BACTERIAL ENDOPHYTES OF PLANTS USED FOR SOIL RECLAMATION

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ABSTRACT

Microorganisms that colonize the plant rhizosphere and root tissues may provide host plants with nutrients, stimulate growth and increase tolerance to abiotic stress. These plant-microbe associations are also being investigated to assist land reclamation in Alberta's oil sands. However, these newly reconstructed landscapes may be limited by several factors that include low soil nutrient levels, reduced microbial activity and the presence of residual hydrocarbons. This study was designed to assess the bacterial root microbiome of plants growing in oil sands reclamation covers and investigate the potential use of bacterial endophytes in phytoremediation. Soil microbial community structure in these areas was mainly driven by soil total and organic carbon, NH_4^+ and organic matter. In addition, an assessment of the bacterial root microbiome associated with sweet clover (*Melilotus albus*) and barley (*Hordeum vulgare*) strongly suggests that plants have the ability to select for certain soil bacterial consortia. Sweet clover plants were more selective and mainly associated with *Sinorhizobium* and *Rhizobium*, whereas *Acholeplasma* was unique to barley. Furthermore, genera such as *Pseudomonas* and *Pantoea* were able to successfully colonize both plants. However, due to the presence of residual hydrocarbons in these areas, plants may rely on association with hydrocarbon degrading endophytes. Therefore, an assessment of unculturable endophytic communities revealed that sweet clover had higher CYP153 gene copy numbers when compared to barley. In addition, a total of 42 endophytic bacteria isolates tested positive for hydrocarbon degrading genes and were further investigated for their application as inoculants. Based on overall growth promoting effects, sweet clover plants and four different bacterial strains were selected for phytoremediation experiments. Despite plant growth inhibition caused by diesel fuel toxicity, an overall higher plant biomass was observed in inoculated plants. However, only at high diesel concentrations did bacterial inoculants enhanced soil hydrocarbon degradation.

In conclusion, bacterial species associated with plants growing in reclamation covers were mainly driven by plant factors and this microbiome harbors endophytes that can be potentially used in phytoremediation. In particular, bacterial endophytes such as *Pantoea* and *Pseudomonas* species in association with sweet clover plants were shown to successfully reduce petroleum hydrocarbons in soil.

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TABLE OF CONTENTS

PERMISSION TO USE i
ABSTRACT ii
ACKNOWLEDGMENTS iii
TABLE OF CONTENTS iv
LIST OF TABLES vii
LIST OF FIGURES x
LIST OF ABBREVIATIONS xiv
1. INTRODUCTION 1
2. LITERATURE REVIEW 5
2.1 The Alberta Oil Sands 5
2.2 Tailings Management 7
2.3 Reclamation Strategies 8
2.4 Soil microbial community function and structure 9
2.5 Plant-microbe interactions 12
2.5.1 The rhizosphere microbiome 13
2.5.2 The endosphere microbiome 15
2.6 Phytoremediation 17
2.6.1 Advantages and disadvantages 18
2.6.2 Use of plants and associated microorganisms for hydrocarbon degradation 19
2.7 Ecology of hydrocarbon degrading bacteria 23
2.7.1 Hydrocarbon degradation 24
2.7.1.1 Aerobic degradation of aliphatic compounds 24
2.7.1.2 Aerobic degradation of aromatic compounds 27
2.7.1.3 Hydrocarbon degrading genes 29
3. MICROBIAL COMMUNITIES ASSOCIATED WITH ANNUAL BARLEY PLANTED ON OIL SANDS RECLAMATION SITES IN ALBERTA, CANADA. 32
3.1 Preface 32
3.2 Abstract 32
3.3 Introduction 33
3.4 Materials and Methods 35
3.4.1 Sample collection and processing 35
3.4.2 Soil Chemical Analyses 36
3.4.3 Survey of rhizosphere bacteria community 39
3.4.4 Survey of endophytic bacteria community 39
3.4.5 Survey of culturable rhizosphere and endophytic bacteria 39
3.4.6 Phospholipid fatty acid (PLFA) analysis 39
3.4.7 DNA Extraction 40
3.4.8 DNA Amplification 41
3.4.9 Denaturing Gel Gradient Electrophoresis (DGGE) 41

3.4.10 Statistical Analysis ... 42
3.5 Results ... 43
3.5.1 Soil Physical-chemical properties ... 43
3.5.2 PLFA Analysis ... 46
3.5.3 DGGE Analysis ... 52
3.5.3.1 DGGE profiles of bulk soil microbial community ... 52
3.5.3.2 DGGE profiles of rhizosphere microbial community ... 55
3.5.3.3 DGGE profiles of endophytic microbial community ... 58
3.5.4 Survey of Endophytic bacterial communities by culture dependent techniques ... 64
3.6 Discussion ... 68
3.7 Conclusions ... 72

4. BACTERIAL ROOT MICROBIOME OF PLANTS GROWING IN OIL SANDS RECLAMATION COVERS ... 73
4.1 Preface ... 73
4.2 Abstract ... 73
4.3 Introduction ... 74
4.4 Materials and Methods ... 76
4.4.1 Sample collection and processing ... 76
4.4.2 Survey of rhizosphere and endophytic bacteria community ... 77
4.4.3 DNA extraction ... 78
4.4.4 High-throughput 16S rRNA amplicon sequencing ... 78
4.4.5 Bioinformatics and statistical analysis ... 78
4.4.6 Data deposition ... 79
4.5 Results ... 79
4.6 Discussion ... 95
4.7 Conclusions ... 100

5. HYDROCARBON DEGRADING GENES IN ROOT ENDOPHYTIC COMMUNITIES ON OIL SANDS RECLAMATION COVERS ... 101
5.1 Preface ... 101
5.2 Abstract ... 101
5.3 Introduction ... 102
5.4 Materials and Methods ... 105
5.4.1 Sample collection and processing ... 105
5.4.2 Survey of unculturable endophytic bacteria community ... 106
5.4.3 DNA Extraction ... 106
5.4.4 Synthesis of qPCR standards ... 106
5.4.4.1 qPCR amplification ... 107
5.4.5 PCR Amplification of culturable isolates ... 111
5.4.6 Statistical Analysis ... 113
5.5 Results ... 113
5.6 Discussion ... 123
5.7 Conclusions ... 128

6. POTENTIAL USE OF ENDOPHYTIC ROOT BACTERIA AND HOST PLANTS TO DEGRADE HYDROCARBONS ... 129
6.1 Preface ... 129

6.2 Abstract ... 129
6.3 Introduction ... 130
6.4 Materials and Methods ... 132
6.4.1 Inoculum preparation ... 132
6.4.2 Seed inoculation ... 133
6.4.3 Plant growth promotion experiments ... 133
6.4.3.1 Seed germination and vigor ... 133
6.4.3.2 Root Elongation ... 133
6.4.3.3 Long-term pot experiment ... 134
6.4.4 Phytoremediation experiment ... 134
6.4.4.1 Experimental setup ... 134
6.4.4.2 Hydrocarbon analysis ... 135
6.5 Results ... 136
6.5.1 Seed germination ... 136
6.5.2 Root Elongation ... 136
6.5.3 Long-term growth promotion ... 139
6.5.4 Phytoremediation experiment ... 142
6.6 Discussion ... 150
6.7 Conclusions ... 155

7. OVERALL DISCUSSION ... 157

8. SUMMARY AND CONCLUSIONS ... 162

9. FUTURE RESEARCH ... 165

10. REFERENCES ... 167

APPENDIX A: Identification of endophytic bacteria isolates associated with annual barley (*Hordeum vulgare*) growing at an oil sands reclamation area. identification is based on 16s rRNA sanger sequencing ... 195

APPENDIX B: Rarefaction curves relating the observed number of sequences to the observed number of operational taxonomic units (OTUs) at 97% rRNA sequence similarity ... 200

APPENDIX C: Sequence genes CYP 153 (339 bp) and *alkb* (548 bp) and *nah* (377 bp) synthesized by Biomatik (cambridge, canada). ... 207

APPENDIX D: Identification of endophytic bacteria isolates associated with annual barley (*Hordeum vulgare*) positive for hydrocarbon degrading genes (CYP 153. *alkb*, *nah*). Identification is based on 16s rRNA sanger sequencing ... 209

APPENDIX E: Effect of endophytic bacteria on sweet clover and barley in seed germination experiments. ... 211

APPENDIX F: Effect of endophytic bacteria on sweet clover in root elongation experiments. 215

APPENDIX G: Preliminary assessment of total extractable F3 and F4 hydrocarbons in soils. . 217

APPENDIX H: Photographs of sweet clover plants in phytoremediation growth chamber experiments using soils amended with diesel fuel at 5,000, 10,000 and 20,000 $mg \cdot kg^{-1}$ ... 219

LIST OF TABLES

Table 2.1 Key microbial processes mediating chemical transformations associated with nutrient cycling in soil (Murphy *et al.*, 2007). 11

Table 2.2 Examples of phytoremediation of hydrocarbon contaminated soil by combined use of microbes. 22

Table 2.3 Examples of hydrocarbon degrading genes and bacterial taxa. 30

Table 3.1 Landforms, elevation and GPS coordinates of sampling locations (UTM Zone 12N). 38

Table 3.2 Soil chemical proprieties of peat mineral samples (0-20 cm) collected in the engineered and standard cover at an oil sands reclamation area. 44

Table 3.3 Total hydrocarbon analysis of samples collected in the engineered and standard cover at an oil sands reclamation area. 45

Table 3.4 Soil PLFA analysis of peat mineral samples (0-20 cm) collected in the engineered and standard cover at an oil sands reclamation area. Different letters indicate significant differences (Tukey HSD $p \leq 0.05$) among the interaction of cover type and slope positions in the soil microbial community... 49

Table 3.5 Correlation indicating Spearman's rank R^2 values relating soil PLFAs and soil parameters. Statistically significant correlations are indicated in bold type, * and ** denote p values ≤ 0.05 and ≤ 0.01, respectively. THC = total hydrocarbons, OM = organic matter, TOC = total organic carbon, TC = total carbon, F:B = fungal/bacterial ratio. 51

Table 3.6 Phylogenetic affiliation of selected endophytic bacteria based on 16S rRNA gene sequences re-amplified from DGGE bands (Figure 3.10).. 62

Table 3.7 Total culturable rhizosphere bacteria associated with annual barley (*Hordeum vulgare*) plants growing on the standard and engineered cover at an oil sands reclamation area (Log $CFU \cdot g^{-1}$ of soil dry weight ± standard deviations). 65

Table 3.8 Total culturable endophytic bacteria associated with annual barley (*Hordeum vulgare*) plants growing on the standard and engineered cover at an oil sands reclamation area (Log $CFU \cdot g^{-1}$ of soil dry weight ± standard deviations). 66

Table 3.9 Analysis of Variance (ANOVA) for total culturable endophytic and rhizosphere bacteria associated with annual barley grown in reclamation areas. 66

Table 4.1 Correlation indicating Spearman's R^2 values relating endophytic bacterial phyla and soil parameters. Statistically significant correlations are indicated in bold type, * and ** denote p values ≤ 0.05 and ≤ 0.01, respectively. OM = organic

matter, TOC = total organic carbon, TC = total carbon and EC = electrical conductivity. 82

Table 4.2 Correlation indicating Spearman's R^2 values relating rhizosphere bacterial phyla and soil parameters. Statistically significant correlations are indicated in bold type, * and ** denote p values ≤ 0.05 and ≤ 0.01, respectively. OM = organic matter, TOC = total organic carbon, TC = total carbon and EC = electrical conductivity. 83

Table 4.3 Alpha richness and diversity of endophytic and rhizosphere communities associated with barley and sweet clover plants. Different letters indicate significant differences (Tukey HSD p ≤ 0.05). 91

Table 5.1 List of primers used for quantitative real-time polymerase chain reaction (qPCR) to assess hydrocarbon degrading genes present within unculturable endophytes and polymerase chain reaction (PCR) for the screening cultured hydrocarbon degrading endophytic bacteria. 109

Table 5.2 Quantitative real-time polymerase chain reaction (qPCR) conditions to assess hydrocarbon degrading genes present within unculturable endophytes in an oil sands reclamation area. 110

Table 5.3 PCR conditions to assess hydrocarbon degrading genes present within culturable endophytes in an oil sands reclamation area. 112

Table 5.4 Analysis of variance (ANOVA) for Log gene copy number$\cdot g^{-1}$ of plant of 16s rRNA, CYP153, *alkB*, *nah* and the ratio of all of hydrocarbon degrading genes relative to 16s rRNA (n=120). 115

Table 5.5 Spearman's rank correlation coefficients (n = 60) between 16s rRNA and Hydrocarbon degrading gene abundance and soil physico-chemical parameters for barley endophytic community. Statistically significant correlations are indicated in bold type, * and * and ** denote p values ≤ 0.05 and ≤ 0.01, respectively. THC = total hydrocarbons, OM = organic matter, EC = electrical conductivity, TOC = total organic carbon, TC = total carbon. 119

Table 5.6 Spearman's rank correlation coefficients (n = 60) between 16s rRNA and Hydrocarbon degrading gene abundance and soil physico-chemical parameters for sweet clover endophytic community. Statistically significant correlations are indicated in bold type, * and ** denote p values ≤ 0.05 and ≤ 0.01, respectively. THC = total hydrocarbons, OM = organic matter, EC = electrical conductivity, TOC = total organic carbon, TC = total carbon. 120

Table 6.1 Effect of endophytic bacteria on barley and sweet clover seed germination rate (%) (total of 10 seeds) at 25ºC. Symbols indicate treatments significantly (LSD p ≤ 0.05) higher (*) and lower (†) when compared to the control. 137

Table 6.2 Sweet clover plant parameters grown in soils amended with diesel after 65 days. Different letters indicate significance at LSD ($p \leq 0.05$). 143

Table 6.3 Effect endophytic bacterial inoculants on the number of secondary shoots and flowering buds of sweet clover plants grown in soils amended with diesel after 65 days. Different letters indicate significance at LSD ($p \leq 0.05$). 143

Table 6.4 Total extractable hydrocarbons in soil after 65 days growth of sweet clover plants inoculated with hydrocarbon degrading endophytic bacteria. Different letters indicate significant differences (Tukey HSD $p \leq 0.05$). 148

Table A.1 Identification of endophytic bacteria isolates associated with annual barley (*Hordeum vulgare*) growing at an oil sands reclamation area. 196

Table D.1 Identification of endophytic bacteria isolates associated with annual barley (*Hordeum vulgare*) positive for hydrocarbon degrading genes (CYP153, *alkB*, *nah*). 210

Table E.1 Effect of endophytic bacteria on barley and sweet clover seed germination rate (%) (Total of 10 seeds) at 10°C. Symbols indicate treatments significantly (LSD $p \leq 0.05$) lower (†) when compared to the control. 212

Table E.2 Effect of endophytic bacteria on barley and sweet clover seed germination vigor (%) (Total of 10 seeds) at 25°C. Symbols indicate treatments significantly (LSD $p \leq 0.05$) higher (*) and lower (†) when compared to the control. 213

Table E.3 Effect of endophytic bacteria on barley and sweet clover seed germination vigor (%) (Total of 10 seeds) at 10°C. Symbols indicate treatments significantly (LSD $p \leq 0.05$) higher (*) and lower (†) when compared to the control. 214

Table G.1 Total extractable F3 and F4 hydrocarbons in soils amended with diesel without plants or addition of bacterial inoculants. 218

LIST OF FIGURES

Figure 2.1 Outline of oil sands deposits (orange) in Alberta and adjacent provinces (Wellstead *et al.*, 2016). 6

Figure 2.2 Biodegradation of alkanes (Leung *et al.*, 2006). 26

Figure 2.3 Biotransformation of benzene to catechol followed by the ortho-cleavage of catechol. Modified from Leung *et al*. (2006). 28

Figure 3.1 Transects of sampling locations along the engineered (E1-E10) and standard cover (S1-S10) at an oil sands reclamation area. 37

Figure 3.2 Boxplot of total PLFA biomarkers concentrations at different slope positions at the standard and engineered cover at an oil sands reclamation area. Median values are indicated by the horizontal bar within the box. The first and third quartiles are denoted by the lower and upper box limits respectively, and the 5th and 95th percentiles by the bars at the end of the vertical bars projecting from the box. Different letters indicate significant differences (Tukey HSD $p \leq 0.05$). 48

Figure 3.3 Non-metric multidimensional scaling (MDS) analysis Non-metric multidimensional scaling (MDS) analysis and multiple response permutation procedure (MRPP) of PLFA profiles from peat-mineral mix (PMM) samples collected in the engineered and standard cover at an oil sands reclamation area. 50

Figure 3.4 Dendrogram analysis using Pearson's correlation coefficient for bulk soil DGGE banding patterns of bacterial 16S rRNA communities. 53

Figure 3.5 Non-metric multidimensional scaling (MDS) analysis of DGGE banding patterns from bulk soil bacterial 16S rRNA communities. 54

Figure 3.6 Dendrogram analysis using Pearson's correlation coefficient for rhizosphere soil DGGE banding patterns of bacterial 16S rRNA communities. 56

Figure 3.7 Non-metric multidimensional scaling (MDS) analysis of DGGE banding patterns from rhizosphere soil bacterial 16S rRNA communities. 57

Figure 3.8 Dendrogram analysis using Pearson's correlation coefficient from DGGE banding patterns of endophytic bacterial 16S rRNA communities. 59

Figure 3.9 Non-metric multidimensional scaling (MDS) analysis of DGGE banding patterns from endophytic bacterial 16S rRNA communities. 60

Figure 3.10 Representative DGGE of PCR-amplified 16S rRNA gene fragments from endophytic bacteria associated with annual barley roots growing on the

standard and engineered cover at an oil sands reclamation area. Arrows indicate sequenced bands whose closest identities are provided in Table 3.6. 61

Figure 3.11 Relative abundance of 140 randomly selected bacterial endophyte isolates (total=316) associated with annual barley and identified by 16S rRNA Sanger sequencing. 67

Figure 4.1 Analysis of root associated bacterial communities (endosphere and the rhizosphere compartments) at phylum level in barley and sweet clover growing in oil sands reclamation areas. 81

Figure 4.2 Venn diagram for endosphere (BE) and rhizosphere (BR) bacterial communities associated with barley and endosphere (CE) and rhizosphere (CR) bacterial communities associated with sweet clover. Numbers indicated shared unique OTUs at 0.03 dissimilarity distances after removing singletons involved. 85

Figure 4.3 Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity between samples for barley endosphere (BE), clover endosphere (CE), barley rhizosphere (BR) and clover rhizosphere (CR). 86

Figure 4.4 Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity between samples at different slope positions (a) and cover managements (b). 87

Figure 4.5 Ternary plot representing the relative occurrence of individual genus (circles) that are members of the five most abundant families in root samples of sweet clover and barley compared with rhizosphere soil. Genera enriched in different compartments are colored by taxonomy of the most abundant families. The size of the circles is proportional to the mean abundance in the community. 89

Figure 4.6 Ternary plot representing the relative occurrence of individual genus (circles) in root samples of sweet clover and barley compared with rhizosphere soil. Genera enriched in different compartments are colored according to habitat in which each genus is most frequently associated based on at least 10% enrichment or depletion of soil microbiota in the endosphere. The size of the circles is proportional to the mean abundance in the community. 90

Figure 4.7 Heatmap based on relative abundance of sweet clover and barley associated endophytic communities. Vertical columns represent samples; horizontal rows represent genera that are 2% most abundant in at least one sample. Clustering of samples (top) is based on genera co-occurrence by Bray-Curtis dissimilarity. Letters (A-F) indicate different clusters at a 70% dissimilarity cut off. 93

Figure 4.8 Heatmap based on relative abundance of sweet clover and barley associated rhizosphere communities. Vertical columns represent samples; horizontal rows represent genera that are 2% most abundant in at least one sample.

Clustering of samples (top) is based on genera co-occurrence by Bray-Curtis dissimilarity. Letters (A-C) indicate different clusters at a 40% dissimilarity cut off. 94

Figure 5.1 Abundance of gene copy numbers within endophytic microbial communities associated with barley (n=60) and sweet clover (n=60) growing in oil sands reclamation soils. Error bars represent standard deviation, and *** indicate significance at $p \leq 0.001$ at Student's two-sample t-test. 116

Figure 5.2 Abundance of relative ratio of hydrocarbon degrading genes compared with 16s rRNA in endophytic microbial communities associated with barley (n=60) and sweet clover (n=60) growing in oil sands reclamation soils. Error bars represent standard deviation, and ** indicate significance at $p \leq 0.01$ at Student's two-sample t-test. 118

Figure 5.3 Neighbor-Joining tree using the Maximum Composite Likelihood method. Symbols represent isolates positive for ○ *alkB*, ▲ CYP 153, ■ *nah* and ◆ both CYP153 and *nah*. 122

Figure 6.1 Effect of endophytic bacteria on root length of sweet clover plants measured at 20 days. Plants were supplemented with water and alternate additions of Hoagland nutrient solution. Error bars represent standard deviations and * indicate significance at LSD ($p \leq 0.05$) compared to control. 138

Figure 6.2 Plant secondary shoots (A), number of flowering buds (B) and height (C) of sweet clover plants inoculated with endophytic bacteria after 65 days. Error bars represent standard deviations and * indicate significance at LSD ($p \leq 0.05$) compared to control. 140

Figure 6.3 Shoot fresh (A) and dry weight (B), root fresh (C) and dry (D) weight of sweet clover plants inoculated with endophytic bacteria after 65 days. Error bars represent standard deviations and * indicate significance at LSD ($p \leq 0.05$) compared to control. 141

Figure 6.4 Effect of endophytic bacterial inoculants EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover (*Melilotus albus*) plant parameters after 65 days. Error bars represent standard deviations (n=5) and different letters indicate significant differences (LSD $p \leq 0.05$). 144

Figure 6.5 Effect of endophytic bacterial inoculants EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover plants (*Melilotus albus*) growing in soils amended with diesel (5,000 $mg \cdot kg^{-1}$) at 65 days after planting. 145

Figure 6.6 Effect of endophytic bacterial inoculants EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5

(*Pseudomonas* sp.) on sweet clover plants (*Melilotus albus*) growing in soils amended with diesel (10,000 mg·kg^{-1}) at 65 days after planting. 146

Figure 6.7 Effect of endophytic bacterial inoculants EA1-17 (*Stenotrophomona*s sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover plants (*Melilotus albus*) growing in soils amended with diesel (20,000 mg·kg^{-1}) at 65 days after planting. 147

Figure 6.8 The F2 and F3 hydrocarbon fractions in soils initially amended with diesel at 5,000 (A), 10,000 (B) and (C) 20,000 mg·kg^{-1} after phytoremediation experiment. Error bars represent standard deviations (n=5) and different letters indicate differences (Tukey HSD p ≤ 0.05). 149

Figure B.1 Rarefaction curves for bacterial OTUs, clustering at 97% rRNA sequence similarity. Curves represent sequences for each sample for endophytic barley (EB), endophytic clover (EC), rhizosphere barley (RB) and rhizosphere clover (RC). 201

Figure F.1 Effect of bacteria on root length of sweet clover measured at 20 days. Plants were supplemented only with water. Error bars represent standard deviations and * indicate significance at LSD (p ≤ 0.05) compared to control. 216

Figure G.1 Effect of endophytic bacterial strains EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover plants (*Melilotus albus*) growing in soils amended with diesel (5,000 mg·kg^{-1}) at 65 days after planting. 220

Figure G.2 Effect of endophytic bacterial strains EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover plants (*Melilotus albus*) growing in soils amended with diesel (10,000 mg·kg^{-1}) at 65 days after planting. 221

Figure G.3 Effect of endophytic bacterial strains EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover plants (*Melilotus albus*) growing in soils amended with diesel (20,000 mg·kg^{-1}) at 65 days after planting. 222

LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylate
alkB	Alkane hydroxylase
alkM	Alkane hydroxylase
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
ATCC	American type culture collection
BaP	Benzo(a)pyrene
BE	Barley endosphere
BLAST	Basic local alignment search tool
*bph*A	Biphenyl dioxygenase subunit alpha
BR	Barley rhizosphere
BSA	Bovine serum albumin
BTEX	Benzene, toluene, ethylbenzene and xylene
C12*O*	Catechol 1,2-dioxygenase
*cat*23	Catechol-2,3-dioxygenase
CCME	Canadian council of ministers of the environment
CE	Clover endosphere
CEC	Cation exchange capacity
CFU	Colony-forming unit
CR	Clover rhizosphere
CT	Consolidated tailings
CYP	Cytochrome P450
DGGE	Denaturing gradient gel electrophoresis
DRO	Diesel Range Organics
EC	Electrical conductivity
EcM	Ectomycorrhizal fungi
EDTA	Ethylenediaminetetraacetic acid
F:B	Fungal/bacterial ratio
FID	Flame ionization detector
GC	Gas chromatography
GCL	Geo-clay liner
Gr-	Gram negative bacteria
Gr+	Gram positive bacteria
HSD	Honest significant difference
LB	Liquid broth
LSD	Least significant difference
MDS	Multidimensional scaling
MFT	Mature fine tailings

MRPP	Multi-response permutation procedures
NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
ndoB	Naphthalene 1,2-dioxygenase
OD	Optical density
OM	Organic matter
OTUs	Operational taxonomic units
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate-buffered saline
PCoA	Principal Coordinate Analysis
PCR	Polymerase chain reaction
PGPR	Plant growth- promoting rhizobacteria
*phn*A	Phosphonoacetate hydrolase
PLFA	Phospholipid fatty acid analysis
PPM	Peat mineral mix
qPCR	Quantitative real-time polymerase chain reaction
RCBD	Randomized complete block design
SD	Standard deviation
SOC	Soil organic carbon
sp.	Species
spp.	Species
TAE	Tris, acetic acid
TC	Total carbon
TCA	Tricarboxylic acid cycle
THC	Total hydrocarbons
TOC	Total organic carbon
*tod*C1	Benzene 1,2-dioxygenase subunit alpha
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UTM	Universal Transverse Mercator
xylE	D-xylose-proton symporter

1. INTRODUCTION

Canada's oil sands in northern Alberta are the world's third largest proven oil reserves after Venezuela and Saudi Arabia (Li *et al.*, 2017). The oil sands are a vital part of the Canadian economy and most of these reserves contain an unconventional heavy and viscous form of petroleum known as bitumen (Government of Alberta, 2017). Due to increased global demand for oil, bitumen production is expected to increase from 2.3 million barrels/day in 2014 to over 6 million barrels/day by 2030 (Bourgès-Gastaud *et al.*, 2017; Wellstead *et al.*, 2016). However, oil sands mining activities creates a landscape scale disturbance that removes vegetation, topsoil, parent geologic material, and overburden to a depth that often exceeds 80 m (Aubertin and McKenna, 2016). In addition, each cubic meter of mined oil sands requires up to 3 m^3 of water and produces approximately 4 m^3 of mine tailings (Fedorak *et al.*, 2002). Since oil sands industries in Alberta operate under a low-discharge policy, mine tailings accumulate in settling ponds where tailing sands are precipitated and the water is recycled (Onwuchekwa *et al.*, 2014; Yergeau *et al.*, 2012a).

Following consolidation processes, tailing sands are buried and capped with plant growth medium in land reclamation strategies (Allen, 2008). Oil sands reclamation activities include soil re-construction and ultimately the re-establishment of vegetation. However, contrary to wildfire and logging, oil sands mining activities nearly always affect physical landform attributes, causing significant challenges to land reclamation (Audet *et al.*, 2015). These challenges start with the nature of the tailing sands, which have low nutrient content, high pH (8–9), low or no organic matter and high concentrations of toxic materials including naphthenic acids, polycyclic aromatic hydrocarbons, phenolic compounds and trace metals (Kwak *et al.*, 2015; Naeth *et al.*, 2011; Lefrançois *et al.*, 2010; Onwuchekwa *et al.*, 2014). Therefore, reclamation practices in the oil sands region have mainly used a peat-mineral mix (PMM) cover to build soil fertility due to the high peat quantities and availability in the mining footprint (Beasse *et al.*, 2015; Quideau *et al.*, 2013). After the addition of a peat-mineral (PMM) cover, barley (*Hordeum vulgare*) is often

sown to provide quick vegetation cover and erosion control. Barley is also used because it is a poor competitor and is readily invaded by local flora within the first few years (Rowland, 2008). The growth and survival of these plant species and a sustainable soil microbial community structure is essential for reaching a long-term ecosystem stability.

Soil microbial communities represent the greatest known reservoir of biological diversity and play an important role in ecosystem functioning (Berendsen *et al.*, 2012). Microorganisms are key determinants of soil physical, biological and chemical characteristics, biogeochemical cycling and other terrestrial ecosystem functions (Prosser, 2015). However, compared to non-rooted bulk soil, the rhizosphere, which is the narrow zone of soil that is influenced by root exudates, is a ‘hot spot’ for numerous organisms and is considered as one of the most complex ecosystems (Raaijmakers *et al.*, 2009; Tkacz *et al.*, 2015; Bakker *et al.*, 2013). Soil microorganisms are chemotactically attracted by and feed on rhizodeposits, and in turn they can have profound effects on plant growth health and nutrition (Philippot *et al.*, 2013). In addition to the rhizosphere, microorganisms are also able to colonize most plant compartments and plants can also function as filters of soil microorganisms (Chen *et al.*, 2010; Berg *et al.*, 2014). Microorganisms that penetrate and colonize the root internal tissues (the endosphere) and that spend at least part of their life cycle inside plants are characterized as endophytes (Turner *et al.*, 2013). Endophytic bacteria colonizing the endosphere can establish beneficial associations and are often recognized as symbionts with a unique and intimate interactions with their host plants (Hardoim *et al.*, 2008; Berg *et al.*, 2014). Endophytic bacterial species are very diverse and they may have the ability to degrade xenobiotics (Phillips *et al.*, 2008), provide protection against invading pathogens (either by antibiosis or via induced resistance) (Mazurier *et al.*, 2009), promote plant growth (Pavlova *et al.*, 2017) and reduce plant stress under adverse conditions (Soleimani *et al.*, 2010; Deng *et al.*, 2011; Khan *et al.*, 2011). Therefore, since tailings sands used in oil sands reclamation strategies may contain residual hydrocarbon products (Lefrançois *et al.*, 2010), plants growing in these areas may rely on the presence and activity of specific plant-associated microorganisms harboring degradation genes required for the enzymatic break-down of these contaminants. In addition, endophytic bacteria have a great biotechnological potential to improve the efficiency of phytoremediation techniques.

Phytoremediation consists of the use of plants to remove pollutants from the environment or to render them harmless (Pilon-Smits, 2005; Salt *et al.*, 1998). This technique uses naturally

occurring processes by which organic and inorganic pollutants are degraded and sequestered. Due to its environmentally friendly, effective, relatively inexpensive and carbon neutral approach for the clean up of toxic pollutants in the environment, phytoremediation has recently gained popularity with the industry and government agencies (Germaine *et al.*, 2013; Glick, 2010). However, the use of plant-based technologies alone has limitations regarding the fact that plants are autotrophs and not ideally suited for the metabolism and breakdown of organic compounds (Dowling and Doty, 2009). Therefore, the exploitation of plant-microbe interactions may overcome these limitations by using plants associated with pollutant-degrading and/or plant growth-promoting microorganisms. The use of endophytic pollutant degraders has also the advantage that any toxic xenobiotics taken up by the plant may be degraded in planta, therefore reducing phytotoxic effects to other organisms (Ryan *et al.*, 2008).

With continually expanding oil industry and a global dependence on fossil fuel, there is an increasing concern with the release of these synthetic compounds into the soil environment. Fortunately, natural microbial communities have a high metabolic diversity which can assist in degradation of these compounds (Pandey *et al.*, 2016). Hydrocarbon degrading bacterial species are also very diverse, which includes *Arthrobacter, Burkholderia, Flavobacterium, Mycobacterium, Pseudomonas, Rhodococcus* and *Stenotrophomonas* spp. (Deka and Lahkar, 2016).

The bacterial root microbiome associated with plants growing in oil sands reclamation covers may harbor a unique set of organisms that could be used as biotechnological tools in future reclamation strategies and phytoremediation applications. The overall objective of this research was to unravel the root associated bacterial microbiome of plants growing in reclamation soils and to assess their applicability in phytoremediation. The specific objectives of this study were to: (*i*) assess the diversity of endophytic root bacteria associated with plants growing on reclamation soils, (*ii*) screen endophytic root bacteria for hydrocarbon degrading genes and (*iii*) investigate the potential use of endophytic root bacteria and host plants to degrade hydrocarbons. To achieve these objectives, a series of studies were designed to address the following hypotheses:

1) Plants select unique populations of endophytic root bacteria that improve their growth and development.

2) Endophytic root bacteria of plants growing on reclamation soils have specific genes that help host plants improve their growth and development.
3) Endophytic bacteria-plant associations can be used to remediate contaminants.

The following research thesis is presented in manuscript-style format in which more than one research study may address one of the above hypotheses. This thesis includes a literature review (Chapter 2) followed by four research studies (Chapter 3, 4, 5 and 6), an overall summary of discussions (Chapter 7), summary of conclusions (Chapter 8) and future work directions (Chapter 9). Chapter 3 begins with a broad assessment of the most dominant plant associated bacterial communities in an oil sands reclamation area identified by culture dependent and independent techniques. Chapter 4 provides an in-depth culture independent analysis of these bacterial communities associated with two plant species growing in an oil sands reclamation area. Chapter 5 focuses on the quantification of hydrocarbon-degrading genes present within these cultured and uncultured bacterial communities. Chapter 6 evaluates growth promotion and phytoremediation capabilities of selected bacterial communities in association with their host plants. Lastly, Chapter 7, 8 and 9 includes a summary of the major findings in this research and suggests future research directions. Each research study chapter has been written to stand alone for submission to peer-reviewed journals, however each chapter includes a preface that provides a transition from one study chapter to another. Due to the manuscript format, some redundant information may occur.

2. LITERATURE REVIEW

2.1 The Alberta Oil Sands

Canada has the world's third largest proven oil reserves and it is in the top five oil producers globally (Wellstead *et al.*, 2016; Natural Resources Canada, 2017). The oil reserves in Alberta accounts for 98% of total Canadian oil reserves and approximately 13% of the global oil reserves. Most of these oil reserves contain an unconventional dense and extremely viscous form of petroleum, which is usually referred to as oil sands or bituminous sands (Government of Alberta, 2017). The province of Alberta contains over 169 billion remaining barrels (27×10^9 m^3) of this type of bitumen and can supply Canada's energy demands for next 475 years (Brown and Ulrich, 2015; Chastko, 2004). The crude oil production in the oil sands is also expected to increase from about 2.3 million barrels per day in 2014 to 3.5–4 million barrels per day in 2025 and over 6 million barrels per day by 2030 (Bourgès-Gastaud *et al.*, 2017; Wellstead *et al.*, 2016). In addition, the overall estimated industry investment in Alberta's oil sands reached over $21.6 billion in 2011. Economically, the oil sands have substantial impact on Canadian energy sector creating a significant number of jobs, a wide variety of cross-disciplinary scientific studies to increase bitumen extraction efficiencies and decreasing environmental impacts (Huang *et al.*, 2016).

Geographically, the oil sands reserve in northern Alberta underlies a total of 142,200 km^2 and are spread in three regions: the Athabasca (40,000 km^2), Cold Lake (22,000 km^2) and Peace River (8,000 km^2) (Natural Resources Canada, 2017). Among these three regions, the Athabasca is the largest in size and contains the largest amount of bitumen (Figure 2.1). After research and development in the early 1900s, mining activities were initiated in 1967. During this year, The Great Canadian Oil Sands Company, later renamed Suncor Energy, began commercial oil production at 12,000 barrels per day (Shaughnessy, 2010). Oil production from bitumen extraction expanded significantly from 1978 to 2003. Roughly, 500 km^2 of the 140,200 km^2 oil sands deposit is currently undergoing surface mining activity (Audet *et al.*, 2015).

Figure 2.1 Outline of oil sands deposits (orange) in Alberta and adjacent provinces (Wellstead *et al.*, 2016).

Approximately 20% of the bitumen oil reserves in the region are close enough to the surface to be mined (CAPP, 2017). The bitumen mining involves the use of shovel excavators and haul trucks similar to other types of large scale open pit mines (*i.e.* coal, copper, iron) (Aubertin and McKenna, 2016). However, most of oil sands mines are up to five to ten times the size of large open pit mining operations. In addition, the oil sand deposits lie under natural forests characterized by bogs and fens dominated by black spruce (*Picea mariana*), trembling aspen (*Populus tremuloides*) and jack pine (*Pinus banksiana*) (Lévesque, 2014). Therefore, surface mining in the oil sands region creates landscape scale disturbance that removes the vegetation, topsoil, parent geologic material, and overburden to a depth that often exceeds 80 m (Aubertin and McKenna, 2016).

Bitumen is extracted from the oil sands using the Clark hot water extraction (CHWE) process which consists mainly of blending hot water and caustic soda to separate the bitumen from the mineral solids (Lévesque, 2014). Each cubic meter of mined oil sands requires up to 3 m^3 of process water and produces approximately 4 m^3 of tailings, which consists of process water, sand, clays, organics and residual bitumen (Fedorak *et al.*, 2002). Since oil sands companies in Alberta operate under a low-discharge policy, mine tailings are accumulated in settling basins known as tailing ponds (Yergeau *et al.*, 2012a).

2.2 Tailings Management

Mine tailings, which must be retained on-site, are deposited in tailings ponds that currently occupy a surface area of approximately 180 km^2 containing more than 800 million m^3 of tailings (Yang *et al.*, 2016). Tailing ponds are engineered dam and dyke systems designed to keep mine tailings from being released into the environment. These ponds are essentially settling basins and as ponds are filled, they are retained indefinitely, pending reclamation (Mohamad Shahimin *et al.*, 2016). A major concern with using tailings ponds as a disposal method is the slow rate of sedimentation and consolidation of the fine tailings, which could take 125-150 years (Fedorak *et al.*, 2002). Initially, sand particles quickly settle to the bottom, then a middle layer of fine materials form, known as mature fine tailings (MFT), which comprises about 70% of water and 30% of fine clay (Suncor, 2017). To speed up fine tailings sedimentation, the oil sands industry has developed the Consolidated tailings (CT) process. CT technology expedites the

process of dewatering tailings through the addition of chemical coagulants such as calcium sulfate (gypsum) or aluminum sulfate (alum) (Renault *et al.*, 2003). Sodium hydroxide is also occasionally added to the separation water to improve bitumen extraction efficiency (Roy *et al.*, 2016). However, as clarified water is then recycled, this practice can further enhance the salinity and hardness of process water and concentrating contaminants (Allen, 2008; Yergeau *et al.*, 2012a). In addition, important concerns have been raised over oil sands development and tailing ponds regarding the size of the disturbed area, which currently occupies 430 km^2 and is projected to increase to 1767 km^2 over the next 10–15 years (MacKenzie and Quideau, 2010). Following mine decommissioning and consolidation processes, fine tailings are dewatered, mixed with sand, buried, and capped with plant growth medium in land reclamation strategies (Allen, 2008).

2.3 Reclamation Strategies

Land reclamation is defined as the process of transforming any disturbed land to its previous land capacity state or better, considering stability and restoration of biological self-sustaining processes (Quoreshi, 2008). Oil sands reclamation activities include soil re-construction and ultimately the re-establishment of vegetation. Contrary to wildfire and logging, oil sands mining activities nearly always affect physical landform attributes, causing significant challenges to land reclamation (Audet *et al.*, 2015). These challenges start with the nature of the tailing sands, which were affected by tailings water and considered generally an inappropriate plant growth medium.

The coarse tailings has low nutrient content, high pH (8–9), low or no organic matter and high concentrations of toxic materials including naphthenic acids, polycyclic aromatic hydrocarbons, phenolic compounds and trace metals (Kwak *et al.*, 2015; Naeth *et al.*, 2011; Lefrançois *et al.*, 2010; Onwuchekwa *et al.*, 2014). These conditions are considered extremely harsh for plant development and the addition of a cover material with suitable reclamation material is necessary (Naeth *et al.*, 2011; Beaudoin-Nadeau *et al.*, 2016). Approximately 30 cm of cover material are applied over tailing sands to support plant growth, supply nutrients and to improve soil properties (Kwak *et al.*, 2015).

Currently, two different types of organic matter commonly used as cover materials include the peat mineral soil mix (PMM) and LFH mineral soil mix (Jamro *et al.*, 2014). These

materials are generally salvaged from upland boreal forests and peatlands drained before mining operations. However, reclamation practices in the Athabasca oil sands region have mainly used PMM to build soil fertility, due to high peat quantities and availability in the mining footprint and the shortage of forest floor (Beasse *et al.*, 2015; Quideau *et al.*, 2013). The use of PMM as cover soils for land reclamation involves the mixing of humic, mesic, and fibric forms of peat materials with generally sandy mineral soils collected from tailings extraction processes or from the B horizons (Ojekanmi and Chang, 2014). Following a stockpiling period, peat is applied based on the regulatory approval from each mining company, most practices use a 20 to 50 cm thick PMM cover to cap the reconstructed soils (Quideau *et al.*, 2013). The application of PMM in land reclamation has been found to increase water holding capacity, cation exchange capacity (CEC), soil organic carbon (SOC) and soil nitrogen (N) dynamics (Ojekanmi and Chang, 2014; Quideau *et al.*, 2013). After the addition of an organic cover, barley (*Hordeum vulgare*) is often sown to provide quick vegetation cover and erosion control. Barley also helps to bulk up soil organic matter content and supply nutrients to desired planted tree species (Rowland, 2008). The growth and survival of these plant species and a sustainable soil microbial community structure is essential for reaching a long-term ecosystem stability after anthropogenic disturbance.

2.4 Soil microbial community function and structure

The soil habitat represents a highly heterogeneous environment in which communities are extremely complex and diverse, with millions of species and billions of individual organisms being found within a single ecosystem. Those communities range from bacteria and fungi, through to larger organisms, such as earthworms, ants and moles (Bardgett and van Der Putten, 2014). Soil has an estimated $2.6 \cdot 10^{29}$ prokaryotic cells and harbor much of the earth's genetic diversity in which one gram of soil contains kilometers of fungal hyphae and more than 10^9 bacterial cells belonging to tens of thousands of different species (Roesch *et al.*, 2007). Although soil macrofauna also play an important role in ecosystem functioning, microorganisms are key determinants of the soil physical, biological and chemical characteristics, biogeochemical cycling, other terrestrial ecosystem functions and the sustainability of soil ecosystems (Prosser, 2015). The vast majority of nutrient cycling processes such as carbon, nitrogen, phosphorus, and sulphur in soil are also carried out by microorganisms (Table 2.1). For example,

chemoautotrophic bacteria such as nitrifiers (*Nitrosomonas* and *Nitrobacter*) are important for nutrient cycling since they oxidize ammonium to nitrate in nitrification processes. Symbiotic (*Rhizobium*) and non-symbiotic free living (*Azotobacter*, *Clostridium*) nitrogen-fixing bacteria greatly increase N supply to the system (Bardgett, 2005). Microorganisms are also involved in decomposition of soil organic matter, degradation of synthetic compounds (*i.e*. pesticides and herbicides) and production of cementing agents that influence soil aggregation (Murphy *et al.*, 2007). Because of their involvement in such key processes, microorganisms are critical to soil function maintenance (Garbeva *et al.*, 2004). Hence, the functional diversity of a microbial community has been defined as the occurrence and distribution of physiological and metabolic traits among members of that community (van Elsas *et al.*, 2006).

Microbes have "functional traits" that assign how well they perform under certain conditions. All organisms have, to a certain degree, the ability to adjust to the environment, however environmental characteristics can impact in their physiology. The environment may alter the conformation of proteins and cell membranes, thermodynamic and kinetic favorability of biochemical reactions in organisms (Morris and Blackwood, 2014).

Table 2.1 Key microbial processes mediating chemical transformations associated with nutrient cycling in soil (Murphy *et al.*, 2007).

Microbial process	Examples of microbial groups involved
Nutrient supply	
Mineralization of organic matter	Heterotrophic microorganisms (Kalbitz *et al.*, 2000)
Solubilisation of minerals	*Penicillium* sp., *Pseudomonas* sp., *Bacillus* sp. (Sharma *et al.*, 2013)
Nutrient Transformations	
Methane oxidation	*Methylococcus* sp., *Methylobacter* sp. (Smith *et al.*, 1997)
Nitrification	
NH_3 to NO_2^-	*Nitrosospira* sp. and *Nitrosomonas* sp. (Bothe *et al.*, 2000)
NO_2^- to NO_3^-	*Nitrobacter* sp. (Lees, 1951)
Non-symbiotic N_2 fixation	*Azospirillum* sp., *Azotobacter* sp. (Steenhoudt and Vanderleyden, 2000)
Symbiotic N_2 fixation	*Rhizobium* sp., *Anabaena* sp. (van Gorkom and Donze, 1971; Wilson *et al.*, 1932)
Sulphur oxidation	*Thiobacillus* sp., Heterotrophic microorganisms (Parker and J., 1953)
Loss of Nutrients	
CO_2 production	Heterotrophic microorganisms (McGill *et al.*, 1975)
Methane (CH_4) production	*Methanobacterium* sp., *Methanosarcina* sp. (Sawayama *et al.*, 2004)
Denitrification (N_2, N_2O)	*Bacillus* sp., *Pseudomonas* sp., *Agrobacterium* sp. (Zumft, 1997)
Reduction of SO_4^{2-} to H_2S	*Desulfovibrio* sp., *Desulfomonas* sp. (Wei *et al.*, 2010)

Soil microbial community structure is based on how organisms become distributed in the environment (Morris and Dress, 2007). This distribution may rely on resources, such as nutrients and energy that could be favorable or inhibitory. Environmental factors that can affect the ecology, activity and structure of microorganisms in soil include: carbon and energy sources, mineral nutrients, ionic composition, available water, temperature, pressure, air composition, electromagnetic radiation, pH, oxidation–reduction potential, surfaces, spatial relationships, microorganism genetics and interaction between microorganisms (Nannipieri *et al.*, 2003; Lu *et al.*, 2014). Mainly in engineered or disturbed ecosystems, microbial communities encounter an ecological challenge. The communities can exhibit 'resistance' to their structure after disturbance, but when such disturbances cause a change in their structure, the rate at which communities recover to their native structure is called 'resilience' (Bora *et al.*, 2014). For example, MacKenzie and Quideau (2010) observed that microbial community structure and mineral nutrient availability were affected by time since reclamation, seasonal variability, inter-annual variability, and vegetation cover at reclamation sites after bitumen exploitation in Alberta. Lefrançois *et al.* (2010) studied *Frankia*-inoculated alders planted on and oil sands reclamation site, and found positive impacts on indigenous soil microbial community structure and function as compared to unplanted soil.

Therefore, greater knowledge concerning the dynamics of soil microbial community structure can benefit the development of new reclamation strategies to enhance plant health and soil ecosystem functioning in disturbed ecosystems.

2.5 Plant-microbe interactions

Microbial development in soil is considered to be inconsistently distributed, where there is a tendency of organisms to live in aggregates and to form active hot spots (Paul *et al.*, 2014). One of the most important soil hot spot is the portion of the soil influenced by the plant root system. Plant roots release a wide variety of compounds into the surrounding soil that create unique environments for the microorganisms living in association with plants (Garbeva *et al.*, 2004). Microorganisms that associate with plants are diverse in their ability to affect plant health, their genotypic and phenotypic characteristics, and their phylogeny (Beattie, 2006). Therefore, a recent approach consists in characterizing plants as holobionts (*i.e.* host and associated microbes)

and that the associated microbes affect plant fitness (Schlaeppi and Bulgarelli, 2015; Vandenkoornhuyse *et al.*, 2015). Other authors (Mercado-Blanco, 2015) have characterized the plant and its microbiome as “super organisms” which work coordinately to shape and sustain an ecosystem.

The plant microbiome has the potential to reduce the incidence of plant disease, increase plant ability to resist abiotic stress, increase agricultural production, reduce chemical fertilizer inputs and reduce greenhouse gas emissions (Turner *et al.*, 2013; Andrews *et al.*, 2010). These plant-microbe interactions may occur in the rhizosphere, endosphere and the phyllosphere.

2.5.1 The rhizosphere microbiome

The rhizosphere is defined as the narrow zone of soil that surrounds and is influenced by plant roots and harbors a vast number of microorganisms (Philippot *et al.*, 2013). It is estimated that the rhizosphere may contain up to 10^{11} microbial cells per gram root and more than 30,000 prokaryotic species, which makes its collective genome much larger than of the plant itself (Berendsen *et al.*, 2012). Plants influence the rhizosphere mainly through rhizodeposition of exudates, mucilage and sloughed cells. Root exudates include low molecular weight (amino acids, organic acids, phenolic acids, sugars, flavonoids, etc.) and high molecular weight (carbohydrates, enzymes, etc.) organic compounds released by roots (Pii *et al.*, 2015; Rasmann and Turlings, 2016). It is estimated that rhizodeposition account for approximately 11% of net photosynthetically fixed carbon and 10–16% of total plant nitrogen depending on plant species and plant age (Bulgarelli *et al.*, 2013). Many different organisms including bacteria, fungi, viruses and archaea are attracted by and feed on rhizodeposits in a phenomenon is known as the “rhizosphere effect” (Philippot *et al.*, 2013; Berendsen *et al.*, 2012).

Soil bacteria attracted to rhizodeposits are mostly organotrophs as they obtain their energy from organic substrates (Bulgarelli *et al.*, 2013). Carbon availability is the most common limiting factor for soil bacteria growth and the availability and accessibility of degradable organic compounds is limited in most soils (Mendes *et al.*, 2013). Therefore, low molecular weight exudates could represent an easy accessible carbon source for these organisms in the rhizosphere where the concentration of these compounds is usually much higher than in the bulk soil. (Pii *et al.*, 2015). The higher abundance in root exudates reflects in a hot spot for microbial

abundance, where the total biomass is 2 to 3 times greater compared to bulk soil (Kuzyakov and Blagodatskaya, 2015).

Proteobacteria usually dominates the rhizosphere microbiota, particularly those of the α and β classes. Other major groups include: *Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Planctomycetes* and *Verrucomicrobia* (Turner *et al.*, 2013; Bulgarelli *et al.*, 2013). Although more abundant, the rhizosphere microbiota is in general less diverse than those of bulk soil (Berendsen *et al.*, 2012). In addition, microbial abundance in the rhizosphere is influenced by plant vegetation cycles, such as annual litter fall in autumn or intensive root growth in spring or even diurnal cycles of photosynthesis (Kuzyakov and Blagodatskaya, 2015). However, root exudates are not the only component of rhizo-deposition, the sloughing of plant root cells and the release of mucilage deposits (including plant cell wall polymers such as cellulose and pectin) are a large part of materials in the rhizosphere. For example, the decomposition of pectin releases methanol, which can be used as a carbon source by other microbes. In addition, plant roots also provide a physical structure where microbes can attach (Turner *et al.*, 2013).

Most members of the rhizosphere microbiome have neutral effects on the plant, by taking advantage of root exudates as nourishment without affecting plants (Raaijmakers *et al.*, 2009). In negative interactions, pathogenic microorganisms may produce metabolites with toxic effects on plants, which results in detrimental effects on plant growth. These organisms are mostly pathogenic fungi, oomycetes, bacteria and nematodes (Raaijmakers *et al.*, 2009; Pii *et al.*, 2015). In positive interactions, known beneficial rhizosphere organisms includes nitrogen-fixing bacteria, mycorrhizal fungi, plant growth- promoting rhizobacteria (PGPR), biocontrol microorganisms, mycoparasitic fungi, and protozoa (Mendes *et al.*, 2013). For example, PGPR can stimulate plant growth, increase yield, reduce pathogen infection, as well as reduce biotic or abiotic plant stress, without conferring pathogenicity (Compant *et al.*, 2010). Hence, plant beneficial microorganisms have been subject of great relevance for agriculture as well as for phytoremediation applications. However, to exert their advantageous traits in the root system, beneficial rhizosphere bacteria must be capable of competing with other microbes for nutrients secreted by roots and for sites that can be occupied on the root (Parray *et al.*, 2016). In addition, plant species can strongly influence the composition of the microbiota living in proximity to them in which only a subset are capable entering and reside inside plant tissues.

2.5.2 The endosphere microbiome

As previously suggested by Hardoim *et al.* (2015) plants do not live alone as single entities, but instead in closely association with the microorganisms present in their neighborhood, and especially with those living internally. Microorganisms that penetrate and colonize the root internal tissues (the endosphere) and that spend at least part of their life cycle inside plants are characterized as endophytes (Turner *et al.*, 2013). The vast majority of these endophytes are arbuscular mycorrhizal fungi (AMF) along with other fungi, bacteria and, to a lesser extent, archaea (Vandenkoornhuyse *et al.*, 2015). However, the meaning of the term "endophyte" has changed in recent years. After the first definition as "any organism occurring within plant tissues" by De Bary (1866), various authors have defined endophytes in different ways (Berg *et al.*, 2014). The term initially characterized fungi living inside plants, but later researchers realized the existence of bacterial endophytes (Hardoim *et al.*, 2015). One the most common definitions of endophytes was described by Hallmann *et al.* (1997), in which authors define endophytes as "microorganisms that can be isolated from surface-disinfested plant tissues that do not cause visible harm to the plant". Endophytes are indeed generally considered to be non-pathogenic, but they may include also latent pathogens that, depending on environmental circumstances and/or host genotype, may cause diseases (Turner *et al.*, 2013). Hence, the definition of an endophyte has now been expanded by many researchers and can include any organisms that live in the endosphere whether neutral, beneficial or detrimental (Backman and Sikora, 2008).

Endophytes that benefit from metabolites produced by plants but have no apparent effects on plant performance are known as commensal endophytes. Beneficial endophytes include microorganisms that confer positive effects to their host plant, such as degradation of xenobiotics (Phillips *et al.*, 2008), protection against invading pathogens (either by antibiosis or via induced resistance) (Mazurier *et al.*, 2009) and plant growth promotion (Varma *et al.*, 1999). A third group of endophytes includes latent pathogens (Hardoim *et al.*, 2015). Other than their effects on host plants, endophytes are also classified as "obligate", requiring plant tissues to complete their life cycle, or "opportunistic", which mainly thrive outside plant tissues and sporadically enter the plant endosphere (Hardoim *et al.*, 2015). In addition, there are several ways in which microorganisms can gain access to the endosphere. Microbes can colonize the plant interior by entering the plant stomata, the leaf surface (by the production of wax degrading enzymes),

through flowers, fruits, stems or cotyledons and seeds (Mercado-Blanco, 2015). However, their main and most studied colonization route occurs by the plant roots in the rhizosphere (Malfanova *et al.*, 2013). Consequently, the density of endophytes is normally higher in roots than other plant organs.

The colonization of endophytes generally starts with the microbial establishment in the rhizosphere following the attachment on the root surface, termed "rhizoplane". While some organisms such as ectomycorrhizal fungi (EcM) remain attached to the rhizoplane, others gain access to the endosphere (Vandenkoornhuyse *et al.*, 2015). Although little is known about the specific sites at which endophytes attach and penetrate into root tissues, it is generally assumed that they gain entry passively by cracks or wounds at lateral root emergence (Turner *et al.*, 2013). Other than lateral root emergence, these cracks can also be formed by arthropod, nematode or microbial activities (Mercado-Blanco, 2015). Although generally in lower concentrations than plant pathogens, many endophytic bacteria express cell-wall-degrading enzymes that facilitate their entry at the endosphere. (Turner *et al.*, 2013). Once inside the plant, endophytes either reside in specific plant tissues such as the root cortex or the xylem, or colonize other plant compartments by transport through the vascular system or the apoplast (Weyens *et al.*, 2009). However, microorganisms that reach the endosphere must contend with triggered plant defense responses, which can function as filters of soil microorganisms, selecting those successful, competent endophytes (Chen *et al.*, 2010; Beattie, 2006). Differently from the rhizosphere microbiota, the plant's endosphere harbors a highly specific set of microbial communities.

In the endosphere, the microbial community structure is very different from the adjacent soil. Overall, diversity is much lower than in the rhizosphere (Vandenkoornhuyse *et al.*, 2015). For example, endophytic bacteria once inside plant tissues rarely exceed 10^7 to 10^8 $CFU \cdot g^{-1}$ of root fresh weight^{-1} and can be as low as 10^3 $CFU \cdot g^{-1}$ of root fresh weight^{-1}, depending on plant age and genotype (Turner *et al.*, 2013). In terms of bacterial community structure, several bacteria have been reported to be endophytic in cultivation based studies. Most of these include *Proteobacteria*, but also *Firmicutes, Actinobacteria* and *Bacteroidetes*. Archaea however does not have an apparent close association with plants (Reinhold-Hurek and Hurek, 2011). In addition, an intimate close association is observed between bacterial endophytes and their host plants. Bacterial endophytes may access nutrients and water more easily than those on the

rhizosphere or the rhizoplane (Beattie, 2006). On the other hand, the beneficial effects of endophytes to their host plants are in general greater than those of rhizobacteria., which might be intensified when the plant is growing under either biotic or abiotic stress conditions (Ma *et al.*, 2011a; Compant *et al.*, 2010). Hence, in addition to their beneficial effects on plant growth, endophytes have a great biotechnological potential to improve the efficiency and applicability of phytoremediation techniques.

2.6 Phytoremediation

The remediation of soils contaminated with organic pollutants is a global issue that consumes considerable economic resources of industries and governments. The estimated costs for the clean-up of contaminated sites with conventional techniques such as landfilling and incineration are very high (Kuiper *et al.*, 2004; Cunningham *et al.*, 1995). Most soils contaminated with organic pollutants are remediated using a diverse set of thermal, chemical, and physical methods that strip the contaminants from the soil (Cunningham *et al.*, 1996). In addition, the numerous classes and types of many potentially toxic compounds increases the challenges on soil remediation (Glick, 2003). Therefore, in the last decades there is an increase in the search for alternative techniques using an environmentally friendly, safe, less expensive and less labor-intensive approach such as phytoremediation (Kuiper *et al.*, 2004).

Phytoremediation is the use of plants and their associated microbes to remove pollutants or render them harmless to the environment (Salt *et al.*, 1998; Pilon-Smits, 2005). This technique uses naturally occurring processes by which organic and inorganic pollutants are degraded and sequestered. Organic pollutants are mostly man made and xenobiotic to organisms, in which many are toxic and some carcinogenic (Pilon-Smits, 2005). Phytoremediation has been used for treating many classes of contaminants including chlorinated solvents (Strand *et al.*, 1998), petroleum hydrocarbons (Soleimani *et al.*, 2010), explosives (Hagan *et al.*, 2016), pesticides (Azab *et al.*, 2016), heavy metals (Wood *et al.*, 2016), radionuclides (Singh *et al.*, 2016) and landfill leachates (Jerez Ch and Romero, 2016). Due to the diverse class of contaminants and where they may occur, there a serval different ways in which phytoremediation can be applied. According to Salt *et al.* (1998), this application is currently divided into the following areas:

- Phytoextraction: the use of pollutant-accumulating plants to remove metals or organics from soil by concentrating them in the harvestable parts.
- Phytodegradation: degradation of organic pollutants by using plants and associated microorganisms.
- Rhizofiltration: the use of plant roots to absorb and adsorb pollutants, mainly metals, from water and aqueous waste streams.
- Phytostabilization: the use of plants to reduce the bioavailability of pollutants in the environment.
- Phytovolatilization: the use of plants to volatilize pollutants; and the use of plants to remove air borne pollutants.

Overall, phytoremediation in general has gained popularity with government agencies and industry in the past years. Although this technique has several advantages, it also has limitations.

2.6.1 Advantages and disadvantages

The main advantages of phytoremediation rely on its high public acceptance and its environmentally friendly approach. Phytoremediation also provides a natural solar-driven technique that is less expensive than physicochemical remediation treatments. Once phytoremediation is established, sites usually require little maintenance and transportation costs associated with moving the contaminated material to a secondary treatment facility (Pilon-Smits, 2005). In addition, phytoremediation is faster than natural attenuation, has fewer air and water emissions and it conserves natural resources (Susarla *et al.*, 2002). However, phytoremediation is frequently slower than conventional physicochemical remediation processes and often limited by plant root depth, since plants must be able to reach the pollutant (Taylor and Gomes, 2012; Pilon-Smits, 2005). Soil pH, salinity, texture, pollutant concentrations and the presence of other toxins must be within the limits of plant tolerance in order for phytoremediation to occur. Contaminants that are highly water soluble may also leach outside the root zone and require additional containment (Cunningham *et al.*, 1995). Despite these limitations, phytoremediation may be suitable for contaminated sites where large surface areas contain relatively immobile

contaminants. However, the use of plant-based technologies alone has limitations regarding the fact that plants are autotrophs and not ideally suited for the metabolism and breakdown of organic compounds (Dowling and Doty, 2009). For example, plants can only absorb a minute quantity of hydrocarbons from the soil and translocate them into their different parts. Although a small fraction can be stored in the vacuole or volatilized into the atmosphere, the majority of the hydrocarbons cannot move considerably into plants from the soil (Khan *et al.*, 2013). Therefore, the exploitation of plant-microbe interactions may overcome these limitations by using plants associated with metal accumulating, pollutant-degrading and/or plant growth-promoting microorganisms.

2.6.2 Use of plants and associated microorganisms for hydrocarbon degradation

Although a few studies have reported that fungi can enhance hydrocarbon degradation can in different plant compartments (Rajtor and Piotrowska-Seget, 2016; Soleimani *et al.*, 2010), bacteria are most important group capable of hydrocarbon degradation (Table 2.2). The potential to degrade organic compounds have been reported in both rhizosphere (Arslan *et al.*, 2014) and endophytic (Yousaf *et al.*, 2011) bacteria in association with host plants. Arslan *et al.* (2014) studied the inoculation of annual ryegrass (*Lolium multiflorum*) with *Pantoea* sp. strain BTRH79 previously characterized for hydrocarbon degradation and 1-Aminocyclopropane-1-Carboxylate (ACC) deaminase activity. In this study, despite that soil diesel contamination overall reduced plant growth and development, hydrocarbon degradation was enhanced in inoculated plants.

Although rhizosphere bacteria play an important role in phytoremediation, endophytic bacteria offer several advantages over rhizosphere bacteria. The bacterial population in the rhizosphere is more difficult to control and, unless the pollutant is selective, desired strains are often reduced by competition with indigenous microbes (Doty, 2008). In contrast, endophytes that naturally inhabit the plant tissues would encounter a less competitive environment. The use of pollutant degrader endophytes also has the advantage that any toxic xenobiotics taken up by the plant may be degraded in planta, therefore reducing phytotoxic effects to other organisms (Ryan *et al.*, 2008).

Therefore, although relatively a new concept, the unique niche in the endosphere for hydrocarbon degradation has been previously reported in the literature. One of the first studies to

report different endophytic bacterial strains associated with plants that were able to degrade hydrocarbons was conducted by Siciliano *et al.* (2001). The results in this study indicated that plants grown in a soil contaminated with different concentrations of aliphatic hydrocarbons and polycyclic aromatic hydrocarbons naturally recruited endophytes with the necessary hydrocarbon degrading genes.

Phillips *et al.* (2008) also studied the endophytic bacteria associated with plants growing on a hydrocarbon contaminated site. These authors found that hydrocarbon degradation was associated to dominant endophytic bacterial species. Bacterial community structure in the endosphere dominated by *Pseudomonas* sp. revealed an increased hydrocarbon degradation potential and activity. In addition, most of the isolated endophytic bacteria in this study exhibited the potential to degrade both aromatic and aliphatic hydrocarbons. Differently from Siciliano *et al.* (2001) and Phillips *et al.* (2008), Barac *et al.* (2004) intentionally inoculated yellow lupine (*Lupinus arboreus*) plants with engineered *Burkholderia cepacia* G4. Results in this study indicated that *Burkholderia cepacia* not only could increase plant tolerance to toluene, but also improve the overall degradation of the contaminant.

Other studies with endophytic bacteria that assessed hydrocarbon degradation were conducted with *Enterobacter* spp. In Yousaf *et al*. (2011), despite a strong reduction in shoot and root biomass due to the presence of diesel, inoculation with *Enterobacter ludwigii* strains significantly reduced this effect compared to non-inoculated plants. This study also assessed CYP153 hydrocarbon degrading genes within *E. ludwigii* strains and found that the expression of this gene varied distinctly between different strains, plants species, plant developmental stages and plant compartments. Soils amended with diesel have also been used by Andria *et al*. (2009) to study to test activities and plant colonization of the endophyte *Pseudomona*s sp. strain ITRI53 in which hydrocarbon degrading alkane monooxygenase (*alkB*) gene abundance and expression was investigated. Plants inoculated with *Pseudomona*s sp. strain ITRI53 showed higher growth and survival in the presence of diesel. Although the increase in diesel concentration decreased the number of culturable bacteria, the inoculation of endophyte strains resulted in higher plant survival. In addition, bacterial strains expressed *alkB* genes indicating their active role in diesel degradation.

Pseudomonas spp. have also been used by Germaine *et al*. (2009) in the phytoremediation of polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, fluorene

and phenanthrene. This study investigated the ability of an endophytic naphthalene degrading bacterium, *Pseudomonas putida* VM1441 (pNAH7), to protect pea plants from the phytotoxic effects of naphthalene, along with its ability to enhance naphthalene removal from contaminated soil. Inoculation with this strain not only resulted in higher (40%) naphthalene degradation rates but also protected plants from the toxic effects of naphthalene. Andreolli *et al.* (2013) inoculated hybrid poplar (*Populus deltoids* and *Populus nigra*) plants with *Burkholderia fungorum* DBT1, a bacterial strain that indicated PAH degradation and plant growth promotion but it was never identified before as an endophytic strain. Results in this study indicate that *B. fungorum* DBT1 can infect the roots of poplar and improve the phytoremediation efficiency of PAHs. The absence of the strain *B. fungorum* DBT1 in the rhizosphere samples implies that PAH removal by this strain occurred within the root tissue.

Table 2.2 Examples of phytoremediation of hydrocarbon contaminated soil by combined use of microbes.

Plant used	Microorganism	Habitat	Microorganism characteristics	Reference
Tall fescue (*Festuca arundinacea*) and meadow fescue (*Festuca pratensis*)	*Neotyphodium*	Endosphere	Plant growth promotion	(Soleimani *et al.*, 2010)
Ryegrass (*Lolium perenne*)	*Pantoea* sp. strain BTRH79	Rhizosphere	Alkane degradation (CYP153 gene) and ACC deaminase activity	(Arslan *et al.*, 2014)
Yellow lupine (*Lupinus arboreus*)	*Burkholderia cepacia* G4	Endosphere	Toluene-degrading endophyte.	(Barac *et al.*, 2004).
Italian ryegrass (*Lolium multiflorum*), birdsfoot trefoil (*Lotus corniculatus*) and alfalfa (*Medicago sativa*)	*Enterobacter ludwigii.*	Endosphere	Alkane degradation (CYP153 gene)	(Yousaf *et al.*, 2011)
Italian ryegrass (*Lolium multiflorum*)	*Pseudomona*s sp. strain ITRI53	Endosphere	Alkane degradation (*alkB* gene)	(Andria *et al.*, 2009)
Pea (*Pisum sativum*)	*Pseudomonas putida* VM1441 (pNAH7)	Endosphere	PAH degradation (*nahY* gene)	(Germaine *et al.*, 2009)
Hybrid poplar (*Populus deltoids* and *Populus nigra*)	*Burkholderia fungorum* DBT1	Endosphere	Capacity of degrading several PAHs	(Andreolli *et al.*, 2013)
Wheat (*Triticum aestivum)*	*Pseudomonas* sp. GF3	Endosphere	Phenanthrene-degrading bacteria	(Sheng and Gong, 2006)
Wheat (*Triticum* sp.) and corn (*Zea. mays*)	*Burkholderia cepacia* strain FX2	Endosphere	Toluene degrading bacteria	(Wang *et al.*, 2010b)

2.7 Ecology of hydrocarbon degrading bacteria

Petroleum (crude oil) is a complex mixture that includes several different chemical components which are mostly hydrocarbons. Bacteria are the most active agents in petroleum degradation. Most hydrocarbon degrading bacteria are widespread in oil-polluted environments but they also occur in pristine environments (Afzal *et al.*, 2013b). These bacterial species are heterotrophic organisms (*i.e.* requiring an organic substrate to feed on) and petroleum derived hydrocarbons can be used as nutrient source by hydrocarbon degrading bacteria. The composition of these hydrocarbons in crude oil can be divided in saturated aliphatic hydrocarbons, aromatic, resins and asphaltenes (Mbadinga *et al.*, 2011).

Most hydrocarbons that are metabolized by bacteria are either aliphatic or aromatic hydrocarbons. Aliphatic hydrocarbons includes both linear or branched chain hydrocarbons, which may be unsaturated (alkenes and alkynes) or saturated (alkanes) (Pandey *et al.*, 2016). Hydrocarbon degrading bacterial species are very diverse, although in the case of alkane-degrading bacteria, almost all bacteria belong to α-, β-, and γ-Proteobacteria and Actinomycetales (Afzal *et al.*, 2013). Based on the number of research studies, the most important alkane-degrading bacteria in both marine and soil environments are *Achromobacter* (Tanase *et al.*, 2013)*, Acinetobacter* (Fatima *et al.*, 2016)*, Alcaligenes* (Kim and Cho, 2006)*, Arthrobacter* (Ferrera-Rodríguez *et al.*, 2013)*, Bacillus* (Tao *et al.*, 2016)*, Burkholderia* (Tara *et al.*, 2014)*, Flavobacterium* (Turner *et al.*, 2015)*, Mycobacterium* (Kim *et al.*, 2015)*, Micrococcus* (Dellagnezze *et al.*, 2014)*, Nocardioides* (Hamamura *et al.*, 2006)*, Nocardia* (Ali *et al.*, 2012) *, Pseudomonas* (van Beilen *et al.*, 2006)*, Rhodococcus* (Stancu, 2015)*, Ralstonia* (Kubota *et al.*, 2008)*, Sphingomonas* (Liu *et al.*, 2016) and *Stenotrophomonas* spp. (Tebyanian *et al.*, 2013).

Aromatic hydrocarbons include mono (*i.e.* benzene, toluene, phenol, etc.) and polycyclic aromatic hydrocarbons (PAHs). Among PAHs, Benzo(a)pyrene (BaP) is considered as the most carcinogenic and toxic (Haritash and Kaushik, 2009). However, different studies have shown bacterial degradation of BaP by *Mycobacterium* sp. (Schneider *et al.*, 1996; Moody *et al.*, 2004). Several other bacterial species are known to degrade PAHs, which most are isolated from contaminated soil or sediments (Haritash and Kaushik, 2009). The simplest and most soluble PAH is Naphthalene. General naphthalene-degrading bacteria include *Pseudomonas* (Jin *et al.*,

2016), *Vibrio* (Geiselbrecht *et al.*, 1996), *Mycobacterium* (Daane *et al.*, 2001), *Marinobacter* (Deppe *et al.*, 2005)., *Sphingomonas* (Pinyakong *et al.*, 2003)., *Rhodococcus* (Allen *et al.*, 1997) and *Micrococcus* spp. (Jegan *et al.*, 2010).

Pseudomonas spp. are the most predominant group of soil bacterial taxa that biodegrade complex organic compounds which includes aliphatic and aromatic hydrocarbons. Hydrocarbon degradation by *Pseudomonas* spp. is a result of different processes that typically requires the collaborative efforts of several different enzymes (Glick, 2010). Phillips *et al.* (2008) isolated endophytic bacteria from different plant species growing in an oil contaminated soil and found that *Pseudomonas* spp. dominated endophytic communities. The presence of *Pseudomonas* spp. was also associated with high alkane degradation potential and activity.

2.7.1 Hydrocarbon degradation

With continually expanding oil industry and a global dependence on fossil fuel, there is an increasing concern with the release of these synthetic compounds into the soil environment. The novel chemical structures in these compounds are often recalcitrant to microbial degradation since microorganisms have not had time to adjust their metabolism to respond and degrade those compounds (Leung *et al.*, 2006). Fortunately, natural microbial communities have a high metabolic diversity which can assist in degradation of these compounds. In addition, many synthetic compounds are similar to naturally-occurring compounds, so that, given the right conditions and adequate time, they can be degraded by bacterial communities. (Leung *et al.*, 2006). Hence, upon exposure to crude oil, virtually any soil or water habitat can ultimately enhance a bacterial population capable of degrading hydrocarbons.

Hydrocarbon degradation by bacteria may occur in anaerobic or aerobic conditions. Although several studies have reported the occurrence of anaerobic degradation (Heider *et al.*, 1998; Aitken *et al.*, 2004), most microbial hydrocarbon degradation occurs in the presence of oxygen.

2.7.1.1 Aerobic degradation of aliphatic compounds

One of the most important aspects that limits biodegradation of oil pollutants in soil is their limited availability to microorganisms. Petroleum hydrocarbons bind to soil components and often have low water solubility (Das and Chandran, 2011). As hydrocarbon molecular

weight increases, water solubility decreases. Although low molecular weight hydrocarbons are sparingly soluble in water to ensure a sufficient transfer to bacterial cell, high molecular weight (medium and long chain n-alkanes) alkanes are virtually water insoluble (Singh *et al.*, 2012). However, solubility of long chain hydrocarbons can be enhanced by the production of biosurfactants, which increase dispersion and solubility of organic compounds in water. Many soil bacteria produce biosurfactants, such as mono-rhamnolipids, which is produced by a *Pseudomonas aeruginosa* strain (Rahman and Gakpe, 2008). Other than biosurfactant production, microorganisms are equipped with metabolic machinery to use both aliphatic and aromatic compounds as a carbon and energy source.

Aliphatic hydrocarbons are in general more readily biodegradable when compared to aromatic. Within aliphatic compounds, branched chain structures (*e.g*. 2-Methylpentane) and substituted compounds such as halogenated ones (*e.g*. Chloroethane) are more persistent to microbial degradation, while medium-sized straight-chain compounds (*e.g*. n-alkanes in the range of C10–C18) are more readily degraded (Leung *et al.*, 2006).

Metabolism of hydrocarbons by microorganisms is a complex process in which consists on converting these metabolically inactive molecules to more active forms for further catalysis (Abbasian *et al.*, 2015). Aerobic alkane degraders can use alkanes as an electron acceptor, an electron donor, as energy source, or as an precursor for other molecules (Afzal *et al.*, 2013b). According to Leung *et al.* (2006), the most common pathway on alkane degradation depends on the action of monooxygenase enzymes which are specific for n-alkanes. This reaction results in the addition of an oxygen atom to the terminal methyl group, producing an alcohol. Subsequently, the alcohol formed is converted first to an aldehyde and then to a fatty acid, which is further metabolized via β-oxidation and the citric acid cycle, a common catabolic pathway in most living cells (Leung *et al.*, 2006; van Hamme *et al.*, 2003). In a second pathway, a dioxygenase enzyme acts on the terminal methyl group of an n-alkane by adding two oxygen atoms. This results in the formation of a peroxide compound that is further converted into a fatty acid (Leung *et al.*, 2006) (Figure 2.2).

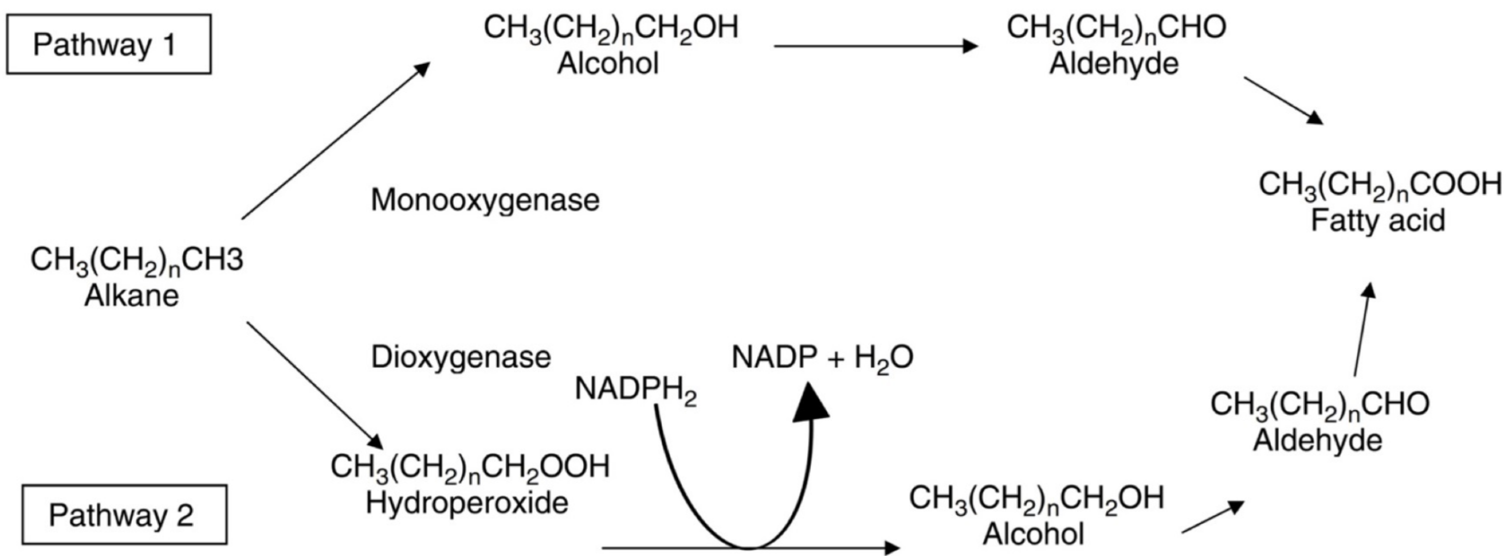

Figure 2.2 Biodegradation of alkanes (Leung *et al.*, 2006).

2.7.1.2 Aerobic degradation of aromatic compounds

Aromatic hydrocarbons are less biodegradable than saturated hydrocarbons and therefore are associated with higher risks to the environment and life forms (Varjani, 2017). Although simple hydrocarbons with one aromatic ring (*e.g.* BTEX) are also present in petroleum, aromatic compounds with two or more aromatic rings, *i.e*. polycyclic aromatic hydrocarbons (PAHs), are a group of significant environmental concern (Leung *et al.*, 2006). The electrochemical stability, persistency, carcinogenic index and resistance towards biodegradation of PAHs increase with an increase number of aromatic rings. Similar to alkanes, hydrophobicity also tends to increase with high molecular weight (Ghosal *et al.*, 2016). Generally, PAHs with low molecular weight (< 3 rings) are more susceptible to biodegradation than those with high molecular weight (> 4 rings). Even though bacteria may also use aromatic hydrocarbons as sole sources of carbon and energy, these are not degraded as easily as alkanes (Leung *et al.*, 2006).

A key step in the degradation of aromatic compounds is the cleavage of the aromatic ring by an initial oxidative attack, which is carried out by dioxygenase enzymes with molecular oxygen as reactant. This results in the formation of cis-dihydrodiols by incorporation of both oxygen atoms of an oxygen molecule and then to formation of catechols (Varjani, 2017). After this step, the benzene ring is cleaved by microorganisms in different ways by appropriate enzymes. Mainly, catechol cleavage can subsequently follow one of two pathways: (*i*) ortho-cleavage, in which the ring is cleaved between the two carbon atoms with hydroxyl groups and the (*ii*) meta- cleavage, in which the ring splits between adjacent carbon atoms with and without a hydroxyl group (Leung *et al.*, 2006; Abbasian *et al.*, 2015). After catechol cleavage, metabolites such as acetate, succinate, pyruvate or acetaldehyde subsequently enter the tricarboxylic acid cycle (TCA) cycle and then are available as energy and carbon sources to the cell (Leung *et al.*, 2006). Essentially, polyaromatic hydrocarbons are degraded one ring at a time, following the same degradation step (Ghosal *et al.*, 2016) (Figure 2.3).

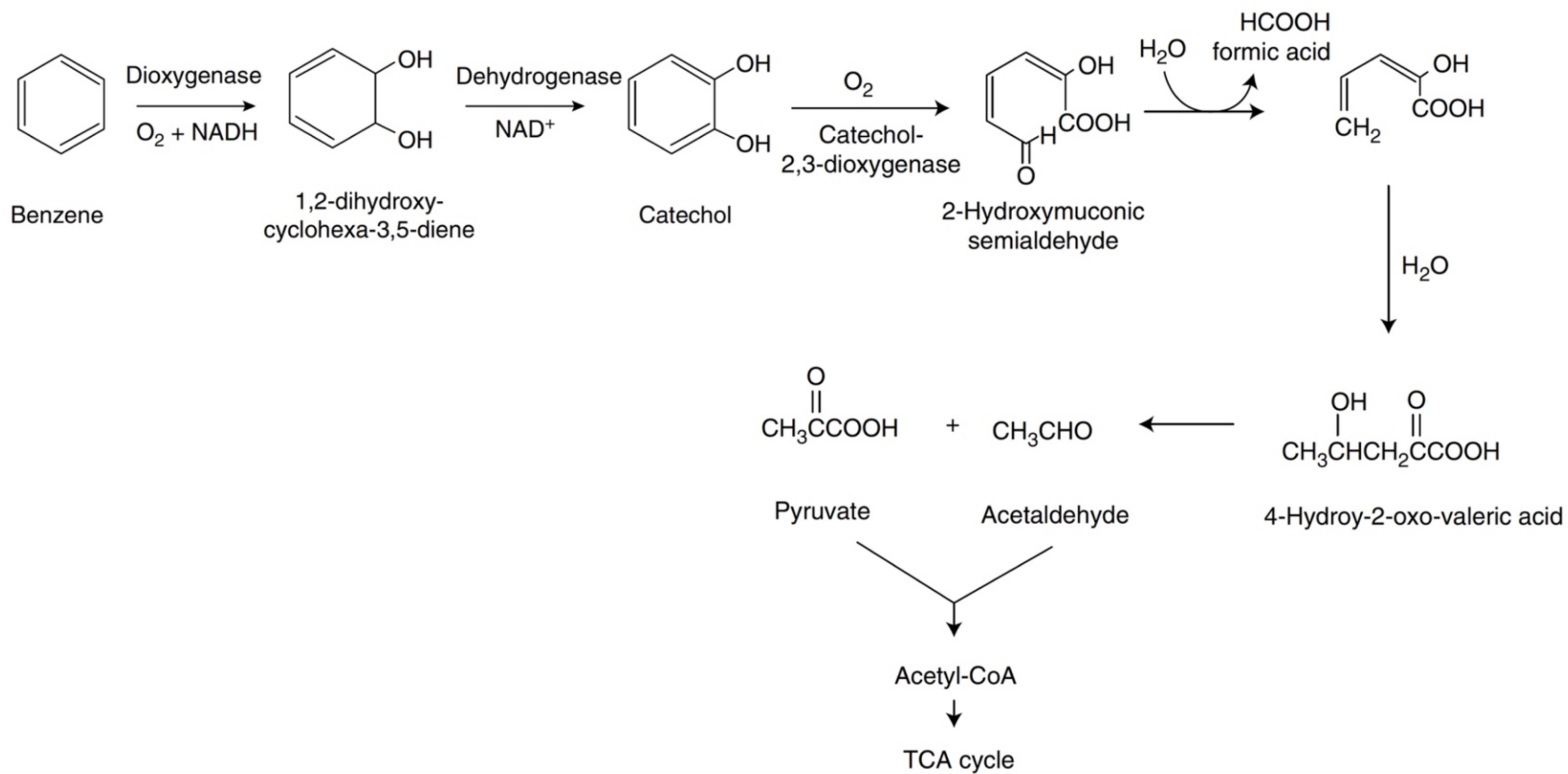

Figure 2.3 Biotransformation of benzene to catechol followed by the ortho-cleavage of catechol. Modified from Leung *et al*. (2006).

2.7.1.3 Hydrocarbon degrading genes

The initial intracellular attack of organic pollutants is an oxidative process (Varjani, 2017). In this process, mono and dioxygenases are key enzymes in the biodegradation of a wide range of hydrocarbons and other environmental pollutants. Genes encoding these enzymes have been characterized in several organisms (Rojo, 2009) (Table 2.3). Most of the genes required for the breakdown of hydrocarbons can often be found on plasmids, which facilitates genetic transfer and acquisition in oil-polluted environments (Brooijmans *et al.*, 2009).

Monooxygenases are classified into rubredoxin-dependent enzymes and in most bacterial species this enzyme is encoded by the gene *alkB* while some bacteria, such as *Acinetobacter* sp., express the enzyme encoded by the *alkM* gene (Abbasian *et al.*, 2016). The best-characterized alkane-degradation pathway (the *alk* system) is encoded by the OCT plasmid of *Pseudomonas putida* GPo1 to convert n-alkanes (C6–C10) into their fatty acids (van Beilen *et al.*, 2001). Other genes encoding for rubredoxin-dependent monooxygenases have been also reported in *Rhodococcus sp*. strain Q15 (Rh *alkB1*,Rh *alkB2*) (Whyte *et al.*, 2002), *P. putida* ATCC 17484 (*ndoB*) (Margesin *et al.*, 2003), *P. putida* F1 (*tod*C1) (Furukawa *et al.*, 1993), *P. putida* ATCC 33015 (*xyl*E, *cat*23) (Luz *et al.*, 2004), and P. *pseudoalcaligenes* KF707 (*bph*A) (Tairazf *et al.*, 1992). Although the *alkB* gene has been the predominant gene in most studies for metabolism of alkanes (Liu *et al.*, 2015b; Wasmund *et al.*, 2009; Wallisch *et al.*, 2014), a second alkane hydroxylase that is alternatively found in bacteria belongs to the CYP153 family of the cytochrome P450 monooxygenases (Kloos *et al.*, 2006; Rojo, 2009).

CYP153 is an enzyme of the CYP superfamily that can also catalyze the hydroxylation of alkanes (Yergeau *et al.*, 2012b). According to van Beilen *et al.* (2006), several strains such as *Gordonia* sp. and *Rhodococcus rhodochrous* were able to degrade hydrocarbons, but did not contain *alkB* homologs. Therefore, these authors identified that many of these strains contain cytochrome P450 enzymes belonging to the CYP153 family. Wang *et al.* (2010a) reported more than 250 *alkB* homologues in 45 bacterial species and approximately 60 homologues of CYP153 in 18 bacterial species have been discovered to date.

Table 2.3 Examples of hydrocarbon degrading genes and bacterial taxa.

Gene name	Source Organism	Reference
Alkane monooxygenase		
alkB	*Pseudomonas putida* GPo1	(van Beilen *et al.*, 2001)
alkM	*Acinetobacter* sp.	(Ratajczak *et al.*, 1998)
Rh *alkB1*, Rh *alkB2*	*Rhodococcus sp*. strain Q15	(Whyte *et al.*, 2002)
Naphthalene dioxygenase		
ndoB	*Pseudomonas putida* ATCC 17484	(Margesin *et al.*, 2003)
nah	*Pseudomonas putida* G7	(Simon *et al.*, 1993)
*phn*A	*Burkholderia* sp. strain RP007	(Laurie and Lloyd-jones, 1999)
Toluene dioxygenase		
*tod*C1	*Pseudomonas putida* F1	(Zylstra and Gibson, 1989)
Biphenyl dioxygenase		
*bph*A	*Pseudomonas pseudoalcaligenes* KF707	(Tairazf *et al.*, 1992)
Cytochrome P450		
CYP153	*Pseudomonas putida* GPo12	(van Beilen *et al.*, 2006)
Catechol dioxygenase		
C12O	*Pseudomonas* sp. strain EST1001	(Kivisaar *et al.*, 1991)
*xyl*E,	*Pseudomonas putida* ATCC 33015	(Nakai *et al.*, 1983)
*cat*23	*Pseudomonas putida* ATCC 33015	(Laramee *et al.*, 2000)

Aromatic hydrocarbons have limited chemical reactivity are normally biodegraded by bacteria with the help of O_2 by oxygenases which forms intermediate compounds such as catechols (Fuchs *et al.*, 2011). A few bacteria (*e.g. Mycobacterium* sp.) are capable of oxidizing aromatic rings via cytochrome P450 monooxygenase enzyme, however most of the PAHs-degrading bacteria oxidize naphthalene using dioxygenases (Ghosal *et al.*, 2016). While the *nah* gene encodes for naphthalene dioxygenases associated with degradation of low-molecular weight PAH (*e.g*. naphthalene, phenanthrene), the *pdo*1 gene encodes for pyrene dioxygenases associated with the degradation of high-molecular weight PAHs (*e.g*. pyrene, benzo(*a*)pyrene) (Han *et al.*, 2014). Other genes involved in PAH degradation are C12*O* and *ndoB*. The gene C12*O* encodes for catechol 1,2- dioxygenase associated with cleavage of the last aromatic ring in the degradative pathway of PAHs (Han *et al.*, 2015). The *ndoB* gene, located on the NAH7 plasmid of *Pseudomonas putida*, also encodes for naphthalene dioxygenase, the first enzyme in the naphthalene degradation pathway (Luz *et al.*, 2004). In addition, Laurie and Loyd-Jones (2000) found that *phn*A genes of *Burkholderia* sp. also encodes for naphthalene dioxygenase despite that this gene has a low level of sequence homology with *nah* genes.

Although several genes have been used to investigate both aliphatic and aromatic hydrocarbon degradation. Alkane monooxygenase (*alkB*) and naphthalene dioxygenase gene (*nah*) are the main genes corresponding to the metabolism of aliphatic and aromatic hydrocarbons respectively (Liu *et al.*, 2015b).

3. MICROBIAL COMMUNITIES ASSOCIATED WITH ANNUAL BARLEY PLANTED ON OIL SANDS RECLAMATION SITES IN ALBERTA, CANADA.

3.1 Preface

Plant associated endophytic root bacteria colonize plant tissues and play a key role in supporting plant health and growth in both managed and natural ecosystems. In northern Alberta, oil sands mining activities result in a large disturbance footprint that causes significant challenges for land reclamation strategies. Therefore, plant-microbe associations can aid the enhancement and establishment of a sustainable plant community cover in these areas. However, the potential use of bacterial endophytes to assist plant growth on oil sands reclamation covers requires an understanding of the diversity and metabolic potential of these endophytes. In order to begin to elucidate how root endophytic bacteria can be used as biotechnological tools to improve land reclamation, we must first investigate the overall bacterial community profile associated with plants currently growing in these areas. In this study, the root associated microbial communities of annual barley (*Hordeum vulgare*) grown at one of the oil sands reclamation areas was investigated.

3.2 Abstract

Microbial communities that colonize plant rhizosphere and the root interior can ameliorate plant stress and promote growth. The use of plant-microbe associations is being investigated as a strategy to assist in reclamation of disturbed soils in northern Alberta. This study assessed the diversity of bacterial endophytes and rhizosphere bacteria associated with annual barley growing at an oil sands reclamation area. Plants and peat-mineral samples were collected at different slope positions in two different reclamation cover managements. Root associated microbial communities were assessed by culture dependent and culture-independent techniques including phospholipid fatty-acid analysis (PLFA) and denaturing gel gradient electrophoresis (DGGE). Results indicate that available nutrients and total hydrocarbons varied

mainly by slope positions. However, no slope effect was observed for most soil chemical parameters. The data in this study also suggests that microbial community structure was mainly driven by soil total and organic carbon, NH_4^+ and organic matter. Rhizosphere and endophytic bacterial community structure analysed by culture independent techniques varied depending on slope positions and cover type. While most bulk and rhizosphere soils differentiated mainly by cover management, endophytic profiles did not cluster by either cover or slope positions. Selected endophytic bacteria bands from DGGE gel closely matched those of growth promoting bacteria and potential hydrocarbon degraders. Culture dependent techniques assessing endophytic bacteria revealed a dominance of the class *Gammaproteobacteria*, in which *Enterobacteriaceae* (44%), *Xanthomonaceae* (30%) and *Pseudomonaceae* (26%) were the most abundant families in this class. Several endophytic isolates also matched those from DGGE profiles. Hence, the results in this study suggest that plants growing on oil sands reclamation covers host a wide range of bacterial endophytes, which potentially could be used to assist plant establishment and growth in these areas.

3.3 Introduction

The oil sands deposits in northern Alberta, Canada contain an estimated 1.7 trillion barrels of recoverable oil within the Peace River, Cold Lake, and Athabasca River regions (Audet *et al.*, 2015; Hsu *et al.*, 2015). These deposits represent the third largest oil reserve in the world and a major resource within Canada's energy sector (Kannel and Gan, 2012). However, the Athabasca's oil sands region are different from other oil deposits, as it predominantly consists of bitumen, which is a dense and extremely viscous form of petroleum found in combination with sand, clay, and water (Yergeau *et al.*, 2012a). Bitumen extraction in the Athabasca was initiated in 1967, expanded significantly from 1978 to 2015, and it is expected to more than double over the next two decades (Audet *et al.*, 2015; CAPP, 2017). Alberta's oil sands lie under a total area of 142,000 km^2 of natural boreal forest and muskeg (peat) which needs to be removed during mining operations (MacKenzie and Quideau, 2010).

In addition to the removal of existing vegetation, top soil and overburden (stockpiled for later use in land reclamation), the current bitumen extraction process is based on a hot water and steam with added sodium hydroxide (NaOH) that separates the bitumen from the sand (Lévesque, 2014). These processes use large quantities of water and generate large volumes of

fine tailings, which consist of sand, clay, organics, and residual bitumen (Greer *et al.*, 2011; Yergeau *et al.*, 2012a). After bitumen extraction, mine tailings are discharged into ponds or dykes where tailing sands settle to the bottom and clarified water is pumped back to processing plant in order to be re-used in bitumen extraction (CAPP, 2017).

Reclamation strategies in the oil sands are considered a long-term endeavor as it takes several years to successfully reclaim disturbed areas to equivalent land capability prior to disturbance. However, land reclamation efforts are challenging due to the nature of the tailing sands, a generally inappropriate plant growth medium with low nutrient content, high pH, low or no organic matter and residual hydrocarbon products (Naeth *et al.*, 2011; Lefrançois, 2009). To improve land reclamation, industries have focused on covering tailing sands with suitable reclamation material. The use of peat–mineral soil mix (PMM) as cover material for land reclamation has become a common practice. Peat-mineral mix (PMM) is used to create a suitable plant growth medium and to provide a source of native plants that can facilitate natural recovery of vegetation in the disturbed areas (Shaughnessy, 2010). In addition, peat-mineral mix will also add organic C-content, improve water-holding capacity and permit aggregation of tailings sands (Rowland, 2008). Planting of seedlings of the dominant boreal forest tree species and the colonization by pioneer species that can tolerate harsh conditions is also essential to improve reclamation strategies and allow the re-establishment of a natural forest (Renault *et al.*, 2004; Lefrançois *et al.*, 2010).

Where soil erosion is likely to occur, annual barley (*Hordeum vulgare*) is often planted in reclamation landscapes to provide a quick vegetation cover and erosion control. Barley is used because it is a poor competitor and is readily invaded by local flora within the first few years (Rowland, 2008). Barley also helps to bulk up soil organic matter content and supply nutrients to the secondary crops; however, due to harsh environmental conditions, plant growth in reclamation areas may depend on symbiotic relationships with microbes.

Plant-associated bacteria may stimulate plant growth, promote stress resistance, suppress diseases and influence plant growth by nutrient mobilization and transport (Berg *et al.*, 2014). The plant microbiome is a key determinant of plant health and growth and has received substantial attention in recent years (Turner *et al.*, 2013). Bacteria that live in the rhizosphere are attracted by and feed on nutrients, exudates, border cells and mucilage released by the plant root (Philippot *et al.*, 2013). Root exudates contain a variety of compounds, predominately organic

acids and sugars, that are key determinants of rhizosphere micro biome structure (Turner *et al.*, 2013). However, plants can also function as filters of soil microorganisms, while selecting those successful, competent endophytes (Chen *et al.*, 2010).

Endophytic bacteria are those that reside for at least part of their lives within plant tissues and often recognized as symbionts with a unique and intimate interaction with the plant (Berg *et al.*, 2014; Hardoim *et al.*, 2008). Endophytes are thought to be a sub-population of the rhizosphere microbiome, generally considered to be non-pathogenic and once inside their hosts they change their metabolism and become adapted to their internal environment (Turner *et al.*, 2013; Germida *et al.*, 1998). Numerous studies have shown that bacterial endophytes can promote their host plant establishment as well as improve plant growth under adverse conditions (Soleimani *et al.*, 2010; Deng *et al.*, 2011; Khan *et al.*, 2011). Endophytic bacteria also may have the ability to control plant pathogens, insects and nematodes, which make them suitable as bio-control agents (Hallmann and Berg, 2006). Recent studies suggest that they may also play an important role in remediation of contaminated soils and water (Chen *et al.*, 2010; Guo *et al.*, 2010; Xiao *et al.*, 2010; Mastretta *et al.*, 2013). However, interactions between microbes and model plants, such as in Rhizobium-legume symbioses, are well understood in agriculture, although the diversity in plant microbiome interactions in engineered soils is not yet well defined.

Therefore, the aim of this study was to assess mechanisms driving annual barley root associated bacterial community structure in an oil sands reclamation area. Specific objectives in this study were to determine the impact of (*i*) cover management (*ii*) slope positions and (*iii*) soil parameters on microbial community structure. Additionally, this study aimed to assess the diversity of endophytic bacteria associated with these plants.

3.4 Materials and Methods

3.4.1 Sample collection and processing

Annual barley (*Hordeum vulgare*) was collected at an oil sands reclamation area of approximately 2.2 km^2 near Fort McMurray, Alberta. Three biological replicates of each plant, attached rhizosphere soil, and bulk soil (0-20 cm depth) were collected at different slope

positions along two transects (20 sampling locations) (Figure 3.1, Table 3.1). The first transect consisted of 10 sampling locations (S1-S10) in the standard cover, which is a cover management area consisting of a 40 cm of peat mineral mixture and 10 cm of sandy loam on the surface of 100 cm of tailing sands. The second transect also consisted of 10 sampling locations (E1-E10) in the engineered cover, an area of 50 cm of a peat mineral mixture on top of 120 cm of tailing sands separated from the bottom 30 cm of tailing sands by a geo-clay liner (GCL). The main objective of the GCL added by the industry is to retain the moisture on the top of the cover to improve plant growth and to prevent seepage from compounds on the bottom of the tailing sands from reaching the surface of the plant cover. Samples were collected during the summer of 2013, transported at 4 °C and stored at -20 °C until processing within the next 48 h.

3.4.2 Soil Chemical Analyses

Soil samples were analyzed for soil total organic carbon (TOC) and total carbon (TC) by the method from Dhillon *et al.* (2015) using a LECO C632 Analyzer (LECO Corporation, St. Joseph, MI, United States). Soil organic Matter (OM), was analyzed using the dry-ash method (McKeague, 1978). Soil pH was measured in a 1:2 soil: water slurry. Soil available ammonium was extracted using a 2 N KCl solution, NH_4^+ in the extract was mixed with hypochlorite and salicylate to form indophenol which was determined colorimetrically at 660 nm (Laverty and Bollo-Kamara, 1988). Soil available nitrate was extracted using a calcium chloride solution and determined colorimetrically at 520 nm according to Laverty and Bollo-Kamara (1988). Available phosphorus and potassium were measured using a modified Kelowna extraction (Qian *et al.*, 1994) and available sulfate by a calcium chloride extraction (McKeague, 1978). Soil total hydrocarbons were measured in accordance with the "Reference Method for the Canada-Wide Standard for Petroleum Hydrocarbons in Soil - Tier 1 Method, Canadian Council of Ministers of the Environment, December 2000. A subsample of the sediment/soil was extracted with a 1:1 hexane: acetone solution using a rotary extractor. The extract was purified using a silica gel clean-up to remove polar compounds. The F2, F3 and F4 fractions were analyzed by Gas Chromatography with a Flame Ionization Detector (GC/FID).

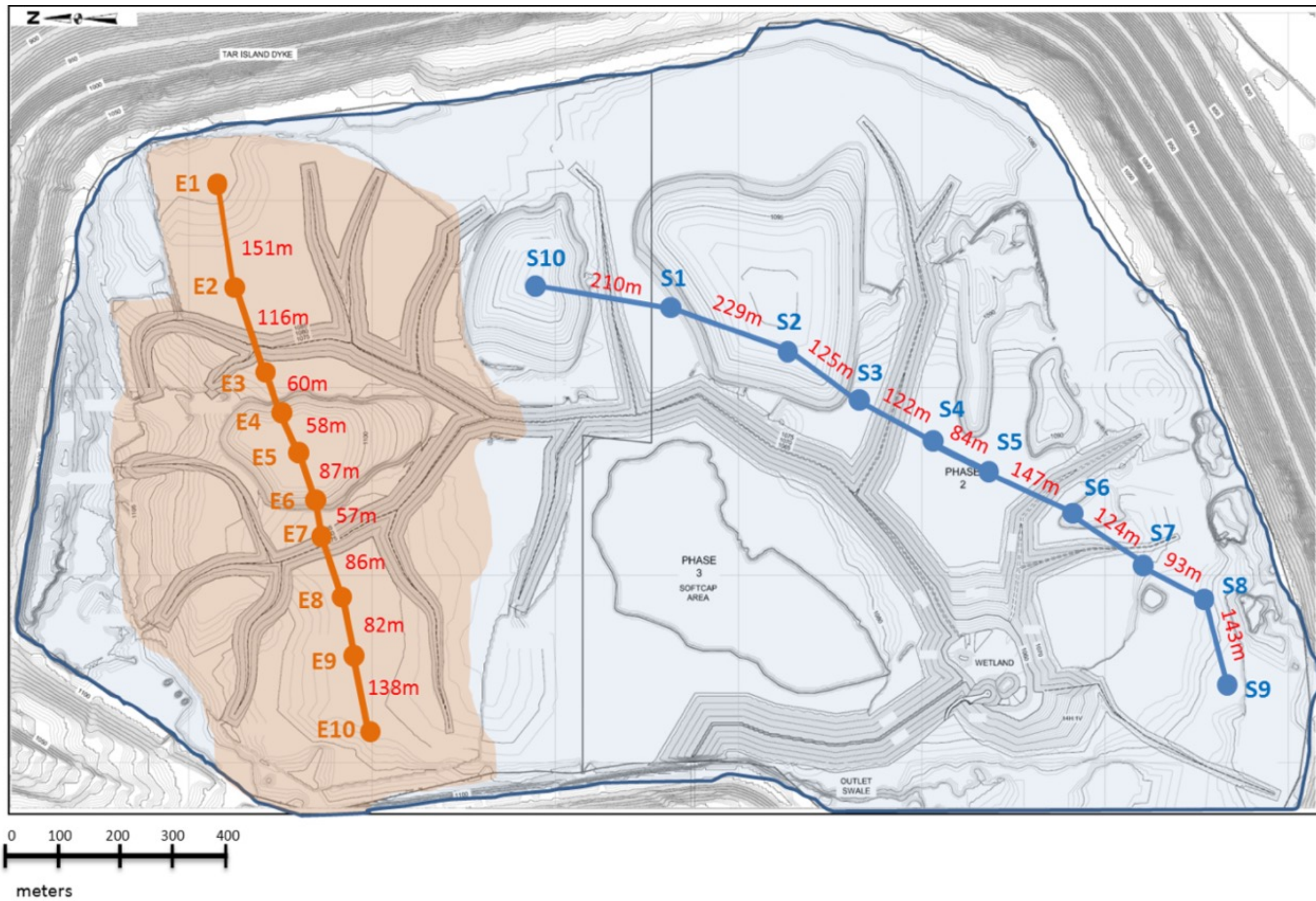

Figure 3.1 Transects of sampling locations along the engineered (E1-E10) and standard cover (S1-S10) at an oil sands reclamation area.

Table 3.1 Landforms, elevation and GPS coordinates of sampling locations (UTM Zone 12N).

Location	Cover	Elevation (m)	Landform	Northing	Easting
S1	Standard	307.89	Midslope	6316283.7	472129.03
S2	Standard	308.25	Midslope	6316064.9	472198.00
S3	Standard	304.14	Depression	6315945.91	472237.29
S4	Standard	304.00	Upslope	6315834.43	472288.00
S5	Standard	305.72	Crest	6315750.54	472284.68
S6	Standard	307.21	Level	6315604.83	472265.09
S7	Standard	304.02	Upslope	6315482.47	472245.15
S8	Standard	304.05	Midslope	6315401.69	472197.33
S9	Standard	308.45	Level	6315276.51	472127.36
S10	Standard	312.23	Upslope	6316462.32	472017.46
E1	Engineered	310.04	Level	6316998.48	471762.05
E2	Engineered	305.23	Crest	6316873.28	471677.31
E3	Engineered	306.00	Lower slope	6316775.94	471614.32
E4	Engineered	310.94	Upslope	6316723.92	471583.75
E5	Engineered	315.26	Crest	6316773.00	471549.08
E6	Engineered	310.04	Midslope	6316615.75	471487.61
E7	Engineered	306.43	Lower slope	6316561.74	471469.25
E8	Engineered	307.16	Level	6316481.71	471438.36
E9	Engineered	305.54	Level	6316411.41	471395.80
E10	Engineered	306.17	Level	6316288.47	471332.41

3.4.3 Survey of rhizosphere bacteria community

Plant roots (entire root system) and adhering soil (5 g) were placed into an Erlenmeyer flask containing 195 mL of phosphate-buffered saline (PBS) (1.2 g of $Na_2HPO_4 \cdot L^{-1}$, 0.18 g of $NaH_2PO_4 \cdot L^{-1}$, 8.5 g of $NaCl \cdot L^{-1}$) buffer and shaken on a rotary shaker (150 rpm) at 22 °C for 25 min. After shaking, dilutions (1:10) in sterile PBS were used for culturable techniques, and the remaining slurry was transferred to a 50 mL Falcon centrifuge tube and centrifuged at 2,000 *g* for 5 min (Dunfield and Germida, 2003). The supernatant containing PBS buffer was discarded and the rhizosphere soil stored -80 °C for DNA extractions.

3.4.4 Survey of endophytic bacteria community

Root material was recovered and transferred into an Erlenmeyer flask containing 100 mL NaClO (1.05 % $v \cdot v^{-1}$) in PBS and placed on a rotary shaker (150 rpm) at 22 °C for 15 min. To remove the remaining NaClO solution, roots were rinsed 10 times with sterile water and 0.1 mL of the final wash was spread on Trypticase soy agar (TSA) plates to check for contamination (Siciliano and Germida, 1999). Sterile roots were chopped aseptically and subdivided in samples for culturable dependent techniques and remaining roots stored in sterile tubes at -80 °C for DNA extraction.

3.4.5 Survey of culturable rhizosphere and endophytic bacteria

Rhizosphere culturable bacteria, from soil/buffer solutions obtained previously and endophytic culturable bacteria, obtained by macerating 2.5 g surface-sterile root in 9 mL of (PBS) buffer using a sterile mortar and pestle, were serially diluted in PBS buffer and spread plated onto 1/10 strength TSA plates containing 0.1 $g \cdot L^{-1}$ of cycloheximide. Plates were incubated for 3 days at 28 °C. After the incubation, endophytic bacteria plates from each treatment replicate and dilution were selected and isolates in varying morphology were streaked twice on new plates to obtain purified strains. Purified isolates were then inoculated on 9 mL of soy broth (TSB) medium. Pure bacterial cultures were stocked in 50% glycerol at -80 °C prior to microbial DNA extractions.

3.4.6 Phospholipid fatty acid (PLFA) analysis

PLFA analysis of soil samples was based on a modified protocol from Helgason *et al.* (2010). Soil samples were sieved, freeze-dried and ground with mortar and pestle to maximize

lipid recovery. Fatty acids were extracted from 4.0 g of lyophilized, ground soil in a methanol/chloroform mixture and then dried down under constant N_2 flow. Neutral, glyco- and phospho- lipids were separated using solid phase extraction columns (0.50 g Si; Varian Inc. Mississauga, ON), sequentially eluted with chloroform ($CHCl_3$), acetone ($(CH_3)_2CO$) and methanol (MeOH) respectively, and the phospholipid fraction dried under N_2 flow. With a solution of 1:1 methanol/toluene and methanolic potassium hydroxide (KOH) at 35 °C the phospholipid fraction was methylated. After methylation, the resulting fatty acid methyl esters (FAMEs) were analyzed using a Hewlett Packard 5890 Series II gas chromatograph with a 25mUltra 2 column (J&W Scientific). Peaks were identified using fatty acid standards and MIDI identification software (MIDI Inc., Newark, DE) and quantified based on the addition of a known concentration of the internal standard methyl nonadecanoate (19:0) (Helgason *et al.*, 2010a; Drenovsky *et al.*, 2004).

Bacterial biomass was determined by biomarker abundance calculated based on the peak area detected for each fatty acid, relative to that of a known quantity of the internal standard. Biomarkers used to represent gram positive bacteria (Gr+) were i14:0, i15:0, a15:0, i16:0, i17:0, a17:0. For gram negative bacteria (Gr-), biomarkers used were 16:1 ω7t, 16:1ω9c, 16:1ω7c, 18:1ω7c, 18:1ω9c, cy17:0, and cy19:0 (Macdonald *et al.*, 2004). Physiological stress biomarker was reported as the ratio of cy19:0 to 18:1ω7c. All biomass values were reported based on dry soil weight in units of $nmol \cdot g^{-1}$ soil derived from individual molecular weights of each fatty acid (Helgason *et al.*, 2010a; Hynes and Germida, 2012).

3.4.7 DNA Extraction

Total endophytic community DNA was extracted from surface disinfected root samples using the PowerPlant® Pro DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). Rhizosphere and bulk soil community DNA was extracted using the MoBio PowerSoil® extraction kit (MoBio Laboratories Inc., Carlsbad, CA). In addition, DNA from microbial cultures in culture dependent techniques was extracted using the UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). All DNA exactions were conducted following the manufacture's protocols. DNA yield was quantified using a NanoDrop2000 Spectrophotometer (Thermo Scientific, Ottawa, ON) and in a SYBR® Safe (Invitrogen) 1% agarose gel by comparison with a high DNA mass ladder (Invitrogen) using a Bio-Rad Gel Doc

XR System (Bio-Rad Laboratories, Mississauga, ON). Microbial DNA was amplified using the primer set EUB 338 (ACTCCTACGGGAGGCAGCAGATT) and EUB 518 (ATTACCGCGGCTGCTGG) (Fierer *et al.*, 2005) and purified PCR products were sequenced by Macrogen Inc. (Seoul-Rep. of Korea).

3.4.8 DNA Amplification

Bulk soil, rhizosphere and endophytic community structure were examined by PCR-amplified bacterial gene 16S rRNA using a primer set U341f-gc (5'-GCG GGC GGG GCG GGG GCA CGG GGG GCG CGG CGG GCG GGG CGG GGG _CCT ACG GGAGGC AGC AG-3') and U758r (3'-CTACCAGGG TATCTAATCC-5') (~417 bp fragment) (Helgason *et al.*, 2010a; Phillips *et al.*, 2010). The optimized PCR reaction consisted of 1 µL of DNA extract; 1 µM of primers U341-gc and U758; 6.25 µg BSA (Invitrogen); and 25 µL units of Hot Start Taq Master Mix (Qiagen); 21.38 µL of RNase-Free H_2O to make a total reaction volume of 50 µL. Amplification of the targeted gene was accomplished using touchdown PCR. Ten cycles of 1 min denaturing at 94 °C, 1 min annealing at 65 to 55 °C and 1 min extension at 72 °C were carried out, followed by 18 repeated cycles using an annealing temperature of 55 °C (Helgason *et al.*, 2010a). PCR amplification product were confirmed with 1.4 % agarose gel stained with SYBR® Safe (Invitrogen) and visualized using a Bio-Rad Gel Doc XR System (Bio-Rad Laboratories, Mississauga, ON). Final DNA concentration was determined using a NanoDrop2000 Spectrophotometer (Thermo Scientific, Ottawa, ON) and corresponding software (Hynes and Germida, 2012).

3.4.9 Denaturing Gel Gradient Electrophoresis (DGGE)

DGGE was performed on 16S rRNA PCR product using a Bio-Rad DCode system (Bio-Rad, Mississauga, ON) as described by Hynes and Germida (2012). Approximately 600 ng of DNA was loaded onto an 8% polyacrylamide gel with a 40–60% denaturing gradient of formamide and urea. Electrophoresis was carried out for 16 h at 80 V and 60 °C. The resulting gels were stained with SYBR® Safe (Invitrogen, Burlington, ON) in 1 × TAE buffer (Tris, acetic acid and EDTA mixture) for 0.5 h, de-stained for 0.5 h and photographed using a Bio-Rad Gel Doc XR System with Image Lab Software (Bio-Rad, Mississauga, ON). Gel picture was analyzed for banding patterns using Bionumerics v.5.1 (Applied Maths, Austin, TX).

Dominant DNA bands were excised, using a sterile scalpel, and eluted in sterile H_2O for 45 min at 37 °C as described in Phillips *et al.* (2009). Eluted DNA was re-amplified using the set of primers (U341f and U758r) and temperature program as in the DNA amplification section above. Confirmation of re-amplified fragments was carried out on 1.5% agarose gel, followed by PCR purification with the QIAquick PCR Purification Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's recommendations. Finally, purified PCR products were sequenced by Macrogen Inc. (Seoul-Rep. of Korea). Nucleotide sequence similarities were determined by Basic Local Alignment Search Tool (BLAST).

3.4.10 Statistical Analysis

Analysis of variance (ANOVA) for PLFA profiles was performed using SAS (version 9.3). Non-metric multidimensional scaling (MDS) analysis of PLFA community composition was carried out using PCOrd v.5.10 (MjM Software Gleneden Beach, OR). PLFA data was transformed to log (mol% + 1) and Sørensen distance measure was selected using the autopilot slow and thorough analysis option in PCOrd V.5.0 (Helgason *et al.*, 2010b; McCune and Grace, 2002). A random starting point was used for initial analysis and then optimized in previous ordinations to achieve the lowest stress. The Monte Carlo test of significance and Multi-Response Permutation Procedure (MRPP) were subsequently used to test for differences between groups.

Analysis of DGGE gel profiles was carried out using Bionumerics v.5.1 software (Applied Maths, Austin, TX). The detection of bands was carried out using a minimum profiling of 5%, a position tolerance of 1.5% and with optimization of 2.0%. Based on densiometric curves and the Ward linkage method, cluster analysis was performed using the Pearson correlation coefficient. Band matching was performed on DGGE profiles and a presence-absence matrix was created. This binary matrix was used in further non-metric multidimensional scaling (MDS) using the same parameters for PLFA profiles (Helgason *et al.*, 2010a; Hynes and Germida, 2012).

3.5 Results

3.5.1 Soil Physical-chemical properties

Although soil physical-chemical properties in samples collected at an oil sands reclamation area indicated no significant differences in soil properties between cover managements, significant differences were observed between slope positions at each cover (Table 3.2). The highest organic matter content was observed in the upslope (15.7 %) followed by level ground (12.9 %) at the engineered cover. Overall, soil organic and total carbon were higher in the lower slope at the engineered cover (13.3 and 14.1 % respectively) followed by the midslope at the standard cover (13 and 14.2 % respectively). In addition, analysis of overall available nutrients revealed higher nutrient content in the engineered lowerslope followed by the standard midslope.

The peat mineral samples were also characterized for total concentration of hydrocarbons (Table 3.3). Although not statistically significant, higher concentrations of hydrocarbons in the standard cover when compared with the engineered cover. Also, differences in the total hydrocarbon concentration by slope positions were observed. In general, samples collected at level ground exhibited higher concentrations in both covers; the samples in the crest and upslope had the lowest concentrations of hydrocarbons.

Table 3.2 Soil chemical proprieties of peat mineral samples (0-20 cm) collected in the engineered and standard cover at an oil sands reclamation area.

						Available				
Cover	Slope	Texture	OM	TOC	TC	NH_4^+	NO_3^-	SO_4^{2-}	PO_4^{2-}	K^+
			----------------- (%) -------------------			------------------- ($mg \cdot kg^{-1}$ of soil) ----------------				
Standard	Crest	Sandy Loam	11.4ab	7.7abc	8.2abc	3.15 ab	6.0a	70.7ab	7.1b	87.0a
	Level	Sandy Loam	4.1b	3.2c	3.7c	2.10b	1.3a	181.3a	7.3b	74.5a
	Midslope	Sandy Loam	11.3ab	13.0a	14.2a	5.17ab	5.3a	43.3b	8.1b	135.7a
	Upslope	Sandy Loam	8.6ab	7.6abc	7.9abc	2.57b	6.3a	96.5ab	4.8b	88.3a
	Depression	Sandy Loam	5.7ab	5.1bc	5.3bc	2.20b	1.6a	33.0b	3.2b	63.0a
Engineered	Crest	Sandy Loam	11.2ab	10.5abc	11.2abc	3.30 ab	12.4a	52.1b	3.3b	76.5a
	Level	Sandy Loam	12.9a	9.7abc	10.2abc	3.60 ab	4.9a	22.4b	7.9b	99.7a
	Midslope	Sandy Loam	7.2ab	6.3abc	5.7bc	2.75 ab	4.2a	20.2b	4.8b	57.0a
	Upslope	Sandy Loam	15.7a	6.8abc	6.9abc	5.80a	12.2a	21.0b	5.5b	62.5a
	Lower slope	Sandy Loam	10.8ab	13.3ab	14.1ab	3.45 ab	8.3a	23.4b	23.6a	94.0a

Different letters indicate significant differences (LSD $p \leq 0.05$). OM = organic matter, TOC = total organic carbon, TC = total carbon.

Table 3.3 Total hydrocarbon analysis of samples collected in the engineered and standard cover at an oil sands reclamation area.

		CCME Total Hydrocarbons ($mg \cdot kg^{-1}$) *					
Cover	Slope	F1 (C_6-C_{10})	F1 BTEX	F2 (C_{10}-C_{16})	F3 (C_{16}-C_{34})	F4 (C_{34}-C_{50})	Total (C_6-C_{50})
Standard	Crest	<10	<10	<30	326	328	654
	Level	<10	<10	<30	635	609	1240
	Midslope	<10	<10	<30	342	287	629
	Upslope	<10	<10	<30	599	531	1130
	Depression	<10	<10	<30	975	813	1790
Engineered	Crest	<10	<10	<30	281	306	587
	Level	<10	<10	<30	618	609	1230
	Midslope	<10	<10	<30	277	318	595
	Upslope	<10	<10	<30	217	262	479
	Lowerslope	<10	<10	<30	486	482	968

*Analysis according to the Canadian Council of Ministers of the Environment (CCME).

BTEX: six compounds, benzene, toluene, ethylbenzene, and ortho, meta and para-xylene.

F1- nC_6 to nC_{10} (hexane to decane range).

F2- nC_{10} to nC_{16} (decane to hexadecane range).

F3- nC_{16} to nC_{34} (hexadecane to tetratriacontane range).

F4- nC_{34} to nC_{50} (tetratriacontane to pentacontane range).

3.5.2 PLFA Analysis

Total PLFA microbial biomass varied significantly between different cover managements and slope positions. At the crest, microbial biomass was 53% higher in the standard cover when compared with the engineered (Figure 3.2). However, in the upslope no difference was observed between covers. The highest biomass in the standard cover was detected at the crest (34.9 $nmol \cdot g^{-1}$ soil) and at level ground in the engineered cover (32.89 $nmol \cdot g^{-1}$ soil). On level ground, the largest difference between the two covers was observed. The highest total microbial biomass was detected in the engineered cover and the lowest in the standard cover (9.92 $nmol \cdot g^{-1}$ soil). Although significant differences were observed in total PLFAs, analysis of variance (Table 3.4) indicated no significant differences in most PLFA biomarkers by cover type and slope. However, differences in microbial profiles were mainly observed between the interaction of cover and slope positions (C x S) as significant differences were observed ($p \leq 0.01$) for Gr+, Gr-, AMF, Stress, Fungal and total PLFA biomarkers.

Absolute values of Gr+, Gr-, AMF and Fungal biomarkers were 4.2, 2.7, 3.5 and 3.5-fold higher in the engineered cover when compared to the standard cover respectively (Table 3.4). Overall, as total PLFA biomarkers increased, Gr+ and Gr- biomarkers also significantly increased. However, with the exception of samples from the level ground at both covers, no significant differences were observed between slope positions in AMF and Fungal biomarkers. Although Gr+ and Gr- biomarkers varied by slope and cover, no significant differences was observed for the relative abundance of these biomarkers (Gr+ mol% and Gr- mol%).

Multi-dimensional scaling (MDS) analysis for PLFA profiles resulted in a 2-dimensional solution and final stress of 11.91 (Figure 3.3). Axis 1 from the ordination analysis represented 83.8% of variability and Axis 2 10.7%. Ordination and MRPP analysis indicated that both cover type and slope positions were a significant determinant of community composition. Although no evident cluster was observed by cover type, MDS analysis indicated a clear division between cover management with a higher variability in samples from the standard cover were when compared to the engineered. Based on slope positions, only samples from crest at the engineered cover clustered.

Significant correlations where observed between soil parameters and microbial PLFAs (Table 3.5). Most positive correlations were observed between Gr+, Gr-, AMF, fungal and total

PLFAs with TOC, TC and NH_4^+. In addition, Gr+ biomarkers also correlated positively with OM ($R^2 = 0.342$, $p \leq 0.01$) and K^+ ($R^2 = 0.280$, $p \leq 0.05$). However, the F:B ratio indicated negative correlations with OM ($R^2 = -0.335$, $p \leq 0.01$) and NO_3^- ($R^2 = -0.453$, $p \leq 0.01$). Similarly, physiological stress biomarkers indicated negative correlations ($p \leq 0.01$) with TOC, TC, NH_4^+. In addition to the absolute PLFA abundance, the relative abundance of Gr+ bacterial biomarkers also indicated positive strong correlations with OM ($R^2 = 0.373$, $p \leq 0.01$); however, the relative abundance of Gr- bacterial biomarkers indicated no positive correlations with soil parameters.

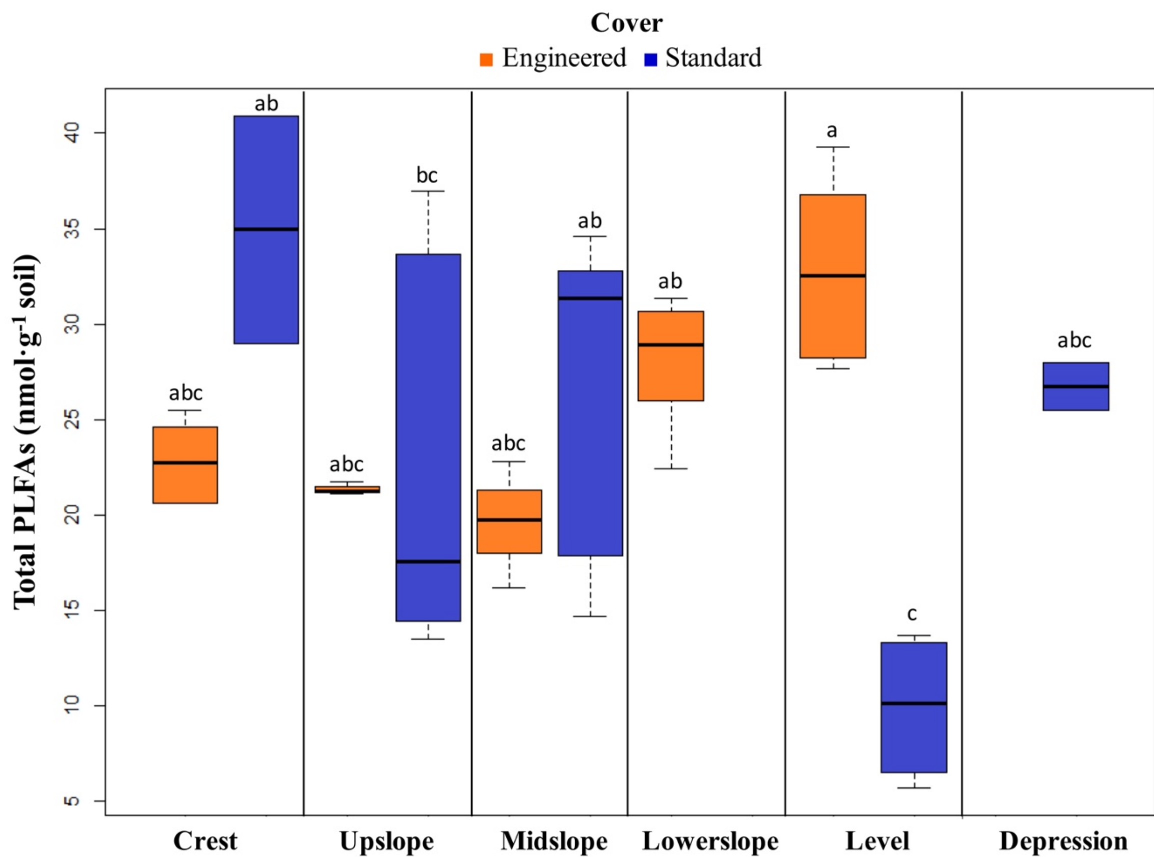

Figure 3.2 Boxplot of total PLFA biomarkers concentrations at different slope positions at the standard and engineered cover at an oil sands reclamation area. Median values are indicated by the horizontal bar within the box. The first and third quartiles are denoted by the lower and upper box limits respectively, and the 5th and 95th percentiles by the bars at the end of the vertical bars projecting from the box. Different letters indicate significant differences (Tukey HSD $p \leq 0.05$).

Table 3.4 Soil PLFA analysis of peat mineral samples (0-20 cm) collected in the engineered and standard cover at an oil sands reclamation area. Different letters indicate significant differences (Tukey HSD $p \leq 0.05$) among the interaction of cover type and slope positions in the soil microbial community.

Cover	Slope	Gr+	Gr-	AMF	Fungal	Stress	Gr+	Gr-
		---------------------- ($nmol \cdot g^{-1}$ soil) ----------------					--------- (mol %)---------	
Standard	Crest	7.09a	7.10ab	1.36ab	1.75ab	0.23b	28.74a	35.07a
	Level	1.48c	2.65b	0.42b	0.57b	0.79a	20.33a	49.42a
	Midslope	4.64ab	6.57a	1.11ab	1.43ab	0.33b	21.34a	37.69a
	Upslope	4.00abc	5.66ab	1.01ab	1.60ab	0.42b	25.90a	42.65a
	Depression	4.09abc	6.37ab	1.44ab	1.96ab	0.21b	22.28a	43.31a
Engineered	Crest	3.80bc	6.21a	1.06ab	1.34ab	0.36b	23.55a	47.20a
	Level	6.25a	7.26a	1.49a	2.01a	0.26b	26.03a	38.55a
	Midslope	3.13bc	4.42ab	0.97ab	1.18ab	0.38b	22.96a	40.13a
	Upslope	4.02b	4.79ab	1.01ab	1.24ab	0.40b	26.07a	38.76a
	Lowerslope	5.28ab	6.34a	1.30ab	1.62ab	0.28b	26.46a	39.53a
ANOVA								
	Cover (C)	0.9747	0.7554	0.2779	0.6548	0.0325	0.6953	0.9847
	Slope (S)	0.0488	0.3196	0.4606	0.7363	0.0006	0.2851	0.5766
	C*S	<0.001	0.0001	0.0030	0.0074	<0.0001	0.0984	0.0102

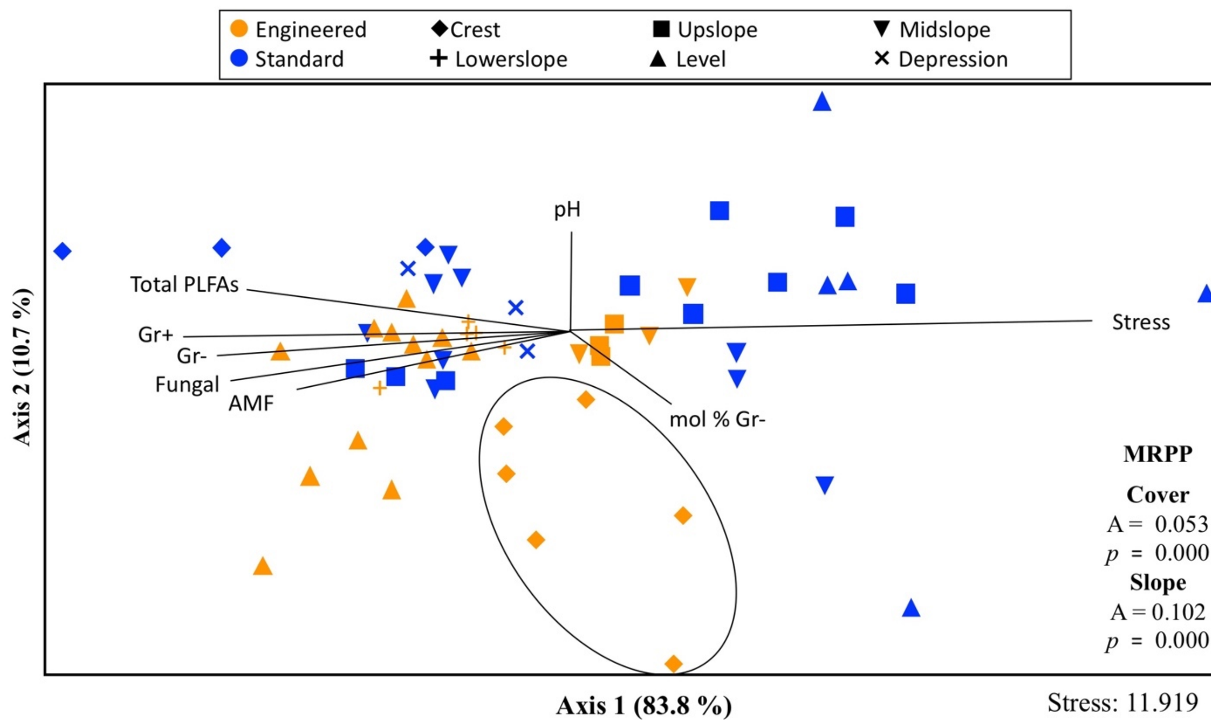

Figure 3.3 Non-metric multidimensional scaling (MDS) analysis Non-metric multidimensional scaling (MDS) analysis and multiple response permutation procedure (MRPP) of PLFA profiles from peat-mineral mix (PMM) samples collected in the engineered and standard cover at an oil sands reclamation area.

Table 3.5 Correlation indicating Spearman's rank R^2 values relating soil PLFAs and soil parameters. Statistically significant correlations are indicated in bold type, * and ** denote p values ≤ 0.05 and ≤ 0.01, respectively. THC = total hydrocarbons, OM = organic matter, TOC = total organic carbon, TC = total carbon, F:B = fungal/bacterial ratio.

	Gr+	Gr-	AMF	Stress	Fungal	F:B	Gr+ (mol %)	Gr- (mol %)	Total PLFAs
THC	0.076	-0.055	0.140	0.002	0.085	0.212	-0.076	-0.183	-0.017
TOC	**0.427****	**0.407****	**0.331****	**-0.344****	**0.330****	-0.103	**0.271***	-0.146	**0.294***
TC	**0.413****	**0.393****	**0.317****	**-0.332****	**0.319****	-0.113	**0.259***	-0.150	**0.281***
NH_4^+	**0.503****	**0.403****	**0.349****	**-0.419****	**0.346****	-0.100	**0.310***	**-0.316****	**0.379****
pH	0.003	0.017	0.052	-0.090	0.091	0.096	0.077	-0.059	0.103
OM	**0.342****	0.191	0.111	-0.219	0.072	**-0.335****	**0.373****	**-0.262***	0.156
NO_3^-	0.025	-0.061	-0.171	0.080	-0.188	**-0.453****	0.256	-0.017	-0.112
SO_4^{2-}	**-0.283***	-0.123	-0.218	0.250	-0.161	-0.053	**-0.310***	0.234	-0.137
PO_4^{2-}	0.079	-0.115	-0.086	-0.024	-0.086	-0.115	0.227	-0.119	-0.061
K^+	**0.280***	0.240	0.092	-0.210	0.157	-0.171	0.184	0.015	0.205

3.5.3 DGGE Analysis

3.5.3.1 DGGE profiles of bulk soil microbial community

Dendrogram analysis of bulk soil microbial fingerprints indicated a high variability by slope position and cover management (Figure 3.4). However, clustering was observed in a few banding patterns. For instance, samples S1 and S2 both from midslope position at the standard cover clustered at 90%. Clustering of the same cover and slope position was only observed at the lower slope and level ground in the engineered cover. In the engineered cover, sampling points E3 and E7 both from the lower slope landform grouped at 60% similarity. This also occurred with samples E9 and E10 both at level ground. Samples E4, E5 and E6 also clustered at 60% similarity as these three sampling points are geographically in the field. Samples E5 and E6, both at midslope landform, have also clustered at 60% similarity. In general, the DGGE profiles from the engineered cover indicated higher similarity by sampling locations when compared the standard cover. In fact, although from different slope positions, a few sampling locations geographically close to each other on the field were somewhat clustered in the standard cover.

Multi-dimensional scaling (MDS) analysis for bulk soil samples resulted in a 3-dimensional solution and final stress of 6.83. Axis 1 from the ordination analysis of 16S DGGE fingerprints represented most variability (65.6%) (Figure 3.5). Axis 2 and 3 accounted for 17.2 and 10.6% respectively of the data variability. Samples were mainly grouped by cover type. In addition, similar to PLFA MDS analysis, bulk soil samples from the standard cover were more variable when compared with the engineered cover. MRPP results indicate that only cover management affected microbial community structure ($A = 0.170$, $p = 0.000$).

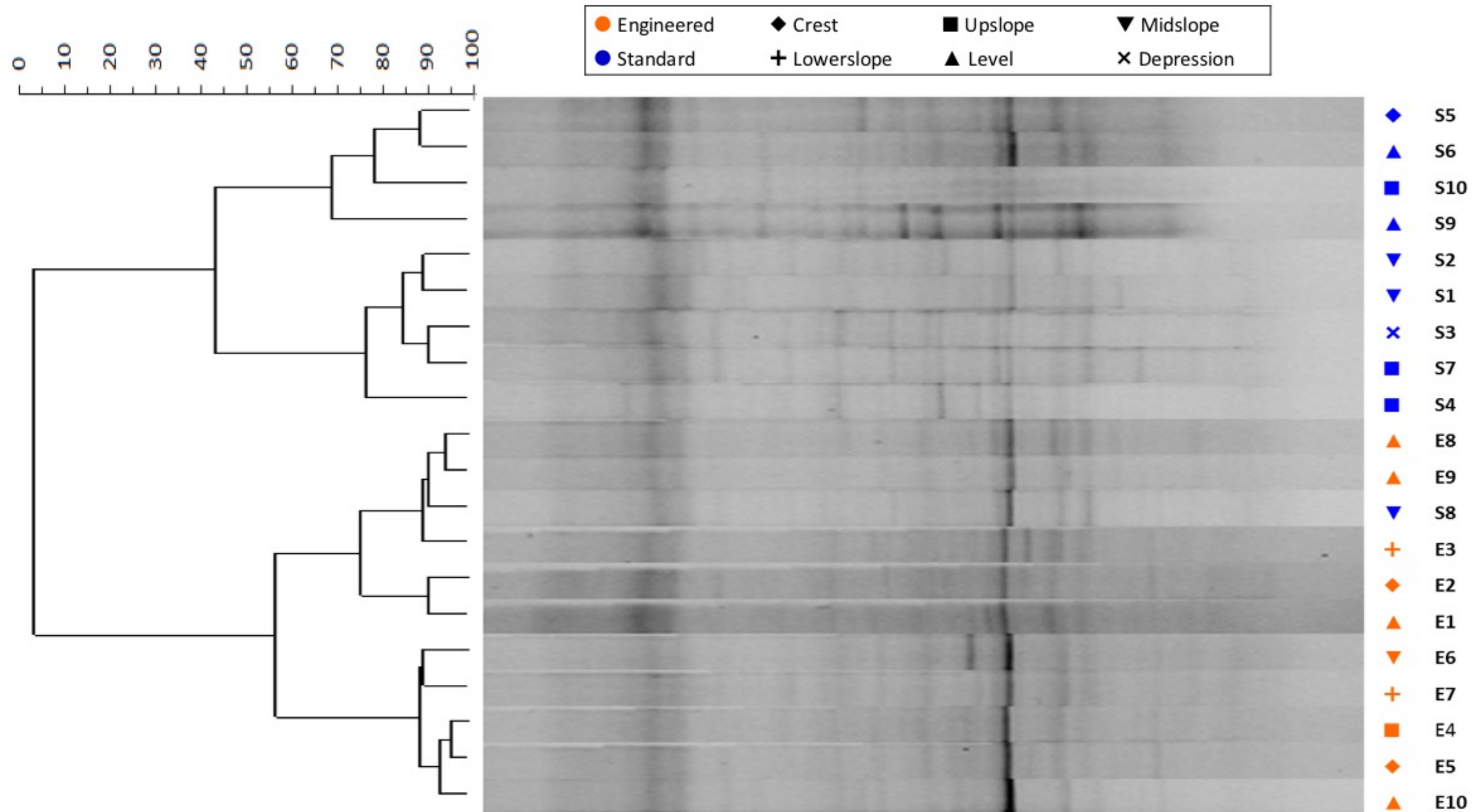

Figure 3.4 Dendrogram analysis using Pearson's correlation coefficient for bulk soil DGGE banding patterns of bacterial 16S rRNA communities.

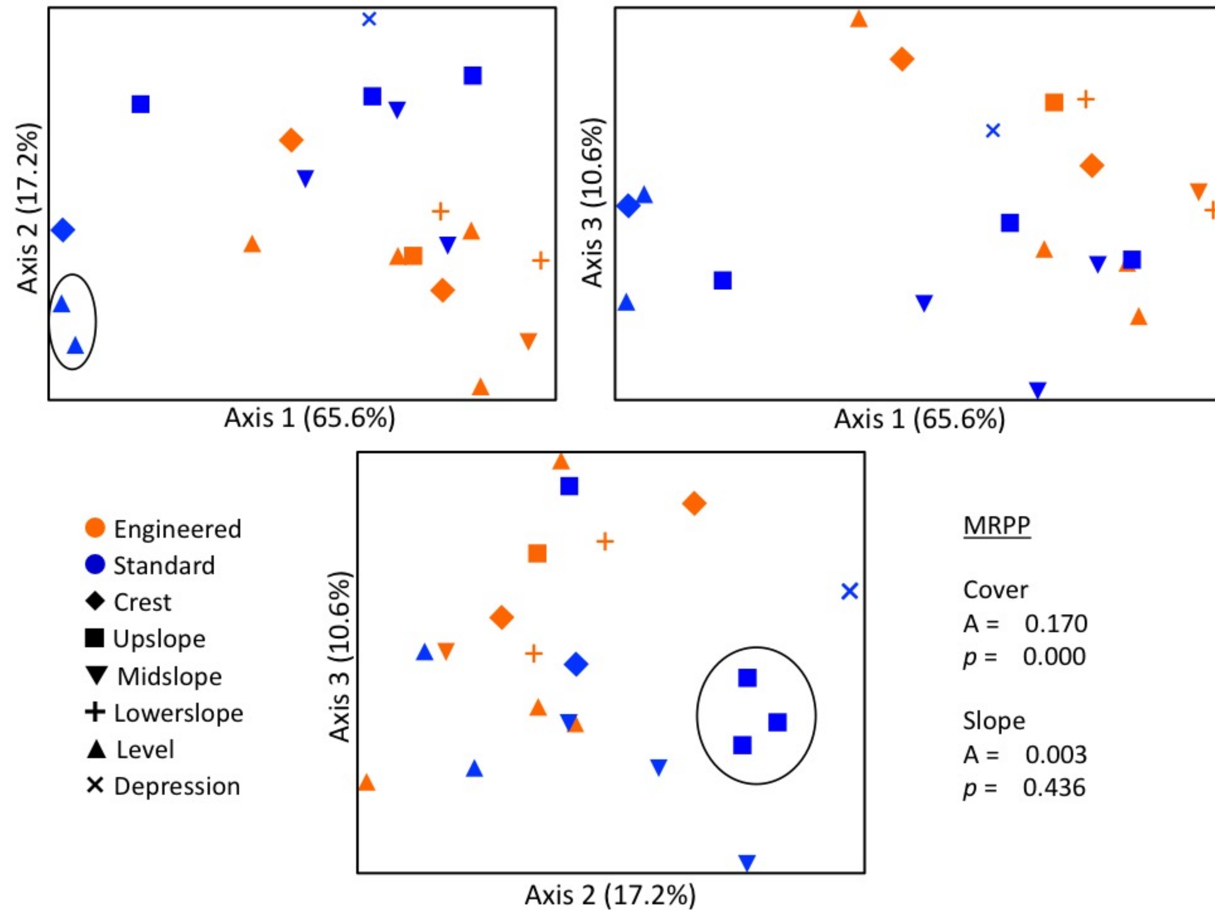

Figure 3.5 Non-metric multidimensional scaling (MDS) analysis of DGGE banding patterns from bulk soil bacterial 16S rRNA communities.

3.5.3.2 DGGE profiles of rhizosphere microbial community

Rhizosphere DGGE profiles mainly clustered by cover type. In some profiles, the banding patterns from the same cover and slope position clustered at approximately 85% of similarity (S7, S10) (Figure 3.6). In other profiles, DGGE banding patters clustered also by slope, but at different cover management, or by the geographical proximity of sampling points in the field. In the standard cover, sampling points S1 and S8 both from midslope position were clustered at approximately 90% similarity. Samples S10 and S7 both from upslope in the standard cover clustered in 85%. Similar to bulk soil, clustering in rhizosphere microbial community profiles were in general based mostly by cover management and in some cases by slope position.

In addition, MDS ordination analysis of rhizosphere profiles resulted in a 3-dimensional solution and final stress of 8.14 in which most variability of the data was accounted in Axis 1 (44.9%) (Figure 3.7). Axis 2 and 3 accounted for 30.3 and 15.7% of the variability in the data. Similar to bulk soil profiles, samples from the standard cover were more variable when compared to the engineered cover. As indicated by the significant MRPP results, only cover management affected the microbial community structure. However, when analyzed by cover type and slope, only one cluster containing samples from the midslope at the standard cover was observed.

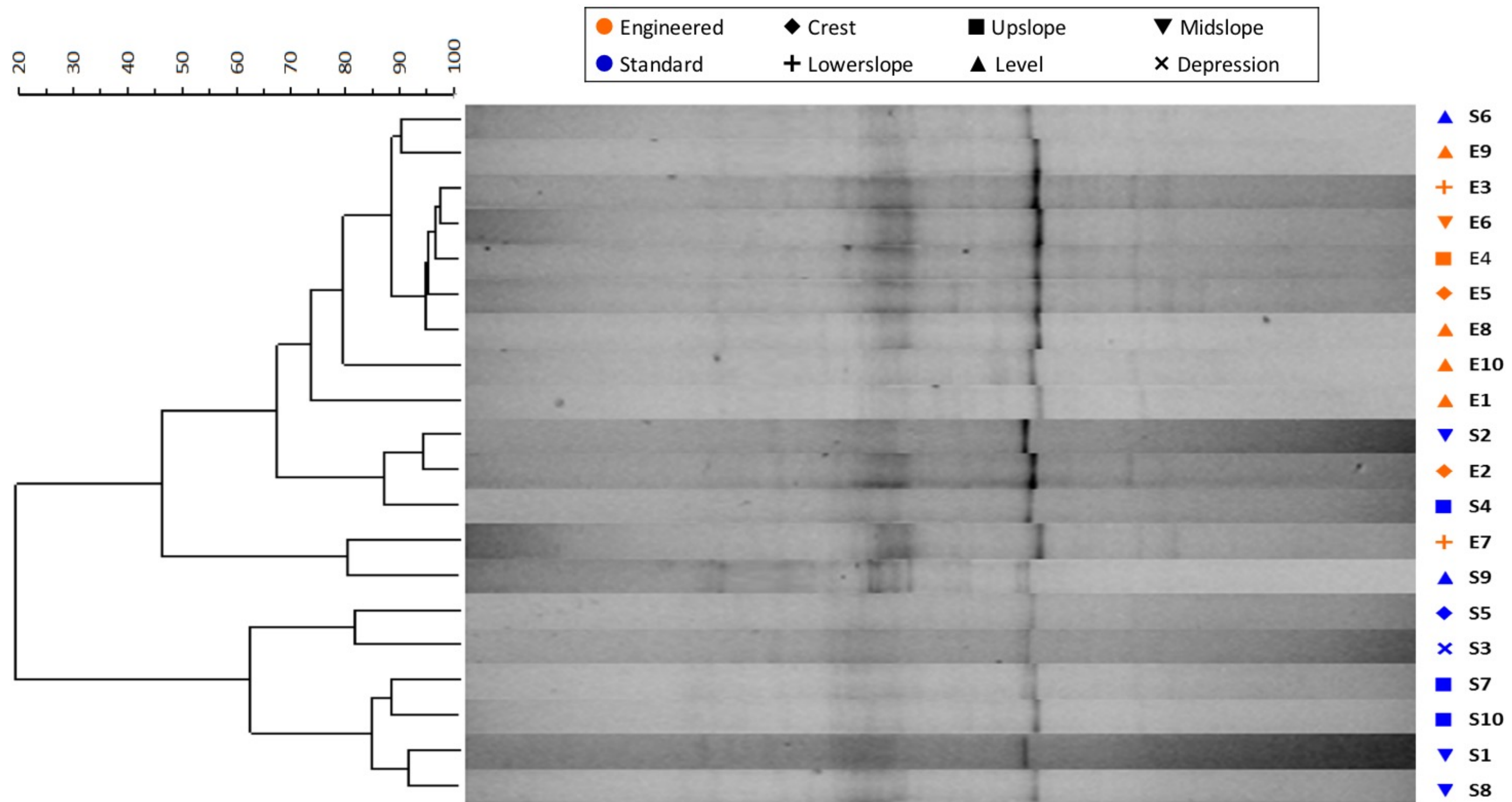

Figure 3.6 Dendrogram analysis using Pearson's correlation coefficient for rhizosphere soil DGGE banding patterns of bacterial 16S rRNA communities.

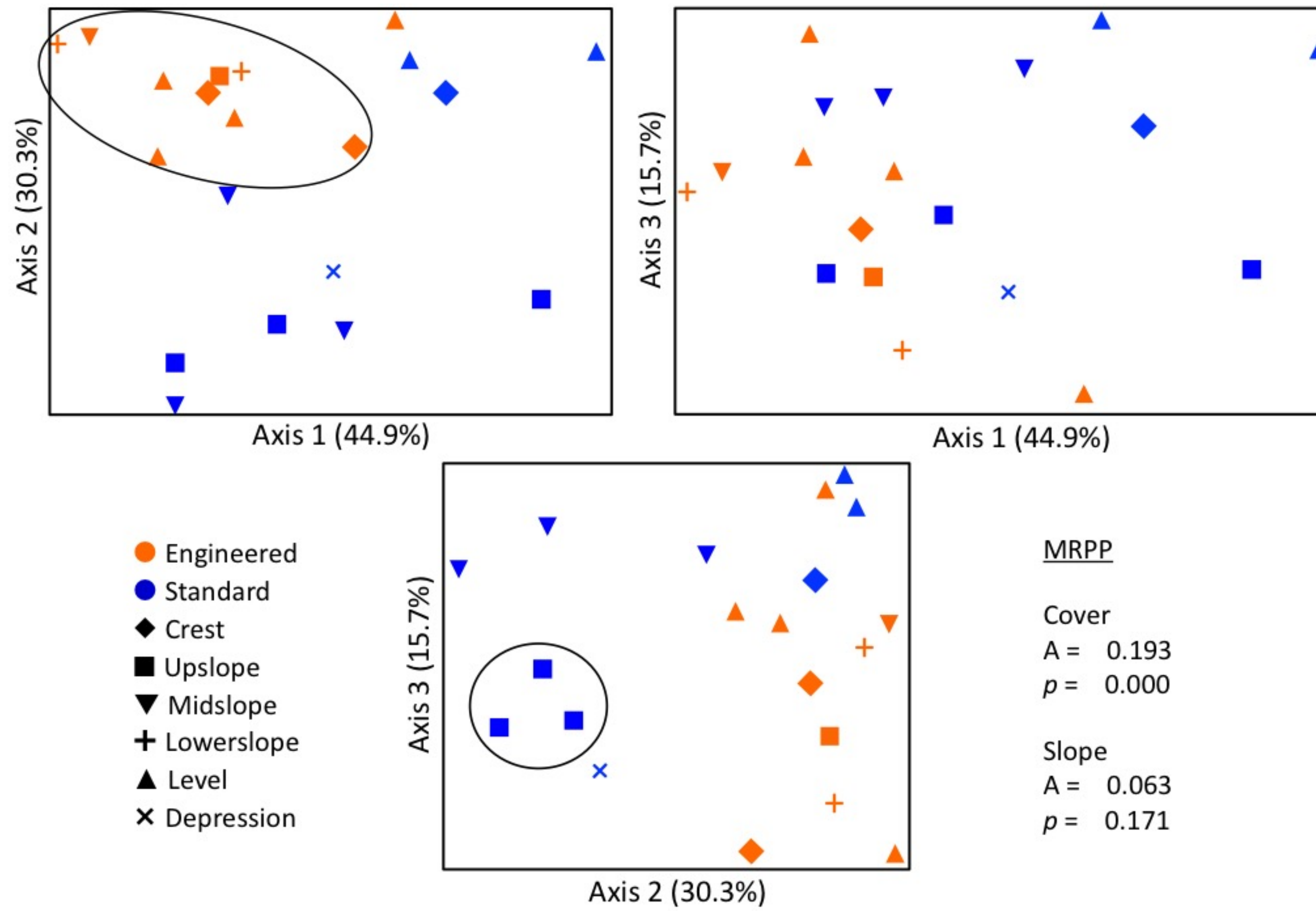

Figure 3.7 Non-metric multidimensional scaling (MDS) analysis of DGGE banding patterns from rhizosphere soil bacterial 16S rRNA communities.

3.5.3.3 DGGE profiles of endophytic microbial community

Different from rhizosphere and bulk soil profiles, where microbial profiles were mainly clustered by cover type, endophytic DGGE community profile dendrogram analysis indicated a high variability between samples and no clustering was observed by slope positions or cover (Figure 3.8). In some profiles, banding patterns from the same cover and slope position clustered together at approximately 60% of similarity. In other profiles, endophytic profiles also clustered by geographical proximity of the sampling points on the field. Sampling points E8 and E10 had a 60% similarity and both collected at level ground. This also occurred with profiles from the points E9 and E1 which had a 65% similarity and were categorized by the same cover and slope position, *i.e.* engineered cover at level ground. In the engineered cover, sampling points E6 and E7, geographically close in the field, clustered at a 60% similarity.

Multi-dimensional scaling (MDS) analysis from endophytic profiles resulted in a 3-dimensional solution and final stress of 8.69 (Figure 3.9). Axis 1 from the ordination analysis of 16S DGGE fingerprints represented most variability (52.2%). Axis 2 and 3 accounted for 16.7 and 20.6% respectively of the data variability. Similar to the dendrogram analysis, samples from both engineered and standard cover were highly variable and no evident grouping was observed either by cover type or slope positions. In addition, MRPP analyses indicated non-significant results.

DGGE fingerprints from endophytic community indicated a high variability between samples (Figure. 3.10). Select DGGE gel bands that were excised and successfully re-amplified were sequenced and their closest identities compared using the Basic Local Alignment Search Tool (BLAST) (Table 3.6). As expected, most DGGE gel bands were identified as rhizosphere and endophytic associated bacteria. In addition, similar bands could be observed between the samples analyzed. For instance, an uncultured *Proteobacterium* clone from agricultural soils (Band 16, Figure. 3.10) was observed in all the profiles analyzed. Other bands identified as *Phytoplasmas* (Band 1, 4, 10, Figure. 3.10), *Actinomycetes* (Band 20, 21, 22, Figure. 3.10), *Flavobacterium* spp. (Band 2, 5, Figure. 3.10) were unique to specific samples.

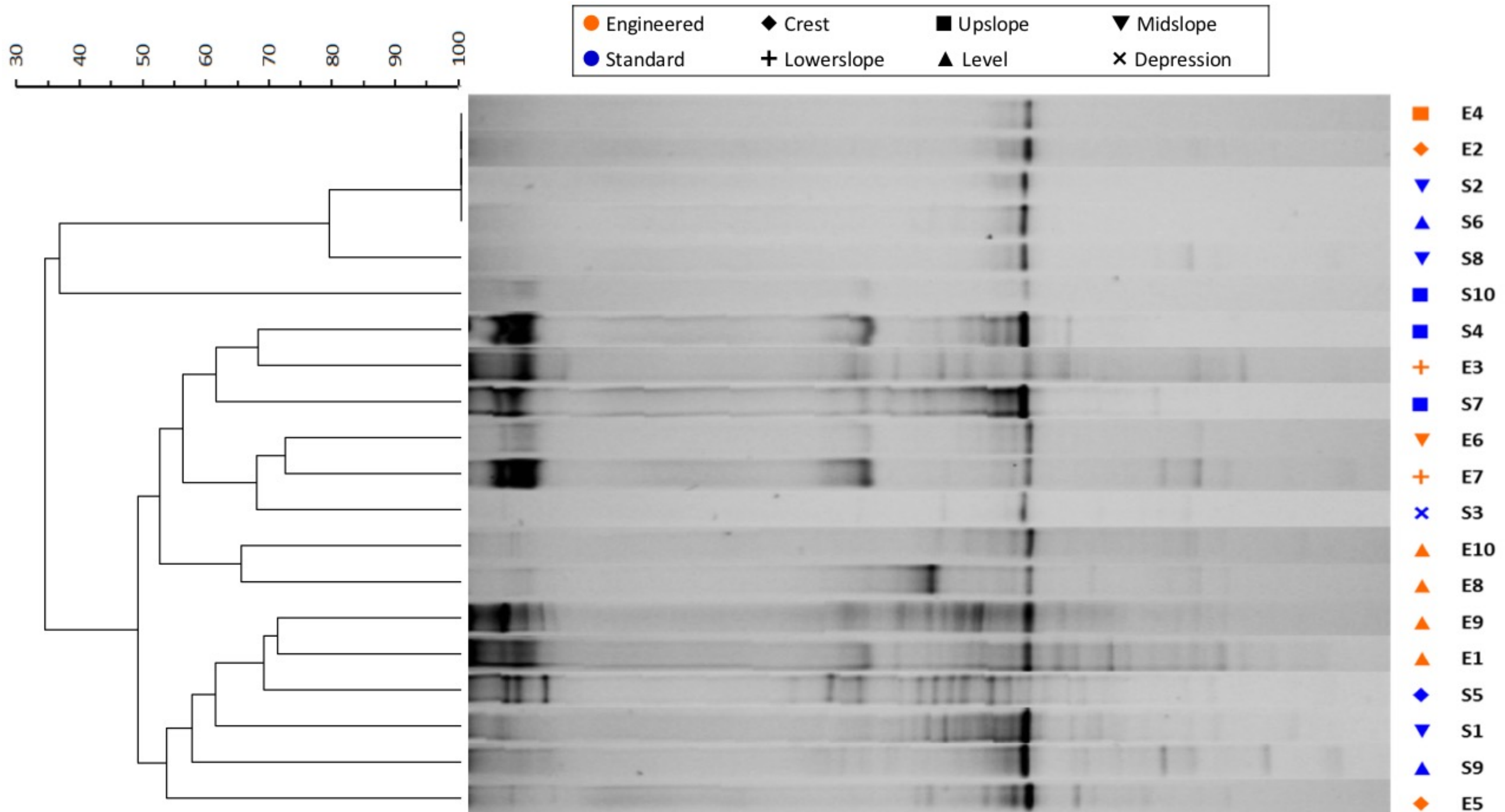

Figure 3.8 Dendrogram analysis using Pearson's correlation coefficient from DGGE banding patterns of endophytic bacterial 16S rRNA communities.

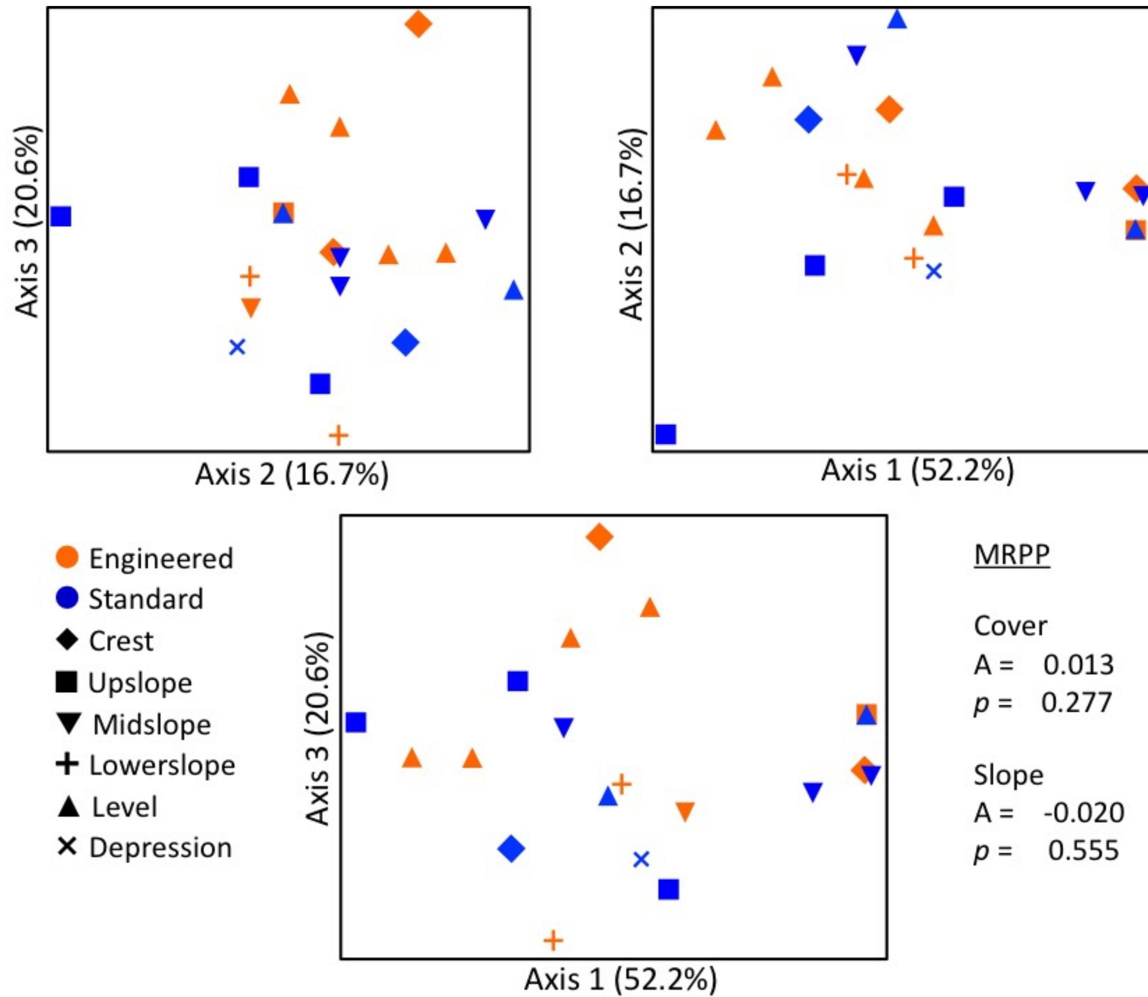

Figure 3.9 Non-metric multidimensional scaling (MDS) analysis of DGGE banding patterns from endophytic bacterial 16S rRNA communities.

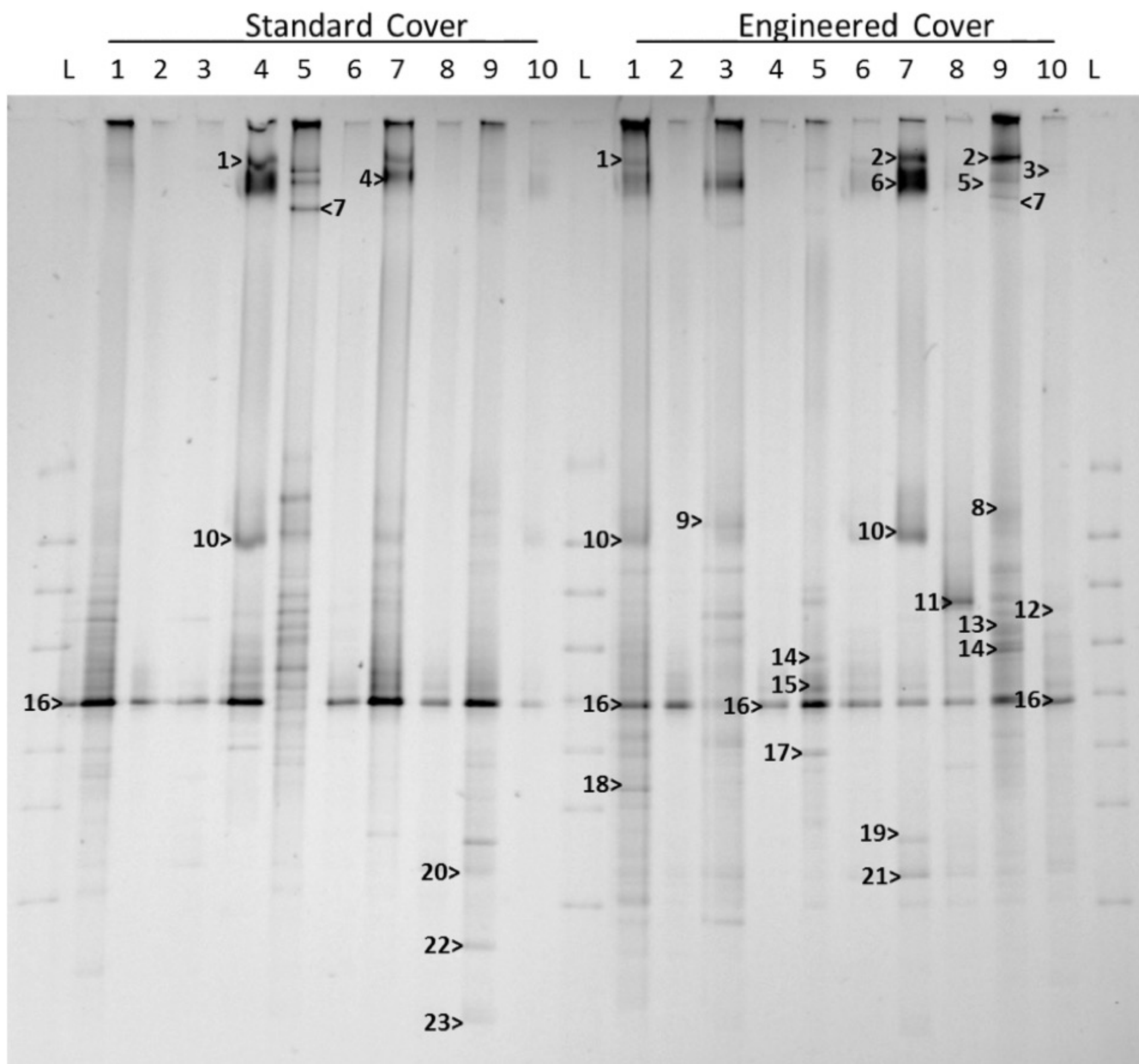

Figure 3.10 Representative DGGE of PCR-amplified 16S rRNA gene fragments from endophytic bacteria associated with annual barley roots growing on the standard and engineered cover at an oil sands reclamation area. Arrows indicate sequenced bands whose closest identities are provided in Table 3.6.

Table 3.6 Phylogenetic affiliation of selected endophytic bacteria based on 16S rRNA gene sequences re-amplified from DGGE bands (Figure 3.10).

DGGE Band	Accession #	Closest match	Similarity (%)	Notes on Original Source	Reference
1	U96616.1	*Phytoplasma* spp. STRAWB2 16S ribosomal RNA gene	98	*Phytoplasmas* associated with diseases of strawberry in Florida	(Jomantiene *et al.*, 1998)
2	KM035977.1	*Flavobacterium* spp. THG-DN8.5 16S ribosomal RNA gene	99	Bacteria isolated from rhizosphere of wild strawberry plant.	Unpublished.
3	JQ374133.2	Uncultured bacterium clone NT61d2_21281 16S ribosomal RNA gene,	95	Bacteria isolated from soils exposed to elevated atmospheric CO_2.	(Dunbar *et al.*, 2012)
4	HQ589192.1	*Psammotettix cephalotes'* flower stunt *Phytoplasma* strain BVK 16S ribosomal RNA gene	98	Determination of 16S rRNA of various *Phytoplasma* strains	Unpublished.
5	JQ977374.1	*Flavobacterium* spp. Eab5 16S ribosomal RNA gene	100	Diversity of culturable plant-associated bacteria	Unpublished.
6	EU133393.1	Uncultured bacterium clone FFCH13347 16S ribosomal RNA gene	91	Novelty and uniqueness patterns of rare members of the soil	(Elshahed and Youssef, 2011)
7	DQ279372.1	*Sphingobacterium* spp. TM14_4 16S ribosomal RNA gene	97	Bacteria associated with ectomycorrhizal symbiont fungus.	(Barbieri *et al.*, 2007)
8	FQ658649.1	Uncultured bacterium 16S ribosomal RNA gene clone P1AB111	100	PAH degrading bacteria.	Unpublished.
9	FJ448595.1	Uncultured bacterium clone D1_KR_030507_A09_65_01 16S ribosomal RNA gene	96	Isolate from earthworm gut microbiota.	(Rudi *et al.*, 2009)
10	HG764368.1	Leafhopper (*Deltocephalinae*) aster yellow *Phytoplasma* partial 16S rRNA gene	100	*Phytoplasma*	(Perilla-Henao, 2013)
11	KM067138.1	*Pseudomonas fluorescens* strain CL14 16S ribosomal RNA gene	100	Rhizosphere bacteria of turmeric plants	Unpublished.

Table 3.6 cont.

DGGE Band	Accession #	Closest match	Similarity (%)	Notes on Original Source	Reference
12	KM125762.1	Uncultured bacterium clone LNH_9_9_11_Pumice.233571 16S ribosomal RNA gene, partial sequence	91	Microbial communities associated with pumice.	Unpublished.
13	EF516828.1	Uncultured bacterium clone FCPP661 16S ribosomal RNA gene	93	Bacteria isolated from grassland soil.	(Cruz-Martínez *et al.*, 2009)
14	HF678325.1	Uncultured *Burkholderiales* bacterium partial 16S rRNA gene, DGGE band IT_B03	90	Grapevine root-associated bacterial communities.	(Marasco *et al.*, 2013)
15	AY799982.1	Uncultured bacterium clone cwr253 16S small subunit ribosomal RNA gene, partial sequence	100	Sulfate-Reducing and Cellulose-Degrading Bacteria in Wetlands and Rhizosphere Environments.	Unpublished.
16	EF664750.1	Uncultured *Proteobacterium* clone GASP-MB1W1_C05 16S ribosomal RNA gene, partial sequence	99	Agricultural soil bacteria.	(Jangid *et al.*, 2011)
17	EU133393.1	Uncultured bacterium clone FFCH13347 16S ribosomal RNA gene, partial sequence	96	Bacterial community in tallgrass prairie soil.	(Elshahed and Youssef, 2011)
18	GU550525.1	*Micromonospora* spp. I08A-00459 16S ribosomal RNA gene, partial sequence	99	Endophytic *Actinobacteria* from Mangroves.	Unpublished.
19	FJ448589.1	Uncultured bacterium clone D1_KR_030507_G03_23_13 16S ribosomal RNA gene, partial sequence	100	Isolate from earthworm gut microbiota.	(Rudi *et al.*, 2009)
20	KF447933.1	*Actinoplanes digitatis* strain OKG1 16S ribosomal RNA gene, partial sequence	100	Soil *Actinomycete*.	Unpublished.
21	EU593726.1	*Lentzea violacea* strain 173540 16S ribosomal RNA gene, partial sequence	100	Isolated soil *Actinomycetes*.	Unpublished.
22	KJ425224.1	*Actinoplanes auranticolor* strain INA01094 16S ribosomal RNA gene, partial sequence	99	Endophytic *Actinomycetes* isolated from medicinal plants.	Unpublished.

3.5.4 Survey of Endophytic bacterial communities by culture dependent techniques

Total culturable rhizosphere bacteria associated with annual barley were higher in locations E1 and E9 (8.52 and 8.47 Log CFU·$g^{-1}$of dry soil respectively) in the engineered cover (Table 3.7). Endophytic bacteria also indicated similar results as the highest culturable bacteria were observed in locations E5 and E9 (6.42 and 6.38 Log CFU·$g^{-1}$of dry root respectively) (Table 3.8). Although the interaction of cover and slope indicated significant differences, no relationship in the number of culturable rhizosphere bacteria by slope position or cover alone was observed (Table 3.9). Differences in culturable endophytic bacteria were both significant by cover and slope, however the interaction between cover and slope indicated no significant differences. A total of 316 endophytic bacteria isolates were selected based on different morphology. Of these isolates, seven isolates from each sampling location (total of 140) were randomly selected and sequenced (Figure 3.11, Appendix A, Table A1). The identification of endophytes was tentatively allocated to species for some isolates, but mostly by genera. Bacteria from the class *Gamma-Proteobacteria* dominated most of the isolates selected, which corresponded to 54% of the total sequenced. This class included 16% of *Enterobacter* spp., 11% of *Xanthomonas* spp. and 26.6% of *Pseudomonas* spp. The class *Flavobacteria* corresponded to 12% of the isolate collection with 61% of *Chryseobacterium* spp., 33% of Flavobacterium spp. and 6% *Riemerella* spp. The class *Sphingobacteri*a represented 10% of the total isolates. In this class, all the isolates selected matched *Pedobacter* spp. in the database. The class *Actinobacteria* represented 7% of the total collection in which it mostly matched *Arthrobacter* spp. (50%) and only 20% of *Microbacterium* spp. The class *Bacilli* corresponded to 12% of the isolates; in this class most of the isolates (47%) matched *Bacillus pumilus* spp. The class *Alphaproteobacteria* corresponded to only 2% of the total endophyte culture collection. Gram negative bacteria represented most of the isolated identified (80%), in which 28% of those were *Enterobacter* spp. Gram positive bacteria corresponded to 20% of total isolates and 44% of these matched *Bacillus* spp. Hence, the endophytic bacteria exhibiting 16S rRNA sequence similarities matched most closely the sequences of species of the genus *Pseudomonas*. This genus corresponded to a total of 20 isolates in which most were closely related to *Pseudomonas fluorescens* (4), *Pseudomonas syringae* (2), *Pseudomonas poae* (2), *Pseudomonas koreensis* (1), *Pseudomonas tolaasii* (1) and unidentified (10).

Table 3.7 Total culturable rhizosphere bacteria associated with annual barley (*Hordeum vulgare*) plants growing on the standard and engineered cover at an oil sands reclamation area (Log $CFU \cdot g^{-1}$ of soil dry weight ± standard deviations).

Cover	Location	Slope	Log CFU
Standard	S1	Midslope	8.26 ± 0.05
	S2	Midslope	7.02 ± 0.04
	S3	Depression	7.46 ± 0.04
	S4	Upslope	7.95 ± 0.04
	S5	Crest	8.04 ± 0.05
	S6	Level	6.73 ± 0.02
	S7	Upslope	7.00 ± 0.04
	S8	Midslope	6.97 ± 0.04
	S9	Level	7.97 ± 0.04
	S10	Upslope	7.59 ± 0.03
Engineered	E1	Level	8.52 ± 0.08
	E2	Crest	8.29 ± 0.34
	E3	Lowerslope	7.70 ± 0.07
	E4	Upslope	7.68 ± 0.05
	E5	Crest	7.48 ± 0.08
	E6	Midslope	8.39 ± 0.03
	E7	Lowerslope	8.24 ± 0.05
	E8	Level	7.36 ± 0.11
	E9	Level	8.47 ± 0.01
	E10	Level	8.03 ± 0.02

Table 3.8 Total culturable endophytic bacteria associated with annual barley (*Hordeum vulgare*) plants growing on the standard and engineered cover at an oil sands reclamation area (Log CFU·$g^{-1}$of soil dry weight ± standard deviations).

Cover	Location	Slope	Log CFU
Standard	S1	Midslope	5.75 ± 0.09
	S2	Midslope	4.34 ± 0.03
	S3	Depression	6.03 ± 0.03
	S4	Upslope	5.64 ± 0.15
	S5	Crest	5.27 ± 0.11
	S6	Level	5.29 ± 0.22
	S7	Upslope	5.38 ± 0.02
	S8	Midslope	5.98 ± 0.08
	S9	Level	5.86 ± 0.03
	S10	Upslope	6.35 ± 0.02
Engineered	E1	Level	5.01 ± 0.15
	E2	Crest	5.06 ± 0.65
	E3	Lowerslope	6.28 ± 0.01
	E4	Upslope	5.25 ± 0.22
	E5	Crest	6.42 ± 0.02
	E6	Midslope	4.45 ± 0.19
	E7	Lowerslope	6.33 ± 0.04
	E8	Level	5.86 ± 0.07
	E9	Level	6.38 ± 0.02
	E10	Level	5.37 ± 0.15

Table 3.9 Analysis of Variance (ANOVA) for total culturable endophytic and rhizosphere bacteria associated with annual barley grown in reclamation areas.

	Rhizosphere Bacteria (Log CFU·g^{-1} of dry soil)	**Endophytic Bacteria** (Log CFU·g^{-1} of dry root)
Cover (C)	$p = 0.178$	$p = 0.012$
Slope (S)	$p = 0.118$	$p = 0.001$
C*S	$p = 0.005$	$p = 0.227$

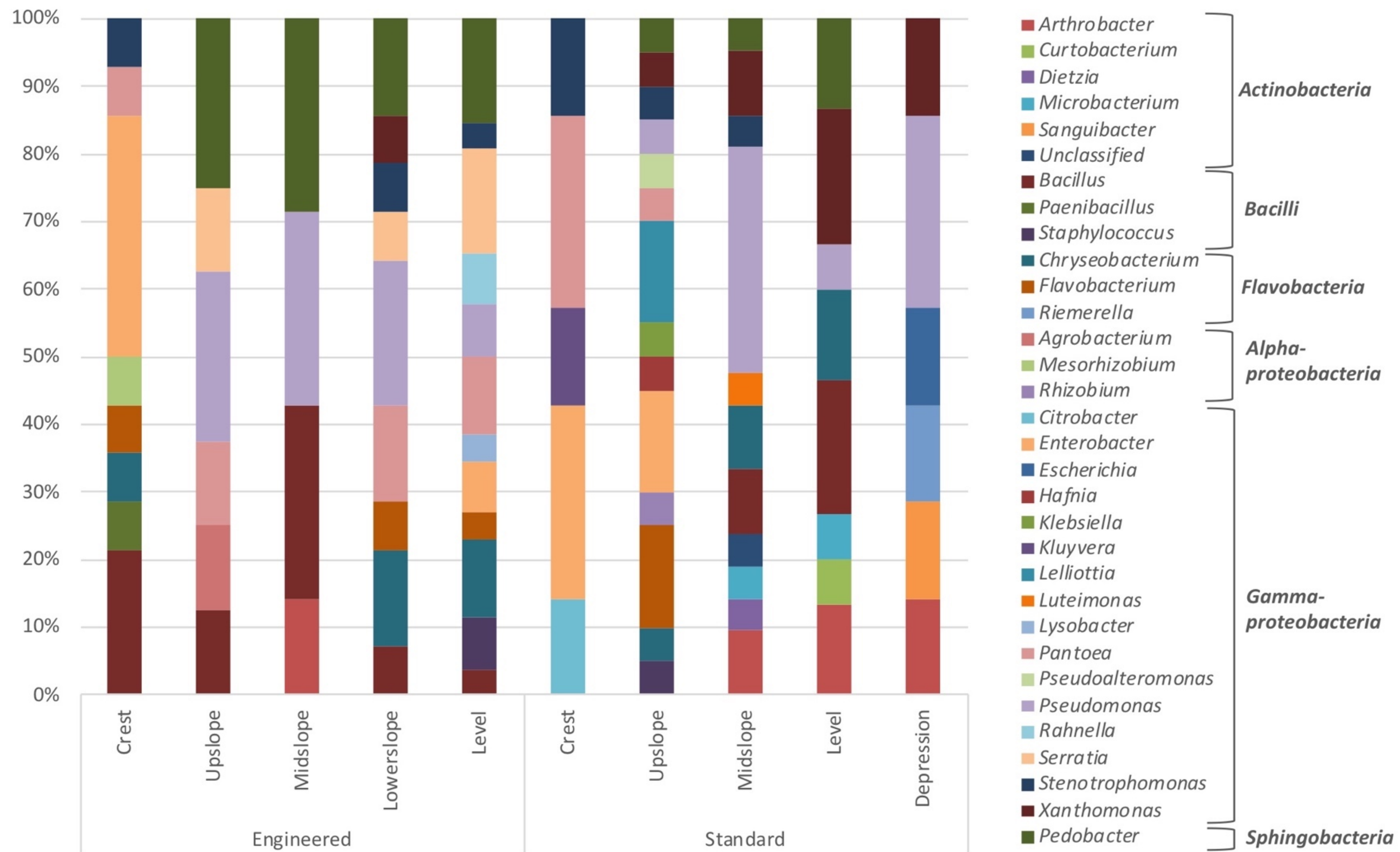

Figure 3.11 Relative abundance of 140 randomly selected bacterial endophyte isolates (total=316) associated with annual barley and identified by 16S rRNA Sanger sequencing.

3.6 Discussion

This study assessed the diversity of dominant endophytic, rhizosphere and bulk soil bacterial communities associated with annual barley currently growing at two different cover managements at an oil sands reclamation area. Nutrient availability (N-P-K-S) are of great importance in the initial stages of reclamation as they can be readily used by plants. In this study, different slope positions at the two cover managements affected nutrient availability; however, the data in this study suggest no linkage between microbial community for most available nutrients. For instance, no slope effect was observed for NO_3^-, which is highly mobile in soils, indicating that soil structure is not yet well established on these sites. Overall the results presented here are in agreement with MacKenzie and Quideau (2010) as these authors also found no slope and available nutrient effect on the microbial community. According to MacKenzie and Quideau (2010), reclamation areas are usually built with heavy equipment at an industrial scale resulting in large variability in PMM placement and depth, which may lead to inconsistent topographical effects. In addition, Leatherdale *et al.* (2012) found that soil water patterns did not respond to slope effects due to heterogeneity of soil physical properties and vegetation. According to these authors, PMM is generally placed on site while still frozen and broken up and evened out to the prescribed application depth, which can create immense spatial variability in the area.

An important soil management practice in oil sands reclamation is the use of peat as a cover material. Peat is available in large areas of the mining footprint and is used to improve soil physical, chemical, and biological properties (Ojekanmi and Chang, 2014; Shaughnessy, 2010). High organic matter content is one of the main reasons peat is often added as capping material in the tailing sands (Mackenzie, 2011). Organic matter and organic carbon content analyzed the current study indicated similar results from the literature (MacKenzie and Quideau, 2010). According to Rowland (2008), peat mineral mixes usually have 2 to 17% of organic C. Similar to MacKenzie and Quideau (2010), significant correlations were observed between TOC, OM and microbial PLFAs. Bradford *et al.* (2017) also observed positive correlations between total PLFA abundance and TOC. Total organic carbon has an important effect on water retention in soils and it has great practical significance in reclamation (Moskal *et al.*, 2001). Smith *et al.* (2014) studied the effects of how microbial communities respond to changes in C pools and nutrient availability and suggested that the abundance of microbial groups is influenced by the

distribution of soil organic matter pool. In the current study, total PLFAs, Gr+ and Gr- bacterial biomarkers were higher at sampling locations where TOC and OM were also high. Wang *et al.* (2016) also found that soil TOC was the major factor influencing bacterial biomass on subtropical forests. Gram-positive bacteria in soil are mainly associated with two phyla, *Firmicutes* (low G+C) and *Actinobacteria* (high G+C) (McCaig *et al.*, 1999). Although *Firmicutes* are considered the second most abundant bacterial phylum in soil, *Actinobacteria* are involved in important processes that includes the decomposition of organic materials in soil (*e.g.* lignin and other recalcitrant polymers) and thereby playing a vital part in the carbon cycle (Heuer *et al.*, 1997; Filippidou *et al.*, 2015). Similar to the current study, Howell and MacKenzie (2017) also found that *Actinobacteria* dominates organic rich peat mixes in reclamation areas. However, soil carbon is also often associated with the presence of gram negative bacteria (Hopkins *et al.*, 2014). According to Koranda *et al.* (2014) labile C substrates are predominantly metabolized by gram-negative bacteria and fungi. One of the largest divisions within gram-negative bacteria comprises the phylum *Proteobacteria*, which contains organisms with a vast level of morphological, physiological and metabolic diversity (Gupta, 2000; Spain *et al.*, 2009).

Although significant differences by slope and cover type in absolute PLFA values were observed, the data in this study indicated no differences in the relative abundance of Gr+ and Gr- biomarkers (mol%). Overall, soil organic matter and organic carbon were slightly higher in the engineered cover, which also has the (GCL) membrane to retain moisture on the surface of the cover, when compared to the standard cover. However, these differences were not significant enough to reflect shifts in the microbial community. These results are in agreement with Bachar *et al.* (2010), in which the authors have suggested that although bacterial abundance is highly associated with soil carbon and moisture, this may not lead to shifts in the microbial community structure. In addition, PLFA physiological stress biomarkers results indicated negative correlations with TOC, TC, NH_4^+ and OM, which may suggest nutrient limiting conditions for microbial growth in these areas as previously suggested by Helgason *et al.* (2010a) in Canadian prairie agroecosystems.

In addition to PLFA, Denaturing Gel Gradient Electrophoresis (DGGE) is a technique that has been extensively used in the characterization of microbial communities associated with plants growing in reclamation soils (Lefrançois *et al.*, 2010; Phillips *et al.*, 2010). Culture independent DGGE analysis of bulk soil microbial profiles in the current study indicated

significant differences by cover management but not by slope positions. Similar results were also observed by MacKenzie and Quideau (2010), in which the authors found inconsistent topographical for soil microbial communities in an oil sands reclamation area. According to MacKenzie and Quideau (2010), this may be due to that reclamation sites are built with heavy equipment at an industrial scale resulting in large variability in PMM placement and depth.

In the rhizosphere DGGE fingerprints, no clustering was observed by slope position, although these profiles were highly differentiated by cover management. This may be due to similar effects influencing bulk soil samples, however more pronounced by plant cover influence in the rhizosphere. In both the bulk soil and rhizosphere samples, only a few strong bands were identified, which is most likely due to the high presence of low-abundance amplicons. Since DGGE detects mostly dominant members of the community, less abundance members might be shown as smears (Jousset *et al.*, 2010). In the three different microbiomes in this study (bulk soil, rhizosphere and endosphere), most rhizosphere and bulk soil DGGE profiles were shown as smears with only a few dominant bands observed. In endophytic gels however, more dominant bands were observed.

DGGE gels from endophytic communities revealed that all sampling locations developed unique endophytic communities containing at least one dominant endophytic bacterium. Most of these bands were re-amplified and identified as endophytic bacteria from other plants. In the endophytic DGGE gel profile, most of bands closely matched DNA from soil and plant associated bacteria. Band 16 (Figure. 3.10) was observed in all the profiles analyzed. There original source from this sequence is a *Proteobacteria* isolated from a successional forest abandoned in 1951 (Jangid *et al.*, 2011). In addition, excised bands (Bands 18, 20, 22, Figure. 3.10) matched endophytic *Actinomycetes*, which were also isolated in culture dependent techniques. The results presented here are consistent with Nimnoi *et al.* (2010), who studied the diversity of *Actinomycetes* endophytes. According to Sardi *et al.* (1992), since *Actinomycetes* live abundantly in the rhizosphere, they have great potential for passive penetration in cracks of root tips, easily infecting plant roots.

Also, in the endophytic bacteria DGGE gels, several bands matched *Phytoplasma* species (Bands 1, 4 and 10, Figure. 3.10). *Phytoplasmas* are recognized as pathogens and are widespread mainly in Europe and North America (Kamińska *et al.*, 2010). Also, *Phytoplasmas* are obligate intracellular parasites of insects, including leafhoppers (Band 10, Figure. 3.10) (Lee *et al.*, 2000).

Despite the progress in molecular biology of these uncultured species, it is still largely unknown how these pathogens manipulate plant host physiology (Cettul and Firrao, 2011). In fact, *Phytoplasmas* may occur in very low concentrations in plant species and may provoke no harm or only mild pathogenic symptoms to host plants (Kamińska *et al.*, 2010).

Flavobacterium spp. (Band 2 and 5, Figure. 3.10) and *Pseudomonas* spp. (Band 11, Figure. 3.10) re-amplified from the DGGE gels correspond to a large number of bacteria isolated and characterized that can potentially metabolize PAHs (Xu-xiang *et al.*, 2006; Germaine *et al.*, 2009). Phillips *et al.* (2009) suggested that when the dominant endophytic population consisted of *Pseudomonas* spp., the ability of plants to metabolize alkane pollutants also improved. Since reclamation sites may contain residual hydrocarbons (Greer *et al.*, 2011), the association of plants with these endophytes could reduce plant stress under the presence of hydrocarbons.

Dendrogram analysis of endophytic bacteria profiles indicated a very diverse community by slope position and cover (Figure 3.8). In some profiles the presence of several bands were observed while in others only a single band was detected. In the dendrogram analysis only few of the profiles where clustered, as these corresponded to the same slope positions or sampling points next to each other. However, for most of the fingerprints no clustering was observed by cover type or slope. In addition, clusters in endophytic microbial profiles were unique when compared to banding patterns from rhizosphere and bulk soil DGGE profiles.

The DGGE banding pattern represents a good approximation of the most dominant bacterial species biodiversity, but it also has limitations such as the co-migration of DNA fragments and low resolution for less abundant microbes (Zheng *et al.*, 2013). However, PCR-DGGE is still currently a powerful method for determining shifts in microbial community composition and rapid method to profile the phylogenetic structure of environmental microbial community without artifacts of culture based techniques. The screening of microbial community profiles using culture independent methods are relevant to improve understanding in the overall community which is highly important for oil sands reclamation strategies. Nevertheless, these strategies may depend on the culturable endophytic bacteria that not only can have beneficial effects on host plants but also can be manipulated and further used in reclamation.

Culturable endophytes selected phenotypically have been analyzed and characterized genotypically using Sanger sequencing and compared those sequences with the BLAST

database. Most of the endophytic bacteria isolated from annual barley plants growing on oil sands reclamation soils corresponded to *Enterobacter, Flavobacterium, Pseudomonas* and *Xanthomonas* spp. Moore *et al.* (2006) isolated endophytic bacteria from poplar trees growing on a BTEX-contaminated site. These authors selected 60 bacterial isolates and *Pseudomonas* spp. was numerically predominant among the isolates collected. In this study, *Arthrobacter, Enterobacter* and *Acinetobacter* spp. were isolated from root material. In the current study, most of the genera identified were similar to those found by Moore *et al.* (2006). In addition, bacterial form the genus *Flavobacterium* and *Pseudomonas* were identified in both culture dependent and independent techniques. These two genera contain species know to be hydrocarbon degraders (Lee *et al.*, 2006), plant growth promoters (Shcherbakov *et al.*, 2013) and to control plant pathogens (Mazurier *et al.*, 2009).

3.7 Conclusions

This study revealed that soil microbial communities in oil sands post-mining landscapes are mainly driven by total and organic carbon, NH_4^+ and organic matter. The results in this study also highlight the limitations in correlating slope effects to nutrient availability and microbial profiles in reclamation areas. Therefore, confirming that slope effects and the potential differences between the two cover managements are not yet well established on these sites. Endophytic bacteria successfully colonized barley plants, although bacterial profiles are highly variable at different sampling locations. Whereas bulk soil and rhizosphere DGGE bacterial profiles differentiated mainly by cover type, endophytic profiles did not clustered either by slope or cover type. In addition, most of selected bacterial endophyte bands from DGGE gel closely matched rhizosphere and soil bacteria from agricultural and grassland soils. Few bands selected from endophytic DGGE profiles matched growth promoting bacteria and potential hydrocarbon degraders, suggesting they may assist plant growth on reclamation covers. Several endophytic isolates from culture dependent techniques also matched those from DGGE profiles. These contain species know to be hydrocarbon degraders and plant growth promoters. Therefore, this study provides an initial screening of dominant bacteria associated with barley plants growing in an oil sand reclamation cover. Further research is required to determine a more detailed characterization of these communities and how they may be influenced by soil or plant related factors.

4. BACTERIAL ROOT MICROBIOME OF PLANTS GROWING IN OIL SANDS RECLAMATION COVERS

4.1 Preface

Chapter 3 provided an initial screening of dominant bacteria associated with barley plants growing in an oil sands reclamation area using culture dependent and independent methods. The results presented indicated that soil microbial communities were driven mainly by total and organic carbon, NH_4^+ and organic matter. Additionally, it was demonstrated that endophytic bacteria successfully colonized barley plants and that a few bacterial profiles matched those of plant growth promoting and potential hydrocarbon degraders. Since barley is commonly planted in reclamation covers, its associated bacterial communities are of high importance to future native plant species. However, an in-depth analysis of bacterial profiles associated with barley and naturally occurring pioneer plant species is still required. To address this, annual barley and white sweet clover were sampled in the following year and 16S rRNA amplicon sequencing was used to characterize the bacterial root microbiome associated with these plants.

4.2 Abstract

Oil sands mining in northern Alberta impacts a large footprint, but the industry is committed to reclaim all disturbed land to an ecologically healthy state in response to environmental regulations. However, these newly reconstructed landscapes may be limited by several factors that include low soil nutrient levels and reduced microbial activity. Rhizosphere microorganisms colonize plant roots providing hosts with nutrients, stimulating growth, suppressing disease and increasing tolerance to abiotic stress. High-throughput sequencing techniques can be used to provide a detailed characterization of microbial community structure. This study used 16S rRNA amplicon sequencing to characterize the bacterial root microbiome associated with annual barley (*Hordeum vulgare*) and sweet clover (*Melilotus albus*) growing in an oil sands reclamation area. The results in this study indicate that *Proteobacteria* dominated the endosphere, whereas other phyla such as *Acidobacteria* and *Gemmatimonadetes* were restricted to the rhizosphere

suggesting that plants have the ability to select for certain soil bacterial consortia. The bacterial community in the endosphere compartments were less rich and diverse compared to the rhizosphere. Furthermore, it was apparent that sweet clover plants were more selective, as the community exhibited a lower richness and diversity compared to barley. Members of the family *Rhizobiaceae*, such as *Sinorhizobium* and *Rhizobium* were mainly associated with clover, whereas *Acholeplasma* (wall-less bacteria transmitted by insects) was unique to barley. Genera from the *Enterobacteriaceae* family, such as *Yersinia* and *Lentzea* were also mostly detected in barley, while other genera such *Pseudomonas* and *Pantoea* were able to successfully colonize both plants. Endophytic bacterial profiles varied within the same plant species at different sampling locations; however, these differences were driven by factors other than slope positions or cover management. The results in the current study suggest that bacterial endophytic communities of plants growing in land reclamation systems are a subset of the rhizosphere community and selection is driven by plant factors.

4.3 Introduction

Soil microbial communities represent the greatest known reservoir of biological diversity (Berendsen *et al.*, 2012). However, compared to non-rooted bulk soil, the rhizosphere, which is the narrow zone of soil that is influenced by root exudates, is a 'hot spot' for numerous organisms and is considered as one of the most complex ecosystems (Raaijmakers *et al.*, 2009; Tkacz *et al.*, 2015; Bakker *et al.*, 2013). The increased microbial abundance and activities in the rhizosphere environment are due to the release of organic carbon by plant root exudation (Bakker *et al.*, 2013). Soil microorganisms are chemotactically attracted to root exudates, which allow them to proliferate in this carbon rich environment (Raaijmakers *et al.*, 2009). In turn, rhizosphere microbiota can also directly and/or indirectly affect the composition and biomass of plant communities in natural and agricultural ecosystems (Philippot *et al.*, 2013). The complexity of plant-microbe interactions has resulted in a number of studies that revealed profound effects on plant growth, development, nutrition, diseases, and productivity (Mendes *et al.*, 2013). Although the majority of research in plant-microbe interaction focuses on the rhizosphere, microorganisms are also able to readily colonize most plant compartments and plants can also function as filters of soil microorganisms (Chen *et al.*, 2010; Berg *et al.*, 2014).

Microbes residing within plant tissues (the endosphere) for at least part of their lives, whether in leaves, roots or stems, are considered endophytes (Kobayashi and Palumbo, 2000; Turner *et al.*, 2013). Endophytes are thought to be a sub-population of the rhizosphere microbiome and/or that once inside their hosts they change their metabolism and become adapted to their internal environment (Turner *et al.*, 2013; Germida *et al.*, 1998). The best evidence suggests that microbial endophytes enter at lateral root junctions, most likely at naturally occurring cracks, however they also have characteristics distinct from rhizosphere inhabiting bacteria, suggesting that not all rhizosphere bacteria can enter plants (Turner *et al.*, 2013). Numerous studies suggest that bacterial endophytes can promote host plant establishment and improve plant growth under adverse conditions (Soleimani *et al.*, 2010; Deng *et al.*, 2011; Khan *et al.*, 2011). Bacterial endophytes may also have the ability to control plant pathogens, insects and nematodes, which make them suitable as biocontrol agents (Hallmann and Berg, 2006). In addition, recent studies also suggest that endophytes may play an important role in remediation of contaminated soils and water (Chen *et al.*, 2010; Guo *et al.*, 2010; Xiao *et al.*, 2010; Mastretta *et al.*, 2013).

Plants and microbes have both adapted to use their close association for their mutual benefit (Edwards *et al.*, 2015). Due to the importance of these associations, interactions between microbes and model plants, such as in Rhizobium-legume symbiosis, have been extensively reported in the literature (Child, 1975; Freiberg *et al.*, 1997). However, the diversity of root associated microorganisms in reclamation soils after mining operations are not well understood.

The Athabasca's oil sands region in northern Alberta are unconventional petroleum deposits where bitumen, a dense and extremely viscous form of petroleum, is found in combination with sand, clay, and water (Yergeau *et al.*, 2012a). Covering an area of over 100,000 km^2, the oil sands yielded 2.3 million barrels of bitumen per day in 2014 (Government of Alberta, 2017). These oil deposits, estimated at 169 billion barrels represent the third largest oil reserve in the world and a major resource within Canada's energy sector (Kannel and Gan, 2012). However, bitumen lies under a total area of 142,000 km^2 of natural boreal forest, which needs to be removed during mining operations (MacKenzie and Quideau, 2010). Following bitumen extraction, mine tailings are accumulated in ponds where tailing sands are settled and the water is recycled (Onwuchekwa *et al.*, 2014; Yergeau *et al.*, 2012a). Land reclamation strategies in the oil sands are challenging due to the nature of the tailing sands, a generally

inappropriate plant growth medium with low nutrient content, high pH, low or no organic matter and residual hydrocarbon products (Naeth *et al.*, 2011; Lefrançois *et al.*, 2010). Hence, land reclamation strategies in the oil sands have focused on covering the tailing sands with suitable reclamation material to improve vegetation establishment. A common practice has been the use of peat-mineral soil mix (PMM) to create a suitable plant growth medium and to provide a source of native plants that can facilitate the vegetation natural recovery (Shaughnessy, 2010). Planting of seedlings of the dominant boreal forest tree species and the colonization by pioneer species is essential to improve reclamation strategies and allow the re-establishment of a natural forest (Renault *et al.*, 2004; Lefrançois *et al.*, 2010). In addition, annual barley (*Hordeum vulgare*) is often planted in reclamation landscapes to provide a quick vegetation cover and erosion control (Audet *et al.*, 2015).

Previous studies on oil sands reclamation sites have focused on the shifts in soil microbial community structure and nutrient profiles (MacKenzie and Quideau, 2010) and the impact of nitrogen fixing *Frankia*-inoculated alders on soil quality and dominant root associated microbial communities (Lefrançois *et al.*, 2010). However, given the challenges of land reclamation in the Alberta's oil sands, and the importance of root associated microbiota for a successful vegetation cover, an in-depth characterization of these microbial profiles is essential to improve current reclamation strategies.

In this study, high-throughput 16S rRNA amplicon sequencing was used to characterize bacterial communities associated with two plant species growing on an oil sands reclamation area. In addition, this study investigated the influences of host plants and landforms on the bacterial community composition and structure. Specifically, this study aimed to determine whether soil or plant specific factors were the main source influencing bacterial colonization in these plants.

4.4 Materials and Methods

4.4.1 Sample collection and processing

Annual barley (*Hordeum vulgare*), as a planted species, and white sweet clover (*Melilotus albus*), as an unplanted native species, were collected at an oil sands reclamation area of approximately 2.2 km^2 near Fort McMurray, Alberta, Canada. Three biological replicates of each plant and attached rhizosphere soil (0-20 cm depth) were collected at different slope

positions along two transects (20 sampling locations) previously mentioned in Chapter 3. The first transect consisted of 10 sampling locations (S1-S10) in the standard cover, which is a cover management area consisting of a 50 cm of peat mineral mixture on the surface of 100 cm of tailing sands. The second transect also consisted of 10 sampling locations (E1-E10) in the engineered cover, an area of 50 cm of a peat mineral mixture on top of 120 cm of tailing sands separated from the bottom 30 cm of tailing sands by a geo-clay liner (GCL). The main objective of the GCL added by the industry is to retain the moisture on the top of the cover to improve plant growth and to prevent seepage from compounds on the bottom of the tailing sands from reaching the surface of the plant cover. Samples were collected during the summer of 2014, transported at 4 °C and stored at -20 °C until processing within the next 48 h. Soil samples were analyzed for soil total organic (TOC) and total carbon (TC) by the method from Dhillon *et al.* (2015) using a LECO C632 Analyzer (LECO Corporation, St. Joseph, MI, United States). Soil organic Matter (OM), was analyzed using the dry-ash method (McKeague, 1978). Soil pH was measured in a 1:2 soil: water slurry. Soil available ammonium was extracted using a 2 N KCl solution, NH_4^+ in the extract was mixed with hypochlorite and salicylate to form indophenol which was determined colorimetrically at 660 nm (Laverty and Bollo-Kamara, 1988). Soil available nitrate was extracted using a calcium chloride solution and determined colorimetrically at 520 nm according to Laverty and Bollo-Kamara (1988). Available phosphorus and potassium were measured using a modified Kelowna extraction (Qian *et al.*, 1994) and available sulfate by a calcium chloride extraction (McKeague, 1978) (Chapter 3).

4.4.2 Survey of rhizosphere and endophytic bacteria community

Plant roots (entire root system) and adhering soil (5 g) were placed into an Erlenmeyer flask containing 195 mL of phosphate-buffered saline (PBS) (1.2 g of $Na_2HPO_4 \cdot L^{-1}$, 0.18 g of $NaH_2PO_4 \cdot L^{-1}$, 8.5 g of $NaCl \cdot L^{-1}$) buffer and shaken on a rotary shaker (150 rpm) at 22 °C for 25 min. After shaking, the remaining slurry was transferred to a 50 mL Falcon centrifuge tube and centrifuged at 2,000 *g* for 5 min. The supernatant containing PBS buffer was discarded and the rhizosphere soil stored at -80 °C for DNA extraction (Dunfield and Germida, 2003). Root material was recovered and transferred into an Erlenmeyer flask containing 100 mL NaClO (1.05% $v \cdot v^{-1}$) in PBS and placed on a rotary shaker (150 rpm) at 22 °C for 15 min. To remove the remaining NaClO solution, roots were rinsed 10 times with sterile water and 0.1 mL of the final wash was spread on Trypticase soy agar (TSA) plates to check for contamination (Siciliano

and Germida, 1999). In addition, a PCR was conducted on the final wash using the 520F/799R2 bacterial primers to ensure root sterilization. Sterile roots were chopped aseptically and stored in sterile tubes at -80 °C for DNA extraction. Root nodules from sweet clover plants were removed prior to DNA extraction.

4.4.3 DNA extraction

Total endophytic community DNA was extracted from surface disinfected root samples using the PowerPlant® Pro DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) and the rhizosphere soil community DNA was extracted using the MoBio PowerSoil® extraction kit (MoBio Laboratories Inc., Carlsbad, CA). DNA exactions were conducted following the manufacture's protocols. The DNA yield was quantified using Qubit® Fluorometric Quantitation (Invitrogen) and in a SYBR® Safe (Invitrogen) 1% agarose gel by comparison with a high DNA mass ladder (Invitrogen) using a Bio-Rad Gel Doc XR System (Bio-Rad Laboratories, Mississauga, ON).

4.4.4 High-throughput 16S rRNA amplicon sequencing

To determine the diversity and bacterial community composition in the endosphere and the rhizosphere, DNA samples were submitted for high throughput sequencing at McGill University and Génome Québec Innovation Centre using Illumina technology. The primer set and PCR protocol used were as described in Edwards *et al.* (2007). Briefly, PCR amplifications were conducted using the 520F (5`-AGCAGCCGCGGTAAT-3`) /799R2 (5`-CAGGGTATCTAATCCTGTT-3`) primer set that amplifies the V4 region of the 16S rRNA gene. Amplicons with attached Illumina flow cell adapter sequences were added in Illumina MiSeq 2.0 platform in equimolar concentrations. Sample libraries were prepared according to the MiSeq reagent kit preparation guide (Illumina, San Diego, CA), and the sequencing protocol from Caporaso *et al.* (2010b).

4.4.5 Bioinformatics and statistical analysis

Sequence reads were analyzed using Mothur v. 1.36.0 (Kozich *et al.*, 2013) and the MiSeq standard operating procedure developed by the same laboratory. This analysis process involves the formation of contigs, removal of error sequences and chimera removal. High-quality reads were down-sampled to the smallest sample size and classified with naïve Bayesian

classifier implemented in MOTHUR (classify.seqs) using SILVA taxonomy provided by the authors. Sequences from chloroplasts, archaea, eukaryotic organisms were also removed before taxonomic classification. All operational taxonomic units (OTUs) were clustered at a cutoff of 0.03 (97% similarity). Rarefaction curves values and Simpson diversity were also generated using Mothur software. Chao1 richness, Shannon diversity and principal coordinate analysis (PCoA) were performed using QIIME (Quantitative Insights Into Microbial Ecology) 1.9.1 (Caporaso *et al.*, 2010a). Heatmap and ternary plots were conducted using by R v.2.15.2 (R Foundation for Statistical Computing; available at http://www.R-project.org) using the VEGAN package (version 2.0–7) and ggtern (version 2.1.4) respectively. Analysis of variance followed by Tukey post hoc test and Spearman's rank correlations were performed using SAS v 9.3 (SAS Institute Inc., Cary, NC, United States).

4.4.6 Data deposition

Metagenomic datasets were deposited in the NCBI sequence read archive (SRA) under the submission ID SUB2526072. The metagenomic project can also be accessed in NCBI under GenomeProject ID 381225 (accession PRJNA381225, http://www.ncbi.nlm.nih.gov/bioproject/381225).

4.5 Results

After quality filtering, all OTU sequences assigned to chloroplast and mitochondrion origins were removed from the dataset according to Caporaso *et al.* (2010b). Taxonomy was assigned to bacterial OTUs against a subset of the Silva database resulting in in the recovery of 5,013,100 sequences and 13,107 unique OTUs (3% dissimilarity) across 120 endophytic and 120 rhizosphere bacterial community samples (Appendix B).

The bacterial community consisted of 19 different phyla; however, for most bacterial communities analyzed, only four different phyla represented at least 80% of the profile. *Proteobacteria* and *Actinobacteria* were the most abundant phyla observed in all samples analyzed (Figure 4.1). In barley plants, *Proteobacteria* represented on average 56% of the endosphere and 49% of the rhizosphere community, whereas they represented 84% and 69% for clover plants, respectively. Although *Proteobacteria* was more abundant in the endosphere, the *Actinobacteria* relative abundance was 24% higher in the rhizosphere for barley and 1.6-fold

higher for clover. At phylum level, the two plants analyzed harboured different bacterial communities. Whereas endophytic profiles for barley plants indicated high abundance of *Tenericutes*, sweet clover plants harboured a low abundance of this phylum. *Tenericutes* corresponded to 12% of barley endosphere profiles whereas less than 1% in the rhizosphere and in both rhizo-compartments (endosphere and rhizosphere) of sweet clover plants. However, sweet clover associated bacterial communities also indicated a higher abundance of *Firmicutes* when compared with barley. Overall the rhizosphere communities showed similar profiles between the two plant species analyzed. In addition, although soil physical and chemical properties indicated differences between sampling locations, no significant strong correlations were observed between the most abundant bacterial endophyte phyla and soil chemical parameters (Table 4.1). However, in rhizosphere communities, the phyla *Actinobacteria* indicated significant positive correlations with organic matter ($R^2 = 0.434$, $p \leq 0.01$) total organic carbon ($R^2 = 0.370$, $p \leq 0.05$), total carbon ($R^2 = 0.348$, $p \leq 0.05$), available ammonium ($R^2 = 0.347$, $p \leq 0.05$) and nitrate ($R^2 = 0.351$, $p \leq 0.05$) (Table 4.2). The phylum *Nitrospira*, although not highly abundant in the rhizosphere compared to *Actinobacteria*, indicated similar positive correlations with soil parameters. *Nitrospira* indicated positive correlations with organic matter ($R^2 = 0.595$, $p \leq 0.01$) total organic carbon ($R^2 = 0.381$, $p \leq 0.05$), total carbon ($R^2 = 0.382$, $p \leq 0.05$), available ammonium ($R^2 = 0.480$, $p \leq 0.05$) and nitrate ($R^2 = 0.452$, $p \leq 0.05$).

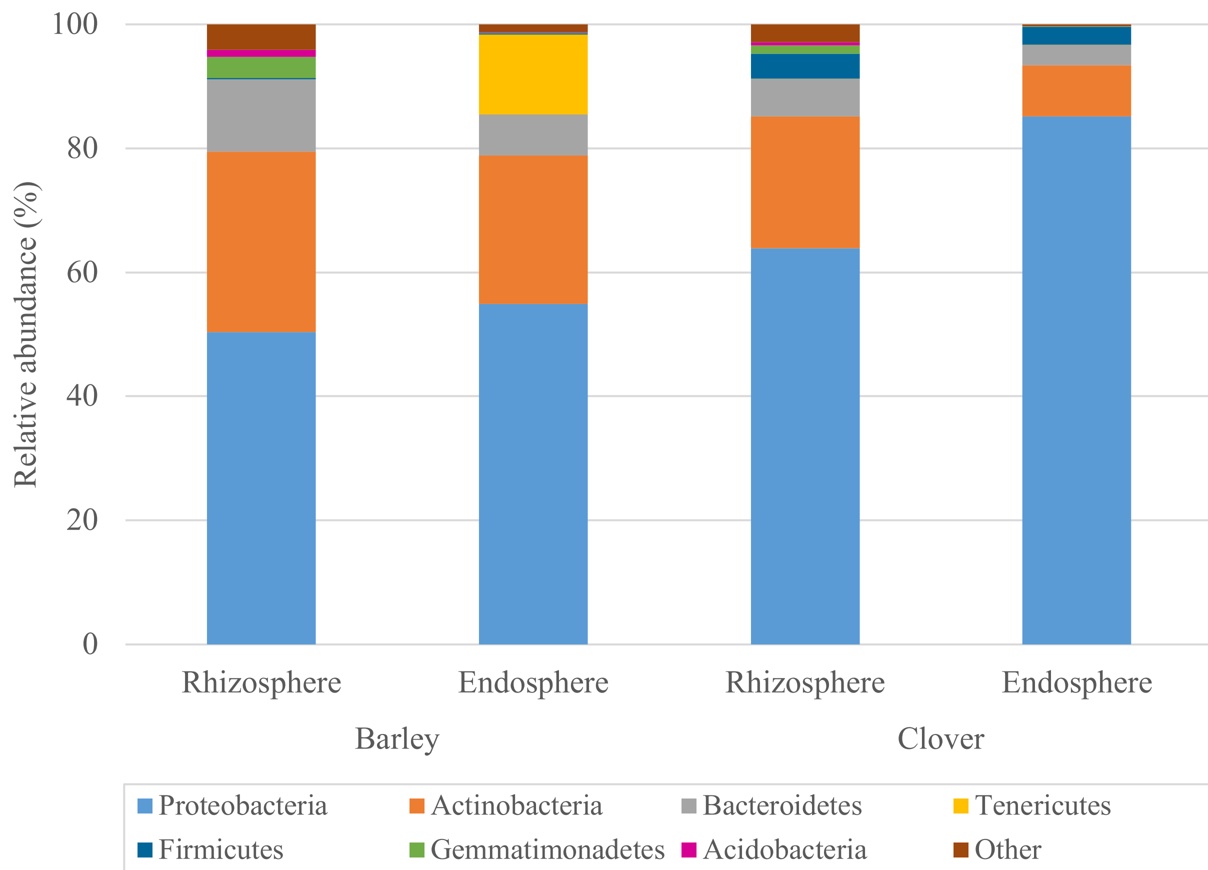

Figure 4.1 Analysis of root associated bacterial communities (endosphere and the rhizosphere compartments) at phylum level in barley and sweet clover growing in oil sands reclamation areas.

Table 4.1 Correlation indicating Spearman's R^2 values relating endophytic bacterial phyla and soil parameters. Statistically significant correlations are indicated in bold type, * and ** denote p values ≤ 0.05 and ≤ 0.01, respectively. OM = organic matter, TOC = total organic carbon, TC = total carbon and EC = electrical conductivity.

	pH	OM	TOC	TC	NH_4^+	NO_3^-	SO_4^{2-}	PO_4^{2-}	K^+	EC
Proteobacteria	0.093	0.044	-0.024	-0.015	0.151	0.105	0.112	-0.216	0.091	0.052
Actinobacteria	0.013	0.031	-0.147	-0.162	-0.140	0.028	-0.085	0.007	-0.158	0.132
Bacteroidetes	0.060	-0.050	-0.146	-0.148	-0.093	0.033	0.309	-0.051	0.175	0.160
unclassified	-0.148	0.003	-0.195	-0.202	-0.064	0.033	0.062	0.033	0.070	0.006
Gemmatimonadetes	-0.120	0.192	-0.139	-0.154	0.037	0.161	0.106	-0.172	0.143	0.164
Firmicutes	0.142	0.009	-0.195	-0.202	0.044	0.143	-0.066	-0.215	-0.244	0.167
Acidobacteria	0.060	0.107	-0.059	-0.066	0.014	0.287	0.119	-0.114	**0.350***	0.102
Verrucomicrobia	0.019	-0.134	-0.268	-0.276	-0.266	-0.011	0.097	0.069	0.292	0.138
Tenericutes	-0.148	0.085	-0.021	-0.016	0.022	0.063	-0.031	0.124	-0.144	-0.115
Armatimonadetes	-0.064	0.045	-0.009	0.005	0.017	0.106	0.069	0.023	0.167	0.076
Nitrospira	-0.089	0.266	0.247	0.246	0.243	0.132	0.076	-0.036	**0.318***	-0.186
Chlamydiae	-0.094	-0.055	0.115	0.137	-0.006	0.110	0.145	0.109	0.116	-0.045
Chloroflexi	0.007	0.118	-0.078	-0.091	0.063	0.081	0.097	-0.193	0.002	-0.055
Planctomycetes	0.004	-0.049	-0.017	-0.017	-0.055	-0.170	0.087	-0.126	-0.076	-0.002
TM7	0.027	-0.014	0.037	0.050	-0.043	0.019	0.127	-0.012	0.179	-0.087
Spirochaetes	0.016	-0.183	0.047	0.047	-0.243	-0.063	-0.067	0.176	0.035	-0.095
Deinococcus–Thermus	-0.008	0.087	-0.003	-0.003	**0.340***	0.105	0.116	**-0.328***	-0.057	-0.180
Fusobacteria	-0.209	-0.125	-0.208	-0.236	-0.209	-0.153	0.042	0.236	0.097	-0.266

Table 4.2 Correlation indicating Spearman's R^2 values relating rhizosphere bacterial phyla and soil parameters. Statistically significant correlations are indicated in bold type, * and ** denote p values ≤ 0.05 and ≤ 0.01, respectively. OM = organic matter, TOC = total organic carbon, TC = total carbon and EC = electrical conductivity.

	pH	OM	TOC	TC	NH_4^+	NO_3^-	SO_4^{2-}	PO_4^{2-}	K^+	EC
Proteobacteria	0.134	-0.115	-0.027	-0.036	-0.034	-0.054	-0.103	0.122	0.092	-0.113
Actinobacteria	-0.098	**0.434****	**0.370***	**0.348***	**0.347***	**0.351***	-0.237	-0.155	0.072	-0.124
Bacteroidetes	0.169	-0.019	-0.025	-0.026	0.065	-0.027	0.070	-0.191	0.077	0.039
unclassified	0.064	0.030	-0.023	-0.008	0.107	-0.058	0.206	-0.240	0.040	0.040
Gemmatimonadetes	0.067	0.039	-0.043	-0.020	0.026	0.108	0.177	-0.183	-0.126	0.206
Firmicutes	0.073	0.023	-0.108	-0.113	0.163	-0.050	-0.017	-0.044	-0.031	-0.195
Acidobacteria	-0.068	0.156	0.167	0.184	0.158	0.002	0.060	0.021	0.137	-0.154
Verrucomicrobia	0.086	0.090	0.006	0.025	0.269	0.034	0.173	**-0.317***	0.002	0.014
Tenericutes	0.254	-0.259	-0.209	-0.202	-0.050	-0.212	0.194	-0.097	-0.096	0.082
Armatimonadetes	0.158	-0.063	0.041	0.062	0.068	-0.035	0.220	-0.106	0.184	-0.087
Nitrospira	-0.323	**0.595****	**0.381***	**0.382***	**0.480****	**0.452***	-0.249	-0.134	-0.009	-0.275
Chlamydiae	0.045	0.040	0.140	0.129	0.065	0.064	-0.013	-0.082	0.059	0.015
Chloroflexi	-0.022	-0.070	-0.101	-0.097	-0.104	-0.126	0.289	-0.097	0.036	0.102
Planctomycetes	-0.142	0.119	0.021	0.018	-0.036	0.057	0.045	-0.003	0.055	0.026
Chlorobi	0.006	**0.325***	0.205	0.205	0.308	**0.344****	-0.220	-0.220	-0.145	0.153
TM7	-0.034	0.063	0.101	0.102	0.055	0.121	0.210	0.276	0.238	-0.290
Spirochaetes	0.028	-0.199	-0.074	-0.093	0.007	-0.178	0.162	-0.019	0.180	-0.278

A Venn diagram revealed that 1,132 OTUs (which represents 8% of the total number of OTUs) were common to all endophytic and rhizosphere bacterial communities (Figure 4.2). However, 1,717 OTUs (13% of total number of OTUs) were shared only in the different rhizosphere communities and only 57 OTUs (0.4% of total) were shared between endophytic communities. The number of shared OTUs among the two endophytic communities was also the lowest number of OTUs shared between communities. In addition, a total of 8,586 OTUs were unique for rhizosphere samples whereas 1,459 OTUs were unique to the endosphere. As expected, the rhizosphere harboured most of the unique OTUs, in which 4,145 and 2,724 where associated only with barley and sweet clover plants, respectively. Within endophytic communities, 532 OTUs were unique for clover plants and 870 for barley.

Similar to Venn diagram, PCoA also indicated differences between bacterial communities (Figure 4.3). PCoA resulted in a 3-dimensional solution in which, PC1 accounted for 9.55% of the variation and PC2 and PC3 for 12.38 and 27.17% respectively. Based on the different communities, rhizosphere samples were clustered in two regions, one which corresponded to sweet clover associated rhizosphere soil only and another with both barley and sweet clover rhizosphere soil. Overall, results indicate a clear division between the endosphere and rhizosphere compartments. Endophytic communities however were more variable between sampling locations when compared to the rhizosphere. In addition, sweet clover endosphere compartments indicated a higher variation among samples when compared to barley. Although clustering was observed based on plant species, no clustering was observed based on cover type and slope positions (Figure 4.4).

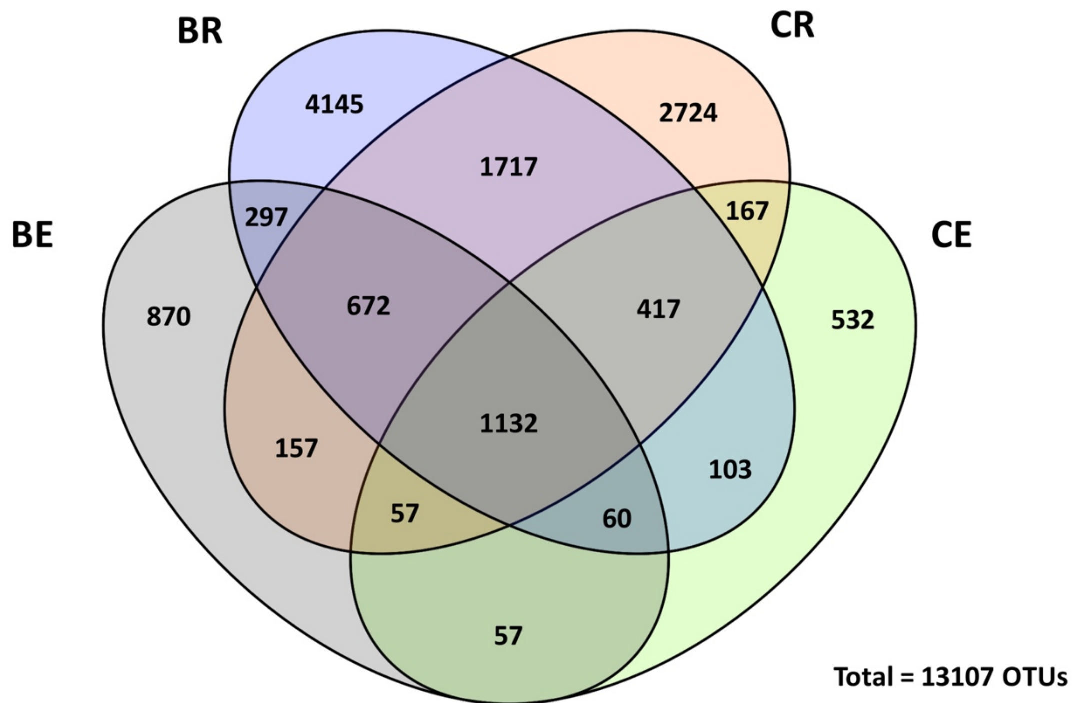

Figure 4.2 Venn diagram for endosphere (BE) and rhizosphere (BR) bacterial communities associated with barley and endosphere (CE) and rhizosphere (CR) bacterial communities associated with sweet clover. Numbers indicated shared unique OTUs at 0.03 dissimilarity distances after removing singletons involved.

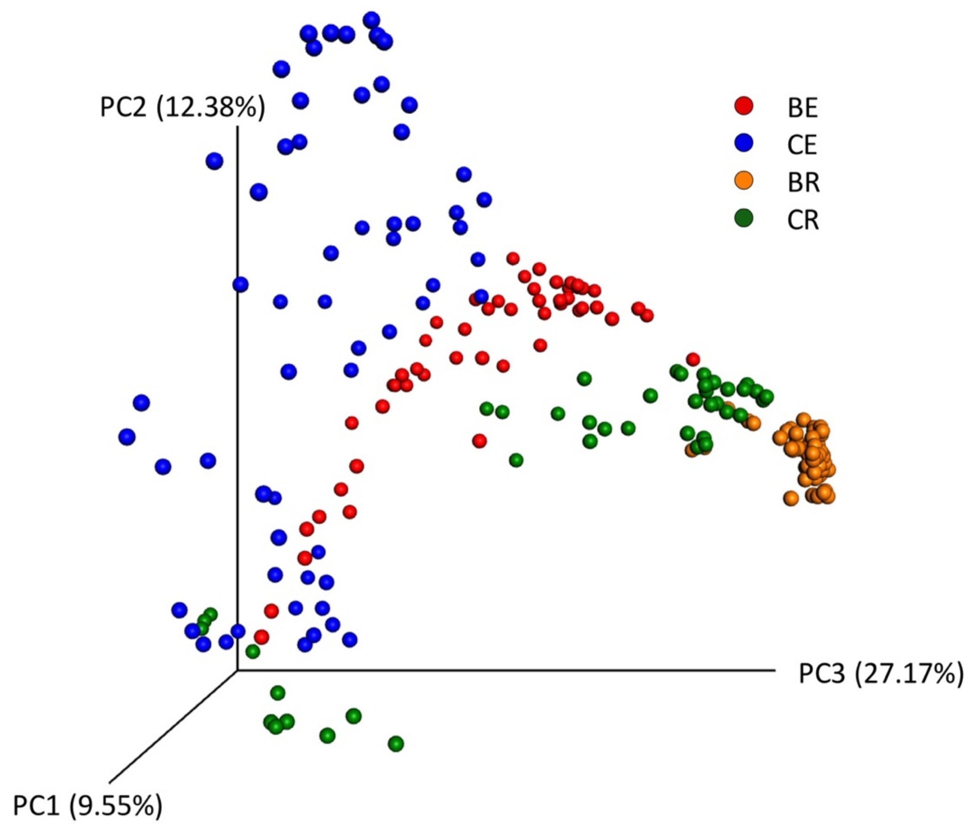

Figure 4.3 Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity between samples for barley endosphere (BE), clover endosphere (CE), barley rhizosphere (BR) and clover rhizosphere (CR).

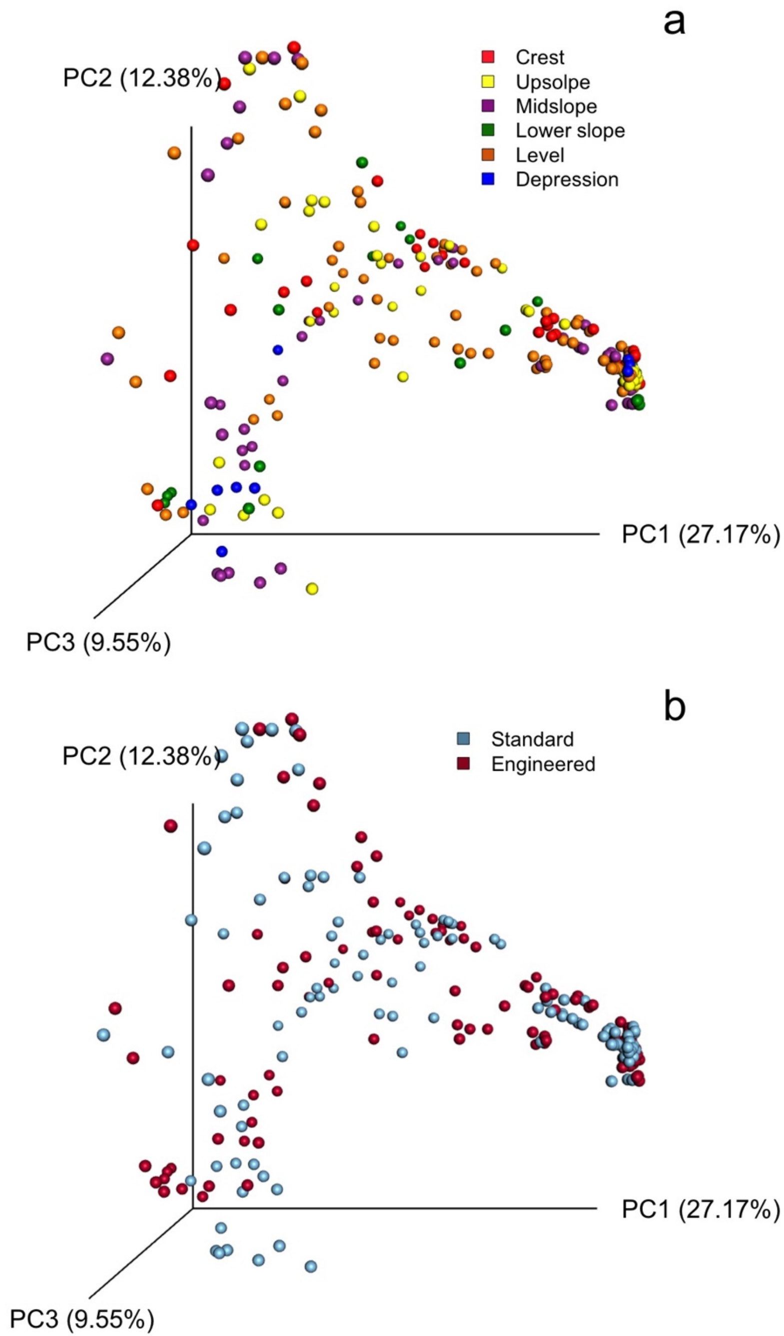

Figure 4.4 Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity between samples at different slope positions (a) and cover managements (b).

Based on shared OTUs between different communities (Figure 4.2) and on PCoA (Figure 4.3), the results in this study indicate that although there are differences in the rhizosphere compartments among the two plants analyzed, most of the differences were within the endosphere. Therefore, the data in this study was analyzed using ternary plots based on three main environments: (*i*) the soil rhizosphere microbiota of both plants, (*ii*) the endosphere compartment of sweet clover and (*iii*) the endosphere of barley (Figure 4.5, Figure 4.6). According to the most abundant families (Figure 4.5), barley plants harboured a high abundance of *Xanthomonadaceae*, whereas clover plants had a high abundance of *Rhizobiaceae*. Genera from the family *Enterobacteriaceae* were mostly associated with the endophytic communities, whereas *Pseudomonadaceae*, *Sphingomonadaceae* were associated with both the rhizosphere and endosphere bacterial communities. A ternary plot was used to assess in which compartment each genus is most abundant or restricted (Figure 4.6). Here, each genus was categorized based on whether there was a 10% increase or decrease on its relative abundance in endosphere compared with rhizosphere. The results in the current study suggest that only a few genera were restricted by rhizo-compartment, since most of genera can be found in both compartments. Interestingly, barley was more effective at recruiting bacterial genera to its rhizosphere and endosphere than clover.

Chao richness and Shannon diversity indices indicated significant differences between rhizo-compartments (Table 4.3). Both indexes show a lower diversity and richness in the endophytic communities. Additionally, barley rhizosphere and endosphere microbiota had higher richness and diversity when compared to sweet clover.

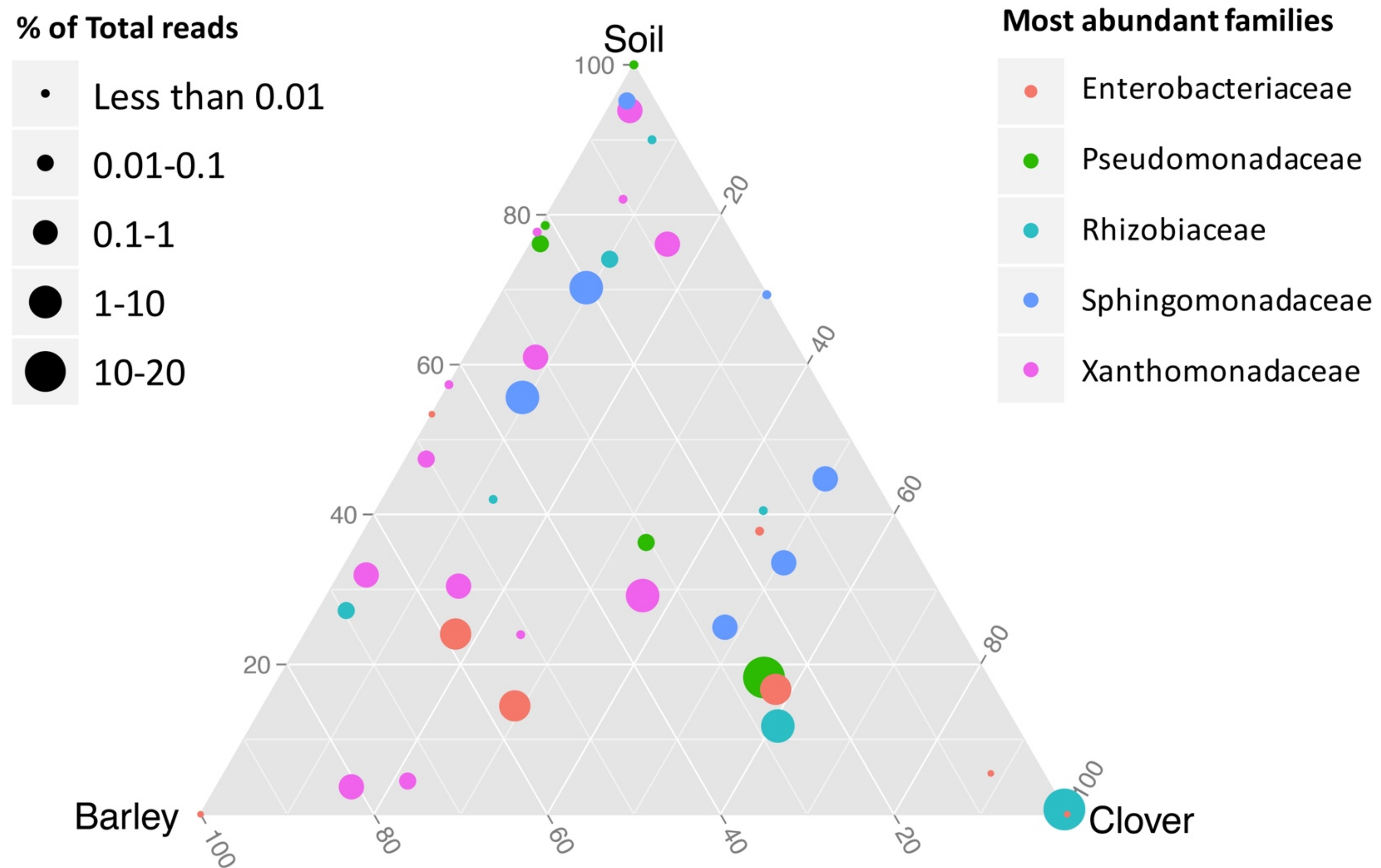

Figure 4.5 Ternary plot representing the relative occurrence of individual genus (circles) that are members of the five most abundant families in root samples of sweet clover and barley compared with rhizosphere soil. Genera enriched in different compartments are colored by taxonomy of the most abundant families. The size of the circles is proportional to the mean abundance in the community.

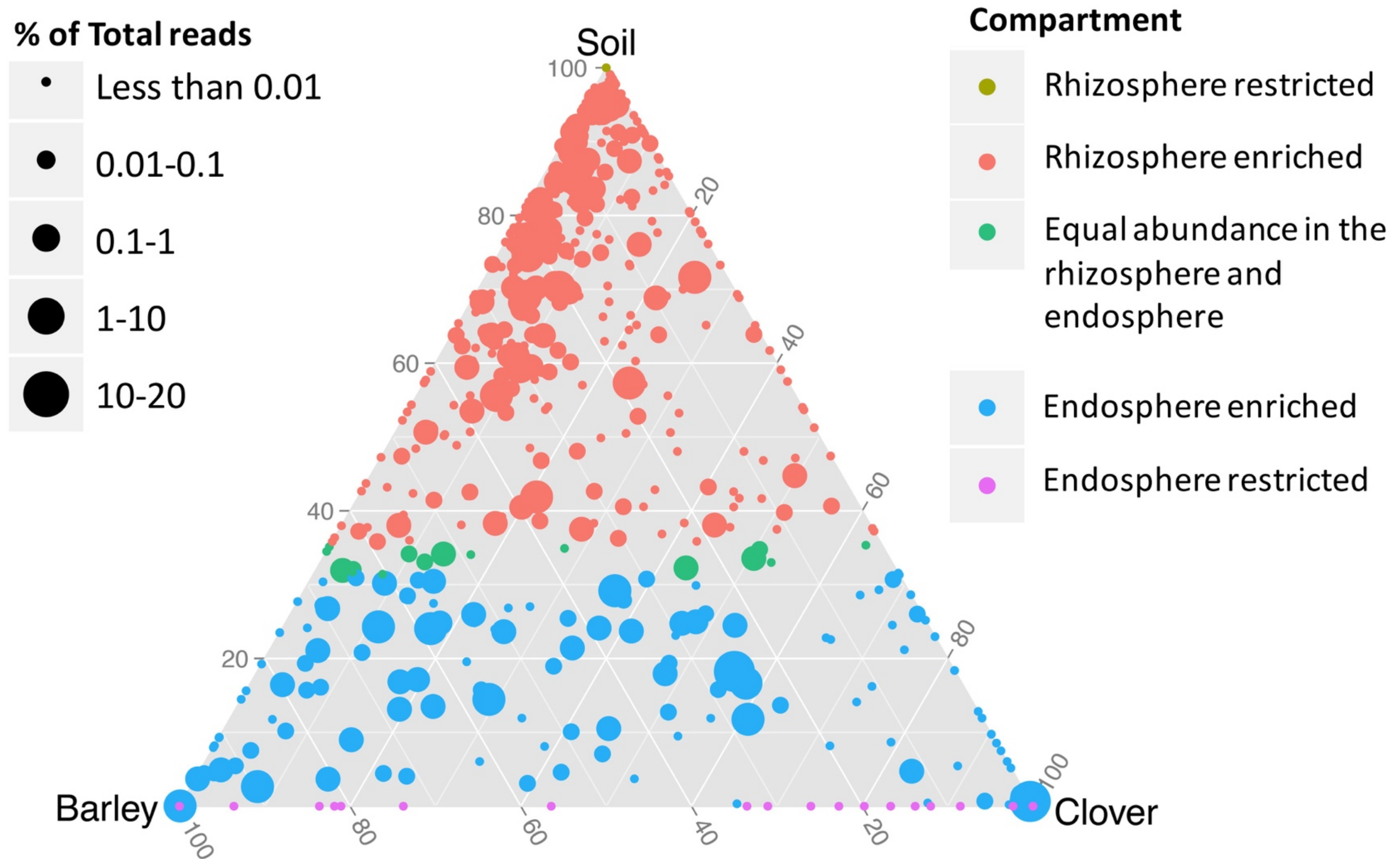

Figure 4.6 Ternary plot representing the relative occurrence of individual genus (circles) in root samples of sweet clover and barley compared with rhizosphere soil. Genera enriched in different compartments are colored according to habitat in which each genus is most frequently associated based on at least 10% enrichment or depletion of soil microbiota in the endosphere. The size of the circles is proportional to the mean abundance in the community.

Table 4.3 Alpha richness and diversity of endophytic and rhizosphere communities associated with barley and sweet clover plants. Different letters indicate significant differences (Tukey HSD $p \leq 0.05$).

		Chao	Shannon
Rhizosphere	Barley (BR)	714.9a	4.90a
	Clover (CR)	611.6b	3.73b
Endosphere	Barley (BE)	244.0c	3.00c
	Clover (CE)	136.4d	1.91d

To investigate the main genera driving differences in the endophytic communities, a heat map using hierarchical cluster based on Bray–Curtis distance was generated using the 2% most abundant genera (Figure 4.7). The results in this study suggest that bacterial profiles mainly clustered by plant species and not by cover type or by different slope positions.

Six main clusters were observed after a 70% dissimilarity cut off between endophytic community profiles. Based on the cluster profiles, indicator species analysis was conducted to confirm the main genera influencing differences in the bacterial community. The first cluster (A) (left to right) consisted on barley endophytic communities from one sampling location at the upslope in the standard cover. This was the smallest cluster detected and on average the endophytic profiles consisted mainly on *Yersinia* (57%), *Pseudomonas* (7.5%), *Lentzea* (5.6%), *Rhizobium* (2.28%) and *Sphingmononas* (1.49%). The main indicator genus of this cluster was *Yersinia*, which in some samples corresponded to 60% of the profile. Cluster B was limited to barley endophytic communities only, in this cluster the most abundant genera were *Acholeplasma* (46.8%), unclassified genus from the family *Enterobacteriaceae* (8.5%), *Lentzea* (4.3%), *Pseudomonas* (3.1%) and *Amycolatopsis* (2.7%). *Acholeplasma* was the indicator genus for this cluster, which in some samples it could represent up to 80% of the profile. Cluster C corresponded mainly to *Lentzea* (10.4%), *Pseudomonas* (6.4%), unclassified genus from the family *Microbacteriaceae* (4.3%), *Rhizobium* (4.3%) and *Acholeplasma* (3.88%). In this cluster *Lentzea* was the indicator genus for this cluster which it could represent up to 50% of the bacterial profile in some samples. In addition, cluster C was one of the clusters that represented most of the endophytic profiles for barley plants. With the exception of a few samples, no particular genus corresponded to more than 50% of the community structure in this cluster. Cluster D consisted of 76% of sweet clover plants and 24% of barley. On average the most

abundant genera in this cluster were *Pseudomonas* (50.1%), *Sinorhizobium* (7.8%), *Pantoea* (7.5%), *Enterococcus* (4.3%) and *Arthrobacter* (3.8%). *Pseudomonas* was also detected as the indicator genus of cluster D. Cluster E was detected as a small cluster limited to sweet clover plants with *Pantoea* as the indicator genus. On average, this cluster mainly consisted by *Pantoea* (45.7%), *Sinorhizobium* (13.4%), *Pseudomonas* (6.87%), *Xanthomonas* (6.71%) and *Rhizobium* (6.61%). Cluster F also was limited to sweet clover plants, however with a dominant *Sinorhizobium* endophytic community. *Sinorhizobium* was the main indicator genus of this cluster and it represented up 90% of the bacterial profile in some samples. The most abundant genera of this cluster were *Sinorhizobium* (44.2%), *Rhizobium* (12.9%), *Pseudomonas* (6.2%), *Hansschlegelia* (2.9%) and unclassified genus from the family *Comamonadaceae* (2.1%).

To investigate rhizosphere associated bacterial communities, a heat map was also generated using hierarchical cluster based on Bray–Curtis distance (Figure 4.8). Rhizosphere bacterial profiles also clustered mainly on different plant species. As expected, clustering dissimilarities were much lower when compared to endophytic communities. Three main clusters were observed after a 40% dissimilarity cut off. Cluster A was limited to sweet clover plant species only, the most dominant genus in this profile is *Pseudomonas* (58%), followed by *Arthrobacter* (12.3%) and *Pantoea* (9.1%). Cluster B also was limited to sweet clover species; however, this cluster was mainly detected by an equal abundance of unclassified genus from the order *Bacillales* (17%) *Pantoea* (15%) and *Acinetobacter* (14%). However, most of rhizosphere profiles were represented in cluster C and interestingly all barley profiles were on this cluster. In cluster C, the most abundant genera detected were *Arthrobacter* (6.5%), *Sphingomonas* (5.3%) and an unclassified genus from the order *Rhizobiales* (4.6%). Also, in this cluster none of the genera identified represented more than 40% of the profile and no dominant genus could be observed. This cluster also included a group of sweet clover associated rhizosphere bacterial profiles mainly differentiated from other samples within the cluster by the abundance of *Stenotrophomonas* (9.1%).

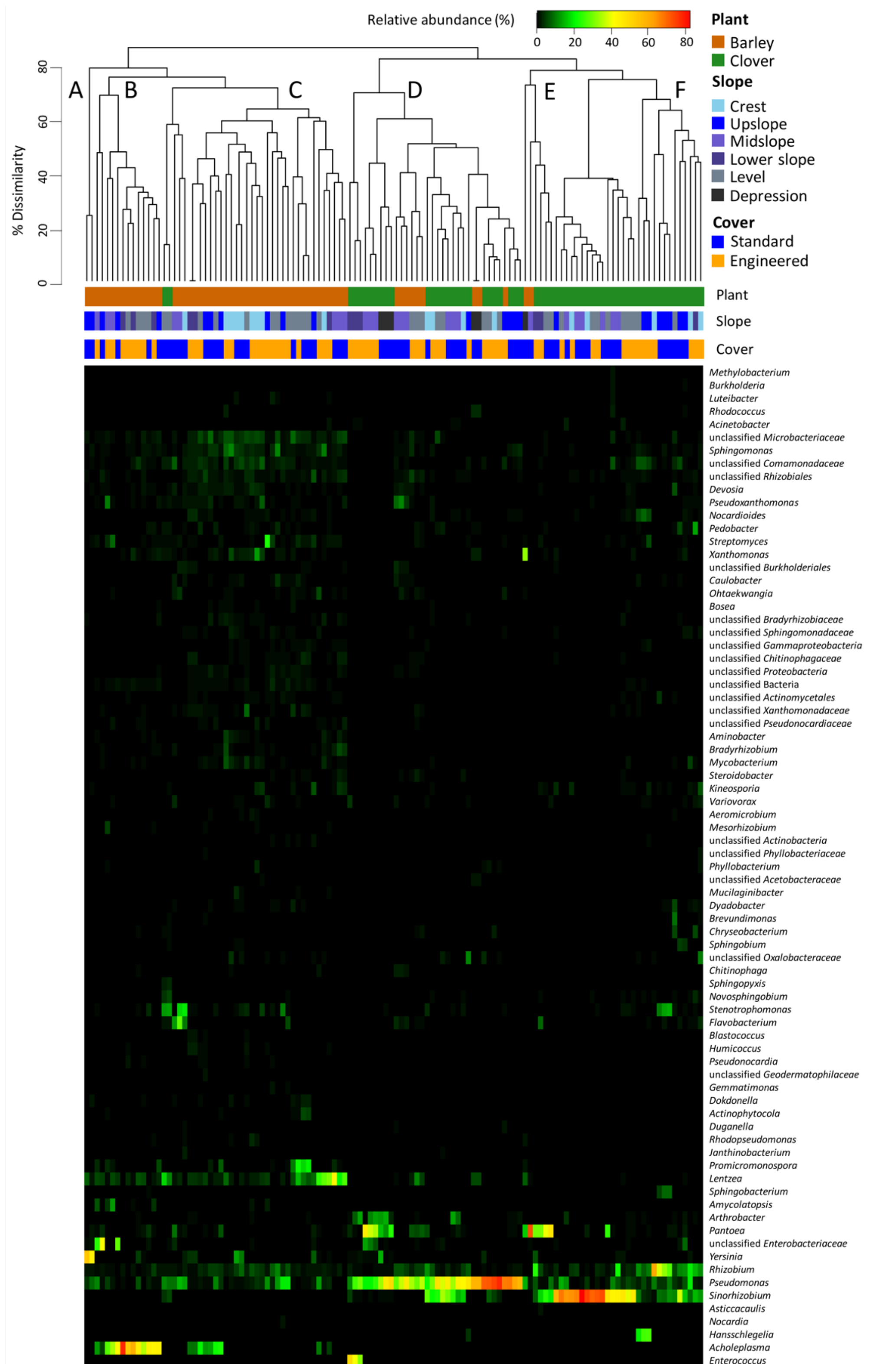

Figure 4.7 Heatmap based on relative abundance of sweet clover and barley associated endophytic communities. Vertical columns represent samples; horizontal rows represent genera that are 2% most abundant in at least one sample. Clustering of samples (top) is based on genera co-occurrence by Bray-Curtis dissimilarity. Letters (A-F) indicate different clusters at a 70% dissimilarity cut off.

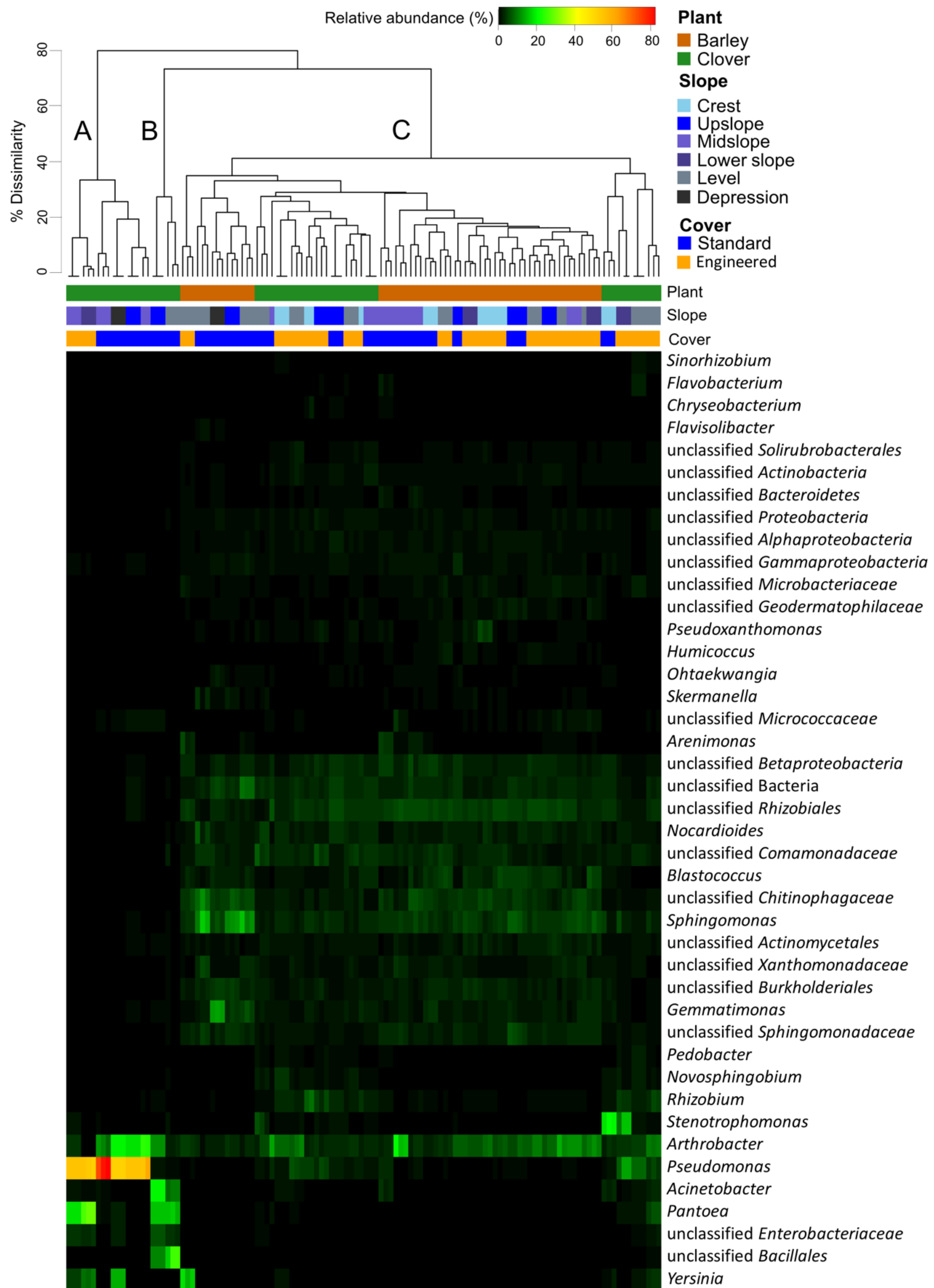

Figure 4.8 Heatmap based on relative abundance of sweet clover and barley associated rhizosphere communities. Vertical columns represent samples; horizontal rows represent genera that are 2% most abundant in at least one sample. Clustering of samples (top) is based on genera co-occurrence by Bray-Curtis dissimilarity. Letters (A-C) indicate different clusters at a 40% dissimilarity cut off.

4.6 Discussion

The data presented here provides new insights on plant microbe interactions in reclamation sites, as most previous studies have focused on the microbial communities in tailing ponds (Yergeau *et al.*, 2012a) and on the overall soil microbial biomass in oil sands reclamation sites (MacKenzie and Quideau, 2010). Furthermore, very few studies focused on endophytic communities in oil sands reclamation covers using culture-independent methods (Lefrançois *et al.*, 2010). In an attempt to unravel the root associated bacterial microbiome of plants growing in reclamation soils, 16S rRNA high-throughput amplicon sequencing was used to characterize endophytic and rhizosphere bacterial communities associated with two plant species in one of the Athabasca oil sands reclamation sites. However, a common challenge in the assessment of bacterial endophytes using molecular methods has been amplification of plant chloroplast 16S rRNA gene by universal bacterial primers (Chelius and Triplett, 2001). Therefore, in order to reduce the number of plant chloroplast sequences, a primer set previously designed by Edwards *et al.* (2007) was used as they have been proven to be successful in removing these sequences.

Illumina MiSeq of PCR amplicons and sequence analyses revealed that both endophytic and rhizosphere bacterial profiles varied considerably across the different sampling locations. The data in this study also suggest that changes in the microbiome are mainly due to different rhizo-compartments (rhizosphere and endosphere) and host plants. Similar results were observed by Ofek-Lalzar *et al.* (2014), who studied the rhizoplane bacterial communities associated with wheat and cucumber and found that variability was correlated with rhizo-compartment at a higher extent and different host plant at a lesser extent. In the current study, it was observed a lower diversity in the endosphere compartment compared to the rhizosphere. Hence, as previously reported in Germida *et al.* (1998) and Edwards *et al.* (2015), the data presented here also suggests that endophytic root colonization is not a passive process and that both sweet clover and barley plants have the ability to select for certain soil microbial consortia. The enrichment for a subset of selected dominant phyla in the endosphere compartment was also consistent with Shannon diversity and the Chao richness analysis. Siciliano and Germida (1999) also reported changes in the abundance of certain genera in the endosphere when compared to the rhizosphere and a lower diversity in the endosphere.

Differences in bacterial community profiles in this study were detected at a broad taxonomic level such as at the phylum level. The results presented here indicate that there was an increase in the relative abundance of *Proteobacteria* in the endophytic community of both plants when compared to the rhizosphere. *Proteobacteria* were previously described as effective rhizosphere and root colonizers in several plants such as rice (Edwards *et al.*, 2015), smooth cordgrass (Hong *et al.*, 2015) and wheat (Ai *et al.*, 2015) due to their high ability to utilize root exudates (Fierer *et al.*, 2007). *Proteobacteria* are known to respond rapidly to carbon sources, and are generally considered to be r-strategists and fast-growing bacteria (Peiffer *et al.*, 2013). The enrichment of *Proteobacteria spp*. in root compartments, mostly in sweet clover plants, was previously suggested in the literature in tomato (Yao and Allen, 2006) and in grapevine (Zarraonaindia *et al.*, 2015) as a response to chemotaxis via photoassimilates secreted by root cells (Bulgarelli *et al.*, 2013).

Similar to other studies using *Arabidopsis thaliana* (Lundberg *et al.*, 2012) and rice plants (Edwards *et al.*, 2015), the current study revealed that several phyla common in the rhizosphere (*Acidobacteria*, *Verrucomicrobia* and *Gemmatimonadetes*), were almost absent in the endosphere. In fact, the results presented here indicate that relative abundance of *Acidobacteria* is below 1% in endophytic profiles at some sampling locations. However, differently from Edwards *et al*. (2015) and Hong *et al*. (2013), the present study found a high abundance of *Actinobacteria* in all rhizosphere profiles, which is in agreement with Bodenhausen *et al*. (2013) and Sugiyama *et al.* (2014). According to Bulgarelli *et al*. (2013), *Actinobacteria* is considered a specific bacterial taxon that responds favorably to organic carbon substrate addition and the high abundance of this phylum has been observed in both rhizosphere and endosphere compartments of different plant species. Also, differently from the *Arabidopsis thaliana* root microbiome studied in Lundberg *et al*. (2012), the present study found that both sweet clover and barley plants harboured a higher relative abundance of *Firmicutes* in rhizosphere profiles when compared with the endosphere. According to Bulgarelli *et al.* (2013), *Firmicutes* dominate both rhizo-compartments, however the dominance in the endosphere can only be observed in certain plants. Furthermore, it was observed that the phylum *Tenericutes* was only detected in the endosphere compartment of barley plants. *Tenericutes* is a phylum that contains the class Mollicutes, characterized by the absence of a cell wall (Montagna *et al.*, 2015).

Recently, Rivera-Tapia *et al*. (2002) have reported that *Tenericutes* are known to colonize the gut of animals, insects and plants.

Although soil chemical analysis revealed significant differences between sampling locations, strong correlations were only observed between these parameters and bacterial phyla in the rhizosphere. Some of these correlations have been previously suggested in the literature, such as positive correlations between soil organic matter, total organic carbon and the abundance of *Actinobacteria* (Li *et al.*, 2012b; Embarcadero-Jiménez *et al.*, 2015). However, in endophytic profiles, the results presented here suggest that these communities may be driven by factors other than the soil chemical parameters analyzed in this study.

The number of shared unique OTUs in shown by Venn diagram suggests that rhizosphere samples contained the majority of OTUs in the dataset, which confirms that soil serves as a primary reservoir for potential endophytes (Germida *et al.*, 1998; Zarraonaindia *et al.*, 2015). Furthermore, differences in rhizo-compartments were also observed in PCoAs. Here, rhizosphere soil samples differentiated from its respective endophytic bacterial communities, as previously reported in the literature (Zarraonaindia *et al.*, 2015; Lundberg *et al.*, 2012). Additionally, bacterial community profiles analyzed by PCoA indicated clustering regions containing a low variability between samples in rhizosphere profiles and a high variability in endophytic profiles. During land reclamation activities, no soil or seed inoculation was conducted, hence bacterial profiles in all rhizo-compartments studied here corresponded to naturally occurring indigenous communities.

Previous studies (Germida *et al.*, 1998; Ofek-Lalzar *et al.*, 2014) suggested that plant factors play a dominating role in the endophytic community composition and bacterial communities vary between plant species. Several studies have suggested that different root exudates produced by different plant species may affect distinct root associated microbial populations (Phillips *et al.*, 2012). Based on these evidences, ternary plots were generated using the mean relative abundance from each genus in each root endosphere compartments and the rhizosphere soil of both plants combined. Here, although plant factors may actively select for certain soil microbial consortia, the results presented here indicate that sweet clover plants were more restrictive when compared to barley. In addition, sweet clover plants were more closely associated with members of the family *Rhizobiaceae* while barley plants harboured a high abundance of *Xanthomonadaceae*. The economic importance of *Rhizobiaceae* and its potential in

nitrogen fixation have been extensively reported in the literature (Ludwig, 1980; Long, 1996) as well as their ability to colonize the root interior of leguminous plants (*e.g*. alfalfa and sweet clover plants) (Bromfield *et al*., 2010). Nitrogen fixers associated with naturally occurring plant species can facilitate vegetation development through addition of atmospheric nitrogen to the system and could alleviate potential nutrient limitation in reclamation areas (Lefrançois *et al.*, 2010). In the dataset of the current study, the family *Xanthomonadaceae* contains the genus *Xanthomonas*, which some are known as plant pathogens (Soares *et al*., 2010), but mainly *Stenotrophomonas*, which are capable of great metabolic versatility and are colonizers of soil and plants (Ryan *et al*., 2009). *Stenotrophomonas* were previously isolated in barley rhizosphere soil (Caesar-TonThat *et al*., 2007), reported as a multifunctional plant growth-promoting rhizobacterium (PGPR) (Alavi *et al*., 2013) and to induce antagonistic behaviour against soil-borne plant pathogens (Dunne *et al*., 1998). The results presented here also suggest a high abundance of the family *Enterobacteriaceae* associated with both endosphere plant compartments. Members of the *Enterobacteriaceae* family are often associated as human pathogens, however this family consists of a large group distributed in many environments (Yousaf *et al*., 2011). *Enterobacteriaceae* are widespread in several plant systems and some have been suggested as beneficial plant-associated bacteria that can promote plant growth (Kämpfer *et al*., 2005) and biocontrol activity (Chernin *et al*., 1995). Therefore, the data presented in the current study strongly suggest that the two plants analyzed supported the enrichment of different bacterial taxa. Alternatively, plant factors such as root exudation, may drive the selection of different bacterial taxa (Phillips *et al*., 2012).

Since most of data presented here indicated differences occurring mainly in endosphere compartments, a heat map analysis was used for a finer and more specific comparison between profiles. The results in this study using heat map also support previous analysis in the dataset in which endophytic bacterial profiles differentiated mainly among plant species. Although these profiles varied within the same plant species at each sampling location, differences in these profiles may be driven to factors other than slope positions or cover managements. Within the endophytic community, *Sinorhizobium*, *Pseudomonas*, *Rhizobium*, *Acholeplasma, Lentzea, Pantoea* and *Yersinia* were the main genera driving these differences. *Sinorhizobium* have been previously reported as nitrogen-fixing bacterial endophytes of alfalfa, beans and sweet clover (Dudeja *et al*., 2012; Bromfield *et al*., 2010). Sweet clover is a fast-growing legume and the

results presented in this study suggest that *Sinorhizobium* corresponds to a significant share of endophytes associated with this plant. *Sinorhizobium* can be free-living in the soil or form nitrogen-fixing nodules on the roots of leguminous plants such as the genera *Melilotus* (Biondi *et al.*, 2009). Sweet clover plants in the current study may rely on the association with *Sinorhizobium* spp. to grow in reclamation soils. However, the high dominance of this genus is not always observed in sweet clover endophytic profiles analyzed in this study. *Pseudomonas* species can successfully colonize both barley and clover plants, although most of the profiles with a high relative abundance of *Pseudomonas* were observed in sweet clover plants. These results were expected, as it was previously observed that *Pseudomonas* ssp. are common colonizers of the plant interior (Moore *et al.*, 2006; Ofek-Lalzar *et al.*, 2014). Although this genus contains pathogenic species, a wide range of *Pseudomonas* ssp. are known for PAH degradation (Germaine *et al.*, 2009), potential heavy metal extraction enhancement (Rajkumar *et al.*, 2009) and PGPR (Bhattacharyya and Jha, 2012). *Rhizobium* species were most commonly found in sweet clover plants and with *Sinorhizobium*, whereas *Acholeplasma* species were restricted to barley plants. *Acholeplasma* are wall-less bacteria from the phylum *Firmicutes* and close relatives of *Phytoplasmas*, whereas *Acholeplasma*s are not known to be pathogenic (Kube *et al.*, 2014). *Acholeplasmas* are also known to colonize the guts and hemolymph of insects (Tully *et al.*, 1988) and transmission to plants occurs when these insects feed on plant tissues (Bonnet *et al.*, 1991). In the current study, barley plants may be more susceptible to insect feeding than clover plants and, hence, a higher incidence of *Acholeplasma* species in barley. Although most of endophytic profiles analyzed corresponded to clusters mainly driven by *Sinorhizobium*, *Pseudomonas*, *Rhizobium* and *Acholeplasma,* it was also observed smaller clusters with a high incidence of *Pantoea, Lentzea* and *Yersinia*. *Pantoea* is a gram-negative bacteria of the family *Enterobacteriaceae* first identified by Gavini *et al.* (1989), which can be human and clinical strains, epi- and endophytes or merely present in water and soil samples (Brady *et al.*, 2008). Previous studies have also demonstrated the application of *Pantoea* in heavy metal biosorption (Ozdemir *et al.*, 2004), plant growth promotion (Feng *et al.*, 2006) and phenolic compounds degradation (Dastager *et al.*, 2009a). Similar to *Pantoea, Lentzea* and *Yersinia* are also members of the family *Enterobacteriaceae*. *Lentzea* is a genus of mesophilic *Actinomycetes* first identified by Yassin *et al.* (1995) and later identified as capable of biodegradation of aliphatic polyester poly(lactide) (Tokiwa and Jarerat, 2004; Jarerat *et al.*,

2002). The genus *Yersinia*, although commonly known as human pathogens (Perry and Fetherston, 1997), consists of 15 species of mostly harmless environmental organisms residing in the plant interior, soil and water (Ayyadurai *et al.*, 2010; Hallmann and Berg, 2006). Lawson and Afenu (2013) have also identified *Yersinia* spp. isolates with potential capabilities of degrading diesel oil. Both *Yersinia* and *Lentze*a were mostly detected in barley endophytic profiles, which may suggest these organisms are adapted to survive as part of their lifecycle in barley, but encounter a less favorable environment in sweet clover.

4.7 Conclusions

This study provided an in-depth analysis of bacterial endophytic profiles of plants growing in oil sands reclamation areas. Consistent with prior findings based on high-throughput amplicon sequencing (Ofek-Lalzar *et al.*, 2014; Bulgarelli *et al.*, 2012), the results presented here confirm that rhizo-compartments produce the strongest differentiation of root associated bacterial communities. In addition, host plants also account as main driving factors affecting the endophytic microbiome. A lower diversity in the endosphere compartment and the depletion or enrichment of certain bacteria strongly suggests that plant factors select for certain soil bacterial consortia. Endophytic profiles studied here also revealed that sweet clover plants were more selective than barley. Whereas members of the family *Rhizobiaceae*, such as *Sinorhizobium* and *Rhizobium* were mainly associated with clover, *Acholeplasma* was unique to barley. *Yersinia* and *Lentzea* were also mostly detected in barley, although *Pseudomonas* and *Pantoea* were able to successfully colonize both plants. Endophytic bacterial profiles also varied within the same plant species at different sampling locations; however, these differences were driven by factors other than the soil parameters analyzed in this study. Future studies will be focused on determining the mechanisms driving root associated communities and functional aspects within this microbiome to improve plant growth in reclamation areas.

5. HYDROCARBON DEGRADING GENES IN ROOT ENDOPHYTIC COMMUNITIES ON OIL SANDS RECLAMATION COVERS

5.1 Preface

An in-depth analysis of barley and sweet clover root associated bacterial profiles at an oil sands reclamation area was provided in Chapter 4. These results suggested that sweet clover plants were more selective than barley in recruiting bacterial endophytes. Although certain endophytes (*e.g. Pseudomonas* and *Pantoea* spp.) colonized both plants, overall, different plants harboured different bacterial communities. Previous research showed that due to water recycling efforts by the oil sands industry, tailings sands used in land reclamation may contain residual hydrocarbons. Hence, plant-microbe associations in these areas may be associated with the hydrocarbon degrading potential within endophytic bacteria. To date, little is known about the hydrocarbon degradation potential in endophytic communities associated with plants growing in oil sands reclamation areas. To address this gap, this study identified the presence and abundance of three hydrocarbon degrading genes (CYP 153, *alkB* and *nah*) in endophytic bacterial communities previously studied in Chapters 3 and 4.

5.2 Abstract

The Canadian oil sands industry has expanded rapidly in the recent years resulting in a large disturbance footprint. The industry is however committed to reclaim such disturbed lands to an equivalent land capability before mining. Overburden materials or tailing sands used in reclamation are not suitable for plant growth due to their low nutrient capabilities and high concentrations of toxic materials including naphthenic acids, polycyclic aromatic hydrocarbons, phenolic compounds and trace metals. Therefore, peat-mineral mix (PMM) is a commonly used cover material in reclamation strategies to regulate water content, increase organic matter content and promote the establishment of microorganisms and vegetation. Whereas chemical fertilization can be used to increase plant growth on a short-term basis, plants growing in this area may rely on their associated microbiota for degradation of potential toxic compounds such as residual

hydrocarbons. Endophytic bacteria are known to ameliorate plant stress and to improve phytoremediation technologies, however little is known about the potential of hydrocarbon degrading endophytic bacteria in oil sands reclamation. In this study, three hydrocarbon degrading genes (CYP 153, *alkB* and *nah*) were quantified in endophytic bacterial communities associated with two plant species growing in an oil sands reclamation area. The aims of this study were to (*i*) to quantify the total hydrocarbon potential of unculturable endophytic bacteria and (*ii*) to assess culturable endophytic bacteria for the presence of hydrocarbon degrading genes. Results revealed higher CYP153 gene copy numbers in sweet clover endophytic communities compared to barley. Conversely, both plants indicate a similar abundance of 16 rRNA, *alkB* and *nah* genes. Analysis of variance indicated significant differences for most variables (cover, slope and sampling locations) and genes analyzed. In addition, results also suggest that total hydrocarbons, pH, soil carbon and nitrogen play an important role in determining hydrocarbon degrading bacterial communities. The assessment of hydrocarbon degrading genes in culturable bacteria previously isolated in Chapter 3 revealed isolates positive for all functional genes analyzed. Out of a total of 316 isolates, 42 isolates were positive for at least one hydrocarbon degrading gene. Most of these isolates were positive for *alkB*, and closely match the database for *Pantoea*, *Pseudomonas* and *Enterobacter* spp. Future studies will investigate plant growth promoting and hydrocarbon degradation effects of these isolates when inoculated to host plants.

5.3 Introduction

The oil sands region in northern Alberta corresponds to the largest known crude bitumen deposits (Audet *et al.*, 2015) and contains the world's second largest petroleum reserve (Wellstead *et al.,* 2016). Oil production in these areas expanded significantly in the last decades and it is projected to double, increasing from 3 million barrels per day in 2010 to over 6 million barrels per day, by 2030 (Audet *et al.*, 2015; Wellstead *et al.*, 2016). Open pit bitumen mining in the oil sands creates landscape scale disturbance which involves a deep and complete removal of vegetation, soil, and subsoil. (Mollard *et al.*, 2013; Beasse *et al.*, 2015). During bitumen extraction, the hot water oil sands treatment produces large volumes of process-affected tailings that are deposited in large basins (tailings ponds) where they are retained until reclamation (Mohamad Shahimin *et al.*, 2016; Dobchuk *et al.*, 2013). These tailing ponds contain oil sands

process water (OSPW) combined sand, slits and clays in suspension as well as soluble organic chemicals such as naphthenic acids (NAs), ammonia, heavy metals and salts (Pouliot *et al.*, 2012; Kovalenko *et al.*, 2013). After settlement, the remaining water in tailing ponds is re-used in bitumen extraction and tailing sands are further used in reclamation strategies (CAPP, 2017).

Reclamation strategies in the oil sands region requires building an ecosystem with equivalent land capability relative to pre-disturbance conditions (Brown and Naeth, 2014). However, tailing sands materials used in reclamation have low nutrient content, high pH, low or no organic matter and contain residual hydrocarbon products (Lefrançois *et al.*, 2010). Due to the nature of the tailings sands, a peat-mineral mix (PMM) is typically used as a surface cover (20 to 50 cm thick organic amendment). Peat is highly available on the mining footprint and it is usually salvaged from nearby upland boreal forests and peatlands drained before the onset of mining (Quideau *et al.*, 2013). Lowland organic peat and underlying mineral soil deposits are then mixed forming a peat-mineral mix (PMM) which is used in reclamation. Peat-mineral mixes have a high organic matter content and a high water holding capacity which may makes it an ideal substrate for seedling establishment (Ojekanmi and Chang, 2014). After the placement of PMM, fertilization is also commonly used to ameliorate soil nutrient deficiencies (Pinno and Errington, 2015). Although nutrient deficiencies may be initially addressed with fertilizer addition, plants growing in these areas rely on the activity of local microbiota to overcome potential stress caused by the presence of residual hydrocarbons.

Soil microorganisms are key to soil health and terrestrial ecosystems functioning since they mediate essential biogeochemical processes responsible for nutrient cycling (Quideau *et al.*, 2013). Symbiotic relationships with microbes may influence how plant species respond to environmental change. Plant-associated microbial communities may also help plants by stimulating growth, suppressing diseases, increasing nutrient acquisition and promoting stress resistance to petroleum hydrocarbons (Berg *et al.*, 2014; Siciliano *et al.*, 2001). In turn, microbes benefit by the deposition of plant mucilage and root exudates (Turner *et al.*, 2013; Hardoim *et al.*, 2008).

Nearly all plant tissues host microbial communities; however, most studies have focused on plant-microbial associations in the rhizosphere and endosphere (Turner *et al.*, 2013; Philippot *et al.*, 2013). Bacteria colonizing the endosphere (endophytic bacteria) can establish beneficial, neutral or detrimental associations and are often recognized as symbionts with a unique and

intimate interaction with the plant (Hardoim *et al.*, 2008; Berg *et al.*, 2014; Turner *et al.*, 2013). In addition, endophytic bacteria may produce several hydrolytic enzymes that are involved in the decomposition of plant compounds. Many of these enzymes have a similar chemical structure to organic toxic pollutants such as petroleum hydrocarbons. (Kukla *et al.*, 2014). These bacteria would likely protect plants from the phytotoxic effects of contaminants (Siciliano *et al.*, 2001). Hence, the efficiency of reclamation processes may rely on the presence and activity of plant-associated microorganisms carrying degradation genes required for the enzymatic break-down of contaminants (Yousaf *et al*., 2011).

Several hydrocarbon degrading genes have been previously detected in bacterial endophytic communities (Kukla *et al.*, 2014; Oliveira *et al.*, 2014). Among these genes, *alkB* has been extensively studied in hydrocarbon contaminated environmental samples (Wallisch *et al.*, 2014; Wasmund *et al.*, 2009) and microbes harboring the *alkB* gene are described as key players in alkane degradation (Wallisch *et al.*, 2014). The *alkB* gene codes for a subunit of the bacterial alkane monoxygenase enzyme and it is an general indicator for alkane degradation in the environment (Pérez-de-Mora *et al.*, 2011). In addition, alkane-degrading bacteria may also contain CYP153 genes encoding cytochrome P450 alkane hydroxylase (van Beilen *et al.*, 2006). Both *alkB* and CYP153 hydroxylase genes are known as the most important genes in the degradation of long chain length alkanes (Yousaf *et al.*, 2010a; Arslan *et al.*, 2014; Wasmund *et al.*, 2009). However, naphthalene dioxygenase gene (*nah*) is also an important PAH-degrading gene which have been detected widely from environments contaminated by aromatic compounds (Baldwin *et al.*, 2003; Yang *et al.*, 2014).

In this study, endophytic bacterial communities associated with two plant species in an oil sands reclamation area were quantified for three hydrocarbon degrading genes (CYP 153, *alkB* and *nah*). The aims of this study were to (*i*) quantify the total potential of unculturable hydrocarbon degrading endophytic bacteria using qPCR and (*ii*) assess culturable endophytic bacteria for the presence of hydrocarbon degrading genes using PCR.

5.4 Materials and Methods

5.4.1 Sample collection and processing

Annual barley (*Hordeum vulgare*) a planted species and white sweet clover (*Melilotus albus*) an unplanted native species were collected at an oil sands reclamation area (UTM Zone 12N, 6316207N 471907E). Three biological replicates of each plant were collected at different slope positions along two transects (Chapter 3). The first transect consisted of 10 sampling locations (S1-S10) in the standard cover, which is a cover management area consisting of a 40 cm of peat mineral mixture and 10 cm of sandy loam on the surface of 100 cm of tailing sands. The second transect also consisted of 10 sampling locations (E1-E10) in the engineered cover, an area of 50 cm of a peat mineral mixture on top of 120 cm of tailing sands separated from the bottom 30 cm of tailing sands by a geo-clay liner (GCL). The main objective of adding the GCL to the cover management is to retain the moisture on the top of the cover to improve plant growth and to prevent seepage from compounds on the bottom of the tailing sands to reach the surface of the plant cover. Samples were collected with help of a shovel, transported at 4 °C and stored at -20 °C until processing within the next 48 h.

Soil samples were analyzed for soil organic (TOC) and total carbon (TC) by the method from Dhillon *et al.* (2015) using a LECO C632 Analyzer (LECO Corporation, St. Joseph, MI, United States). Soil organic Matter (OM), was analyzed using the dry-ash method (McKeague, 1978). Soil pH was measured in a 1:2 soil: water slurry. Soil available ammonium was extracted using a 2 N KCl solution, NH_4^+ in the extract was mixed with hypochlorite and salicylate to form indophenol which was determined colorimetrically at 660 nm (Laverty and Bollo-Kamara, 1988). Soil available nitrate was extracted using a calcium chloride solution and determined colorimetrically at 520 nm according to Laverty and Bollo-Kamara (1988). Available phosphorus and potassium were measured using a modified Kelowna extraction (Qian *et al.*, 1994) and available sulfate by a calcium chloride extraction (McKeague, 1978). Soil total hydrocarbons were measured in accordance with the "Reference Method for the Canada-Wide Standard for Petroleum Hydrocarbons in Soil - Tier 1 Method, Canadian Council of Ministers of the Environment, December 2000. A subsample of the sediment/soil was extracted with a 1:1 hexane: acetone solution using a rotary extractor. The extract was purified using a silica gel

clean-up to remove polar compounds. The F2, F3 and F4 fractions were analyzed by Gas Chromatography with a Flame Ionization Detector (GC/FID).

5.4.2 Survey of unculturable endophytic bacteria community

Root material (entire root system) collected was transferred into an Erlenmeyer flask containing 100 mL NaClO (1.05% $v \cdot v^{-1}$) in PBS (1.2 g of $Na_2HPO_4 \cdot L^{-1}$, 0.18 g of $NaH_2PO_4 \cdot L^{-1}$, 8.5 g of $NaCl \cdot L^{-1}$) and placed on a rotary shaker (150 rpm) at 22 °C for 15 min. To remove the sodium hypochlorite solution, roots were rinsed 10 times with sterile water and 0.1 mL of the final wash spread in TSA plates to check for contamination after 72h (Siciliano and Germida, 1999).

5.4.3 DNA Extraction

Total genomic DNA was extracted from surface disinfected root samples using the PowerPlant® Pro DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). Purified isolates from culture dependent techniques in Chapter 3 were re-inoculated on 9 mL of soy broth (TSB) and DNA from microbial cultures was extracted using the UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). DNA yield was quantified using Qubit® Fluorometer (Qubit 2.0 Fluorometer, Life Technologies). Total genomic DNA was analyzed for total 16S rRNA abundance and the presence of hydrocarbon degrading genes using a one-step quantitative PCR (qPCR) (Kim and Gu, 2006). DNA extracted from bacterial isolates were analyzed for the presence of hydrocarbon degrading genes using PCR techniques (Table 5.1).

5.4.4 Synthesis of qPCR standards

Total bacterial 16s rRNA standards were synthesized from *Pseudomonas stutzei* previously obtained from the culture collection of the Soil Microbiology Laboratory at the Department of Soil Science, University of Saskatchewan. Using microbial DNA from these isolates, the 16S rRNA gene was amplified using PCR (Fierer *et al.*, 2005) and subsequently verified in a 1.0% agarose gel. The bands of interest were excised under UV light and gel purified using the Qiagen QIAquick gel extraction kit (Qiagen Inc., Toronto, Ontario) following the manufacturer’s instructions. The target insert was then quantified using a Qubit Fluorometer (Qubit 2.0 Fluorometer, Life Technologies). Target 16S rRNA genes were subsequently ligated in a vector using TOPO TA Cloning Kit® (Thermo Fisher Scientific Inc., Waltham, MA).

Standards for *alkB*, CYP153 and *nah* were synthesized by Biomatik (Cambridge, Canada), including flanking restriction sites for subcloning into PUC18 plasmids (Appendix C). After the plasmid containing the desired insert was prepared, it was ligated into the plasmid vector using Invitrogen TOPO TA Cloning Kit® (Thermo Fisher Scientific Inc., Waltham, MA) with One Shot ® MAX efficiency™ DH5α –T1R *Escherichia coli*. Transformed *E. coli* cells were then spread on liquid broth (LB) media with 50 $\mu g \cdot mL^{-1}$ of ampicillin and X-gal for 16 h at 37 ºC. Successful ligation was confirmed as white colonies which were grown in 3 ml LB broth (16 h at 37 ºC, 150rpm) containing 50 $\mu g \cdot mL^{-1}$ of ampicillin. Plasmid for *E. coli* cells were extracted using the Qiagen QIAprep Spin Miniprep kit (Qiagen Inc., Toronto, Ontario). After plasmid purification, the plasmid products were sequenced by Macrogen Inc. (Seoul-Rep. of Korea) to validate the target gene. Prior to use as standards, plasmids with inserts were linearized using HindIII (EUB, *alkB*, *nah*) and SphI (CYP153) as a restriction enzymes (Thermo Fisher Scientific Inc., Waltham, MA). The linearized product was verified in an agarose gel and bands of interest were excised and purified using the Qiagen QIAquick gel extraction kit (Qiagen Inc., Toronto, Ontario). Linearized and purified plasmids containing the inserts were quantified using Qubit Fluorometer (Qubit 2.0 Fluorometer, Life Technologies), and subsequently used in a dilution series to form a standard curve.

Efficiency of qPCR (E) and correlation coefficient (R^2) were determined based on the slopes of the standard curves generated using serial 10-fold dilutions of DNA standards. All qPCR efficiencies were calculated as follows: $E (\%) = (10^{(-1/slope)} - 1) \times 100$. A five-point standard curve using technical triplicates was generated along with triplicate negative controls for each run.

5.4.4.1 qPCR amplification

Serial plasmid dilutions (carrying the 16S rRNA gene) from 10^7 to 10^2 gene copies$\cdot \mu L^{-1}$ were used as standards. Quantitative PCRs were performed in three technical replicates for each sample. The PCR master mix contained 0.75 µL of each primer (10 µM), 0.4 µL of Rox reference dye (1:10), 0.625 µL of bovine serum albumine (BSA) (10 $\mu g \cdot \mu l^{-1}$) (Thermo Fisher Scientific Inc., Waltham, MA), 10 µL of Platinum SYBR 2X mix (Invitrogen, Burlington, ON), 4 µL of template DNA (10 $\mu g \cdot \mu L^{-1}$) and ultra-pure H_2O for a total volume of 20 µL. The number of copies of the target CYP 153 gene was determined according to Arslan *et*

al. (2014) in which serial dilution were prepared from 10^7 to 10^2 gene copies·μL^{-1}. Reaction mixtures contained 10 µL of Platinum SYBR green qPCR SuperMix-UDG (Invitrogen), 2 µL of BSA (10 mg·mL^{-1}), 0.4 µL of each primer (10 µM), 0.4 µL of Rox reference dye (1:10), 4 µL of template DNA (10 µg·μL^{-1}) and ultra-pure H_2O for a total volume of 20 µL. For the *alkB* gene, serial plasmid dilutions from 10^6 to 10^1 gene copies·μL^{-1} were used as standards. Real-time PCR assays was carried out according to Wallisch *et al.* (2014). Real time PCRs was performed using the Platinum SYBR green qPCR SuperMix-UDG kit (Invitrogen). The PCR mix contained 10 µL of Platinum SYBR green qPCR SuperMix-UDG (Invitrogen), 0.4 µL of $MgCl_2$ (50 mM), 0.4 µL of each *alkB* specific forward and reverse primers (10 µM), 0.4 µL of purified BSA (10 mg·mL^{-1}), 0.4 µL of Rox reference dye (1:10), 4 µL of template DNA (10 µg·μL^{-1}) and ultra-pure H_2O for a total volume of 20 µL. Copy numbers of *nah* gene in endophytic microbial communities was assessed according to Han *et al*. (2014). Standard dilutions were prepared from concentrations 10^7 to 10^2 gene copies·μL^{-1}. The PCR reaction mix contained 10 µL of Platinum SYBR green qPCR SuperMix-UDG (Invitrogen), 0.4 µL of purified BSA (10 mg·mL^{-1}), 0.4 µL of each primer (10 µM), 0.4 µL of Rox reference dye (1:10), 4 µL of template DNA (10 µg·μL^{-1}) and ultra-pure H_2O for a total volume of 20 µL.

The assays for q-PCR were conducted using three technical replicates for each sample (Table 5.2). In addition, PCR products were checked on a 1% agarose gel to ensure there was no contamination and that gene fragments were the expected sizes. The amplification data was expressed as gene copy number per gram of fresh root weight.

Table 5.1 List of primers used for quantitative real-time polymerase chain reaction (qPCR) to assess hydrocarbon degrading genes present within unculturable endophytes and polymerase chain reaction (PCR) for the screening cultured hydrocarbon degrading endophytic bacteria.

Primer	Target	Sequence (5`-3`)	Expected fragment size (bp)	Reference
EUB 338	16S rRNA	ACTCCTACGGGAGGCAGCAGATT	200	(Fierer *et al.*, 2005)
EUB 518		ATTACCGCGGCTGCTGG		
alkB-F	Alkane monooxygenase (*alkB*)	AAYACIGCICAYGARCTIGGICAYAA	550	(Wasmund *et al.*, 2009; Wallisch *et al.*, 2014)
alkB-R		GCRTGRTGRTCIGARTGICGYTG		
P450fw1	Cytochrome P450 hydroxylase (CYP153)	GTSGGCGGCAACGACACSAC	339	(Yousaf *et al.*, 2011; Arslan *et al.*, 2014)
P450rv3		GCASCGGTGGATGCCGAAGCCRA		
nah-F	Naphthalene dioxygenase (*nah)*	CAAAARCACCTGATTYATGG	377	(Han *et al.*, 2014; Baldwin *et al.*, 2003)
nah-R		AYRCGRGSGACTTCTTTCAA		

Table 5.2 Quantitative real-time polymerase chain reaction (qPCR) conditions to assess hydrocarbon degrading genes present within unculturable endophytes in an oil sands reclamation area.

Target	qPCR conditions	Reference
16S rRNA	95 °C for 2 min followed by 40 cycles (denaturation at 95 °C for 1 min, annealing at 53 °C for 30s, and extension at 72 °C for 1 min) with fluorescence signal data acquisition in an additional step of 80 °C for 1 min at the end of each cycle.	(Fierer *et al.*, 2005)
Cytochrome P450 hydroxylase (CYP153)	95 °C for 2 min followed by 40 cycles (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s). with fluorescence signal data acquisition in an additional step of 80 °C for 1 min at the end of each cycle.	(Yousaf *et al.*, 2011; Arslan *et al.*, 2014)
Alkane monooxygenase (*alkB*)	95 °C for 2 min followed by a touchdown PCR (5 cycles of 45 s 95 °C, 1 min 62 °C (stepwise reduced to 57 °C) and 45 s 72 °C, followed by 40 cycles of 45 s 95 °C, 1 min 57 °C and 45 s 72 °C; final extension of 10 min at 72°C.	(Wasmund *et al.*, 2009; Wallisch *et al.*, 2014)
Naphthalene dioxygenase (*nah)*	94 °C for 1 min followed by a touchdown PCR (5 cycles of 94 °C for 20 s, 60 °C (reduced by 1 °C per cycle) for 30 s, 72 °C for 30 s; and 35 cycles of 94 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s).	(Han *et al.*, 2014; Baldwin *et al.*, 2003)

Real-time PCR cycling conditions included an initial UDG incubation step at 50 °C for 2 min before enzyme activation step and a melting curve analysis consisting of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s.

5.4.5 PCR Amplification of culturable isolates

Polymerase chain reaction was used to assess the presence of hydrocarbon degrading genes in bacterial endophytes previously isolated in culture dependent techniques (Chapter 3). As a positive control, DNA plasmids linearized from qPCR assays containing each gene of interest were used. In addition, the same primers used for qPCR were used (Table 5.1). DNA from bacterial cultures positive for at least one hydrocarbon degrading gene were further sequenced by Macrogen Inc. (Seoul-Rep. of Korea).

PCR reaction mix for the analysis of CYP153 gene consisted of 12.5µL of Hot Start Master Mix (Qiagen), 1 µL of each primer (25mM), 0.625 of purified BSA (10 $mg{\cdot}mL^{-1}$), and 1 µL purified genomic DNA and purified PCR grade water to 25µL (van Beilen *et al.*, 2006). For the detection of *alkB* gene, PCR mix contained a total of 12.5 µL of Hot Start Master Mix (Qiagen), 2.8 µL of each primer (25mM), 0.625 of purified BSA (10 $mg{\cdot}mL^{-1}$), 10 ng of purified genomic DNA and purified PCR grade water to 25µL (Tanase *et al.*, 2013). In addition, PCR *nah* gene analysis was conducted as previously described by Baldwin *et al.* (2003). Briefly, the PCR reaction mix consisted of 12.5 µL of Hot Start Master Mix (Qiagen), 1 µL of each primer (25mM), 0.625 of of purified BSA (10 $mg{\cdot}mL^{-1}$) and 1µL purified genomic DNA and purified PCR grade water to 25µL.

Table 5.3 PCR conditions to assess hydrocarbon degrading genes present within culturable endophytes in an oil sands reclamation area.

Target	PCR conditions	Reference
Cytochrome P450 hydroxylase (CYP153)	Initial denaturation step of 5 min at 95 °C; 25 cycles of 45 s at 95 °C, 1 min at 58 °C, and 1 min at 72 °C and a final elongation step of 10 min at 72 °C.	(van Beilen *et al.*, 2006).
Alkane monooxygenase (*alkB*)	Initial denaturation for 5 min at 95 °C, followed by 25 cycles of 60 s at 90 °C; 60 s at 50 °C, 2 min at 72 °C; and with a final elongation for 10 min at 72 °C.	(Tanase *et al.*, 2013).
Naphthalene dioxygenase (*nah)*	Initial denaturation step of 10 min at 95 °C; 30 cycles of 1 min at 95 °C, 1 min at 47 ºC and 2 min at 72 °C, and a final extension step of 10 min at 72 °C	(Baldwin *et al.*, 2003).

5.4.6 Statistical Analysis

Statistical Analysis for qPCR gene copy numbers was performed using SAS (version 9.3). The Shapiro-Wilk statistic was used to test the normality of the data. Analysis of variance (ANOVA) and Student's two-sample t-test was used to determine significant differences between the variables analyzed. Spearman's rank correlation was used to determine relationships between gene abundance of 16S rRNA, CYP153, *alkB*, *nah* and soil properties. After sequencing of culturable isolates, multiple sequence alignment of the sequences was conducted using CLUSTAL X 2.0 (Larkin *et al.*, 2007) and a phylogenetic tree was constructed using MEGA 7.0 (Kumar *et al.*, 2016). The phylogenetic and evolutionary relations were inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Nodal robustness of the tree was assessed using 1000 bootstrap replicates.

5.5 Results

Real-time PCR was used to quantify the 16S rRNA gene and three hydrocarbon degrading genes (CYP153, *alkB*, *nah*). In addition, the relative abundance of hydrocarbon degrading genes compared with 16S rRNA was also considered (*i.e.* the ratio between functional genes and 16S rRNA). The results in this study indicated an overall reaction efficiency of 85 to 100% for 16S rRNA qPCR assays, 90-100% for CYP 153, 85-95% for *alkB* and 90-95% for *nah*. In addition, all standard curves were linear ($R^2 > 0.99$). The gene copy numbers ranged from 10^3 to 10^4 for 16S rRNA, 10^2 to 10^4 for CYP 153, 10^2 to 10^4 for *alkB* and 0 to 10^4 for *nah*.

Analysis of variance (ANOVA) revealed significant differences in the genes analyzed by most variables (cover, slope, plant species and sampling locations) (Table 5.4). Differences in CYP 153 gene copy numbers were also significant either at $p \leq 0.05$, $p \leq 0.01$ or at $p \leq 0.001$ for all variables analyzed. However, no significant differences were observed between different plant species and gene copy numbers of 16s rRNA, *alkB*, *nah* and the relative abundance of *alkB* and *nah*. Cover type was also not significant for gene copy numbers of 16S rRNA and *nah*. In addition, significant differences in the interactions between variables were observed in all genes analyzed.

Overall, functional genes CYP, *alkB* and *nah* indicated a lower gene copy number when compared to 16s rRNA (Figure 5.1). However, the average CYP153 gene copy number was 3% higher than 16s rRNA in sweet clover and 22% lower in barley. In sweet clover plants, *alkB* gene

copy numbers were 17% lower when compared to CYP153. Similar to 16 rRNA and CYP153, *alkB* gene copy numbers were also more variable in sweet clover endophytic bacteria when compared to barley. Analysis of *nah* gene copy numbers indicated the lowest values among all genes analyzed. The variability of each sample can be observed in the high standard deviations (Figure 5.1) as *nah* gene quantification for most of the sampling locations was below detection levels and, in some locations, it was detected up to 10^4 gene copy numbers. Analysis of gene copy numbers using a two-sample t-test in indicated significant differences ($p \leq 0.001$) between different plant species only for CYP153. Overall, CYP 153 gene copy numbers were 27% higher in sweet clover plants when compared to barley. However, no statistical differences were observed for 16s rRNA, *alkB* and *nah* gene copy numbers by plant species.

Table 5.4 Analysis of variance (ANOVA) for Log gene copy number·g^{-1} of plant of 16s rRNA, CYP153, *alkB*, *nah* and the ratio of all of hydrocarbon degrading genes relative to 16s rRNA (n=120).

	16s	**CYP 153**	***alkB***	***nah***	**Ratio (CYP/16s)**	**Ratio (*alkB*/16s)**	**Ratio (*nah*/16s)**
Cover (C)	NS	*	***	NS	***	***	NS
Slope (S)	***	**	***	***	***	***	**
Plant (P)	NS	***	NS	NS	***	NS	NS
Location (L)	***	***	**	***	***	***	***
C x S	***	***	***	***	**	***	***
P x C	***	***	***	***	***	***	***
P x S	***	***	***	***	***	***	***
P x L	***	***	***	***	***	***	***
L x S	***	***	***	***	***	***	***
L x C	***	***	***	***	***	***	***
P x C x S	***	***	***	***	***	***	***
P x L x C	***	***	***	***	***	***	***
P x L x S	***	***	***	***	***	***	***
P x L x S x C	***	***	***	***	***	***	***

*, **, *** Significant at p ≤0.05, 0.01, and 0.001, respectively.

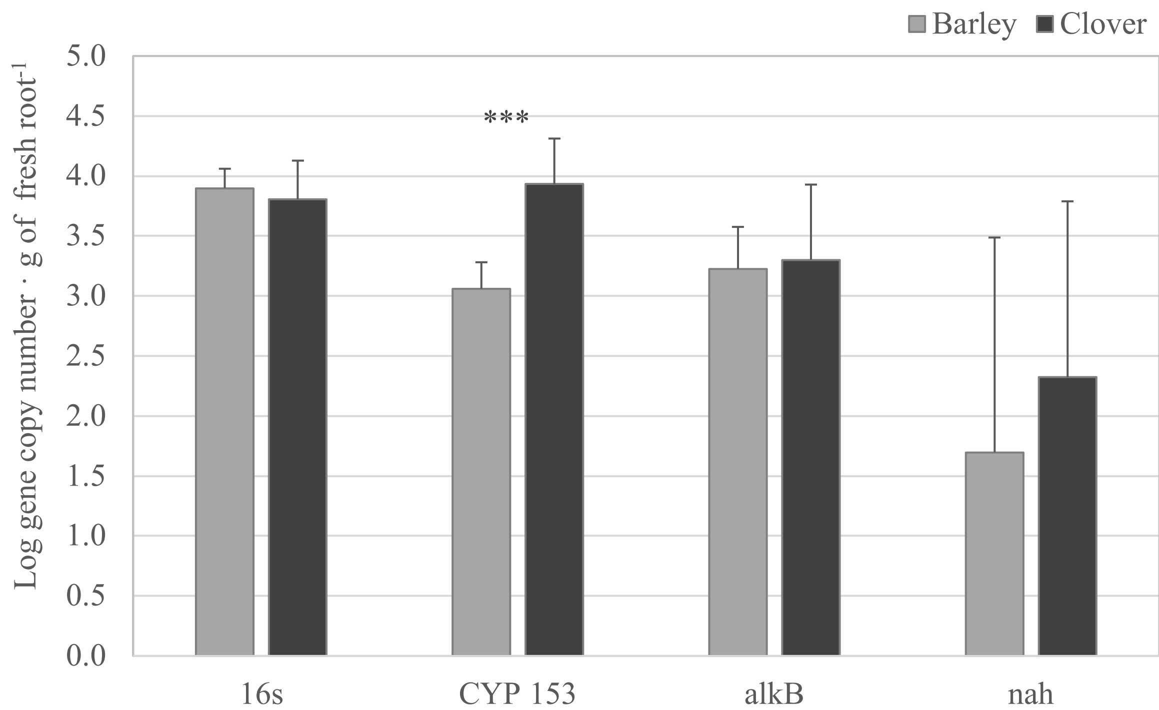

Figure 5.1 Abundance of gene copy numbers within endophytic microbial communities associated with barley (n=60) and sweet clover (n=60) growing in oil sands reclamation soils. Error bars represent standard deviation, and *** indicate significance at $p \leq 0.001$ at Student's two-sample t-test.

In order to investigate hydrocarbon degradation potential among endophytic bacterial communities, this study also analyzed the ratio of each hydrocarbon degrading gene relative to 16 rRNA. The results suggest that, similar to absolute gene copy numbers, significant differences were only observed in the ratio CYP153/16s rRNA, which was 30% higher in sweet clover plants when compared to barley (Figure 5.2). Although not statistically significant, sweet clover endophytic communities exhibited a higher ratio of *alkB*/16s rRNA (4%) and *nah*/16s rRNA (39%) when compared to barley.

Since previous analyses indicated differences in gene copy numbers by plant species, a correlation analysis between soil physical-chemical parameters and endophytic communities was conducted for each plant (Table 5.5, 5.6). Overall, no significant correlations were observed between sweet clover endophytic 16s rRNA gene copy numbers and soil parameters; however, the abundance of this gene negatively correlated with organic matter (OM) ($R^2 = -0.27$, $p \leq 0.05$), NO_3^- ($R^2 = -0.30$, $p \leq 0.05$), NH_4^+ ($R^2 = -0.36$, $p \leq 0.01$), total organic carbon (TOC) ($R^2 = -0.28$, $p \leq 0.05$) and total carbon (TC) ($R^2 = -0.26$, $p \leq 0.05$) in barley plants. In addition, CYP153 also negatively correlated to NH_4^+ ($R^2 = -0.41$, $p \leq 0.01$) in barley plants. Conversely, CYP 153 correlated negatively with THC ($R^2 = -0.28$, $p \leq 0.05$), but positively with OM ($R^2 = 0.29$, $p \leq 0.05$), TOC ($R^2 = 0.23$, $p \leq 0.05$) and TC ($R^2 = 0.25$, $p \leq 0.05$) in sweet clover plants.

Correlations between *alkB* gene copy numbers and soil parameters also indicated differences between plants. Whereas barley plants indicated positive correlations with pH ($R^2 = 0.34$, $p \leq 0.01$) and total hydrocarbons (THC) ($R^2 = 0.46$, $p \leq 0.01$), sweet clover plants exhibited significant correlations with organic matter ($R^2 = 0.33$, $p \leq 0.05$), TOC ($R^2 = 0.26$, $p \leq 0.05$) and TC ($R^2 = 0.28$, $p \leq 0.05$). Similar to *alkB*, *nah* gene copy numbers indicated positive correlations with THC ($R^2 = 0.30$, $p \leq 0.05$) in barley plants, but negative correlations ($R^2 = -0.47$, $p \leq 0.01$) in sweet clover plants. In addition, positive correlations between *nah* gene copy numbers and NH_4^+ ($R^2 = 0.61$, $p \leq 0.01$), TOC ($R^2 = 0.43$, $p \leq 0.01$) and TC ($R^2 = 0.44$, $p \leq 0.01$) were also observed in sweet clover plants.

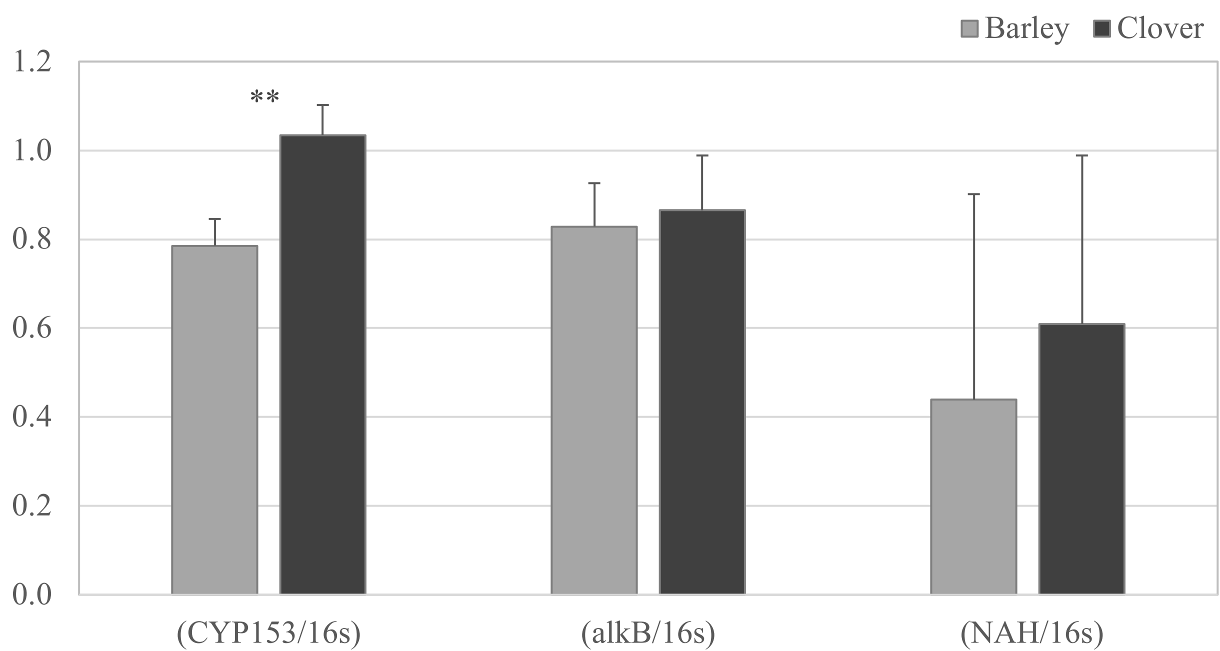

Figure 5.2 Abundance of relative ratio of hydrocarbon degrading genes compared with 16s rRNA in endophytic microbial communities associated with barley (n=60) and sweet clover (n=60) growing in oil sands reclamation soils. Error bars represent standard deviation, and ** indicate significance at $p \leq 0.01$ at Student's two-sample t-test.

Table 5.5 Spearman's rank correlation coefficients (n = 60) between 16s rRNA and Hydrocarbon degrading gene abundance and soil physico-chemical parameters for barley endophytic community. Statistically significant correlations are indicated in bold type, * and * and ** denote p values ≤ 0.05 and ≤ 0.01, respectively. THC = total hydrocarbons, OM = organic matter, EC = electrical conductivity, TOC = total organic carbon, TC = total carbon.

	16s	CYP 153	*alkB*	*nah*	Ratio: CYP 153/16s	Ratio: *alkB*/16s	Ratio: *nah*/16s
pH	0.15	0.11	**0.34****	0.19	0.09	**0.32***	0.19
THC	0.16	0.11	**0.46****	**0.30***	0.10	**0.43****	**0.28***
OM	**-0.27***	-0.24	-0.09	-0.23	-0.21	0.04	-0.23
NO_3^-	**-0.30***	-0.24	**-0.34****	-0.13	-0.18	-0.22	-0.11
SO_4^{2-}	0.05	0.14	-0.05	0.15	0.19	-0.10	0.16
PO_4^{3-}	0.08	0.03	0.24	**0.26***	-0.05	0.19	0.24
K^+	-0.08	-0.09	0.19	**0.33***	-0.12	0.19	**0.35****
NH_4^+	**-0.36****	**-0.41****	-0.21	-0.16	**-0.42****	-0.08	-0.14
EC	-0.05	-0.02	-0.24	0.00	0.06	-0.24	0.01
SAND	0.14	0.07	-0.02	0.06	-0.03	-0.05	0.05
SILT	-0.25	-0.14	0.05	0.12	0.02	0.10	0.14
CLAY	0.16	0.10	0.07	-0.08	0.06	-0.01	-0.07
TOC	**-0.28***	-0.24	-0.16	-0.23	-0.18	-0.08	-0.21
TC	**-0.26***	-0.22	-0.18	**-0.26***	-0.15	-0.10	-0.23

Table 5.6 Spearman's rank correlation coefficients (n = 60) between 16s rRNA and Hydrocarbon degrading gene abundance and soil physico-chemical parameters for sweet clover endophytic community. Statistically significant correlations are indicated in bold type, * and ** denote p values ≤ 0.05 and ≤ 0.01, respectively. THC = total hydrocarbons, OM = organic matter, EC = electrical conductivity, TOC = total organic carbon, TC = total carbon.

	16s	CYP 153	*alkB*	*nah*	Ratio: CYP 153/16s	Ratio: *alkB*/16s	Ratio: *nah*/16s
pH	-0.18	-0.17	-0.20	-0.24	0.00	-0.24	-0.16
THC	-0.19	**-0.28***	-0.24	**-0.47****	-0.17	**-0.27***	**-0.37****
OM	-0.06	**0.29***	**0.33***	**0.43****	**0.48****	**0.53****	**0.46****
NO_3^-	-0.01	0.20	0.21	**0.39****	**0.28***	**0.30***	**0.40****
SO_4^{2-}	**0.39****	0.02	-0.04	-0.14	**-0.53****	**-0.41****	**-0.26***
PO_4^{3-}	0.06	0.12	0.03	**-0.29***	0.19	0.08	-0.34
K^+	0.04	0.02	0.15	0.03	-0.01	0.15	0.03
NH_4^+	-0.09	0.19	0.30	**0.56****	**0.36****	**0.53****	**0.61****
EC	**-0.30***	**-0.26***	-0.23	-0.21	0.01	-0.20	-0.09
SAND	0.05	0.07	-0.02	-0.08	0.04	0.03	-0.15
SILT	-0.06	-0.10	-0.03	0.00	-0.13	-0.07	0.06
CLAY	-0.16	-0.19	-0.11	-0.18	-0.02	-0.11	-0.08
TOC	0.20	**0.23***	**0.26***	**0.43****	0.07	**0.26***	**0.35****
TC	0.24	**0.25***	**0.28***	**0.44****	0.06	0.25	**0.34****

Assessment of functional genes using endpoint PCR revealed barley bacterial isolates positive for all genes analyzed. A total of 316 isolates were tested for the presence of hydrocarbon degrading genes and purified PCR products from positive isolates were sequenced by Sanger sequencing. The identification of endophytes was tentatively allocated to species, but mostly by genera. Out of 316 isolates, 42 (13%) were positive for at least one hydrocarbon degrading gene. Among these isolates, 16 harbor only the *alkB* gene, 12 the CYP 153 gene and 12 the *nah* gene. Interestingly, two isolates were positive for both the CYP153 and *nah* gene, these isolates were identified as *Serratia liquefaciens* and *Rahnella* sp.

Most of these isolates closely match the database for *Pantoea* spp. (6 isolates), followed by *Pseudomonas* spp. (5 isolates), *Enterobacter* spp. (5 isolates), *Chryseobacterium* spp. (4 isolates), *Serratia* spp. (4 isolates), *Bacillus* spp. (3 isolates), *Ewingella* spp. (2 isolates), *Flavobacterium* spp. (2 isolates), *Pedobacter* spp. (2 isolates) *Rahnella* spp. (2 isolates), *Staphylococcus* spp. (2 isolates), *Stenotrophomonas* spp. (2 isolates), *Kluyvera* sp. (1 isolate) and *Xanthomonas* sp. (1 isolate) (Figure 5.3, Appendix D, Table D.1).

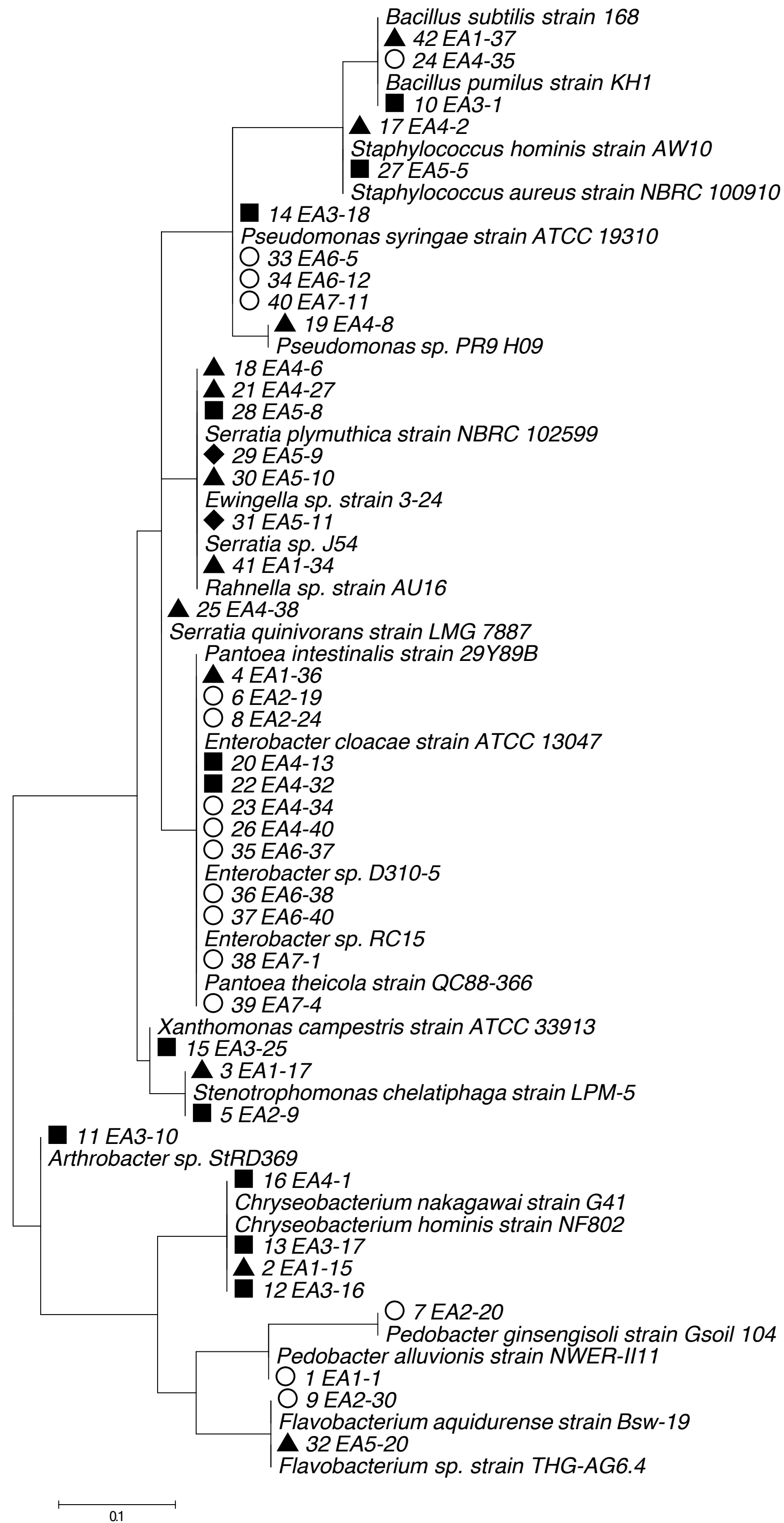

Figure 5.3 Neighbor-Joining tree using the Maximum Composite Likelihood method. Symbols represent isolates positive for ○ *alkB*, ▲ CYP 153, ■ *nah* and ◆ both CYP153 and *nah.*

5.6 Discussion

Most studies that have investigated the potential hydrocarbon degrading bacterial communities focused on soil (Yang *et al.*, 2014; Jurelevicius *et al.*, 2012; Wallisch *et al.*, 2014; Bell *et al.*, 2013) or water (Wasmund *et al.*, 2009; Wang *et al.*, 2010a) habitats. A few studies have assessed hydrocarbon degraders on the plant interior (Yousaf *et al.*, 2010a, 2011; Siciliano *et al.*, 2001).

This study investigated the presence of hydrocarbon degrading genes in culturable isolates from annual barley plants isolated in Chapter 3. Furthermore, a finer analysis assessing the abundance of unculturable endophytic hydrocarbon degraders in sweet clover and barley plants was conducted based on samples collected in Chapter 4. To analyze for the hydrocarbon degrading bacterial endophytes associated with these plants, qPCR was the method of choice for nucleic acid quantification due to its common use for the investigation of hydrocarbon degrading functional genes (Wasmund *et al.*, 2009; Yergeau *et al.*, 2012; Arslan *et al.*, 2014). Several genes such as *ndoB* (Margesin *et al.*, 2003), *tod*C1 (Furukawa *et al.*, 1993), *bph*A (Tairazf *et al.*, 1992) and C120 (Kivisaar *et al.*, 1991) have been previously studied in bacterial hydrocarbon degradation. However, this study focused on three hydrocarbon degrading genes most commonly reported in the literature (CYP 153, *alkB*, *nah*) with the addition of a total bacterial 16S rRNA gene.

Overall, the results in this study indicates that potential hydrocarbon degrading bacterial endophytes are highly dependent on plant factors and may behave differently than soil bacterial communities previously investigated in the literature. Although not significant, analysis of 16s rRNA revealed higher gene copy numbers in barley plants when compared to sweet clover. In Chapter 4 it was suggested that sweet clover species were more restrictive in selecting bacterial endophytes when compared to barley. In this study, except for plant species and cover type, significant differences of 16s rRNA gene copy numbers were observed for all variables analyzed. However, no significant correlations were observed between this gene and soil THC. Yergeau *et al.* (2009) conducted studies in an ex-situ and in-situ bioremediation treatment soils in the Canadian high Arctic and found no significant differences in the amount of 16S rRNA between contaminated and uncontaminated soils. In contrast to Wu *et al.* (2017), who investigated agricultural soils impacted by acidification and PAH pollution, and Clark *et al.* (2012), who

assessed the influence of different long-term fertilization in soils, the current study indicated negative correlations between barley endophytic communities and soil C, N and OM. However, these effects were not observed in sweet clover. Previous studies in Chapter 4 suggested that plant factors play a key role in determining the endophytic community composition and soil parameters had no significant effect in endophytic communities. In this study, although both plants harbor equal 16S rRNA gene abundance, these communities interact differently with soil parameters.

The primers designed by van Beilen *et al.* (2006) were used to assess CYP153 genes in alkane-degrading bacterial strains. This gene encodes for cytochrome P450 enzymes that have been characterized as alkane hydroxylases responsible for bacterial oxidation of n-alkanes. Several organisms including *Mycobacterium spp., Sphingomonas spp.* and *Proteobacteria spp.* are known for the presence of this gene (van Beilen *et al.*, 2006) and several studies have used CYP for assessing hydrocarbon degrading communities (Arslan *et al.*, 2014; Yousaf *et al.*, 2010a; Khan *et al.*, 2013). In addition, CYP 153 has been previously studied in endophytic communities (Yousaf *et al.*, 2011) and in rhizoplane of grasses (Tsuboi *et al.*, 2015). In the current study, a few samples of sweet clover endophytic DNA indicated higher gene copy numbers of CYP153 when compared to 16s rRNA. This is most likely due to the presence of multiple alkane hydroxylases (AH) in one single strain, as previously been documented by van Beilen *et al.* (2006). For the integral membrane AHs; the majority of strains actually contains more than one AH. In addition, endophytic communities from sweet clover plants harbored significantly higher absolute and relative abundance of CYP153 when compared to barley. Similar to Yousaf *et al.* (2010) and Siciliano *et al.* (2001), the data in the current study suggests that the enrichment of selected hydrocarbon-degrading bacteria in the endosphere compartment depends on the presence of the contaminant and on different plant species. Plant root exudates can determine which microorganisms colonize the endosphere and also affect phytoremediation (Phillips *et al.*, 2012). Therefore, these results suggest that the root interior of sweet clover species permitted a more suitable habitat for bacteria containing the CYP153 gene.

Based on previous studies assessing bacterial community structure in these plants (Chapter 4), sweet clover endophytic community profiles are mainly associated with a high abundance of *Pseudomonas* and *Sinorhizobium* spp. *Pseudomonas* spp. has been extensively reported in the literature for alkane degradation (Kukla *et al.*, 2014; Germaine *et al.*, 2009;

Milcic-Terzic *et al.*, 2001). However, since *Pseudomonas* spp. are also common colonizers of the root interior, they could be associated with several functions other than hydrocarbon degradation (Hardoim *et al.*, 2012; Ma *et al.*, 2011b). Unlike *Pseudomonas, Sinorhizobium* spp. are most commonly associated with nitrogen fixation. However, Muratova *et al*. (2014) have used *Sinorhizobium meliloti* isolated from the surface of roots of *Phragmites australis* for phenanthrene degradation.

ANOVA revealed significant effects in CYP153 gene copy numbers by all parameters analyzed. In barley plants, no significant correlations were observed between CYP153 and THC, which is in agreement with Powell *et al.* (2010) who found no difference in hydrocarbon gene abundance between an unaffected and a fuel affected sub Antarctic soils. In addition, Margesin *et al.* (2003) suggested that hydrocarbon derived from gram negative bacteria such as *Pseudomonas* and *Acinetobacter* spp. are enriched following hydrocarbon contamination, however these authors found no significant correlation in gram-positive bacteria such as *Rhodococcus* and *Mycobacterium* spp. Previous studies in Chapter 4 revealed that barley endosphere root compartments harbor higher abundance of *Actinobacteria* spp. when compared to sweet clover. Therefore, most of CYP153 degrading genes in barley may be associated with *Actinobacteria* spp. which these results suggest that the abundance of this phylum is less affected by the presence of hydrocarbons in soil. Unlike barley plants, sweet clover endophytic communities harboring the gene CYP153 indicated negative correlations with THC, which may indicate an inhibition of hydrocarbon degraders. However, the ratio CYP153/16s rRNA indicated no significant correlations with THC, which was also observed in barley plants. In addition, CYP153 gene abundance indicated negative correlations with soil NH_4^+ in barley plants. Yang *et al.* (2014) also found similar results with hydrocarbon degrading genes and total-N. However, Powell *et al.* (2010) found positive correlations between NH_4^+ and the presence of alkane mono-oxygenase genes, which is in disagreement with the results for barley endophytes in this study but in agreement with sweet clover. According to Powell *et al.* (2010), although the levels of hydrocarbons may influence the overall microbial community structure, other factors, such as nutrients, are key in selecting for alkane-degrading micro-organisms. Therefore, although CYP153 is an important gene used for the detection of hydroxylase genes in recent studies (Yousaf *et al.*, 2010a), most studies have used the *alkB* gene (Wasmund *et al.*, 2009; Wallisch *et al.*, 2014; Pérez-de-Mora *et al.*, 2011; Jurelevicius *et al.*, 2013).

Degenerate *alkB* primers designed by Kloos *et al*. (2006) based on *Pseudomonas putida*, *Bacillus subtilis* and *E. coli* were used in the current study as it has been proven to be successful for the detection of the alkane monooxygenase. Analysis of variance of *alkB* gene copy numbers revealed significant differences for all variables analyzed with the exception of plant species. Similar to other genes investigated in this study, although no significant differences were observed in *alkB* gene abundance, barley and sweet clover endophytic communities indicated different correlations with soil parameters. Whereas overall *alkB* barley endophytic gene abundance indicated positive correlations with soil THC, the ratio *alkB*/16s rRNA in sweet clover endophytes indicated negative correlations. Positive correlations between *alkB* with hydrocarbons have been previously observed in the literature (Powell *et al.*, 2010; Jurelevicius *et al.*, 2012; Schulz *et al.*, 2012). According to Jurelevicius *et al.* (2012), THC is a key factor influencing *alkB* diversity in soils with similar physicochemical properties. However, in the current study, although both plant species were collected at the same sampling locations, different effects were observed between *alkB* endophytes and soil THC depending on plant species. In addition to THC, barley plants indicated positive correlations with soil pH. Soil pH is a key determinant of bacterial community diversity and composition which has significant impacts on soil ecological processes driven by bacteria (Wu *et al.*, 2017). Yang *et al.* (2014) and Pérez-de-Mora *et al.* (2011) also found positive, although not significant, correlations between hydrocarbon degrading genes and soil pH. Barley endophytic *alkB* gene abundance indicated no significant correlations with soil carbon, which is in agreement with Wasmund *et al.*, (2009). However, similar to CYP513, sweet clover endophytic communities containing *alkB* positively correlated to OM, NO_3^- , NH_4^+, TC and TOC. According to Powell *et al.* (2010), the combination of TC and pH is the most influential for microbial communities as TC also determines soil water holding capacity which influences soil oxygen availability.

Naphthalene dioxygenase (*nah*) primers previously designed by Baldwin *et al*., (2003) has been widely used in environments with a high incidence of aromatic compounds. The *nah* gene encodes naphthalene dioxygenases associated with degradation of low-molecular weight polycyclic aromatic hydrocarbons (PAHs) (Han *et al.*, 2014; Baldwin *et al.*, 2003). However, the information on the abundance of PAH metabolic genes in soil (Yang *et al.*, 2014) and among endophytic communities are very limited. In this study, a high variability of this gene was observed throughout sampling locations. In addition, no significant differences in the overall or

relative (*nah*/16S rRNA) gene abundance was observed between plant species and cover management. These results also indicate similar correlations between *nah* and soil parameters compared to CYP153 and *alkB*. Although mostly no significant correlations between *nah* and soil nutrients was observed in barley endophytic communities, several positive correlations were found between both absolute and relative *nah* gene abundance in sweet clover plants with nitrogen and carbon. Han *et al.* (2014) also found significant positive correlations between *nah* with total carbon and nitrogen in soils contaminated with PAH in a coke factory area. Li *et al.* (2012) has previously suggested that the efficiency of remediation in PAH-polluted soils depends on the application of organic substances and thus increasing inputs of TC, TN, and OM. Although significant positive correlations for other hydrocarbon degrading genes and OM were also observed in sweet clover plants, *nah* absolute gene abundance correlated at $p \leq 0.01$ with OM. According to Han *et al.* (2014), in addition to providing nutrients for microorganisms, OM may increase the solubility and bioavailability of PAHs which may be a key factor determining PAHs degrading bacteria. Although the overall assessment of hydrocarbon degrading genes in uncultured endophytes are important for future reclamation and remediation strategies, this study assessed culturable hydrocarbon degrading endophytes as these can be used as potential inoculants in contaminated sites.

Analysis of the presence of hydrocarbon degrading genes in culturable barley endophytic isolates revealed that most of these isolates closely match the database for *Pantoea*, *Pseudomonas* and *Enterobacter* spp. *Pantoea* is a known genus of hydrocarbon degrading bacteria (Phillips *et al.*, 2012; Oliveira *et al.*, 2014; Khan *et al.*, 2013). Arslan *et al.* (2014), investigated diesel fuel phytoremediation using ryegrass inoculated with *Pantoea* sp. BTRH79. These authors found that *Pantoea* enhanced plant growth and development in contaminated soils which was further enhanced by the application of nutrients. In addition, Tara *et al.* (2014) also inoculated carpet grass (*Axonopus affinis*) with *Pantoea* sp. BTRH79 (positive for CYP153 genes and also showing ACC deaminase activity) in a diesel-polluted soil and found an increase plant biomass and phytoremediation activity in inoculated plants. In the current study, although isolate (4) EA1-36 was positive for CYP153, most of the isolates closely related to *Pantoea* spp. where positive for *alkB*.

Previous studies (van Beilen *et al.*, 2006; Wallisch *et al.*, 2014) identified *alkB* in *Pseudomonas* spp. This genus have been used in several inoculation studies assessing

hydrocarbon degradation (Tara *et al.*, 2014; Khan *et al.*, 2014; Gałazka *et al.*, 2012; Tang *et al.*, 2010). Ivanova *et al.* (2015) studied barley inoculation with *Pseudomonas* putida F701 previously isolated from oil contaminated soil samples and found that *Pseudomonas putida* F701 is a good colonizer of plant roots and effective in hydrocarbon degradation when plants are grown in soils mixed with oil. In addition, Ivanova *et al.* (2015) also concluded that hydrocarbon degradation is enchanced when *P. putida* is inoculated in combination with other hydrocarbon degrading bacteria.

Endophytic *Enterobacter* ssp. containing the CYP153 gene has been previously assessed for hydrocarbon degradation (Yousaf *et al.*, 2011). The genus comprises a range of beneficial plant-associated bacteria showing plant growth promotion and hydrocarbon degradation (Yousaf *et al.*, 2011). Sheng *et al.* (2008) studied pyrene degradation using Wheat (*Triticum aestivum*) and corn (*Zea mays*) inoculated with *Enterobacter* sp. 12J1 and concluded that *Enterobacter* was able to promote plant biomass and pyrene removal. In addition, the survival of *Enterobacte*r sp. 12J1 strains was higher when in association with plants.

5.7 Conclusions

This study assessed hydrocarbon degrading genes in endophytic communities associated with plants growing in oil sands reclamation soils. Three hydrocarbon degrading genes (CYP153, *alkB* and *nah*) were quantified using qPCR and significant differences between plant species, cover management and sampling locations were observed. The results in this study suggest that whereas both plants analyzed indicated similar 16s rRNA, *alkB* and *nah* gene abundance, sweet clover species harbor a higher abundance of CYP153. In addition, this study suggests that total hydrocarbons, pH, soil carbon and nitrogen play an important role in determining hydrocarbon degrading communities. However, since previous studies indicated that the two plants analyzed harbor different bacterial endophytes, plant factors may also play an important role in selecting hydrocarbon degrading bacteria. The assessment of potential culturable hydrocarbon degrading bacteria previously isolated from barley plants revealed a that total of 42 isolates were positive for at least one hydrocarbon degrading gene. Most of these isolates were positive for the presence of *alkB* and closely match the database for *Pantoea*, *Pseudomonas* and *Enterobacter* spp. Future studies will assess the application of these isolates in phytoremediation.

6. POTENTIAL USE OF ENDOPHYTIC ROOT BACTERIA AND HOST PLANTS TO DEGRADE HYDROCARBONS

6.1 Preface

Chapter 5 provided an assessment of hydrocarbon degrading genes in endophytic communities associated with plants growing in oil sands reclamation soils. The results suggested that although both plants analyzed had similar 16s rRNA, *alkB* and *nah* gene abundance, sweet clover plants harbored bacterial species with a higher abundance of CYP153 genes. In addition, a total of 42 bacterial isolates were positive for at least one hydrocarbon degrading gene analyzed. Most of these isolates closely match the database for *Pantoea*, *Pseudomonas* and *Enterobacter* spp. Since one of the main objectives of my research was to determine the potential use of endophytic root bacteria and host plants to degrade hydrocarbons, this study was designed to assess the feasibility of these applications. Therefore, Chapter 6 is focused on evaluating (*i*) plant growth promotion effects of selected isolates and (*ii*) their soil hydrocarbon degradation potential in association with plants.

6.2 Abstract

Microbe-assisted phytoremediation depends on competent root associated microorganisms that enhance remediation efficiency of organic compounds. Endophytic bacteria are a key element of the root microbiome and may assist plant degradation of contaminants. The objective of this study was to (*i*) investigate plant growth promotion and compatibility of selected endophytic hydrocarbon degrading bacteria in barley (*Hordeum vulgare*) and sweet clover (*Melilotus albus*) plants and (*ii*) investigate the application of plant-bacterial associations in a phytoremediation experiment using soils amended with diesel at 5,000, 10,000 and 20,000 $mg \cdot kg^{-1}$. Seed germination experiments revealed that specific associations between endophytic bacteria (used as inoculants) and host plants varied from plant growth stimulation to inhibition. Since most growth promotion effects were observed in sweet clover, this plant was selected for a

root elongation and long-term growth promotion assays. After 65 days, sweet clover inoculation with EA1-36 (*Pantoea* sp.) and EA3-16 (*Chryseobacterium* sp.) exhibited a significant increase in the number of secondary shoots and root biomass respectively. Based on overall growth promoting effects, bacterial strains: EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) were selected for a phytoremediation experiment. In that study plant growth inhibition caused by diesel fuel toxicity was overcome in inoculated plants, which showed significantly higher plant biomass. Analysis of soil F2 and F3 hydrocarbon fractions also revealed that these soils were remediated by inoculated plants when diesel was applied at 10,000 $mg \cdot kg^{-1}$ and 20,000 $mg \cdot kg^{-1}$. Thus, plants inoculated with select hydrocarbon degrading bacteria could be a strategy to increase plant tolerance and hydrocarbon degradation in contaminated (*e.g.* diesel fuel) soils.

6.3 Introduction

Petroleum derived fuels and chemicals are extensively used by modern society (Singh *et al.*, 2012; Khan *et al.*, 2013; Margesin *et al.*, 2003). However, the processes of extraction, refining, transport, use and storage of petroleum and its derivatives are prone to leaking and/or spilling accidents, resulting in widespread environmental contaminations (Meyer *et al.*, 2014). Besides its harmful effects on human health, hydrocarbon contamination also affects plant growth and development (Saraeian *et al.*, 2017). Therefore, a broad range of physical, chemical and biological methods are used for remediation of water and soil contaminated with hydrocarbons (Khan *et al.*, 2014). Conventional physicochemical soil remediation techniques, although highly efficient, are often expensive and environmentally invasive, causing changes in soil structure, decreasing microbial activity and the depletion of essential nutrients for plant development (Rajtor and Piotrowska-Seget, 2016; Afzal *et al.*, 2014). To overcome these challenges, one of the most promising technologies for cleaning up hydrocarbon contaminated soils is phytoremediation (Płociniczak *et al.*, 2017).

Phytoremediation is defined as the use of plants to remove pollutants from the environment or to render them harmless (Pilon-Smits, 2005; Salt *et al.*, 1998). This technology provides an environmentally friendly, effective, relative inexpensive and carbon neutral approach for the clean up of toxic pollutants in the environment (Germaine *et al.*, 2013; Glick, 2010). In order to be suitable for phytoremediation, plants should be adapted to the polluted environment,

however, the presence of soil organic pollutants normally reduces plant growth and subsequent phytoremediation efficiency (Afzal *et al.*, 2014). After uptake, organic compounds may be metabolized by plants; however, plants can only absorb minute quantities of soil hydrocarbons and translocate them into their different compartments, where they can be stored or volatilized into the atmosphere (Khan *et al.*, 2013). In addtion, one of the main disadvantages of using plans alone for phytoremediation is that by being photoautotrophic, plants do not rely on organic molecules as a source of energy or carbon (McCutcheon and Schnoor, 2003). Unlike microorganisms, plants have not developed the ability to degrade recalcitrant molecules, resulting in a more limited spectrum for their use in remediation of contaminated soils (Weyens *et al.*, 2009). Therefore, exploitation of plant–bacteria partnerships may overcome these limitations.

The plant microbiome includes fungal, archaeal and bacterial communities associated with their host plants in the rhizosphere (narrow zone of soil that surrounds and is influenced by plant roots), phyllosphere (aerial surface of plants) and endosphere (interior tissue of plants) (Tardif *et al.*, 2016). Bacteria isolated from the rhizosphere and the endosphere (endophytic bacteria) are known to enhance the accumulation of heavy metals and degradation of organic compounds (Germaine *et al.*, 2013; Sessitsch *et al.*, 2013). Plants release exudates from their roots that enhance microbial bioremediation in the rhizosphere, *i.e.* phytoremediation ex-planta (Salt *et al.*, 1998). Microbial abundance and activity can be 5–100 times greater in the rhizosphere when compared to bulk soil (Germida *et al.*, 2002). Therefore, competent microorganisms that produce hydrocarbon degrading specific enzymes are also enriched in the rhizosphere. Although rhizosphere bacteria have been sucessfully used to degrade hydrocarbons (Al-Baldawi *et al.*, 2017; Liu *et al.*, 2015a), endophytic bacteria have a more intimate relationship with their host plants, which may result in a higher phytoremediation efficiency (Li *et al.*, 2012a).

Endophytic bacteria reside at least part of their lives within plant tissues (Turner *et al.*, 2013). These microorganisms, while actively colonizing plant tissues, may enhance the plant's adaptation and growth in polluted soils (Tardif *et al.*, 2016; Afzal *et al.*, 2014). Endophytic bacteria are also known to provide direct plant growth promoting mechanisms such as nitrogen fixation (Knoth *et al.*, 2014), auxin (Shi *et al.*, 2011), siderphore (Rungin *et al.*, 2012) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Barnawal *et al.*, 2016) prodution.

Moreover, endophytic bacteria can indireclty benefit plant growth by acting as biocontrol agents (Chen *et al.*, 1995), increasing heavy metal accumulation (Sheng *et al.*, 2008a) and by hydrocarbon degradation (Andreolli *et al.*, 2013; Phillips *et al.*, 2008).

Although a few studies report that fungi can colonize different plant compartments and enhance HC degradation (Soleimani *et al.*, 2010; Dai *et al.*, 2010), bacteria are the most important group capable of enhancing phytoremediation of petroleum hydrocarbons (Khan *et al.*, 2013). Previous studies also report that endophytic bacteria harboring hydrocarbon degrading genes are able to promote plant growth and degrade hydrocarbons in soil (Yousaf *et al.*, 2011; Andria *et al.*, 2009). For example, Andria *et al.* (2009) studied two bacterial strains (containing the *alkB* hydrocarbon degrading gene) islolated from the endosphere and rhizosphere of Italian ryegrass (*Lolium multiflorum*) and found that inoculation of the endophytic strain resulted in better establishment of plants growing in hydrocarbon contaminated soils. Khan *et al.* (2013) and Tara *et al.* (2014) also suggested that bacteria having pollutant-degrading and/or plant growth-promoting activities can play an important role in phytoremediation of contaminated soils.

Therefore, the objectives of this study were to (*i*) assess plant growth promotion and compatibility of selected endophytic hydrocarbon degrading bacteria inoculated in barley (*Hordeum vulgare*) and sweet clover (*Melilotus albus*) plants and (*ii*) evaluate their use as inoculants to enhance phytoremediation of soils amended with diesel fuel.

6.4 Materials and Methods

6.4.1 Inoculum preparation

Forty-two bacterial isolates positive for hydrocarbon degrading genes (Chapter 5) were grown in 300 mL Erlenmeyer containing 1/2 strength Trypticase Soy Broth (TSB) medium at 28˚C for 48 h on a rotary shaker (150 rpm) until a density of 10^9 CFU·mL^{-1} was reached (OD_{660} of 1). Cells were harvested by centrifugation (15 min at 6000 *g*), washed three times in sterile phosphate-buffered saline (PBS) (1.2 g of Na_2HPO_4·L^{-1}, 0.18 g of NaH_2PO_4·L^{-1}, 8.5 g of NaCl·L^{-1}) buffer and re-suspended in sterile tap water to 1/10 of the original volume of PBS buffer to obtain an inoculum with a cell density of 10^{10} CFU·mL^{-1} (Barac *et al.*, 2004; Ho *et al.*, 2013).

6.4.2 Seed inoculation

Seeds were surface sterilized by soaking in ethanol (65% v·v^{-1}) for 3 min and in a NaClO solution (10% v·v^{-1}) for 5 min, followed by 10 rinses in sterile water (Vincent, 1970). Surface sterilized seeds were placed in a bacterial suspension for 4 h, allowing the bacteria to penetrate the seed ruptures in order to ensure colonization during seed germination. Seeds and bacterial suspensions were placed in sterile plastic bags containing 3 mL of 1 % (w·v^{-1}) methylcellulose and mixed with 15 g of talc. Subsequently, seeds were mixed in this formulation until uniformly coated and air dried overnight (de Freitas *et al.*, 1993). This procedure resulted in a final bacterial concentration of 10^8- 10^9 CFUs per seed. Control seeds were coated as previously described using a mixture of autoclaved bacterial strains.

6.4.3 Plant growth promotion experiments

6.4.3.1 Seed germination and vigor

Bacterial isolates were assessed for seed germination by placing coated seeds onto sterile filter paper moistened with 4 mL of sterile distilled water (10 seeds per plate and 5 replicates) and incubated at 10 and 25 ºC. Germination rate (%) was estimated according to Wu *et al.* (2016): (Gt/T) × 100, where Gt is the total number of germinated seeds within 7 days and T is the total number of seeds. Germination vigor (%) was determined as: (Gmax/T) × 100, where Gmax is the maximum number of seeds germinated in the first day during the 7 days of germination assay and T is as defined previously.

6.4.3.2 Root Elongation

The root elongation assays were conducted using growth pouches as described previously (Belimov *et al.*, 2002; de Freitas *et al.*, 1993). Plastic pouches (16.5 × 18 cm) containing chromatographic filter paper (Mega International, Minneapolis, MN, United States) and 20 mL of sterile 1/5 strength Hoagland's nutrient solution (Hoagland and Arnon, 1938) were wrapped in aluminum foil and autoclaved prior to seeding. Ten inoculated seeds were placed in each seed-pack growth pouch and five replicate pouches were used for each treatment and control. After germination, pouches were thinned to 5 seeds per pouch. Growth pouches were covered with aluminum foil to prevent light in plant roots and incubated upright on a wooden tray in a growth chamber with a 16 h/25°C day (1500 µmol.m^{-2}) and 8 h/15°C night cycle. The moisture content

in the pouches was kept constant throughout the experiment by two treatments: (*i*) additions of sterile distilled water when required or (*ii*) additions of sterile distilled water alternated with 1/5 strength Hoagland's nutrient solution every 2 days. All water/nutrient solution additions were conducted under aseptic conditions. After 20 days, plants were removed from the pouches for root length measurement. The roots were scanned using an Epson (Perfection V700) scanner with a resolution of 600 dpi and processed using WinRhizo 2013e (Regent Instruments, Canada).

6.4.3.3 Long-term pot experiment

Plants were grown in pots containing 1.5 kg of Dark Brown Chernozem silty clay agricultural soil mixed with silica sand (1:2 ratio). The properties of the soil and silica sand mix were as follows: 23.5 mg·kg^{-1} available inorganic N (NO_3^-+ NH_4^+); 5.3 mg kg^{-1} of $NaHCO_3$ extractable phosphorus; 88.5 mg·kg^{-1} of CH_3COONH_4 extractable K; and 1.42% of $K_2Cr_2O_7^-$ H_2SO_4 determined organic matter content (ALS Laboratory Group, Saskatoon, Canada). Inoculated and control plants were thinned to 1 plant per pot and arranged in a randomized complete block design (RCBD). Each treatment was replicated six times. Pots were rotated daily and watered to maintain approximately 50% gravimetric water content. Plants were harvested at flowering stage (65 days) and the number of flowering buds, number of secondary shoots, plant height, shoot and root biomass was measured.

6.4.4 Phytoremediation experiment

6.4.4.1 Experimental setup

Plants were grown in 1.5 kg pots containing a Dark Brown Chernozem silty clay agricultural soil amended with 5,000, 10,000 and 20,000 mg·kg^{-1} of diesel fuel purchased from a gasoline station. The four best performing bacterial strains in previous assays (section 6.4.3) were selected for a phytoremediation experiment using the seed inoculation method previously described in section 6.4.2. Uninoculated control plant (CU) treatment consisted of seeds coated with a mixture of autoclaved bacterial strains in equal amounts. Control soil (CS) treatment consisted of contaminated soil with diesel amendments without plants or addition of inoculants. Each treatment combination was replicated five times. Pots were arranged in a fully randomized block design (RCBD), rotated daily and watered to maintain approximately 50% gravimetric water content. One week after seed germination, pots were thinned to two plants per pot and an additional 100 ml inoculant suspension (10^9 CFU·mL^{-1}) was added to ensure bacterial

inoculation (Yousaf *et al.*, 2011; Afzal *et al.*, 2013a). The CU treatment was inoculated with a suspension (10^9 CFU·mL^{-1}) of a mixture containing equal numbers of the autoclaved bacterial strains. The CS treatment was watered with additional 100 mL of sterile tap water. Pots were maintained in growth chamber conditions with a 16 h/25°C day (1500 μmol.m^{-2}) and 8 h/15°C night cycle and harvested after 65 days. After harvest, plants were analyzed for plant height, number of secondary shoots, root and shoot biomass. Soils collected from each pot were analyzed for F2-F3 hydrocarbon fractions.

6.4.4.2 Hydrocarbon analysis

Diesel fuel contains three fractions differential based on component molecular weight: F1 (nC6 to nC10), F2 (nC10 to nC16) and F3 (nC16 to nC34) hydrocarbons. The F1 fraction consists of volatile hydrocarbon mixtures and are usually lost in the sample collection and preparation processes. Consequently, soils were only analyzed for the F2 and F3 fractions using the modified method from Schwab *et al.* (1999) and Siddique *et al.* (2006) followed by GC-FID analysis. Briefly, 2 g of soil sample was added into a 45 mL glass vial and mixed 2 g with anhydrous Na_2SO_4 treated with dichloromethane and 30 mL of a hexane/acetone solution (1:1, v·v^{-1}). Glass vials were sealed with a Teflon-lined cap and shaken overnight on an orbital shaking incubator (200 rpm). After shaking, samples were centrifuged for 10 min (1,000 rpm) and the extract was recovered using a glass pipette. The extracts were cleaned using a column procedure to remove polar organic compounds (Siddique *et al.*, 2006). A silica gel column was prepared for each treatment using approximately 63.5 mm of 70–230 mesh Grade 60 Å activated (heated to 101°C for 12 h) silica gel followed by approximately 24.5 mm of anhydrous Na_2SO_4 (dried at 400°C for 4 h). After conditioning the column with hexane, the extract was passed through the silica gel column, and the column was flushed with additional solvent to ensure all compounds of interest were collected. Samples were dried under N_2 flow and diluted to a final volume of 1.5 mL with hexane to fit the GC calibration range. The F2 and F3 hydrocarbon fractions were measured using a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector (FID). The capillary column was a Zebron™ ZB-FFAP, 30 m long × 0.32mm width × 0.25 μm film thickness (Phenomenex Inc., Torrance, CA, United States). The carrier gas was helium (purity = 99.999%). The injector and detector temperature were 250°C and 320°C, respectively. The oven temperature program consisted of an initial temperature of 40°C for 2 min, which was increased at a rate of 12°C min^{-1} to 320°C, and held

for 10 min. The quantification of F2 and F3 fraction were accomplished by an eight-point external calibration curve using peak area. External standards consisted of serial dilutions of Diesel Range Organics (DRO) GC grade (Sigma-Aldrich, Oakville, ON, Canada). In addition, a reference matrix blank (silica sand + solvent) and a matrix spiked (silica sand + a known diesel concentration) was analyzed every 20 samples to confirm extraction efficiency.

6.5 Results

6.5.1 Seed germination

As an initial screening for plant compatibility, barley and sweet clover seeds were inoculated with hydrocarbon-degrading endophytic bacteria (n=42) and their effects on seed germination rate evaluated (Table 6.1, Appendix E). In general, inoculants had no significant ($p \leq 0.05$) stimulation on barley seed germination. However, 20 strains significantly inhibited barley seed germination.

Inoculation of sweet clover seeds with strain EA3-25 (*Xanthomonas* sp.) significantly increased germination, with 98% of the seeds having germinated after 7 days compared to 86.6% in control treatments. Almost all bacterial strains tested (40/42) had no significant effect on seed germination rate; however, strain EA5-9 (*Rahnella* sp.) inhibited seed germination (*i.e.* 54% germinated).

Overall, while most inhibitory effects due to bacterial inoculation were observed in barley, stimulation of seed germination was only observed in sweet clover. Therefore, sweet clover was selected as the test crop. Based on their overall effects on sweet clover, a total of 30 hydrocarbon-degrading endophytic strains were selected for further studies.

6.5.2 Root Elongation

Root elongation was assessed in experiments with water and alternate additions of Hoagland nutrient solution. Bacterial strains EA1-1 (*Pedobacter* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.), EA6-5 (*Pseudomonas* sp.) and EA7-4 (*Pantoea* sp.) significantly ($p \leq 0.05$) increased sweet clover root length by 42 to 68% (Figure 6.1, Appendix F). Although not significant, inoculation with 14 other strains resulted in an average root length greater than control plants. In contrast, strains EA4-1 (*Chryseobacterium* sp.), EA4-6 (*Serratia* sp.), EA4-13 (*Enterobacter* sp.) and EA4-27 (*Ewingella* sp.) inhibited ($p \leq 0.05$) root elongation (Figure 6.1).

Table 6.1 Effect of endophytic bacteria on barley and sweet clover seed germination rate (%) (total of 10 seeds) at 25ºC. Symbols indicate treatments significantly (LSD $p \leq 0.05$) higher (*) and lower (†) when compared to the control.

	Barley		Sweet clover	
	(%)			
Strain	**Mean**	**SD**	**Mean**	**SD**
Control	92.0	8.4	86.0	7.5
EA1-1	78.0	21.7	84.0	5.5
EA1-15	60.0†	21.2	90.0	14.1
EA1-17	72.0	27.8	88.0	11.0
EA1-36	80.0	7.1	82.0	11.0
EA2-9	78.0	23.9	88.0	8.4
EA2-19	66.0	27.0	80.0	10.0
EA2-20	66.0†	16.1	80.0	15.8
EA2-24	84.0	20.7	84.0	15.2
EA2-30	92.0	8.4	76.0	15.2
EA3-1	84.0	11.4	92.0	8.4
EA3-10	60.0†	18.7	92.0	13.0
EA3-16	92.0	8.4	92.0	8.4
EA3-17	62.0	27.6	92.0	8.4
EA3-18	48.0†	14.8	88.0	11.0
EA3-25	18.0†	13.0	98.0*	4.5
EA4-1	18.0†	14.8	94.0	5.5
EA4-2	4.0†	5.5	90.0	10.0
EA4-6	14.0†	15.2	77.0	31.3
EA4-8	22.0†	8.4	72.0	24.9
EA4-13	6.0†	8.9	80.0	7.1
EA4-27	58.0†	17.9	73.0	8.9
EA4-32	58.0†	11.0	82.0	4.5
EA4-34	30.0†	30.0	88.0	8.4
EA4-35	54.0	39.1	86.0	11.4
EA4-38	24.0†	13.4	94.0	5.5
EA4-40	92.0	13.0	90.0	10.0
EA5-5	66.0	27.0	90.0	7.1
EA5-8	70.0	18.7	84.0	11.4
EA5-9	18.0†	13.0	54.0†	8.9
EA5-10	12.0†	16.4	74.0	11.4
EA5-11	74.0	27.0	68.0	13.0
EA5-20	60.0	25.5	86.0	8.9
EA6-5	70.0†	10.0	88.0	4.5
EA6-12	74.0	15.2	92.0	4.5
EA6-37	36.0†	11.4	82.0	19.2
EA6-38	68.0	30.3	84.0	16.7
EA6-40	72.0	24.9	78.0	13.0
EA7-1	88.0	16.4	84.0	15.2
EA7-4	66.0	34.4	96.0	5.5
EA7-11	88.0	13.0	88.0	11.0
EA1-34	64.0†	16.1	82.0	8.4
EA1-37	46.0†	23.0	82.0	11.0

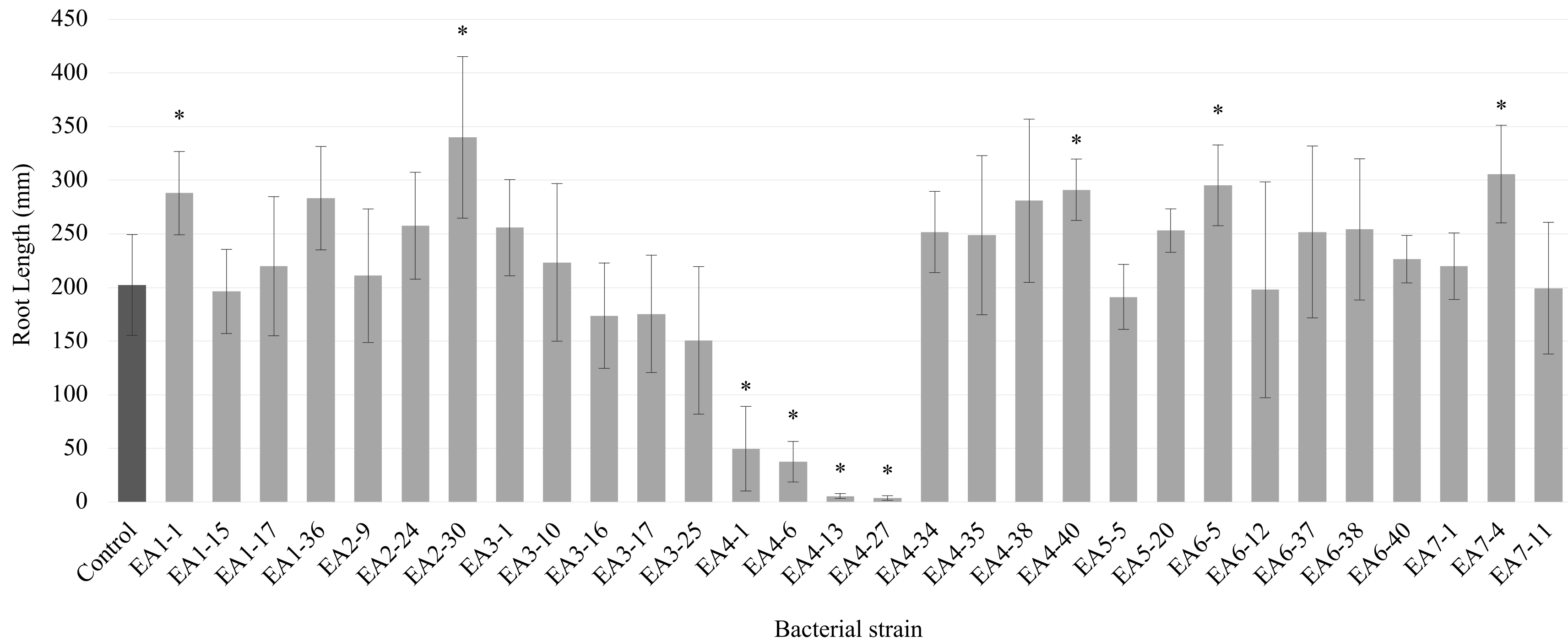

Figure 6.1 Effect of endophytic bacteria on root length of sweet clover plants measured at 20 days. Plants were supplemented with water and alternate additions of Hoagland nutrient solution. Error bars represent standard deviations and * indicate significance at LSD ($p \leq 0.05$) compared to control.

6.5.3 Long-term growth promotion

Based on seed germination results, 30 endophytic bacterial strains were tested for plant growth promotion assessed at 65 days post treatment (Figure 6.2). Inoculation with strain EA1-36 significantly increased the average number of secondary shoots (n=16) compared to control plants (n=14.3) (Figure 6.2a). The number of flowering buds; however, was not affected by plant inoculation, and none of the bacterial inoculants increased plant height (Figure 6.2b, Figure 6.2c). Moreover, inoculation with strains EA4-34 and EA7-1, both *Pantoea* sp., significantly reduced plant height by 17 and 18%, respectively.

Overall, most bacterial strains had no effect on shoot fresh and dry weight (Figure 6.3a, Figure 6.3b). Inoculation with strain EA7-1 (*Pantoea* sp.), however, inhibited root and shoot growth. Inoculation of sweet clover with strains EA6-5 (*Pseudomonas* sp.), EA6-12 (*Pseudomonas* sp.), EA6-37 (*Enterobacter* sp.) and EA6-38 (*Kluyvera* sp.) also led to a significant decrease in shoot fresh weight. Whereas strains EA1-15 (*Chryseobacterium* sp.), EA1-17 (*Stenotrophomonas* sp.), EA6-12 (*Pseudomonas* sp.), EA6-37 (*Enterobacter* sp.) and EA7-1 (*Pantoea* sp.) significantly decreased root dry weight, strain EA3-16 (*Chryseobacterium* sp.) significantly increased root fresh and dry weight by 42 and 55%, respectively.

Based on plant growth promotion, seven endophytic bacterial strains [EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA3-1 (*Bacillus* sp.), EA3-16 (*Chryseobacterium* sp.), EA3-25 (*Xanthomonas* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.)] were selected for further study. Among these strains, isolates EA3-1 (*Bacillus* sp.), EA3-16 (*Chryseobacterium* sp.) and EA3-25 (*Xanthomonas* sp.) tested positive for the *nah* gene, which is involved in the degradation of aromatic hydrocarbons (Chapter 5). Since most studies on hydrocarbon degradation use diesel fuel as a model compound (which contains mostly aliphatic hydrocarbons) the subsequent phytoremediation experiment focused on bacteria capable of enhancing degradation of aliphatic hydrocarbons. Thus, three isolates positive for the *alkB* gene [EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.), EA6-5 (*Pseudomonas* sp.)] and one isolate positive for the CYP153 gene [EA1-17 (*Stenotrophomonas* sp.)] were tested in a phytoremediation experiment.

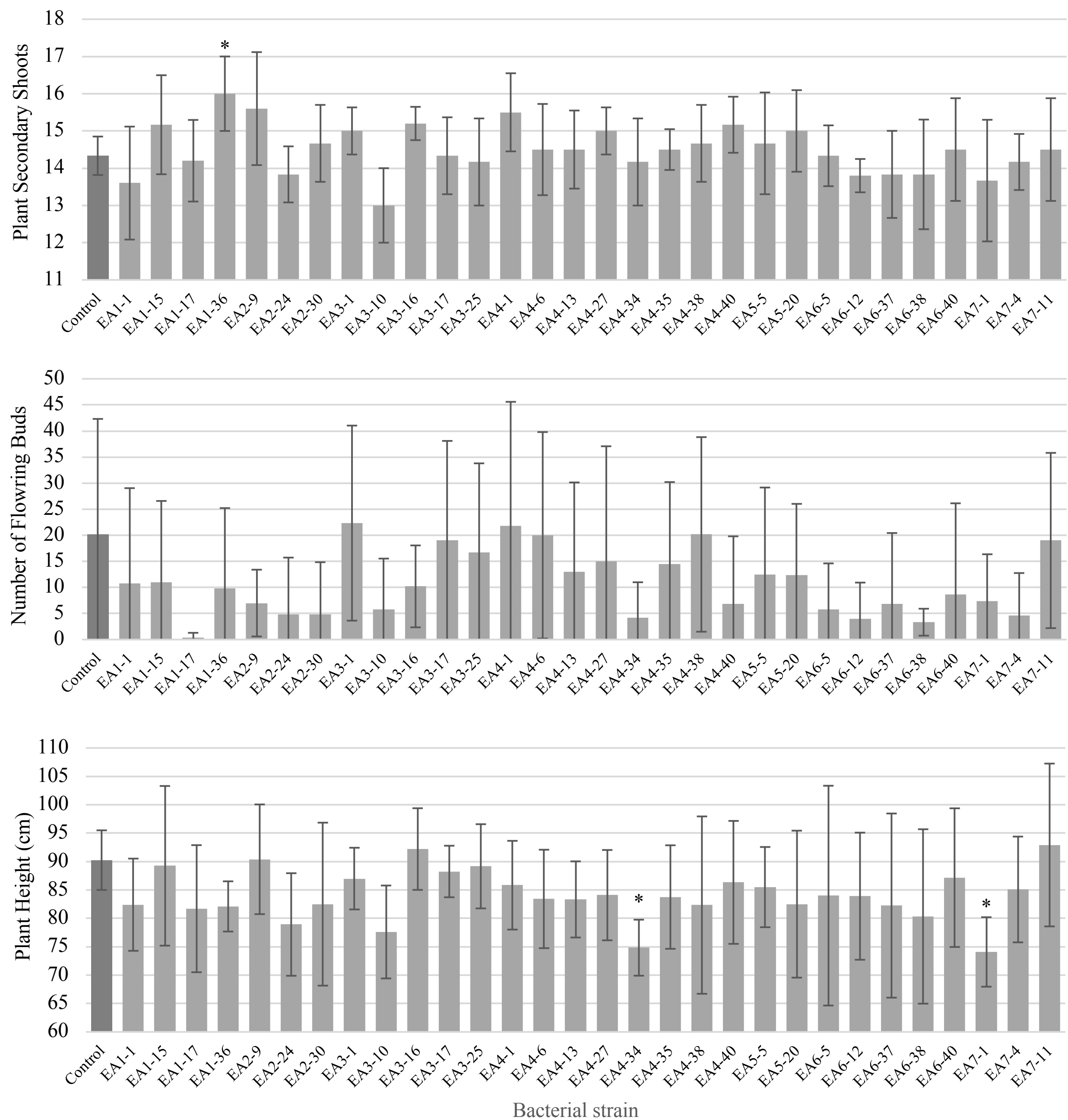

Figure 6.2 Plant secondary shoots (A), number of flowering buds (B) and height (C) of sweet clover plants inoculated with endophytic bacteria after 65 days. Error bars represent standard deviations and * indicate significance at LSD ($p \leq 0.05$) compared to control.

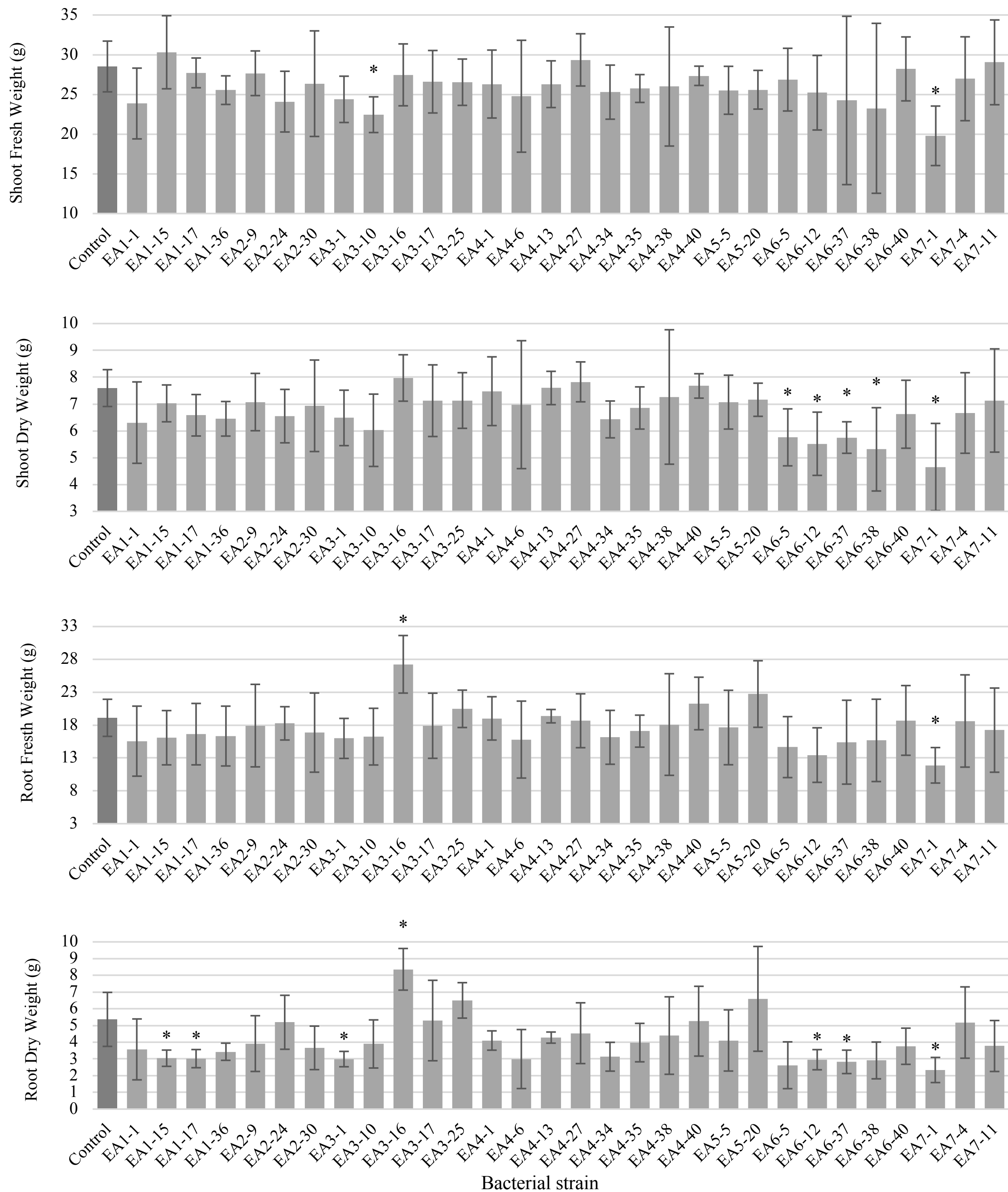

Figure 6.3 Shoot fresh (A) and dry weight (B), root fresh (C) and dry (D) weight of sweet clover plants inoculated with endophytic bacteria after 65 days. Error bars represent standard deviations and * indicate significance at LSD ($p \leq 0.05$) compared to control.

6.5.4 Phytoremediation experiment

As expected, an increase in soil diesel concentration led to a decrease in plant biomass (Table 6.2, Appendix H). However, plants inoculated with endophytic bacteria had an overall greater plant biomass compared to control treatments. Inoculation with most bacterial strains increased the number of secondary shoots in diesel contaminated soil, but had no effect on the number flowering buds (Table 6.3). In soils amended with diesel at 5,000 mg·kg^{-1}, all inoculated plants had a significantly greater average number of secondary shoots compared to control plants. At this diesel concentration, plants inoculated with strain EA1-17 (*Stenotrophomonas* sp.) exhibited the highest average number of secondary shoots (N=12.7) followed by plants inoculated with strains EA4-40 (*Pantoea* sp.) (N=12.3) and EA2-30 (*Flavobacterium* sp.) (N=12.2). In soils amended with diesel at 10,000 mg·kg^{-1}, a significant increase in the number of secondary shoots was also observed in all inoculated plants except for plants inoculated with strain EA4-40 (*Pantoea* sp.). However, only plants inoculated with strains EA1-17 (*Stenotrophomonas* sp.) and EA6-5 (*Pseudomonas* sp.) had a significantly higher number of secondary shoots in soils amended with diesel at 20,000 mg·kg^{-1}. Strain EA1-17 (*Stenotrophomonas* sp.) also enhanced the number of flowering buds in plants grown in soils amended with diesel at 5,000 mg·kg^{-1}, but there was no significant difference in plants grown in soils amended with 10,000 and 20,000 mg·kg^{-1} diesel (Table 6.4).

Overall, inoculation of plants with hydrocarbon degrading endophytic bacteria led to an increase in plant height, shoot and root fresh weight (Figures 6.4, 6.5, 6.6 and 6.7). For example, all inoculated plants had significantly greater plant height and shoot fresh weight compared to control plants in soils amended with 5,000 mg·kg^{-1} diesel. Strains EA1-17 (*Stenotrophomonas* sp.) and EA4-40 (*Pantoea* sp.) significantly increased root fresh weight, and plant height was 72, 60, 75 and 60% higher in plants inoculated with strains EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.), respectively. In addition, inoculation of plants with strains EA1-17 (*Stenotrophomonas* sp.) and EA4-40 (*Pantoea* sp.) led to a 2-fold increase in shoot fresh weight.

Inoculation with sweet clover with hydrocarbon degrading endophytic bacteria enhanced plant biomass in soils amended with diesel at 10,000 mg·kg^{-1} and statistical differences between inoculated treatments were also observed. For example, plant height, shoot and root fresh weight

were greater in plants inoculated with EA1-17 (*Stenotrophomonas* sp.), and EA6-5 (*Pseudomonas* sp.) compared to control plants and other inoculated treatments. The average plant height in sweet clover inoculated with strains EA1-17 (*Stenotrophomonas* sp.) and EA6-5 (*Pseudomonas* sp.) was 21.4 and 18.5 cm, respectively, compared to 5.67 cm to control plants.

Among all diesel concentrations, the lowest plant biomass was observed in soils amended with diesel at 20,000 $mg{\cdot}kg^{-1}$. At this concentration, the inoculation with strains EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) increased plant height ($p \leq 0.05$). In addition, whereas shoot fresh weight was greater in all inoculated treatments, no statistical differences in root fresh weight were observed.

Table 6.2 Sweet clover plant parameters grown in soils amended with diesel after 65 days. Different letters indicate significance at LSD ($p \leq 0.05$).

Soil diesel Concentration ($mg{\cdot}kg^{-1}$)	Number of Secondary Shoots	Number of Flowering Buds	Shoot Fresh Weight (g)	Plant Height (cm)	Root Fresh Weight (g)
5,000	11.2a	1.0a	13.14a	50.86a	6.81a
10,000	4.8b	0.1b	1.57b	13.13b	0.59b
20,000	0.8c	0.0b	0.05c	4.77c	0.04c

Table 6.3 Effect endophytic bacterial inoculants on the number of secondary shoots and flowering buds of sweet clover plants grown in soils amended with diesel after 65 days. Different letters indicate significance at LSD ($p \leq 0.05$).

Soil diesel Concentration ($mg{\cdot}kg^{-1}$)	Inoculant	Number of Secondary Shoots	Number of Flowering Buds
5,000	Control	8.4c	0.0b
	EA1-17	12.7a	4.4a
	EA2-30	12.2a	0.0b
	EA4-40	12.3a	0.5b
	EA6-5	10.5b	0.0b
10,000	Control	2.0c	0.0a
	EA1-17	6.4ab	0.0a
	EA2-30	4.6ab	0.0a
	EA4-40	4.1bc	0.0a
	EA6-5	6.9a	0.4a
20,000	Control	0.0cd	0.0a
	EA1-17	1.1ab	0.0a
	EA2-30	0.0d	0.0a
	EA4-40	0.9bc	0.0a
	EA6-5	1.8a	0.0a

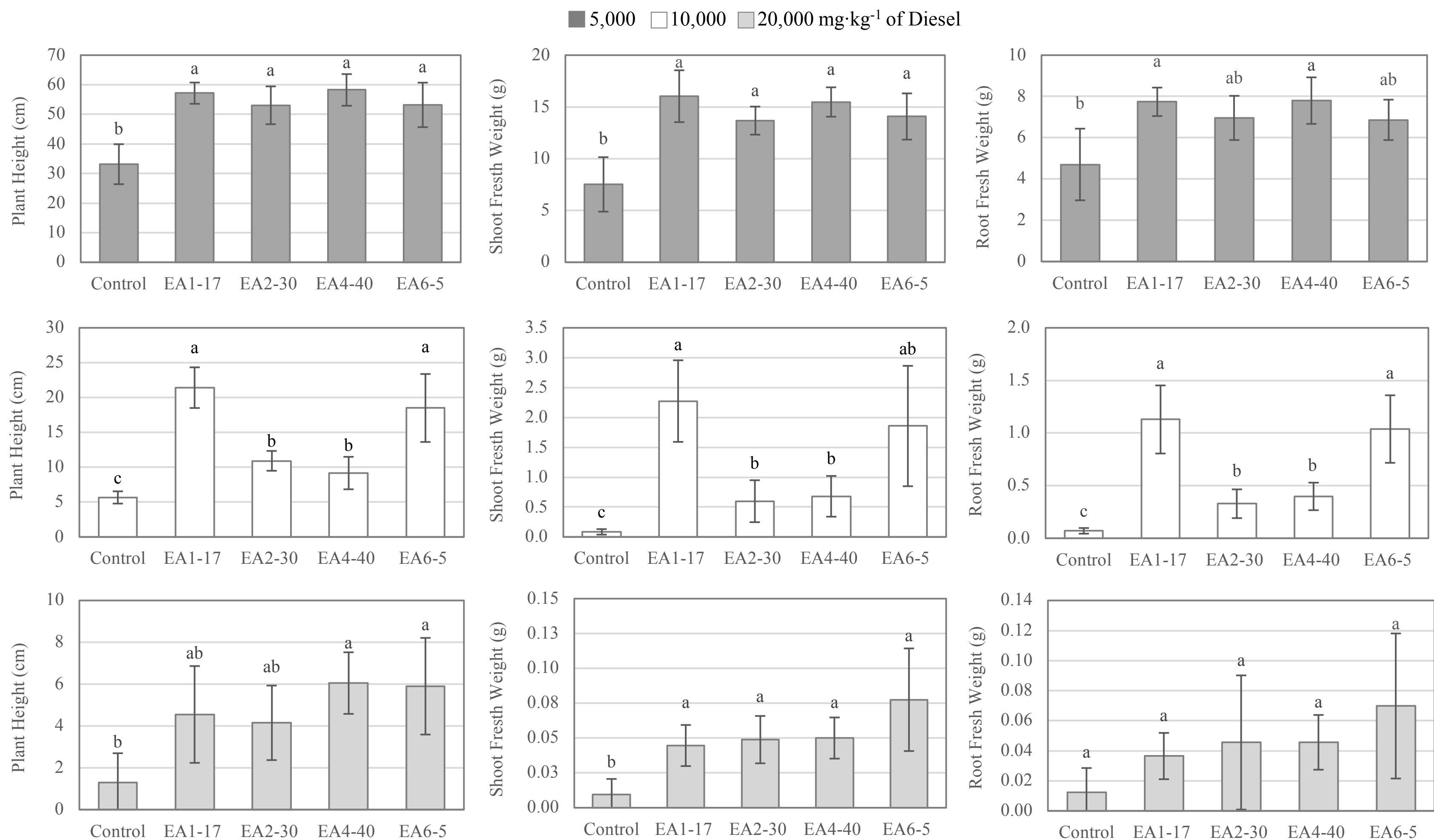

Figure 6.4 Effect of endophytic bacterial inoculants EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover (*Melilotus albus*) plant parameters after 65 days. Error bars represent standard deviations (n=5) and different letters indicate significant differences (LSD $p \leq 0.05$).

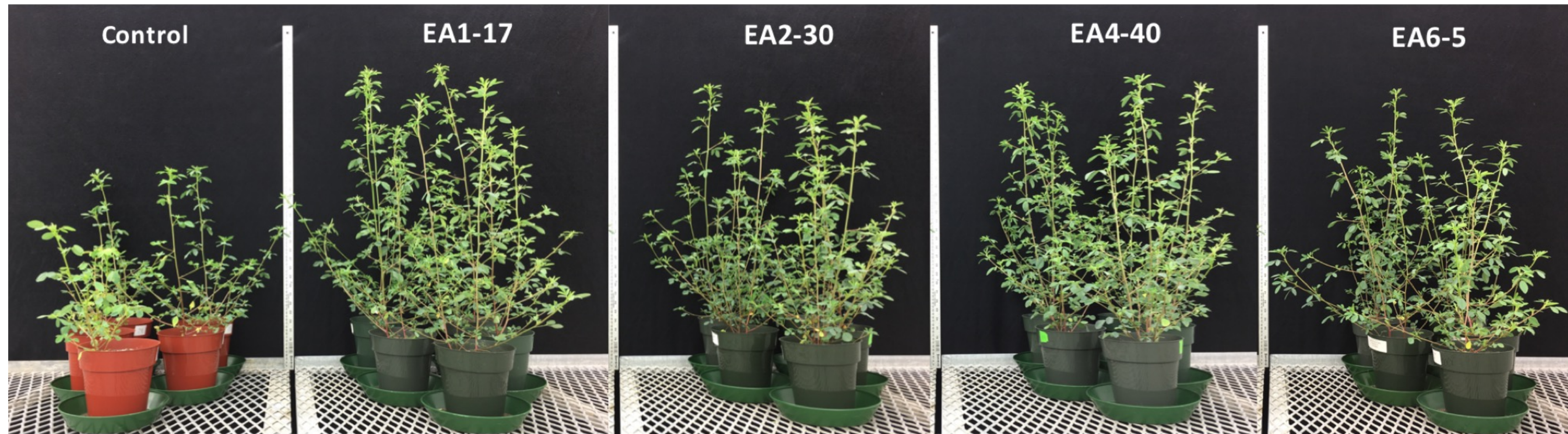

Figure 6.5 Effect of endophytic bacterial inoculants EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover plants (*Melilotus albus*) growing in soils amended with diesel (5,000 $mg \cdot kg^{-1}$) at 65 days after planting.

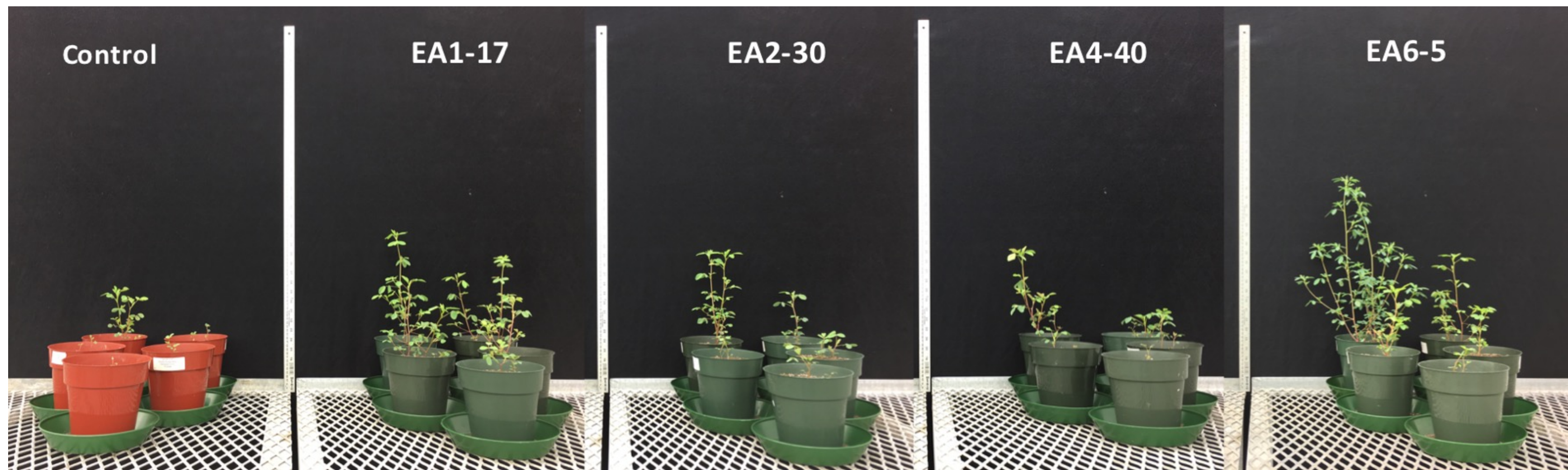

Figure 6.6 Effect of endophytic bacterial inoculants EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover plants (*Melilotus albus*) growing in soils amended with diesel (10,000 $mg \cdot kg^{-1}$) at 65 days after planting.

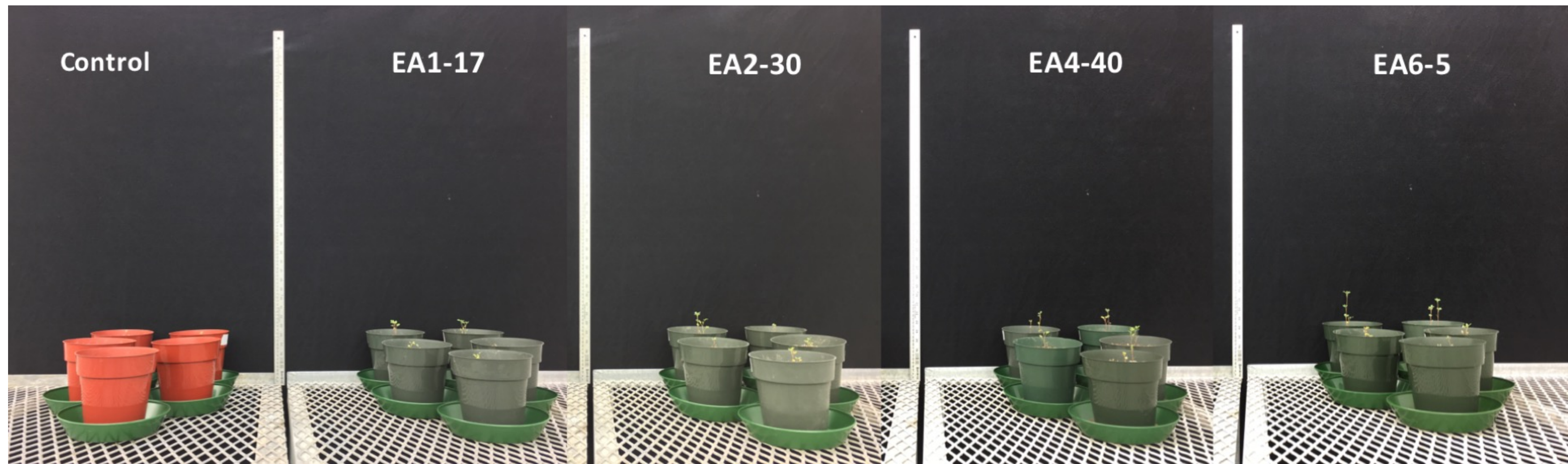

Figure 6.7 Effect of endophytic bacterial inoculants EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover plants (*Melilotus albus*) growing in soils amended with diesel (20,000 mg·kg^{-1}) at 65 days after planting.

In a preliminary experiment (Appendix G), analysis of the soil F2 and F3 hydrocarbon fractions immediately after soil amendment with diesel at 10,000 and 20,000 mg·kg^{-1} revealed a recovery of 10,930 and 21420 mg·kg^{-1} respectively. After 65 days, phytoremediation experiments revealed that all plants reduced soil hydrocarbons to some extent (Table 6.4, Figure 6.8). However, the overall soil hydrocarbon concentration was significantly lower in inoculated treatments.

In soils amended with diesel at 5,000 mg·kg^{-1}, there was no significant difference in total soil hydrocarbon concentration between treatments. In contrast, all inoculated treatments caused a significant decrease in total soil hydrocarbon concentration in soils amended with 10,000 mg·kg^{-1} diesel (Table 6.4). This decrease was a specific reduction in the F3 hydrocarbon fraction, which was 60 to 68% lower in inoculated treatments compared to uninoculated control plants (CU) (Figure 6.8b). In soil amended with diesel at 20,000 mg·kg^{-1}, significantly lower total hydrocarbon concentrations were observed in treatments inoculated with strains EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.), in which the final hydrocarbon concentration was 7625 and 4163 mg·kg^{-1}, respectively, compared to 12952 mg·kg^{-1} in the CU treatments (Table 6.4). There was no significant reduction in the F2 hydrocarbon fraction in most inoculated treatments, except for treatments inoculated with strain EA6-5 (*Pseudomonas* sp.) where a significant 71% reduction in this F2 fraction was observed. In addition, the F3 fraction concentration was 48 and 66% lower ($p \leq 0.05$) in treatments inoculated with strains EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.), respectively.

Table 6.4 Total extractable hydrocarbons in soil after 65 days growth of sweet clover plants inoculated with hydrocarbon degrading endophytic bacteria. Different letters indicate significant differences (Tukey HSD $p \leq 0.05$).

	Diesel Concentration (mg·kg^{-1})		
	5,000	10,000	20,000
Treatment	-------------- Total extractable hydrocarbons (mg·kg^{-1}) ----------------		
CS	430.69a	5086.69a	12974.22a
CU	418.70a	4882.57a	12952.72a
EA1-17	429.78a	1849.09b	10976.27ab
EA2-30	468.93a	1877.28b	10261.35ab
EA4-40	431.32a	2010.31b	7625.73bc

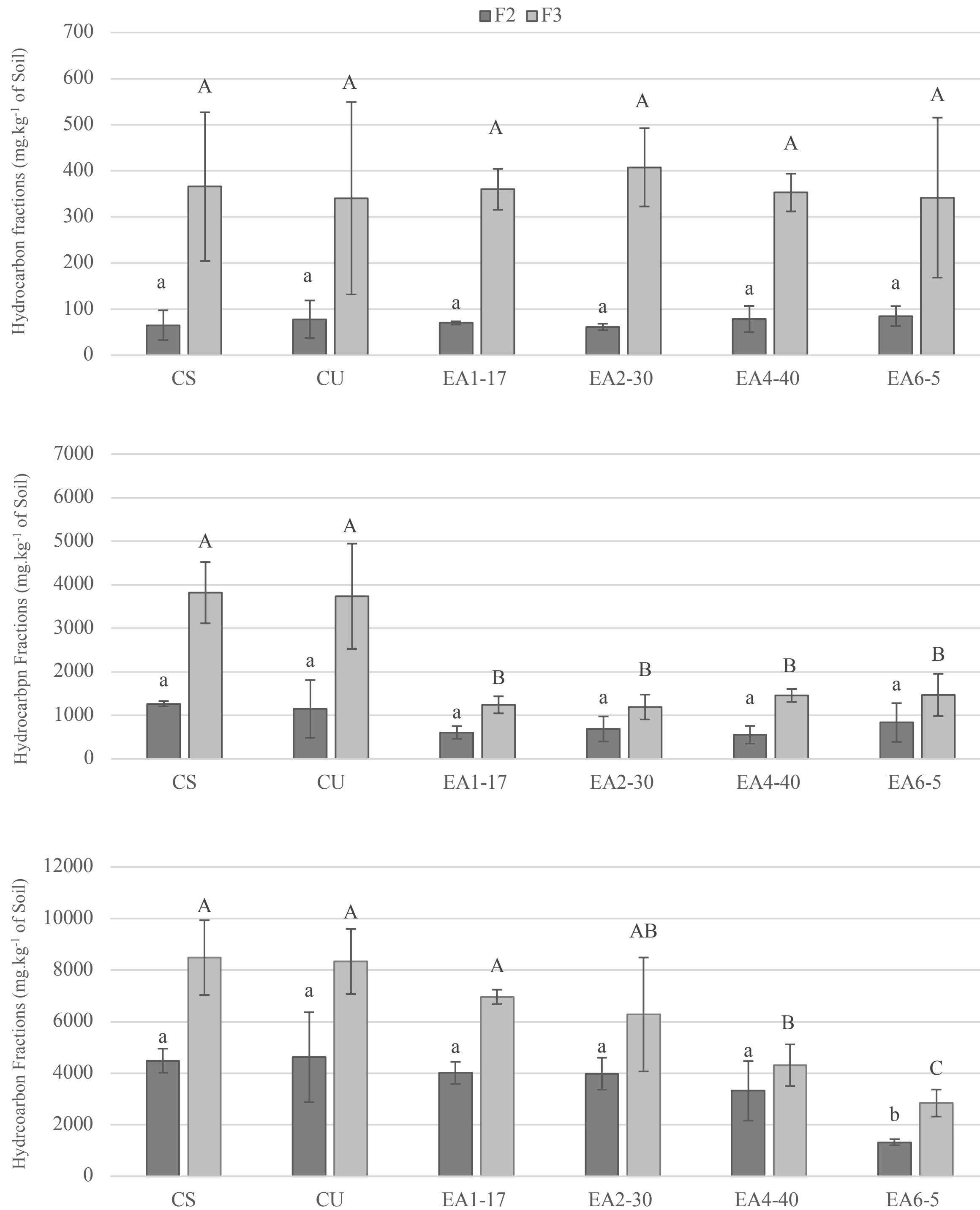

Figure 6.8 The F2 and F3 hydrocarbon fractions in soils initially amended with diesel at 5,000 (A), 10,000 (B) and (C) 20,000 mg·kg^{-1} after phytoremediation experiment. Error bars represent standard deviations (n=5) and different letters indicate differences (Tukey HSD $p \leq 0.05$).

6.6 Discussion

This study investigated the use of hydrocarbon degrading endophytic bacteria to enhance phytoremediation. In an initial screening to select bacteria better suited for phytoremediation, endophytic isolates were assessed for their effects on seed germination rate. A total of 42 isolates were inoculated on barley and sweet clover seeds, some of which stimulated or inhibited seed germination. This is a common phenomenon as other researchers found similar effects. For example, Long *et al.* (2008) studied plant growth promotion effects of bacterial endophytes associated with black nightshade (*Solanum nigrum*) and found that, out of 37 isolates, 22 significantly stimulated seed germination, but one isolate inhibited seed germination. Moreover, previous studies have also investigated the effects of bacterial inoculation on barley seed germination (Cardinale *et al.*, 2015; Harper and Lynch, 1980; Kang *et al.*, 2007; Guglielmetti *et al.*, 2013). Guglielmetti *et al.* (2013) reported that whereas barley inoculation with *Luteibacter rhizovicinus* had no effect on seed germination, *Pseudomonas chlororaphis* inhibited seed germination. Kang *et al.* (2007) also observed significant inhibitory effects of *P. chlororaphis* on barley seed germination, but no inhibition in cucumber or rice seeds. In the current study, bacterial inoculation mostly inhibited barley seed germination; however, strain EA3-25 stimulated sweet clover seed germination. This strain, previously identified as *Xanthomonas* sp., tested positive for the presence of naphthalene dioxygenase (*nah*) gene (Chapter 5). *Xanthomonas* is most commonly known to cause plant diseases (Soares *et al.*, 2010), but this genus also contain species known for polycyclic aromatic hydrocarbons (PAHs) degradation (Deka and Lahkar, 2016).

Barley plants have been used for phytoremediation of oil contaminated soils (Mikolasch *et al.*, 2016; Asiabadi *et al.*, 2015); however, most studies used other gramineous (Afzal *et al.*, 2011; Arslan *et al.*, 2014) and leguminous (Yateem *et al.*, 2000; Muratova *et al.*, 2010) plants. Sweet clover are forage legumes that fix nitrogen in symbiotic association with *Sinorhizobium meliloti* (Bromfield *et al.*, 2010). According to Hall *et al.* (2011) and Franco and Balieiro (2000), members of *Fabaceae* family, such as sweet clover, might be better suited to phytoremediation of petroleum hydrocarbons due to their low implementation costs and high capacity for adaptation in nutrient limited, degraded and oil contaminated soils. In the current study, sweet clover responded more favorably than barley to bacterial inoculants tested and, therefore, it was

selected for additional growth promoting (*i.e.* root elongation and long-term growth chamber experiments) and phytoremediation experiments.

Several studies have investigated the effects of bacterial inoculation on root elongation (Sheng *et al.*, 2008a; Bal *et al.*, 2013; Glick *et al.*, 1994). In this study, five bacterial strains stimulated root elongation in sweet clover plants. Among these isolates, the highest growth promoting effects were observed with EA2-30 (*Flavobacterium* sp.) and EA7-4 (*Pantoea* sp.). Pant *et al.* (2016) studied *Flavobacterium* inoculation in physic nut (*Jatropha curcas*) and found that this genus exhibited multiple plant growth promoting traits and improved biodegradation of 1,4-dichlorobenzene. Gontia-Mishra *et al.* (2016) also reported that inoculation of *Flavobacterium* sp. may increase drought tolerance in wheat plants. In addition, plant growth promotion by endophytic *Pantoea* spp. have also been reported in sugarcane (Quecine *et al.*, 2012), rice (Feng *et al.*, 2006) and pepper (Kim *et al.*, 2012) plants. *Pantoea* spp. may promote plant growth by increasing nitrogen supply (Asis and Adachi, 2004; Feng *et al.*, 2006), solubilizing phosphorus (Chen *et al.*, 2014; Wang *et al.*, 2016a) and producing phytohormones (Apine and Jadhav, 2011; Omer *et al.*, 2004). As both *Flavobacterium* sp. and *Pantoea* sp. enhanced root elongation in in sweet clover, these results indicate that, in addition to hydrocarbon degrading genes, these bacteria may possess plant growth promoting traits that could improve phytoremediation of contaminants. If true, this could be a strategy to enhance phytoremediation.

In addition to growth promotion at early stages of plant development (*i.e.* seed germination and root elongation), a long-term growth chamber experiment was used to assess growth promoting effects at plant flowering stage. In this study, while no significant differences in the number of flowering buds were observed between control and inoculated plants, inoculation of sweet clover with strain EA1-36 (*Pantoea* sp.) significantly increased the number of secondary shoots. Bakhshandeh *et al.* (2017) studied the inoculation of phosphate-solubilizing *Pantoea* spp. in rice and reported that *Pantoea* promoted plant growth in all parameters analyzed. According to Dastager *et al.* (2009), *Pantoea* also has significant growth inhibitory activity against phytopathogenic fungi and a wide tolerance to environmental conditions such as temperature, salt concentration and pH. However, Chen *et al.* (2017) reported that although some *Pantoea* species may confer beneficial effects to their plant hosts, other species may inhibit plant growth. In the current study, although sweet clover inoculation with EA1-36 (*Pantoea* sp.)

significantly increased the number of secondary shoots, strain EA4-34 (*Pantoea* sp.) significantly decreased plant height. According to de Souza *et al.* (2015) and Hol *et al.* (2013), the effect of bacteria on plant growth promotion depends on many factors, which not only depend on plant species and nutrient availability but also on bacterial species and strains. In some cases, variation in these factors can even lead to opposite effects on plant traits (Hol *et al.*, 2013). In the current study, inoculation with EA3-16 (*Chryseobacterium* sp.) also enhanced sweet clover root biomass. Since plant essential soil nutrients are taken up by roots, root growth is considered a prerequisite for the enhancement of plant development (Mills and Jones, 1996). Hence, many plant growth promoting bacteria stimulates root growth (Adesemoye *et al.*, 2009). Marques *et al.* (2010) found significant root growth in *Zea mays* plants when inoculated with *Chryseobacterium* sp. This genus has been previously known for its ability to solubilize phosphate (Chen *et al.*, 2006) and has been reported to increase root and shoot biomass, chlorophyll content, nitrate reductase activity, phosphorus content and crop yield in horse gram (*Macrotyloma uniflorum*) (Singh *et al.*, 2013). Therefore, plant growth promotion of sweet clover observed after inoculation with *Pantoea* sp. and *Chryseobacterium* sp. in this current study might be associated with the ability of these strains to solubilize phosphate.

Bacterial strains EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) were selected for the phytoremediation experiment based on their ability to promote plant growth in the absence of hydrocarbons. Diesel fuel was selected as the model compound as other studies have used it to assess hydrocarbon degradation (Andria *et al.*, 2009; Ferrera-Rodríguez *et al.*, 2013; Arslan *et al.*, 2014). The results in this study revealed that plant growth decreased as soil diesel concentrations increased. Similar results were observed by Wang *et al.* (2011) in reed (*Phragmites australis*) plants growing in soils amended with diesel concentrations ranging from 1,000 to 20,000 $mg \cdot kg^{-1}$. These authors concluded that root growth and total chlorophyll content significantly decreased in soils amended with diesel at concentrations higher than 10,000 $mg \cdot kg^{-1}$. According to Tara *et al.* (2014) and Chen *et al.* (2013), plant growth inhibition in the presence of petroleum hydrocarbons might be due to hydrocarbon toxicity and hydrophobicity, which can adversely affect plant health and development.

In addition, the C:N ratio in hydrocarbon contaminated soils is typically high due to the high input of carbon (C) into the system (Bento *et al.*, 2012). As nitrogen is essential for plant

and bacterial growth, large inputs of carbon sources results in a rapid depletion of nitrogen pools. This imbalance may cause adverse effects on soil microbial populations, as well as on the ability of plants to survive in contaminated soils (Hall *et al.*, 2011). However, sweet clover plants, due to their association with N-fixing bacteria, may withstand those adverse conditions in contaminated soils and also support the survival of hydrocarbon degrading bacteria (Hall *et al.*, 2011). In the current study, inoculation of sweet clover with hydrocarbon degrading endophytic bacteria enhanced plant growth in diesel amended soils, even though diesel concentration inhibited plant growth without the inoculant.

At low diesel concentration (soils amended with diesel at 5,000 $mg \cdot kg^{-1}$), inoculation of plants with endophytic bacteria significantly enhanced plant biomass, however, no significant differences in soil F2-F3 hydrocarbons concentrations were observed between control and inoculated treatments. Kaimi *et al.* (2007) also studied phytoremediation efficiency of twelve plant species in diesel-contaminated soils without the addition of bacterial inoculants. These authors found a significant hydrocarbon reduction in most planted soils; however, treatments with alfalfa (*Medicago sativa*) at flowering stage exhibited similar hydrocarbon concentrations to unplanted control soils. According to Liu *et al.* (2011), soil hydrocarbon reduction in control treatments are mainly due to volatilization, eluviation, photolysis and biodegradation by indigenous microorganisms. Therefore, the results in the current study suggests that although bacterial inoculants might alleviate plant stress, hydrocarbon reduction in soils amended with diesel at 5,000 $mg \cdot kg^{-1}$ might be attributed to volatilization and/or biodegradation by indigenous microorganisms. In addition, a noticeable diesel smell was observed throughout the duration of this experiment suggesting hydrocarbon volatilization.

Similar to soils amended with diesel at 5,000 $mg \cdot kg^{-1}$, bacterial inoculation also enhanced plant biomass in soils amended with diesel at 10,000 $mg \cdot kg^{-1}$. These results are in agreement with Tara *et al.* (2014), in which the authors studied the inoculation of hydrocarbon degrading bacteria in carpet grass (*Axonopus affinis*) growing in soils amended with diesel at 10,000 $mg \cdot kg^{-1}$. These authors found that plant inoculation with *Pseudomonas* sp. and *Pantoea* sp. led to an increase in plant biomass. In the current study, inoculation of sweet clover with bacterial strains EA1-17 (*Stenotrophomonas* sp.) and EA6-5 (*Pseudomonas* sp.) enhanced plant biomass. *Pseudomonas* spp. are well known to dominate the rhizosphere and several studies have shown their ability to improve phytoremediation (Germaine *et al.*, 2009; Afzal *et al.*, 2011).

Stenotrophomonas spp. also have been previously used in phytoremediation (Binks *et al.*, 1995) and are highly adaptable to nutrient-limited environments (Ryan *et al.*, 2009). In addition to differences in plant biomass, bacterial inoculation also improved soil F3 hydrocarbon degradation. Hydrocarbons in the non-volatile F3 fraction (C_{16}-C_{34}) are more resistant to microbial degradation, while the F2 (C_{10}-C_{16}) fraction consisting of semi-volatile medium-sized-chain compounds are more bioavailable and therefore more readily degraded (Leung *et al.*, 2006; Gomez and Sartaj, 2013). Gomez and Sartaj (2013) compared microbial biodegradation efficiency of different hydrocarbon fractions and their results indicated that F2 fractions had considerably higher biodegradation rate compared to F3 fractions. In the current study, analysis of soils amended with diesel at 10,000 $mg{\cdot}kg^{-1}$ suggests that whereas F2 fraction degradation might have been achieved by indigenous soil microorganisms, degradation of the F3 fraction was significantly enhanced only by bacterial inoculants. Moreover, although all inoculated treatments significantly reduced soil F3 hydrocarbons, inoculation of sweet clover with EA1-17 (*Stenotrophomonas* sp.) and EA6-5 (*Pseudomonas* sp.) also enhanced overall plant biomass.

Plant growth inhibition was observed in all plants grown in soils amended with diesel at 20,000 $mg{\cdot}kg^{-1}$, thus suggesting that diesel at this concentration may be excessive for sweet clover development. Similarly, Hou *et al.* (2016) reported that nut grass (*Cyperus rotundus*) growth was inhibited in soils amended with diesel at concentrations greater than 15,000 $mg.kg^{-1}$. According to Hou *et al.* (2016), high diesel amendments could change soil physical properties (*e.g*. soil aeration and water infiltration), thus affecting plant root development, which is essential for stimulating hydrocarbon-degrading microbes in the rhizosphere (Germida *et al.*, 2002).

In this study, bacterial inoculants were applied following the procedure reported by Afzal *et al.* (2013), who compared several inoculation methods including soil, seed and foliar inoculation in diesel phytoremediation studies. Afzal *et al.* (2013) concluded that soil inoculation of *Burkholderia phytofirmans* PsJN resulted in greater ryegrass (*Lolium multiflorum*) biomass, bacterial colonization and hydrocarbon degradation. Other authors *e.g*. Sheng and Gong (2006), Gałazka *et al.* (2012), Tara *et al.* (2014) and Shabir *et al.* (2016) also reported a significant increase in phytoremediation activity when soil was inoculated with hydrocarbon-degrading bacteria. In the current study, soil inoculation with EA1-17 (*Stenotrophomonas* sp.) and EA2-30 (*Flavobacterium* sp.) had no significant effects on sweet clover biomass and hydrocarbon

degradation. Similarly, inoculation with strains EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) had no effect on plant biomass, but significantly reduced total soil hydrocarbon concentration. Yousaf *et al.* (2010) reported similar results when Italian ryegrass (*Lolium multiflorum* var. Taurus) was inoculated with *Pantoea* sp. and *Pseudomonas* sp. Therefore, the results in the current study suggests that hydrocarbon reduction in inoculated treatments occurred exclusively by bacterial degradation in soil, and not as a result of phytoremediation activity. Additionally, although strains EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) may be competitive and/or efficient hydrocarbon degraders, their application as inoculants may be limited in soils with high diesel concentrations, as they were unable to reduce plant hydrocarbon toxicity. According to Yousaf *et al.* (2010), introduced inoculants undergo competition with the native microflora following introduction into the soil. The introduced inoculant is rapidly out-competed by indigenous soil microorganisms, thus, in addition to their degradation capacity, their competitive ability is an important criterion for phytoremediation applications.

Overall, the endophytic strain EA6-5 (*Pseudomonas* sp.), which harbored hydrocarbon-degrading genes, was the most effective candidate in phytoremediation experiments. *Pseudomonas* spp. are considered useful for biotechnological applications due to their ease in cultivation and stress resistance (Louvado *et al.*, 2015). Additionally, *Pseudomona*s are ubiquitous in various environments and contain species that play important roles in hydrocarbon degradation (Liu *et al.*, 2016). The *alkB* gene organization found in strain EA6-5 encodes for enzymes required in alkane degradation. In *Pseudomonas* spp., the *alkB* gene as well as other hydrocarbon degrading genes (*e.g*. *nah*, *pah* and *phn*) can often be found on plasmids, which facilitates genetic transfer and acquisition by other bacteria in oil-polluted environments (Brooijmans *et al.*, 2009; Abbasian *et al.*, 2016). Therefore, the results of the current study suggest that strain EA6-5, which tested positive for the *alkB* gene, potentially could transfer its hydrocarbon degrading genes to other indigenous soil microbes.

6.7 Conclusions

In order to obtain bacterial strains better suited for phytoremediation applications, this study investigated plant compatibility and growth promoting effects of previously isolated hydrocarbon degrading endophytic bacteria. The results demonstrate that inoculation of seeds with specific endophytic bacterial strains increased seed germination rate, whereas some bacteria

inhibited seed germination. As sweet clover plants responded more favorably than barley to bacterial inoculants tested, this crop was selected for further root elongation and a long-term growth chamber study. Whereas a total of 7 bacterial strains enhanced sweet clover root elongation, plant inoculation with EA1-36 (*Pantoea* sp.) and EA3-16 (*Chryseobacterium* sp.) resulted in a significant increase in the number of secondary shoots and root biomass, respectively after 65 days. Based on overall growth promoting effects, four different bacterial strains, EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.), were selected for phytoremediation studies. These bacterial inoculants enhanced overall plant biomass even though soil diesel concentrations inhibited plant growth. In addition, whereas no significant differences in total hydrocarbon concentrations were observed in soils amended with diesel at 5,000 $mg \cdot kg^{-1}$, a significant F3 hydrocarbon fraction reduction was observed in soils amended with diesel at 10,000 $mg \cdot kg^{-1}$. Inoculation with strains EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) also significantly reduced F2 and F3 hydrocarbons in soils amended with diesel at 20,000 $mg \cdot kg^{-1}$. Therefore, these results indicate that hydrocarbon degrading bacteria could be used as inoculants to increase plant tolerance and hydrocarbon degradation (*e.g.* diesel fuel) in soils.

7. OVERALL DISCUSSION

Most of Canada's vast oil reserves are in the form of oil sands, a mixture of sand, water, clay and bitumen. Oil sands mining operations impacts a large footprint, but the industry is committed to reclaim all disturbed land to an ecologically healthy state in response to governmental regulations. The challenges of oil sands reclamation start with the nature of the tailing sands, a remaining waste product after bitumen extraction, which is affected by tailings water and considered generally an inappropriate plant growth medium. Therefore, a successful vegetation reestablishment in these areas may depend on natural, synergistic relationships among plants, microorganisms and the environment. Root associated microorganisms colonize plant roots and may provide hosts with nutrients, stimulating growth, suppressing disease and increasing tolerance to abiotic stress. The potential use of plant-microbe interactions to assist plant growth on oil sands reclamation covers requires an understanding of the diversity and metabolic potential of these root associated organisms. To date, most studies have focused on the microbial communities in tailing ponds (Yergeau *et al.*, 2012a) and on the overall soil microbial biomass in oil sands reclamation sites (MacKenzie and Quideau, 2010). Thus, the primary objective in the current study was to unravel the root associated bacterial microbiome of plants growing in reclamation soils.

This study investigated root associated microbial communities of annual barley (*Hordeum vulgare*) grown at one of the oil sands reclamation areas (Chapter 3). Since an important soil management practice in oil sands reclamation is the use of peat as a cover material to increase soil carbon, significant positive correlations were observed between organic carbon, organic matter and soil microbial PLFAs. Rhizosphere and bulk soil DGGE fingerprints were highly differentiated by cover management. However, only a few dominant bands were identified in these profiles as other less abundant communities were shown as smears. This is in agreement with Jousset *et al.* (2010), who suggested that DGGE detects mostly dominant members of the community. Conversely, barley endophytic bacterial profiles indicated less, although more dominant bands, compared to those of soil profiles. Most of these bands were re-

amplified and closely matched DNA from bacteria found in agricultural soils and plant associated bacteria. Several bands matched *Phytoplasma* spp., which causes mildly pathogenic symptoms to host plants or may cause no harm (Kamińska *et al.*, 2010). Bands also matched those of *Flavobacterium* and *Pseudomonas* spp., which may contain potential PAHs degraders (Xu-xiang *et al.*, 2006; Germaine *et al.*, 2009). Culturable bacterial endophytes isolated from annual barley plants growing on oil sands reclamation soils corresponded mostly to *Enterobacter, Flavobacterium, Pseudomonas* and *Xanthomonas* spp. In addition, *Flavobacterium* and *Pseudomonas* spp. were identified in both culture dependent and independent techniques. These two genera contain species know to be hydrocarbon degraders (Lee *et al.*, 2006), plant growth promoters (Shcherbakov *et al.*, 2013) and to control plant pathogens (Mazurier *et al.*, 2009).

Although DGGE banding pattern represents a good approximation of the most dominant bacterial species biodiversity, this technique limited the resolution of low abundance microbes due to the co-migration of DNA fragments (Zheng *et al.*, 2013). Hence, annual barley (as a planted species) and white sweet clover (as an unplanted native species) were sampled in the following year and 16S rRNA amplicon sequencing was used to characterize the bacterial root microbiome associated with these plants (Chapter 4). The results of this study suggests that changes in the microbiome were mainly due to different rhizo-compartments (rhizosphere and endosphere) and host plants. As previously reported in Germida *et al.* (1998), endophytic root colonization is not a passive process and the current study suggests that both sweet clover and barley plants have the ability to select for certain soil microbial consortia. Differences in bacterial community profiles were detected even at broad taxonomic levels such as phylum level, in which there was an increase in the abundance of *Proteobacteria* in the endosphere compartment of both plants. *Proteobacteria* have been previously described as effective rhizosphere and root colonizers due to their high ability to utilize root exudates (Fierer *et al.*, 2007). In addition, sweet clover plants were more restrictive than barley, which confirms previous findings by Germida *et al.* (1998) and Ofek-Lalzar *et al.* (2014), in which the authors suggest that plant factors play a dominating role in selecting bacterial endophytes. Members of the family *Rhizobiaceae* were more closely associated with sweet clover plants, while barley plants harboured a high abundance of *Xanthomonadaceae*. The family *Rhizobiaceae* contains known nitrogen fixing bacteria that could alleviate potential nutrient limitation in reclamation

areas (Lefrançois *et al.*, 2010). *Xanthomonadaceae* contains the genus *Xanthomonas*, but mainly *Stenotrophomonas*, which are capable of great metabolic versatility and are colonizers of soil and plants (Ryan *et al.*, 2009). At genus level, most differences within endophytic bacterial profiles were due to the abundance of *Sinorhizobium*, *Pseudomonas* and *Acholeplasma.* The association with *Sinorhizobium,* a nitrogen-fixing bacteria (Bromfield *et al.*, 2010), was restricted to sweet clover, suggesting that these plants may rely on this association to grow in reclamation soils. In addition, only barley plants harboured *Acholeplasma* endophytes. *Acholeplasma* are wall-less bacteria known to colonize the guts and hemolymph of insects and their transmission to plants occurs when these insects feed on plant tissues (Bonnet *et al.*, 1991; Tully *et al.*, 1988). However, *Pseudomonas* species successfully colonized both plants. *Pseudomonas* ssp. are common colonizers of the plant interior (Ofek-Lalzar *et al.*, 2014) and contain species with a wide range of metabolic capabilities including PAH degradation (Germaine *et al.*, 2009) and plant growth promotion (Bhattacharyya and Jha, 2012). Therefore, analyzes in Chapter 4 revealed that different plants harboured distinct bacterial communities which may have essential metabolic capabilities that can improve plant growth in reclamation areas.

Previous studies have shown that tailings sands used in land reclamation may contain residual hydrocarbons (Quoreshi, 2008; Lefrançois *et al.*, 2010) and therefore, plant microbial selection in these areas may be associated with the presence of hydrocarbon degrading bacteria. Hence, Chapter 5 investigated the abundance of hydrocarbon degrading genes (CYP 153, *alkB*, *nah*) and bacterial 16S rRNA gene. Overall, significant differences between plants were only observed in the quantification of the CYP153 gene, in which sweet clover endophytic bacterial communities harbored a higher abundance of this gene compared to barley. Whereas overall *alkB* gene abundance in barley endophytic bacteria indicated positive correlations with soil THC, negative correlations were observed between THC and the *alkB*/16s rRNA ratio in sweet clover endophytes. According to Jurelevicius *et al.* (2012), THC is a key factor influencing *alkB* diversity in soils with similar physicochemical properties. Sweet clover endophytic bacterial communities containing *alkB* also positively correlated to OM, NO_3^-, NH_4^+, TC and TOC. According to Powell *et al.* (2010), the combination of TC and pH is the most influential for microbial communities as it determines soil water holding capacity which influences soil oxygen availability. In addition to unculturable endophytes, the current study assessed the presence of hydrocarbon degrading genes in previously isolated barley endophytic bacteria. Most of these

isolates were identified as *Pantoea*, *Pseudomonas* and *Enterobacter* spp. Tara *et al.* (2014) studied carpet grass (*Axonopus affinis*) inoculation with *Pantoea* sp. BTRH79 in diesel-polluted soils and observed an increase plant biomass and phytoremediation activity in inoculated plants. *Pseudomonas* spp have also been used in several inoculation studies that assessed hydrocarbon degradation (Tara *et al.*, 2014; Khan *et al.*, 2014). Endophytic *Enterobacter* ssp. containing the CYP153 gene has been previously investigated for hydrocarbon degradation (Yousaf *et al.*, 2011) as this genus comprises a range of beneficial plant-associated bacteria with plant growth promotion and hydrocarbon degradation effects (Yousaf *et al.*, 2011).

Since the third main objective of this research was to investigate the potential use of endophytic root bacteria and host plants to degrade hydrocarbons, Chapter 6 assessed these applications with previously selected bacterial isolates. Inoculation of barley and sweet clover seeds with bacteria led to either growth promoting or detrimental effects on seed germination rate/vigor. Although no positive effects were observed in barley, bacterial strain EA3-25 (*Xanthomonas* sp.) enhanced seed germination in sweet clover. *Xanthomonas* it is mostly known to cause plant diseases (Soares *et al.*, 2010), but also contain species known for polycyclic aromatic hydrocarbons (PAHs) degradation (Deka and Lahkar, 2016). Since most growth promoting effects were detected in sweet clover, this plant was selected for a root elongation, full potting experiment and phytoremediation experiments. Sweet clover plants, as forage legumes that fix nitrogen in symbiotic association with *Sinorhizobium meliloti* (Bromfield *et al.*, 2010), may be better suited to adapt in nutrient limited and oil contaminated soils (Hall *et al.*, 2011; Franco and Balieiro, 2000). In addition, inoculations of sweet clover strains with EA2-30 (*Flavobacterium* sp.) and EA3-1 (*Bacillus* sp.) enhanced root elongation. Pant *et al.* (2016) inoculated *Jatropha curcas* with *Flavobacterium* sp. and found that this isolate promoted multiple plant growth promoting effects and biodegradation of 1,4-dichlorobenzene. *Bacillus pumilus* have also enhanced plant growth and induced the proliferation of other rhizosphere bacteria on root surfaces of *Atriplex lentiformis* growing in mineral ore mine tailings (de-Bashan *et al.*, 2010). In addition to the early stages of plant development, the current study also investigated promoting effects at plant flowering stage. At this stage, the results in this study highlights the ability of sweet clover plants to develop in nutrient limited conditions. According to Ogle *et al.* (2008), sweet clover, like other legumes, increases nitrogen content in soils and have an easy establishment in reclamation areas. The inoculation of sweet clover with EA1-36

(*Pantoea* sp.) and EA3-16 (*Chryseobacterium* sp.) led to a significant increase in the number of secondary shoots and root biomass respectively. Since plant essential soil nutrients are taken up by roots, root growth is considered a prerequisite for enhanced plant development (Mills and Jones, 1996). Although bacterial inoculants promoted plant growth in soils without the addition of contaminants, phytoremediation studies revealed similar results as reported by Arslan *et al.* (2014), in which high diesel concentrations caused detrimental effects to all plants. According to Chen *et al.* (2013), due to its toxicity and hydrophobicity, hydrocarbons can adversely affect plant health and development. In addition, the C:N ratio in hydrocarbon contaminated soils is typically high due to the high input of carbon (C) into the system (Bento *et al.*, 2012), however, due to their association with N-fixing bacteria, sweet clover plants may withstand those adverse conditions and also support the survival of hydrocarbon degrading bacteria (Hall *et al.*, 2011). Overall, inoculation of sweet clover with hydrocarbon degrading bacteria enhanced plant biomass in diesel contaminated soils compared to uninoculated control plants. However only in soils amended with diesel at10,000 and 20,000 $mg{\cdot}kg^{-1}$ an increase in soil hydrocarbon degradation was observed. In soils initially amended with diesel at 20,000 $mg{\cdot}kg^{-1}$, inoculation with strains EA6-5 (*Pseudomonas* sp.) and EA4-40 (*Pantoea* sp.) significantly decreased soil hydrocarbon concentrations. *Pseudomonas* spp., which are considered to be ubiquitous in various environments, contains species that play an important role in hydrocarbon degradation (Liu *et al.*, 2016). In addition, due to their easy cultivation and stress resistance *Pseudomonas* spp. are ideal candidates for biotechnological applications (Louvado *et al.*, 2015).

The plant microbiome plays an important role in the growth and development of plants growing in reclamation soils. In this context, my research demonstrated that bacteria associated with barley and sweet clover were mainly driven by plant factors and this microbiome harbors endophytes that can be potentially used in phytoremediation. In particular, bacterial endophytes such as *Pantoea* spp. and *Pseudomonas* spp. in association with sweet clover plants were proven to successfully reduce petroleum hydrocarbons in soil.

8. SUMMARY AND CONCLUSIONS

The first objective of this study was to assess the diversity of endophytic root bacteria associated with plants growing on reclamation soils. An initial assessment of dominant soil microbial communities in oil sands post-mining landscapes revealed that total and organic carbon, NH_4^+ and organic matter are the main factors influencing these communities. Additionally, endophytic bacteria also successfully colonized barley plants and most of selected bacterial endophytes closely matched rhizosphere and soil bacteria from agricultural and grassland soils. However, a few endophytes matched those of growth promoting bacteria and potential hydrocarbon degraders, suggesting they may assist plant growth on reclamation covers. Since barley is commonly planted in reclamation covers, to provide quick vegetation cover and erosion control, its associated bacterial communities are of high importance to future native plant species. Therefore, an in-depth analysis of bacterial profiles associated with barley and naturally occurring white sweet clover (*Melilotus albus*) plants was also conducted in order to elucidate a better understanding within this research objective.

Consistent with prior findings in the literature, the results in this study confirmed that rhizo-compartments (endosphere and rhizosphere) indicated the strongest differentiation of root associated bacterial communities. In addition, plant species account as main driving factors affecting the endophytic microbiome. Endosphere compartments also indicated a lower diversity and the depletion or enrichment of certain bacteria when compared with the rhizosphere, which strongly suggests that plant select for certain soil bacterial consortia. Endophytic profiles also revealed that sweet clover plants were more selective than barley. Although *Pseudomonas* and *Pantoea* were able to successfully colonize both plants, members of the family *Rhizobiaceae*, such as *Sinorhizobium* and *Rhizobium* were mainly associated with sweet clover, whereas *Acholeplasma* was unique to barley. Endophytic bacterial profiles also varied within the same plant species at different sampling locations; however, these differences were driven by factors other than the soil parameters analyzed in in this study.

Previous studies have shown that tailings sands used in land reclamation may contain residual hydrocarbons (Quoreshi, 2008; Lefrançois *et al.*, 2010). It is possible, therefore, that plant selection of root microbial communities might depend on the microbes ability to degrade hydrocarbons. Thus, the second research objective of this study was to screen endophytic root bacteria for hydrocarbon degrading genes (CYP 153, *alkB* and *nah*) and a universal bacterial gene (16S rRNA). The abundance of hydrocarbon degrading genes within endophytic communities was influenced by the interaction of plant species, cover management and sampling locations. Whereas both plants analyzed had similar 16s rRNA, *alkB* and *nah* gene abundance, sweet clover species harbor higher abundance of CYP153, which suggest that plant factors play an important role in selecting hydrocarbon degrading bacteria. In addition, soil total hydrocarbons, pH, soil carbon and nitrogen play an important role in determining hydrocarbon degrading communities. Previously isolated endophytic bacterial cultures revealed a total of 42 isolates that were positive for at least one hydrocarbon degrading gene, in which most of these isolates were positive for the presence of *alkB* and closely matched *Pantoea*, *Pseudomonas* and *Enterobacter* spp.

The third objective of this study was to investigate the potential use of endophytic root bacteria and host plants to degrade hydrocarbons. In order to obtain bacterial strains better suited for phytoremediation applications, plant compatibility and growth promoting effects were investigated without the use of contaminants prior to phytoremediation applications. Here, unique associations between bacteria strains and host plants varied from seed germination stimulations to inhibitions. In addition, most growth promoting effects were observed in sweet clover plants and based on overall growth promoting effects, four different bacterial strains, EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.), were selected for phytoremediation experiments. The increase in diesel fuel concentration in soils inhibited overall plant growth; however, plants inoculated with endophytic bacteria had significantly greater plant biomass. At lower diesel concentrations, analysis of soil hydrocarbon fractions revealed no significant reduction in hydrocarbons between treatments. However, in soils amended with diesel at 10,000 and 20,000 $mg.kg^{-1}$ there was a significant reduction in hydrocarbons observed in treatments inoculated with EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.).

Overall, both barley and sweet clover plant cultivars used in this research harbored a diverse group endophytic bacteria, which suggests that despite the adverse conditions in oil sands reclamation areas, the root microbiome remains similar to those found in agricultural and natural landscapes. Although differences in sampling locations and cover management were observed, plant factors played a dominant role in selecting bacterial endophytes. Sweet clover plants were more restrictive when compared to barley, supporting the enrichment of select bacterial taxa. Sweet clover association with nitrogen fixers (*e.g. Sinorhizobium* spp.) and *Pseudomonas* spp. (which includes several known hydrocarbon degraders) may play an important role on the ability of this plant to successfully colonize reclamation soils. In addition, sweet clover uncultured bacterial endophytes harboured a higher abundance of the gene CYP153, which encodes for hydrocarbon degrading enzymes. Finally, bacterial endophytes, such as *Pantoea* spp. and *Pseudomonas* spp. isolated from oil sands reclamation areas can be successfully used in association with sweet clover plants in phytoremediation of petroleum hydrocarbons.

9. FUTURE RESEARCH

Tailing sands are characterized by the presence of residual hydrocarbons, low nutrient content and the oil sands process-affected water (OSPW) associated with the tailing sands has high salinity. Previous studies have investigated the use of nitrogen-fixing *Actinobacteria* (Lefrançois *et al.*, 2010) and Ectomycorrhizal (ECM) fungi (Beaudoin-Nadeau *et al.*, 2016) in association with alders as a way to remediate oil sands reclamation areas. However, little is known about how other plants and their associated microbial communities, such as endophytic bacteria might perform at mine reclamation sites. Khan *et al.* (2017) studied inoculation of plants with endophytic bacteria in high saline soils and Tara *et al.* (2014) used a combination of alkane-degrading and bacteria possessing ACC deaminase activity to enhance diesel phytoremediation. Similar studies should be conducted at oil sands reclamation areas.

Peat mineral mixes are the most commonly used covering material in oil sands reclamation; however, the LFH soil horizon is also used by the industry. Mackenzie and Naeth (2009) studied the establishment of native boreal plant species on these two covers and found that LFH improves plant nutrient availability and creates a more diverse ecosystem. Similarly, Howell and MacKenzie (2017) studied soil microbial communities in these two cover types and found that although no significant differences were observed in community level physiological profiles (CLPP), phospholipid fatty acid (PLFA) profiles indicated significant differences by cover materials. These studies, however, failed to address root associated microbial communities. Consequently, more studies are needed to assess functional and genetic microbiome variability in these covers. For example, future studies might focus on comparing the same planted species growing in these two covers to determine how their associated bacterial communities differentiate due to cover type.

Other plants such as jack pine (*Pinus banksiana*), white spruce (*Picea glauca*) and slender wheatgrass (*Elymus trachycaulus*) are commonly used in oil sands reclamation strategies. Therefore, an analysis of the root microbiome of these plants and other plants growing in these

reclamation areas could contribute to a better understanding of the factors controlling microbial community structure and function.

The phytoremediation growth chamber experiments in this study were limited in scope. Future studies should assess bacterial inoculant survival on inoculated seeds and in soil. Furthermore, fields studies should be conducted to determine if results observed in the lab also occur in the field. It would also be important to understand how bacterial inoculants impact indigenous microbial communities. Hence, future phytoremediation studies could focus on the use of bacterial strains EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.), EA6-5 (*Pseudomonas* sp.), and a consortium of these isolates, in a field phytoremediation experiment. Moreover, different plant species should be tested with and without fertilizer applications for their effectiveness as for remediating a variety of soil contaminants including crude oil.

Finally, some of the isolated bacteria used in this study, such as EA3-1 (*Bacillu*s sp.), EA3-16 (*Chryseobacterium* sp.) and EA3-25 (*Xanthomonas* sp.), tested positive for the *nah* gene and demonstrated promising plant growth promotion effects, but were not used in phytoremediation experiments due to time constraints. It would be worthwhile for these and other promising endophytic bacteria to be investigated as phytoremediation tools.

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APPENDIX A: Identification of endophytic bacteria isolates associated with annual barley (*Hordeum vulgare*) growing at an oil sands reclamation area. Identification is based on 16S rRNA Sanger sequencing.

Table A.1 Identification of endophytic bacteria isolates associated with annual barley (*Hordeum vulgare*) growing at an oil sands reclamation area.

Sample Code	Location	Accession #	Closest match	Similarity (%)
EA1-1	E1	HF936841.1	*Pedobacter alluvionis*	100
EA1-10	E1	JF494826.1	*Pantoea agglomerans*	94
EA1-13	E1	NR_104943.1	*Enterobacter cloacae*	100
EA1-3	E1	KF053337.1	*Serratia* sp.	97
EA1-8	E1	KJ482776.1	*Pedobacter sp.*	96
EA1-9	E1	KJ482776.1	*Pedobacter sp.*	96
EA1-11	E1	NR_025461.1	*Curtobacterium herbarum*	98
EA1-15	E2	LK054576.1	*Chryseobacterium* sp.	100
EA1-17	E2	NR_074977.1	*Stenotrophomonas chelatiphaga*	100
EA1-18	E2	JN699021.1	*Bacillus pumilus*	91
EA1-19	E2	KM059525.1	*Enterobacter* sp.	91
EA1-26	E2	NR_113627.1	*Paenibacillus pabuli*	99
EA1-34	E2	NR_074777.1	*Enterobacter* sp.	95
EA1-35	E2	NR_074777.1	*Enterobacter* sp.	99
EA1-36	E3	NR_132712.1	*Pantoea theicola*	100
EA1-37	E3	NR_118381.1	*Bacillus pumilus*	100
EA1-38	E3	NR_116808.1	*Serratia glossinae*	93
EA2-6	E3	KJ831451.1	*Pseudomonas* sp.	93
EA2-7	E3	JF494826.1	*Pantoea agglomerans*	93
EA2-9	E3	NR_132712.1	*Stenotrophomonas pavanii*	100
EA2-11	E3	EU375390.1	*Pedobacter* sp.	89
EA2-17	E4	JQ966939.1	*Serratia* sp.	90
EA2-19	E4	GU413438.1	*Pantoea theicola*	99
EA2-20	E4	KT375349.1	*Pedobacter ginsengisoli*	99
EA2-21	E4	KJ579161.1	*Pedobacter* sp.	99
EA2-22	E4	KJ846483.1	*Pseudomonas* sp.	94
EA2-26	E4	GQ254771.1	*Bacillus pumilus*	99
EA2-27	E4	AY972422.1	*Pseudomonas* sp.	93
EA2-28	E5	NR_102966.1	*Pantoea vagans*	99
EA2-29	E5	AF130886.2	*Enterobacter agglomerans*	99
EA2-30	E5	KR088356.1	*Flavobacterium aquidurense*	96
EA2-33	E5	DQ659031.1	*Mesorhizobium* sp.	92
EA2-37	E5	HF566234.1	*Enterobacter hormaechei*	97
EA2-38	E5	JN699022.1	*Bacillus pumilus*	95
EA2-41	E5	EF123221.1	*Bacillus* sp.	88
EA3-1	E6	KC595863.1	*Bacillus pumilus*	99

Table A.1 cont.

EA3-11	E6	LK021068.1	*Pseudomonas koreensis*	94
EA3-12	E6	JX908960.1	*Pedobacter* sp.	96
EA3-13	E6	KJ482715.1	*Arthrobacter*	92
EA3-14	E6	KM092525.1	*Bacillus subtilis*	88
EA3-7	E6	NR_044382.1	*Pedobacter alluvionis*	98
EA3-9	E6	AY965083.1	*Pseudomonas* sp.	92
EA3-16	E7	NR_126257.1	*Chryseobacterium hominis*	100
EA3-18	E7	KP299228.1	*Pseudomonas syringae*	95
EA3-21	E7	FR682741.1	*Pedobacter* sp.	88
EA3-25	E7	NR_121770.1	*Xanthomonas campestris*	100
EA3-26	E7	NR_108537.1	*Flavobacterium myungsuense*	99
EA3-27	E7	NR_102514.1	*Pseudomonas poae*	97
EA3-32	E7	NR_042647.1	*Chryseobacterium gregarium*	99
EA4-1	E8	NR_126257.1	*Chryseobacterium nakagawai*	100
EA4-2	E8	NR_134713.1	*Staphylococcus hominis*	99
EA4-3	E8	NR_121749.1	*Staphylococcus pasteuri*	98
EA4-4	E8	NR_027221.1	*Lysobacter antibioticus*	89
EA4-5	E8	NR_044382.1	*Pedobacter alluvionis*	89
EA4-6	E8	NR_134019.1	*Serratia plymuthica*	100
EA4-8	E8	CP012830.1	*Pseudomonas* sp.	100
EA4-25	E9	EF363766.1	*Flavobacterium* sp.	98
EA4-32	E9	NR_104943.1	*Enterobacter* sp.	100
EA4-34	E9	KT375349.1	*Pantoea theicola*	100
EA4-35	E9	FJ973535.1	*Bacillus subtilis*	100
EA4-38	E9	NR_102514.1	*Serratia quinivorans*	100
EA4-39	E9	NR_102514.1	*Pseudomonas poae*	98
EA4-40	E9	EF173381.1	*Pantoea theicola*	100
EA4-44	E10	KM009133.1	*Chryseobacterium jejuense*	97
EA5-1	E10	GQ148962.1	*Rahnella* sp.	96
EA5-2	E10	GQ232463.1	Bacteria unclassified	99
EA5-3	E10	KM009133.1	*Chryseobacterium jejuense*	99
EA5-4	E10	KJ482773.1	*Stenotrophomonas* sp.	99
EA5-8	E10	NR_074820.1	*Serratia plymuthica*	100
EA5-9	E10	KT862774.1	*Rahnella* sp.	99
EA5-12	S1	KC108940.1	*Pedobacter sp.*	95
EA5-14	S1	LK021101.1	*Pantoea septica*	99
EA5-16	S1	JX657047.1	*Flavobacterium pectinovorum*	97
EA5-18	S1	JQ977617.1	*Agrobacterium* sp.	91

Table A.1 cont.

EA5-19	S1	JX047449.1	*Staphylococcus* sp.	99
EA5-20	S1	KM513968.1	*Flavobacterium* sp.	100
EA5-27	S1	NR_108382.1	*Flavobacterium macrobrachii*	92
EA5-35	S2	EF428995.1	*Pseudomonas fluorescens*	93
EA5-36	S2	NR_042647.1	*Chryseobacterium gregarium*	97
EA5-37	S2	AY561586.1	*Agrobacterium* sp.	93
EA5-38	S2	AB772879.1	*Pseudomonas* sp.	97
EA5-39	S2	KJ733896.1	*Xanthomonas* sp.	92
EA5-42	S2	LK021199.1	*Xanthomonas cynarae*	89
EA5-44	S2	KC108940.1	*Pedobacter* sp.	94
EA6-10	S3	NR_074432.1	*Riemerella anatipestifer*	99
EA6-11	S3	NR_074716.1	*Sanguibacter keddieii*	99
EA6-12	S3	KP259552.1	*Pseudomonas* sp.	98
EA6-3	S3	KM009990.1	*Escherichia hermannii*	98
EA6-5	S3	KP259552.1	*Pseudomonas* sp.	100
EA6-7	S3	NR_121770.1	*Xanthomonas fuscans*	99
EA6-9	S3	NR_042252.1	*Arthrobacter tecti*	99
EA6-16	S4	KM355416.1	*Enterobacter* sp.	92
EA6-20	S4	KF835769.1	*Enterobacter aeros*	89
EA6-21	S4	KM458060.1	*Lelliottia* sp.	96
EA6-22	S4	NR_044729.2	*Hafnia alvei*	98
EA6-25	S4	KM458060.1	*Lelliottia* sp.	89
EA6-27	S4	KJ846496.1	*Klebsiella* sp.	91
EA6-28	S4	KF516260.1	*Lelliottia nimipressuralis*	96
EA6-37	S5	LC034140.1	*Enterobacter* sp.	100
EA6-38	S5	JN251781.1	*Kluyvera intermedia*	91
EA6-39	S5	JN251781.1	*Citrobacter* sp.	93
EA6-40	S5	KP852523.1	*Enterobacter* sp.	93
EA7-1	S5	KC834342.1	*Pantoea theicola*	91
EA7-2	S5	DQ530153.1	*Stenotrophomonas maltophilia*	94
EA7-4	S5	KF679345.1	*Pantoea intestinalis*	100
EA7-20	S6	FN386709.1	*Arthrobacter nitroguajacolicus*	95
EA7-22	S6	KM035942.1	*Microbacterium* sp.	92
EA7-29	S6	KM117168.1	*Arthrobacter protophormiae*	96
EA7-30	S6	NR_074977.1	*Bacillus pumilus*	97
EA7-31	S6	NR_074977.1	*Bacillus pumilus*	94
EA7-36	S6	NR_114481.1	*Pseudomonas tolaasii*	97
EA7-37	S6	NR_074977.1	*Bacillus pumilus*	98

Table A.1 cont.

EA8-12	S7	NR_113713.1	*Rhizobium galegae*	96
EA8-14	S7	DQ122300.1	*Xanthomonas campestris*	97
EA8-16	S7	HF912854.1	*Pseudoalteromonas*	99
EA8-17	S7	JX514422.1	*Pseudomonas syringae*	96
EA8-18	S7	KJ481222.1	*Enterobacter* sp.	94
EA8-6	S7	DQ530153.1	*Stenotrophomonas maltophilia*	97
EA8-8	S7	KJ959612.1	*Chryseobacterium*	92
EA8-19	S8	EU434528.1	*Pseudomonas fluorescens*	91
EA8-20	S8	AB772879.1	*Pseudomonas* sp.	95
EA8-21	S8	EF428995.1	*Pseudomonas fluorescens*	91
EA8-22	S8	KJ513869.1	*Luteimonas* sp.	98
EA8-23	S8	NR_042647.1	*Chryseobacterium gregarium*	98
EA8-25	S8	EU434528.1	*Pseudomonas fluorescens*	91
EA8-27	S8	AB772879.1	*Pseudomonas* sp.	95
EA8-30	S9	NR_042647.1	*Chryseobacterium gregarium*	98
EA8-33	S9	NR_042647.1	*Chryseobacterium gregarium*	96
EA8-34	S9	KJ482776.1	*Pedobacter* sp.	97
EA8-38	S9	HF566366.1	*Xanthomonas campestris*	92
EA8-41	S9	DQ122300.1	*Xanthomonas campestris*	97
EA8-43	S9	KJ482776.1	*Pedobacter* sp.	97
EA8-44	S9	HF566366.1	*Xanthomonas campestris*	92
EA7-22	S10	KM035942.1	*Microbacterium* sp.	92
EA7-24	S10	KM023314.1	*Bacillus safensis*	94
EA7-25	S10	KM023314.1	*Bacillus safensis*	90
EA7-26	S10	FN386709.1	*Arthrobacter nitroguajacolicus*	94
EA7-27	S10	FN386709.1	*Arthrobacter nitroguajacolicus*	96
EA7-28	S10	NR_112775.1	*Dietzia timorensis*	91
EA8-30	S10	NR_121739.1	*Stenotrophomonas rhizophila*	99

APPENDIX B: Rarefaction curves relating the observed number of sequences to the observed number of operational taxonomic units (OTUs) at 97% rRNA sequence similarity.

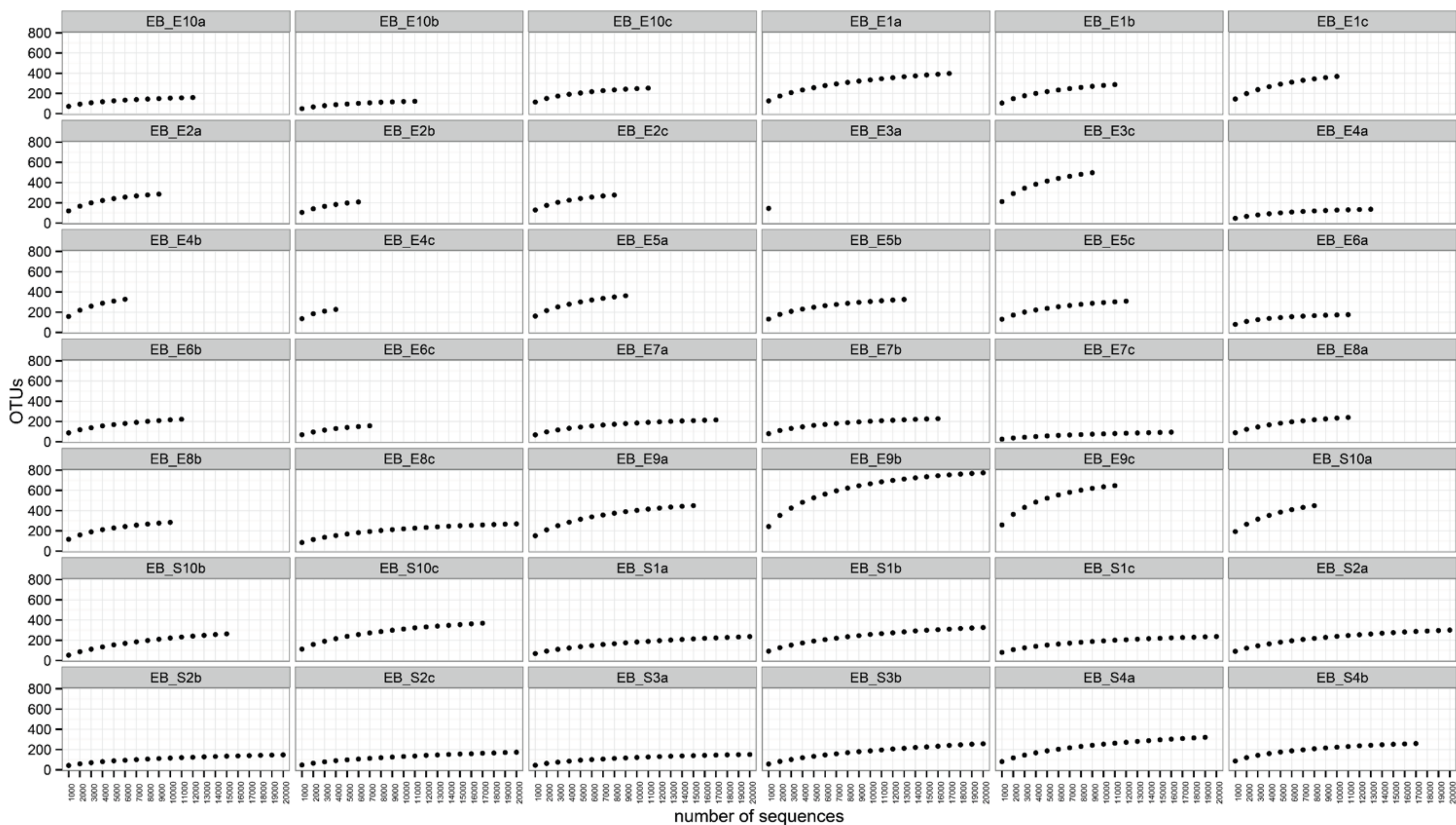

Figure B.1 Rarefaction curves for bacterial OTUs, clustering at 97% rRNA sequence similarity. Curves represent sequences for each sample for endophytic barley (EB), endophytic clover (EC), rhizosphere barley (RB) and rhizosphere clover (RC).

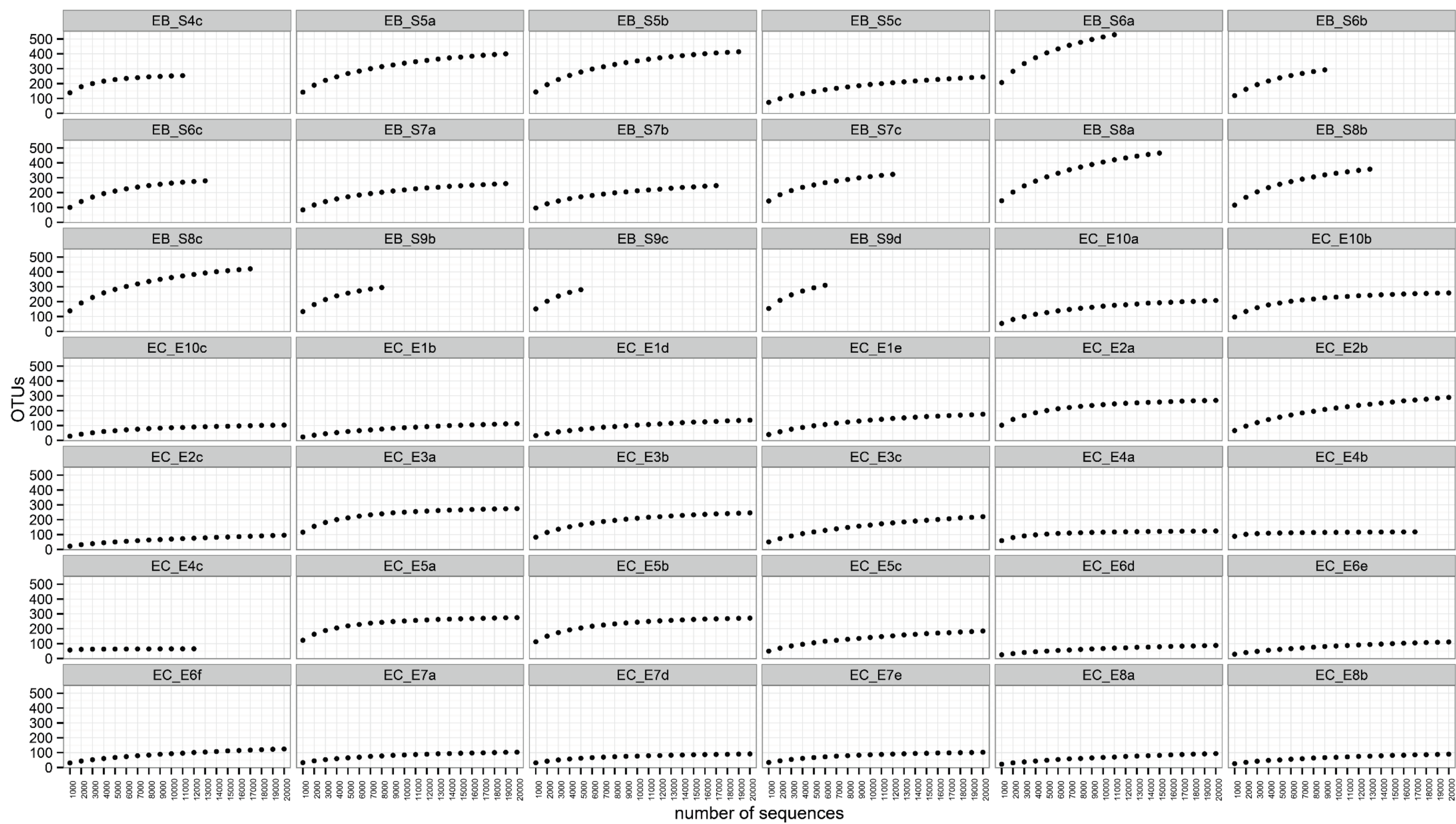

Figure B.1 cont.

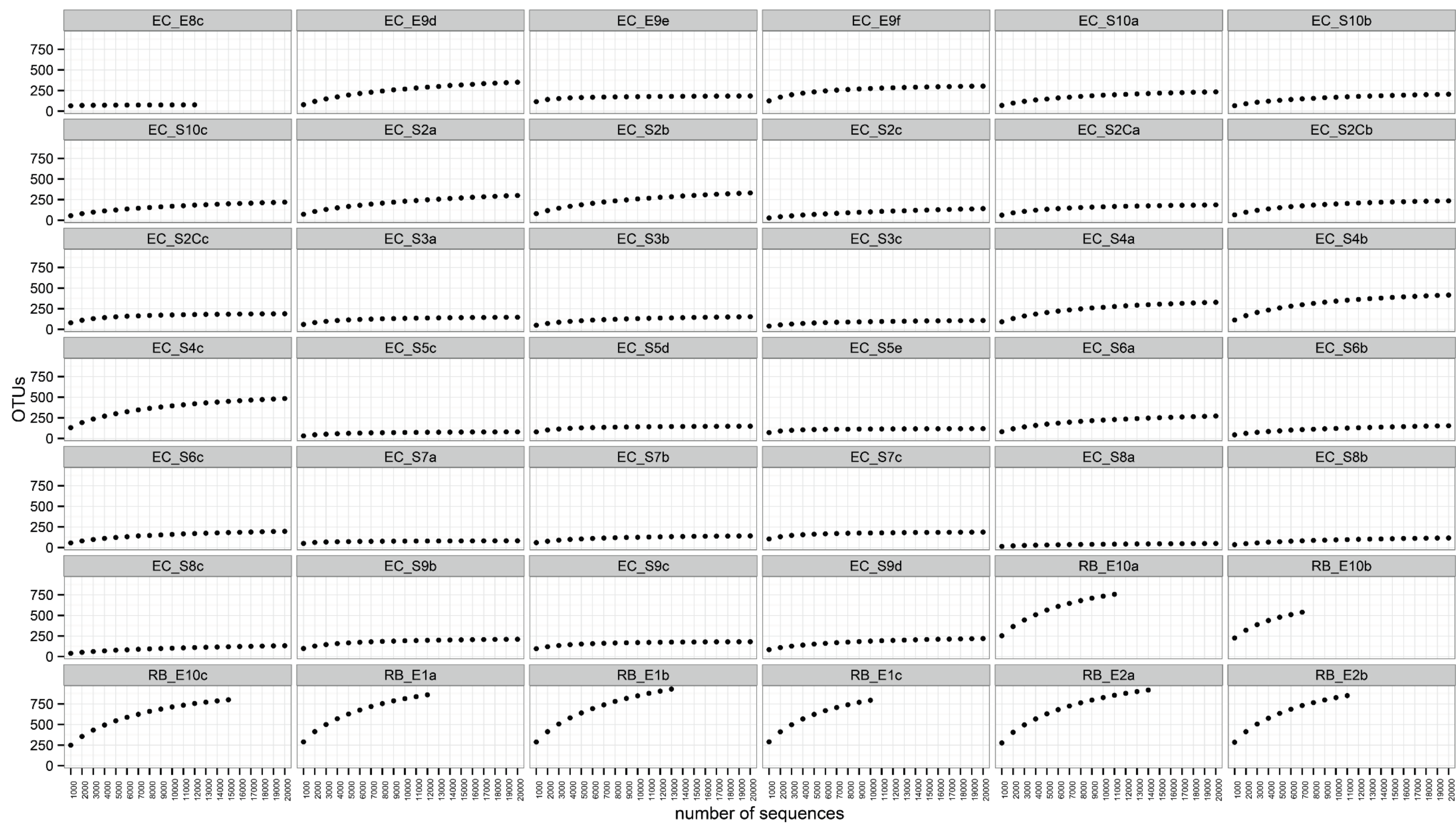

Figure B.1 cont.

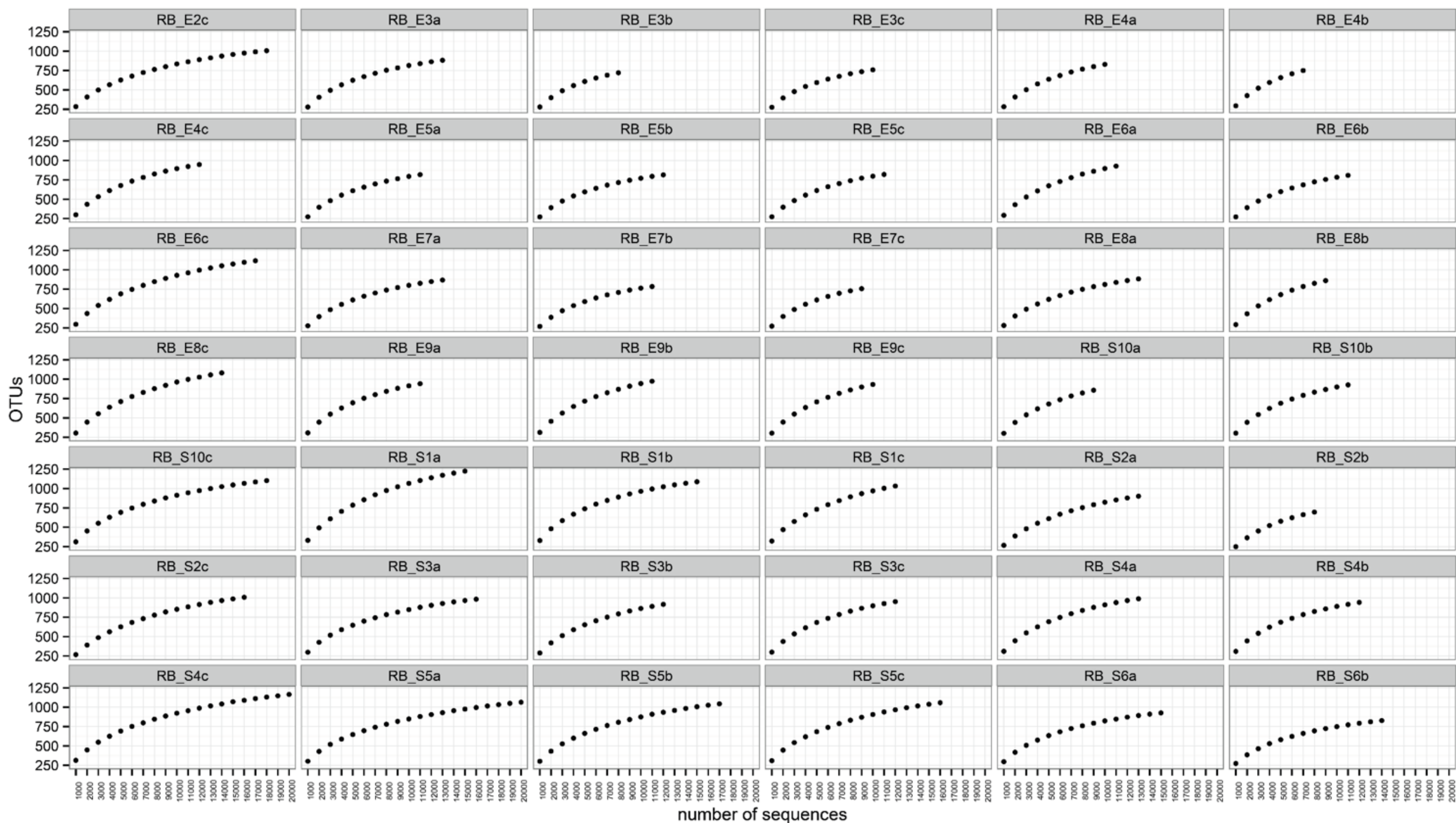

Fig. B.1 cont.

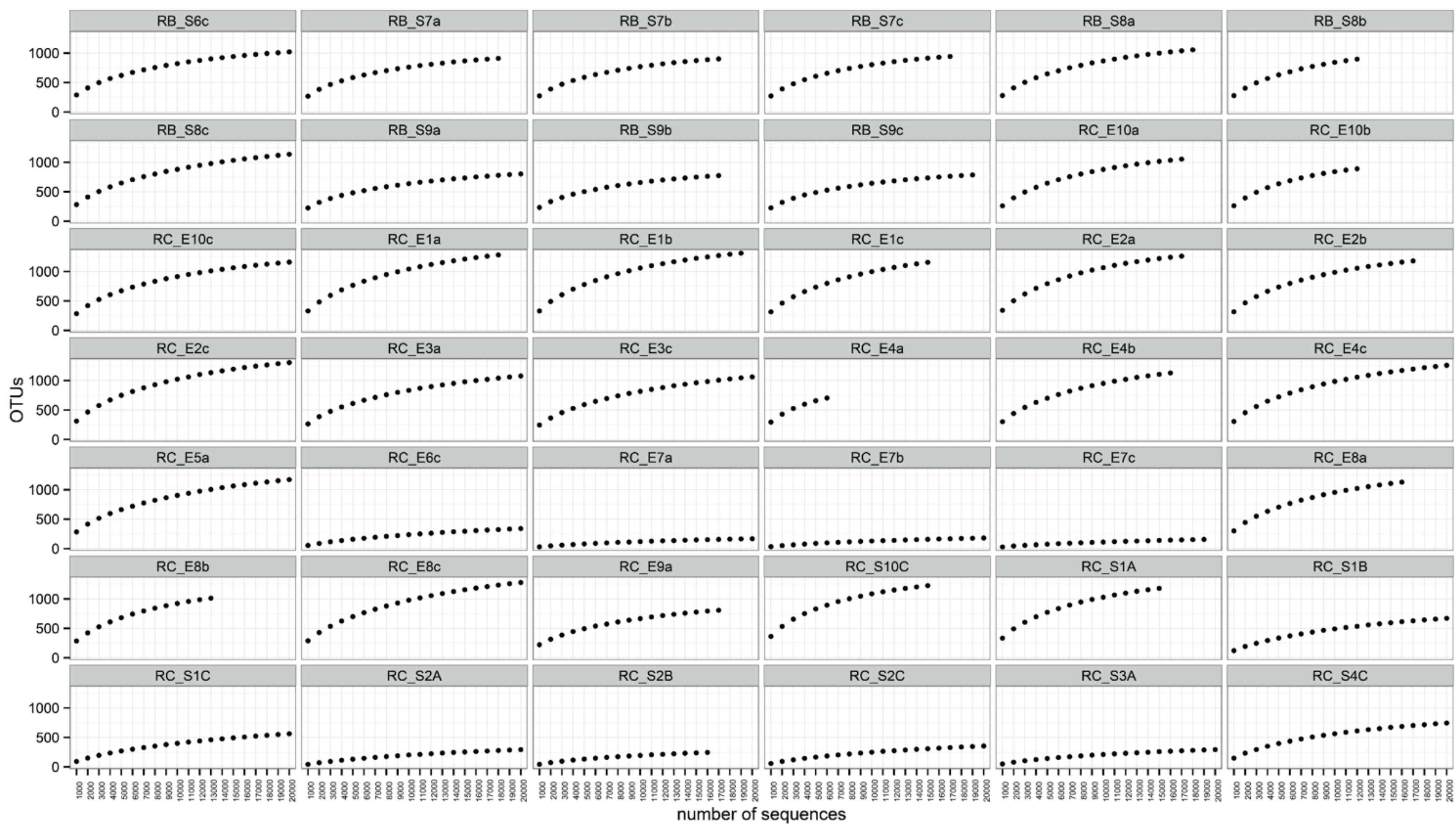

Fig. B.1 cont.

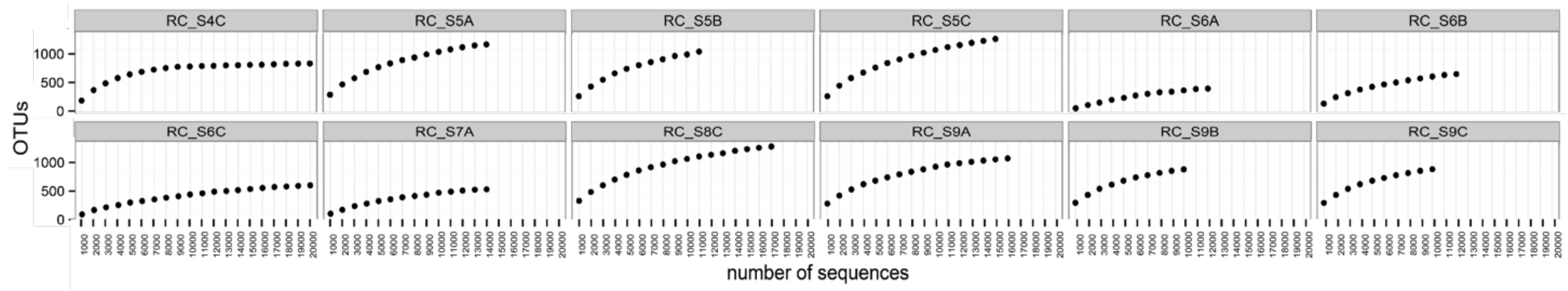

Fig. B.1 cont.

APPENDIX C: Sequence genes CYP153 (339 bp) and *alkB* (548 bp) and *nah* (377 bp) synthesized by Biomatik (Cambridge, Canada).

CYP153 (339 bp)

```
  1  gtgggcggca  acgacaccac  tcgaaactcg  atgagtggcg  gtgtgcttgc  gctcaaccgg
 61  ttccccgacc  agttcgagaa  gctgaaggcg  aaccccgacc  tgatccccaa  catggtctcg
121  gagatcatcc  ggtggcagac  cccgctggcc  tacatgcgcc  ggatcgccaa  gaccgacacc
181  atgctgaagg  gccagttcat  ccgcaagggc  gacaaagtcg  tgatgtggta  cgcctcgggc
241  aaccgcgacg  aaagcgtgtt  cgaacggccc  gatgagttga  tcatcgatcg  gagcaacgcc
301  cgccaccaca  tctccttcgg  cttcggcatc  caccgctgc
```

alkB (548 bp)

```
  1  aacacggcgc  acgaactagg  gcacaagaag  accgccatcg  aacgctggct  ggccaagctg
 61  gccctggcgc  cgaccggcta  tggccatttc  tgtatcgagc  acaaccgcgg  gcatcaccgg
121  gacgtggcca  cgccggaga  ttccgcctca  tcacgtatgg  gcgagagcta  ctatcgcttc
181  atcaagcgcg  agatccccgg  ggccttccgc  cgcgcctggg  ccatcgaggg  cgatcgcatg
241  gcccgcaagg  gactgagccg  ctggtcgttg  cagaacgata  tcgtccacac  ggccctggta
301  accgtcgtgc  tgtggggcgg  gctgattttc  tggctgggtc  tcgcggtgct  gcccttcctg
361  ctgctgcagg  cgctgatcgc  ctattcgctg  ctttcctccg  cgaactacgt  agagcattac
421  ggactgttgc  gccagagatt  ggccagcggt  cgctacgaac  gccccgagcc  gcgccactcc
481  tggaacagca  atcatgtgct  gtcgaacatc  ctcctctatc  agctccagcg  ccactcggac
541  caccacgc
```

nah (377 bp)

```
  1  caaaagcacc  tgattcatgg  cgatgaagaa  cttttccagc  acgaactgag  aaccattttt
 61  gcgcggaact  ggctttttct  cactcatgac  agcctgattc  catcccccgg  cgactatgtt
121  accgcaaaaa  tgggtattga  cgaggtcatc  gtctctcggc  agagcgacgg  ttcgattcgt
181  gccttcctga  acgtttgtcg  gcaccgtggc  aagacgctgg  ttaacgcgga  agccggcaat
241  gccaaaggtt  tcgtttgcag  ctatcacggc  tggggcttcg  gctccaacgg  tgaactgcag
301  agcgttccat  tcgaaaaaga  gctgtacggc  gagtcgctca  acaaaaaatg  tctggggttg
361  aaagaagtcg  ctcgcgt
```

APPENDIX D: Identification of endophytic bacteria isolates associated with annual barley (*Hordeum vulgare*) positive for hydrocarbon degrading genes (CYP153. *alkB*, *nah*). Identification is based on 16S rRNA Sanger sequencing.

Table D.1 Identification of endophytic bacteria isolates associated with annual barley (*Hordeum vulgare*) positive for hydrocarbon degrading genes (CYP153, *alkB*, *nah*).

#	Code	Description	Gene	Similarity (%)	Accession #
1	EA1-1	*Pedobacter alluvionis*	*alkB*	100%	HF936841.1
2	EA1-15	*Chryseobacterium* sp.	CYP153	100%	LK054576.1
3	EA1-17	*Stenotrophomonas chelatiphaga*	CYP153	100%	NR074977.1
4	EA1-36	*Pantoea theicola*	CYP153	100%	NR132712.1
5	EA2-9	*Stenotrophomonas pavanii*	*nah*	100%	NR132712.1
6	EA2-19	*Pantoea theicola*	*alkB*	100%	GU413438.1
7	EA2-20	*Pedobacter ginsengisoli*	*alkB*	100%	KT375349.1
8	EA2-24	*Enterobacter cloacae*	*alkB*	100%	KJ579161.2
9	EA2-30	*Flavobacterium aquidurense*	*alkB*	100%	KR088356.1
10	EA3-1	*Bacillus pumilus*	*nah*	100%	KC595863.1
11	EA3-10	*Arthrobacter* sp.	*nah*	100%	NR134699.1
12	EA3-16	*Chryseobacterium hominis*	*nah*	100%	NR126257.1
13	EA3-17	*Chryseobacterium hominis*	*nah*	100%	NR126257.1
14	EA3-18	*Pseudomonas syringae*	*nah*	100%	KP299228.1
15	EA3-25	*Xanthomonas campestris*	*nah*	100%	NR121770.1
16	EA4-1	*Chryseobacterium nakagawai*	*nah*	100%	NR126257.1
17	EA4-2	*Staphylococcus hominis*	CYP153	100%	NR134713.1
18	EA4-6	*Serratia plymuthica*	CYP153	100%	NR134019.1
19	EA4-8	*Pseudomonas* sp.	CYP153	100%	CP012830.1
20	EA4-13	*Enterobacter cloacae*	*nah*	100%	NR104943.1
21	EA4-27	*Ewingella* sp.	CYP153	100%	NR133716.1
22	EA4-32	*Enterobacter* sp.	*nah*	100%	NR104943.1
23	EA4-34	*Pantoea theicola*	*alkB*	100%	KT375349.1
24	EA4-35	*Bacillus subtilis*	*alkB*	100%	FJ973535.1
25	EA4-38	*Serratia quinivorans*	CYP153	100%	NR102514.1
26	EA4-40	*Pantoea theicola*	*alkB*	100%	EF173381.1
27	EA5-5	*Staphylococcus aureus*	*nah*	100%	NR074956.1
28	EA5-8	*Serratia plymuthica*	*nah*	100%	NR074820.1
29	EA5-9	*Rahnella* sp.	CYP153/*nah*	100%	KT862774.1
30	EA5-10	*Ewingella* sp.	CYP153	100%	NR133716.1
31	EA5-11	*Serratia liquefaciens*	CYP153/*nah*	100%	KT862774.1
32	EA5-20	*Flavobacterium* sp.	CYP153	100%	KM513968.1
33	EA6-5	*Pseudomonas* sp.	*alkB*	100%	KP259552.1
34	EA6-12	*Pseudomonas* sp.	*alkB*	100%	KP259552.1
35	EA6-37	*Enterobacter* sp.	*alkB*	100%	LC034140.1
36	EA6-38	*Kluyvera intermedia*	*alkB*	100%	KP852523.1
37	EA6-40	*Enterobacter* sp.	*alkB*	100%	KP852523.1
38	EA7-1	*Pantoea theicola*	*alkB*	100%	KC834342.1
39	EA7-4	*Pantoea intestinalis*	*alkB*	100%	KF679345.1
40	EA7-11	*Pseudomonas syringae*	*alkB*	100%	JX514422.1
41	EA1-34	*Rahnella* sp.	CYP153	100%	KX450471.1
42	EA1-37	*Bacillus pumilus*	CYP153	100%	KX856184.1

APPENDIX E: Effect of endophytic bacteria on sweet clover and barley in seed germination experiments.

Table E.1 Effect of endophytic bacteria on barley and sweet clover seed germination rate (%) (Total of 10 seeds) at 10°C. Symbols indicate treatments significantly (LSD p ≤ 0.05) lower (†) when compared to the control.

	Barley		Sweet clover	
	(%)			
Strain	Mean	SD	Mean	SD
Control	98.0	4.5	73.8	38.1
EA1-1	86.0	8.9	73.8	37.0
EA1-15	86.0	16.7	77.3	39.8
EA1-17	74.0†	15.2	88.0	11.0
EA1-36	86.0	16.7	58.9	47.4
EA2-9	76.0†	11.4	80.0	7.1
EA2-19	82.0	14.8	74.0	16.7
EA2-20	80.0†	12.2	88.0	8.4
EA2-24	82.0	13.0	96.0	5.5
EA2-30	94.0	8.9	69.8	35.0
EA3-1	84.0	15.2	92.0	11.0
EA3-10	88.0	8.4	96.0	8.9
EA3-16	90.0	10.0	92.0	8.4
EA3-17	34.0†	16.7	84.0	13.4
EA3-18	46.0†	18.2	88.0	11.0
EA3-25	32.0†	16.4	90.0	10.0
EA4-1	22.0†	8.4	88.0	8.4
EA4-2	40.0†	20.0	67.5	35.4
EA4-6	48.0†	23.9	94.0	5.5
EA4-8	30.0†	14.1	72.0	24.9
EA4-13	8.0†	13.0	82.0	14.8
EA4-27	62.0†	17.9	74.0	5.5
EA4-32	50.0†	14.1	78.0	13.0
EA4-34	24.0†	15.2	94.0	5.5
EA4-35	20.0†	12.2	94.0	8.9
EA4-38	36.0†	16.7	94.0	5.5
EA4-40	92.0	13.0	90.0	10.0
EA5-5	68.0†	8.4	90.0	7.1
EA5-8	68.0†	17.9	90.0	10.0
EA5-9	24.0†	13.4	68.0	23.9
EA5-10	14.0†	11.4	42.0	29.5
EA5-11	76.0†	11.4	74.0	8.9
EA5-20	70.0†	18.7	82.0	8.4
EA6-5	90.0	10.0	86.0	5.5
EA6-12	96.0	5.5	82.0	11.0
EA6-37	82.0†	8.4	84.0	16.7
EA6-38	88.0	8.4	84.0	18.2
EA6-40	78.0†	8.4	84.0	15.2
EA7-1	94.0	8.9	80.0	7.1
EA7-4	76.0†	5.5	84.0	15.2
EA7-11	76.0†	5.5	90.0	7.1
EA1-34	32.0†	20.5	86.0	5.5
EA1-37	38.0†	11.0	66.0	15.2

Table E.2 Effect of endophytic bacteria on barley and sweet clover seed germination vigor (%) (Total of 10 seeds) at 25°C. Symbols indicate treatments significantly (LSD $p \leq 0.05$) higher (*) and lower (†) when compared to the control.

	Barley		Sweet clover	
	----- (%) -----			
Strain	Mean	SD	Mean	SD
Control	68.0	13.0	48.0	15.7
EA1-1	58.0	11.0	44.0	5.5
EA1-15	22.0†	8.4	26.0†	5.5
EA1-17	38.0†	12.8	70.0*	5.5
EA1-36	62.0	8.4	40.7	15.5
EA2-9	38.0	19.2	50.0	25.5
EA2-19	36.0	20.7	46.0	15.2
EA2-20	34.0†	11.9	58.0	19.2
EA2-24	54.0	32.1	78.0	17.9
EA2-30	44.0†	5.2	56.0	15.2
EA3-1	44.0†	9.7	78.0	16.4
EA3-10	24.0†	23.0	74.0	21.9
EA3-16	68.0	17.9	84.0*	11.4
EA3-17	46.0	42.2	74.0	15.4
EA3-18	36.0†	8.9	16.0	18.2
EA3-25	6.0†	5.5	84.0*	11.4
EA4-1	12.0†	8.4	54.0	18.2
EA4-2	4.0†	5.5	4.0†	5.5
EA4-6	10.0†	12.2	45.6	21.5
EA4-8	10.0†	10.0	0.0†	0.0
EA4-13	6.0†	8.9	70.0	10.0
EA4-27	36.0†	18.2	58.0	8.4
EA4-32	32.0†	14.8	60.0	10.0
EA4-34	20.0†	18.7	70.0	15.8
EA4-35	52.0	36.3	76.0	27.0
EA4-38	10.0†	7.1	54.0	18.2
EA4-40	46.0†	8.9	48.0	17.9
EA5-5	50.0	27.4	84.0*	8.9
EA5-8	52.0	17.9	68.0	4.5
EA5-9	14.0†	11.4	22.0	17.9
EA5-10	0.0†	0.0	26.0	15.2
EA5-11	52.0	21.7	42.0	11.0
EA5-20	50.0	23.5	74.0*	5.5
EA6-5	60.0	12.2	62.0	16.4
EA6-12	56.0	19.5	78.0*	11.0
EA6-37	32.0†	13.0	42.0	31.1
EA6-38	48.0	14.8	50.0	17.3
EA6-40	60.0	25.5	54.0	27.0
EA7-1	72.0	17.9	62.0	21.7
EA7-4	62.0	32.7	60.0	12.2
EA7-11	78.0	17.9	68.0	21.7
EA1-34	54.0	26.1	68.0	16.4
EA1-37	22.0†	21.7	50.0	15.8

Table E.3 Effect of endophytic bacteria on barley and sweet clover seed germination vigor (%) (Total of 10 seeds) at 10°C. Symbols indicate treatments significantly (LSD $p \leq 0.05$) higher (*) and lower (†) when compared to the control.

	Barley		Sweet clover	
	---- (%) ----			
Strain	**Mean**	**SD**	**Mean**	**SD**
Control	76.0	11.4	17.7	13.3
EA1-1	30.0†	10.0	4.0	8.9
EA1-15	42.0	28.6	0.0†	0.0
EA1-17	18.0†	19.2	70.0*	10.0
EA1-36	42.0†	21.7	0.0†	0.0
EA2-9	28.0†	26.8	0.0†	0.0
EA2-19	22.0†	14.8	14.0	11.4
EA2-20	32.0†	13.0	0.0†	0.0
EA2-24	18.0†	14.8	2.0	4.5
EA2-30	24.0†	20.7	39.0*	7.5
EA3-1	42.0†	20.5	18.0	8.4
EA3-10	8.0†	8.4	0.0†	0.0
EA3-16	40.0†	15.8	74.0*	15.2
EA3-17	8.0†	8.4	12.0	16.4
EA3-18	32.0†	14.8	16.0	18.2
EA3-25	12.0†	16.4	0.0†	0.0
EA4-1	6.0†	5.5	4.0	5.5
EA4-2	26.0†	23.0	2.0	4.5
EA4-6	28.0†	27.7	0.0†	0.0
EA4-8	14.0†	16.7	0.0†	0.0
EA4-13	2.0†	4.5	32.0	19.2
EA4-27	28.0†	19.2	20.0	15.8
EA4-32	26.0†	16.7	4.0	8.9
EA4-34	22.0†	13.0	6.0	13.4
EA4-35	14.0†	11.4	18.0	14.8
EA4-38	0.0†	0.0	0.0†	0.0
EA4-40	46.0†	8.9	48.0*	7.9
EA5-5	52.0	17.9	26.0	29.7
EA5-8	28.0†	13.0	6.0	5.5
EA5-9	2.0†	4.5	2.0	4.5
EA5-10	4.0†	5.5	0.0†	0.0
EA5-11	32.0†	13.0	10.0	12.2
EA5-20	46.0	26.1	16.0	15.2
EA6-5	64.0	24.1	54.0*	18.2
EA6-12	86.0	8.9	22.0	11.0
EA6-37	34.0†	21.9	50.0*	17.3
EA6-38	62.0	11.0	14.0	15.2
EA6-40	40.0	27.4	2.0	4.5
EA7-1	66.0	19.5	4.0	5.5
EA7-4	32.0†	8.4	2.0	4.5
EA7-11	38.0†	21.7	6.0	8.9
EA1-34	14.0†	16.7	4.0	8.9
EA1-37	0.0†	0.0	0.0†	0.0

APPENDIX F: Effect of endophytic bacteria on sweet clover in root elongation experiments.

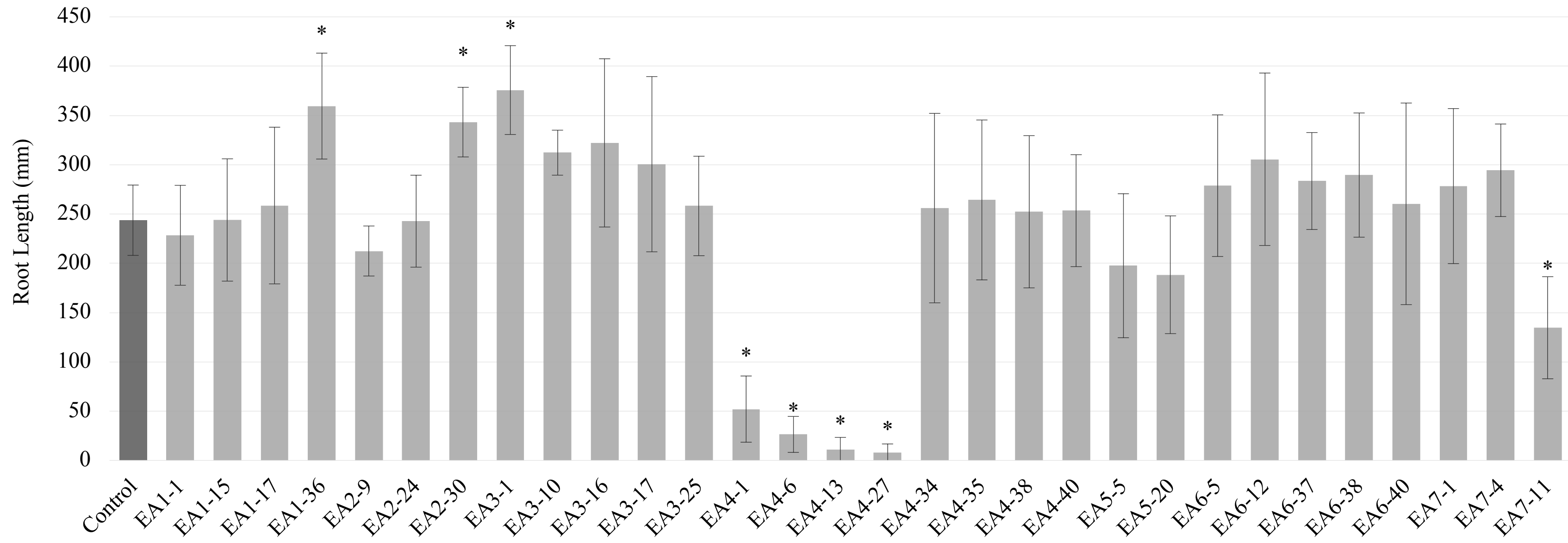

Figure F.1 Effect of bacteria on root length of sweet clover measured at 20 days. Plants were supplemented only with water. Error bars represent standard deviations and * indicate significance at LSD ($p \leq 0.05$) compared to control.

APPENDIX G: Preliminary assessment of total extractable F3 and F4 hydrocarbons in soils.

Table 4 Total extractable F3 and F4 hydrocarbons in soils amended with diesel without plants or addition of bacterial inoculants.

Diesel concentration ($mg \cdot kg^{-1}$)	Time	---------------------- Hydrocarbons ($mg \cdot kg^{-1}$)-------------------		
		F2 (C10-C16)	F3 (C16-C34)	Total (F2 and F3)
10,000	(T=0)	6700	4230	10930
	(T= 7 days)	4640	3180	7820
20,000	(T=0)	13500	7920	21420
	(T= 7 days)	11000	7230	18230

APPENDIX H: Photographs of sweet clover plants in phytoremediation growth chamber experiments using soils amended with diesel fuel at 5,000, 10,000 and 20,000 mg·kg^{-1}.

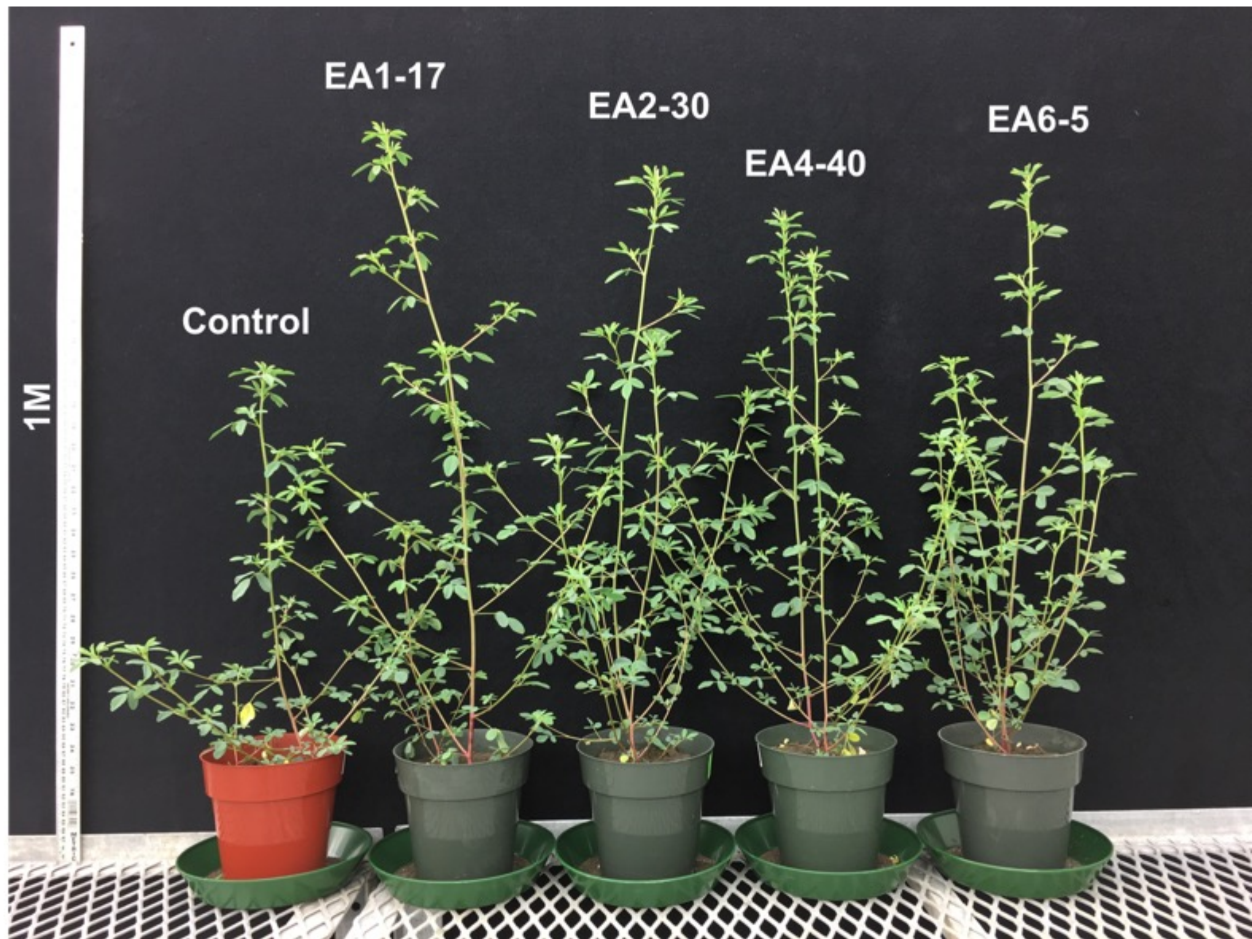

Figure G.1 Effect of endophytic bacterial strains EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover plants (*Melilotus albus*) growing in soils amended with diesel (5,000 $mg \cdot kg^{-1}$) at 65 days after planting.

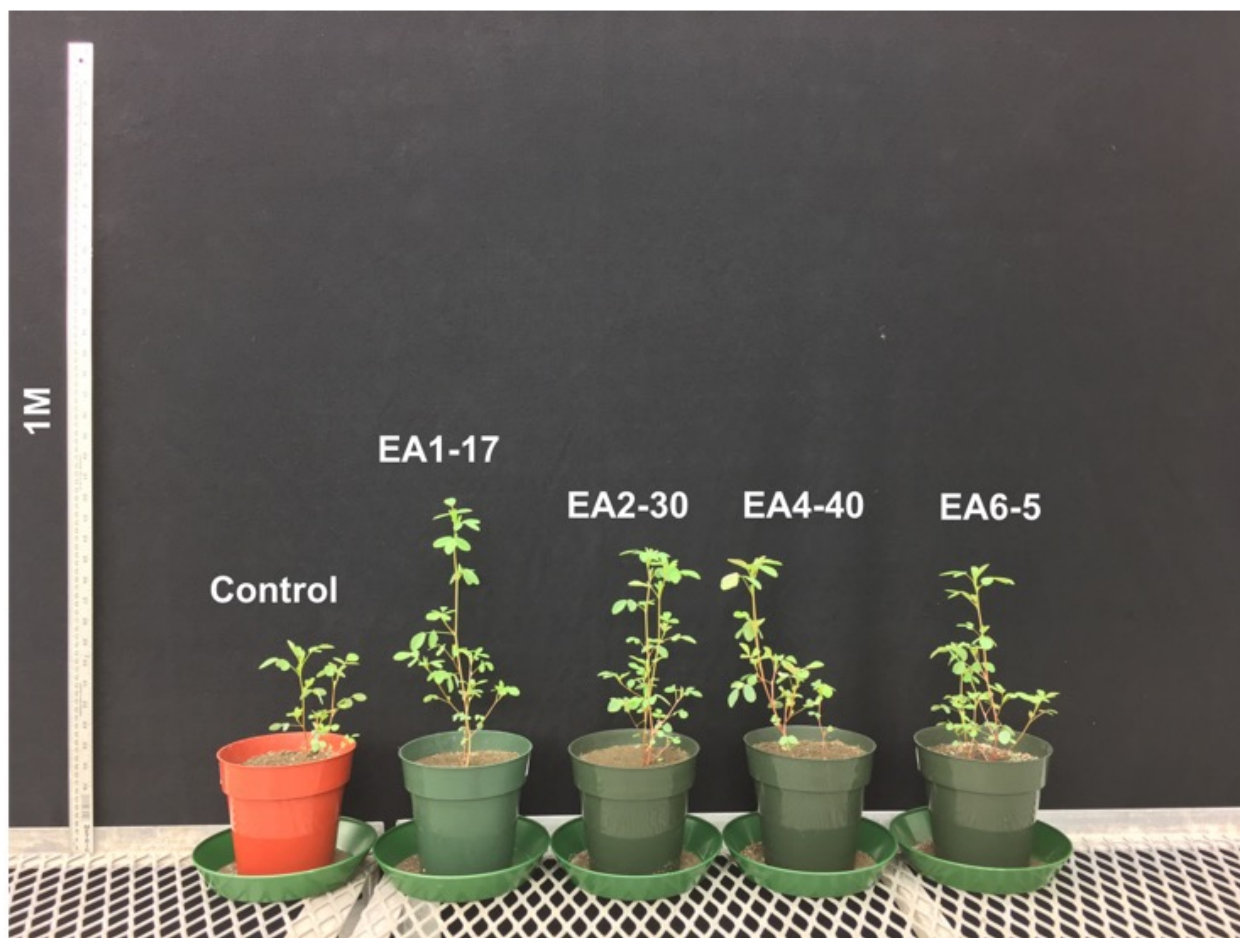

Figure G.2 Effect of endophytic bacterial strains EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover plants (*Melilotus albus*) growing in soils amended with diesel (10,000 $mg \cdot kg^{-1}$) at 65 days after planting.

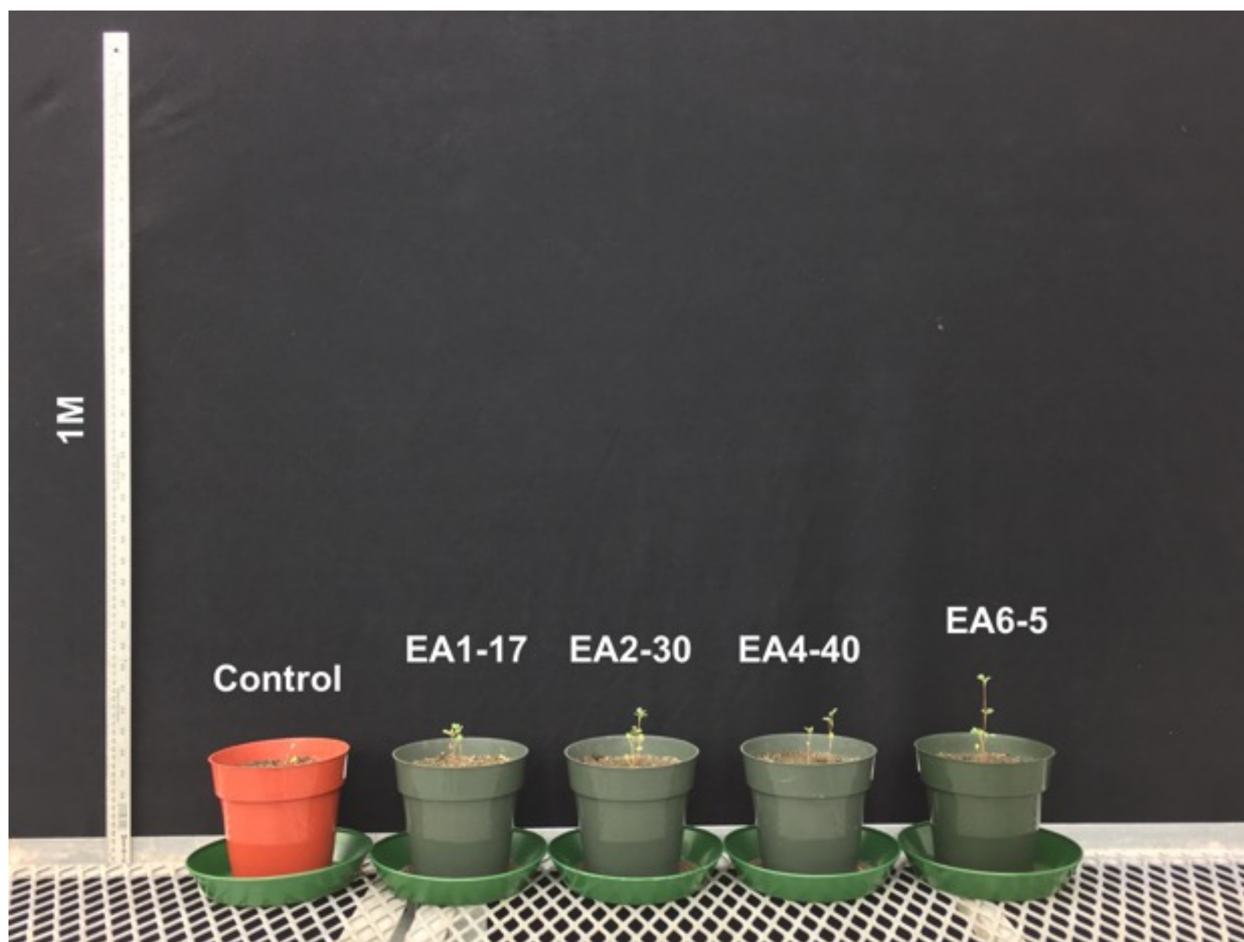

Figure G.3 Effect of endophytic bacterial strains EA1-17 (*Stenotrophomonas* sp.), EA2-30 (*Flavobacterium* sp.), EA4-40 (*Pantoea* sp.) and EA6-5 (*Pseudomonas* sp.) on sweet clover plants (*Melilotus albus*) growing in soils amended with diesel (20,000 mg·kg^{-1}) at 65 days after planting.