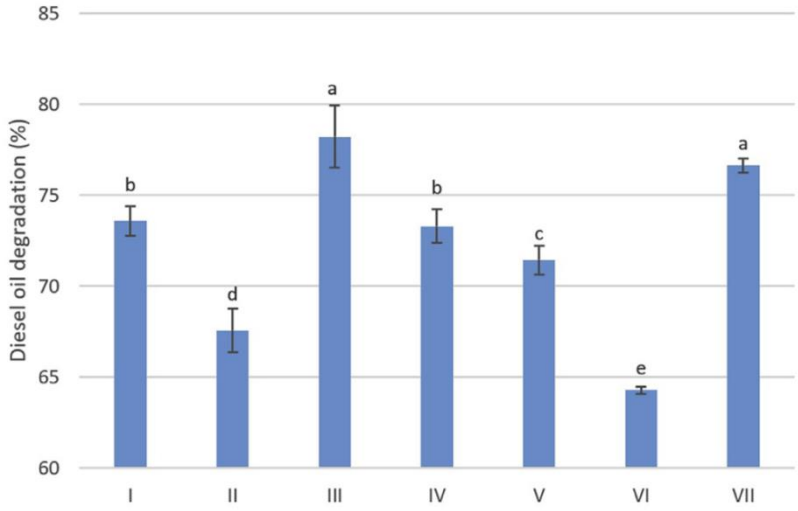

Figure 6.1 Percentage of diesel fuel decrease after 21 days for all tested variants relative to the control (Paper I). I: enrichments from dry digestate; II: enrichments from soil and dry digestate; III: enrichments from composted digestate; IV: enrichments from soil and composted digestate; V: enrichments from fresh digestate; VI: enrichments from soil and fresh digestate; VII: enrichments from soil. Mean (n = 3) and standard deviation. Values followed by the same letter are not statistically different (ANOVA; $p > 0.05$).

The obtained results highlight a good potential of digestate as a source of hydrocarbons degrading bacteria. High degradation efficiency of obtained enrichment is comparable with those observed by other authors. For example, hydrocarbons degrading consortium isolated from soil was able to remove 87 % of PHCs from the medium within 30 days (Raju et al. 2016) or 69 % of diesel within one week (Souza et al. 2015). The obtained results proved that bacteria originating from digestate has similar PHCs degradation potential as bacteria originating from PHCs contaminated soils.

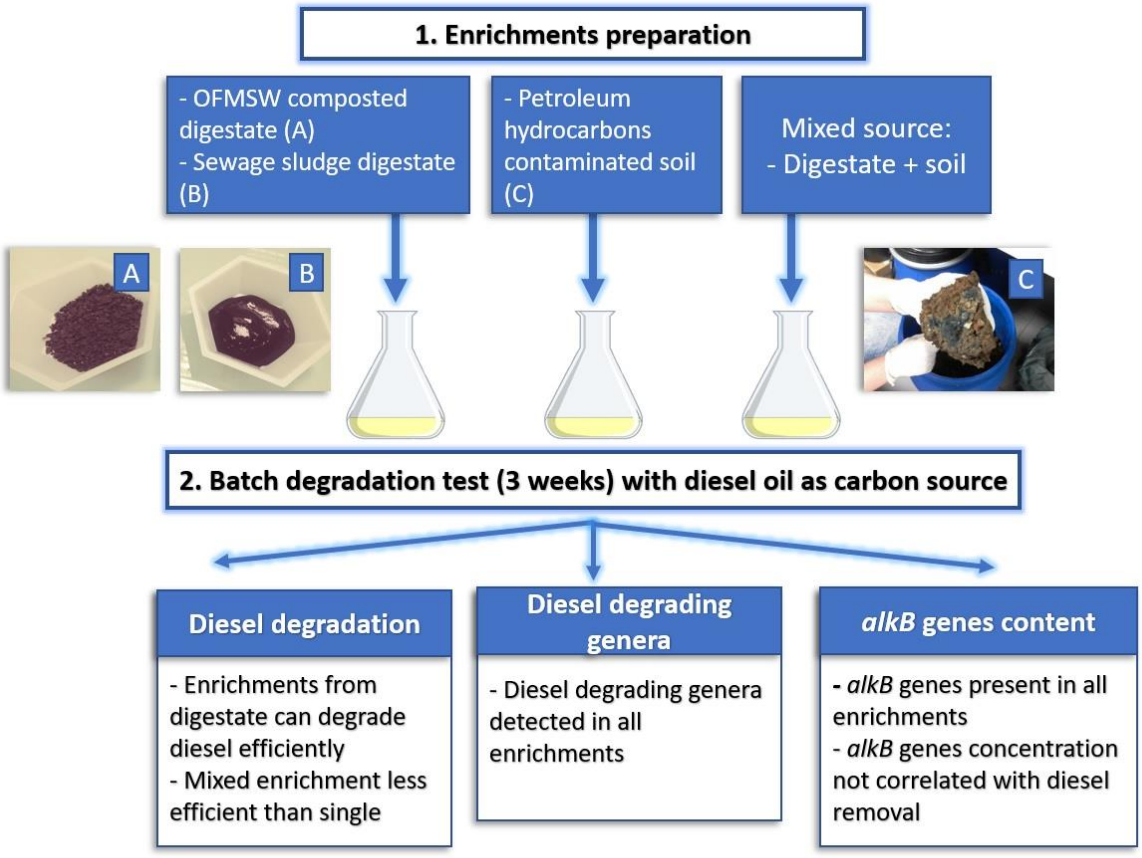

Figure 6.2 Preparation of enrichments and main outcomes of the enrichment experiment (paper I). All microbial enrichments were able to grow on diesel fuel as sole carbon and energy source. Diesel fuel degradation was observed in all flasks.

6.1.2 Hydrocarbons removal under microcosm conditions (Paper II)

During microcosm experiment, no direct relation was observed between application of fresh sewage sludge digestate and hydrocarbons removal from both contaminated soils tested (paper II). Interestingly, combination of digestate with other amendments like saw dust and bacteria immobilized on biochar has resulted in significant increase of hydrocarbons degradation efficiency. After 30 days, in clay rich soil (S1) control, treatment with mineral fertilizer and

treatment with digestate has reached around 10 % of hydrocarbons removal and did not differ significantly (ANOVA; $p > 0.05$) (Figure 6.3). In sandy soil (S2) also the mentioned treatments did not differ significantly (ANOVA; $p > 0.05$), reaching around 24 % of hydrocarbons removal. After addition of sawdust (as bulking agent to increase soil porosity) together with digestate, hydrocarbons removal reached respectively 28 and 37 % in soil S1 and S2, respectively. Addition of bacteria immobilized on biochar together with digestate resulted in 23 and 35 % of hydrocarbons removal in soil S1 and S2, respectively. Interestingly, in treatment with mineral nutrients and bacteria immobilized on biochar no significant hydrocarbons decrease was observed (ANOVA; $p > 0.05$), which suggest that digestate positively affected conditions for bacterial proliferation. The decrease of hydrocarbons in control was probably caused by natural attenuation (Megharaj et al. 2011).

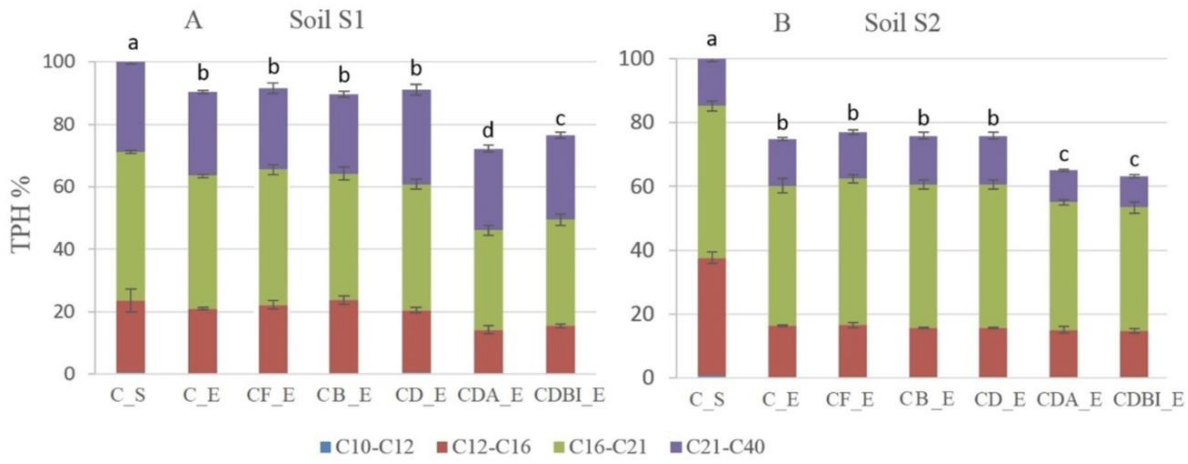

Figure 6.3 Percentage of TPH in clay (panel A) and sandy (panel B) soil after 30 days of treatments. C: soil + water; CF: soil + mineral nutrients; CD: soil + digestate; CDA: soil + digestate + bulking agent; CDBI: soil + digestate + bacteria immobilized on biochar; S: day 1; E: day 30. Mean (n = 3) and standard deviation. The same letters represent no significant differences among treatments for each soil, respectively (one-way ANOVA; p N 0.05).

According to the highest hydrocarbon degradation levels obtained for conditions CDA and CDBI, the use of sawdust and bacteria immobilized onto biochar together with digestate was further evaluated under bioreactor experiment.

6.1.3 Monitoring of hydrocarbons concentration in bioreactors setup (Paper III)

In bioreactor treatment (paper III) addition of mineral nutrients resulted in 56.5 % of hydrocarbons removal after 63 days, while addition of digestate as a source of nutrients and bacteria revealed 73.7 % removal (Figure 6.4). The most efficient treatment was combination of digestate addition and soil bioaugmentation with bacteria immobilized on biochar with 94.5 % of contaminant removal. In treatments containing digestate, hydrocarbons removal was faster than in treatment with mineral nutrients. In both treatments with digestate 50 % of hydrocarbons was removed in the first month of the treatment, which is in accordance with other studies analyzing the effect of organic amendments on soil bioremediation performance (Wang et al. 2011). In all of the treatments, a decrease of characteristic unresolved chromatographic peak was observed together with hydrocarbons removal which points for removal of weathered polar substances of recalcitrant nature (Bruckberger et al. 2018).

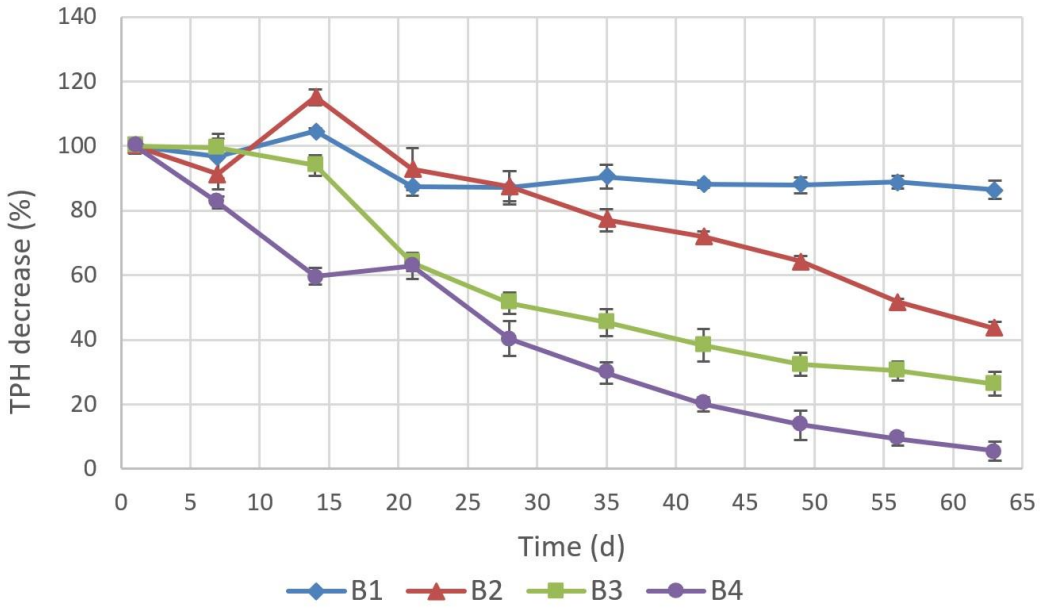

Figure 6.4 Hydrocarbons removal during 63 days of treatment under bioreactor experiment (paper III). B1: soil + sawdust; B2: soil + sawdust + mineral nutrients; B3: soil + sawdust + digestate; B4: soil + sawdust + digestate + immobilized bacteria. Results are presented as a mean of samples collected from two reactors and analyzed in triplicate with standard deviation.

6.1.4 Lesson learned: digestate as fertilizer in soil bioremediation

Performed experiments proved that digestate contains hydrocarbon degrading bacterial strains able to degrade diesel fuel under broth-cultivation conditions and can contribute to PHCs removal during soil bioremediation. Due to mass transfer disturbance, degradation of petroleum product is slower in soil environment than in broth conditions. PHCs degradation efficiency in soil may vary from weeks to months or even years, depending on contamination characteristics

(e.g. weathered or fresh contaminant, complexity and molecular weight of petroleum components) and the scale of the experiment (Megharaj et al. 2011; Dados et al. 2014; Agnello et al. 2016). In batch experiment (paper I) diesel fuel was greatly removed within 3 weeks. In microcosm experiment (paper II) slow contaminant decrease was observed and after one month of treatment less than 50 % of contaminant was removed, while during soil treatment in bioreactors 50 % of contaminant was removed after one month of treatment with digestate and within the second month almost 95 % of hydrocarbons was removed for the most efficient treatment (B4). The differences among both experiments are probably caused by the differences in the oxygen supply. In microcosm experiment the air exchange was passive which could result in oxygen depletion in the bottom of the bottles. In bioreactors experiment, active air supply limited oxygen deficits which was reflected by faster hydrocarbons removal. Also, different characteristics of soil used in both experiments could affect contaminant degradation efficiency. In microcosm experiment soil S1 contained high clay content with relatively low hydrocarbons level (6 100 mg TPH g^{-1} soil) which was affecting contaminant bioavailability while in sandy soil S2 high concentration of hydrocarbons (soil S2: 32 600 mg TPH g^{-1} soil) provoked a toxicological response (initial bacterial growth inhibition). Soil used in bioreactor experiment had sandy texture and TPH level of 13 200 mg g^{-1} soil which could affect fast response for applied treatment.

6.2 Digestate in soil: effect of treatments on soil microbial abundance and activity

In microcosm experiment (paper II) microbial respiration and activity was affected by soil texture and applied treatment. Summary of this experiment is presented on the Figure 6.5. Addition of digestate has improved microbial density and respiration in all treatments, however it was not directly correlated with hydrocarbons removal. Application of digestate in both soils has significantly increased bacterial density. Despite differences between both soils in treatments containing digestate, bacterial density was comparable which suggests that digestate had major effect on soil microbial community structure. Application of digestate has also unified pH values in both soils. Initially soil S1 had alkaline pH of 8.3 (± 0.2) while soil S2 has acid pH of 5.9 (± 0.2). After digestate application (25 % w/w ratio) the pH of soil S1 and S2 was 7.1 (± 0.3) and 6.8 (± 0.4), respectively (both values are not significantly different; ANOVA; $p > 0.05$). In both soils, only the addition of another amendment (sawdust or bacteria immobilized on biochar) together with digestate has resulted in significant decrease of

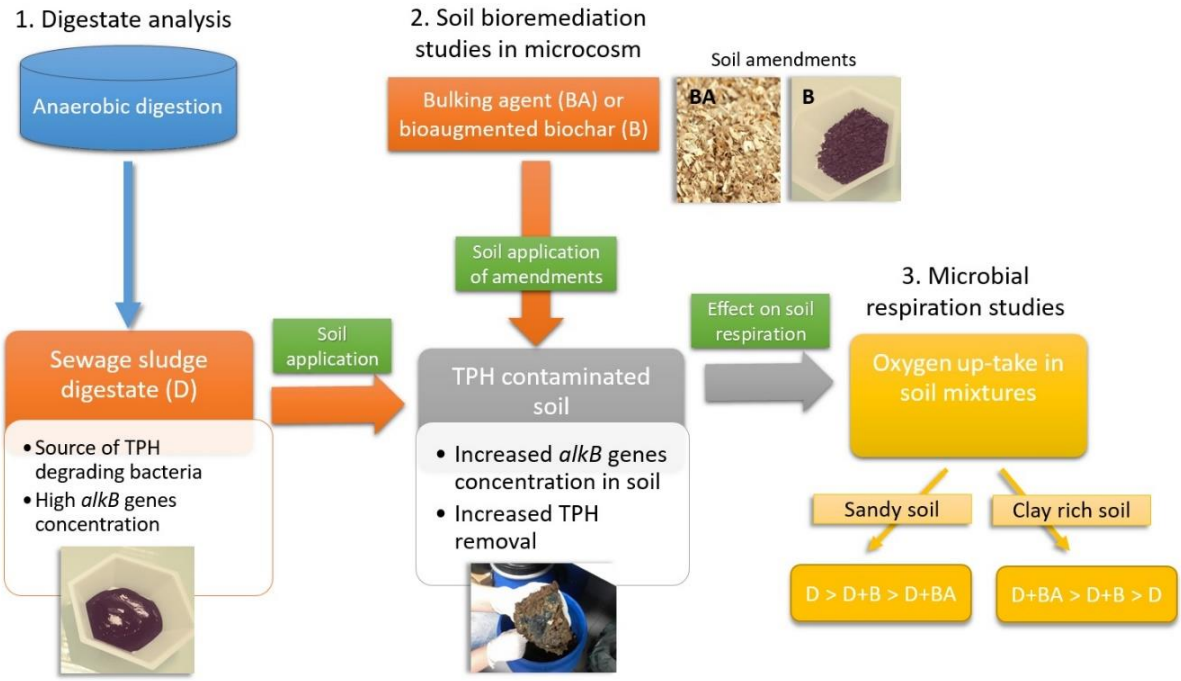

Figure 6.5 Structure and main results of microcosm experiment (paper II). The experiment examined the effect of digestate, saw dust and immobilized inoculum on PHCs bioremediation efficiency in two types of contaminated soils.

hydrocarbons concentration. In clay rich soil (S1) due to formation of aggregates, addition of bulking agent (saw dust) together with digestate has improved soil porosity and additionally increased respiration rate. In this treatment also the highest hydrocarbons removal was observed for this soil type. The observations are in accordance with other studies in which oxygen and nutrient transfer were inhibited in clay rich soils affecting activity of hydrocarbon degrading bacteria (Masy et al. 2016) and further stimulated by the addition of bulking agent (Alvim and Pontes, 2018). Interestingly, in sandy soil (S2) addition of bulking agent together with digestate showed lower respiration rate than in treatment with digestate alone. Soil S2 had a porous texture of fine sand and no aggregates formation was observed, thus addition of saw dust could not evidently affect oxygen transfer and transport. However, addition of saw dust together with digestate has significantly increased hydrocarbons removal. This decrease may be a result of contaminant dilution due to sorption on sawdust and decrease of soil toxicity. This is in accordance with performed sorption tests which showed that one third of contaminant from soil was sorbed onto the sawdust. For both soils addition of PHCs degrading bacteria immobilized on biochar together with digestate has significantly increased hydrocarbons degradation. In addition, application of inoculated biochar together with mineral nutrient has not resulted in hydrocarbons concentration changes comparing to control. Differences in oxygen up-take among both soils are connected with soil texture which affect porosity and impact oxygen

transfer (Yeh and Young, 2003).

In bioreactor experiment (paper III) measured CO_2 evolution was correlated with contaminant removal which suggests that biodegradation was the major force driving hydrocarbons removal. The highest mineralization extent was observed in treatment containing saw dust, digestate and inoculated biochar, after in treatment with saw dust and digestate and in treatment with sawdust and mineral nutrients. Overview of the experiments is presented on the Figure 6.6. Application of digestate resulted in double CO_2 evolution value compared to mineral fertilizer. These results are in agreement with studies of other authors reporting higher mineralization rates in treatments with organic amendments in comparison with mineral fertilizers (Tahhan and Abu-Ateih, 2009; Cerqueira et al. 2014). Values of CO_2 evolution observed during the experiment are in accordance with observations of other authors reporting that 10 g CO_2 kg^{-1} soil was produced during the first month of soil treatment with sewage sludge in similar amendment ratio (Namkoong et al. 2002). Density of bacterial population was measured at the beginning, in the middle and at the end of the treatment, however, no correlation was found between hydrocarbons removal efficiency and 16S rDNA concentration.

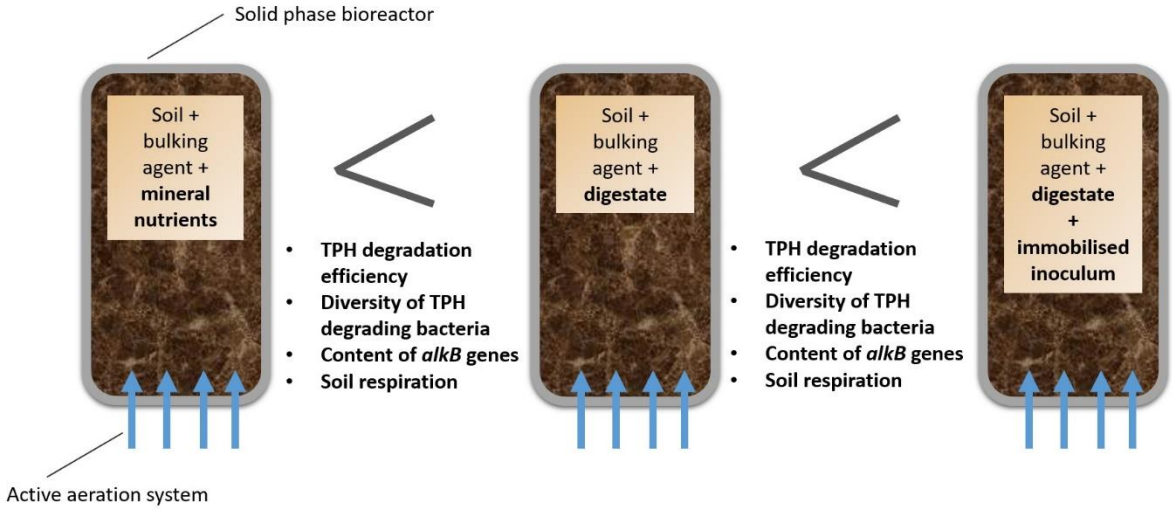

Figure 6.6 Performed treatments and main conclusions of the bioreactor experiment (paper III). The experiment confirmed enrichment in PHCs degrading bacteria and increased abundance of *alkB* genes in soil amended with digestate. Frequent sampling allowed to monitor contaminant degradation together with microbial activity changes and *alkB* genes abundance.

For both microcosms and bioreactor experiments, addition of digestate influenced microbial activity represented by soil respiration in higher extent than mineral nutrients. Digestate addition provoked changes in soil environment, beneficial for development of soil microbiota, by stabilization of soil pH, increase of soil water holding capacity and supplementation of new

nutrient and carbon source. Increased soil respiration after digestate addition was thus a result of increased active microbial population, degrading PHCs as well as organic fractions of digestate.

6.3 Microbial communities' characterisation

6.3.1 Presence of *alkB* genes in digestate and amended soils

In seven tested soil and digestate bacterial enrichments (paper I) *alkB* genes were detected (Figure 6.7). In all enrichments, initial concentration of *alkB* genes has increased during 21 days of incubation with diesel fuel exceeding 10^7 gene copies mL^{-1} at the end of the incubation time. These results prove that: (i) digestate contains bacteria caring *alkB* genes able to degrade diesel fuel, (ii) bacteria caring *alkB* genes were promoted during incubation. In this study the correlation between initial or final *alkB* genes concentration and diesel removal was not found, which is not in accordance with previous studies (Salminen et al. 2008).

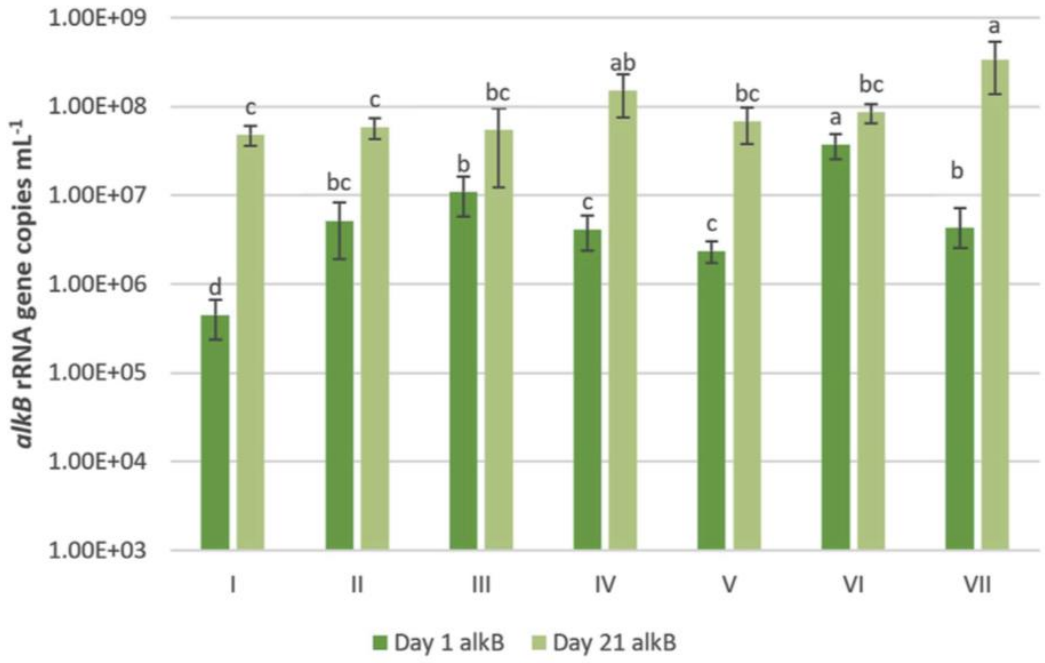

Figure 6.7 Copy number of alkB genes at the be beginning and at the end of the experiment. I: enrichments from dry digestate; II: enrichments from soil and dry digestate; III: enrichments from composted digestate; IV: enrichments from soil and composted digestate; V: enrichments from fresh digestate; VI: enrichments from soil and fresh digestate; VII: enrichments from soil. Mean (n = 3) and standard deviation. Values that are annotated with the same letter among one sampling time are not significantly different (Tukey's multiple range test with p = 0.05).

In microcosm experiment (paper II) high concentration of *alkB* genes was detected in fresh

digestate (over 10^6 copies g^{-1} DW digestate). In analyzed treatments (containing digestate and saw dust or digestate and inoculated biochar) for both tested soils, addition of digestate as well as inoculated biochar has significantly increased the content of *alkB* genes in soil in comparison to control (Figure 6.8). In soil S1 initial copy number of *alkB* genes was increased from 10^5 copies g^{-1} soil in control treatment to over 10^6 copies g^{-1} soil. Similar concentrations of *alkB* genes were observed during bioremediation treatments in other studies (Sutton et al. 2013; Masy et al. 2016). In soil S2, certainly due to high initial concentration of hydrocarbons and low content of soil organic matter low microbial counts were observed, and *alkB* genes were not detected as already observed in other studies (Sutton et al. 2013).

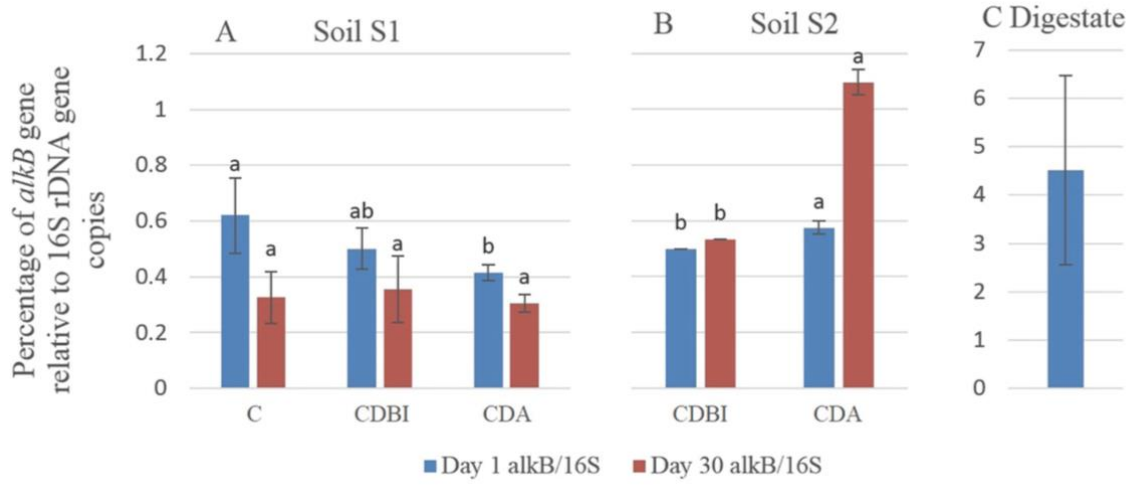

Figure 6.8 Percentage of alkB genes relative to the total bacteria represented by 16S rRNA genes in clay soil (panel A), sandy soil (panel B), and digestate (panel C) at the beginning and end of incubation. C: soil + water; CDA soil + digestate + bulking agent; CDBI: soil + digestate + bacteria immobilized on biochar. Mean (n = 3) and standard deviation. Separate ANOVAs were performed according to the sampling time. Values that are annotated with the same letter among one sampling time are not significantly different (Tukey's multiple range test with p = 0.05). In samples C from soil S2, alkB genes were below the detection limit due to low quantity of extracted DNA.

Percentage value of *alkB* genes relative to total 16S rDNA in soil S1 decreased with time for control (from 0.6 % to 0.3 %) and treatments (from 0.4 % to 0.3 % for treatment with digestate and bulking agent and from 0.5 % to 0.4 % for treatment with digestate and inoculated biochar). The concentration of *alkB* genes is connected to the amount of bioavailable PHCs fraction (Powell et al. 2006; Sutton et al. 2013). In the clay rich soil with high sorption affinity to hydroponic molecules and relatively low concentration of PHCs, bioavailable fraction was expected to be depleted quite fast, thus observed decrease was not surprising. In sandy soil S2 the *alkB* genes percentage has significantly increased for treatment with digestate and bulking

agent and reached the highest value observed in the study of 1.1 % while for treatment with digestate and inoculated biochar the percentage remained stable at 0.5 %. Observed ratio of *alkB* genes is in accordance with other studies (Powell et al. 2006; Sutton et al. 2013). Ratio of *alkB* genes in digestate was higher than observed in digestate amended soils and was equal to 4.5 % which surpass value observed in the literature and proves that digestate as soil amendment can increase soil bioremediation potential by increasing the concentration of functional genes (Kim et al. 2014).

Bioreactor experiment (paper III) has confirmed observation from microcosm study (paper II) and enrichments experiment (paper I). For all treatments containing digestate the amount of *alkB* genes was significantly higher in comparison to control or treatment supplied with mineral nutrients at all measuring points (the beginning, middle part and the end of the experiment) and exceeded 10^7 copies g^{-1} soil. For all treatments, excluding control, concentration of *alkB* genes was the lowest at the beginning of the experiment, increased in the middle phase and slightly decreased at the end. Addition of inoculated biochar has additionally increased the copy number of *alkB* genes to 10^8 copies g^{-1} soil (Figure 6.9). Such a high concentration of *alkB* genes during bioremediation was much higher than values observed in the literature (Sutton et al. 2013; Masy et al. 2016; Shahi et al. 2016). Obtained values of *alkB* genes concentration were in accordance with hydrocarbons removal efficiency.

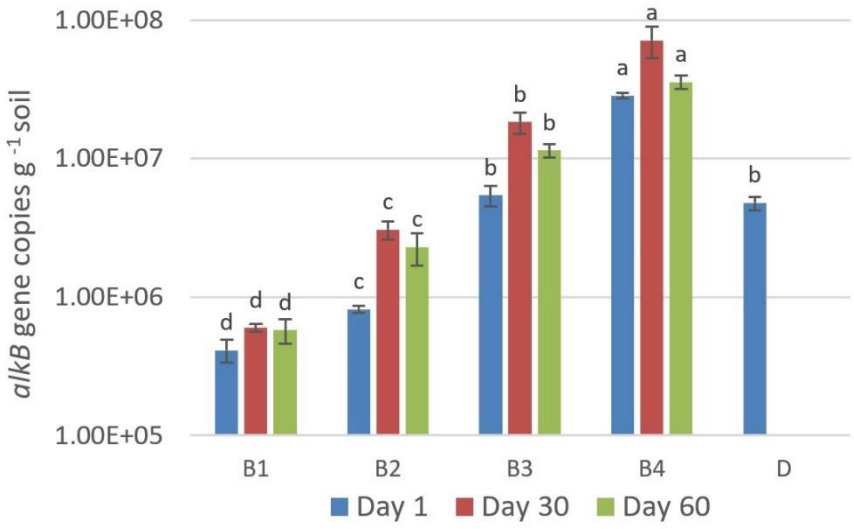

Figure 6.9 Logarithm of *alkB* genes copy number during the treatment under bioreactor experiments (paper III). D – digestate. B1: soil + sawdust; B2: soil + sawdust + mineral nutrients; B3: soil + sawdust + digestate; B4: soil + sawdust + digestate + immobilized bacteria. Results are presented as an average value of three samples collected from the same reactor with standard deviation. The same letters represent no significant differences among treatments (ANOVA; $P \leq 0.05$). Values that are annotated with the same letter among one sampling time are not significantly different (Tukey's multiple range test with p = 0.05).

Percentage ratio of *alkB* genes to total 16S rDNA was also greatly higher in treatments containing digestate and was additionally increased by application of inoculated biochar. For example, in control and treatment with mineral nutrients the initial *alkB* genes ratio was 1.4 % and 0.4 %, respectively while for treatment with digestate and digestate with inoculated biochar the ratio was 4.2 and 17.6 %, respectively. The highest noted *alkB* genes ratio was observed for treatment with digestate and inoculated biochar in the middle of the treatment and reached 60.1 %. Final values of the ratio were 2.0 and 1.3 % for control and treatment with mineral nutrients, respectively, and 11.6 and 11.9 % for treatment with digestate and digestate with inoculated biochar. The results confirm that digestate can supplement and maintain high level of *alkB* genes in the soil.

6.3.2 Microbial taxa identification

6.3.2.1 Bacterial taxa detected during enrichment experiment (paper I)

In enrichment experiment, many hydrocarbon degrading genera were detected (Figure 6.10). *Rhodococcus* and *Achromobacter* were the most common genera and were found in all samples in different densities. *Rhodococcus* genus is well known from hydrocarbons degrading capacities (Cappelletti et al. 2017). Species within the genera possess wide spectrum of alkane monooxygenases and can utilize wide range of organic contaminants as primary substrates or as co-substrates. Bacteria within *Rhodococcus* genus are also capable of biosurfactant production which facilitate the contact and availability with hydrophobic compounds (Cappelletti et al. 2017). *Achromobacter* species were already isolated from crude oil contaminated soils and have proved ability to metabolize petroleum products (Marecik et al. 2015), such as *Achromobacter xylosoxidans* growing on alkanes (Tanase et al. 2013). Best diesel degradations efficiency was achieved by consortia in which *Rhodococcus* and *Achromobacter* were dominant.

Among other hydrocarbon degrading bacteria, *Stenotrophomonas* species were detected in samples I, II, III, VI and VII and dominate in IV and V enrichments. This genus was already found in diesel contaminated environments (Martin-Sanchez et al. 2018) and members of this genus were detected during crude oil degradation in batch treatments (Jin and Kim 2017). P*seudomonas* species were detected in samples II, IV, V, VI. This genus is also rich in well recognized hydrocarbons degraders, carrying various types of *alkB* genes and having ability to produce biosurfactants (Wang et al. 2013). Detected during this study *Pseudomonas veronii*

(cultures II, IV, V, VI) was observed to degrade wide spectrum of hydrocarbons including aliphatic hydrocarbons, BTX and various PAHs (Máthé et al. 2012). Interestingly the species was not detected in the most efficient diesel removing cultures (VII, III and I), suggesting that process efficiency depends not only on the presence of diesel degraders but also on their specific metabolic properties. Members of *Gordonia* genus were detected only in samples enriched from sewage sludge fresh digestate (V) and constituted the most abundant bacterial group in this enrichment. This genus is ubiquitous in soils and has ability to degrade a wide range of organic compounds including petroleum products as well as can produce biosurfactants (Cappelletti et al. 2017). Members of *Bordetella* genus were detected only in samples enriched from dry sewage sludge digestate (I). Species of *Bordetella* were previously isolated from PHCs contaminated soils and are associated with hydrocarbons degradation (Wang et al. 2016). Species belonging to *Microbacterium* genus were detected in all samples excluding enrichment from mixed soil and composted digestate (IV). This genus contains bacterial species with crude oil degrading capacities (Muthukamalam et al. 2017). However, *Microbacterium oxydans* which was detected during the present study was observed to degrade complex polysaccharides like alginic acid, which is widely distributed in cell walls (Jung et al. 2013). This may suggest growth on dead biomass produced during incubation. Members of *Rhodanobacter* genus were detected within samples V and VI, corresponding with enrichment originating from sewage sludge fresh digestate and sewage sludge fresh digestate with soil. This genus contains known hydrocarbon utilizing bacterial strains (Abass et al. 2018). *Rhodanobacter lindaniclasticus*, identified in samples V, was previously observed to degrade phenol (Felföldi et al. 2010) and benzo(a)pyrene (Reyes-Sosa et al. 2018).

Species of *Raoultella* were found in samples II IV VI VII. This genus was previously isolated from crude oil contaminated sites and was associated with phenols biodegradation (Kaczorek et al. 2016). *Raoultella ornithinolytica* strains, found in samples II, IV, VI and VII, could degrade diesel fuel and exhibited strong oil emulsification activity (Morales-guzmán et al. 2017). Some species of *Pusillimonas* were detected in samples I, enriched from dry sewage sludge digestate. Various strains of *Pusillimonas* genera were able to grow using diesel as sole carbon source including *Pusillimonas noertemannii* (T3-5) and *Pusillimonas oleiphila* (T7-7) previously isolated from oil-polluted sea-bed mud (Huang et al. 2008). *Pusillimonas sp. 5HP*, detected here, was previously detected within specialized microbial community inside a biotrickling filter for the removal of pharmaceutical recalcitrant VOCs (Hu et al. 2016).

Presence of genes belonging to the family of alkane monooxygenases was previously reported in the species belonging to all the detected genera excluding *Rhodanobacter*, *Raoultella* and *Pusillimonas*. It may mean that these genera utilize other metabolic pathway to degrade alkanes *e.g.* cytochrome P450 or were degrading other components of diesel fuel e.g. phenols or PAHs (Wang et al. 2011). It is also possible for bacteria cells to obtain functional genes from the environment due to horizontal gene transfer, thus bacterial strains can get ability to degrade diesel even if previously it was not observed (Shahi et al. 2016).

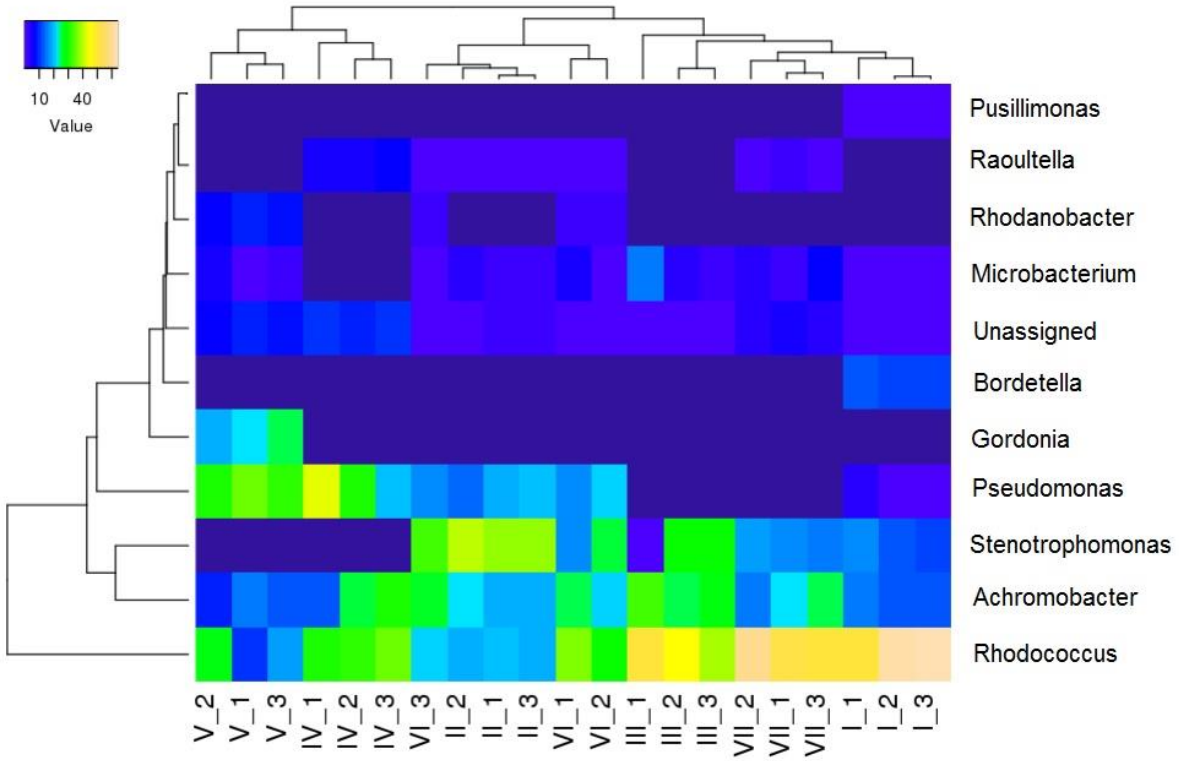

Figure 6.10 Heatmap profile presenting samples diversity in enrichment experiment (paper I) on genus level based on relative abundance. I: enrichments from dry digestate; II: enrichments from soil and dry digestate; III: enrichments from composted digestate; IV: enrichments from soil and composted digestate; V: enrichments from fresh digestate; VI: enrichments from soil and fresh digestate; VII: enrichments from soil. Numbers 1, 2 and 3 indicate the three replicates. Clustering was based on Pearson correlation.

6.3.2.2 Bacterial taxa detected during microcosm experiment (paper II)

Clustering of genera has shown that digestate and amended soils had high level of similarity, especially at the beginning of the treatment (Figure 6.11). For example, *Psychrobacter*, *Mycobacterium* and *Acinetobacter* were the most abundant genera (> 2 %) in digestate and amended soils at the beginning of the treatment while in S1 control soil the most common were

Bacillus, *Agromyces*, *Patulibacter*, *Leptolinea* and *Longilinea*. At the end of the treatment, in S1 control soil the main genera were *Pseudoxanthomonas*, *Agromyces*, *Thiobacillus*, *Pseudomonas* and *Acinetobacter*. With time, differences in bacterial community diversity became also visible between the two soils. In soil S1 for CDA treatment the most abundant genera were *Arenimonas*, *Arthrobacter*, *Thermomonas* and *Mycobacterium* while for treatment CDBI *Arenimonas*, *Thermomonas* and *Mycobacterium*. In soil S2 the dominant genera in CDA treatment were *Dietzia*, *Mycobacterium*, *Halomonas* and *Stenotrophomonas* while in CDBI treatment *Dietzia*, *Stenotrophomonas*, *Mycobacterium*, *Halomonas* and *Microbacterium*.

In S1 control soil, genera not associated with PHCs degradation were more often present at the beginning of the study and replaced with time by taxa containing some known species capable of alkane degradation. For instance at the beginning, among the top genera only *Bacillus* has known ability to degrade hydrocarbons (Barra Caracciolo et al. 2015; Reyes-Sosa et al. 2018), while after 30 days new genera appeared among which hydrocarbons degraders are recognized *e.g. Pseudoxanthomonas*, *Pseudomonas* (*alkB* genes expression) and *Acinetobacter* (*alkB* genes expression) (Liu et al. 2011; Pepi et al. 2011; Nie et al. 2014; Barra Caracciolo et al. 2015; Reyes-Sosa et al. 2018). Species belonging to *Thiobacillus* were also found in control soil, presence of this bacteria was already detected in petroleum reservoirs (Reyes-Sosa et al. 2018). Within genera present in digestate and consequently in amended soils at the beginning, *Psychrobacter* was the most abundant. This genus was not previously assigned to hydrocarbons degradation activity and surprisingly it was linked with mercury resistance (Pepi et al. 2011). Among other top genera, *Acinetobacter* species were shown to be able to use alkanes as a carbon and energy source and possess *alkB* genes (Liu et al. 2011; X. B. Wang et al. 2011; Nie et al. 2014), while *Mycobacterium* was previously observed to catalyze different reaction and express wide range of catabolic genes including *alkB* genes (Nie et al. 2014; Wang et al. 2016).

In treatments amended with digestate also an increase of taxa containing recognized hydrocarbons degraders was observed. In S1 soil for treatments CDA and CDBI *Mycobacterium* species were still present, however new dominant genera appeared including *Arenimonas* which was previously observed in hydrocarbon degrading cultures and associated with oil degradation (Wang et al. 2016; Reyes-Sosa et al. 2018), *Arthrobacter* (for CDA only) able to degrade crude oil components (Reyes-Sosa et al. 2018) and *Thermomonas* which was observed in microbial communities under intensive oil degradation (Al-Kharusi et al. 2016). In S2 soil, microbial diversity evolved differently and the most abundant genera detected after 30 days were: *Dietzia*, known to express *alkB* like genes and having ability to degrade a wide range

of hydrocarbons (X. B. Wang et al. 2011), *Halomonas* with ability to degrade aliphatic hydrocarbons (Reyes-Sosa et al. 2018), *Stenotrophomonas* which was previously observed in soils amended with digestate (Wolters et al. 2018), *Microbacterium* previously described as phenanthrene degrader (Reyes-Sosa et al. 2018) and *Mycobacterium*. At the end of the treatment, increase of other bacterial genera connected with hydrocarbon degradation was observed. For instance, in S2 soil treatment DB, a 2 % increase of *Gordonia* known to possess *alkB* genes was observed (Liu et al. 2011).

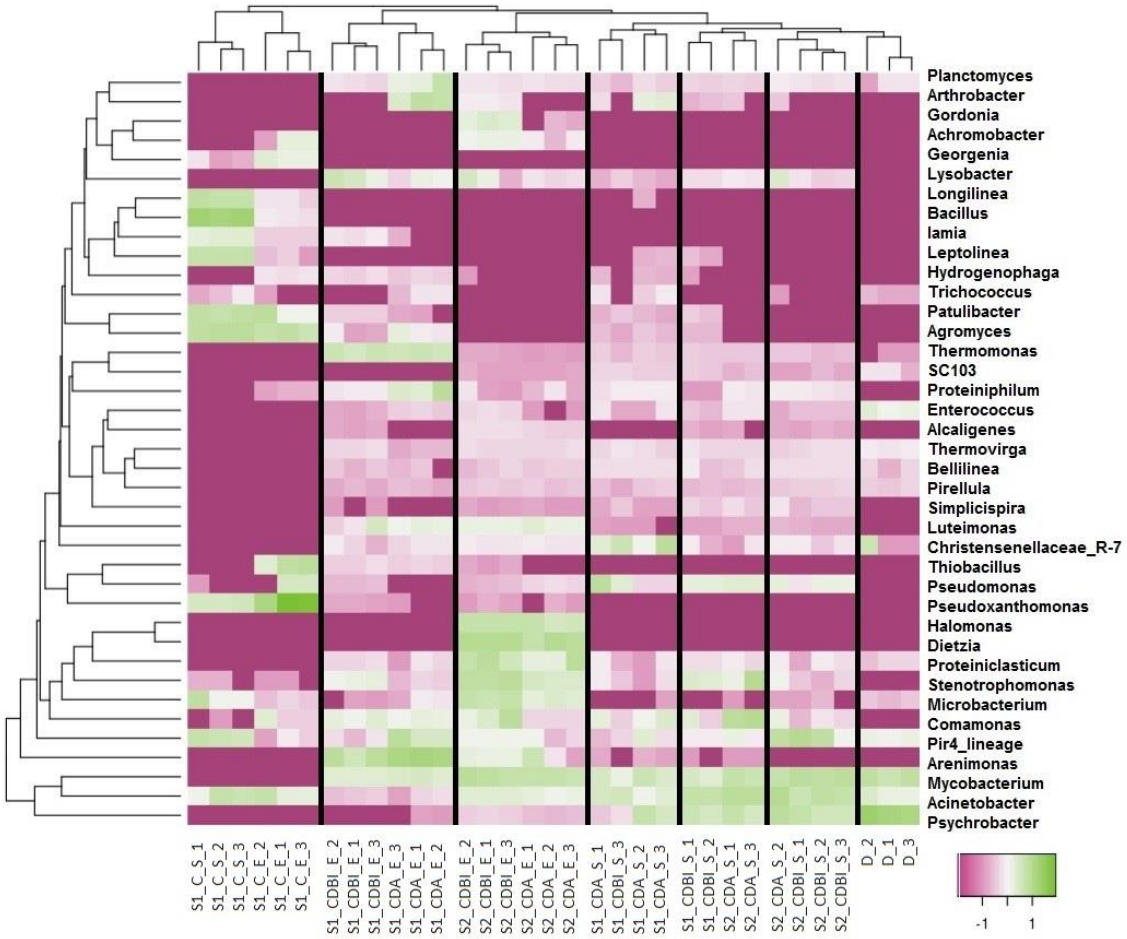

Figure 6.11 Heatmap profile showing dominant (≥ 0.1%) bacterial genera in microcosm experiment (paper II), based on relative abundance in logarithmic values, in digestate (D) and treatments of soil S1 and soil S2. C: soil + water; CDA: soil + digestate + sawdust; CDBI: soil + digestate + bacteria immobilized on biochar. Letters S and E indicate time of sampling with S: start (day 1), and E: end (day 30). Numbers 1, 2 and 3 indicate the three replicates. Treatment CDBI contained immobilized bacteria enriched respectively from both soils. Samples C from soil S2 were not included in analysis due to low quantity of extracted DNA.

6.3.2.3 Bacterial taxa detected during bioreactor experiment (paper III)

Clustering analysis revealed similarity between samples B1_S and B2_S, B1_M and B1_E as well as B2_M and B2_E indicating that mineral nutrients shaped the bacterial composition (Figure 6.12). Samples B3_E showed similar population shift as B2_E. This observation is interesting as initially B3_S and B3_M were more similar to treatments B4_S. B4_M and B4_E showed similarity to each other however not to B4_S what may suggest an adaptation step in the first part of the experiment.

In the control (B1), the most abundant genera at the beginning of the experiment were *Pseudomonas* (47 %), *Pseudoxanthomonas* (10 %), *Lutibacter* (7 %) and *Patulibacter* (5 %). After 30 days, the population of *Pseudomonas* decreased to 1.6 % and remained low, *Thermomonas* reached a concentration peak (10 %) and other genera became dominant at the end of incubation: *Pseudoxanthomonas* (15 %), *Microbacterium* (11 %), *Opitutus* (6 %) and *Nocardioides* (5 %). During the treatments, species associated with hydrocarbons degradation with time became more diverse compared to initial state. For example, initially in treatment B1 among hydrocarbons degrading genera only *Pseudomonas* was abundant, with time *Pseudoxanthomonas*, *Microbacterium* and *Nocardioides* were promoted (Yu et al. 2018; Reyes-Sosa et al. 2018; Liu et al. 2011). *Opitutus* genus contains facultative anaerobic soil bacteria species, however its ecological functions still need to be studied (Tegtmeier et al. 2018).

In the treatment B2, *Pseudomonas* were dominant in the first days of the study (90 %) and dropped to 4% after 30 days. At the end of the incubation the dominant genera included *Microbacterium* (24 %), *Cellvibrio* (14 %), *Dietzia* (12 %) and *Georgenia* (6 %). Within *Dietzia* genus promoted in treatment B2 in the second part of the incubation, interestingly, most members of were assigned to *Dietzia maris*. This species was identified as a hydrocarbon degrader [38]. During the treatment B2 also the diversity of hydrocarbons degraders increased. The dominant presence of *Pseudomonas* was replaced in time by genera like *Microbacterium* and *Dietzia* containing hydrocarbons degrading strains [38].

Pseudomonas genus is a common soil bacterial taxa and contains many well-known hydrocarbons degrading species (Liu et al. 2011; Reyes-Sosa et al. 2018). At the beginning of the experiment, high abundance of this genus was observed in reactors B1 and B2 and which has significantly decreased with time. Species assignment within the genus revealed that

Pseudomonas stutzeri constituted 38 and 87 % of the total bacteria, respectively for treatments B1 and B2. It was previously observed that *Pseudomonas stutzeri* is associated with aerobic denitrification process (Sun et al. 2017). It may indicate that in the present study *Pseudomonas* was not a key genus in hydrocarbons degradation. Further, the decrease of this genus with time could be a consequence of intensive growth of hydrocarbon degrading species favored by the high content of PHCs and stimulated by nutrient or oxygen supplementation. High abundance of one species in reactors B1 and B2 was connected with respectively low diversity measures.

In soil amended with digestate (B3), the most common genera at the beginning were *Pseudomonas* (33 %), *Acinetobacter* (32 %) and *Rhodococcus* (12 %), all of these genera contain known species with ability to degrade hydrocarbons. The abundance of *Pseudomonas* remained high during the first month of the treatment while at day 63 it decreased to 7 %. Abundance of *Acinetobacter* consequently decreased with time while *Rhodococcus* remained stable during the incubation. Interestingly, in the middle part of the process, *Alkanindiges* reached peak concentration of 18 % and almost totally disappeared after the next 30 days (0.2 %). *Alkanindiges* genera contains many hydrocarbons degrading species. The increase of the population in the middle part of the treatment may be connected with the phase of intensive degradation while decrease at the end of the treatment could be connected with depletion of bioavailable hydrocarbons. Genera observed at the end of the process included *Cellvibrio* (43.6 %) and *Microbacterium* (15 %).

In case of treatment B4, at the beginning, the dominant genera were *Rhodococcus* (42 %), *Acinetobacter* (13 %) and *Psychrobacter* (6 %) where the first two are associated with hydrocarbons degradation. After 63 days, the relative importance of *Rhodococcus* decreased to 15.8 % and other hydrocarbons degrading genera became dominant including *Alcanivorax* (21 %), *Microbacterium* (8 %) and *Arenibacter* (5 %) (Reyes-Sosa et al. 2018). Other promoted genera with no clear implication in hydrocarbons metabolism included *Cellvibrio* (12 %), *Rhodanobacter* (7 %) and *Castellaniella* (7 %). In treatments amended with digestate, initial richness of hydrocarbons degraders and overall diversity measures were higher compared to reactors B1 and B2.

Bacterial community composition was initially influenced by digestate addition however it had no crucial effect on the direction of microbial community changes. As mentioned in other studies, the sequence of the bacterial succession in amended soils does not only depend strictly on the composition of organic matter but also on soil type and environmental conditions (Pérez-Piqueres et al. 2006).

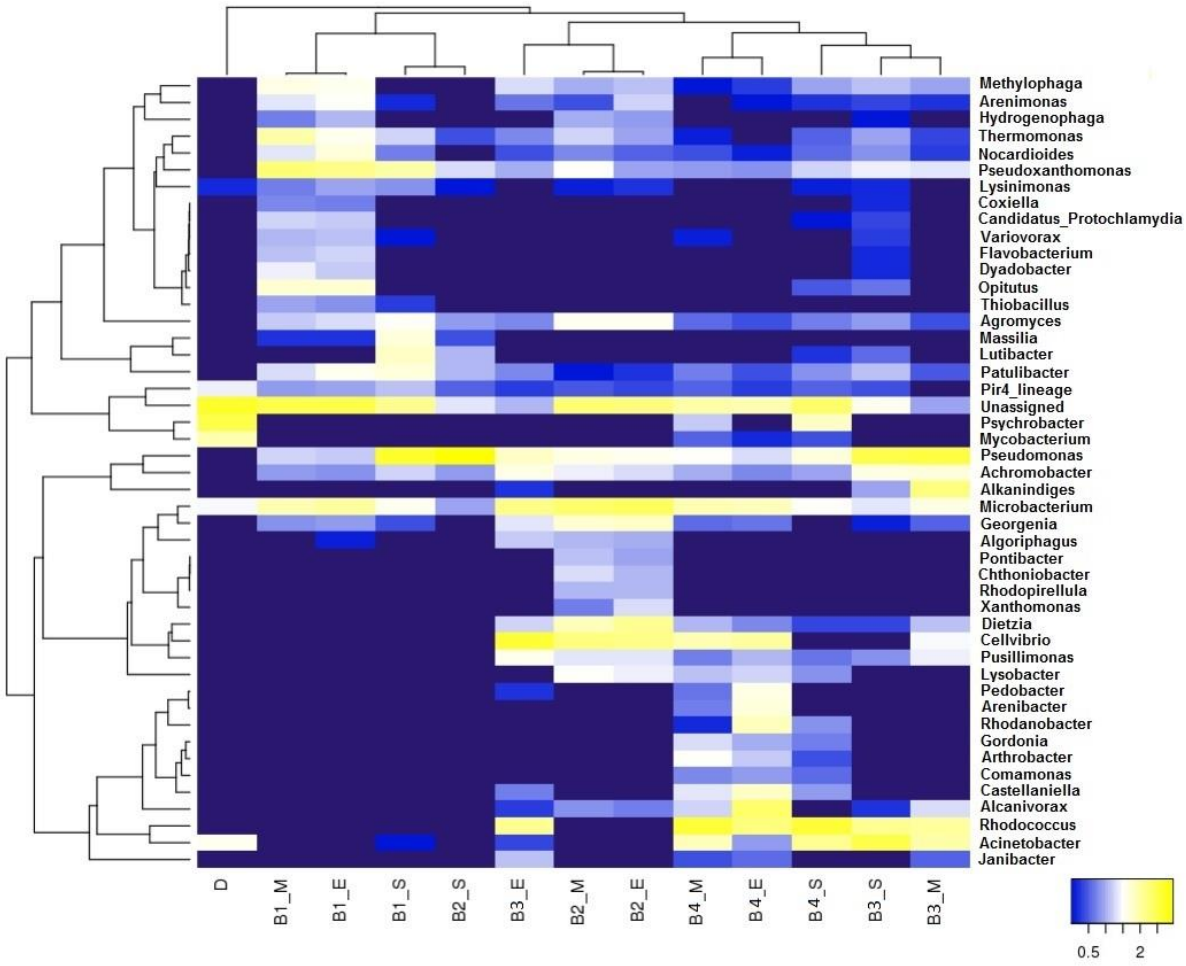

Figure 6.12 Heat map of the top genera (> 0.1 %) detected during bioreactor experiment (paper III), based on logarithm of relative abundance. B1: soil + sawdust; B2: soil + sawdust + mineral nutrients; B3: soil + sawdust + digestate; B4: soil + sawdust + digestate + immobilized bacteria; D: digestate. Samples were taken within 3 times points: S – start (1 d); M –middle (30 d); E – end (63 d). Results are presented as a mean of three samples collected from the same reactor. To show similarities between samples Pearson clustering method was employed. D – digestate. Samples were taken within 3 times points: S – start (1 d); M –middle (30 d); E – end (63 d).

6.3.3 New look on organic amendments: contribution and filling the knowledge gaps

Performed experiments for the first time proved the presence of hydrocarbon degrading bacteria in digestate (paper I) as well as high concentration of *alkB* genes responsible for degradation of alkanes (papers II and III). Application of digestate has increased soil bioremediation potential due to increase and maintenance of high content of these functional genes. In previous studies, the presence of *alkB* genes in soil was observed to correspond with the amount of soil organic matter (SOM) (Sutton et al. 2013). Soil application of digestate positively affected soil properties (stabilization of pH, increase of water content capacity) and lead to increase of SOM content which positively affected bacterial counts and the content of functional genes.

Table 6.1 presents bacterial genera containing *alkB* genes possessing species observed during all experiments. During enrichment experiment, diesel degrading bacteria have been enriched from samples of digestates as well as digestate mixed with contaminated soil and contaminated soil alone (paper I). All the enrichment had unique bacterial composition. Different genera containing species carrying *alkB* genes were found in dry digestate enrichments (6 genera), composted digestate (4 genera), fresh digestate (5 genera) and contaminated soil (4 genera). The experiment confirmed presence of diverse hydrocarbons degrading species in digestate enrichments. Summary of the main results from 3 experiments is presented on the Figure 6.13.

Table 6.1 Number of detected bacterial genera (> 0.1 %) including *alkB* genes carrying species in all performed experiments. Paper I - I: enrichments from dry digestate; II: enrichments from soil and dry digestate; III: enrichments from composted digestate; IV: enrichments from soil and composted digestate; V: enrichments from fresh digestate; VI: enrichments from soil and fresh digestate; VII: enrichments from soil. Paper II - C: soil + water; CDA: soil + digestate + bulking agent; CDBI: soil + digestate + bacteria immobilized on biochar; S: day 1; E: day 30. Paper III - B1: soil + sawdust; B2: soil + sawdust + mineral nutrients; B3: soil + sawdust + digestate; B4: soil + sawdust + digestate + immobilized bacteria; D: digestate. Samples were taken within 3 times points: S – start (1 d); M –middle (30 d); E – end (63 d). Mean of triplicate and standard deviation (in brackets).

Enrichment experiment (paper I)	Number of genera	Microcosm experiment (paper II)	Number of genera	Bioreactor experiment (paper III)	Number of genera
I	6 (0.0)a	S1_C_S	6 (1.0)cd	B1_S	7 (0.0)e
II	5 (0.0)b	S1_C_E	8 (0.6)b	B1_M	6 (0.0)f
III	4 (0.0)c	S1_CDA_S	6 (0.6)cd	B1_E	6 (0.0)f
IV	3 (0.0)d	S1_CDA_E	6 (0.6)cd	B2_S	7 (0.0)e
V	5 (0.0)b	S1_CDBI_S	6 (0.0)d	B2_M	8 (1.0)d
VI	5 (0.0)b	S1_CDBI_E	7 (0.6)bc	B2_E	7 (0.6)de
VII	4 (0.0)c	S2_C_S	n.d.	B3_S	10 (0.0)c
		S2_C_E	n.d.	B3_M	10 (0.0)c
		S2_CDA_S	6 (0.6)cd	B3_E	10 (0.0)c
		S2_CDA_E	10 (1.2)a	B4_S	11 (0.0)b
		S2_CDBI_S	6 (0.6)cd	B4_M	12 (0.0)a
		S2_CDBI_E	10 (0.0)a	B4_E	11 (0.6)b
		D	4 (0.0)	D	4 (0.0)

Extraction of meta-DNA from digestate samples revealed the presence of 4 genera containing *alkB* genes possessing species (papers II and III). The effect of digestate application to soil on the composition of microbial community was dependent on the soil type and experiment scale. In microcosm (paper II) in initial control soil S1 (clay rich soil) 6 genera with *alkB* gene species were detected. Addition of digestate to soil, together with bulking agent and inoculated biochar did not change this initial number. At the end of the experiment for treatment with digestate and bulking agent the number of specific petroleum degrading genera remained unchanged, while for control and other treatments it increased. Interestingly, in soil S2 (sandy soil), due to

low quantity of bacteria and thus also extracted DNA, control treatment could not be properly characterised, while in treatments containing digestate initial number of specific genera was 6 for both treatments. At the end of the experiment, number of specific *alkB* gene possessing genera in both treatments increased to 10. These results suggest that digestate application may be interesting bioremediation strategy for exploited soils.

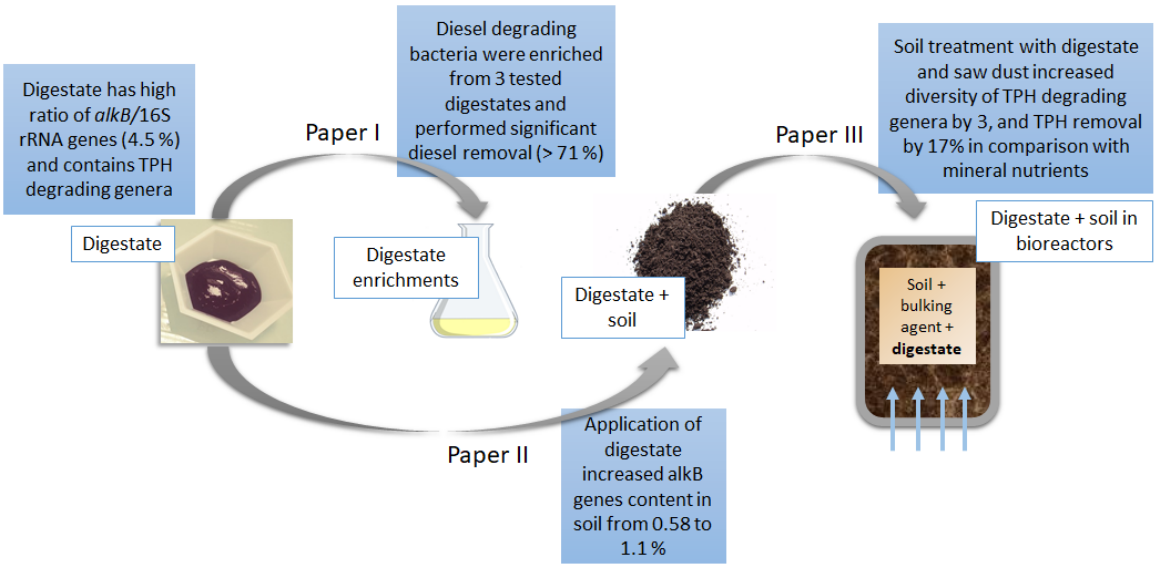

Figure 6.13 Summary of the main results obtained during the thesis. Soil application of digestate positively affected precence of *alkB* genes in soil and abundance of PHCs degrading bacterial genera. The use of digestate as soil organic amendment was more beneficial for hydrocarbons bioremediation than mineral fertilizer.

In bioreactor experiment the presence of *alkB* genes possessing genera reflected PHCs degradation efficiency for all treatments. In control, 7 specific genera were observed at the beginning and 6 in the middle and at the end of the treatment. In treatment with addition of mineral nutrients 7 specific genera were observed initially, 8 in the middle and 7 at the end. Addition of digestate increased initial number of specific genera to 10 and this number did not change throughout the treatment. Application of digestate together with inoculated biochar resulted in 11 initial specific genera, 12 in the middle and 11 at the end. Obtained results clearly present that addition of digestate to contaminated soil has significantly increased the diversity of hydrocarbons degrading bacterial taxa.

7 Conclusions

During this PhD thesis, complex analysis of the indigenous digestate microflora in terms of potential to degraded petroleum hydrocarbons was performed. The presence of petroleum degrading bacteria, possessing *alkB* genes in digestate was confirmed. Microbial enrichments originating from digestate were characterised and bacterial genera were classified. Digestate enrichments showed significant diesel fuel removal and high content of *alkB* genes which were promoted during incubation. In all enrichments, specific hydrocarbons degrading bacterial taxa were present. Diesel fuel degradation levels did not reveal significant differences in PHCs removal for bacteria origination from digestate (composted OFMSW digestate) and microbes native to petroleum contaminated soils. Relation between PHCs degrading bacteria from soil and digestate was studied. Microbial enrichments from mixed sources showed lower degradation rates in comparison with single source enrichments which suggests that longer time is needed for establishment of new microbial population structure after population disturbance.

The content of *alkB* genes in digestate was surprisingly high and surpassed values observed for hydrocarbon contaminated soils. After digestate application to contaminated soil, the level of *alkB* genes in soil was significantly increased and maintained. Digestate may be successfully used in bioremediation to increase concentration of *alkB* genes in the soil as well as the density and diversity of hydrocarbons degrading taxa in soil. Monitoring of the concentration of *alkB* genes during the treatment reflected actual PHCs removal rates and therefore can be seen as a useful tool for assessing the biodegradation efficiency. Soil application of digestate increased PHCs bioremediation level in a significantly greater extent than mineral fertilizers. Obtained results indicate that soil supplemented with digestate provides suitable environment for bacterial development. Soil application of digestate increased overall bacterial activity represented by microbial respiration in greater extent than mineral fertilizers as well as improved soil bioremediation potential. This thesis highlights that organic amendments like digestate can improve hydrocarbons metabolic capacity of soil by increasing the copy number of functional genes and hydrocarbons degrading taxa. All performed experiments revealed a great potential of digestate as sustainable source of nutrients and bacteria in bioremediation of hydrocarbons contaminated soils.

Results obtained during this PhD thesis gives a solid basis for further development of proposed

bioremediation treatments in full scale operations. Proved higher efficiency in soil bioremediation as well as lower costs of digestate comparing to mineral fertilizers should encourage decision makers and contractors to promote and use sustainable nutrient sources during full scale land treatments.

8 Recommendations for future research

To recognize the bioaugmentation potential of organic fertilizers in soil bioremediation, other organic restudies should be studied in terms of functional genes crucial for bioremediation of organic contaminants. Digestate can be further studied in terms of potential content of functional genes necessary for degradation of other emerging organic contaminants (*e.g.* dioxygenases enabling PAHs degradation).

As the chemical composition of organic fertilizers and amendmends is the factor affecting the metabolic capacities of indigenous microbial communities, future studies can focus on the relation between amendment characteristics and the content of specific genes. Composition, the level of easily available carbon (biological stability of fertilizer), the concentration and bioavailability of nutrients should be analysed together with abundance of functional microbial species.

To understand the process of bacterial adaptation and shifts of the key degraders after the use of organic fertilizers, future studies could focus on the kinetics of functional genes spreading in soil and fluctuation of functional genes concentration according to the available contaminant concentrations. Application of techniques enabling monitoring of functional genes expression in relation to bioavailable contaminant would allow a deeper understanding of microbial activity during bioremediation treatments.

As digestate in more concentrated in comparison to raw feedstock also its trace elements content is significantly higher. Further studies shouls consider the environmental impact of soil digestste application including possible risk for groundwater contamination as well as spreading and speciation of trace elements originating from digestate in soil.

Presented thesis contains developed bioremediation treatment strategies which could be further optimized under field scale operations. Application of digestate in large scale treatments can be performed as treatment in biopiles, as landfarming or enrichments originating from digestate can be further studied in bioaugmentation strategies.

Performed experiments were focusing on solid and sludge digestate. However, due to high content of ammonia, liquid digestate can be further valorised as a medium for bacteria growth

in bioaugmentation strategies. Liquid digestate can be also analysed as nutrient rich water phase in slurry-phase soil bioreactors. Liquid digestate may be an interesting solution for slurry-phase bioreactors providing nutrients and microorganisms at the same time. An adaptation step may be here considered including enrichment of liquid digestate microflora in the presence of the contaminant of interest in order to increase biodegradation capacities of native microorganisms.

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