http://researchcommons.waikato.ac.nz/

Research Commons at the University of Waikato

Copyright Statement:

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

The thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of the thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from the thesis.

A Microbial Community description of a heavy metal contaminated soil site

A thesis submitted in partial fulfilment

of the requirements for the degree

of

Masters of Science in Biological Sciences

at

The University of Waikato

by

LIN TAN

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

The University of Waikato

2012

Abstract

Tui mine area, with extremely low pH (2.9-3.3) values and high-metal concentrations, is one of the most contaminated sites in NZ. The structure of the microbial community in different soil layers (Top, Middle and Bottom) of the Tui mine tailing was investigated using culture-based and molecular approaches. The zinc resistance of an *Acidocella* strain, isolated previously, was determined by culturing methods. Growth curves of *Acidocella* indicated that it could grow at up to 250mM $ZnSO_4$, and in the presence of 250mM $MgSO_4$, both growth and Zn^{2+} resistance were greatly enhanced with tolerance up to 750mM Zn^{2+}. Acidophilic bacteria were isolated and enumerated on solid media, and were found to be related to *Actinobacteridae* and *Gammaproteobacteria*. By contrast, cloning and pyrosequencing analysis of the 16S rRNA genes revealed that *Leptospirillum ferrooxidans* and *Acidithiobacillus ferrooxidans* were the most common strains that occurred in the soil environment in all layers. From a clone library of the Top layer DNA, 69 out of 118 clone sequences were phylogenetically affiliated with the iron-oxidizing *Leptospirillum ferrooxidans* within the Nitrospira and 16 with *Acidithiobacillus ferrooxidans* within the *Proteobacteria.* A broadly similar result was obtained from pyrosequencing analysis. The most abundant OTU (21.9%) was affiliated with *Leptospirillum ferrooxidans* and the second most abundant (8.4%) affiliated with *Acidithiobacillus ferrivorans*. With the Bottom layer soil, 61.5% of the amplicons sequences in the pyrosequencing run had the closest match with *Leptospirillum ferrooxidans* and the second most abundant OTU (27.2% of sequences) were affiliated with *Acidithiobacillus ferrivorans SS3*. Both molecular approaches indicate that a diverse population of bacterial species (128 different species from pyrosequencing) exist in the Top layer soil with the Bottom layer soil having a lower in diversity (49 different species from pyrosequencing).

Acknowledgements

There are many people to whom I am indebted in helping me complete this thesis. Firstly I would like to thank my supervisor, Huge Morgan, who offered me the opportunity to work on this exciting project and to conduct research on this topic in New Zealand. I also thank him for the encouragement and patient he has shown throughout this study.

I am thankful to Eric Bottos in sharing his ideas and helping with various aspects of my lab work especially from the very beginning of my project. Many thanks to Roanna Richards for helping me doing 454 sample preparation and teaching me how to build the phylogenetic tree. Thank you also to John Longmore for providing me the sequencing results.

I am grateful to many people in the Thermopile Research Unit who treat me like their family. In particular， I would like to thank Charles lee and Stephen Archer， for their help with my 454 result analysis. Also to be thanked is Lynne Parker, who not only offers me the equipment and reagent, but also provides technical assistance.

Last but not least, I would like to address a special thanks to my parents and my partner. I would like to thank my dad for his pride and financial support. Then I would like thank my mom for her psychological support. To my partner, who has always been with me from the very start, I could never thank you enough for the love and support you have given to me.

Table of Content

Abstract ii

Acknowledgements iii

Table of Content iv

List of Figures vii

Abbreviations x

1. Introduction 1

1.1 Tui mine Environment 1

1.1.1 Heavy metals 1

1.1.2 pH 5

1.2 Microorganisms associated with the mining environment 9

1.3 Methods of microbial community analysis 13

1.3.1 Culture based methods 13

1.3.2 Culture independent methods 14

1.3.3 Clone library analysis 15

1.3.4 454 Pyrosequencing 16

1.4 Aims and outline of Thesis 17

2. Material and Methods 19

2.1 Analysis of Tui Soil Environment 19

2.1.1 pH 21

2.2 Cultivation of Microorganisms 22

2.2.1 Pre-incubation Preparation 22

2.2.1.1 Sample preparation 22

2.2.1.2 Media preparation 22

2.2.2 Cultures incubation and observation 23

2.2.3 Microorganisms storage 25

2.3 Community analysis by molecular methodology 26

2.3.1 DNA extraction 26

2.3.1.1 CTAB-based DNA extraction from pure cultures 26

2.3.1.2 Bead beating and CTAB-based DNA extraction from soil sample 27
2.3.1.3 DNA extraction by The NucleoSpin® Soil Kit 27
2.3.1.4 DNA extraction by the MOBIO kit 29
2.3.2 DNA Quantification 30
2.3.3 Polymerase Chain Reaction (PCR) 30
2.3.4 Agarose gel Electrophoresis 32
2.3.5 DNA Purification 32
2.3.6 Restriction endonuclease digestion 33
2.3.7 Cloning 34
2.3.7.1 Electro-competent cells preparation 35
2.3.7.2 Ligation 36
2.3.7.3 Transformation 37
2.3.7.4 Colony selection and analysis 37
2.3.7.5 Plasmid DNA preparation by alkaline lysis 37
2.3.7.6 Plasmid DNA Analysis 38
2.3.8 Sanger sequencing and data analysis 39
2.3.9 454 Pyrosequencing 39
2.3.9.1 454 Pyrosequencing 40
2.3.9.2 454 Pyrosequencing data processing 44

3. Results and Discussion 45

3.1 pH of environmental samples 45

3.2 Culture-based results 45

3.2.1 Environmental samples analysis 45
3.2.1.1 Culturing results 46
3.2.1.2 Molecular analysis 51
3.2.2 Acidocella growth 54

3.3 Molecular-based analysis 57

3.3.1 Clone library analysis 58

3.4 454 Pyrosequencing analysis 68

4. Conclusion 71

5. References 72

6. Appendices 83

6.1 Appendix A: Culture Mediums 83

6.2 *Appendix B: Agarose gel electrophoresis* 87

6.3 *Appendix C: Map of 1kb plus ladder* 88

6.4 *Appendix D: Procedure of Isopropanol clean up method* 89

6.5 *Appendix E: Procedure of QuickClean II Gel Extraction Kit* 90

6.6 *Appendix E: Procedure of QuickClean II PCR Purification Kit* 92

6.7 Appendix F: Map of pCR™ 4-TOPO® 93

6.8 *Appendix G: EMA Treatment* 94

6.9 *Appendix H: Procedure of UltraClean® 15 DNA Purification Kit* 95

6.10 *Appendix I: Protocol of QuBit dsDNA HS Kit* 97

6.11 *Appendix J KAPA qPCR analysis* 98

6.12 Appendix K: 16S rRNA gene sequences 100

6.12.1 DNA sequences from pure culture 100

6.12.2 DNA sequences from clone libraries 103

List of Figures

FIG 1 LOCATION OF THE TUI MINE SITE, TE AROHA, NEW ZEALAND (MORRELL, *ET AL*, 1996) 6

FIG 2 MAIN TUI MINE AREA (FROM GOOGLE MAP) 7

FIG 3 THE TUI AND TUNAKOHOIA STREAMS FLOW INTO THE WAIHOU RIVER (WAIKATO REGIONAL COUNCIL, 2011) 7

FIG 4 MAP OF THE TE AROHA AND WAIHI REGIONS SHOWING STREAMS AND RIVERS 8

FIG 5 ELECTRON FLOW DURING Fe^{2+} OXIDATION BY THE ACIDOPHILE *ACIDITHIOBACILLUS FERROOXIDANS*. 11

FIG 6. 16S RRNA MOLECULE (GARRETT & GRISHAM, 2005) 15

FIG 7 FLOW DIAGRAM OF THE MAIN METHODS IN THIS STUDY 18

FIG 8 SITES OF SAMPLES COLLECTION FROM MAIN TAILING AREA IN TUI MINE, TE AROHA 19

FIG 9 THREE SOIL SAMPLES FROM SAME SITE WITH DIFFERENT DEPTH. 20

FIG 10 THREE LIQUID SAMPLES FROM TUI MINE, TE AROHA. 21

FIG 11 OUTLINE OF DNA EXTRACTION BY THE NUCLEOSPIN® SOIL KIT 28

FIG 12 CLONE LIBRARY EXPERIMENTAL OUTLINE 35

FIG 13 454 SAMPLE PREPARATION 40

FIG 14 AMPLIFICATION OF 16S RRNA FROM BACTERIAL ISOLATES (USING 1492R AND 27F) 51

FIG 15 GEL ELECTROPHORESIS OF RESTRICTION DIGESTION RESULTS OF 15 AMPLIFIED 16S RRNA AMPLICONS WITH *HAE* III AND *AFA* I 52

FIG 16 *ACIDOCELLA* GROWTH CURVES AND GLUCOSE CONSUMPTION IN DIFFERENT MEDIA COMPOSITIONS (1A) 54

FIG 17 *ACIDOCELLA* GROWTH CURVES AND GLUCOSE CONSUMPTION IN DIFFERENT MEDIA COMPOSITIONS (1B) 55

FIG 18 *ACIDOCELLA* GROWTH RESPONSE TO ADD ZN 56

FIG 19 *ACIDOCELLA* GROWTH RESPONSE TO ZN AND MG ADDITIONS TO THE MEDIUM 57

FIG 20 GEL ELECTROPHORESIS OF DOUBLE-ENZYME RESTRICTION DIGESTION RESULTS OF AMPLIFIED 16S RRNA AMPLICONS FROM THREE SOIL CLONE LIBRARIES SAMPLES 59

FIG 21 FREQUENCY OF BACTERIAL 16S RFLP RESTRICTION PATTERNS 61

FIG 21 TOP LAYER BACTERIAL DIVERSITY 67

FIG 23 BACTERIAL DIVERSITY WITH THE TOP AND BOTTOM LAYER SOIL SAMPLE AT THE PHYLUM LEVEL 68

FIG 24 VENN DIAGRAM SHOWING SHARED OTUS BETWEEN TOP AND BOTTOM LAYER SOIL SAMPLE. 70

List of Tables

TABLE 1 HEAVY-METAL CONCENTRATION IN WATYER 3
TABLE 2 SUMMARY OF DATA FOR HEAVY METALS CONCENTRATIONS FROM THE TUI AND TUNAKOHOIA STREAMS AND FROM THE WAIHOU RIVER. 4
TABLE 3 MEDIUM COMPOSITION 24
TABLE 4 NEAR-FULL LENGTH BACTERIAL 16S RDNA PCR 31
TABLE 5 RESTRICTION ENDONUCLEASES USED IN THIS STUDY 33
TABLE 6 DOUBLE-ENZYME RESTRICTION REACTION MIX 33
TABLE 7 SINGLE ENZYME RESTRICTION REACTION MIX 34
TABLE 8 CLONING REACTION MIX 36
TABLE 9 KAPA HIFI HOTSTART PCR REACTION 41
TABLE 10 PH VALUE OF SAMPLES 45
TABLE 11 (A, B & C) ISOLATION RESULTS FROM THREE SOIL SAMPLES 48
TABLE 12 (A, B & C) ISOLATION RESULTS FROM THREE LIQUID SAMPLES 49
TABLE 13 BLAST RESULTS OF SEQUENCES FROM PURE CULTURES 53
TABLE 14 BLAST RESULTS OF SEQUENCES FROM THE CLONE LIBRARIES 62

Abbreviations

BME	β -mercaptoethanol
BSA	bovine serum albumin
CCD	charge-coupled device
CTAB	cetyltrimethylammonium bromide
EDTA	ethylene diamine tetra-acetic acid
EMA	ethidium monoazide
kb	Kilobase
LB	luria broth
Milli-Q	Millipore Corporation
NCBI	National Centre of Biotechnology Information
NZ	New Zealand
OD	optical density
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
RFLP	Restriction Fragment Length Polymorphism
SDS	sodium dodecyl sulphate
TAE	Tris-acetic acid EDTA buffer
TE	Tris EDTA buffer
UV	ultra violet
V	volts

1. Introduction

Mining activities always result in deterioration of environmental quality and this environmental degradation resulting from the mine tailings oxidation has been recognised as a serious issue in many mining areas around the world (Johnson, *et al,* 2003, Morrell, *et al*, 1996 & Nabi Bidhendi, *et al*, 2007). Acid mine and rock drainages (AMD) caused by active and abandoned mines can be easily catalysed by "atmospheric, hydrological or biological weathering" (Luís, *et al*, 2009).

1.1 Tui mine Environment

The Tui mine sits on the western flank of Mount Te Aroha to the northeast of Te Aroha town centre (Fig 1). Even though it has not been worked for nearly 40 years, the tailings area is defined as an extreme environment characterised by a low pH and high-metal concentration, and described as among the most heavily metal-polluted environments in New Zealand (Morrell, *et al,* 1996). Nutrients (N and P) are poor in mine tailings because of the lack of aggregate structure or organic matter (Mendez, *et al,* 2008). Oxidation of tailings generates acidic drainage which greatly decreases the environment pH and this enhances the release of metals to toxic levels, which diminishes colonisation by plants. In total, Tui mine is extremely acidic and has poor substrates and high concentration of heavy metals.

1.1.1 Heavy metals

Metal contamination can be dangerous or lethal to components of the biota such as "phytoplankton, zooplankton, benthos, mangroves, fish, birds, mammals and finally to humans" (Amado Fiilho, *et al*, 1997, Correa Junior, *et al*, 2000, Junior, *et al,* 2002 & Almeida, *et al,* 2009). Heavy metals have long-term hazardous

impacts on the health of soil ecosystems and deleterious influences on soil biological processes (Bhattacharyya, *et al,* 2008, Khan, *et al,* 2010, Pérez-de-Mora, *et al,* 2006 & Wang, *et al,* 2007). Although there are no major physical limitations, no vegetation can colonise in the main Tui mine area (Fig 2).

Copper, lead and zinc sulphides were the main mineral components extracted from the underground Tui Mine in the 1960's (Craw, D. 2003). During that period, over 20,000 tonnes of copper-lead-zinc concentrate and 2.5 tonnes of gold-silver concentrate were produced from 160,000 tonnes of ore, which contained about 17% zinc, 7% lead and 0.6% copper (Sabti, *et al,* 2000). At the same time, an approximate 100.000 m^3 of sulphide-bearing railings were deposited on sites (Morrell, *et al,* 1996). As a result of mining activities and the deposition of mine tailings nearby, the concentration of sulphides minerals such as chalcopyrite ($CuFeS_2$), galena (PbS), sphalerite (ZnS) and pyrite (FeS_2) is very high and this subsequently developed the acid mine drainage (AMD) which is defined as one of the most significant issues around the world mining regions (Craw, D., 2003; Bradshaw, *et al,* 1980; Ritcey, 1989 & Weissberg, *et al,* 1970).

A PhD thesis from Massey University detailed the concentration of heavy metals in the surface tailings including As, Cu, Pb, and Zn which are 254 mg/kg, 26-991 mg/kg, 1503-27416 mg/kg and 123-2333mg/kg, respectively. X-ray diffraction (XRD) analysis of the Tui tailings by Morrell (1996) showed that the "entire" concentration of metals tend to be much higher with increasing depth, which originally had the same sulphide minerals content detected by SEM micrographs. The heavy metal fraction (HMF) result from 0-200mm tailing sample is 1.45-1.55%, which is only a quarter of HMF from 200-300mm sample (7.23%) and one-tenth from 500-600mm sample (16.25%).

Besides the main mine tailing site, the concentration of heavy metals were also extremely high in sediments from the Tui and Tunakohoia Streams, which lie on the left and right flanks of the centre tailing site (Fig. 3). Some more mobile minerals like gold were also detectable downstream at the Waihou River and the further downstream at the Waitekauri and Ohinemuri Rivers (Fig. 4). Sabti *et al*

Table 1 Heavy-metal concentration in watyer (µg/L=ppb) from the Tui (Tui) and Tunakohoia (Tk) streams and from the Waihou (Wa) River. See Fig. 4 for locations (Sabti, *et al,* 2000)

	Cu	Zn	Cd	Pb	As
Tui 1	28	740	2.0	51	26
Tui 2	36	790	4.0	60	12
Tui 3	570	46,400	272	649	42
Tui 4	32	3,000	6.0	0.04	12
Tui 5	570	57,600	286	426	14
Tui 6	12	11,300	0.1	0.20	3
Tui 7	8	1,300	2.6	0.20	22
Tui 8	6	850	1.1	0.02	21
Tui 9	6	810	1.6	0.03	19
Tui 10	5	820	1.3	0.04	15
Tui 11	4	600	0.7	0.03	29
Tui 12	7	370	0.1	1.04	18
Tui 13	8	140	0.1	1.00	25
Tk 1	10	2,740	4.5	1.00	10
Tk 2	12	14,000	18	0.2	17
Tk 3	13	13,000	21	0.09	17
Tk 4	18	9,900	23	0.24	9
Tk 5	16	9,600	20	119	19
Tk 6	310	11,300	73	24	19
Tk 7	8	12	0.1	1.00	19
Tk 8	5	5,600	13	0.17	10
Tk 9	20	2,200	6.0	0.09	17
Tk 9A	16	360	4.0	0.08	15
Tk 10	17	530	1.8	0.10	35
Tk 11	15	200	1.0	0.2	14
Wa 1	10	25	0.1	0.06	18
Wa 2	9	24	0.1	0.05	19
Wa 3	11	16	1.0	0.16	12
Wa 4	14	19	0.4	0.06	16
Wa 5	13	15	0.1	0.03	14
Wa 7	13	15	0.1	0.03	14

(1996) summarised the data of elemental concentrations in those streams and rivers (Tables 1 & 2).

From these results, it can see that the upper reaches of the Tui and Tunakahoia Streams contain much higher levels of heavy minerals than the limits of potable water values, which are not suitable for domestic water supply. Among all heavy metals, Zinc has a much higher average concentration than others and the greater mobility was shown by a long dispersal pattern in two streams with concentrations extending as far as the confluences with the Waihou River. The highest zinc concentrations were in sample sites Tui3, Tui5, Tui6, TK2, TK3 and TK6 with concentration above 11,300 µg/L (potable water should be less than 3000 µg/L). Figure 4 indicates that all these 6 sites were closest to the mine tailings and this corresponds with the heavy metals concentrations in the main site. Tui3 and Tui5, located just on

the edge of mine tailing, have the greatest amount of metal with Zn concentration being nearly 20 times higher than the potable limit. Although the average concentrations of Cd and Pb are much lower than Zn, they are also major contamination factors in the liquid phase as the potable limits of Cd and Pb in water were only 3μg/L and 10μg/L, respectively. Similarly to the zinc result, concentrations of cadmium and lead in Tui3 and Tui5 were more than 40 times higher than the limits.

Table 2 Summary of data for heavy metals concentrations (mg/kg) in <0.5mm and 0.5-1.0mm (in parentheses) size fractions of stream sediments from the Tui and Tunakohoia Streams and from the Waihou River (Sabti, *et al,* 2000).

	Cu	Zn	Cd	Pb	As
Tui Stream (n=7)					
Arithemic Mcan	143(107)	2171(1894)	6.1(6.6)	88(75)	3.3(3.6)
Median	96(110)	2141(1940)	6.3(7.3)	95(69)	3.3(3.0)
Geometric Mean	123(104)	2116(1831)	5.5(5.7)	82(74)	3.2(3.0)
Maximum (0.5mm size)	363	2782	9	112	5.1
Minimum (0.5mm size)	70	1447	2.4	29	1.2
Tunakohoia Stream (n=8)					
Arithemic Mcan	3281(2816)	6961(5250)	22.2(9.6)	1201(975)	18(18)
Median	345(183)	3766(2428)	6.3(4.2)	1.0(2.5)	17(12)
Geometric Mean	217(139)	2342(1694)	5.6(3.1)	5.4(7.0)	13(13)
Maximum (0.5mm size)	21,423	23,624	74	9352	38
Minimum (0.5mm size)	0.6	87	<0.3	0.1	1.8
Waihou River (n=5)					
Arithemic Mcan	1.5(2.0)	43.6(53.9)	0.3 (<0.3)	4.0(6.0)	3.4(2.8)
Median	1.5(1.8)	38.0(56.0)	0.3 (<0.3)	4.0(7.0)	1.2(1.4)
Geometric Mean	2.8(2.0)	41.9(51.0)	0.3 (<0.3)	3.4(4.5)	1.6(1.7)
Maximum (0.5mm size)	17	71	0.3	7.0	2.0
Minimum (0.5mm size)	14	38	0.3	1.0	0.5

Compared with levels in the streams, significant amounts of heavy metals were contained in the sediments from the upper reaches of the Tui and Tunakahoia

Streams near the Tui Mine. Sabti, *et al* (2000) indicates that the greatest concentrations in both Tui and Tunakohoia stream were adjacent to, or just below, the main tailings. Further downstream of the Tui and Tunakohoia stream (Tui 10-13 and TK12, see Fig. 4), the elemental concentrations in the sediments were also unacceptably high while the Tunakohoia stream was once a water source to town Te Aroha.

From all the data, it is easy to see that among all the heavy metals, zinc was has the highest concentration in both sediments and water. Cadium and lead concentrations are not as high as zinc concentration, but still much higher than the potable values.

Concentrations of metals in the soil are the key ecological and toxicological concern, which greatly reduce soil respiration and microbial biomass, and block waste decomposition and de-toxification. It has a damaging effect on the activities and functions of microorganisms such as N mineralization (Chander, *et al,* 1995; Hu, *et al,* 2006 & Zhou, *et al,* 2009). With concentration of Zn above 1165µg/g, the toxicological characteristics will play an important role on microbes (Martínez, *et al,* 2007; Pierzynski, *et al,* 1993 & Zhou, *et al,* 2009). Over long periods of exposure to pollution, microbes are exposed to selective pressures and will develop heavy metal resistance (Díaz, *et al,* 2006; Hu, *et al,* 2006; Zhou, *et al,* 2009 & Zouboulis, *et al,* 2004). The microbial community structure can be mostly determined by the heavy metals in the soil ecosystem (Khan, *et al,* 2010).

1.1.2 pH

pH is another important factor that influences local ecosystems. Sulphide oxidation has been considered the main reason for the pH decrease and the depletion of Cu, Fe and Zn in the surface tailings (Morrell, *et al,* 1996). The low pH environment in this area enhances metal mobilization and toxicity in both soil and mine wastewaters (Edwards, *et al,* 2000 & Sabti, *et al,* 2000). In the town of Te Aroha, both surface and ground water nearby the Tui Mine have been contaminated by the acid mine drainage (AMD) and heavy metals are leaching

from the tailings dam into the Tui and Tunakohoia streams (Fig. 3). The acidity of the surface soil (0-200mm) has been greatly increased by the oxidation of poly-sulphidic tailings where the pH is now between 2.3 and 4.0 (Morrell, *et al*, 1996).

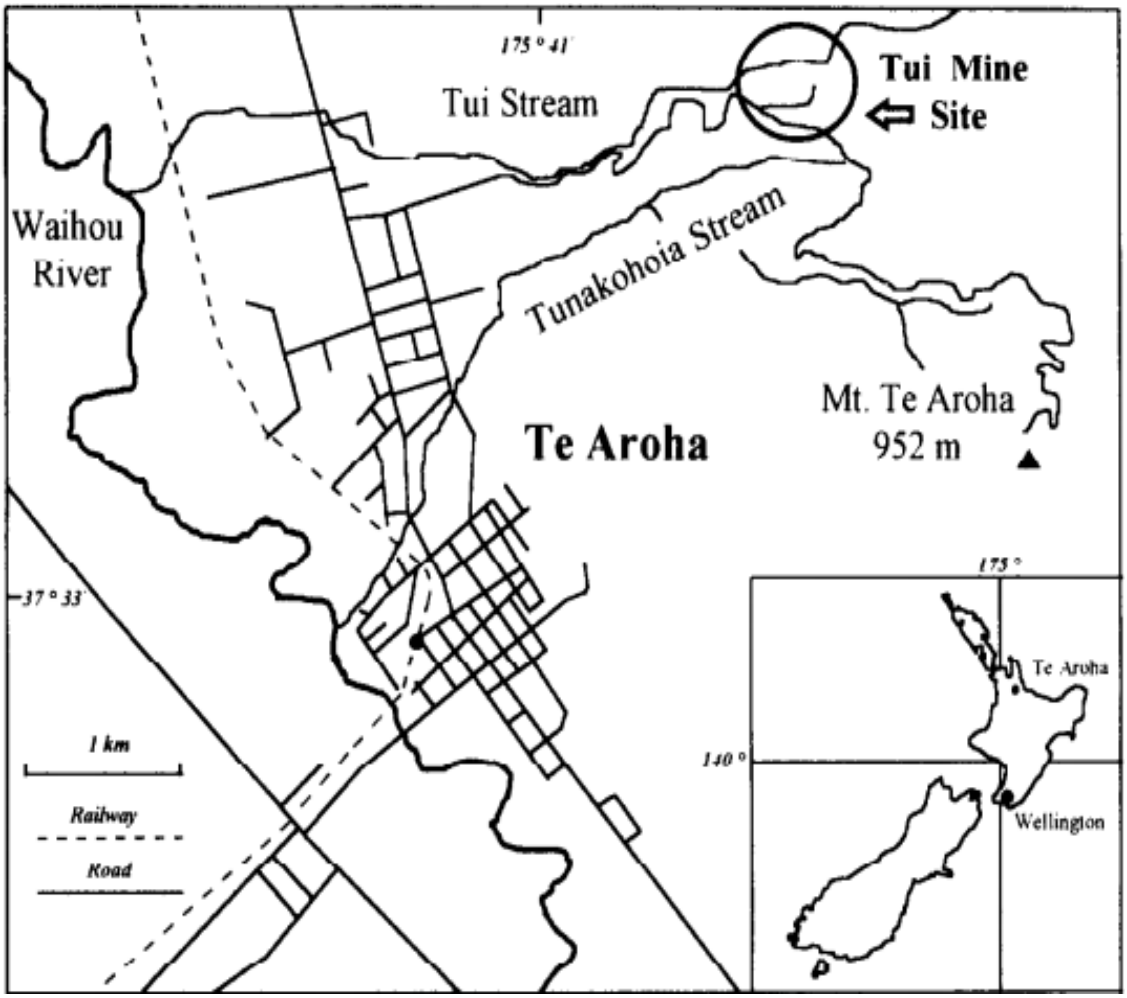


Fig 1 Location of the Tui Mine site, Te Aroha, New Zealand (Morrell, *et al,* 1996)

Fig 2 Main Tui Mine Area (From Google Map)

Fig 3 The Tui and Tunakohoia streams flow into the Waihou River (Waikato regional council, 2011)

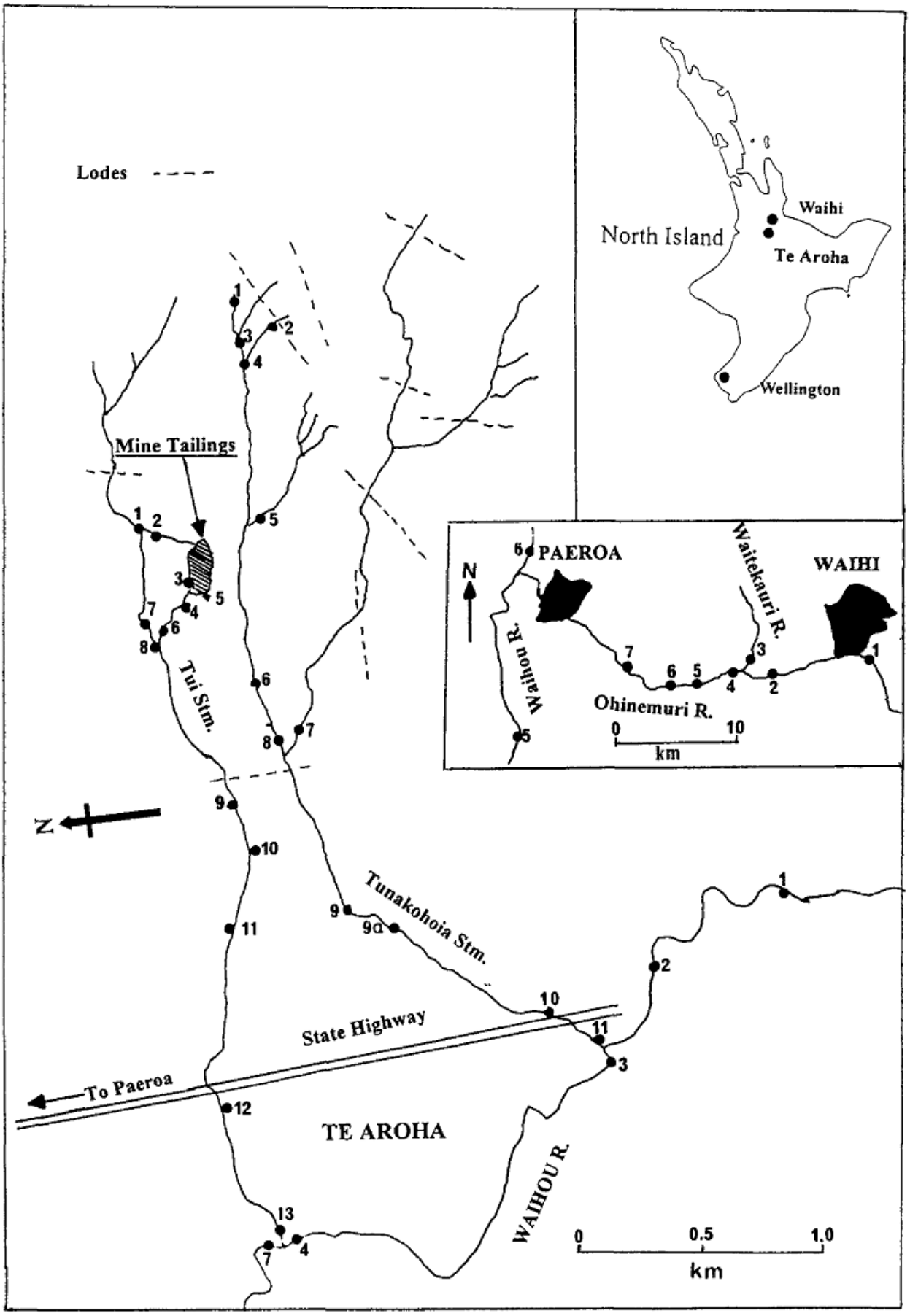


Fig 4 Map of the Te Aroha and Waihi regions showing streams and rivers

1.2 Microorganisms associated with the mining environment

Soil quality can be indicated by the soil biological characteristics as they are very sensitive to the soil physico-chemical changes. (Brookes, 1995; Hinojosa, *et al.*, 2004 & Khan, *et al*, 2010). Previous articles established that species with least detoxification abilities will disappear from the harsh ecosystem and only those microbes having the efficient responses to deal with the stresses can survive (Stolz, *et al,* 1999 & Zhou, *et al,* 2009). The normal functioning of a soil microbial community around heavy metals are greatly weakened but over time, microbes will develop heavy metal resistance.

A diverse range of microorganisms have been isolated from many mining areas around the world (Madigan, *et al*, 2009). Due to the harsh physico-chemical factors (low pH, poor substrates and heavy metals) in the mining tailings, microbial diversity in the environment was expected to be very low (Almeida, *et al*, 2009; Irene, 2011 &Krzaklewski, 2002).

Organisms which grow optimally at acidic environment, typically below pH 4.0, are identified as acidophiles (Madigan, *et al*, 2009). Because of the low pH in mining areas, acidophiles are expected to be the key organisms that can grow in the tailings. A wide range of acidophiles belong to various phyla of the prokaryotes and eukaryotes. Compared with fungi, bacteria are defined as a group to be less acid tolerant (Madigan, *et al,* 2009). However, most of the work in this MSc project only focused on the bacteria and archaea.

In the past few years, numerous studies have been undertaken to isolate and describe the microbes in various acidic mine tailings and most results showed that bacteria have a high degree of biodiversity in mine tailings. Typical, representatives found in various phyla include *Proteobacteria*, *Firmicutes*, *Acidobacteria*, *Actinobacteria* and *Nitrospira* (Xie, *et al*, 2011, Monica, *et al*, 2008; Hallberg, *et al*, 2001; Huang, *et al*, 2011, Johnson, *et al.*, 2001; Tan, *et al*, 2006 & Zhang, *et al*, 2007) and the predominant organisms in abandoned Zn-Pb tailings sites were *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*,

which were in the *Gamaproteobacteria* class and *Nitrospira* family, respectively. (Kelly, *et al*, 2000 & Madigan, *et al*, 2009).

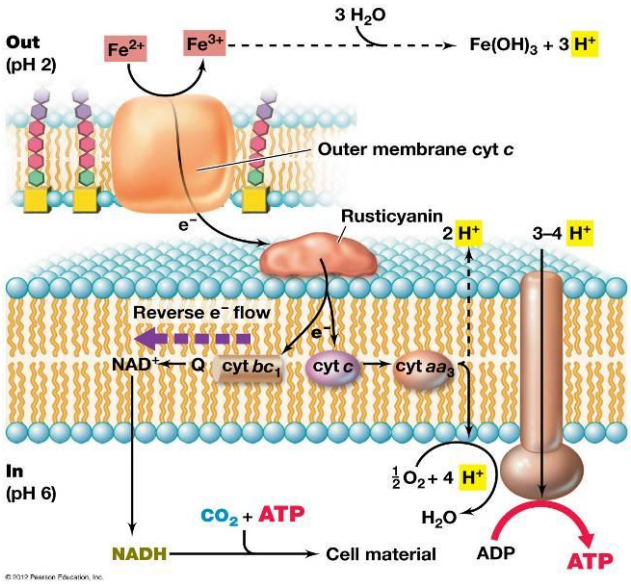
Clone result from Tan, *et al*, (2006) indicates that 104 out of 141 clones were phylogenetically affiliated to the iron-oxidizing *Leptospirillum* group III within the Nitrospira and thirty clones were affiliated with the *Proteobacteria*, half of which belonged to organisms related to *Alphaproteobacteria* species capable of ferric iron reduction. Similar results from Zhou, *et al* (2011), in his TS sample (defined as high concentrations of sulphate, iron and heavy metals with pH at 1.8 and collected from Teishu resercoir, China), which was characterized by low pH, high sulphate and high iron, *Acidithiobacillus ferrooxidans and Leptospirillum ferrooxidans* constituted 98.22% of the entire microbial community.

Acidithiobacillus ferrooxidans and *Leptospirillum ferrooxidans* are two iron oxidizing bacteria most commonly isolated from acidic soils and they are widely regarded as the greatest contributors in the acid generation process of mine tailings (Fortin, *et al*, 1996; Huang, *et al*, 2011; Kelly, *et al*, 2000; Schippers, *et al*, 1995; Southam, *et al*, 1992 & Tan, *et al*, 2006). Previous researches also indicate that *Acidithiobacillus ferrooxidans* is found in significant numbers being involves in the sulphur cycle (using S^0 or metal sulphides as electron donor) and it is one dominant group of CO_2-fixing microorganisms in acid mine tailings (Baker, *et al*, 2003; González-Toril, *et al*, 2003; Madigan, *et al*, 2009; Xie, *et al*, 2011).

In nature, pyrite (FeS_2) is one of the most common forms of iron generated from the reaction of sulfur with ferrous sulphide (FeS). When pyrite is first exposed to air, a slow chemical reaction called initiator reaction happens with developing acidic conditions oxidizing the sulphide to sulphate ($2FeS_2 + 7O_2 + 2H_2O \rightarrow 2Fe^{2+} + 4\ SO_4^{2-} + 4H^+$), and then the generated ferrous iron is oxidized by O_2 ($4Fe^{2+} + 4H^+ + O_2 \rightarrow 4\ Fe^{3+} + 2H_2O$). Fe^{2+} does not be oxidized spontaneously in air at low pH values, but *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* carry out the oxidation and form the precipitate (mainly $Fe(OH)_3$) with the typical yellowish-red colour. With more acidic condition, the Fe^{3+} becomes soluble with the reaction occurring spontaneously to generate ferrous ions and sulphuric acid ($FeS_2 + 14\ Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2\ SO_4^{2-} + 16H^+$). *Acidithiobacillus*

ferrooxidans and *Leptospirillum ferrooxidans* grow autotrophically at optimal pH 2 to 3 and even at pH values below pH 1 by using ferrous iron (iron pyrite (FeS_2) as the major source of Fe^{2+}) as the electron donor and synthesising ATP with consumption of the proton motive force across the cytoplasmic membrane.

The electron flow and key processes during Fe^{2+} oxidation by *Acidithiobacillus ferrooxidans* is shown in Fig. 5. As an autotrophic bacterium, CO_2 fixation by the Calvin cycle consumes much energy in reverse electron flow reactions to obtain the reducing power (NADH). Compared with the large energetic demands for biosynthesis, very little energy is generated in ferrous oxidation, and this means in order to grow, huge amount of Fe^{2+} could be oxidized by only a small number of bacteria.



$$4Fe^{2+} + 4H^{+} + O_2 \rightarrow 4Fe^{3+} + 2\,H_2O$$

$$4Fe^{3+} + 12H_2O \rightarrow 4Fe(OH)_3 + 12H^{+}$$

Fig 5 Electron flow during Fe^{2+} oxidation by the acidophile *Acidithiobacillus ferrooxidans.* The periplasmic copper-containing protein rusticyanin is the immediate acceptor of electrons from Fe^{2+}. From here, electrons travel a short electron transport chain resulting in the reduction of O_2 to H_2O. Reducing power to drive the Calvin cycle comes from reverse electron flow. (Madigan, *et al*, 2009)

Besides those two species, some other bacteria are also found in acid mine tailings environments and as mentioned before, they are mostly represented in various phyla including *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *Nitrospira* and

Proteobacteria. The *Acidobacteria,* specifically related to *Acidobacteria capsulatum* (aerobic and gram-negative chemoorganotroph), are characterized as the moderately acidophilic heterotrophs at an optimal pH 3 to 6 and have been widely isolated from acid mine tailings. In acid environments with high concentration of iron, they use Fe^{3+} as an electron acceptor to oxidize organic matter in microbial metabolism (Baker, *et al,* 2003; Irene, *et al*, 2011 & Madigan, *et al,* 2009). *Actinobacteria*, defined as a second major group of gram-positive bacteria, contains high-GC in their DNA. Some researchers found that ferrous iron oxidation and ferric iron reduction were undertaken by some uncultured microorganisms with 90% similarity to *Acidimicrobium ferrooxidans* (Irene, *et al,* 2011). In contrast, *Actinobacteria* and *Firmicutes* are defined as a group of bacteria with low-GC group. In He *et al,* (2008) results, the organisms identified as members of Firmicutes were mostly affiliated with the genus *Sulfobacillus* which is important in the leaching of ores.

In Tui mine tailings, *Acidocella* was isolated from previous work. This organism belongs to genus *Acidocella,* class *Alphaproteobacteria* and it is an acidophilic heterotroph (Coupland, K & Johnson, D. B., 2007). Researchers isolated many strains of the genus *Acidocella* and found they exhibit extremely high resistance to Zn, Al and Cd with its inheritance on their plasmids and this property could be transferred to other acidophilic bacterium (Coupland, *et al*, 2007; Ghosh, *et al*, 1997; Wakao, *et al*, 2002). In a study by Ghosh (1997), *Acidocella* provided extremely high resistance of $CdSO_4$ and $ZnSO_4$ at 1M/L for each, $NiSO_4$ at 200mM and $CuSO_4$ at 150mM. However, the metal salts extended the lag and log phase of growth and greatly decreased its growth rate and final cell yield. With aluminium ion (Al^{3+}), *Acidocella* could be tolerant to the concentration up to 100mM and their growth was extraordinarily enhanced by aluminium with the reason of this enhanced growth seemed to be a chemical buffering action of the aluminium (Wakao, *et al*, 2002).

As with *Bacteria* occurring in the acid mine tailings, some species of *Archaea* have also been indentified from previous work. *Ferroplasma* (a chemolithotroph) is one of the commonest acidophilic iron oxidizers belonging to the *Archaea*. In mine tailings containing pyrite (FeS), which its energy source, *Ferroplasma* can

even grow at pH values below 0. *Ferroplasma* uses CO_2 as carbon source oxidizes Fe^{2+} to Fe^{3+}, which generates acid ($Fe^{3+} + H_2O \rightarrow Fe(OH)_3 + 3H^+$). Acidophilic archaea, mostly *Ferroplasma acidiphilum*, were predominant in the oxidized zones and the oxidation front, indicating their importance to generation of acid mine drainage. (Almeida, *et al*, 2009; Baler, *et al*, 2003; Huang, *et al*, 2011; Irene , *et al*, 2011 & Madigan, *et al*, 2009)

1.3 Methods of microbial community analysis

Two main strategies were used to investigate the microbial community of the Tui mine soil environment which are culture-dependent and culture-independent methods. A combination of both traditional cultivation approaches and molecular techniques can provide a more intensive description of the microbial communities and the functional roles they play in the AMD ecosystem (González-Toril, *et al*, 2003 & Tan, *et al*, 2006)

1.3.1 Culture based methods

Culture based methods refer to the growth of microorganisms under laboratory conditions with defined media to determine the physiological and functional properties of organisms in the environment. Their salient property can be determined directly by using microscopy, spectrophotometer and also by varying physical parameters like pH, temperature, nutrient content, etc. However, these methods do have some limitations. First and the biggest problems is that not all microbes from the sample can be grown on a single media with specific substrates and nutrients (Sjoling & Cowan, 2003 & Kaeberlein, *et al*., 2002). Moreover, any different media compositions and culture conditions will lead to different selective pressures and finally change the growing community structure. Because of this enrichment bias, the original community cannot be represented by the development of a community structure in growth media (Dunbar, *et al.*, 1997; Santegods, *et al*, 1996 & Wagner, *et al.*, 1993). With standard methods, it is

suggested that more than 99% of microbes in natural environments cannot be cultivated (Amann, *et al*, 1995).

1.3.2 Culture independent methods

Culture-independent techniques are mainly based on the development of the polymerase chain reaction (PCR), dideoxy terminator sequencing and the use of specific genetic biomarkers (Hao, *et al.*, 2008; Mullis, *et al.*, 1987; Sanger, 1988 & Woese, *et al*., 1990). DNA extraction from environment samples, PCR amplification of target gene and sequencing are commonly involved in these molecular-based methods. They have progressed the detection, identification and characterization of microbial communities and provide a quicker and ostensibly less-biased method of community analysis (Hao, *et al.*, 2008).

Although the new techniques avoid the intrinsic bias caused by culture-based experiments, biases introduced by PCR, the first step in almost all molecular-based studies, cannot be ignored. Typically, the small subunit 16S ribosomal RNA gene (Fig. 6) is used as a genetic marker to detect the bacterial diversity from environmental samples, as it is present in all bacteria and has both conserved regions and variable regions. The conserved regions in 16S rRNA can be used to design primers for PCR amplification and the variable regions can be used for phylogenetic analysis to indicate the taxonomic relationship between microbes. (Madigan, *et al*, 2008; Ward, *et al*, 1990)

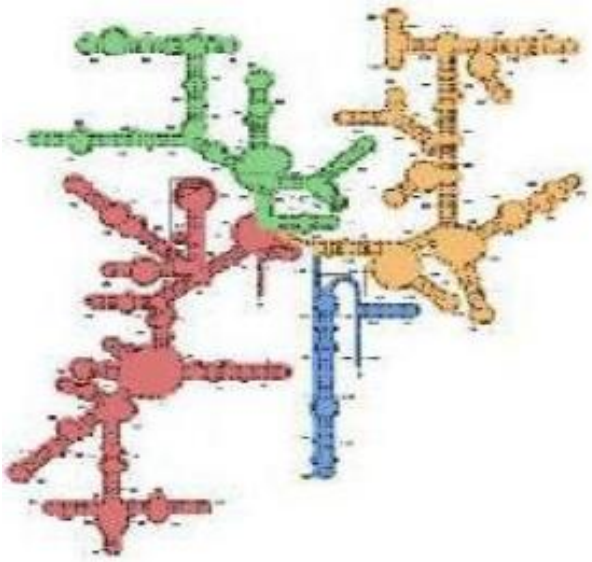


Fig 6. 16S rRNA molecule (Garrett & Grisham, 2005)

Red corresponds to the 5" domain (nucleotides 27-556), green to the central domain (nucleotides 564-912), yellow (923-1391) and blue (1392-1541) correspond to the 3" domain.

1.3.3 Clone library analysis

Based on the PCR amplification of specific genes, normally the 16S rRNA gene for bacterial analysis, clone libraries can be built by inserting the target PCR amplicons into a vector and then transforming into a bacterial host by electroporation or chemical protocol. In this study, a TOPO TA cloning kit (Invitrogen, CA, USA) was used to build the library. PCR reactions with the target samples were run first to get the amplifications and then the amplicons were inserted into the TOPO® vectors and ligated. Vectors with inserts were introduced into the DH5α™-T1^{R} Competent *E.coli* Cells by electro-transformation and finally grew on the LB medium with kanamycin at 50ug/ml as the selective. Only cells with ligated-vector are selected to can grow on this selective medium. Because each bacterial transformant contains a single copy of 16S rRNA, microbes from the original environment can be then identified by each cloned amplicon. Although a large number of microorganisms can be easily identified by this method, it will take a long time and high cost with cloning and sequencing. To reduce the sequencing costs, plasmid DNA amplicons can be digested by restriction endonucleases first to identify common patterns of restriction and thus decrease the duplication.

1.3.4 454 Pyrosequencing

454 sequencing, which is defined as a next-generation technology, can process hundreds of thousands of sequences simultaneously on single DNA molecules attached to beads in separated wells. By using bar-coded primers for each sample, even DNA from different environments can be run together without interference. The key breakthroughs of this technology are "high throughput, simplified all in *vitro* sample preparation and the miniaturization of sequencing chemistries, enabling massively parallel sequencing reactions to be carried out (Rothberg, J. M. & Leamon, J. H., 2008)". This method is based on DNA polymerase base incorporation (generates inorganic pyrophosphate (PPi)), ATP sulfurylase (converts PPi to ATP), luciferase (converts luciferin to oxyluciferin to generate light by using the ATP) and light detection by the charge-coupled device (CCD) (Nyrén, 1987; Petrosino, *et al*, 2009; Ronaghi, 1996; Rothberg, *et al*, 2008 & Williams, 2011). In the 454 system, each single DNA molecule is attached to individual beads. The microscopic beads are put into a fiber-optic plate containing more than one million wells, and each well holds only one bead. Time and costs can be greatly saved because all sequencing reactions can be carried out individually at one run by each well. (Amend, *et al*, 2011; Hirsch, *et al*, 2010; Kwon, 2010; Madigan, *et al*, 2009; Roh, *et al*, 2010 & Zhang, 2011)

1.4 Aims and outline of Thesis

Although quite a few researches have focused on the surface and ground water quality which is impacted by the Tui mining operation, no study has been taken to analysis the microorganisms in Tui mine soil (Tay, 1980, Livingston, 1987 & Pang, 1995). This study focused on the Tui mine soil which supposedly is of low biomass and less diverse than normal due to the low pH and high heavy metal content of the soil.

The aim of this study is to identify bacteria by aligning them to closely related species and build phylogenetic trees and consequently describe the bacterial diversity in Tui Mine soil which is defined as an extreme environment.

There are three main strategies used in my study. The first is based on the culture-dependent method which uses different media to cultivate and separate microbes, and then get DNA from all pure colonies and sequence them. The second and third strategies are based on the molecular methods, ie- clone library and 454 pyrosequencing are used to analysis the microbial diversity directly from soil DNA. The major structure of my project is shown in Fig. 7.

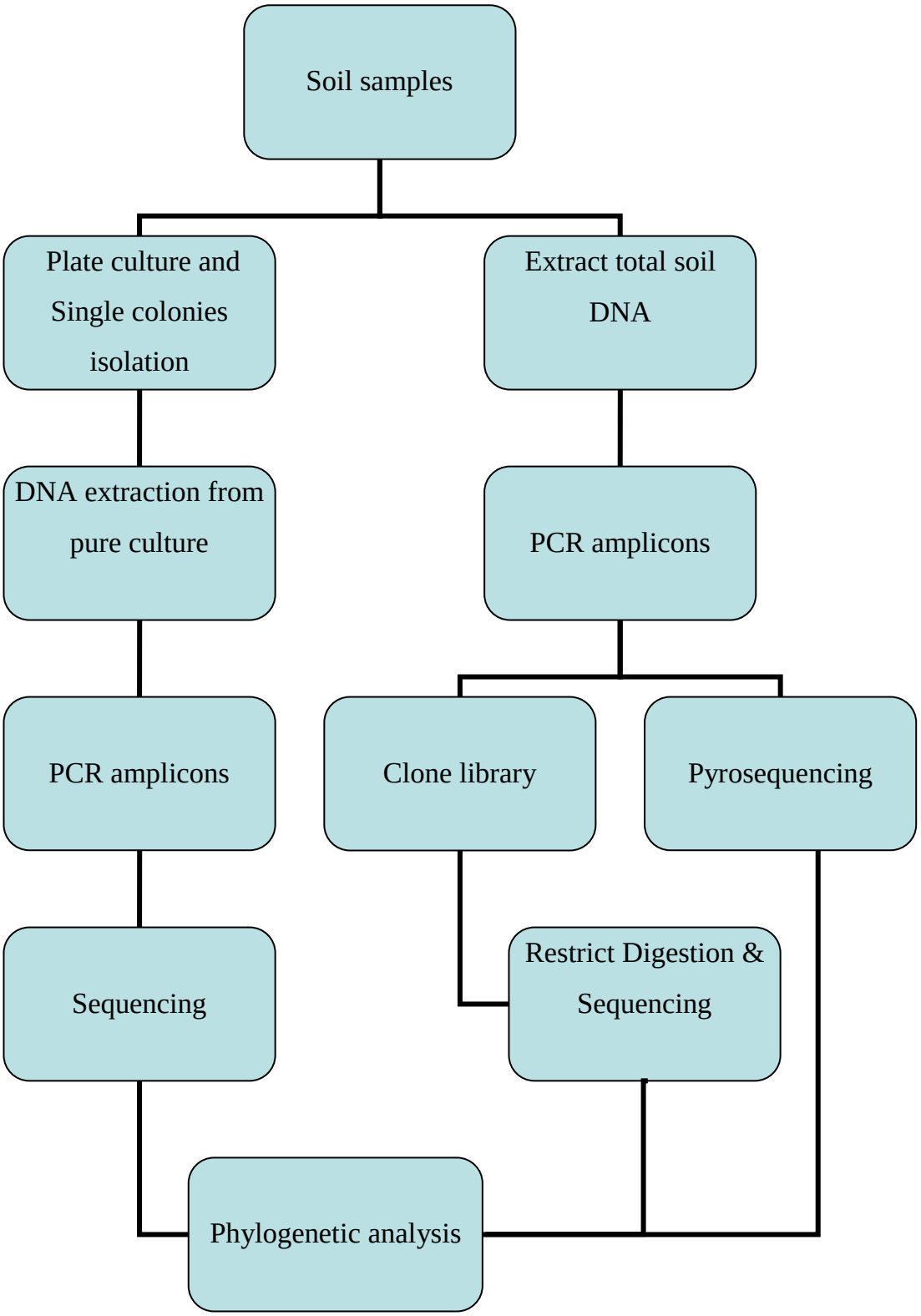


Fig 7 Flow diagram of the main methods in this study

2. Material and Methods

2.1 Analysis of Tui Soil Environment

Samples were collected from the Tui mine main tailing site in Te Aroha, Waikato, New Zealand (NZ) by Prof. Hugh Morgan and Lin Tan in July 2010. Permission to access and sample the mine tailings was obtained from Environmental Waikato. Soil samples are collected from three different points (Fig. 8, labelled as A, B and C) in the main mine tailing area and then well mixed. A, B and C points locate at the same site, which should have the same compositions. Sampling with more than one site can greatly decrease the biases introduced from just single site and better represent the whole tailing environment.

Fig 8 Sites of samples collection from main tailing area in Tui mine, Te Aroha

With each sites, three different layers of soil samples were extracted with different depths by spade and auger. Approximate 1kg of each sample was collected into plastic bags and labelled as top-layer (0-200mm) soil sample, middle-layer (200-400mm) soil sample and bottom-layer (400-600mm) soil sample. These layers are clearly distinguished by their different colours (Fig 9). Top layer soil sample, which is yellow-coloured, is assumed as an aerobic layer as it from the surface of the tailing; with white in colour, the Middle layer soil sample is from 200mm-400mm deep and is supposed to be the mixed aerobic and anaerobic condition; Bottom layer soil sample is the deepest sample and dark gray in colour and is an anaerobic layer. About 100g of mixed soil samples for each slayer were filled into two 50ml-Falcon tubes and stored at -20℃ immediately on return to the laboratory and were used for DNA extraction. The remaining soil samples were stored at 4℃ for later use.

Fig 9 Three soil samples from same site with different depth.

Besides soil samples, 1L of each of three liquid samples were also collected from the small pool, the brown stream and green stream near the main tailings site (Fig 10) for later study. When liquid was taken back to the laboratory, 50ml of each sample was centrifuged at 10,000×*g* for 20min. After discarding the upper liquid, sediment was re-suspended with another 50ml liquid sample and the centrifuge step repeated. Sediment was finally well mixed with 10ml liquid sample and transferred to a 15ml-Falcon tubes followed by centrifugation at 10,000×*g* for 20min. Supernatant was tipped off and sediments were ready to use for extracting DNA. For long-term storage, all tubes were stored at -20℃.

Fig 10 Three liquid samples from Tui Mine, Te Aroha.

2.1.1 pH

The *in situ* pH of the liquid samples was measured using pH strips (Merck KgaA, Darmstadt, Germany) and after return to the laboratory, the pH determined using a pH meter (Jenway 4330, Essex, England). For this, 5 grams of soil sample was

mixed with 45ml of Milli-Q water and after shaking for 20 minutes, the pH was recorded.

2.2 Cultivation of Microorganisms

For all cultivation work standard aseptic procedures were employed. All sample handling was performed in a sterile flow cabinet and all media and equipment sterilised by autoclaving prior to use.

2.2.1 Pre-incubation Preparation

2.2.1.1 Sample preparation

Sample suspensions were made from 1 gram of each Top layer, Middle layer and Bottom layer soil sample by vortexing in 9ml sterile mineral salt solution (0.1g KCl, 2.0g $(NH_4)_2SO_4$, 0.25g K_2HPO_4, 0.25g $MgSO_4 \cdot 7H_2O$ in 1 liter Milli-Q water at pH 4.0). Liquid samples were directly used by vortexing and then transferred into sterile tubes. Serial tenfold dilutions were performed from 10-1 to 10-5 for each of the six sample, ie three soil suspensions and three liquid samples.

2.2.1.2 Media preparation

With culture-based method, only heterotrophic microbes will be isolated on the media used, while both heterotrophs and autotrophs will be detected by the molecular-based methods ie 16S clone library analysis and 454 pyrosequencing analysis. All media used for environmental microbial cultivation in this project are listed in Appendix A. They are all based on the Acidobacterium Medium (0.1g KCl, 2.0g $(NH_4)_2SO_4$, 0.25g K_2HPO_4, 0.25g $MgSO_4 \cdot 7H_2O$, 1.0g Glucose, 0.1g

Yeast extract, and Milli-Q water to final 1 Liter at pH 3.0). For solid medium, agar or gelrite were used to solidify the plates. Since agar does not form a gel at low pH (normally lower than 5.0), gelrite was used with those medium at pH lower than 4.5. Before using for microbial incubation, media were sterilised by autoclaving (121℃, 1bar pressure) for 20 min. After autoclaving, about 20ml of agar or gelrite medium was poured into a sterile petri-dish at temperature above 70℃. With gelrite medium, 0.10% (w/v) $MgSO_4 \cdot 7H_2O$ was added and mixed into every 1L medium before pouring to help solidifying. For those gelrite medium already containing 250mM $MgSO_4$, no Mg^{2+} was needed and medium was poured at a higher temperature at about 90℃ as it is quite easy to solidify with high concentration of magnesium.

2.2.2 Cultures incubation and observation

0.1ml of each sample (three from soil and three from each liquid) was diluted by serial tenfold dilutions (10^{-1} to 10^{-5}) and inoculated onto the agar or gelrite plates of all 9 different solid media and spread plated (named as 4-0-0(3.5), 4-0-0(4.5), 4-0-0 (5.0), 4-Mg-0 (3.5), 4-Mg-0 (4.5), 4-Mg-0 (5.0), 4-Mg-Zn (3.5), 4-Mg-Zn (4.5) and 4-Mg-Zn (5.0), Table 3, also see appendix A for details) in duplicate. Plate enumeration was as follows: First no. indicates gms glucose per litre, second no. indicates concentration of Magnesium in the medium with 0 being Mg^{2+} omitted and Mg being 250mM, third no. indicates Zn concentration with Zn indicating 250mM Zn^{2+}. Plates were placed upside down and incubated under both aerobic and anaerobic (using AnareoGen™ sachets (Oxoid, UK) and an anaerobic incubator jar) conditions at 28℃ for 1-2days. For medium containing high concentrations of heavy metal, incubation time was extended to 3-10 days.

Table 3 Medium Composition

Medium	Compositions
4-0-0 (3.5)	0.1g KCl, 2.0g $(NH_4)_2SO_4$, 0.25g K_2HPO_4, 0.25g $MgSO_4 \cdot 7H_2O$, 4.0g Glucose, 0.4g Yeast extract, and Milli-Q water to final 1 Liter at pH 3.5
4-0-0 (4.5)	Based on the 4-0-0 (3.5) medium with increasing pH to 4.5
4-0-0 (5.0)	Based on the 4-0-0 (3.5) medium with increasing pH to 5.0
4-Mg-0 (3.5)	Based on the 4-0-0 (3.5) medium with adding 250mM/L $MgSO_4 \cdot 7H_2O$
4-Mg-0 (4.5)	Based on the 4-0-0 (3.5) medium with adding 250mM/L $MgSO_4 \cdot 7H_2O$ and increasing pH to 4.5
4-Mg-0 (5.0)	Based on the 4-0-0 (3.5) medium with adding 250mM/L $MgSO_4 \cdot 7H_2O$ and increasing pH to 5.0
4-Mg-Zn (3.5)	Based on the 4-0-0 (3.5) medium with adding 250mM/L of each $MgSO_4 \cdot 7H_2O$ and $ZnSO_4$, and increasing pH to 5.0
4-Mg-Zn (4.5)	Based on the 4-0-0 (3.5) medium with adding 250mM/L of each $MgSO_4 \cdot 7H_2O$ and $ZnSO_4$, and increasing pH to 4.5
4-Mg-Zn (5.0)	Based on the 4-0-0 (3.5) medium with adding 250mM/L of each $MgSO_4 \cdot 7H_2O$ and $ZnSO_4$, and increasing pH to 5.0

In a growing culture, turbidity can be used as a measure of cell numbers and the microbial mass (turbidity) is measured with a spectrophotometer (at 450nm) which gives readings in optical density (O.D) units. Typical growth curves can be drawn with different O.D readings via time of growing. In my study, O.D values

of cultures were record from the beginning of inoculation and checked every eight hours until to the stationary phase of growth was attained.

Concentration of glucose remainning after growth was also measured by the spectrophotometer with DNSA regent (13.6g sigma-Dinitrosalicylic acid, 2.7g Phenol, 0.68g sodium sulphite, 13.6g of sodium hydroxide and 273g potassium sodium tartarate in 1 liter MQ-water). With every 100 µ l of glucose solution, 1ml of DNSA solution were added and incubated in boiling water for 6 min. Tubes were transferred to cold water bath for cooling and then centrifuged at 13,200rpm for 4min. The optical density of supernatant was measured by a spectrophotometer at 575nm and glucose concentration determined from a standard curve constructed with glucose concentrations over the range from 0.0 to 1.0 mg mL^{-1} (Frost, L. D., 2004).

Visible colonies of bacteria and fungi enumerated and the cell morphology confirmed by using a 100× phase-contrast objective lens (Olympus, BH-2, Japan) and photos were taken by a Nikon CoolPix 4500 digital camera (Nikon, Tokyo, Japan). Pure cultures were obtained after repeated streak plating onto fresh plates.

2.2.3 Microorganisms storage

Isolated pure cultures were stored at 4℃ for short term storage and transfers to fresh agar plates made every three weeks. For long term storage, Liquid cultures (about 40ml) were centrifuged at 800 to 1500 *g* for 15 minutes to harvest cells. Pellets were washed with 1× fresh sterile medium, and then spun down again. Cells were gently re-suspended in 2ml fresh medium and 1ml sterile glycerol (at about 30% of total volume). The cell suspension was transferred to six separate sterile eppendorf tubes with individual 0.5ml amount and stored at -80℃.

2.3 Community analysis by molecular methodology

2.3.1 DNA extraction

All DNA extraction methods used in this project are described below. For pure cultures grown on solid media, a single colony was transferred into fresh liquid medum of the same composition as the solid medum only without agar or gelrite. Cell suspensions were collected during the stationary phase of growth with almost the highest optical density and then concentrated by centrifugation at 12,000 *g* for 15 minutes. The cell pellet was re-suspended in cetyltrimethylammonium bromide (CTAB) buffer for DNA extraction as described in *section 2.3.1.1*.

A CTAB method and two DNA extraction kits were used to extract the DNA directly from the soil samples. All detailed information were shown in *Section 2.3.1.2, Section 2.3.1.3 and Section 2.3.1.4*

2.3.1.1 CTAB-based DNA extraction from pure cultures

DNA from pure colonies growing in the liquid media was extracted by a modified CTAB extraction method (Demoster, *et al*, 1999). Cell pellets were re-suspended in 200µl CTAB buffer (100mM Tris-HCl, 1.4M NaCl, l20mM EDTA (ethylenediaminetetraacetic acid), 2% (w/v) CTAB, 1 % (w/v) PVP (polyvinylpyrrolidone), pH 8.0 and 0.4% (w/v) BME (β -mercaptoethanol) added just before use) and incubated shaken at 100rpm & 60℃ for 30 minutes. After cooling, 1000µl chloroform/isoamyl alcohol (24/1, v/v) was added and mixed by repeated inversion. A further 15 seconds vortex was also needed before centrifugation (13200 rpm for 5 minutes). The upper aqueous layer was transferred into a new 1.5 ml sterile Eppendorf tube by adding 500µl of chloroform and isoamyl alcohol mixture (24 ml chloroform mixed with 1ml isoamyl alcohol). After vortexing the tube for 10 seconds, the sample was incubated on a rocking bed for 20 minutes at room temperature and then

centrifuged at 13200 rpm for 5 minutes. The upper aqueous layer was removed to a new 1.5 ml sterile Eppendorf tube and 7 M ammonium acetate was added to a final concentration of 2.5M (approximate 360µl in 650µl of sample). Liquid was mixed gently by repeated. If a precipitate developed then the tube needs to be centrifuged again at 13200rpm for 5 minutes and upper liquid layer was transferred to a new sterile Eppendorf. 0.54 volumes of isopropyl alcohol was added and mixed gently by repeated inversion, then the tube was incubated at -70℃ for at least 1 hour (overnight is best). After refridgeration, the tube was centrifuged at 13200 rpm for 20 minutes. The pellet (may not be visible) was kept in the tube after discarding the supernatant. 1ml 70% ethanol was used to wash the pellet and then the pellet was dried in a laminar flow hood for about 1 hour. Finally, the pellet was re-suspended in 20µl sterile LO-TE (3mM Tris-HCl, pH8.0; 0.2mM EDTA) and stored at -70℃ until use.

2.3.1.2 Bead beating and CTAB-based DNA extraction from soil sample

0.6-0.7 grams of environmental soil sample was weighted out into a 1.5ml screw-capped conical bottomed polypropylene tube containing 0.5g each of 0.1mm and 2.5mm silica-zirconia beads (Biospec Products Inc., OK, USA). A 300µl aliquot of phosphate buffer (100mM NaH_2PO_4, pH 7.4) followed by 300µl SDS lysis buffer (100mM NaCl, 500mM Tris, 10% (w/v) sodium dodecyl sulphate (SDS), pH 8.0) were added to it. The tube was shaken in Fast Prep® FP 120 Cell Disrupter (Bio101 Instruments, Irvine, CA, USA) at 4.0 ms^{-1} for 30 seconds then centrifuged at 13200 rpm for 3 minutes. The liquid was removed and transferred to a 1.5ml sterile Eppendorf tube. 200µl CTAB buffer (BME added just before use) was added into the dispensed liquid and incubated at 100rpm & 60℃ for 30 minutes and CTAB extraction of DNA performed as outlined in *Section 2. 3. 1. 1.*

2.3.1.3 DNA extraction by The NucleoSpin® Soil Kit

To purify DNA from soil, The NucleoSpin® Genomic DNA soil kit (MACHEREY-NAGEL GmbH & Co. KG) was used. The procedure (Fig. 11) was as outlined in the kit manual.

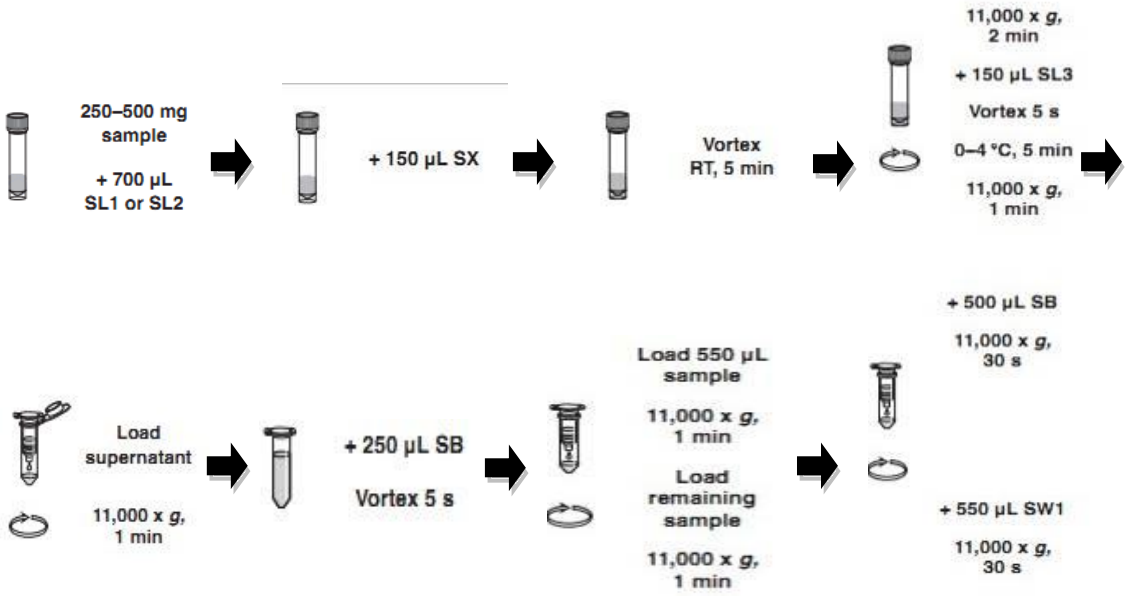


Fig 11 Outline of DNA extraction by The NucleoSpin® Soil Kit

250-500 mg soil sample material was transferred to a NucleoSpin® Bead Tube containing the ceramic beads and the total volume in this step should not be higher than the 1ml marker. 700 µl Buffer SL1 (lysis buffer) and 150 µl Enhancer SX were added to the tube. Sample was mixed by a vortex mixer at full speed and room temperature for 5 min. The tube was then centrifuged for 2 min at 11,000×*g* to eliminate the foam caused by the detergent. 150 µl Buffer SL3 was added to the tube followed by a short-time vortex (5 sec). Mixture was incubated at 0-4℃ for 5 min and then spun at 11,000×*g* for 1min. Up to 700 µl clear supernatant was loaded onto a NucleoSpin® Inhibitor Removal Column (red ring) which was placed in a 2ml Collection Tube (with lid) and centrifuged for 1min at 11,000×*g*. The NucleoSpin® Inhibitor Removal Column was discarded and 250 µl Buffer SB was added to the 2ml Collection Tube followed by a 5-sec vortex. 550 µl sample was loaded onto the NucleoSpin® Soil Column (green ring) which

was placed in a 2 ml Collection Tube (No lid). Column was centrifuge at 11,000 ×*g* for 1min. After discarding the flow-through, column was placed back into the collection tube and the remaining sample was loaded onto the column to repeat the last step. DNA was bound with the column at this moment and a 4-step washing was needed to clean the DNA binding membrane. First wash, 500 µl Buffer SB was added to the NucleoSpin® Soil Column with 30sec centrifugation at 11,000×*g*. Flow-through was discarded and column was placed back into the collection tube; second wash, 550 µl Buffer SW1 was added to the column with the same procedure as the first wash step; third wash, 700 µl Buffer SW2 was added to the NucleoSpin® Soil Column and the lid closed for a 2-sec vortex then centrifuged as previously. After the washing step, column was centrifuged again at 11,000×g for 2min to dry the silica membrane. Finally, the NucleoSpin® Soil Column was placed into a new micro-centrifuge tube and 30 µl Buffer SE (elution buffer) added. After 1 min incubation at room temperature, the sample was centrifuged at 11,000×g for 30 sec. The DNA solution was recovered in the micro-centrifuge tube its concentration of DNA checked by Nano-Drop stored at -20℃.

2.3.1.4 DNA extraction by the MOBIO kit

To extract soil DNA, PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc. US) was used. 0.25 grams of soil sample was dispensed to the PowerBead Tube and gently vortexed to mix. 60 µl of Solution C1 was added to the tube and inverted several times. The PowerBead Tube was horizontally put onto the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1) at maximum speed for 10 min, then centrifuged at 10,000 × *g* for 30 sec at room temperature and the supernatant transferred to a clean 2 ml Collection Tube. 250 µl of Solution C2 was added and vortexed for 5 seconds followed by a 5 min incubation at 4℃. Tube was then re-centrifuged at room temperature for 1 minute at 10,000 × *g*. Avoiding the pellet, up to 600 µl supernatant was transferred to a clean 2ml Collection Tube followed by 200 µl of Solution C3 and brief votex to

mix. The sample was incubated at 4℃ for 5 minutes and then centrifuged at room temperature for 1 minute at 10,000 × *g*. Supernatant (up to 750 µ l) was dispensed into a clean 2 ml Collection Tube and 1200 µ l of Solution C4 (shaking to mix before use) was added followed by a 5-sec vortex. Approximately 675 µ l of this mixture was loaded onto a Spin Filter and centrifuged at 10,000 × *g* for 1 minute at room temperature. Flow-through was discarded and another 675 µ l sample was loaded to repeat the centrifugation step. The remaining supernatant was finally loaded on the Spin Filter for centrifuging and the flow-through discarded. 500 µ l of Solution C5 was added to the Spin Filter with a 30-sec centrifugation at 10,000 × *g*. After discarding the flow-through, the column was centrifuged again at room temperature for 1 minute at 10,000 × *g*. The Spin Filter was carefully placed in a clean 2ml Collection Tube avoiding splashing any Solution C5 onto the column. 100 µ l of Solution C6 was added to the centre of the white filter membrane and centrifuged at room temperature for 30 seconds at 10,000 × *g*. Discarding the Spin Fliter, the soil DNA was in the tube and ready for use. For long-term storage, DNA was frozen at -20℃ to -80℃.

2.3.2 DNA Quantification

The extracted DNA was quantified using a Nanodrop ND-1000 at 260nm (NanoDrop Technologies, Montchanin, DE, USA). The purity of nucleic acids was estimated by the ratio of readings taken at 260nm and 280nm wavelengths as proteins absorb UV light at 280nm. A 260/280 nm ratio was considered to represent DNA and RNA proportion. Normally, 260/280 value between 1.8 and 2.0 were regarded as of sufficiently good quality to use the DNA for PCR.

2.3.3 Polymerase Chain Reaction (PCR)

All PCR reactions were run on the Bio-Rad DNA Engine® (PTC-200) Peltier Thermal Cycler (Bio-Rad Laboratories Inc, Hercules, CA) or Eppendorf

Mastercycler gradient thermocycler (Eppendorf AG, Hamburg, Germany). Every 25 μl PCR reaction contained 0.5 μM of each primer 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') and 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') (Ikner, *et al*, 2007), 1×PCR buffer (Invitrogen Ltd), 0.2 mM dNTPs (Roche Diagnostics), 1 U of Platinum *Taq*, 1.5mM $MgCl_2$, 0.032 mg/ml BSA, 20 ng of template DNA and the entire reaction was made up to 25 μl with Milli-Q (MQ) H_2O (Table 4). One pair of primers, as so-called universal primer, cannot cover all the 16S rRNSA amplification. Another reverse primer 1522R (5'-AGA GTT TGA TCC TGG CTG AG-3') coupled with 27F were also tried for complementation.

Table 4 Near-Full length bacterial 16S rDNA PCR

PCR component	**Volume (μl)**
Water	To 25
$MgCl_2$ (50mM)	0.75
10×PCR buffer	2.5
dNTPs (2mM)	2.5
Forward Primer (27F)(10 μM)	0.75
Reverse Primer (1492R)(10 μM)	0.75
Taq polymerase(5U/μl)	0.2
BSA	2.5
Template DNA	～20ng

Thermal cycling conditions were: an initial denaturation at 94℃ for 2 minutes, then 35 cycles of 94℃ for 30 sec, primer annealing at 55℃ for 30 sec and primer extension at 72℃ for 2 min, followed by a final extension at 72℃ for 5 min. PCR products were checked by running on a 1% agarose gel to ensure amplification success.

2.3.4 Agarose gel Electrophoresis

The electrophoresis of DNA fragments was used to separate the PCR products and restriction endonuclease DNA digests. 1-2% (w/v) of agarose was added to the 1 ×TAE buffer (1L of 50× TAE buffer contained: 242g Tris, 57.1 ml glacial acetic acid, 100ml 0.5M EDTA, pH8.0, also see appendix B) and heated until all agarose was dissolved. 10 μl of SYBR® Safe DNA Gel Stain was added for every 100ml agarose solution when it cooled to approximately 55℃. Agarose solution was well mixed and then poured onto the gel electrophoresis platform until set. Before running the gel, adequate buffer was added to ensure buffer recirculation between the anode and cathode reservoirs. Each 4 μl of DNA samples was mixed with 1 μl 10 ×gel-loading dye (BlueJuice™ Gel Loading Buffer, Invitrogen Ltd) before loading the samples into the agarose well. A 1Kb *Plus* DNA Ladder (Invitrogen Ltd) was always used as a standard to display the approximate fragment sizes. The map of this ladder and how to make it are shown in appendix C. 1% Agarose electrophoresis runs were generally undertaken at 10V/cm for 40min. For restriction endonuclease DNA digests, 2% agarose gels were routinely used running at 40V for 120min. The DNA in the gels added SYBR® Safe DNA Gel Stain was visualised and photographed under UV (260nm) light with an AlphaImager System (Alpha Innotech, CA, USA). For gels without SYBR® Safe DNA Gel Stain, they were stained with ethidium bromide (0.5 mg/L) for 10 to 40 min and destained in RO water for up to 30min before viewing.

2.3.5 DNA Purification

All PCR products need to be cleaned up before proceeding to the next step. For those samples with only one clear band in the gel, PCR products were purified by either by Isopropanol Clean-up method or QuickClean II PCR Purification Kit (GenScript, NY, USA). If more than one band occurred in the gel, the whole PCR product needs to be run in the gel and the desired size DNA amplicons were cut from the gel. QuickClean II Gel Extraction Kit (GenScript, NY, USA) was used to purify the DNA

form the cut and extracted gel fragment. Procedures of the three methods are detailed in appendices D, E & F.

2.3.6 Restriction endonuclease digestion

Table 5 Restriction endonucleases used in this study

Enzyme	Buffer	Cleavage Site	Incubation Temp. (℃)	Supplier
Xba I	Buffer M	5′-T ↓ CTAG A-3′ 3′-A GATC ↑ T-5′	37	Invitrogen, CA, USA
Afa I	Buffer T	5′-GT ↓ AC-3′ 3′-CA ↑ TG-5′	37	Invitrogen, CA, USA
Hae III	Buffer M	5′-GG ↓ CC-3 3′-CC ↑ GG-5′	37	Invitrogen, CA, USA

Three different restriction enzymes (Table 5) were used in this study. Because there were more than hundred samples obtained from clone library, a double enzyme digestion (Table 6) was used to increase the accuracy. With PCR product from pure cultures, single enzyme digestion was used to perform analysis in parallel and the master mix of the digestion components is shown in Table 7. All chemicals were mixed prepared prior to adding PCR products. Mixtures were incubated at 37℃ for 3 hours and the enzymes were inactivated by heating at 65℃ for 20 min. All reactions were run on the Eppendorf Mastercycler gradient thermocycler

Table 6 Double-enzyme Restriction Reaction Mix

Component	Volume (μl)
Xba I	0.5
Hae III	0.5
10×Buffer M	2
0.1 % BSA	2
Substrate DNA	≤1 μg
Sterile water	To 20

(Eppendorf AG, Hamburg, Germany) and the digestion results were checked with gel electrophoresis by using 2% agarose gel with TAE buffer.

Table 7 Single enzyme Restriction Reaction Mix

	Enzyme	Buffer	BSA	Substrate	Water
***Xba* I**					
	Xba I	10×Buffer M	0.1 % BSA	Substrate DNA	Sterile water
	1μl	2μl	2μl	≤1μg	To 20μl
***Afa* I**					
	Afa I	10×Buffer T	0.1 % BSA	Substrate DNA	Sterile water
	1μl	2μl	2μl	≤1μg	To 20μl
***Hae* III**					
	Hae III	10×Buffer M		Substrate DNA	Sterile water
	1μl	2μl		≤1μg	To 20μl

2.3.7 Cloning

Clone libraries were generated from the ～1.5Kb 16S rRNA gene products, using a TOPO® TA cloning kit (Invitrogen, CA, USA) according to the manufacturer's directions. The outline of the experimental steps is shown in Figure 12. After running PCR reactions first, the 16S rDNA amplicons were inserted into the TOPO® vectors (Appendix G) and introduced into the DH5α™-T1^{R} Competent *E.coli* cells by electro-transformation. A 50μl of PCR mix was prepared to obtain sufficient quantity of the 16S rDNA amplicon. Reactions were undertaken by the normal PCR cycling ending with a 7-30min extension at 72℃ to ensure all PCR products were full length and 3'-adenylated. PCR products were purified by QuickClean II PCR Purification Kit (GenScript, NY, USA) and checked by agarose gel electrophoresis before use.

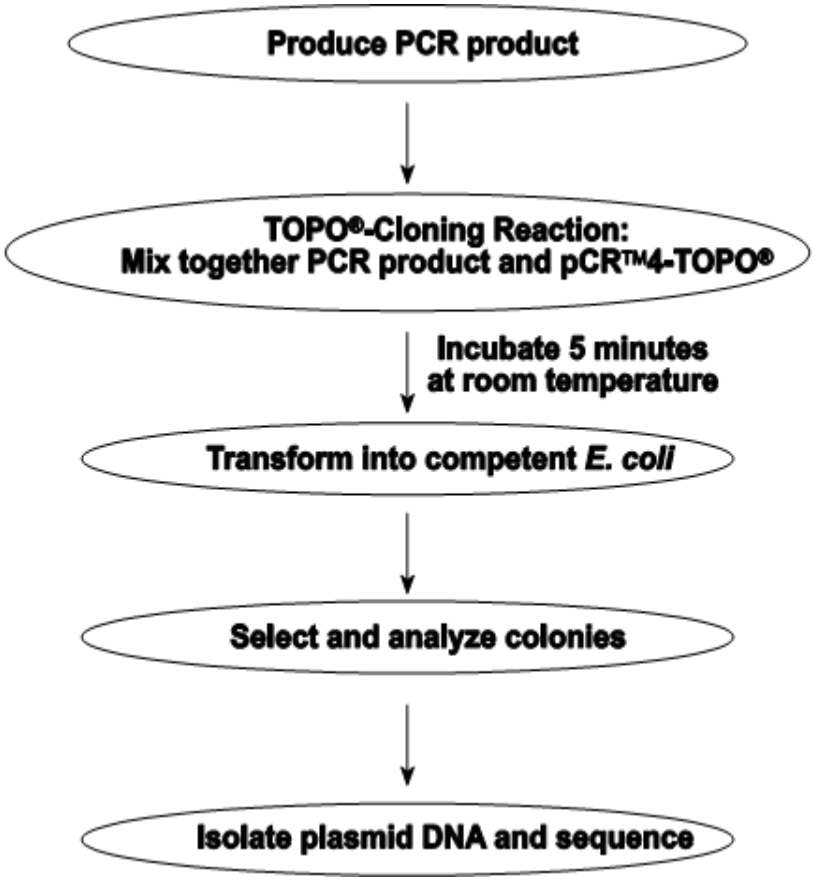


Fig 12 Clone Library Experimental Outline

2.3.7.1 Electro-competent cells preparation

DH5α^{TM}-T1^{R} Competent *E. coli* contains a mutation in the *ton*A locus and resists the lytic bacteriophages T1 and T5 (DH5α^{TM}-T1^{R} Competent Cells Manual (Invitrogen, CA, USA) & Killmann *et al*., 1996). It is highly sensitive with the changes in temperature or mechanical lysis. Cells was gently dissolved on ice just before transformation

To harvest the competent cells, a single colony was selected from a fresh LB (1% Bacto tryptone, 0.5% Bacto yeast extract and 1.0% NaCl) plate and transferred into 50-ml of liquid LB medium and grown overnight at 37℃ with 200rpm shaking. 1 liter of L-broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5%

NaCl) with 1/100 volume of the fresh overnight culture was incubated at 37℃ with vigorous shaking until an OD_{600} of 0.5 to 1.0 was reached, and the cell suspension was transferred into chilled centrifuge tubes and centrifuged in a cold rotor at 4000 × g for 15min. After discarding the supernatant, cells were re-suspended in 1 liter of cold sterile water and re-centrifuged as previously. 0.5 liter cold water and then 20ml of 10% cold sterile glycerol were used to repeat washing the cells and finally, the pellet was re-suspended in 2-3ml of 10% sterile glycerol 50 μl volumes dispensed into sterile Eppendorf tubes for storage at -80℃ until use.

2.3.7.2 Ligation

The TOPO® cloning reactions were set up by using the reagents shown in Table 8. For each sample, two different amounts of PCR products (0.5 and 4 μl) were used to perform the ligation. After mixing the PCR products, salt solution and water, 1 μl of TOPO® vector was added and gently mixed then incubated at room temperature (22-23℃) for 25min. Ligation reaction was stopped by placing the tube on ice and may be stored at -20℃ overnight.

Table 8 Cloning Reaction Mix

Reagent	**Electrocompetent *E.coli***
Fresh PCR product	0.5 to 4 μl
Diluted Salt Solution	1 μl
Sterile Water	Add to final volume of 5 μl
TOPO® vector	1 μl
Final Volume	**6 μl**

2.3.7.3 Transformation

The competent cells were gently thawed at room temperature and kept cold on ice. 2µl of the TOPO® Cloning Reaction were well mixed with 40µl of the cell suspension in a cold, 1.5ml Eppendorf tube with 1 min incubation on ice. The Gene Pulser apparatus was set at 25µF and 2.5 kV and the Pulse Controller was put to 200 Ω. The mixture of cells and DNA was transferred to a cold 0.2cm electroporation cuvette with shaking of the suspension to the bottom of the cuvette. The cuvette was then placed in a chilled safety chamber slide and inserted into the chamber until the seating between the contacts in the base of the chamber. Pulse was produced with a time constant of 4 to 5 msec. Cuvette was removed from the chamber and 1 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) immediately added to the cunvette. Cells were re-suspended as quickly as possible for maximizing the recovery of transformants. Suspension was then transferred into a 15ml-falcon tube and incubated at 37℃ with 225rpm shaking for at least 1 hour. After incubation, cells were inoculated to plates of selective medium containing 50µg/ml kanamysin (see appendix A) and incubated at 37℃ overnight.

2.3.7.4 Colony selection and analysis

After overnight incubation, several hundred colonies were grown on the selected plate. Most single colonies were selected and transferred to fresh selective plates for overnight incubation. Single colonies grown on the new plates were transferred to 10ml-liquid LB medium (with added kanamycin) to incubate again overnight. All the steps should be well labelled as several hundred samples were obtained in these two incubation steps.

2.3.7.5 Plasmid DNA preparation by alkaline lysis

This method was a derivative alkaline lysis method (Birnboim & Doly, 1979 & Niderberger, T. D., 2005). Cells were harvested by transferring 1ml of overnight culture suspension grown in the kanamycin selective medium into a 1.5ml tube and then spinning at 16,000×*g* for 20 sec. After discarding the supernatant, the cell pellet was re-suspended in 100µl of "Buffer 1 (GET)" (50mM Glucose, 10mM EDTA, 25mM Tris, pH 8.0) by vortexing. 200µl of "Buffer 2 (Lysis)" (1% SDS and 0.2M NaOH) was then added to the tube and mixed by inverting the tube a few times (Do Not Vortex-Lysis). Mixture was incubated on ice for 5 minutes and 150µl of "Buffer 3 (Neutral)" (3.0M Potassium Acetate at pH 5.5) was added with mixing by inverting tubes a few times (Neutralisation). Tube was chilled on ice for 5 minutes and spun at 16,000×*g* for 5 min. The supernatant was then transferred into a clean 1.5ml tube and 1µl RNase A (10mg/ml) was added and incubated at 37℃ for 15min. 200µl Chloroform was added and mixed vigorously by vortexing followed by a 15-min centrifugation at 16,000×*g*. Supernatant was transferred to a fresh tube to which an equal volume of isopropanol had already been added. Tube was mixed vigorously and spun at 16,000×*g* for 5 min. Supernatant was carefully removed and discarded (DNA pellet is usually visible at this point). The small pellet was washed by adding 200µl of ice-cold 70% ethanol and centrifuged at 16,000×*g* for 5 min. DNA pellet was dried by incubating tube in the laminar-flow for at least 10 min. Finally, DNA was re-suspended in 20µl of TE Buffer (10mM Tris, 1mM EDTA, pH 8.0) and stored at -20℃.

2.3.7.6 Plasmid DNA Analysis

The plasmid DNA was amplified by PCR Reaction by using the M13 primers (every 25µl PCR reaction contained 0.75 µl of each 10mM primer M13F (5' -GTA AAA CGA CGG CCA G-3') and M13R (5'-CAG GAA ACA GCT ATG AC-3'), 2.5µL of 10×PCR buffer, 2.5µL of 2mM dNTPs, 2.5µL of 0.2mg/ml BSA, 0.2µL of 5U/µl, 20 ng of template DNA and the entire reaction was made up to 25 µl with Milli-Q H_2O) and following PCR (initial denaturation at 94℃

for 2 minutes, then 25 cycles of 94℃ for 1min, primer annealing at 55℃ for 1min and primer extension at 72℃ for 1min, followed by a final extension at 72℃ for 7 min) was then checked by 1% agarose gel electrophoresis. For clones having the expected amplicon size, amplicons were extracted and subjected to the double restriction endonuclease digestion (0.5 μl *Xba* I, 0.5 μl *Hae* III, 2 μl 10×Buffer M, 10 μl Substrate DNA and sterile water to 20 μl, also see *section 2.3.6*) was done for colonies selection and finally all clones with different restrict digest patterns were sent for sequencing.

2.3.8 Sanger sequencing and data analysis

Purified PCR samples from pure cultures and clone libraries were sent to the University of Waikato DNA sequencing facility for sequencing using 1492R and 27F primers, M13F and M13R primers, respectively. Template DNA was prepared using DYEnamicET dye terminator chemistry from Amersham Biosciences. A MegaBACE 500 DNA Analysis System was applied to resolve DNA sequences with loading linear polyacrylamide Long Read Matrix (Amersham Biosciences).

Sequences were first identified and compared with the Genebank data base using Basic Local Alignment Search Tool (BLASTn). Sequences with greater than 97% pairwise identity were recognized as the same species and with greater than 95% pairwise identity were considered as the same genus. Those sequences with lower than 95% pairwise identity were considered as possibly new species. After initial analysis, sequences were edited and aligned by using the Geneious Pro software package (Williams, T, 2011). Pairwise evolutionary distances were calculated from percent similarities using Jukes and Cantor correction, and phylogenetic trees were constructed by the Neighbour-joining method.

2.3.9 454 Pyrosequencing

To identify the bacterial community in the Top layer and Bottom layer soil samples from Tui mine site, the 454 Pyrosequencing with a double-PCR preparation method was used in this study.

2.3.9.1 454 Pyrosequencing

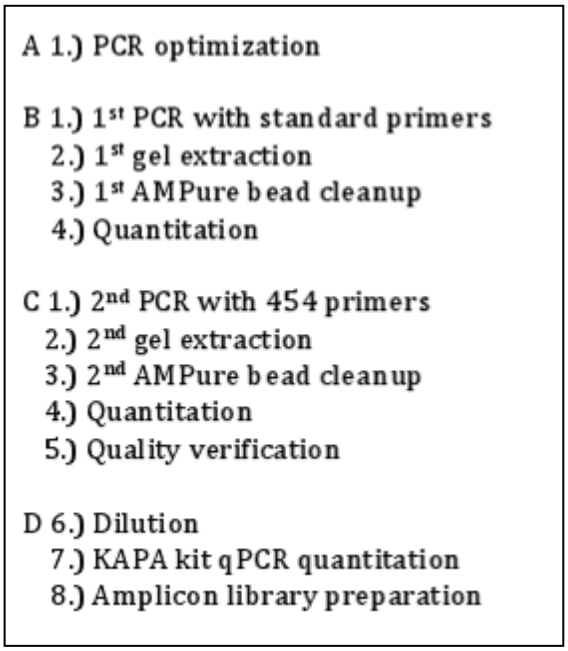


Fig 13 454 Sample Preparation

There are four main steps to get the samples ready in this protocol (Fig. 13). The first part of this protocol is to optimize the PCR to gain the cleanest amplification with fewest number of cycles. In the 454 preparation, a different polymerase (KAPA HiFi^{TM} Hot Start DNA Polymerase) and primers were used from the cloning protocol and the master mix of PCR reaction is shown in Table 9. The primers used in the first PCR reaction were unadapted primer Tx9F (5‘-GGA TTA GAW ACC CBG GTA GTC-3’) and 1391R (5‘-GAC GGG CRG TGW GTRCA-3’) (Cardinale *et al*., 2004). Before making the master mix, all tubes, MQ-water, PCR buffer were sterilized under the UV light for 15 min. Except primers and template DNA, all the remaining components were mixed and treated with EMA (ethidium monoazide) to ensure that any potential contaminating double-stranded DNA in the master mix does not get amplified. The procedure of EMA Treatment is in Appendix H.

Table 9 KAPA HiFi HotStart PCR Reaction

PCR component	Volume (μl)
Water	To 30
5×KAPA HiFi GC Buffer (contains 2.0mM Mg^{2+} at 1×)	6.0
KAPA dNTP Mix (10mM)	0.9
Forward Primer (Tx9F)(10 μM)	0.9
Reverse Primer (1392R)(10 μM)	0.9
Template DNA	～20ng
KAPA HiFi™ Hot Start DNA Polymerase (1U/μl)	0.6

Five different PCR cycling conditions (an initial denaturation at 95℃ for 3 minutes, then 10, 18, 24, 30 or 35 cycles of 98℃ for 20 sec, primer annealing at 52℃ for 15 sec and primer extension at 72℃ for 30 sec, followed by a final extension at 72℃ for 3 min.) were tried to find the fewest cycles that could be used in the next steps. For each run, a positive control (E.*coil* 16S DNA as template DNA) and a negative control (no template DNA added) were used and the negative control was always run for 35 cycles to check for contamination. The PCR products were checked by running on a 1% agarose gel to ensure amplification success.

After PCR optimization, a 30-cycle PCR Reaction was finally used in the following part of the 454 sample preparation. For each sample, 3 ×30μl PCR reactions were mixed after EMA treatment and run with 30 cycles. A 2% TAE gel with large and wide wells (stained with 10μl SybrSafe/100ml agarose solution) was used for electrophoresis. All three reactions of each sample were mixed together with 10μl of 5×loading dye and then loaded into the wells. Gel was run slowly at 80V for about 55min to allow for good separation of bands. After the electrophoresis, the anticipated size PCR products (～680bp) were removed into pre-weighted 1.7ml Eppendorf tubes from the gel by using the SafeImager and cutting with new microscope cover slips. All tubes were weighted again individually to calculate the gel weight and then cleaned up by using the MO BIO

UltraClean® 15 DNA Purification Kit (MO BIO Laboratories, Inc. US). The detailed protocol of the extraction kit is in appendix I. The DNA extracted from gel was cleaned again by using the AMPure XP beads. To clean the amplicons, 1.8 ×volume of AMPure XP beads was added to the PCR products and mixed by pipetting 10 times. Mixtures were incubated at room temperature for 5 min and then the tubes were placed into magnetic collector with 2 min incubation at room temperature. After incubation, the clear solution was removed and transferred to another tube (in case the clean up did not work). The pellet contained in the tube with magnetic beads was washed with 200µl freshly made 70% research grade ethanol and incubated for 30 sec at room temperature. Ethanol was discarded and the wash step repeated. After two washes, tubes were removed from the magnet and 20µl of TE buffer was added to mix with the pellet by 10 times pipetting. Tubes were placed on the magnet again with 1 min incubation and at this moment, all DNA was dissolved in the TE buffer. Leaving the magnetic beads in the tube, all liquid was transferred to new tubes. Most DNA was in this TE buffer and quantitated by using the QuBit Reading (Invitrogen, CA, USA). Appendix I shows the protocol of QuBit dsDNA HS Kit. With the Qubit result, samples were diluted to about 20 ng/µl by using the TE buffer. When the concentration was lower than 20ng/µl, samples were not diluted.

The second time PCR used the adapted primers (BacX-Tx9F (5`-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG -MID GGA TTA GAW ACC CBG GTA GTC-3`) & BacB-1391R (5`-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG–GAC GGG CRG TGW GTR CA-3`)) and ～20ng of DNA from the previous step as the template. The PCR program was identical to the 1st PCR program but with only 10 cycles. After PCR, amplicons were cleaned by the same methods as previously (using MO BIO UltraClean® 15 DNA Purification Kit and AMPure XP Bead Cleanup Kit) and quantitated by QuBit Flurometer (Invirogen). Samples were also analyzed by using the Alilent 2100 Bioanalyzer to verify the quality.

The final part of 454 sample preparation was to dilute the DNA, quantitate by KAPA qPCR analysis and prepare an amplicon library. Samples were diluted to

2×10^8 theoretical molecules/µl (modified from the Roche Amplicon Library Preparation Method Manual):

Convert the concentration of DNA from ng/µl to theoretical molecules/µl:

$$\text{theoretical molecules}/\mu\text{l} = \frac{\text{sample conc.}\,(\text{ng}/\mu\text{l}) \times 6.022 \times 10^{23}}{656.6 \times 10^9 \times \text{amplicon length} * (\text{bp})}$$

* Amplicon length includes forward and reverse adapters and MID.

*Molecules/µL calculated using the above equation is termed theoretical since not all DNA molecules contain both adapters and will amplify in emPCR. Actual (i.e., amplifiable) molecules/µL can only be estimated using the Kapa qPCR kit.

Calculate the volume of TE required to dilute 2 µl of PCR amplicon sample to 2×10^8 theoretical molecules/µl:

$$\text{volume of TE} = \frac{2 \times \text{theoret. molecules}/\mu\text{l}}{2 \times 10^8} - 2$$

qPCR using a KAPA Library Quantification Kit for Roche 454 Titanium/Universal (Kapa Biosystems, Woburn, MA, USA) was used to check the 1x109 dilution, and was adjusted accordingly for making the Amplicon library. The protocol of this Kit is shown in appendix J. The final number of amplifiable molecules needed for emPCR was 4×10^8 in 200µl. For each sample, the total number of amplifiable molecules was calculated based on the percentage of the run assigned for the sample and finally, the required amount of each sample calculated (if lower than 1µl, dilute the sample by 10 first). All samples were mixed together with the final 200µl in total and a copy per bead ratio of 2 was

used in setting up the emPCR reaction. Sequencing was performed using the GS Junior Titanium Sequencing Kit, PicoTiterPlate Kit and GS Junior System (Roche 454 Life Sciences, Branford, CT, USA).

2.3.9.2 454 Pyrosequencing data processing

AmpliconNoise v1.0 was used for analyzing 454 PCR Amplicon pyrosequencing data (Quince *et al.*, 2011). Briefly, raw flow-grams (.sff files) with perfectly matching primer and barcode sequences were filtered for a minimum flow-gram length of 360 cycles (including primer and barcode sequences) before the first noisy signal (i.e., 0.5-0.7 or no signal in all four nucleotides). All flow-grams were then truncated at 360 bases and clustered to remove sequencing noise using PyroNoise (Quince *et al*., 2011; Quince *et al*., 2009). PCR noise was removed using SeqNoise (Quince *et al*., 2011), and PCR chimeras were removed using Perseus (Quince *et al*., 2011). The resulting de-replicated sequences were processed using Mothur 1.17.0 (Schloss *et al*., 2009) to create a unique sequence and names file, which could then be pairwise distance aligned using Espirit. Mothur was also used to calculate sequence distance matrices and cluster sequences into operational taxonomic units (OTUs) defined at the furthest neighbor Jukes-Cantor distance of 0.05 (OTU0.05). Rank-abundance data were generated for each sample as well as Venn diagrams. For phylogenetic assignments, representative sequences of all identified OTU0.05 were analyzed using the Classifier function provided by the Ribosomal Database Project (RDP) Release 10, Update 15. Taxonomic assignment threshold was set at 80%.

3. Results and Discussion

3.1 pH of environmental samples

The pH of the six samples are presented in Table 10. Five out of six samples were found to be naturally acidic having pH values lower than 3.5. Of the three soil samples, the surface soil (Top layer soil) has the lowest pH value and this is likely due to post-mining oxidation with both microbes and atmosphere (sufficiently exposed to oxygen) which generates large amount of acid. The higher pH of the "Brown Stream" sample was due to its location furthest from the main tailing area. The little pool, at which the "pool site" sample was collected, was the nearest liquid phase around the main tailing site and the Green stream ran from this pool.

Table 10 pH value of samples

	pH
Soil sample	
Top layer soil	2.92
Middle layer soil	3.01
Bottom layer soil	3.32
Liquid sample	
Pool site	2.60
Green stream	2.71
Brown stream	5.5

3.2 Culture-based results

3.2.1 Environmental samples analysis

To enrich microbes from all six samples, 9 different media were used in this study and the compositions of all these media are listed in Appendix A. Under anaerobic conditions, no growth was observed even with 20 days incubation, despite the grey appearance of the soil which is normally associated with anaerobiosis. The number of colonies (both bacteria and fungi) enriched on the media under aerobic culture are shown in Tables 11 & 12. From all the different media, 32 bacterial and 48 fungal colonies were regarded as distinct and isolated and purified by repeated streaking and plating (bacterial isolates are labelled in alphabetical order (A to AF) and fungal isolates labelled in numerical order (1 to 48)). Microscopically bacterial isolates displayed varying morphologies, predominantly rods and cocci with different sizes. Due to the limitation of time for my study, only bacteria were analysed further.

Among all 32 bacterial isolates, 3 of them (isolates C, N and W) were un-culturable in the first incubation step and the DNA of a further 10 isolates could not be extracted. The PCR results for the remaining 19 pure cultures indicated that only 16 DNA samples could be amplified and finally, 15 different restriction digest patterns were obtained. Amplicons from these 15 bacterial isolates and a previously isolated *Acidocella* strain were sent for sequencing.

3.2.1.1 Culturing results

Compared with an *Acidocella* strain isolated from previous work, isolate B grown on 4-0-0(3.5) medium from the Top layer soil sample had a similar morphology and was initially defined as the same organism. Further sequencing results would examine this to see if it is correct.

The plate count results indicate that more than 90% of microbes on the media were fungi (Table 11 & 12). Among all 6 samples, the Top layer soil sample had the largest number of microbes and they could grow on all 9 different media. In contrast, no enrichment or colony development occurred on most of the media (6 out of 9) with the Bottom layer soil sample. Although total numbers of colonies

from three liquid samples were quite similar, more bacteria were isolated from the Brown Stream sample than the other two samples.

Of the 34 isolated bacteria, 15 isolates were from the Top layer soil sample, 3 isolates were from the Middle layer and 5 from the Bottom layer soil; from the three liquid samples, no bacteria were obtained from the Green Stream, only 1 bacterium was isolated from the Pool Site and 8 from the Brown Stream. With all plates inoculated with the Bottom layer soil sample, isolates O, P, Q and R were unusually distributed in that they grew on only one of the triplicate plates with 4-0-0 (4.5) medium and they were considered as either contaminations or low populations in the environmental sample. Comparing all bacterial isolates, the Top layer soil sample was considered as the one with the highest diversity and largest population among all six samples.

Of the nine media used in this study only those without added Mg^{2+} or Zn^{2+} supported the isolation of bacterial colonies and the use of only two medium (4-0-0 pH 4.5 and 4-0-0 pH 5.0 would have resulted in all the isolates finally obtained. Thus no obligately heavy metal dependent species exist in these soils, all are adapted to high heavy metal concentrations but not dependent on them for growth. High concentrations of Mg^{2+} are supposed to ameliorate the inhibitory effects of other heavy metals, but this was not observed in this case.

From the three liquid samples, no bacterium grew on the media at pH 3.5 or the media with Mg and Zn. Bacteria from the Pool site sample were only detected on the 4-0-0 (4.5) and 4-Mg-0(4.5) medium, while bacteria on the 4-Mg-0(4.5) medium had been isolated from 4-0-0 (4.5) medium. Unexpectedly, no bacteria were obtained on any of media from the Green Stream sample in contrast to the diversity of bacteria cultured on the 4-0-0(5.0) media from the Brown Stream samples.

Table 11 (a, b & c) Isolation Results from three soil samples

Table 11a. Culture diversity and density from the Top layer soil sample (pH 2.9)				
Medium	**Total bacteria (per 1g soil)**	**Bacterial diversity[a] and isolates[b]**	**Total Fungi (per 1g soil)**	**Fungal isolates**
4-0-0 (3.5)	1.8×10^3	2 (**A, B**)	5.3×10^4	1-10
4-Mg-0(3.5)	0	/	6.65×10^4	11,12
4-Mg-Zn(3.5)	2×10^2	1 (**C**)	2.8×10^4	/
4-0-0 (4.5)	2.2×10^3	6 (A, B, **D, E, F, AE**)	4.75×10^4	/
4-Mg-0(4.5)	0	/	4.95×10^4	13
4-Mg-Zn(4.5)	0	/	1.3×10^4	/
4-0-0 (5.0)	3.5×10^3	11 (A, D **G, H, I, J, K, L, M, AF** AE)	4.7×10^4	14
4-Mg-0(5.0)	0	/	2.8×10^4	/
4-Mg-Zn(5.0)	0	/	1.7×10^4	15-16

[a]Bacterial diversity- different colony types on each plate are indicated by number; [b]Isolates selected are indicated by bold.

Table 11b. Culture diversity and density from the Middle layer soil sample (pH3.0)				
Medium	**Total bacteria (per 1g soil)**	**Bacterial diversity and isolates**	**Total Fungi (per 1g soil)**	**Fungal isolates**
4-0-0 (3.5)	1.5×10^2	3 (A, F, **AC**)	3.1×10^3	17-19
4-Mg-0(3.5)	0	/	2.2×10^3	20, 21
4-Mg-Zn(3.5)	0	/	0.8×10^3	/
4-0-0 (4.5)	3×10^2	3 (B, E, **AD**)	2.2×10^3	/
4-Mg-0(4.5)	0	/	1.7×10^3	/
4-Mg-Zn(4.5)	0	/	0.7×10^3	/
4-0-0 (5.0)	10	1 (**N**)	1.1×10^3	/
4-Mg-0(5.0)	0	/	0.5×10^3	23
4-Mg-Zn(5.0)	0	/	0	/

[a]Bacterial diversity- different colony types on each plate are indicated by number; [b]Isolates selected are indicated by bold.

Table 11c. Culture diversity and density from the Bottom layer soil sample (pH3.3)				
Medium	**Total Bacteria (per 1g soil)**	**Bacterial diversity and isolates**	**Total Fungi (per 1g soil)**	**Fungal isolates**
4-0-0 (3.5)	0	/	0	/
4-Mg-0(3.5)	0	/	0	/
4-Mg-Zn(3.5)	0	/	0	/
4-0-0 (4.5)	3×10^2	4 (**O, P, Q, R**)	3×10^2	/
4-Mg-0(4.5)	0	/	0.2×10^2	24
4-Mg-Zn(4.5)	0	/	0	/
4-0-0 (5.0)	1×10^2	1 (**S**)	1×10^2	/
4-Mg-0(5.0)	0	/	0	/
4-Mg-Zn(5.0)	0	/	0	/

[a]Bacterial diversity- different colony types on each plate are indicated by number; [b]Isolates selected are indicated by bold.

Table 12 (a, b & c) Isolation Results from three liquid samples

Table 12a Culture diversity and density from the Pool site (pH 2.6)				
Medium	**Total bacteria (per ml)**	**Bacterial diversity and isolates**	**Total fungi (per ml)**	**Fungal isolates**
4-0-0 (3.5)	0	/	5.4×10^2	/
4-Mg-0(3.5)	0	/	7.8×10^2	32
4-Mg-Zn(3.5)	0	/	6.5×10^2	33
4-0-0 (4.5)	11	3 (E, D, **T**)	5.0×10^4	/
4-Mg-0(4.5)	10	1 (T)	1.3×10^3	/
4-Mg-Zn(4.5)	0	/	1.0×10^2	34
4-0-0 (5.0)	0	/	3.7×10^2	/
4-Mg-0(5.0)	0	/	5.6×10^2	35, 36
4-Mg-Zn(5.0)	0	/	2.9×10^2	37

[a]Bacterial diversity- different colony types on each plate are indicated by number; [b]Isolates selected are indicated by bold.

Table 12b Culture diversity and density from the Green stream (pH2.7)				
Medium	**Total bacteria (per ml)**	**Bacterial diversity and isolates**	**Total fungi (per ml)**	**Fungal isolates**
4-0-0 (3.5)	0	/	1.0×10^2	25
4-Mg-0(3.5)	0	/	8.0×10^2	26, 27
4-Mg-Zn(3.5)	0	/	5.13×10^2	/
4-0-0 (4.5)	0	/	5.75×10^2	/
4-Mg-0(4.5)	0	/	2.1×10^3	/
4-Mg-Zn(4.5)	0	/	2.85×10^2	28, 29
4-0-0 (5.0)	0	/	6.4×10^2	30
4-Mg-0(5.0)	0	/	3.2×10^2	31
4-Mg-Zn(5.0)	0	/	5.3×10^2	/

[a]Bacterial diversity- different colony types on each plate are indicated by number; [b]Isolates selected are indicated by bold.

Table 12 c Brown Stream (pH 5.5)				
Medium	**Total bacteria** (per 1g soil)	**Bacterial diversity and isolates**	**Total fungi** (per ml)	**Fungal isolates**
4-0-0 (3.5)	0	/	9.0×10^2	/
4-Mg-0(3.5)	0	/	5.0×10^2	/
4-Mg-Zn(3.5)	0	/	1.6×10^2	/
4-0-0 (4.5)	20	2 (E, **U**)	5.6×10^2	40, 41
4-Mg-0(4.5)	39	3 (T, U, **V**)	$6.45.\times10^3$	42
4-Mg-Zn(4.5)	0	/	2.9×10^2	/
4-0-0 (5.0)	6×10^2	12 (E, F, T, U, V, **W, X, Y, Z, AA, AB** AC)	7.2×10^3	43-47
4-Mg-0(5.0)	0	/	4.4×10^2	48
4-Mg-Zn(5.0)	0	/	1.5×10^2	/

[a]Bacterial diversity- different colony types on each plate are indicated by number; [b]Isolates selected are indicated by bold.

3.2.1.2 Molecular analysis

DNA of bacterial isolates were extracted using the CTAB method. Of the original 32 isolates which were subcultured, three did not survive (isolates C, N and W). Of the remaining 29, DNA was only extractable from 19, the remaining 10 did not yield good quality DNA despite at least three attempts with CTAB and harvesting cells at different stages of growth from early to late log phase. The reason for no DNA being extracted may be that the capsule or cell wall fragments were too difficult to be removed or DNA was combined with some possible polysaccharides (Zeng, *et al*, 2008). Fig. 14 exhibits the results of 16S rRNA PCR amplification of the 19 isolates. DNA from isolates H, T and V failed to produce amplicons even with two different reverse primers (1522R and 1492R). When the 1522R and 27F primer set was used, only 9 of the 18 isolates produces amplicons of the expected size, whereas the 27F and 1492R primer set seemed to be more "universal" with 16 amplifications out of 18 isolates.

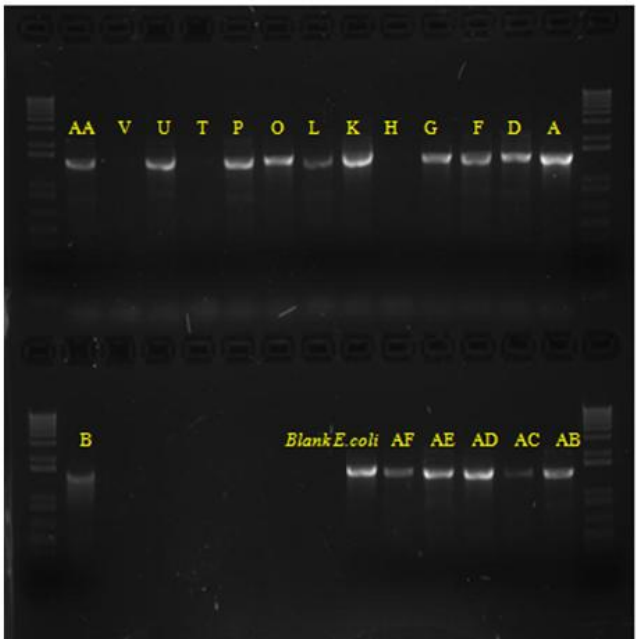


Fig 14 Amplification of 16S rRNA from bacterial isolates (using 1492R and 27F)

The amplified 16S rRNA gene amplicons from the 15 (excluding isolate B) bacterial isolates were subjected to restriction digestion using two different enzymes (*Hae* III and *Afa* I). The digestion results are shown in Fig15. The gel picture clearly showed that isolates D and F had the same restriction patterns with both enzymes and were therefore considered as the same organism. Except for D

and F, all the remaining isolates had unique restriction fragment lengths and were recognized as different bacteria. After analysis by digestion, sixteen amplified 16S rRNA samples from these isolates were sent to the University of Waikato DNA sequencing facility for sequencing. A repeat of the digestion (double-enzyme with *Xba* I and *Hae* III) of the 16S amplicons of 16 pure isolates and the isolated *Acidocella* strain were done combined with clone library (Fig. 19). Restriction result indicated that isolate B and *Acidocella* had a similar RFLP pattern.

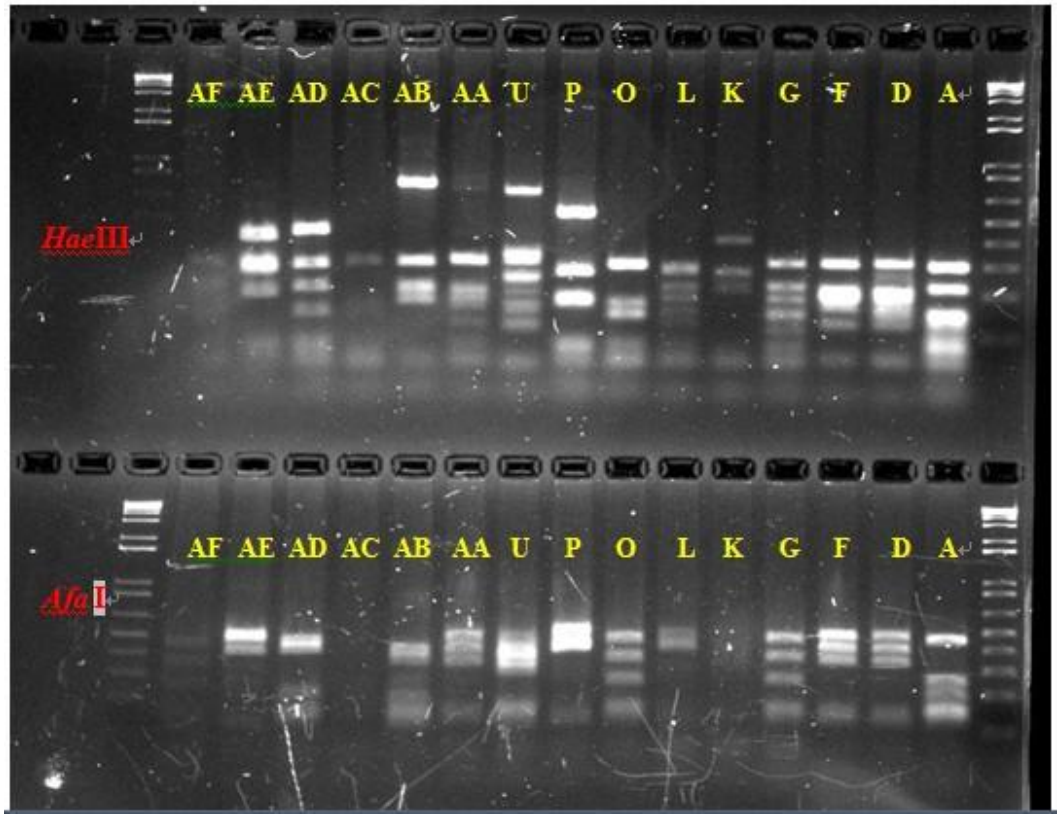


Fig 15 Gel electrophoresis of restriction digestion results of 15 amplified 16S rRNA amplicons with *Hae* III and *Afa* I

Sequences with low qualities and less than 300 base pair were excluded the further analysis. Of the 17 (16 isolates from this study and 1 strain of isolated *Acidocella*), only 10 sequences were reliable and the BLAST result are shown in Table 13. There were no sequences obtained from both isolates B and *Acidocella* even after attempts at sequencing. Only isolates G, AE and AF could be identified

to the species level as only those share more than 98% gene sequence identity. Of the 10 isolates, 6 (A, D, G, L, AE and AF) were only isolarted from the Top layer soil sample and isolates A, G and AF were affiliated to *Leifsonia xyli subsp. xyli str* which is an uncommon species that grow on the acidic mine tailing. *Leifsonia xyli subsp. xyli str* is the gram-positive sugarcane pathogen belonging to the *Actinobacteria* (Sutcliffe, *et al*, 2007). The remaining 3 from the Top layer soil and 4 from other environmental fell within the *Proteobacteria.*

Table 13 BLAST results of sequences from pure cultures

Name	Blast Result	Max Iden.
A	*Leifsonia xyli subsp. xyli str. CTCB07* *Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Clavibacter.*	97%
D	*Frateuria aurantia DSM 6220* *Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Frateuria.*	95%
G	*Leifsonia xyli subsp. xyli str. CTCB07* *Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Leifsonia.*	99%
L	*Novosphingobium aromaticivorans DSM 12444* *Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Novosphingobium.*	95%
P	*Phenylobacterium zucineum HLK1* *Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Phenylobacterium.*	89%
AA	*Frateuria aurantia DSM 6220* *Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Frateuria.*	85%
AB	*Janthinobacterium sp. Marseille* *Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Janthinobacterium.*	94%

AD	*Streptomyces hygroscopicus subsp. jinggangensis 5008* *Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales* *Streptomycineae; Streptomycetaceae; Streptomyces.*	89%
AE	*Burkholderia xenovorans LB400* *Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;* *Burkholderiaceae; Burkholderia.*	99%
AF	*Leifsonia xyli subsp. xyli str. CTCB07* *Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;* *Micrococcineae; Microbacteriaceae; Leifsonia.*	98%

3.2.2 Acidocella growth

From a previous project, *Acidocella* had been isolated from the mine tailings and grown under lab. conditions. The typical growth curve of *Acidocella* on 1-0-0 (3.0) medium is shown in Fig. 16.

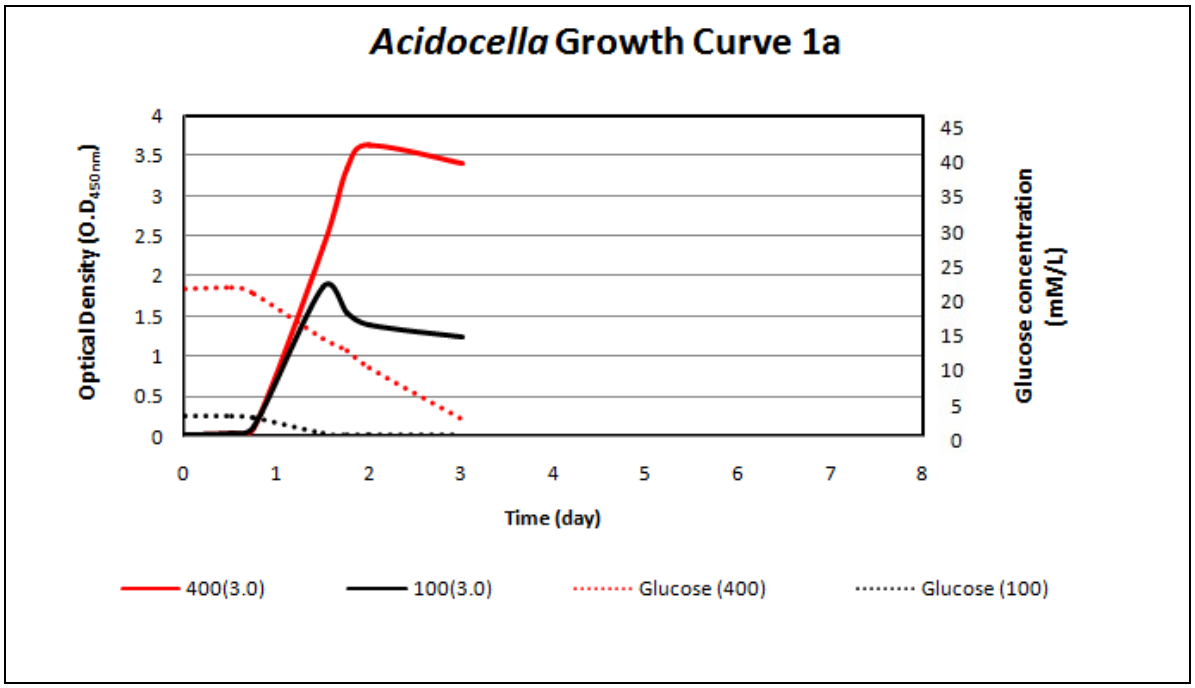


Fig 16 *Acidocella* growth curves and glucose consumption in different media compositions (1a)

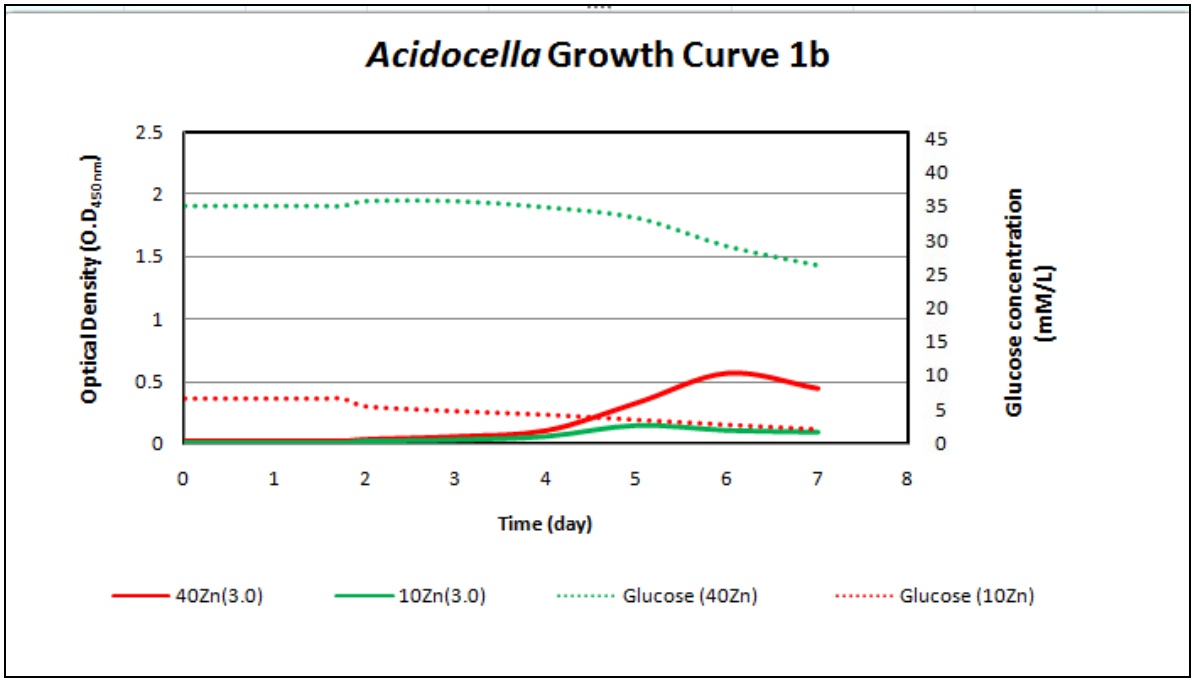


Fig 17 *Acidocella* growth curves and glucose consumption in different media compositions (1b)

Acidocella was first grown on the normal Acidobacterium Medium 1-0-0 (3.0) and 4-0-0 (3.0) medium with 4×glucose and yeast extract. With higher levels of substrate, *Acidocella* growth was greatly enhanced with $O.D_{450nm}$ values to almost 4.0 with 4 grams of glucose. Zn^{2+} resistance with *Acidocella* was demonstrated by adding 250mM/L $ZnSO_4$ into the 1-0-0 (3.0) and 4-0-0 (3.0) media, which is more than ten times higher than the Zn^{2+} concentration in the Tui mine environment. The growth curve in Figure 17 indicates that *Acidocella* was still able to grow at these Zn^{2+} concentrations, albeit with reduced growth rate and final cell yield, compared to media with no added Zn^{2+}. Fig.16 & 17 also gives the information of bacteria growth *vs* the glucose consumption. Without zinc, the glucose was almost completely consumed before stationary phase of growth especially with the 1-0-0 (3.0) medium. With 4-0-Zn (3.0) and 1-0-Zn (3.0) media, more than 70% and 25% of the total amount of glucose remained in the media after reaching the stationary phase of growth. These results demonstrate that zinc has an inhibitory effect on the growth of *Acidocella* regardless of substrate availability.

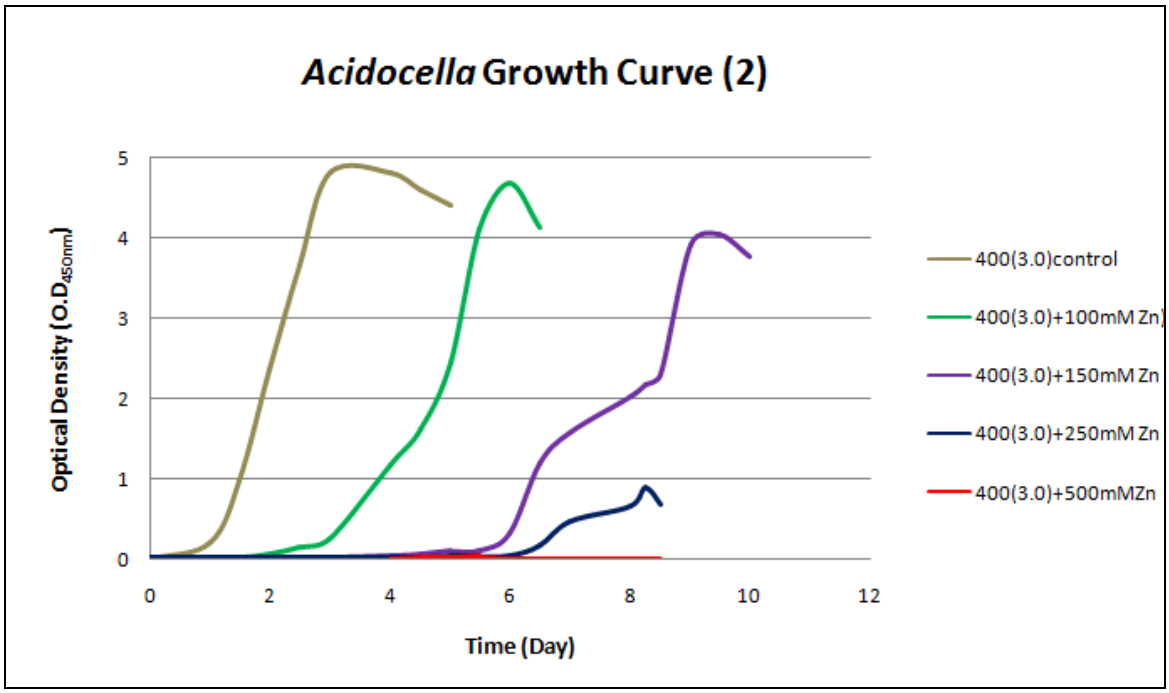


Fig 18 *Acidocella* growth response to add Zn

Previous results indicated *Acidocella* could resist Zn^{2+} at the concentration of 250mM. With increasing Zn^{2+}, the lag phase of *Acidocella* was extended and biomass in the stationary phase decreased (Fig 18). No growth was recorded at Zn^{2+} concentrations higher than 500mM even though Ghosh (1997) had described *Acidocella* isolates as tolerating 1.0 M of Zn^{2+}. Further culturing results indicated that *Acidocella* could not adjust to high concentration of Zn^{2+} even when the inoculum was feom a 250mM culture medium. With 250mM Zn^{2+} medium, the lag phase of growth was always longer than 6 days even for transfers from the same medium.

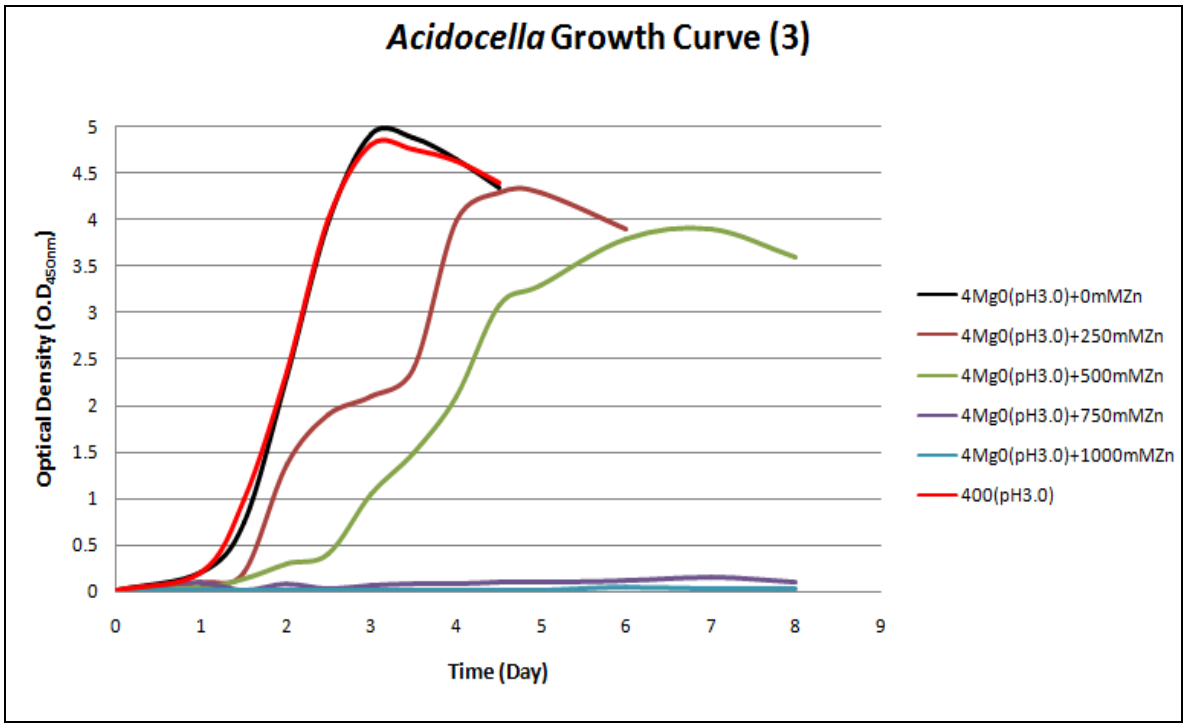


Fig 19 *Acidocella* growth response to Zn and Mg additions to the medium

Beside Zn resistance in *Acidocella*, growth with 250mM Mg^{2+} medium was also measured. Fig.19 indicates that the presence of Mg^{2+} seems to offset the inhibitory effects of other heavy metals. Resistance to Zn^{2+} was greatly ameliorated by the presence of Mg^{2+}. In the presence of 250M Mg^{2+} growth was recorded at concentration of 500mM Zn^{2+} and the extended lag recorded in the absence of Mg++ was now greatly reduced

3.3 Molecular-based analysis

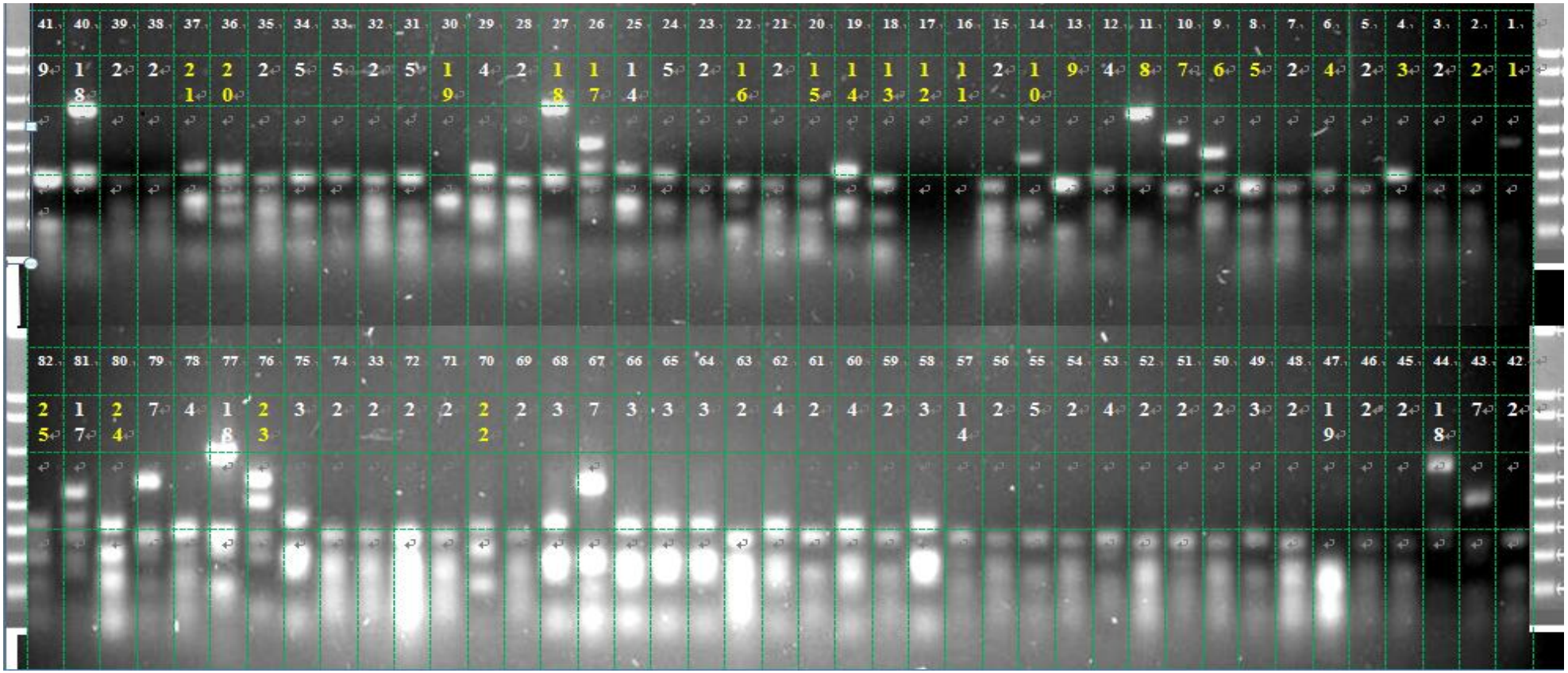
To isolate DNA from soil samples, various methods were attempted to find the best protocols; however, most attempts failed yielding no quantifiable DNA and, with no or inconsistent bacterial 16S rRNA amplification being obtained. Of the three methods of extraction used (CTAB-based method, MOBIO kit and

NucleoSpin® Soil Kit), only the MOBIO method produced amplifiable DNA from all three soil samples. Even then, the concentrations of DNA from the Middle layer and the Bottom layer soils were lower than 5ng/ul, but were sufficient to obtain PCR products for clone library analysis and 454 pyrosequencing.

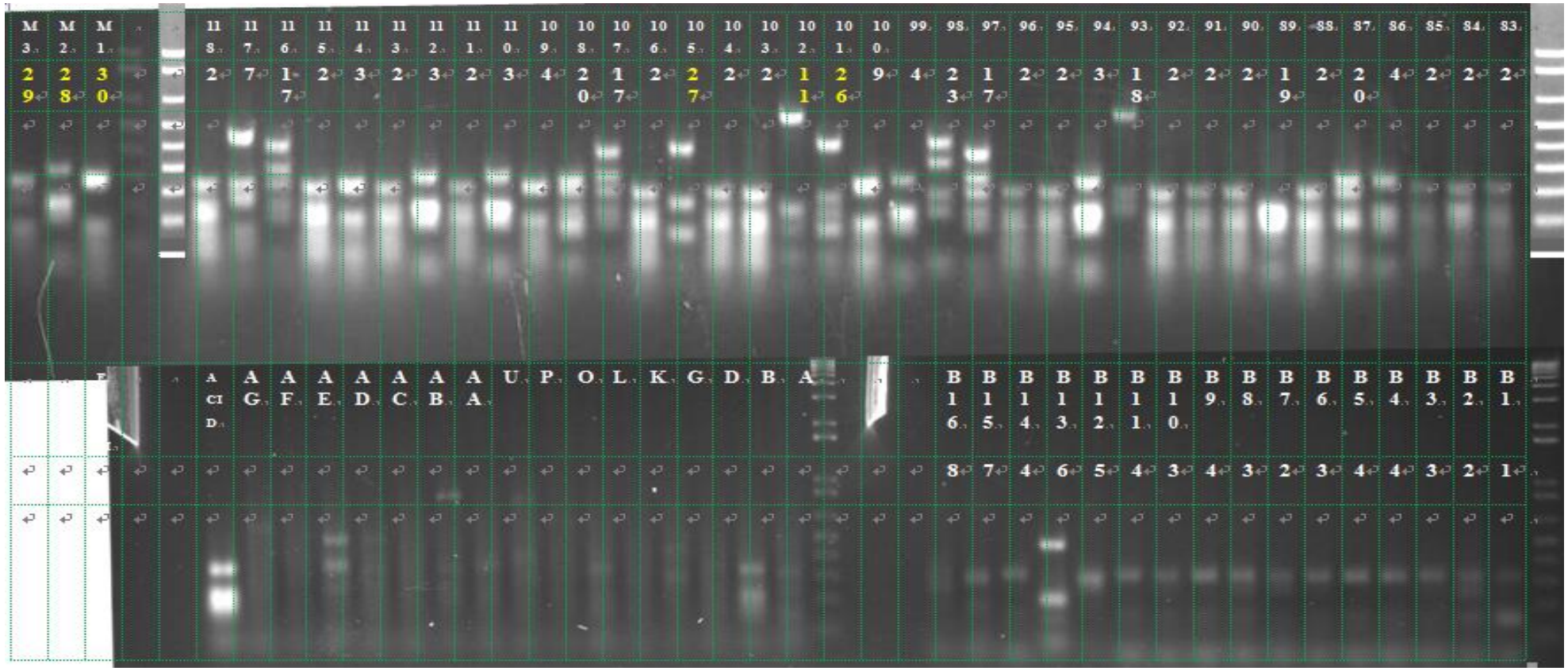
3.3.1 Clone library analysis

In order to compare the results from 454 sequencing and pure cultures (bacterial isolates), three clone libraries of full length bacterial 16S rRNA genes from the Top, Middle and Bottom layer soil samples were constructed. A total of 302 (Top), 94 (Middle) and 39 (Bottom) clones were randomly selected from these three clone libraries. Clones with amplified DNA fragments less than 300bp were excluded from subsequent analyses and finally, a total of 118 (Top), 3 (Middle) and 16 (Bottom) clones were selected, and inserts were subjected to restriction endonuclease analysis, resulting in a total 27, 3 and 6 different RFLP pattern respectively (Fig 20). The frequency of bacterial 16S RFLP restriction patterns and the BLAST results are shown in Fig 21 & 22.

Fig 20 Gel electrophoresis of double-enzyme restriction digestion results of amplified 16S rRNA amplicons from three soil clone libraries samples

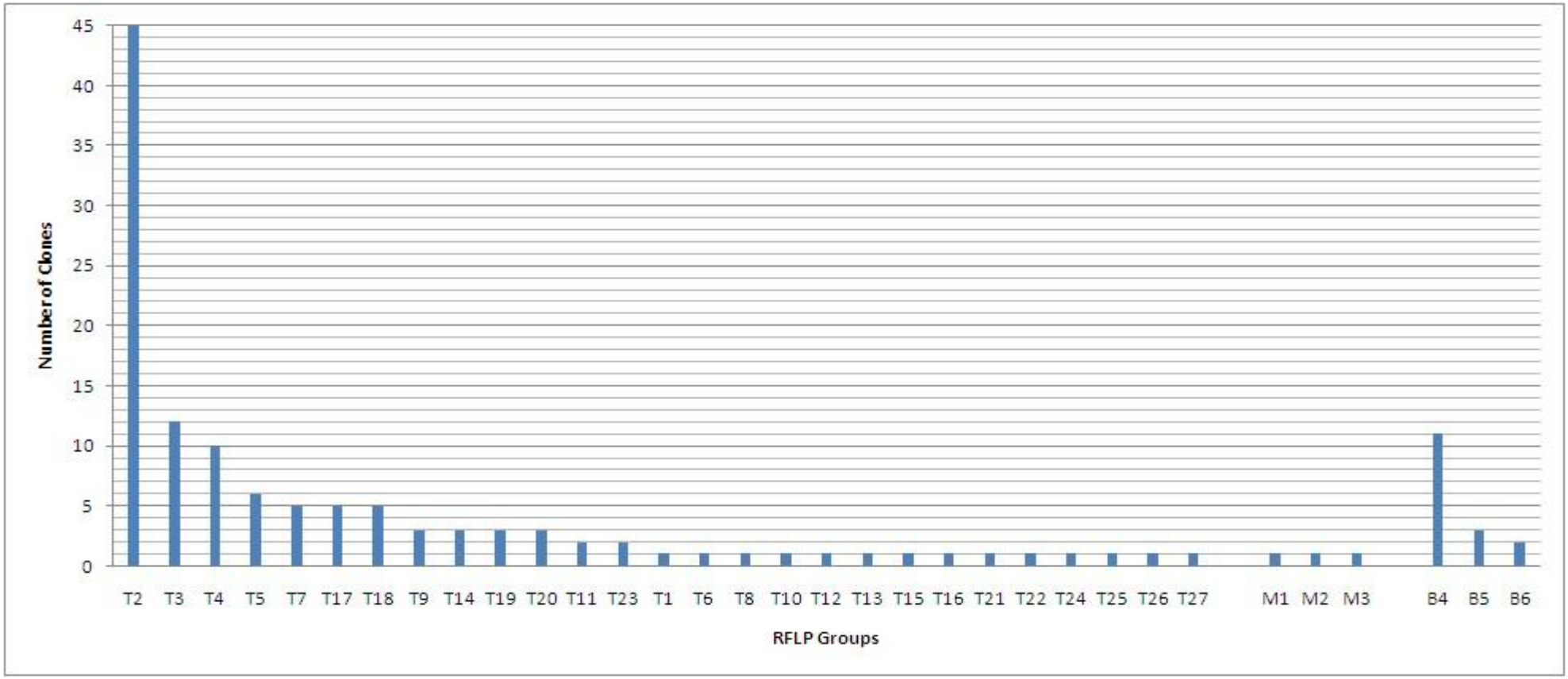


***RFLP results of 1-82 clone isolates from the Top layer soil sample. Unique RFLP patterns are yellow-labeled**



*** RFLP results of 83-118 clone isolates from the Top layer soil sample (First Right side) and 3 clone isolates from the Middle layer soil sample (First Left side) 1-16 clone isolates from the Bottom layer soil sample (right side) and RFLP results from 15 pure cultures and *Acidocella.* Unique RFLP patterns are yellow-labeled**

Fig 21 Frequency of bacterial 16S RFLP restriction patterns



RFLP Groups labeled with “T” are from the Top layer clone library, while “M” and “B” means from the Middle layer clone library and the Bottom layer clone library.

Table 14 BLAST results of sequences from the clone libraries

Name	Frequency	Blast results	Max Iden.
T1	1	*Alicyclobacillus acidocaldarius subsp. acidocaldarius Tc-4-1.* *Bacteria; Firmicutes; Bacillales; Alicyclobacillaceae; Alicyclobacillus*	93
T2	45	*Leptospirillum ferrooxidans C2-3* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.*	99
T3	12	*Acidithiobacillus ferrivorans SS3* *Bacteria; Proteobacteria; Gammaproteobacteria; Acidithiobacillales;* *Acidithiobacillaceae; Acidithiobacillus.*	92
T4	10	*Frateuria aurantia DSM 6220* *Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales;* *Xanthomonadaceae; Frateuria.*	89
T5	6	*Leptospirillum ferrooxidans C2-3* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.*	100
T6	1	*Frateuria aurantia DSM 6220* *Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales;* *Xanthomonadaceae; Frateuria.*	98
T7	5	*Marinobacter hydrocarbonoclasticus ATCC 49840* *Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales;* *Alteromonadaceae; Marinobacter.*	89
T8	1	*Acidobacterium capsulatum ATCC 51196* *Bacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae;*	95

		Acidobacterium.	
T9	3	*Leptospirillum ferrooxidans C2-3* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.*	98
T10	1	*Leptospirillum ferrooxidans C2-3* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.*	98
T11	2	*Acidobacterium capsulatum ATCC 51196* *Bacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae; Acidobacterium.*	94
T12	1	*Leptospirillum ferrooxidans C2-3* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.*	98
T13	1	*Leptospirillum ferrooxidans C2-3* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.*	99
T14	3	*Frateuria aurantia DSM 6220* *Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales;* *Xanthomonadaceae; Frateuria.*	98
T15	1	*Leptospirillum ferrooxidans C2-3* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.*	98
T16	1	*Leptospirillum ferriphilum ML-04* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.*	98
T17	5	*Alkalilimnicola ehrlichii MLHE-1* *Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales;* *Ectothiorhodospiraceae; Alkalilimnicola.*	92
T18	5	*Leptospirillum ferriphilum ML-04* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum*	98
T19	3	*Leptospirillum ferriphilum ML-04*	97

		Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum	
T20	3	*Acidithiobacillus ferrivorans SS3 Bacteria; Proteobacteria; Gammaproteobacteria; Acidithiobacillales; Acidithiobacillaceae; Acidithiobacillus.*	92
T21	1	*Frateuria aurantia DSM 6220 Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Frateuria.*	88
T22	1	*Acidimicrobium ferrooxidans DSM 10331 Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium.*	93
T23	2	*Thioalkalivibrio sulfidophilus HL-EbGr7 Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Ectothiorhodospiraceae; Thioalkalivibrio.*	87
T24	1	*Leptospirillum ferriphilum ML-04 Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum*	99
T25	1	*Acidithiobacillus ferrivorans SS3 Bacteria; Proteobacteria; Gammaproteobacteria; Acidithiobacillales; Acidithiobacillaceae; Acidithiobacillus.*	99
T26	1	*Marinobacter hydrocarbonoclasticus ATCC 49840 Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Marinobacter.*	89
T27	1	*Streptomyces venezuelae ATCC 10712 Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.*	96
M1	1	*Leptospirillum ferriphilum ML-04*	98

		Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.	
M2	1	*Cupriavidus taiwanensis LMG 19424* *Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Cupriavidus.*	91
M3	1	*Leptospirillum ferrooxidans C2-3* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.*	98
B4	11	*Leptospirillum ferrooxidans C2-3* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.*	98
B5	3	*Leptospirillum ferrooxidans C2-3* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.*	98
B6	2	*Leptospirillum ferrooxidans C2-3* *Bacteria; Nitrospirae; Nitrospirales; Nitrospiraceae; Leptospirillum.*	99

One to two representative clones in each unique RFLP pattern were sequenced, and the final sequence types (e.g. those share more than 98% gene sequence identify were considered as the same species). This reduced the number of different sequences from the Top, Middle and Bottom layer clone libraries to 22, 2 and 1, respectively. Comparative analysis of the retrieved sequences showed that all the clones could be grouped into the domain *Bacteria* and no archaeal rRNA gene sequences were detected in the clone library. The reason of no archaeal isolation is due to the PCR primers used for 16S rRNA genes amplification from environmental DNA samples were not archaeal-specific and this resulted in the lack of successful amplification of archaeal rRNA gene.

Leptospirillum ferrooxidans C2-3 was considered as the most common stain that occurred in all three clone libraries, while all 16 clones from the Bottom layer soil clone library and 2 of 3 (M1 & M3) clones from the Middle layer soil clone

library were affiliated with this stain. In the Top layer soil clone library, *L. ferrooxidans C2-3* was also the most commonly recovered phylotype at 58.4% (69 out of 118) of the total clones obtained from. *Acidithiobacillus ferrivorans SS3,* made up 13.6% of the Top layer soil clone library, was the second most abundant strain. *A. ferrooxidans*, and *Leptospirillum spp*. were widely isolated from different acid mine tailings around the world and they were well-known as the iron oxidizing bacteria. In the Top layer soil clone library, these two strains were collectively accounted for greater than 70% (85 out of 118) of the total clones. Of the remaining 33 bacterial 16S rRNA sequences, 3 were affiliated with *Acidobacterium capsulatum* which also one common bacteria occurring in the acid mine environment. 14 of clones with relatively lower pairwise identity (97%-95%) were related to *Frateuria aurantia DSM 6220,* within genus *Xanthomonadaleas.* Number of sequence types indicates that the total taxonomically distinct groups of bacterial community in three soil samples were low and finally, the phylogenetic analyses placed most sequence types in the *Nitrospira, Proteobacteria,* and *Acidobacteria* groups. Figure 21 illustrates the diversity of bacteria present in the Top layer soil clone library.

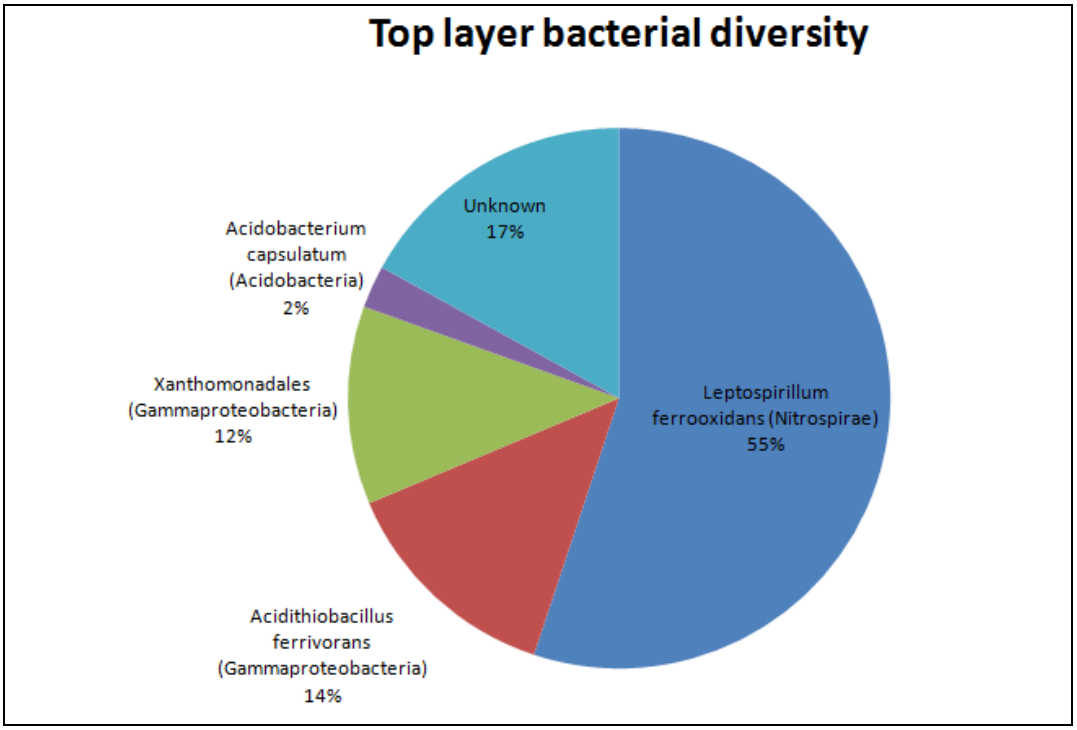


Fig 22 Top layer bacterial diversity

Based on RFLP and Sanger sequencing data derived from a library of 118 clones containing full length bacterial 16S rRNA genes.

3.3.2 454 Pyrosequencing analysis

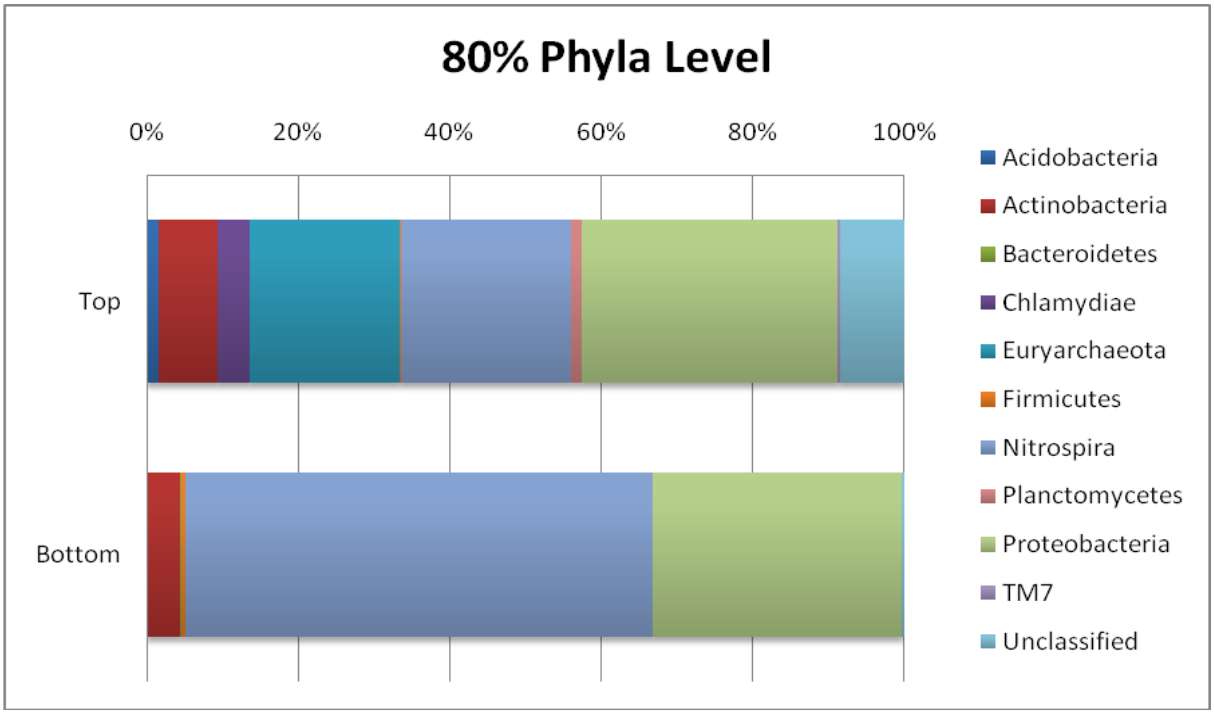


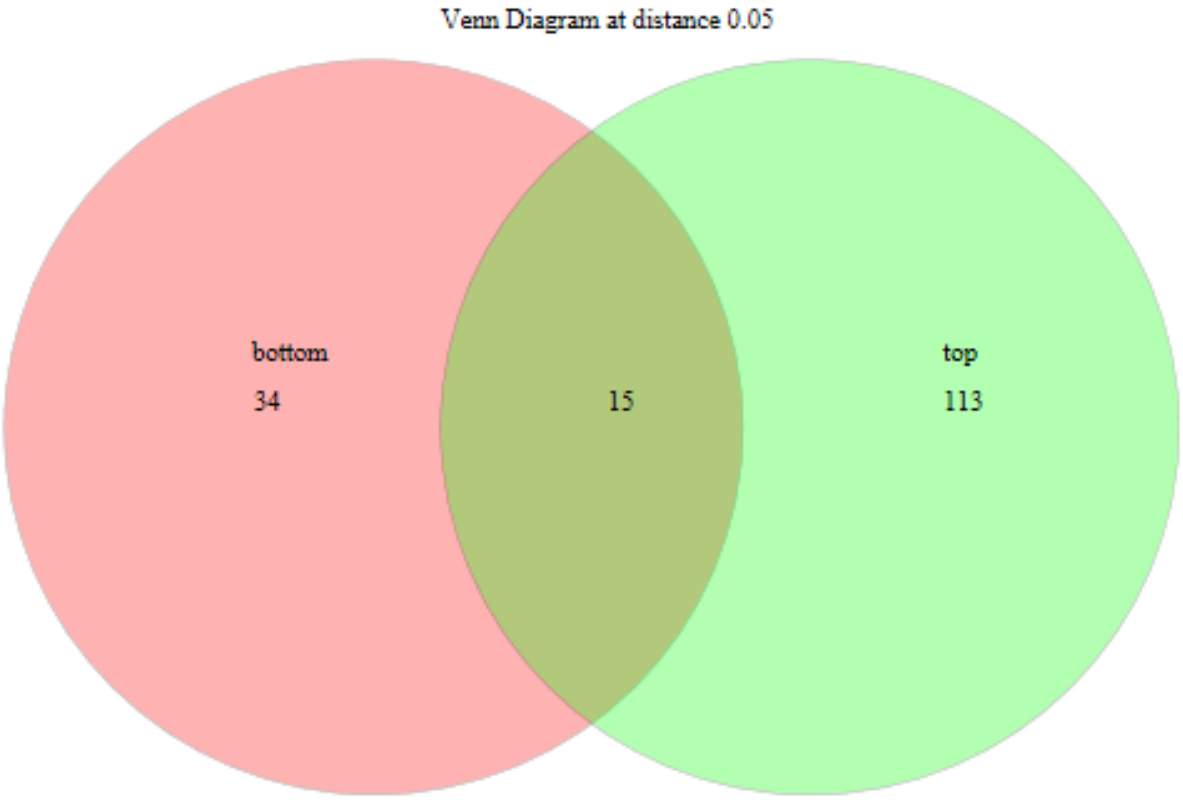
Fig 23 Bacterial diversity with the Top and Bottom layer soil sample at the phylum level

Fig 23 shows the Top and Bottom layer soil bacterial diversity at the phylum level. In the Top layer sample, the largest groups were *Proteobacteria* (33.8%), *Nitrospira* (22.5%), *Euryarchaeota* (19.8%) and *Actinobacteria* (7.7%). In Bottom layer, groups of *Proteobacteria* (32.8%) and *Nitrospira* (61.9%) were the two predominantly phyla. The large representation from these groups is consistent with other acid mine microbiomes (Baker, *et al*, 2003; He, *et al*, 2008; Irene, *et al*, 2011; Madigan, *et al*, 2009 & Tan, *et al*, 2006). Figures 24 shows the community similarity between two samples. Compared with total 128 species existing in the Top layer soil sample, only 49 species were detected from the Bottom layer. The bacterial diversity in the Top layer soil sample is greatly different from the Bottom layer with only 15 shared species. The low bacterial diversity of the Bottom layer soil sample due to the deepest soil sample in acid mine tailings with

lower level of oxygen, higher levels of heavy metal concentrations and lower pH than the Middle and Top layer environments. The Bottom layer is largely defined as an anaerobic condition.

In Top layer sample, one of the most abundant OTU (21.9%) was affiliated with *Leptospirillum ferrooxidans C2-3*, which is greatly similar with the results from Top clone library. The second most abundant OTU (18.2%) was closest matched with 95% pairwise identity value to *Frateuria aurantia DSM 6220*, an acetogen that grow in acidic environment (Johnson, *et al*, 2001 & Swings, *et al*, 1984). This species belonged to *Xanthomonadaceae* at genus level and was also obtained from both pure cultures and clone library with the moderately high proportion (11.9%). OTU with the third largest numbers was *Archaea* with 92% sequence similarity to *Thermoplasma volcanium GSS1*, which was widely collected from many acidia geothermal hot spring environments (Baker, *et al*, 2003 & Burton, *et al*, 2000). *T. volcanium* has moderately thermophilic (45-67℃) growth ranges and was found in some AMD environments (Darland, *et al*, 1970 & Segerer, *et al*, 1988). Another abundant OTU containing about 10% of total number of sequences, affiliated with *Xanthomonadaceae* at genus level, could not be assigned to any species associated with acid environment (sequence similarity was lower than 90%). Compared with 14% clones in the Top layer clone library, *Acidithiobacillus ferrivorans* obtained from pyrosequencing was only 8.4%. Although *Leptospirillum ferrooxidans* and *Acidithiobacillus ferrivorans* were the most common species shared more than 70% of total number clones from the Top layer clone library, no sequences were affiliated to those in the pure isolates. This due to the limitation of culture isolation, while no available iron or sulfur could be used as they are part of iron- or sulfur-oxidizing bacteria.Compared with BLAST results from clone libraries and Pyrosequencing, only isolate D, affiliated with *Xanthomonadales*, was found at the genus level.

In the Bottom layer soil sample, no *Archaea* was obtained and more than 90% of OTUs were affiliated with *Nitrospira* and *Proteobacteria*. Only less than 6% OUTs were from the other phyla with. Of all OTUs representative, the most abundant species were *Leptospirillum ferrooxidans C2-3* (61.5%) and *Acidithiobacillus ferrivorans SS3* (27.2%).



The number of species in group bottom is 49

The number of species in group top is 128

The number of sepecies shared between groups bottom and top is 15

Percentage of species that are shared in groups bottom and top is 9.25926

The total richness for all groups is 162

Fig 24 Venn diagram showing shared OTUs between Top and Bottom layer soil sample.

4. Conclusion

This research has revealed the bacterial diversity in the Top, Middle and Bottom layer of soil from the main Tui mine tailings. Differences between the bacterial communities in the top, middle and bottom soil layers was assessed by the pure culture isolation, a clone library and a pyrosequencing run. The results of the clone library and the pyrosequencing run of the Top layer soil indicated the same predominant bacteria *Leptospirillum ferrooxidans C2-3* and *Acidithiobacillus ferrivorans* were obtained at a high phylogentic level. In the Bottom layer, predominant bacteria were same with Top layer with more than 90% of sequences affiliated with *Leptospirillum ferrooxidans C2-3 and Acidithiobacillus ferrivorans.* However, the Bottom layer bacteria diversity (49 species) obtained from pyrosequencing was much lower than the Top layer (128 species) and the structure of the community was greatly different with only 15 shared species. Results from both Top and Bottom layer samples indicated that pyrosequencing recovered sequences from a broader range of bacterial groups at a lower phylogentic level, Compared with cloning method, pyrosequencing, with identifying many more OTUs, is more powerful in dissecting the structure of microbial communities. Although with the culture-based method, greatly different results were obtained from the molecular methods, it can detect species below the resolution of 454, ie *Acidocella*. It is worth to use the culturing approach, but the molecular approaches give a far more comprehensive picture of the overall diversity.

5. References

Almeida, W. I., Vieira, R. P., Cardoso, A. M., Silveira, C. B., Costa, R. G., Gonzalez, A. M., Paranhos, R., Medeiros, J. A., Freitas, F. A., Albano, R. M. & Martins, O. B. (2009). Archaeal and bacterial communities of heavy metal contaminated acidic waters from zinc mine residues in Sepetiba Bay. Extremophiles. 13, 263-271

Amado Filho, G. M. , Karez, C. S., Andrade, L. R., Yoneshigue-Valentin, Y. & Pfeiffer, W. C. (1997). Effects on growth and accumulation of zinc in six seaweed species. Ecotoxicol Environ Saf, 3, 223–228

Amann, R. I., Ludwig, W. & Schleifer, K. H. (1995). Phylogenetic identification and *in-situ* detection of individual microbial cells without cultivation. *Microbiological Reviews*, 59, 143-169.

Amend, A. S., Seifert, K. A. & Bruns, T. D. (2010). Quantifying microbial communities with 454 pyrosequencing: does read abundance count? *Molecular Ecology*, 19, 5555 – 5565.

Baker, B. J. & Banfield, J. F. (2003). MiniReview: Microbial communities in acid mine drainage. *FEMS Microbiology Ecology*, 44, 139-152.

Bhattacharyya, P., Tripathy, S., Chakrabarti, K., Chakraborty, A. & Banik, P. (2008). Fractionation and bioavailability of metals and their impacts on microbial properties in sewage irrigated soil. *Chemosphere*, 72, 543–550.

Birnboim, H. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, 7, 1513-1523.

Burton, N. P. & Norris, P. R. (2000). Microbiology of acidic, geothermal springs of Montserrat: environmental rDNA analysis. *Extremophiles*, 4, 315-320.

Bradshaw, A. D. & Chadwick, M. J. (1980). *The Restoration of Land: The Ecology and Reclamation of Derelict and Degraded Lund (Studies in Ecology)*, 6. Blackwell Scientific, Oxford, UK.

Cardinate, M., Brusetti, L., Quatrini, P., Borin, S., Puglia, A.M., Rizzi, A., Zanardini, E., Sorlini, C., Corselli, C., and Daffonchio, D. (2004). Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Applied and Environmental Microbiology*, 70, 6147 - 6156.

Chander, K., Brookes, P. C., Harding, S. A. (1995). Microbial biomass dynamics following addition of metal-enriched sewage sluges to a sandy loam soil. *Soil Biol Biochem*, 27, 1409-1421.

Correa-Junior, J. D., Allodi, S., Amado-Filho, G. M. & Farina, M. (2000). Zinc accumulation in phosphate granules of Ucides cordatus hepato-pancreas. *Braz J Med Biol Res*, 33, 217-221.

Coupland, K. & Johnson, D. B. (2008). Evidence that the potential for dissimilatory ferric iron reduction is widespread among acidophilic heterotrophic bacteria. *FEMS Microbiol Lett*, 279, 30-35.

Craw, D. (2003) Metals in the environment. Retrived 24/04/2013 from http://www.otago.ac.nz/geology/research/environmental_geology/metals/sourcetext.html

Dempster, E. L., Pryor, K. V., Francis, D., Young, J. E., & Rogers, H. J. (1999). Rapid DNA extraction from ferns for PCR-based analyses. *Biotechniques*, 27, 66-68.

Darland, G., Brock, T. D., Samsono, W. & Conti, S. F. (1970). A thermophilic, acidophilic mycoplasma isolated from a coal refuse pile. Science 170, 1416-1418.

Díaz, S., Martín-González, A. & Gutiérrez, J. C. (2006). Evaluation of heavy metal acute toxicity and bioaccumulation in soil ciliated protozoa. *Environ. Int.* 32, 711–717.

Dunbar, J., White, S., & Forney, L. (1997). Genetic diversity through the looking glass: effect of enrichment bias. *Applied and Environmental Microbiology,* 63, 1326-1331.

Edwards, K. J., Bond, P. L., Gihring, T. M. & Banfield, J. F. (2000). An archaeal iron-oxidizing extreme acidophile important in acid mine drainage. *Science.* 287, 1796–1799.

Fortin, D., Davis, B. & Beveridge, T. J. (1996). Role of *Thiobacillus* and sulfate-reducing bacteria in iron biocycling in oxic and acidic mine tailings. *FEMS Microbiol Ecol*, 21(1), 11–24.

Garrett, R. & Grisham, C. (2005). Biochemistry (3rd edtion). Thomson Brooks/Cole, Belmont, California.

Ghosh, S., Mahapatra, N. R. & Banerjee, P. C. (1997). Metal resistance in *Acidocella* strains and plasmid-mediated transfer of this characteristic to A*cidiphilium multivorum* and *Escherichia coli. Applied and Environmetal Microbiology,* 63(*11*), 4523-4527.

González-Toril, E., Llobet-Brossa, E., Casamayor, E. O., Amann, R. & Amils, R. (2003). Microbial ecology of an extreme acidic environment, the Tinto River. *Appl Environ Microbiol* 69: 4853–4865.

Hallberg, K. B. & Johnson, D. B. (2001). Biodiversity of acidophilic prokaryotes. *Adv Appl Microbiol,* 49: 37–84.

Hao, D. C., Ge, G. B. & Yang, L. (2008). Bacterial diversityof Taxus rhizosphere: culture-independent and culture-dependent approaches. *Federation of European Microbiological Societies (FEMS),* 284,204-212.

Hirsch, P. R., Mauchline, T. H. & Clark, I. M. (2010). Culture-independent molecular techniques for soil microbial ecology. *Soil Biology & Biochemistry*, 42, 878-887.

Hu, Q., Qi, H. Y., Zeng, J. H. & Zhang, H. X. (2006). Bacterial diversity in soils around a lead and zinc mine. *Journal of Environmental Sciences,* 19, 74-79.

Huang, L. N., Zhou, W. H., Hallberg, K. B., Wan, C. Y., Li, J. & Shu. W. S. (2011). Spatial and temporal analysis of the microbial community in the tailings of a Pb-Zn mine generating acidic drainage. *Applied and Environmental Microbiology*, 77(*15*), 5540-5544.

Irene, S., Nuria, R, Riacardo, A. & José, L. S. (2011). Microbial diversity in anaerobic sediments at Río Tinto, a naturally acidic environment with high heavy metal content. *Applied and Environmental Microbiology*, 77(*17*), 6085-6093.

Johnson, D. B. & Hallberg, K. B. (2003). The microbiology of acidic mine waters. *Res Microbiol,* 154, 466–473.

Johnson, D. B., Rolfe, S., Hallberg, K. B. & Iversen, E. (2001). Isolation and phylogenetic characterization of acidophilic microorganisms indigenous to acidic drainage waters at an abandoned Norwegian copper mine. Environ Microbiol 3: 630–637.

Junior, R. G. S. L., Ar**aújo, F. G.,** Maia, M. F. & Pinto, A. S. S. B. (2002). Evaluation of heavy metals in fish of the Sepetiba and Ilha Grande Bays, Riode Janeiro, Brazil. *Environ Res,* 89, 171–179.

Kaeberlein, T., Lewis, K. & Epstein, S. S. (2002). Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science,* 296:1127-1129.

Kelly, D. P. & Wood, A. P. (2000). Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. *International journal of systematic and evolutionary microbiology,* 50: 511–516.

Khan, S., Hesham, A. E., Qiao, M., Rehman, S. & He, J. Z. (2010). Effects of Cd and Pb on soil microbial community structure and activities. *Environ Sci Pollut Res,* 17, 288-296.

Killmann, H., Benz, R., & Braun, V. (1996). Properties of the FhuA channel in the *Escherichia* coli outer membrane after deletion of FhuA portions within and outside the predicted gating loop. *J Bacteriol,* 178, 6913-20.

Krzaklewski, W., and M. Pietrzykowski. (2002). Selected physico-chemical properties of zinc and lead ore tailings and their biological stabilisation. *Water Air Soil Pollut.* 141, 125–142.

Kwon, S., Kim, T.S., Yu, G.H., Jung, J.H. & Park, H.D. (2010). Bacterial community composition and diversity of a full-scale integrated fixed-film activated sludge system as investigated by pyrosequencing. *J Microbiol Biotechnol,* 20, 1717–1723.

Livingston, M. E. (1987). Preliminary studies on the effects of past mining on the aquatic environment, Coromandel Peninsula, Water & Soil Miscellaneous Publication No.104. Water and Soil Directorate, Ministry of Works, Wellington, New Zealand.

Luís, A. T., Teixeira, P., Almeida, S. F. P., Ector , L., Matos, J. X. & Ferreira da Silva, E. A. (2009). Impact of Acid Mine Drainage (AMD) on Water Quality, Stream Sediments and Periphytic Diatom Communities in the Surrounding Streams of Aljustrel Mining Area (Portugal). *Water Air Soil Pollut,* 200, 147–167.

Madigan, M. T., Martinko, J. M., Dunlap, P. V. & Clark, D. P. (2009). Brock of biology of microorganisms (12th Ed.). San Franciso, USA: Pearson Benjamin Cummings.

Martínez, C. E., Yáñez, C., Yoon, S. J. & Bruns, M. A. (2007). Biogeochemistry of metalliferous peats: sulfur speciation and depth distributions of dsrAB genes and Cd, Fe, Mn, S, and Zn in soil cores, *Environ. Sci. Technol.* 41,5323–5329.

Mendez, M. O., Neilson, J. W. & Maier, R. M. (2008), Characterization of a bacterial community in an abandoned semiarid lead-zinc mine tailing site. *Applied and Environmental Microbiology,* 74(12), 3899-3907.

Morrell, W. J., Stewart, R. B., Gregg, P. E. H., Bolan, N. S. & Horn, D. (1996). An assessment of sulphide oxidation in abandoned base-metal tailings, Te Aroha, New Zealand. *Environmental Pollution,* 94(2), 217-225.

Morrell, W. J. (1997). An assessment of the revegetation potential of base-metal tailings from the Tui mine, Te Aroha, New Zealand. Retrived 24/01/2012 from http://www.massey.ac.nz/massey/home.cfm.

Mullis, K. B. & Faloona, F. A. (1987). specific synthesis of DNA invitro via a polymerase catalysed chain-reaction. *Methods in Enzymology,* 155, 335-350.

Nabi Bidhendi, G. R., Karbassi, A. R., Nasrabadi, T. & Hoveidi, H. (2007). Influence of copper mine on surface water quality. *Int. J. Environ. Sci. Tech.*, 4 (1), 85-91.

Niderberger, T. D. (2005). *The microbial ecology and colonisation of surfaces in selected hot spring – a molecular- and culture- based approach.* Ph.D. Thesis, University of Waikato.

Nyrén, P. (1987). Enzymatic method for continuous monitoring of DNA polymerase activity. *Analytical Biochemistry,* 167: 235-238.

Pang, L. (1995). Contamination of groundwater in the Te Aroha area by heavy metals from an abandoned mine. *New Zealand J. Hydrol.*, 33(l), 17-34.

Pérez-de-Mora, A., Burgos, P., Madejón, E., Cabrera, F., Jaeckel, P. & Scholter, M. (2006). Microbial community structure and function in a soil contaminated by heavy metals: effects of plant growth and different amendments. *Soil Biol Biochem.* 38, 327–341.

Petrosino, J. F., Highlander, S., Luna, R. A., Gibbs, R. A. & Versalovic, J. (2009). Metagenomic pyrosequencing and microbial identification. *Clinical Chem.*, 55, 856–866.

Pierzynski, G. M. & Schwab, A. P. (1993). Bioavailability of zinc, cadmium, and lead in a metal-contaminated alluvial soil, *J. Environ. Qual.* 22, 247–254.

Quince, C., Lanzén, A., Curtis, T. P., Davenport, R. J., Hall, N., Head, I.M., Read, L. F. & Sloan, W. T. (2009). Accurate determination of microbial diversity from 454 pyrosequencing data. Nature Methods, 6, 639–627

Quince, C., Lanzén, A., Davenport, R. J., Turnbaugh, P. J. (2011). Removing noise from pyrosequenced amplicons. *BMC Bioinformatics*, 12: 38 doi: 10.1186/ 1471–2105–12–38.

Ritcey, G. M. (1989). Tailings Management. *Problems and Solutions in the Mining Industry (1st edn),* 6. Elsevier, Amsterdam, The Netherlands.

Roh, S.W., Kim, K.H., Nam, Y. D., Chang, H. W., Park, E.J. & Bae, J. W. (2010). Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. *ISME J,* 4, 1 ‑ 16

Ronaghi, M., Karamohamed, S., Pettersson, B., Uhlen, M. & Nyren, P. (1996). Real-time DNA sequencing using detection of pyrophosphate release. *Anal. Biochem.*, 242, 84–89.

Rothberg, J. M. & Leamon, J. H. (2008). The development and impact of 454 sequencing. *Natural Biotechnol.* 26:1117–1124.

Sabti, H., Hossain, M. M., Brooks, R. R. & Sterwart, P. B. (2000). The current environmental impact of base-metal mining at the Tui Mine, Te Aroha, New Zealand. *Journal of the Royal Society of New Zealand*, 30 (2), 197-208.

Sanger, F. (1988). DNA sequencing with chain terminating inhibitors. *Current Contents/Life Sciences*, 23-23.

Santegods, C., Nold, S. & Ward, D. (1996). Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from a hot spring cyanobacterial mat. *Applied and Environmental Microbiology*, 62, 3922-3928.

Schippers, A., Hallmann, R., Wentzien, S. & Sand, W. (1995). Microbial diversity in uranium mine waste heaps. *Applied Environmental Microbiology*, 61: 2930 – 2935.

Schloss, P. D. (2009). A high-throughput DNA sequence aligner for microbial ecology studies. PLoS One, 4, e8230.

Segerer, A., Langworthy, T. A. & Stetter, K. O. (1988). Thermoplasma acidophilum and Thermoplasma volcanium sp. nov. from Solfatare Fields. Syst. *Appl. Microbiol.* 10, 161-171.

Sjoling, S. & Cowan, D. A. (2003). High 16S rDNA bacterial diversity in glacial meltwater lake sediment, Bratina Island, Antarctica. *Extremophiles*, 7, 275-282.

Southam, G. & Beveridge, T. J. (1992). Enumeration of *Thiobacilli* within pH-neutral and acidic mine tailings and their role in the development of secondary mineral soil. *Applied Environmental Microbiology*, 58: 1904–1912.

Stolz, J. F. & Oremland, R.S. (1999). Bacterial respiration of arsenic and selenium, *FEMS Microb.* 23, 615–617.

Sutcliffe, I. C., & Hutchings, M.I. (2007) Putative lipoproteins identified by bioinformatic genome analysis of Leifsonia xyli ssp. xyli, the causative agent of sugarcane ratoon stunting disease. Mol Plant Pathol 8: 121–128.

Swings, J., De Ley, J. & Gillis, M. (1984). Genus *Frateuria* Swings, Gillis, Kersters, De Vos, Gossele and De Ley. In *Bergery's Manual of Systematic Bacteriology*, 210-213.

Tan, G. L., Shu, W. S., Hallberg, K. B., Li, F., Lan, C. Y. & Huang, L. N. (2006). Cultivation-dependent and cultivation-independent characterization of the microbial community in acid mine drainage associated with acidic Pb/Zn mine tailings at Lechang, Guangdong, China. *FEMS Microb.* 59, 118–126.

Tay, K. A. (1980). Geochemistry and Environmental Impact of the Discharge of Heavy Metals from the Tui Mine and its Tailings. *MSc. Thesis*, University of Waikato, Hamilton, New Zealand.

Wakao, N., Yasuda, T., Jojima, Y., Yamanaka, S. & Hiraishi, A. (2002). Enhanced groth of *Acidocella facilis* and related acidophilic bacteria at high concentration of aluminium. *Microbes and Environments*, 17(*2*), 98-104.

Waikato regional council. (2011). Tui mine remediation project. Retrived 24/01/2012 from http://www.waikatoregion.govt.nz/ .

Wagner, M., Amann, R., Lemmer, H., & Schleifer, K. H. (1993). Probing activated-sludge with oligonucleotides specific for Proteobacteria -

Inadequacy of culture-dependent methods for describing microbial community structure. 59, 1520-1525.

Wang, Y. P., Shi, Y. J., Wang, H., Lin, Q., Chen, X. C. & Chen, Y. X. (2007). The influence of soil heavy metals pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter. *Ecotoxicol Environ Saf,* 67, 75–81.

Ward, D., Weller, R. & Bateson, M. (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature,* 345(*6270*), 63-65.

Weissberg, B. G. & Wodzicki, A. (1970). Geochemistry and hydrothermal alteration and origin of sulphide mineralisation at the Tui mine, Te Aroha, New Zealand. New Zealand Journal of Science, 13, 36-60.

Williams, T. C. (2011). *The Microflora of the Huhu Grub*. Mater Thesis, University of Waikato.

Woese, C. R., Kandler, O. & Wheelis, M. L. (1990). Towards a natural system of organisms - proposal for the domains archaea, bacteria, and eucarya. *Proceedings of the National Academy of Sciences of the United States of America*, 87, 4576-4579.

Xie, J., He, Z., Liu, X. X., Liu, X. D., Van Nostrand, J. D., Deng, Y., Wu, L., Zhou, J. & Qiu, G. (2011). GeoChip-Based analysis of the functional gene diversity and metabolic potential of microbial communities in acid mine drainage. *Applied and Environmental Microbiology,* 77(3), 991-999

Zhang, H. B., Yang, M. X., Shi, W., Zheng, Y., Sha, T. & Zhao, Z. W. (2007). Bacterial diversity in mine tailings compared by cultivation and cultivation-independent methods and their resistance to lead and cadmium. *Microbial Ecology,* 54, 705-712.

Zhang, X. J., Yue, S. Q., Zhong, H. H., Hua, W. Y., Chen, R. J., Cao, Y. F. & Zhao, L. P. (2011). A diverse bacterial community in an anoxic quinoline-degrading bioreactor determined by using pyrosequencing and clone library analysis. *Applied Microbiology Biotechnology,* 91(*2*), 425-434.

Zhou, Y., Yao, J., Martin, M. F., Chen, Y., Chen, H. Mohammad, R., Zhuang, R., Chen, H. Wang, F., Maskow, T. & Zaray, G. (2009). A combination method to study microbial communities and activities in zinc contaminated soil. *Journal of Hazardous Materials*, 169, 875–881.

Zouboulis, A. I., Loukidou, M. X. & Matis, K. A. (2004). Biosorption of toxicmetals fromaqueous solutions by bacteria strains isolated from metal-polluted soils, *Process Biochem,* 39, 909–916.

6. Appendices

6.1 Appendix A: Culture Mediums

1. 100(3.0) Medium (Acidobacterium Medium 1)

$(NH_4)_2SO_4$	2.0 g
KCl	0.1 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Glucose	1.0 g
Yeast Extract	0.1 g
Distilled water	1.0 L

Adjust pH to 3.0 with HCl before sterilization.

2. 400(3.0) Medium (Acidobacterium Medium 2)

$(NH_4)_2SO_4$	2.0 g
KCl	0.1 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Glucose	4.0 g
Yeast Extract	0.4 g
Distilled water	1.0 L

Adjust pH to 3.0 with HCl before sterilization.

3. 4Mg0(3.0) Medium (Medium 3)

Same with 400(3.0) medium with adding 250mM/L of $MgCl_2$

4. 40Zn(3.0) Medium (Medium 4)

Same with 400(3.0) medium with adding 250mM/L of $ZnSO_4$

5. **4MgZn(3.0) Medium (Medium 5)**

   Same with 400(3.0) medium with adding 250mM/L of each $MgCl_2$ and $ZnSO_4$

6. **400(4.5) Medium (Medium 6)**

   Same with 400(3.0) medium with increasing the pH to 4.5

7. **400(5.0) Medium (Medium 7)**

   Same with 400(3.0) medium with increasing the pH to 4.5

8. **4Mg0(4.5) Medium (Medium 8)**

   Same with 400(3.0) medium with increasing pH to 4.5 and adding 250mM/L of $MgCl_2$

9. **4Mg0(5.0) Medium (Medium 9)**

   Same with 400(3.0) medium with increasing pH to 5.0 and adding 250mM/L of $MgCl_2$

10. **40Zn(4.5) Medium (Medium 10)**

    Same with 400(3.0) medium with increasing pH to 4.5 and adding 250mM/L of $ZnSO_4$

11. **40Zn(5.0) Medium (Medium 11)**

    Same with 400(3.0) medium with increasing pH to 5.0 and adding 250mM/L of $ZnSO_4$

12. **4MgZn(4.5) Medium (Medium 12)**

    Same with 400(3.0) medium with increasing pH to 4.5 and adding 250mM/L of each $MgCl_2$ and $ZnSO_4$

13. **4MgZn(5.0) Medium (Medium 13)**

    Same with 400(3.0) medium with increasing pH to 5.0 and adding 250mM/L of each $MgCl_2$ and $ZnSO_4$

14. **400(3.5)-G Medium (Medium 14)**

Same with 400(3.0) medium with increasing pH to 3.5 and adding 1% of gelrite to solidify (for gelrite plate, 1ml of $Mg^{2+}$was added before pour the plate, this will help solidify)

15. **400(4.5)-G Medium (Medium 15)**

    Same with 400(3.0) medium with increasing pH to 4.5 and adding 1% of gelrite to solidify

16. **400(5.0)-A Medium (Medium 16)**

    Same with 400(3.0) medium with increasing pH to 5.0, adding 250mM/L of $MgCl_2$ and 1.5% of agar to solidify

17. **4Mg0(3.5)-G Medium (Medium 17)**

    Same with 400(3.0) medium with increasing pH to 3.5, adding 250mM/L of $MgCl_2$ and 1% of gelrite to solidify

18. **4Mg0(4.5)-G Medium (Medium 18)**

    Same with 400(3.0) medium with increasing pH to 4.5, adding 250mM/L of $MgCl_2$ and 1% of gelrite to solidify

19. **4Mg0(5.0)-A Medium (Medium 19)**

    Same with 400(3.0) medium with increasing pH to 5.0, adding 250mM/L of $MgCl_2$ and 1.5% of agar to solidify

20. **40Zn(3.5)-G Medium (Medium 20)**

    Same with 400(3.0) medium with increasing pH to 3.5, adding 250mM/L of $ZnSO_4$ and 1% of gelrite to solidify

21. **40Zn(4.5)-G Medium (Medium 21)**

    Same with 400(3.0) medium with increasing pH to 4.5, adding 250mM/L of $ZnSO_4$ and 1% of gelrite to solidify

22. **40Zn(5.0)-A Medium (Medium 22)**

    Same with 400(3.0) medium with increasing pH to 5.0, adding 250mM/L of $ZnSO_4$ and 1.5% of agar to solidify

23. 4MgZn(3.5)-G Medium (Medium 23)

Same with 400(3.0) medium with increasing pH to 3.5, adding 1% of gelrite to solidify and 250mM/L of each $MgCl_2$ and $ZnSO_4$

24. 4MgZn(4.5)-G Medium (Medium 24)

Same with 400(3.0) medium with increasing pH to 4.5, adding 1% of gelrite to solidify and 250mM/L of each $MgCl_2$ and $ZnSO_4$

25. 4MgZn(5.0) Medium (Medium 25)

Same with 400(3.0) medium with increasing pH to 5.0, adding 1.5% of agar to solidify and 250mM/L of each $MgCl_2$ and $ZnSO_4$

26. LB medium (Medium 26)

Tryptone	10.0 g
Yeast Extract	5.0 g
NaCl	10 g
Distilled water	1.0 L

Adjust pH to 7.0 with HCl and NaOH before sterilization.

27. LB-A medium (Medium 26)

Same with LB medium with adding 1.5% of agar to solidify

28. LB-K medium (Medium 26)

Same with LB medium with adding 1ml of 50mg/ml kanamycin in 1 liter of medium after autoclave at about 55℃.

29. LB-A-K medium (Medium 26)

Same with LB medium with adding 1.5% of agar to solidify and finally adding 1ml of 50mg/ml kanamycin in 1 liter of medium after autoclave at about 55℃

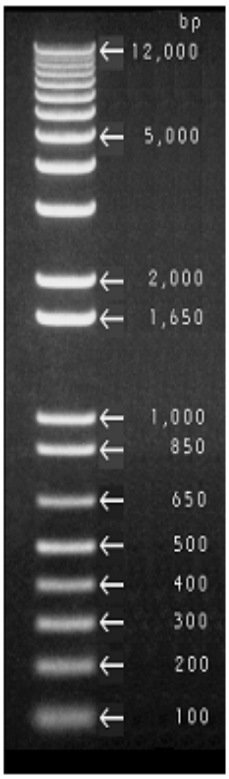
6.2 *Appendix B: Agarose gel electrophoresis*

50× TAE Buffer (~pH 8.5)

Tris(hydroxymethyl) aminomethane.....242g
Glacial acetic acid 57.1ml
0.5M EDTA (pH 8.0).....100ml

Make up to 1L with Milli-Q water and check pH. Dilute to 1×working concentration with MilliQ water.

6.3 *Appendix C: Map of 1kb plus ladder*



1 Kb Plus DNA Ladder
0.7 µg/lane
0.9% agarose gel
stained with ethidium bromide

Structure of Fragments in 1-Kb Increments:

5′-GATCC ——— G
G ——— CCTAG-5′
$(996\ bp)_n$

Notes:
During 1% agarose gel electrophoresis with Tris-acetate (pH 7.5) as the running buffer, bromophenol blue migrates together with the 500 bp band.

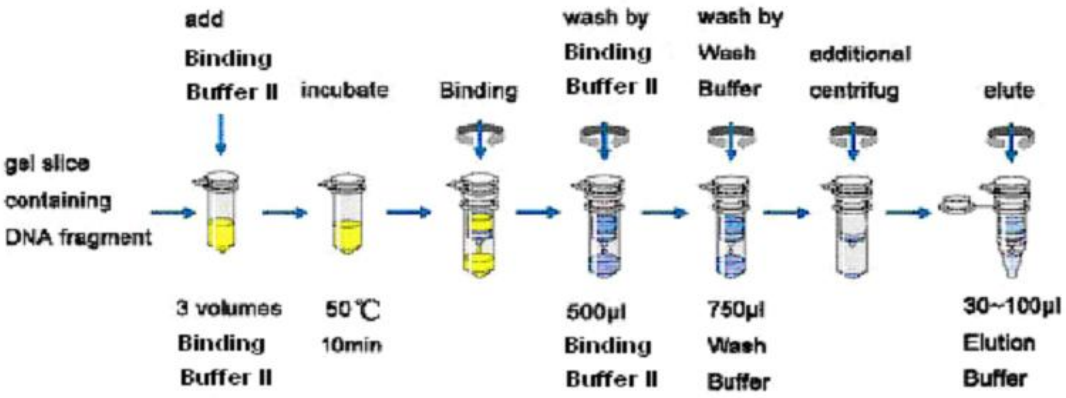
The 1650 bp band is generated from pUC. The bands smaller than 1000 bp are derived from lambda DNA.

6.4 *Appendix D: Procedure of Isopropanol clean up method*

This is for 10 µ l, the speeds are also for the plate centrifuge we use

1. Add 32µL (3.2x sample volume) of 70% Isopropanol to each well, spin down, and mix well
2. Centrifuge the plate at 3000 RPM for 30 minutes
3. Carefully inverse spin (330 RPM for 1 minute) to remove supernatant
4. Gently add 100µl of 70% ethanol
5. Centrifuge the plate at 3000 RPM for 5 minutes
6. Carefully inverse spin (330 RPM for 1 minute) to remove supernatant
7. Gently add 100µl of 70% ethanol
8. Centrifuge the plate at 3000 RPM for 5 minutes
9. Carefully inverse spin (330 RPM for 1 minute) to remove supernatant
10. Air dry away from light for 10 minutes

6.5 *Appendix E: Procedure of QuickClean II Gel Extraction Kit*



1. Excise the DNA band from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
2. Weigh the gel slice. Then Add 3 volumes of Binding Buffer II to 1 volume of gel slice (100mg≈100ul),the gel slice should not be more than 400mg per test)
3. Incubate at 55℃for 10 minutes with occasional vortexing or until the gel slice has been completely dissolved. (Note: 50℃ is used for low-melt agarose).
4. Transfer the sample to Spin column, centrifuge at 6,000×g for 1 min. Discard the flow through until the sample is processed completely. If the sample volume is more than 750 µl, load and centrifuge again using the same column.
5. Add 500 µl Binding Buffer II to Spin column, centrifuge at 12,000 ×g for 30 ~ 60s. Discard all flow through liquid.
6. Add 750 µl Wash Buffer to Spin column, centrifuge at 12,000 ×g for 30~60s. Discard all flow through liquid.
7. Centrifuge at 12,000 ×g for an additional 1 minute and transfer the Spin column to a sterile 1.5ml microcentrifuge tube.
8. Add 30~100 µl Elution Buffer, ddH2O or TE Buffer to the Spin column and let it stand for 1 minute at room temperature. DNA pellet was not sufficiently washed by using less than 20 µl of Elution Buffer. Adjust the volume of elution buffer according to needs if necessary.

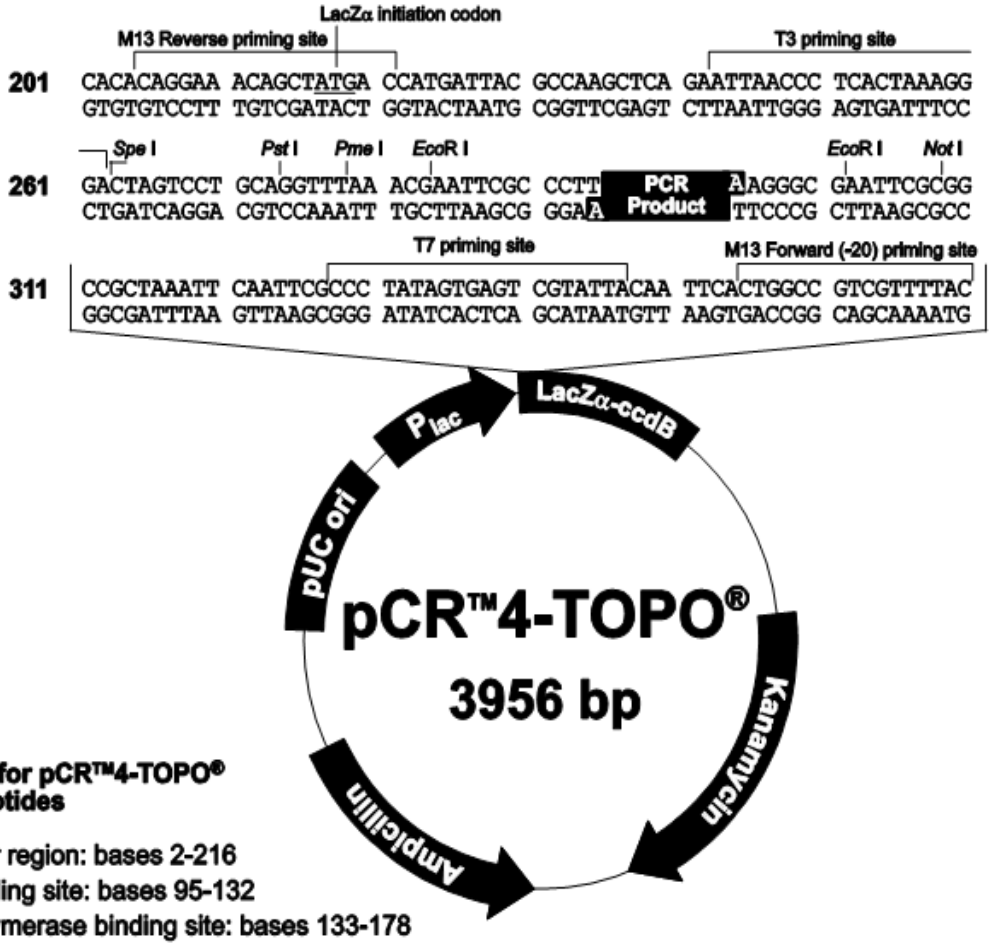
9. Centrifuge at 12,000 ×g for 1 minute. The buffer in the micro-centrifuge tube contains the DNA. The extracted DNA can be stored at -20℃ if not used immediately.

6.6 *Appendix E: Procedure of QuickClean II PCR Purification Kit*

1. Transfer the PCR or enzymatic reaction product to 1.5 ml micro-centrifuge tubes.
2. Add 2 volumes of Binding Buffer to 1 volume of the PCR or enzymatic reaction product and gently mix well. Ensure that the maximum binding buffer input every time does not exceed 200 µl.
3. Apply all above mixture to the Spin column by decanting or pipetting. And centrifuge for only 1 min at 6,000 × g.
4. Discard all flow-through and place the column back in the same tube.
5. Wash the Spin column by 650 µl Wash Buffer in centrifuging for 30-60 seconds at 12,000×g. Discard the flow-through liquid and repeat Step 5 again.
6. Centrifuge for an additional 1 minute at 12,000×g and transfer the Spin column to a sterile 1.5 ml microcentrifuge tube. By this step, there will be no residual liquid in the column.
7. Add 50 µl Elution Buffer, ddH2O or TE Buffer to the center of the Spin column and let it stand for 1 minute at room temperature. Then centrifuge for 1 minute at 12,000×g. The volume of Elution Buffer should be adjusted if necessary.
8. Store the micro-centrifuge tube containing purified plasmid DNA at -20℃ if not use immediately.

6.7 Appendix F: Map of pCR™ 4-TOPO®

The following map shows the features of pCR™4-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The sequence of pCR™4-TOPO® is available from www.lifetechnologies.com or by contacting Technical Support (page 28).



Comments for pCR™4-TOPO®
3956 nucleotides

lac promoter region: bases 2-216
- CAP binding site: bases 95-132
- RNA polymerase binding site: bases 133-178
- Lac repressor binding site: bases 179-199

Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
- LacZα portion of fusion: bases 217-497
- *ccd*B portion of fusion: bases 508-810

T3 priming site: bases 243-262
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
Kanamycin promoter: bases 1021-1070
Kanamycin resistance gene: bases 1159-1953
Ampicillin (*bla*) resistance gene: bases 2203-3063 (c)
Ampicillin (*bla*) promoter: bases 3064-3160 (c)
pUC origin: bases 3161-3834

(c) = complementary strand

6.8 *Appendix G: EMA Treatment*

1. In the dark*, add 1 µL EMA (10mg/µL) to 199 µL MQ-H2O and vortex to mix. (complete darkness except for a small amount of light from a computer screen or torch)
2. Add this working dilution to the PCR master mix at a concentration of 1%. (if the master mix is 500 µL, add 5 µl diluted EMA.)
3. Vortex master mix (still in the dark).
4. Incubate tube on ice in complete darkness for 1 min (allowing EMA to bind dsDNA).
5. Incubate by lying tube on side on ice and place it 20 cm below a strong incandescent light for 1 min (deactivates unbound EMA).
6. Place tube directly back into ice.
7. Proceed by aliquoting master mix into PCR tubes and adding DNA.

6.9 *Appendix H: Procedure of UltraClean® 15 DNA Purification Kit*

1. Determine weight of agarose gel band slice in a 1.5-2.0 ml tube.
2. Add 3 volumes of **ULTRA SALT** (example: if gel weight = 0.1 grams, add 0.3 ml ULTRA SALT).
3. Mix well.
4. Incubate at 55℃ to melt agarose gel. Mix occasionally by shaking thoroughly. Completely melt gel before proceeding (approximately 5 minutes).
5. Resuspend **ULTRA BIND** by vortexing at highest speed with **ULTRA BIND** tube in a horizontal position until homogeneous (this takes about 30 seconds to 1 minute).
6. Add **ULTRA BIND**: 5 µl plus 1 µl per µg of DNA you expect to recover (e.g. for 0-1µg of DNA use 6µl).
7. Incubate 5 minutes at room temperature. **Important:** Mix several times during this binding step by flicking the tube for small volumes or shaking and inverting for large volumes.
8. Centrifuge 5 seconds. Remove supernatant and set aside. (If DNA does not bind, it can be recovered. See Hints and Troubleshooting Guide).
9. Ensure 250 ml of 100% ethanol was added to the **ULTRAWASH before using for the first time**. Re-suspend the pellet in 1 ml of **ULTRA WASH/EtOH** by vortexing 5-10 seconds.
10. Centrifuge 5 seconds. Discard supernatant.
11. Centrifuge 5 seconds again. Remove all traces of **ULTRAWASH/EtOH** by aspirating with a narrow pipet tip.
12. Resuspend the pellet in water or TE (10 mM Tris, 0.1 mM EDTA). Use a volume twice that of the ULTRA BIND silica used in step 6, and resuspend by pipetting until homogeneous. Don’t vortex. Disrupt pellet with the pipet tip and then pipet up and down to make sure the entire pellet is re-suspended.
13. Incubate up to 5 minutes at room temperature.

14. Centrifuge 1 minute.
15. Remove the supernatant immediately and transfer it to a new tube. If white silica is carried over, spin again and transfer the supernatant to a new tube. DNA is now ready to use.

For the 454 Preparation, always perform the final spin twice to ensure all beads are removed.

6.10*Appendix I: Protocol of QuBit dsDNA HS Kit*

Proceed as per manufacturer's instructions, including Standards 1 and 2 to calibrate the instrument.

1. Add 10 µl of each standard to 190 µl HS buffer.

2. Use 1-5 µl of DNA and 199-195 µl of HS buffer for sample quantitation, depending on the volume of sample available and the concentration expected. (e.g., if expecting a concentration of 2.5 ng/µl, then adding 5 µl would result in a concentration higher on the standard curve, which could be more accurate. If only 10 µl DNA is available, only 1 µl of DNA may be spared for quantitation.

3. Record the raw values from QuBit (total amount of DNA in the reaction tube in ng/ml) and calculate the final concentrations using the formula obtained from fitting a linear regression through the standard curve in Excel*. Take into consideration the volume of sample DNA used. The standards are represented by the total amount of DNA in the reaction (e.g., 10 µl of 10 ng/µl = 100 ng standard).

6.11 *Appendix J KAPA qPCR analysis*

1. Dilute the 2 x 108 theoretical molecules/µl amplicon 1:100 (2ul to 200 µl) in 10 mM Tris-HCl, pH 8.0 + 0.05% Tween 20.
2. Dilute the 2 x 108 theoretical molecules/µl amplicon 1:200 (2ul to 400 µl) in 10 mM Tris-HCl, pH 8.0 + 0.05% Tween 20.
3. Run all 6 standards provided with the kit and all amplicon dilutions in triplicates
4. For a 10 µl reaction, add 6 µl Master Mix and 4 µl DNA. Use the following cycling conditions:

Activation: 95°C for 5 min. 35 cycles: Denaturation: 95°C for 30s Annealing/Extension/Data Acquisition: 60°C for 45s (90s for templates over 700bp or for Rapid Prep libraries)

5. To start a run:

Open a previous run in the KAPA folder in ICTAR on Sol00FSEN server Click “New”, “Perform Last Run” Set the Gain Manually on the Green channel to 7.67. Close the Set Gain box and start the run.

6. Analyze the results of the run, allowing the threshold to be set automatically.
7. Confirm 90-110% reaction efficiency for standards.
8. Check to see that the triplicates are accurate and remove any the look likeoutliers
9. Paste the results into an Excel Spreadsheet to analyze.
10. Use the “2011-16-09 Kapa run (analysis) – use as template” Excel spreadsheet in the KAPA Sarah Folder in the KAPA folder in ICTAR as a template for calculations
11. From the Calculated Concentration obtained from the RotorGene 6000 Software, calculate the average undiluted amplifiable sample concentrations:

Actual amplicon concentration = (avg. copies/µl x 460 x dilution factor)/(average fragment length) eg. Dilution factor for 1:100 diluted sample = 100. Average the 100 and 200 dilution concentrations.

2 x 108 theoretical molecules/µl as quantified by QuBit or plate reader translates approximately 4 x 107 amplifiable molecules/µl quantified by Kapa.

6.12 Appendix K: 16S rRNA gene sequences

6.12.1 DNA sequences from pure culture

>Pure_A
TCTAATACTGGATATGACAACTGATCGCATGGTCTGGTTGTGGAAAGATTTTTTGGTTGGGGATGGACTCGCGGCCTA
TCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGC
AACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGTAGGGAAGAAGCGAAAGTGACGGTACCTG
CAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTATTGG
GCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAATCCCGAGGCTCAACCTCGGGCTTGCAGTGGGTACG
GGCAGACTAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACC
GATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGTTGGGCGCTAGATGTAGGGACCTTTCCACGGTTTCTGTGTCGTAGCTAACGCATTA
AGCGCCCCGCCTGGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGA

>Pure_B
CCGTAAGTCGTAACAAGGTACCGTAAGGTAACCGTAGATTTGATCCTGGCTCAGGATAAGTCGTAACAAGGTAACCG
TAAGTAATCCCTAGTGATCCCTGAGGCTCAAGGCGTTGTCGCCTTGGAAGGAGCCTGCGTCTGATTAGCTTGTTGGTG
GGGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGC
CCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGTGT
GAAGAAGGTCTTCGGATTGTAAAGCACTTTTGGCAGGGACGATGATGACGGTACCTG

>Pure_D
TACTAGCGATTCCGACTTCACGAAGTCGAGTTGCAGACTTCGATCCGGACTGGGATCGGCTTTCTGGGATTGGCTCCA
CCTCGCGGTATTGCAACCCTCTGTACCGACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGGGCCATGAGGACTTG
ACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTTCCCACCATTACGTGCTGGCAACTAAGGA
CAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTT
CCGATTCCCGAAGGCACTCCCGCATCTCTGCAGGATTCCGGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCAT
CGAATTAAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCC
CAGGCGGCGAACTTAACGCGTTAGCTTCGACACTGATCTCCGAGTTGAGACCAACATCCAGTTCGCATCGTTTAGGG
CGTGGACTACCAGGGTATCTAATTCCTGTTTGCTCCCCACGCTTTTCGTGCTTTCAGCGTCAGTG

>Pure_G
GGATACGAACTGCGGAGGCATCTCCAGCAGTTGGAAAGAACTTCGGTCAAGGATGGACTCGCGGCCTATCAGGTAG
TTGGTGAGGTAAAGGCTCACCAAGCCTACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGC
GTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAAAA
GCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAG
AGCTCGTAGGCGGTCTGTCGCGTCTGCTGTGAAAACCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACT
AGAGTGCGGTAGGGGAGAATGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGA
AGGCAGTTCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGTTGGGGCGCTAGATGTGGGGACCATTCCACGGTTTCCGTGTCGCAGCTAACGCATTTAAGCGC
CCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGGCCCGCACAAG

>Pure_K
AACTTAGGTACCTTGGTACAACTTACGGTACCTTGTTACGACTTATGGTTACCCTGAAACAAATTATGGTTACCTTAC
GCTTATCTTGGTACAACTTAAGCTTACCCTGGTACCACTTAGGTTGCCCCTTGCACTCCGAACTGAGACCAGCTTTGA
TAGGATTGGCTCCATCTCGCGACTTCGCTTCCCGTTGTACTGGCCATTGTAGTACGTGTGTAGCCCAAGTCATAAGGG

GCATGATGATTTGACGTCATCCCCGCCTTCCTCCGGTTTGTCACCGGCAGTCATTCTAGAGTGCCCAGCCTTACCTGT
GGGCAACTAAAACCAAGGGTTGGGCTCGTTGCGGAACTTAACCCAACATCTCAGAACACAAGCTGACAACAACCAT
GCACCACCTGCCTCCTCTGTCCCAAAGGCCGCCACTATCTCTAGGGAATTCGAAGGGATGTCAAGACTTGTTAAGGT
TCTTCGGGTTGCTTCAAATTAAACCACATACTCCACTGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTCCAGTCTGG
CAACCGTACTCCCCCAGGCGAAATGCTTAATGTGTTACTTTCGGCCCCAAGGGTATCAAAACCCCCTAACACCTAGA
ATTCATCGTTTACGGCTGTGAATTACCAGGGTATCTAATCTGGTTTGCTCCCCACCCTTTCGCGCCTCCCGTCATTTAC
AGCCCAAGAGAGTCCCTTTCCCACTGGTGTTCTCCCCATATATCTACGCATTTCACGCGCTACACGGGAAATTTTCAC
TCTCTCCTTCGGCACACAAGTCCCCCAAGTTTCCAGGGAAACCAAGGTTTGAGCCTTTGCCTT

>Pure_L
CTTACGGTACCTTGGTACAACTTACGGCTACCTTGTTACGACTTATGGTTAGCCTGGAACCAATTATACTACGCCTTG
CTGCTACGTTATTACTACTTATTCCTACTTTGTGACCACTTATTGCAAAGGAGAATCCGAACTGAGACGGCTTTTTGG
GATTAACCCACTGTCACCGCCATTGTAGCACGTGTGTAGCCCAGCGTATAAGGGCCATGAGGACTTGACGTCATCCC
CACCTTCCTCCGGCTTATCACCGGCGGTTTCCTTAGAGTGCCCAACTGAATGATGGCAACTAAGGACGAGGGTTGCG
CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTCTCTACGTCCCCGA
AGGGAACTCCAAATCTCTCTGGATAGCGTAGGATGTCAAACGCTGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACC
ACATGCTCCACCGCTTGTGCAGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGATAA
CTTAATGCGTTAGCTGCGCCACCCAAACACCATGTGCCCGGACAGCTAGTTATCATCGCTTACGGCGTGGACTACCA
GGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCACGTCAATACTTGTCCAGCGAGTCGCCTTCGCCACTGG
TGTTCTTCCGAATATCTACGAATTTCACCTCTACACCCGGAATTC

>Pure_O
TCGACGGTGATGTCAGAGCTTGCTCTGGCGGATCATGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCCGACT
CTGGGATAACTGCTAGAAATGGTAGCTAATACCGGATATGACGACTGGCCGCATGGTCTGGTCGTGGAAAGAATTTC
GGTTGGGGATGGACTCTCGGCCTATCACGTTGTTGGTGAGGTAATGGCTCACCTTTGCTACGACGGTTGGCCGGCCTG
AGAGGGTGACCAGCCACAGTGGGACTGAAACACAGCCCATACTCCTATTCCAGGCAGTTGAGGGGAATATTGCCCT
ATGAGTTCCCGCCTGTTGCAGCTGCCCCCCGTGAGGGATGACTGCCTTCGGTTTGTAACTTTCTTTTACTACGCAAGA
AGCGAAATGGACGGTACCTGGAGAAAACTGTATACCTAACTTGCGGCCAGCAGATTTCTTAATGTTTCCGGTATATG
CGTTGTCTTGAATTATTTCTTCTCGTTGCTCGTATTAATCCTGTCGCTCCTGCGCTTGTGCCCGCCCCCGTCAATTCCTT
TGAGTTTTAGCCTTGGGGCCGTACTCCCCAGGCGGGGGAACTTTATGCGCCTGCTGCGACACGGAGATCGTGGAATG
TTGCCCACATCTACTTCGCCACGTTTACGGCGTGGGGACCACGGGATCTACTAATGTTCGCTCTCATGCTTTCTCTCCT
CATCGTCGGCTGTGGGGCACAGAGCTGCTGTCTTCGTCAGAGTTGCTCCTGATAATTATCGGCGTCTTCCCGCTGCAG
CACGA

>Pure_P
CTTAGGTACCTTGGTACACTTACGGTACCTTGGTACGACTTAGGTTAGCCTGGTACCAATTTTACCGCGCCTTGCTGA
TACGCTATTACTACCTATTCCAACTTCATGCACTCGAGTTGCAGAGTGCAATCCGAACTGAGACGACTTTTAAGGATT
GGCTAACCTTCGCAGGATCGCAGCCCTCTGTAGTCGCCATTGTAGCACGTGTGTAGCCCACCCTGTAAGGGCCATGA
GGACTTGACGTCATCCCCACCTTCCTCCGGCTTACCACCGGCGGTCCCTCTAGAGTGCCCAACTAAATGATGGCAACT
AGAGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACC
TGTGTTCCAGTCCCCGAAGGGAAAGCTGAATCTCTCCAGCGGTCCGGACATGTCAAAAGGTGGTAAGGTTCTGCGCG
TTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGT
ACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCGTCACCGAACGGCATGCCGCCCGACAACTAGCACTCATCGTTT
ACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAGCGTCAGTAATGGACCAGTAA
GTCGCCTTCGCCACCGGTGTTCTTCCGGAATATCTACGAATTTCACCTCTACACTCGGAGTTCCACTTACCTCTTCCCA
TACTCAAGACCGCCAGTATTTGAAGGCAATTTCCGAGGTTGAGCCCCGGGGCTTTCCACCCCCAATTAAACGGGTCC
CGCCTACGCACCCTTTTACGCCCAGTAAATCCCGAAGCCAACGGCTAGCCCCCCTATCGGAATTACC

Pure_U
TAGTGCACTCGTAGCCGTAAGTCGTACTGGTACCGTAAAGTGACCCTAAATTGCATCCCGGCTCAGTAAACTTCCTTA
ATCGGGGGGGATTAACTCTCCAAAAAAATATTAAAACCGCATAACATTATGATGTGGCATCTGATTATTCGCAAATAT
TTATAGGACTAAGATGGGCATGCGCTGATTAGCTAGTTGGGGTGGTAACGCCCTACCAAGGCTACAATGTTTACGGG
ATCTGAAAGGATGACCCCCCACACTGGTACTGAAACCCGGACCAAATCCCTACGGAAGGCACCATGAAGGAATATT
GGTCATGGGACGGAACTCGGAACCACCCTTGCCCCGGGCAGAAAAACGGCCTTATGGTTTGTAAACTGTTTTTGCAG
GAAAATAAACCTTCTTACATGTAAGAAGTTGAATGTACCTTGATAATAAGGATCGGCTAACTCCGTGCCTGCAGCCG

CGGTAGTACGGAGGATCCGAGCGTTATCCGGATTTATTGACTTTAAAGGGTGCGTACGCGGCCTGTTGAGTCAGGCG
TGAAAGACATTAGCTCAATTATCGCAGTGCCCTTGATACTGATGGCCTTGATTATACGAGAGGTAGGCGGAATGAGA
CCAGTATGGGTGAAATGCATAGATATGTCTGAGAACACCAATTGCGAAGGCAGCTTACTATGTGATGATTGACGCTG
AGGCACGAAAGCGTGGGTGATCGAACAGGATTAAATACCCTGGTAGTCCACCCCTACACAGATGAACACTCGATGTT
GGCGATATACGGTCAGCGTCCTAAACGCAAGCCGTTCAGTGTTCCACCTGGGGCTGTACGCCCGCCAGGGGCGAAAA
CTACAAAGGGAATTTGACGGTGGGC

>Pure_AA
CCGTAAGTCGTAACAAGGTTCCGTAAGGGAACCGTAGATTTGATCCTGGTTAAGGGTAAGGAGCTACACGGAAGTG
GGGGATAACGTAGGGAAATTTACGCTAATACCGCATACGTCCTACGGGAGAAAGCGGGGGATCGCAAGACCTCGCG
CGGTTGGATGGACCGATGTGCGATTAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCGCTAGCTGGTCTG
AGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
ATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCCTCGGGTTGTAAAGCACTTTTATCAGGAGCG
AAATCTGCAAGGTTAATACCTTTGCAGTCTGACGGTACCTGAGGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCG
CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGTAGGCGGTTCGTTAAGTCTGTTG
TGAAAGCCCCGGGCTCAACCTGGGAATGGCAATGGATACTGGCGAGCTAGAGTGTGTCAGAGGATGGTGGAATTCC
CGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAACATCAGTGGCGAAGGCGGCCATCTGGGGACAACACTGACG
CTGAGGCACGAAAAGCGTGGGGGAGCAAAACAGGATT

>Pure_AB
AATACCGCATACGATCTAAGGATGAAAGTGGGGGATCGCAAGACCTCATGCTCGTGGAGCGGCCGATATCTGATTA
GCTAGTTGGTAGGGTAAAAGCCTACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAAC
TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAAT
GCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGAGAGCTAATATCTCTTG
CTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGT
TAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGATGTGAAATCCCCGGGCTCAACCTGGG
AATTGCATTGGAGACTGCAAGGCTAGAATCTGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGA
TATGTGGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGCACGAAAGCGTGAGGAGCA
AACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCTACTAGTTGTCGGGTCTTAATTGACTTGGTAACGC
AGCTAACGCGTGAAGTAGACCGCCTGGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGC
ACAAGCGGTGGGATGATGTGGATTAATTTCGATGCAACGCGAAAAACCTTACCCTACCCTTTGACATGGCTGGGAAT
CCCCGAGAGATTG

>Pure_AC
GGATCCACTTCTACTCGACGGCTCCTCCCACAAGGGTTAGGACACCGGCTTCGGGTGTTACCGACTTTCATGACTTGA
CCGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCA
TGAGGTCGAGTTGCAGACCTCAATCCGAACTGAGACCGGCCTTTTGGGATTCGCTCCACCTTACGGTATCGCATCCCT
TTGTACCGGCCATTGTATCATGCGTGAAGCCCAAGACATAAGGTGCATGAAGATTTGACCTCATCCCCACTTTTCTCC
GATTTGACTTCGGTAGTCTCCTATGATTCCTCGGCATATTCCTTTGGCA

>Pure_AD
GATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGG
GCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
CGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGC
GAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCG
TTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCG
GGTCTGCAGTCGATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAG
ATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGC
GAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGG
TGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGGAGTACGGCCGCAAAGGCTAAAACTCAAAGGAATTGACGGG
GGGCCCGCACAAGCAGCCGGAGCATGTGGCTTAATTTCGACGCCAACGCGAAAGAACCTTACCAAGGGCTTGAACA
TAACACCGGGAAAAGGCATT

>Pure_AE

AAAGGGGGGGATCATATGACCTCCCGCTACAGGGGCGGCCGATGGCAGATTAGCTAGTTGGTGGGGTAAAGGCCTA
CCAAGGCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTC
GGGTTGTAAAGCACTTTTGTCCGGAAAGAAAACTTCTGCCCTAATACGGCGGGAGGATGACGGTACCGGAAGAATA
AGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA
GCGTGCGCAGGCGGTCCGTTAAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGGCGGG
CTAGAGTATGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACCCGATGG
CGAAGGCAGCCCCCCTGGGCCAATACTGACGCTCATGCACGAAAGCGTGGGGAGCCAAACAGGGATTAGATACCCC
TGGTTAGTCCACGCCCTAAACGATGTCACTAGTTGTTGGGTTCTTCATTGAACTTAGTAACC

>Pure_AF
CCGTAGTCGTAACAAGGTATCCGTAAGGTAAGCGTAAATTTGAACCTGGCTCAGGATAAGTCGTACCTGGGTCTGGG
TAAACCTCCGGAAACTTAAGCTAATACCGGATACGAACTGCGGAGGCATCTCCAGCAGTTGGAAAGAACTTCGGTCA
AGGATGGACTCGCGGCCTATCAGGTAGTTGGTGAGGTAAAGGCTCACCAAGCCTACGACGGGTAGCCGGCCTGAGA
GGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGAAAGCCTGATGCAGCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGTAGGGAAGAA
GCGAAAGTGACGGTACCTGCAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAG
CGTTGTGCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTCTGTCGCGTCTGCTGTGAAAACCCGAGGCTCAACCT
CGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGAATGGAATTCCTGGTGTAGCGGTGGAATGCGC
AGATATCAGGAGGAACACCGATGGCGAAGGCAGTTCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGCGTGGGGA
GCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCGCTAGATGTGGGGGACCATTCCACGGTTTC
CGTGTCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTTGACGG
GGGCCCGCACAAGCGGCGGGAGCATGCGGATTAAATTCGATGCAACGCGAAAGAACCCTTACCCAAGGGCTTGGAC
ATATACCGAGAACGGGGCTAGAAATAGTCAACTCTTTTGGACACTCGT

6.12.2 DNA sequences from clone libraries

>T 1 (Top layer soil sample clone)
GGATCCGCATACCCTCACTAAAGGGACTAGTCCTGCAGGTTTAAACGAATTCGCCCTTTACGGCTACCTTGTTACGAC
TTCACCCCAATCGCGAGCCCCACCTTCGGCGGCTGGCCCTCTTGCGAGTTACCTCACCGACTTCGGGTGTTACCCACT
CTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGTCGTGCTGATCCGCGATTACTAGCAA
TTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTACGAACGGCTTTCTTGGGTTTGGCTCCACCTCGCGG
TCTTGCGTCCCGTTGTACCGCCCATTGTAGCACGTGTGTCGCCCAGGACATAAGGGGCATGATGATTTGACGTCATCC
CCGCCTTCCTCCGGTTTGTCACCGGCAGTCCTCTGTGAGTCCCCGCCTTCACGCGCTGGTAACACAGAGCAAGGGTTG
CGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCTAGTCTGCCCC
GAAGGGAAGGAATATCTCTACCCCGTTCAGACCGATGTCAAGCCCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAA
CCACATGCTCCACTGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAG
TGCTTTATTGGGTTTCCTTCGGCACCGGAGGTGGGTACCCTCCGACACCTAGCACTCATCGTTTACCGCGTGGACTAC
CAGGGTATCTAATTCCTGTTTGCTCCCCACGCCTTCGCGCCCTCAGCGTCAGGTTTCCGTCCAGTCAGGCGCCTTCCG
CTCAC

>T 2 (Top layer soil sample clone)
TGATTTAGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGAACGAACGCTGGTGGCGTGCCTAACACATGC
AAGTCCAACGTGAAAGGGGAGCAATCCCCCGGTAGGGTGGCAAACGGGTGAGTAATACATGGGTGATCTACCTTAG
GGATGGGGATATCCTTCCGAAAGGAGGGGCAATACCGAATATTGTCCGGAACCGTGAAGGGTTTCGGGGAAAGGGA
GGCCTCTGACACAAGCTTTCGCCTTAAGATGAGCCCATGGCCCATCAGCTAGTTGGTAGGGTAAAGGCCTACCAAGG
CTACGACGGGTCGCTGGTCTGAGAGGACGACCAGCCACACTGGCACTGAGATACGGGCCAGACTCCTACGGGAGGC
AGCAGTGAGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGTGGGAAGAAGGCCTTCGGGTCG
TAAACCACTTTTACTCGGGACGAAAAAGGGATATCAAACAAATATCCCCGATGACGGTACTGTGAGAATAAGCCAC
GGCTAACTCTGTGCCAGCAGCCGCGGTAAGACAGAGGTGGCAAGCGTTGTTCGGAATTACTGGGCGTAAAGAGTCT
GTAGGTGGTTTGTCAAGTCTTTGGTGAAAGGTCGTAGCTTATCTATGGGAATGCCGAAGAGACTGGCAGGCTGGAGG

CTGGGAGAGGGAAGCGGAATTTCTGGTGTAGCGGTGAAATGCGTAGATATCAGAAGGAAGGCCGGTGGCGAAGCGG
CTTCCTGGAACAGTCCTGACACTGAGAGACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACCC
CCCTAAACCGATGGGTACTAAGTGTGGGGGGGGTTTAAACCCTCCGTGCCG

>T 3 (Top layer soil sample clone)
TGATTTAGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTAACACATGC
AAGTCGAACGCGAAAGGGGGCAACCCCGAGTAGAGTGGCGAACGGGTGCGTAACACGTAGGAATCTACCCAGTAGC
GGGGGATAACCCGGCGAAAGCCGGGCTAATACCGCATACGCCCCACGGGGGAAAGCAGGGGATCTTCGGACCTTGC
ACTATTGGATGAGCCTGCGGCGGATTAGCTAGTTGGTAGGGTAAAAGCCTACCAAGGCGACGATCCGTAGCTGGTCT
GAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTTCGC
AATGGGGGCAACTCTGACGAAGCAATGCCGCGTGGATGAAGAAGGCCTTCGGGTTGTAAAGTCCTTTCGTGGGGGA
CGAAAAGGTGGTTCCTAATACGAGCTGCTGTTGACGTGAACCCAAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
CCGCGGTAATACGGGGGGTGCAAGCGTTAATCGGAATCACTGGGCGTAAAGGGTGCGTAGGCGGTACGTTTAGGTC
TGTCGTGAAATCCCCGGGCTCAACCTGGGAATGGCGGTAGAAACCGGCGCACTAGAGTATGGGAGAGGGTGGTGGA
ATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGGAGGAACATCATTGGCGAAACGCGGCCACCCTGGCCCAAT
ACTGA

>T 4 (Top layer soil sample clone)
GGACTAGTCCTGCAGGTTTAAACGAATTCGCCCTTAGATTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTA
ACACATGCAAGTCGAGCGGCAGCGCGGGGGCGACCCTGGCGGCGAGCGGCGGACGGGTGAGTAAGGCATCGGAAT
CTGCCCTGATGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCGAGAGGTGAAAGCGGGGGACC
GAAAGGCCTCGCGCAGCAGGATGAGCCGATGTCCGATTAGCTGGTTGGTGGGGTAACGGCCTACCAAGGCGACGAT
CGGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCA
CTTTTATCGGGAGCGAAACGCGCTGGGTGAATACCCCGGTGAACTGACGGTACCCGAGGAATAAGCACCGGCTAAC
TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGTAGGCG
GTGAGTGAAGTCTGCTGTGAAATCCCTGGGCTCAACCTGGGAATGGCGGTGGATACTGGCTTGCTGGAGTACGGCAG
AGGAAGGTGGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAACATCAGTGGCGAAGGCGACCTTCT
GGGCCAGTACTGACGCTGAGGCACGAAAAGCGTGGGGAGCAAACAGGATTAGATACCCC

>T 5 (Top layer soil sample clone)
ATTGCCTCCGTCTGTACCGGCCATTGTTGCACGTGTGTGGCCCTAGGCATAAAGGCCATGATGACTTGACGTCATCCT
CTCCTTCCTCCAACTTGTCGCTGGCAGTCCCCTTAGAGTGCCCGGCTTTACCCGATGGCAACAAAGGGCGAGGGTTGC
GCTCGTTGCGGGACTGAACCCAACACCTCACGGCACGAGCTGACGACAGCCATGCAGCACCTGTGTGATTGCCCGAT
TTGACGGGTCGCTCCCCTTTCGGTTCGCTACTGACCACATGTCAAGCCTAGGTAAGGTTCCTCGCGTTGCGTCGAATT
AAACCACATGCACCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGCCGTACTCCCCAGGCG
GGGTACTTACTGCGTTTGCTGCGGCACGGAGGGTTTAACCCCTCCACACTTAGTACCCATCGTTTAGGGCGTGGACTA
CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTCTCTCAGTGTCAGGACTGTTCCAGGAAGCCGCCTTCGCCA
CCGGCCTTCCTTCTGATATCTACGCATTTCACCGCTACACCAGAAATTCCGCTTCCCTCTCCCAGCCTCCAGCCTGCCA
GTCTCTTTGGCATTCCCATA

>T 6 (Top layer soil sample clone)
AGCGGCCGCGAATTCGCCCTTTACGGTTACCTTGTTACGACTTCACCCCAGTCATGAACCACACCGTGGTCGGCGTCC
CCCTTGCGGTTAGACTACCGGCTTCTGGTGCAGCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAA
CGTATTCACCGCGGCATGGCTGATCCGCGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACCCCAATCC
GGACTGAGATCGGCTTTCTGGGATTGGCTCCACCTCGCGGTCTCGCAACCCTCTGTACCGACCATTGTAGTACGTGTG
TAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCCTAGA
GTTCCCACCGTTACGTGCTGGCAACTAGGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAGCCATGCAGCACCTGTGTTCCGATTCCCGAAGGCACTCCCGCATCTCGGCAGGATTCCGGACATG
TCAAGGCCAGGTAAGGTTCTGCGCGTTGCATCGAATTAAACCACATACTCCACCCGCTTGTGCGGGCCCCCGTCAAT
TCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCGAACTTTAACGCGTTAGCTTCGAGACTGCGTGCCCAA
GTTGCACCCAACCTCCAGTTCGCATCGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCAAGCTTT
CGTGCCTCA

>T 7 (Top layer soil sample clone)

AGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTAACACATGCAAGTCG
AACGCGAAAGGGGGCAACCCCGAGTAGAGTGGCGGATGGGTGAGTAACGCGTAGGAATCTGCCCAGTAGTGGGGG
ATAACCCGGCGAAAGCCGGGCTAATACCGCATATGCCCTATGGGGCAAAGCAGGGGATCTTCGGACCTTGCGCTATT
GGATGAGCCTGCGTCGGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGACGATCCGTAGCTGGTCTGAGAG
GATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGG
GGGAAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAAGAAA
AAAGTCTGGCTAATATCCAGGCTCTTGACGGTACCTAAAGAAGAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGT
AATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTCGGTAAGTCAGATGTGA
AAGCCCTGGGCTTAACCTGGGAATTGCGTTTGATACTGCCGGACTAGAGTTTGGTAGAGGGAAGTGGAATTCCACGT
GTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTCCTGGACTAAAACTGACACTGA
GGCACGAAAGCGTGGGGAGCAAAACAGGATTAGATACCCTGGTTAGTCCACGCCGTAAACGATGGGTACTAAGACG
TTGGGGAGGGTTTAACCTTTC

>T 8 (Top layer soil sample clone)
AGGGACTAGTCCTGCAGGTTTAAACGAATTCGCCCTTAGATTTGATCCTGGCTCAGAATCAACGCTGGCGGCGTGCC
TAACACATGCAAGTCGAACGAGAAAGTGGAGCAATCCATGAGTAAAGTGGCGCACGGGTGAGTAACACGTGACTAA
CCTACCCTTGAGTGGGGAATAACCTAGGGAAACCTGGGCTAATACCGCATAACACCTTCGGGTCAAAGGAGCAATTC
GCTTAAGGAGGGGGTCGCGGCCGATTAGTTAGTTGGCGGGGTAATGGCCCACCAAGACGATGATCGGTATCCGGCCT
GAGAGGGCGCACGGACACACTGGAACTGAAACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGC
AATGGGGGAAACCCTGACGCAGCAACGCCGCGTGGAGGATGAAGTCCCTTGGGACGTAAACTCCTTTCGACCGGGA
CGATAATGACGGTACCGGAAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAGGGGGGCGAGC
GTTGTTCGGAATTATTGGGCGTAAAGGGTGCGTAGGCGGTTTGGTAAGTCTTGTGTGAAATCTTCGGGCTCAACTCG
AAGTCTGCATGAGAAACTGCCGGGCTTGAGTATGGGAGAGGTGAGTGGAATTCCCGGTGTAGCGGTGAAATGCGTA
GATATCGGGAGGAACACCTGTGGCGAAAGCGG

>T 9 (Top layer soil sample clone)
TAGTCCTGCAGGTTTAAACGAATTCGCCCTTAGATTTGATCCTGGCTCAGAACGAACGCTGGCGGCGTGCCTAACAC
ATGCAAGTCCGACGTGAAAGGGGAGCAATCCCCCGGTAGGGTGGCAAACGGGTGAGTAAGACATGGGTGATCTACC
CTGGAGATGGGGATATCCCTCCGAAAGGGGGGGCAATACCGAATAGAGTCCGGTTCCGTGAAGGGGACCGGGGAAA
GGGAGGCCTCTGGAACAAGCTTCCGCTCCTGGATGAGCCCATGGCCCATCAGCTAGTTGGTAGGGTAAAGGCCTACC
AAGGCGACGACGGGTAGCTGGTCTGAGAGGACAACCAGCCACACTGGCACTGAGACACGGGCCAGACTCCTACGGG
AGGCAGCAGTGAGGAATATTGCGCAATGGGGGCAACCCTGACGCAGCAACGCCGCGTGTGGGAAGAAGGCTTTCGG
GTTGTAAACCACTTTTGCCCGGGACGAAAAGGGGCGTCAGAATACGGCGCTTCGATGACGGTACCGGGAGAATAAG
CCACGGCTAACTCTGTGCCAGCAGCCGCGGTAAGACAGAGGTGGCAAGCGTTGTTCGGAGTTACTGGGCGTAAAGA
GTCTGTAGGTGGTTTGTCAAGTCTTTGGTGAAAGGCCGTGGCTTAACCATGGGAATGCCCAAAGAGACTGGCAGACT
GGAGGCTGGGAGAGGGAAGCGGAATTTCTGGTGTAGCGGTGAAATGCGTAGATATCAGAAGGAAGGCCGGTGGCG
AAGGCGGCTTCCTGGAAACAGGCCCTGACACTGAGAGACGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGG
TAATCCACGCCC

>T 10 (Top layer soil sample clone)
TTAGCGGCCGCGATTCGCCCTTAGATTTGATCCTGGCTCAGAACGAACGCTGGCGGCGTGCCTAACACATGCAAGTC
CAACGTGAAAGGGGAGCAATCCCCCGGTAGGGTGGCAAACGGGTGAGTAATACATGGGTGATCTACCTTAGGGATG
GGGATATCCTTCCGAAAGGAGGGGCAATACCGAATATTGTCCGGAACCGTGAAGGGTTTCGGGGAAAGGGAGGCCT
CTGACACAAGCTTTCGCCTTAAGATGAGCCCATGGCCCATCAGCTAGTTGGCAGGGTAAAGGCCTACCAAGGCTACG
ACGGGTCGCTGGTCTGAGAGGACGACCAGCACACTGGCACTGAGATACGGGCCAGACTCCTACGGGAGGCAGCAGT
GAGGAATATTGCGCAATGGGGGAAACCCTGAC

>T 11(Top layer soil sample clone)
TTAGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGAATCAACGCTGGCGGCGTGCCTAACACATGCAAGT
CGAACGAGAAAGTGGAGCAATCCATGAGTAAAGTGGCGCACGGGTGAGTAACACGTGACTAACCTACCTTTTAGTG
GGGGATAACCTAGGGAAACCTGGGCTAATACCGCATAATACCTACGGGTCAAAGCAGCAATGCGCTGAAAGAGGGG
GTCGCGGCCGATTAGCTAGTTGGTAGGGTAATGGCCCACCAAGGCAGTGATCGGTATCCGGCCTGAGAGGGCGCAC
GGACACACTGGAACTGAAACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGGGAAAC
CCTGACGCAGCAACGCCGCGTGGAGGATGAAGTCCCTTGGGACGTAAACTCCTTTCGATCGGGACGATAATGACGGT
ACCGGAAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAGGGGGGCAAGCGTTGTTCGGAATT

ATTGGGCGTAAAGGGTGCGTAGGTGGTTCGGCAAGTCTTGTGTGAAATCTTCAGGCTCAACTTGAAGTCTGCACAAG
AAACTGCCGGGCTTGAGTATGGGAGAGGTGAGTGGAATTTCCGGTGTAGCGGTGAAATGCGTAGATATCGGAAGGA
ACACCTGTGGCGAAAGCGGCTCACTGGACCATTACTGACACTGAGGCACGAAAGCTAGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCTAGCCCTAACGATGATCGCTTGTGTGCCGGGTATCCAACCCTGCCGTGCCGAAGCTAACGCG
TTTAGCGATCCGCCCTGGG

>T 12 (Top layer soil sample clone)
TGATTTAGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGAACGAACGCTGGCGGCGTGCCTAACACATGC
AAGTCCAACGTGAAAGGGGAGCAATCCCCCGGTAGGGTGGCAAACGGGTGAGTAATACATGGGTGATCTACCTTAG
GGATGGGGATATCCTTCCGAAAGGAGGGGCAATACCGAATATTGTCCGGGACCGTGAAGGGTTTCGGGGAAAGGGA
GGCCTCTGACACAAGCTTTCGCCTTAAGATGAGCCCATGGCCCATCAGCTAGTTGGTAGGGTAAAGGCCTACCAAGG
CTACGACGGGTCGCTGGTCTGAGAGGACGACCAGCCACACTGGCACTGAGATACGGGCCAGACTCCTACGGGAGGC
AGCAGTGAGGAATTTTGCACAATGGGGGCAACCCTGATGCAGCAACGCCGCGTGTGGGAAGAAGGCTTTCGGGTTG
TAAACCACTTTTGCGAGGGACGAAACCCCGGGGTTGAATACACCGTGGGGGTGACGGTACCTCGCGAATAAGCCAC
GGCTAACTCTGTGCCAGCAGCCGCGGTAAGACAGGGGTGGCAAGCGTTGTTCGGAGTGACTGGGCGTAAAGGGTCT
GTAGGTGGTTTGTCAAGTCTTTTGGTGAAAGGCCGTAGCTTAACTATGGGAATGCCCAAGAGACTGGCAGGCTGGCA
GGCCTGGGAGAGGGAAGCCGGAATTTCTGGTGTAGCGGTGAAA

>T 13 (Top layer soil sample clone)
TGATTTAGCGGCCGCGAATTCGCCCTTTACGGTTACCTTGTTACGACTTCACCCCAATCATCGGCCATACCTTGGGCG
GCTCTCTCCTCTTGCGAGGTTAAGGCACCGACTTCGGGTACAACAAACTTTCGTGGTGTGACGGGCGGTGTGTACAA
GGCCCGGGAACGTGTTCACCGCATCGTTCTGATATGCGATTACTAGCGATTCCGACTTCATGAGGTCGAGTTGCAGA
CCTCAATCCGAACTGGGGCCGGTTTTCTCGGGTTTGCTCCGCCTCTCGGCATTGCCTCCGTCTGTACCGGCCATTGTTG
CACGTGTGTGGCCCTAGGCATAAAGGCCATGATGACTTGACGTCATCCTCTCCTTCCTCCAACTTGTCGCTGGCAGTC
CCCTTAGAGTGCCCGGCTTTACCCGATGGCAACAAAGGGCGAGGGTTGCGCTCGTTGCGGGACTGAACCCAACACCT
CACGGCACGAGCTGACGACAGCCATGCAGCACCTGTGTGATTGCCCCGATTTGACGGGTCGCTCCCCCTTTCGGTTC
GCTACTGACCACATGTCAAGCCCTAGGTAA

>T 14 (Top layer soil sample clone)
TTTAGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTAACACATGCAAG
TCGAACGCGAAAGGGGGCAACCCCGAGTAGAGTGGCGAACGGGTGCGTAACACGTAGGAATCTACCCAGTAGCGGG
GGATAACCCGGCGAAAGCCGGGCTAATACCGCATACGCCCCACGGGGGAAAGCAGGGGATCTTCGGACCTTGCACT
ATTGGATGAGCCTGCGGCGGATTAGCTAGTTGGTAGGGTAAAAGCCTACCAAGGCGACGATCGGTAGCTGGTCTGA
GAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAA
TGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTATCGGGAGCGA
AACGCGCTGGGTGAATACCCCGGTGAACTGACGGTACCCGAGGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCG
CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGTAGGCGGTGAGTGAAGTCTGCT
GTGAAATCCCTGGGCTCAACCTGGGAATGGCGGTGGATACTGGCTTGCTGGAGTACGGCAGAGGAAGGTGGAATTC
CCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAACATCAGTGGCGAAGGCGACCTTCTGGGCCAGTACTGACG
CTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGAG
GTTGGGTGCAACTTGGCACGCAGTCTCGAAGCTAACGCGTTAAGTTCGCCCGCCTGGGGAGTACGGTCGCAAGACTG
AAACTCAAAGGAAATTGACCGGGGGCCCGCA

>T 15 (Top layer soil sample clone)
AGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAATACATGCAAGTCG
AACGAGGTCCAACCGGTGGCAACACTGGTGAAGACCTGGTGGCGAACGGGTGAGTAGCACGTGAGCAACCTGCCCC
GAAGACTGGGATAACACCGGGAAACCGGTGCTAATACCGGATGCCCCCATCGGATCGCATGATCTGATGAGGAAAG
GATTCTGCTTCGGGAGGGGCTCGCGGCCTATCAGCTTGATGGTGAGGTAACGGCTCACCATGGCAACGACGGGTAGC
TGGTCTGAGAGGACGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATC
TTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGTGGGAAGAAGGCCTTCGGGTCGTAAACCACTTTTACT
CGGGACGAAAAAGGGATATCAAACAAATATCCCCGATGACGGTACCGTGAGAATAAGCCACGGCTAACTCTGTGCC
AGCAGCCGCGGTAAGACAGAGGTGGCAAGCGTTGTTCGGAATTACTGGGCGTAAAGAGTCTGTAGGTGGTTTGTCA
AGTCTTTGGTGAAAGGCCGTAGCTTAACTATGGGAATGCCAAAGAGACTGGCAGGCTGGAGGCTGGGAGAGGGAAG
CGGAATTTCTGGTGTAGCGGTGAAATGCGTAGATATCAGAAGGAAGGCCGGTGGCGAATGCGGCTTCCTGGAACAG

TCCTGACACTGAGAGACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCCTAAACGATGG
GTA

>T 16 (Top layer soil sample clone)
TTAGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGAACGAACGCTGGCGGCGTGCCTAACACATGCAAGT
CCGACGTGAAAGGGGAGCAATCCCCCGGTAGGGTGGCAAACGGGTGAGTAAGACATGGGTGATCTACCCTGGAGAT
GGGGATATCCCTCCGAAAGGGGGGGCAATACCGAATAGAGTCCGGTTCCGTGAAGGGGACCGGGGAAAGGGAGGC
CTCTGGAACAAGCTTCCGCTCCTGGATGAGCCCATGGCCCATCAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGA
CGACGGGTAGCTGGTCTGAGAGGACAACCAGCCACACTGGCACTGAGACACGGGCCAGACTCCTACGGGAGGCAGC
AGTGAGGAATATTGCGCAATGGGGGCAACCCTGACGCAGCAACGCCGCGTGTGGGAAGAAGGCTTTCGGGTTGTAA
ACCACTTTTGCCCGGGACGAAAAGGGGCGTCAGAATACGGCGCTCCGATGACGGTACCGGGAGAATAAGCCACGGC
TAACTCTGTGCCAGCAGCCGCGGTAAGACAGAGGTGGCAAGCGTTGTTCGGAGTTACTGGGCGTAAAGAGTCTGTAG
GTGGTTTGTCAAGTCTTTGGTGAAAGGCCGTGGCTTAACCATGGGAATGCCAAAGAGACTGGCAGACTGGAGGCTGG
GAGAGGGAAGCGGAATTTCTGGTGTAGCCGGTGAAATGCGTAGATATCAGAAGGAAGGCCGGTGGCGAAGGCGG

>T 17 (Top layer soil sample clone)
TAGTCCTGCAGGTTTAAACGAATTCGCCCTTTACGGCTACCTTGTTACGACTTCACCCCAGTCATTGACCATACCGTG
GTAGGCGCCCTCCTTGCGGTTAGGCTACCTGCTTCTGGTACAGCCAACTCCCATGGTGTGACGGGCGGTGTGTACAA
GGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCATGGAGTCGAATTGCAGA
CTCCAATCCGGACTACGACCGGCTTTCTGAGATTAGCTCCCCCTCGCGGGTTGGCAACCCTCTGTACCGACCATTGTA
GCACGTGTGTAGCCCTGCCCATAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCGGT
CCTCTTAGAGTGCCCAACTGAATGATGGCAACTAAGAGCAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTC
ACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTGAGGTTCCCTTGCGGGCACCCCCCCATCTCTGGGGGGTT
CCTCACATGTCAAGGGCAGGTAAGGTTTTTCGCGTTGCATCGAATTGAACCACATGCTCCACCGCTTGTGCGGGCCCC
CGTCAATTCCTTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGGGTACTTATTGCGTTAGCTGCGACACTAAGA
AGTTAGACTTCCCAGCGTCTTAGT

>T 18 (Top layer soil sample clone)
TTAGCGGCCGCGAATTCGCCCTTTACGGCTACCTTGTTACGACTTAACCCCAATCATGAACCACACCTTGGACGGCTG
CTCCCTTGCGGTTAGCGCACCGGCTTCTAGTGCAACCCACTTTCGTGATTTGACGGGCGGTGTGTACAAGGCCCGGG
AACGTATTCACCGCAGCATTCTGATCTGCGATTACTAGCGATTCCAGCTTCATGCAGTCGAGTTGCAGACTGCAATCC
GAACTGAGGCCGGCTTTTTCCGATTAGCTCCCCCTCGCGGGTTTGCAGCGGTTTATACCGGCCATTGTAGCACGTGTG
TAGCCCTGGACATAAAGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCCCGTTATCCGAGGCGGTTTCGCCAG
AGTGCCCAACTGAATGATGGCAACTGGAGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACG
AGCTGACGACAGCCATGCAGCACCTATATAGCGACCTATTGCTAGGAAGGGATATTTCTACCCCTGTCCACTACATTT
CGAGCCCAGGTAAGGTTCTTCGCGTTGCGTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCC
TTTGAGTTTCAGCCTTGCGACCGTACTCCCCAGGCGGATCGCTTAACGCGTTAGCTTCGGCACGGCAGGGTTTGGATA
CCCCGCCCACACCAA

>T 19 (Top layer soil sample clone)
AGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGAACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCC
AACGTGAAAGGGGAGCAATCCCCCGGTAGGGTGGCAAACGGGTGAGTAATACATGGGTGATCTACCTTAGGGATGG
GGATATCCTTCCGAAAGGAGGGGCAATACCGAATATTGTCCGGGACCATGAAGGGTTCCGGGGAAAGGGAGGCCTC
TGATACAAGCTTTCGCCTTAAGATGAGCCCATGGCCCATCAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCTACGA
CGGGTCGCTGGTCTGAGAGGACGACCAGCCACACTGGCACTGAGATACGGGCCAGACTCCTACGGGAGGCAGCAGT
GAGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGTGGGAAGAAGGCCTTCGGGTCGTAAACC
ACTTTTACTCGGGACGAAAAAGGGATATCAAATAAATATCCCCGATGACGGTACCGTGAGAATAAGCCACGGCTAA
CTCTGTGCCAGCAGCCGCGGTAAGACAGAGGTGGCAAGCGTTGTTCGGAATTACTGGGCGTAAAGAG

>T 20 (Top layer soil sample clone)
AGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTAACACATGCAAGTCG
AGCGGCAGCGCGGGGGCGACCCTGGCGGCGAGCGGCGGACGGGTGAGTAAGGCATCGGAATCTGCCCTGATGTGGG
GGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCGAGAGGTGAAAGCGGGGGACCGAAAGGCCTCGCGCA
GCAGGATGAGCCGATGTCCGATTAGCTGGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCGGTAGCTGGTCTGA
GAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTTCGCAA

TGGGGGCAACCCTGACGAAGCAATGCCGCGTGGATGAAGAAGGCCTTCGGGTTGTAGAGTCCTTTCGTGGGGGACG
AAAAGGTGGTTCCTAATACGAGCTGCTGTTGACGTGAACCCAAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCC
GCGGTAATACGGGGGGTGCAAGCGTTAATCGGAATCACTGGGCGTAAAGGGTGCGTAGGCGGTACGTTAGGTCTGT
CGTGAAATCCCCGGGCTCAACCTGGGAATGGCGGTAGAAACCGGCGCACTAGAGTATGGGAGAGGGCGGTGGAATT
CCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAACATCAGTGGCGAAGGCGGCCACCTGGCCCCAATACTGA
CGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCCCGCCCCTAAACGATGAATACTA
GATGTTTG

>T 21 (Top layer soil sample clone)
TTAGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTAACACATGCAAGT
CGAGCGGCAGCGCGGGGGCGACCCTGGCGGCGAGCGGCGGACGGGTGAGTAAGGCATCGGAATCTGCCCTGATGTG
GGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCGAGAGGTGAAGGCGGGGGACCGAAAGGCCTCGC
GCAGCAGGATGAGCCGATGTCCGATTAGCTGGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCGGTAGCTGGTCT
GAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGAC
AATGGAGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTATCGGGAGC
GAAACGCGCTGGGTGAATACCCCGGTGAACTGACGGTACCCGAGGAATAAGCACCGGCTAACTCCGTGCCAGCAGC
CGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGTGTGCGTAGGCGGTGAGTGAAGTCTG
CTGTGAAATCCCTGGGCTCAACCTGGGAATGGCGGTGGATACTGGCTTGCTGGAGTACGGCAGAGGAAGGTGGAAT
TCCCGGTGTAGCGGTGAAATTGCGTAGAGATCGGGAGGAACATCAGTGGCGAAGGCGACCTTCTGGGCCCAGTACTT
GACGCCTGAGGCACCGAAAGCCGTGGGGAGCAAACAGGATTAGATACCCCTTGGTAGTCCACGCCCCTAAACGATG
CGAA

>T 22 (Top layer soil sample clone)
TCCTGCAGGTTTAAACGAATTCGCCCTTTACGGTTACCTTGTTACGACTTAACCCCAATCACCGACCCCACCTTCGAC
GGCTCCCTCCCAAAAGGGTTGGGTGACCGGCTTCGGGTGTTGCCGACTTTCGTGGTTTGACGGGCGGTGTGTACAAG
GCCCGGGAACGTATTCACCCCGGCGTTGCTGATCCGGGATTACTAGCGACTCCGACTTCACGCAGTCGAGTTGCAGA
CTGCGATCCGAACTGAGACCGGCTTTATGGGATTCGCTCACTCTCGCGAGATAGCAGCCCTTTGTACCGGCCATTGTA
GCATGTGTGCAGCCCTGGACGTAAGGGGCATGATGACTTGACGTCGTCCCCACCTTCCTCCGAGTTGACCCCGGCAG
TCTCCTACGAGTCCCCGGCATTACCCGCTGGCAACATAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACAT
CTCACGACACGAGCTGACGACAGCCATGCACCACCTGTGCGGGATCCCGAAGATCAGCGTATCTCTACGCCTTAACC
CCGCATGTCAAGCCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAGCCACATGCTCCGCCGCTTGTGCGGGCCCCC
GTCAATTCCTTTGAGTTTTTAGCCTTGCGGCCGTACTTCCCCAGGCGGGGCACTTAATGCGTTAGC

>T 23 (Top layer soil sample clone)
AGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGATTGAACGCTGGCGGCGTGCCTAACACATGCAAGTCG
AGCGGTAACAGGTGTAGCAATACATGCTGACGAGCGGCGAACGGGTGAGTAATGCTTCGGAATCTACCCACGGGTG
GGGAATAACCAACCGAAAGGTTGGCTAATACCGCATACGTCCTACGGGGGAAAGCGGGGGATCGAAAGACCTCGCG
CCGGTGGATGAGCCGAAGCCGGATTAGCTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGATCCGTAGCTGGTCTG
AGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
ATGGGGGAAACCCTAATCCAGCGACGCCGCGTGTGTGAAGAAGGCCTGCGGGTTGTAAAGCACTTTTAGTGGGGAT
GAAAAGCCTGGAACTAATACTTCCGGGTCTTGACCTAACCTACAGAAAAAGCACCGGCTAACTCTGTGCCAGCAGCC
GCGGTAATACAGAGGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAAGCGTGCGTAGACGGTCGTGCAAGTCG
GGTGTGAAAAGCCCCGGGCTCAACCTGGGA

>T 24 (Top layer soil sample clone)
TTTAGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGGATTAACGCTGGCGGCGTGCCTGACACATGCAAG
TCGAACGAGGTCGCAAGACCTAGTGGCGGACGGGTGAGTAACACGTGACCAACCAACCTCGAAGTTGGGAATAGCT
CTGCGAAAGCAGGGGTAATACCGAATGTGGCCCGGCGCGGACATCCGCACCGGTCTAAAGATTTATCGCCTCGAGA
CGGGGTCGCGGCCTATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGATGATGGGTAGCTGGTCTGAGAGGACG
ACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGAGGAATCTTCCGCAATGGGCGA
AAGCCTGACGGAGCAACGCCGCGTGTGGGATGAAGACTCTAGGGTTGTAAACCACTGTCGGAGGGGACGATCGTGA
CGGTACCCTCCAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCGAGCGTTATCCGG
ATTTACTGGGCGTAAAGCGCGTTAAGGCGGATGGCCAAGTTGGCGGTGAAATTTCGGGGCTCAACCCCGAAACTGCC
GCCAAAACTGGTCGTCTAGAGTATGGGAGAGG

>T 25 (Top layer soil sample clone)
TGATTTAGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTAACACATGC
AAGTCGAACGGTAACAGGTCTTCGGATGCTGACGAGTGGCGGACGGGTGAGTAATGCGTAGGAATCTGTCTTTTAGT
GGGGGACAACCCAGGGAAACTTGGGCTAATACCGCATGAGCCCTGAGGGGGAAAGCGGGGGATCTTCGGACCTCGC
GCTAAGGGAAGAGCCTACGTCTGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCT
GAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTTCGC
AATGGGGGCAACCCTGACGAAGCAATGCCGCGTGGATGAAGAAGGCCTTCGGGTTGTAAAGTCCTTTCGTGGGGGA
CGAAAAGGTGGTTCCTAATACGAGCTGCTGTTGACGTGAACCCAAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
CCGCGGTAATACGGGGGGTGCAAGCGTTAATCGGAATCACTGGGCGTAAAGGGTGCGTAGGCGGTACGTTAGGTCT
GTCGTGAAATCCCCGGGCTCAACCTGGGAATGGCGGTAGAAACCGGCGCACTAGAGTATGGGAGAGGGTGGTGGAA
TTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAACATCAGTGGCGAAGGCGGCCACCTGGCCCAATACTG
ACGCTGAGGCACGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAATACT
AGATGTTTGGTACCTAGCGT

>T 26 (Top layer soil sample clone)
TGATTTAGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTAACACATGCA
AGTCGAACGCGAAAGGGGGCAACCCCAAGTAGAGTGGCGGACGGGTGAGTAACGCGTAGGAATCTACCCAGTAGTG
GGGGATAACCCGGCGAAAGCCGGGCTAATACCGCATATGCCCTACGGGGCAAAGCAGGGGATCTTCGGACCTTGCG
CTATTGGATGAGCCTGCGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTG
AGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
ATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCGGTGGGGAA
GAAAAAAGCTTGGCTAATATCCAAGCTCTTGACGGTACCTAAAGAAGAAGCACCGGCTAACTCTGTGCCAGCAGCC
GCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTCGGTAAGTCAGA
TGTGAAAGCCCTGGGCTCAACCTGGGAACTGCATTTGATACTGTCGAGCTAGAGTTTGGTAGAGGGAAGTGGGAATT
CCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGGCTTCCTTGGACTAAAACTGA
CACTGAGGCACGAAAGCGTGGGGGAGCAAACAGGATTAGATACCTCTGGTAGTTCCCCGCCGTAAACGA

>T 27 (Top layer soil sample clone)
TTTAGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAACACATGCAAG
TCGAACGAGGTTCATTCGGTAGCAATACTGGAGAAGACCAAGTGGCGAACGGGTGAGTAGCACGTGAGCAACCTGC
CCCGAAGACCGGGACAACACCGGGAAACCGGTGCTAATACCGGATATCCCCACCAGATCGCATGGTTCGGTGAGGA
AATGGATTCCGCTTCGGGAGGGGCTCGCGGCCTATCAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGTCGACGGG
TAGCTGGTCTGAGAGGACGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG
AATCTTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGGGACGAAGGCTTTCTGAGTTGTAAACCTCTT
TCAGCAGGGACGATAATGACGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGGTGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGGCAAGTCGGATGTGAAACCTCCAG
GCTCAACCTGGAGTCGCCATTCGATACTGCTATGGCTAGAGTTTGGTAGGGGACCACGGAATTCCTGGTGTAGCGGT
GAAAATGCGCAGATATCAGGAGGAACACCCAGCGGCGAATGCGGTGGTCTGGGCCAATACTGACGCTGAGGAGCGA
AAGCGTGGGGGAGCGAACAGGATTAGATACCCTGGGTAGTCCCAC

>M 1 (Middle layer soil sample clone)
GCGGCCGCGAATTCGCCCTTTACGGTTACCTTGTTACGACTTCACCCCAATTACCGGCCATACCTTGGGCGGCTCCCT
CCGTTTCCGGTTGGGGCACCGACTTCAGGTACAACAGACCTTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGA
ACGTATTCACCGCGTCGTGCTGATACGCGATTACTAGCGATTCCGACTTCATGAGGTCGAGTTGCAGACCTCAATCCG
AACTGGGACCGGCTTTCTCGGATTTGCTCCACCTCTCGGTCTCGCATCCGTCTGTACCGGCCATTGTTGCACGTGTGT
GGCCCTGGGCATAAGGGCCATGATGACTTGACGTCATCCTCTCCTTCCTCCAACTTGTCGCTGGCAGTCCCCTCAGAG
TGCCCGGCATGACCCGGTGGCAACAGAGGGCGAGGGTTGCGCTCGTTGCGGGACTGAACCCAACACCTCACGGCAC
GAGCTGACGACAGCCATGCAGCACCTGTTCCGCTTCCGGACTGAACCGGTCGGTCCCCTTTCAGTTCCCTACTCGCGG
TATGTCAAGCCCAGGTAAGGTTCTTTCGCGTTGCGTCGAATTAAAACCCACATGCACCACCGCTTTGTGCGGGCCCCC
GTCAATTCCCTTTGAAGTTTTCA

>M 2 (Middle layer soil sample clone)
GCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTAACACATGCAAGTCGA
GCGGCAGCGCGGGGGCGACCCTGGCGGCGAGCGGCGGACGGGTGAGTAAGGCATCGGAATCTGCCCTGATGTGGGG
GATAACGTAGGGAAACTTACGCTAATACCGCATACGACCGAGAGGTGAAAGCGGGGGACCGGAAGGCCTCGCGCAG

CAGGATGAGCCGATGTCCGATTAGCTGGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCGGTAGCTGGTCTGAG
AGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAAT
GGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTATCGGGAGCGAA
ACGCGCTGGGTGAATACCCCGGTGAACTGACGGTACCCGAGGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGC
GGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGTAGGCGGTGAGTGAAGTCTGCTG
TGAAATCCCTGGGCTCAACCTGGGAATGGCGGTGGATACTGGCTTGCTGGAGTACGGCAGAGGAAGGTGGAATTCC
CCGGTGTAGCGGTTGAAATGCGTAGAGATCGGGAGGAACATCAGTCGGCGA

>M 3 (Middle layer soil sample clone)
TTAGCGGCCGCGAATTCGCCCTTAGATTTGATCCTGGCTCAGAACGAACGCTGGCGGCGTGCCTAACACATGCAAGT
CCGACGTGAAAGGGGAGCAATCCCCCGGTAGGGTGGCAAACGGGTGAGTAAGACATGGGTGATCTACCCTGGAGAT
GGGGATATCCCTCCGAAAGGGGGGGCAATACCGAATAGAGTCCGGTTCCGTGAAGGGGACCGGGGAAAGGGAGGC
CTCTGGAACAAGCTTCCGCTCCTGGATGAGCCCATGGCCCATCAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGA
CGACGGGTAGCTGGTCTGAGAGGACAACCAGCCACACTGGCACTGAGACACGGGCCAGACTCCTACGGGAGGCAGC
AGTGAGGAATATTGCGCAATGGGGGCAACCCTGACGCAGCAACGCCGCGTGTGGGAAGAAGGCTTTCGGGTTGTAA
ACCACTTTTGCCCGGGACGAAAAGGGGCGTCAGAATACGGCGCTTCGATGACGGTACCGGGAGAATAAGCCACGGC
TAACTCTGTGCCAGCAGCCGCGGTAAGACAGAGGTGGCAAGCGTTGTTCGGAGTTACTGGGCGTAAAGAGTCTGTAG
GTGGTTTGTCAAGTCTTTGGTGAAAGGCCGTGGCTTAACCATGGGAATGCCAAAGAGACTGGCAGACTGGAGGCTGG
GAGAGGGAAGCGGAAATTTCTGGCGTAGCG

>B 4 (Bottom layer soil sample clone)
TGTGTGGCCCTAGGCATAAAGGCCATGATGACTTGACGTCATCCTCTCCTTCCTCCAACTTGTCGCTGGCAGTCCCCT
TAGAGTGCCCGGCTTTACCCGATGGCAACAAAGGGCGAGGGTTGCGCTCGTTGCGGGACTGAACCCAACACCTCACG
GCACGAGCTGACGACAGCCATGCAGCACCTGTGTGATTGCCCGATTTGACGGGTCGCTCCCCTTTCGGTTCGCTACTG
ACCACATGTCAAGCCTAGGTAAGGTTCCTCGCGTTGCGTCGAATTAAACCACATGCACCACCGCTTGTGCGGGCCCC
CGTCAATTCCTTTGAGTTTCAACCTTGCGGCCGTACTCCCCAGGCGGGGTACTTACTGCGTTTGCTGCGGCACGGAGG
GTTTAACCCCTC

>B 5 (Bottom layer soil sample clone)
CACTGGCACTGAGATACGGGCCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGCGCAATGGGGGAAACCCTGA
CGCAGCAACGCCGCGTGTGGGAAGAAGGCCTTCGGGTCGTAAACCACTTTTACTCGGGACGAAAAAGGGATATCAA
ACAAATATCCCCGATGACGGTACCGTGAGAATAAGCCACGGCTAACTCTGTGCCAGCAGCCCGCGGTAAGACAGAG
GTGGCAAGCGTTGTTCGGAATTACTGGGCGTAAAGAGTCTGTAGGTGGTTTTGTCAAGTCTTTGGTGAAAGGCCGTA
GCTTTAACTATGGGAATGCCAATGAGACTTGGCAGGCTGGAGGCTGGGAGAGGGAAGCGGAAT

>B 6 (Bottom layer soil sample clone)
CCTCTCCTTCCTCCAACTTGTCGCTGGCAGTCCCCTTAGAGTGCCCGGCTTTACCCGATGGCAACAAAGGGCGAGGGT
TGCGCTCGTTGCGGGACTGAACCCAACACCTCACGGCACGAGCTGACGACAGCCATGCAGCACCTGTGTGATTGCCC
GATTTGACGGGTCGCTCCCCTTTCGGTTCGCTACTGACCACATGTCAAGCCTAGGTAAGGTTCCTCGCGTTGCGTCGA
ATTAAACCACATGCACCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGCCGTACTCCCCAG
GCGGGGTACTTACTGCGTTTGCTGCGGCACGGAGGGTTTAACCCCTCCACACTTAGTACCCATCGTTTAGGGCGTGG
ACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTCTCTCAGTGTCAGGACTGTTCCAGGAAGCCGCCTTC
GCCACCGGCCTTCCTTCTGATATCTACGCATTTCACCGCTACACCAGAAATTCCGCTTCCCTCTCCCAGCCTCCA