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Environmental monitoring and microbial degradation of onshore shale gas activity chemicals and fluids

Nai Tran-Dinh, Se Gong, Kaydy Pinetown, Tania J Vergara, Richard Schinteie, Carla Mariani, Paul Greenfield and David J Midgley

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We also pay our respects to the cultural authority of Aboriginal and Torres Strait Islander people and their nations in the Northern Territory, as well as those across Australia.

Executive summary

The aims of this project were to:

Establish microbial community baselines in aquifers and soils of sites proximal to prospective unconventional gas activities in the Beetaloo sub-Basin.

Understand the microbial degradation of a range of chemicals likely to be used in unconventional gas activities, in both the aquifers and soils of the Beetaloo sub-Basin.

Key findings

Physicochemical data of soils and aquifers of the region

Soil Chemistry of the Beetaloo Region

- It is important to note that while considerable soil chemistry data are likely held by pastoralists of the region, little data is publicly available on soil chemistry in the Beetaloo Region outside these privately held holdings. Some, primarily inorganic, chemistry data related to metals are available through the Australian Microbiome Project. In practice this means that soil maps of the Beetaloo sub-Basin region (from the Northern Territory government) use extrapolations of soil type from topological data and these private data to designate a soil at a location as kandosol, vertosol, or chromosol (for instance). Data presented in this report demonstrate that at the local scale, these maps are likely not very reliable. As such, while the authors of this report have elected to keep the chromosol, kandosol, rudosol, tenosol and vertosol as descriptors of soils, matching the locations described of online soil maps of the Northern Territory, these designations may be incorrect.
    - The current study includes a detailed soil chemistry study of five soil samples, collected at geographically distant points and nominally representing chromosol, kandosol, rudosol, tenosol and vertosol soils of the region, noting the concerns above regarding soil-type maps of the region.
    - These data demonstrate that the chromosols and rudosols were chemically distinct from the kandosol, tenosol and vertosols of the region.
    - Soils were acidic (minimum pH 5.1) through to neutral (pH 7.0). They were poorly conductive indicating an absence of readily soluble salts in the soils. All soils, except the tenosol, were high in iron (23.2g – 57.0 g kg^{-1}). In addition to iron, manganese

also differed between the soils with the chromosols having ~180X less manganese than the rudosols.

- In terms of nutrients of importance to living organisms, inorganic nitrogen was generally low with between 1.8-21.1 mg kg^{-1} of total nitrate in the five soils. Total nitrogen was somewhat higher and is most likely associated with the soil organic matter. It should be noted that organic nitrogen is typically not plant available.
- Soil organic carbon content in these soils was estimated using loss on ignition with a conversion factor of 50% of the organic matter being organic carbon. It should be noted that this is likely a significant overestimate for soils with significant clay content.

Aquifer Chemistry of the Beetaloo Region

- The chemistry data presented in this report extend data available from bore-completion logs (typically pH & EC) and other studies in the region, notably GISERA W16. This study provides detailed chemistry information for 38 bores of the region including those intersecting the formations of Gum Ridge, Tindall Limestone, Anthony Lagoon and Montijinni Limestone.
  - In broad terms, aquifers of the region are neutral to moderately alkaline in pH (7.21 to 8.03) with low salinity (650-2140 µS cm^{-1}).
  - Chemically, bores that intersect the Tindall Limestone split into two groups. The first group is distinct from the remainder of the bore samples in this study including other samples from the Tindall Limestone.
    - This split between the Tindall Limestone samples has a rough east/west division, with samples from further northwest being distinct from the remainder of samples in this study.
    - In contrast, samples from bores in the Gum Ridge, Montijinni and Anthony Lagoon form a continuum of water chemistry that does not separate by formation. This could be related to different subsurface connectivity or discontinuities, be related to recharge rates and sources, or be a result of casing issues in the bores resulting in water from multiple sources/formations mixing.
  - In terms of chemistry related to living organisms, organic carbon content was very low (mean ~1 mg L^{-1}), similarly nitrogen content was mostly low (0.1 - 1 mg L^{-1}).

Some bores, notably Motel Bore and the Stuart Plains Homestead Bore, had levels of nitrogen greater than 2 mg L^{-1}.

Microbial baselines for soils of the Beetaloo Region

- It should be noted that sampling for this project occurred at the end of the dry season in the region (August). Soils of the region are subject to meagre rainfall May through September, and this will markedly impact the soil microbial community data of the region.
- Microbiome data for the Beetaloo Region is very limited. Prior to this report, the only soil microbiome data in the public domain were part of the Australian Microbiome Study. Samples from the Australian Microbiome study largely survey the eastern edge of this project's study region. No data, for example, are available west of the Stuart Highway. Data from the current study thus provide significantly more soil microbiome information for the region.
- Microbiome data mirror the soil chemistry data in that soil type does not appear to be a predictor of soil microbiology. Instead, geographic (i.e. how proximally located two samples are) is a better indicator of shared microbiology.
- *Prokaryotic data*
    - Over 3000 OTUs (≈ 3000 species) were detected from the soil samples examined in the present study. This is likely a significant underestimate as OTU clustering necessarily combines similar taxa. Of these ~3000 OTUs, ~1250 abundant OTUs (present at more than 0.1% relative abundance) were detected across the soil samples examined in the present study.
    - The microbiology of the region appears to be broadly consistent with observations from the Australian Microbiome dataset.
    - Most of the detected OTUs were bacterial, with few archaeal taxa detected (though these Archaea are very abundant in some samples).
    - Numerous lineages were detected that are specialised for high-temperature, arid environments or have UV tolerance characteristics.
    - Numerous novel lineages were detected, suggesting that the microbiomes of these soils include unique and poorly described species that should be the subject of further research.
- *Fungal data*

- Just as for prokaryotic data, soil fungal community composition is more likely to be affected by geographic proximity and not soil type.
- In total, ~250 OTUs were detected in the soils of the Beetaloo region. These included ascomycetes (56%), basidiomycetes (19%), glomeromycetes (3%), chytridiomycete (3%) and zygomycetes (<1%) with a large number of potentially novel fungal phyla detected.
- Fungal microbiomes presented in this study are similar to those in the Australian Microbiome, thus extending our understanding of the microbiology of the region.
- Many samples failed to yield amplicons for the fungal PCR, but yielded amplicons for the prokaryotic PCR indicating the probable low abundance of fungi in some samples or highly resistant resting structures that preclude DNA extraction from these fungi. On balance, however, it would seem the edaphic conditions of these soils are not highly conducive to fungal growth (outside of the wet season).
- Those fungi that were detected, were often related to other desert or semi-arid species. Numerous wood rot and other saprobic species were detected along with some plant pathogenic taxa and plant symbiotic species (e.g. ecto- and arbuscular mycorrhizal species).
- A large number of the fungi of the region appear to be novel species.

Microbial baselines for aquifers of the Beetaloo Region

- Knowledge of aquifer microbiomes in the region is largely lacking. This study provides important early data on living organisms in these environments and compliments the work already completed on larger eukaryotes (stygofauna) in this habitat (GISERA W18[1]).
- Low cell number is a feature of the aquifers of the region. Likely related to low availability of biologically important elements or compounds (e.g. organic carbon, nitrogen and potentially oxygen).
- In total, 1452 OTUs were detected across the 38 bores. Most of these OTUs were bacterial, however, some –numerically abundant– archaeal OTUs were also detected.
- Four phyla made up more than 50% of all OTUs detected (Proteobacteria, Actinobacteria, Firmicutes and Bacteroides).

[1] https://gisera.csiro.au/wp-content/uploads/2021/03/GISERA-Project18-Stygofauna_final-report-20201208.pdf

- The bores have a diverse array of oxygen status, this is reflected in the microbial data. Some bores are dominated by specialist anaerobic communities while others have aerobes or microaerophilic taxa.
- Distinct microbiomes were observed in the Tindall Limestones bores that matched the east/west division seen in the chemistry data.
- In broad terms, bores tended to feature a range of autotrophic taxa (those species capable of fixing organic carbon from carbon dioxide). In addition, numerous microbes with enzymatic activities towards hydrocarbons were also detected.

Degradation of alcohols and glycols by microbes in soil and aquifer environments of the Northern Territory.

- All chemicals tested for degradation using commercially available accredited tests (ethylene glycol, propylene glycol, butoxyethanol, isopropanol and methanol) were undetectable in soils after 34 days of incubation (~22 C, 12 hour day/night cycle), likely due to microbial degradation.
    - Degradation may have occurred at a faster rate, however, the single test conducted after 34 days precludes characterising the rate of degradation further.
- In aquifer microcosms experiments, microbes were able to degrade all chemicals to some extent, however, this degradation was not complete during the time period examined (3 months) for any chemical/aquifer combination.

Effect of chemicals on microbial communities in soils and aquifers of the Beetaloo Region

- The microcosm experimental controls revealed that there were significant changes to the microbiome due to wetting the soils followed by storage through the 34 day course of the experiment.
- For prokaryotes in soils and aquifers, more often than not, addition of individual chemicals significantly altered microbial community composition. In contrast, in broad terms, fungal communities were generally not affected by the addition of chemicals.
    - This effect on microbial communities was not characterised by a loss of diversity or a loss of specific groups or lineages.
    - Biocides in soils and aquifers appeared to have a biostatic effect, i.e. the relative abundance of most taxa from aquifers and soils resembled the “pre-experimental” state. In contrast, alcohols, hydrocarbons, and other chemicals did not exhibit this biostatic effect.
    - Changes in microbial communities for the most part appear to be associated with the growth of a small number of probable catabolisers of that chemical. Often these changes are marked by the very significant increase in relative abundance of one or two taxa in response to a chemical addition.
    - In broad terms, microbes detected to increase in relative abundance in response to chemical additions tended to be novel, poorly taxonomically-characterised species.
    - Relatively few taxa responded negatively (i.e. reduced in relative abundance) in a universal way (i.e. were sensitive to all the chemical additions). That said, a small

number of lineages in the soil (both prokaryotes and fungi) and aquifers (prokaryotes only) were identified as putative indicator taxa for environmental disturbance as they respond negatively to many of the chemical additions.

- It should be noted that the chemical concentrations used in this study reflect those used by industry in regular activities and their addition, at these concentrations, does not result in a loss of diversity or loss of specific lineages. Exposure to higher concentrations of chemicals would likely result in different outcomes.

Catabolism of chemicals by microbes from the soils as a sole source of carbon

- All chemicals could be used by soil microbes as a sole source of carbon (except for eicosane for which the treatment became contaminated and no result was determined). Under stringent conditions where turbidity was observed and individual chemicals were the only possible source of carbon.
- In contrast to those experiments completed in the soil microcosms, catabolising taxa detected here were generally taxonomically well resolved (i.e. well known and characterised species).
    - Further, the majority of taxa detected come from linages where catabolism of complex xenobiotics has been well documented in the literature. Particularly, for example, *Methylobacterium* (*senso lato*) and numerous actinobacterial genera.
    - Some of the linages observed in the sole carbon source experiments were also observed in the soil microcosm experiments, suggesting these taxa play an important role in specific chemical degradation in those soils. Conversely, some taxa detected in the sole carbon source trials were not detected in the soil microcosm experiments suggesting these taxa may not be those that immediately respond to the addition of the chemical in those soils. They do, however, represent an additional suite of microorganisms capable of catabolising chemicals if these chemicals were introduced into soils.

Options for future work

- The baselining undertaken in this study provides an important set of data from the soils and aquifers of the region towards the end of the dry season. It should be noted that the soil microcosm experiments demonstrated that very significant change in microbial community structure occurs in soil during wetting events, as such, further sampling for baselining would be beneficial at different times of the year. In addition, the flush of plant

growth that occurs during the onset of the wet season would further alter the microbiomes in these soils and efforts to understand the microbiology of the region should capture these seasonal, temporal and plant-associated variations.

- The current study demonstrates that at the concentrations of chemicals tested herein, microbes are likely able to readily degrade all the chemicals in soils, and similarly in aquifers, microbes are able to degrade the chemicals, albeit at a slower rate. It would be beneficial to determine the concentration of chemicals for which significant community or other stakeholder concern exists and ascertain at what concentration catabolism of these chemicals would be inhibited.

Concluding remarks

- This study has provided important initial data for chemistry and microbiomes for Northern Territory aquifers and soils in the Beetaloo Region.
- This study demonstrates that the microbiomes of both Northern Territory aquifers and soils in the Beetaloo Region contains a significant cohort of indigenous microorganisms capable of biodegrading the chemicals used for onshore gas activities.
- The rates of biodegradation of chemicals used in onshore gas activities was observed to be slower in aquifers than in soils.

1 Introduction

As Australia shifts towards renewable energy sources, natural gas is a key transitional fuel bridging the gap between higher greenhouse gas emitting fossil fuels and renewable sources of energy. Onshore gas activities utilise a range of chemical products in their operation. The risks associated with these chemicals have been the focus of numerous reviews, which have identified potential environmental and human health impacts[2] and are a key focus of the Northern Territory's inquiries into the potential environment impacts of onshore gas activities (https://frackinginquiry.nt.gov.au/inquiry-reports/final-report). Specifically, the Northern Territory Government inquiry into hydraulic fracturing outlines in a report released May 2018:

- 135 recommendations that if adopted and implemented in full would mitigate to acceptable levels the identified risks associated with any onshore shale gas development in the Northern Territory
- A total of 34 recommendations made relating directly to safeguarding water and the environment
- The inquiry identified four high priority issues:
    - Unsustainable groundwater use
    - Contamination of groundwater from leaky bores
    - Contamination of groundwater by surface spills of fracking fluid chemicals (transit or storage) and wastewater
    - Effects on surface or groundwater-dependent ecosystems

This project, GISERA W17, focused on biodegradation of chemicals used in onshore gas activities in the Northern Territory. For the main, chemicals used by the onshore gas industry are well studied and have low risk profiles. This report, therefore, chooses to focus on chemicals that pose the greatest risk to human or environmental health. Chief amongst these compounds are biocides, alcohols and, to a lesser extent hydrocarbons, though this report also considers a small number of other chemicals. While most of these compounds have been comparatively well studied in terms

[2] (Department of the Environment and Energy, 2017; *National assessment of chemicals associated with coal seam gas extraction in Australia. Report 4: Hydraulic fracture growth and well integrity*, 2017); https://frackinginquiry.nt.gov.au/inquiry-reports/final-report

of their biodegradation, comparatively little is known about the migration and degradation of these compounds in edaphic and subsurface environments of the Northern Territory. This project was commissioned to understand the microbial degradation potential of chemical compounds used during onshore gas production, to identify organisms involved in these degradations and to identify potentially sensitive taxa that may be useful indicators of contamination events in soils and aquifers of the Beetaloo Basin region.

1.1 Chemicals used during onshore gas production

Community concerns about environmental contamination during onshore conventional gas exploration, production and enhancement focus primarily on potential contamination of soil and aquifers. As part of Project GISERA W15, an extensive literature review was conducted investigating the chemicals applied to conventional Australian onshore gas activities and to provide estimates of chemical concentrations used, potential biodegradation pathways and associated policy frameworks (Schinteie et al., 2019; report number EP19349).

The chemicals used during onshore gas production play important roles in extraction of the resource. It should be noted that in general, these chemicals comprise relatively small fractions of the fluids used in onshore gas activities. Their use also varies by class. For example, in drilling certain additives may be used in all activities, while others like biocides may be used situationally. Some chemicals serve multiple functions, however, in general chemicals can be classified into broad industry applications depending on their use.

(i) **Biocides**

Biocides are a diverse group of compounds with activities against living organisms. Unsurprisingly, these compounds have significant impacts on microbes and 'higher' organisms. They have a diverse range of structures (Figure 1 - Figure 5) and mode of actions against cells. Some biocides impact a narrow range of organisms, while others have what is called 'broad spectrum' activity and impact most forms of life.

(ii) **Buffers**

Buffers are compounds that control the pH of an environment. That is, they resist changes to the acidity or alkalinity of a fluid (or the environment). Artificial buffers come in various forms.

(iii) **Corrosion inhibitors**

Corrosion inhibitors, as the name suggests, are a group of chemicals that prevent oxidation (corrosion) of materials associated with onshore gas activities. The mode of activity of these compounds can differ, but often these compounds either scavenge oxygen or alter the surface properties of oxidisable materials to prevent corrosion.

(iv) **Epoxy resins**

Epoxy resins are often used to join or protect materials used in onshore gas activities. Typically, these come as multi part mixtures with one part polymerising on contact with other components. In general, polymerised components are biologically inert, however, unused components not bound in the resin may affect biological activity.

(v) **Friction reducers.**

Friction reducers reduce friction between fluids and the wall of the pipe in which they are being pumped. These are particularly important when pumping is being undertaken at high rates.

(vi) **Surfactants**

Surfactants are compounds that interfere with the interfacial tension between surfaces or maintain emulsions (mixtures of different phases). For example, some polar fluids struggle to interact with hydrophobic rock surfaces and surfactants make it easier for these two phases to interact.

(vii) **Viscosity management**

Viscosity management chemicals alter the viscosity (the thickness or thinness) of solutions. This is particularly important if the fluid is carrying other materials. For example, during drilling, thicker solutions can carry particulate matter back to the surface, similarly, fluids carrying proppants (normally silica) need to be able to maintain an even distribution of these particles within the fluid.

For the purposes of the present study, chemicals of interest have been divided into the following categories, based on chemistry[3]: alcohols, biocides, glycols, hydrocarbons, polymers, aldehyde/ketone and amines. It is noteworthy, however, that while chemicals in these groups serve the same purpose, the structures of the individual chemicals in these groups can vary markedly (Figure 1 - Figure 5). For instance, bronopol, methylisothiazolinone and glutaraldehyde are all used as biocides but have very different chemical structures (Figure 2).

[3] Biocides are chemically a diverse group of compounds, but all have activity against microorganisms and have been considered together in the present study.

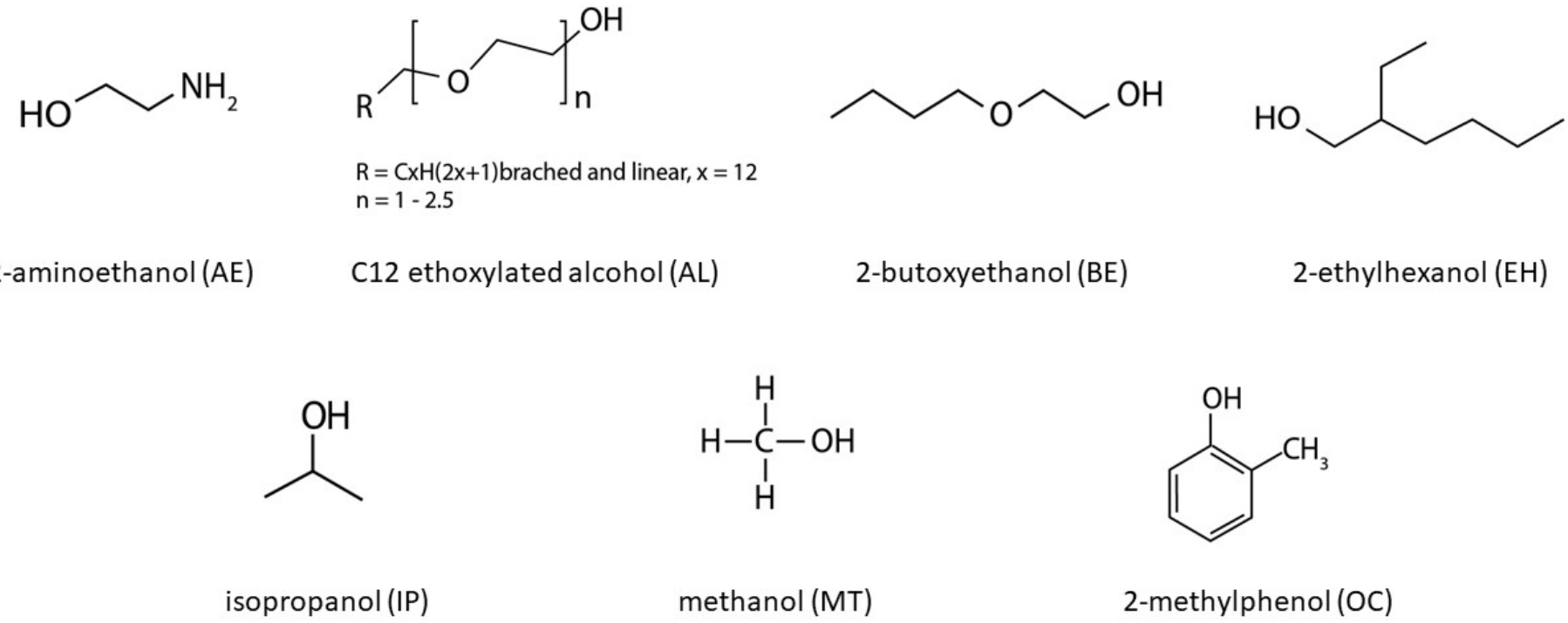
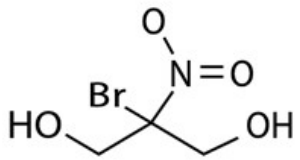
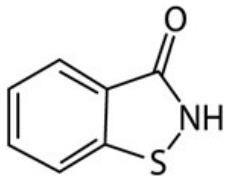


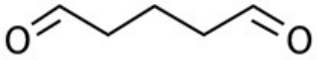
Figure 1: Structures of chemicals of interest (alcohol group)



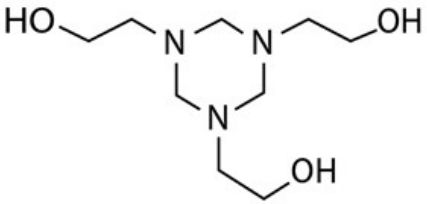
bronopol (BR)



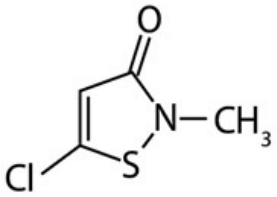
benzisothiazolinone (BZ)



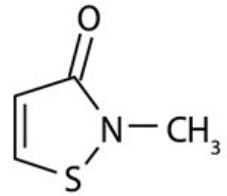
glutaraldehyde (GA)



hexahydro-1,3,5-tris(2-hydroxyethyl) sym-triazine (HT)



methylchloroisothiazolinone (MC)



methylisothiazolinone (MI)

Figure 2: Structures of chemicals of interest (biocide group)

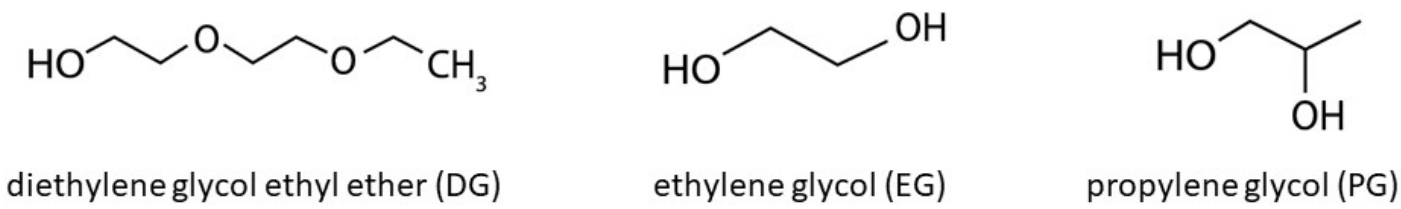


Figure 3: Structures of chemicals of interest (glycol group)

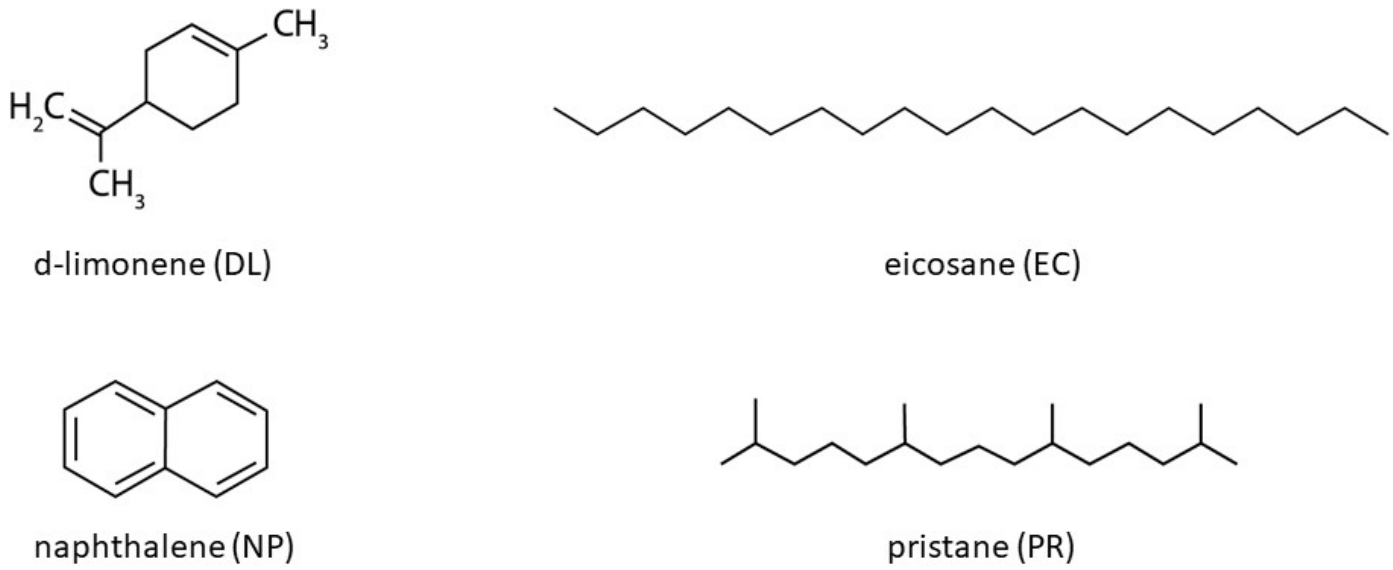


Figure 4: Structures of chemicals of interest (hydrocarbon group)

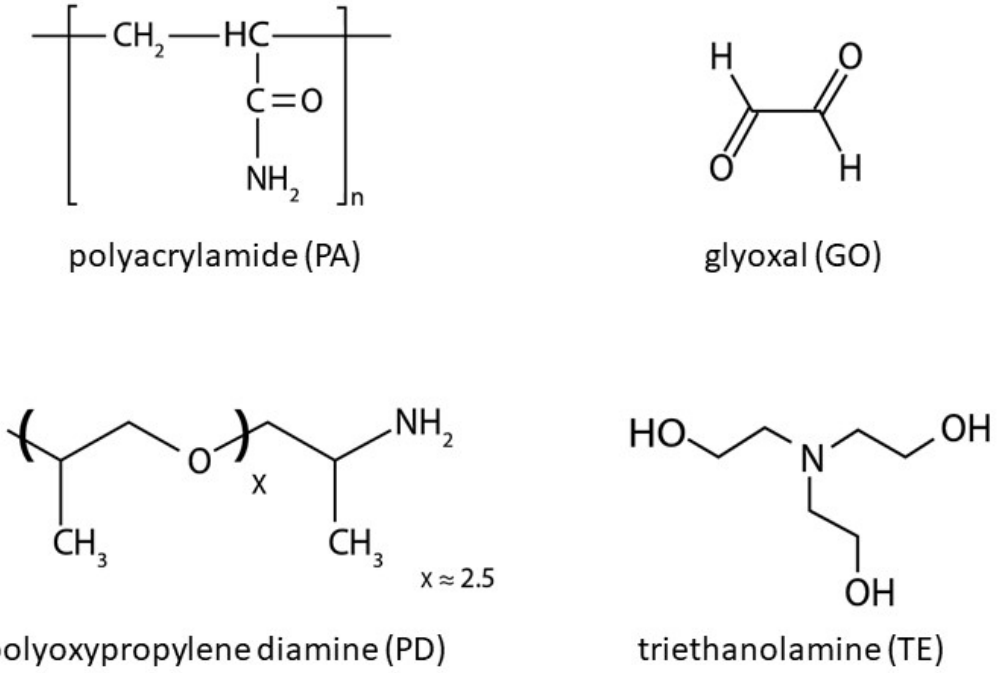


Figure 5: Structures of chemicals of interest (polymer | aldehyde/ketone | amine group)

1.2 The Beetaloo Region

The study region is a large area approximately 2,800,000 hectares in size, situated about 500 kilometres south-east of Darwin, Northern Territory. The region stretches from Mataranka in the North to Elliot in the south (Figure 6). In terms of landscape class, the majority of region comprises loamy and sandy plains, interspersed with moderate amount of clay plains. The eastern region differs slightly with more undulating country in the area around Santos' Tanumbirini exploration site.

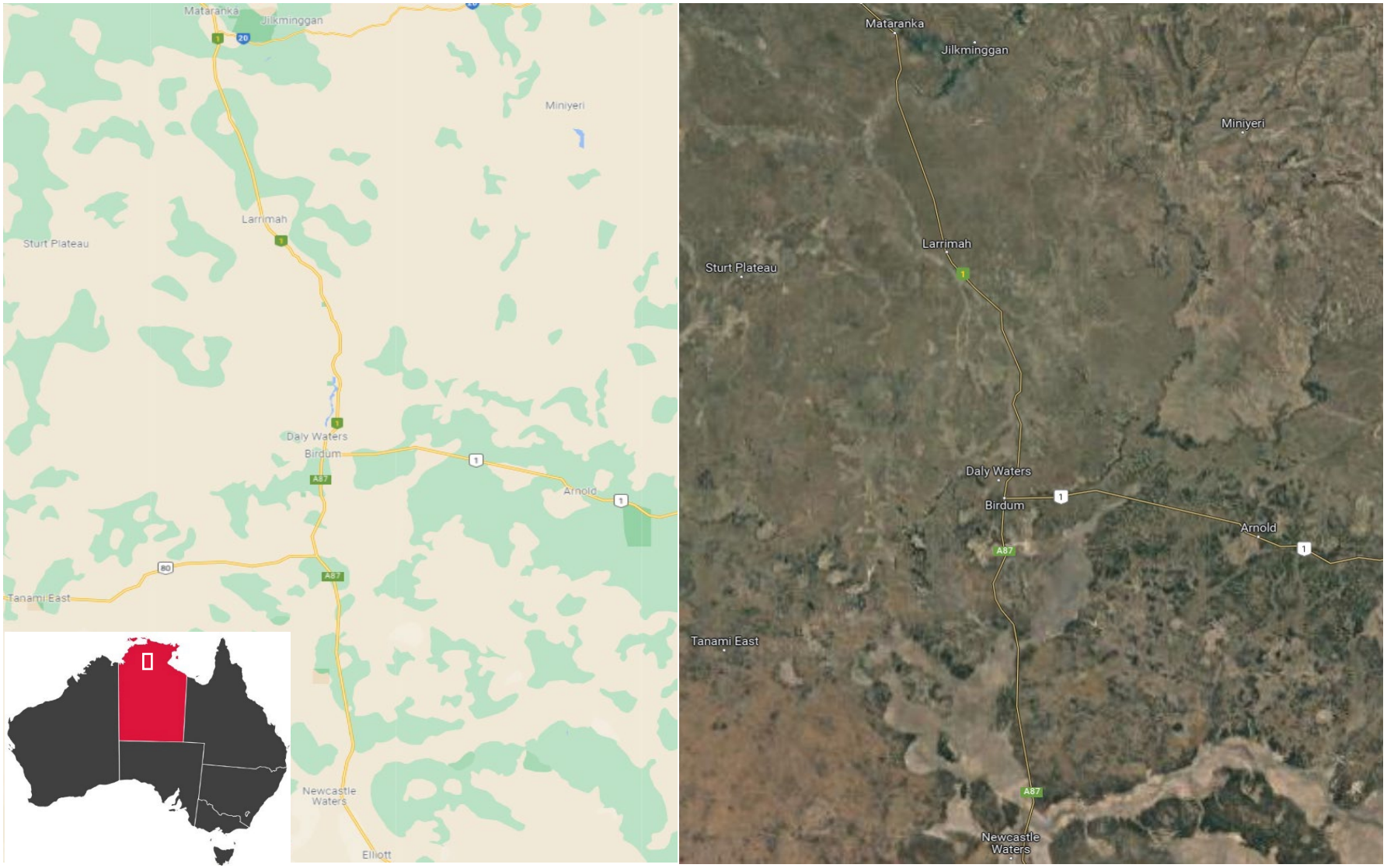


Figure 6: Map of the study region

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1.2.1 Soils

The majority of topsoils of the Beetaloo Basin are visibly similar though they constitute distinct soil types (Figure 7). Indeed, most of the topsoil within the area is sandy and includes significant iron oxide content making the soils a red colour. The most abundant soil type of the region is a kandosol (Figure 7). These red soils are widespread in the Northern Territory and vary with respect to their loam and sand content. Desert kandosols in the Beetaloo Region have a high silica and iron content. In addition to this soil type, this project also sampled putative rudosols, tenosols, vertosols and a chromosol from the western region of the Beetaloo Basin.

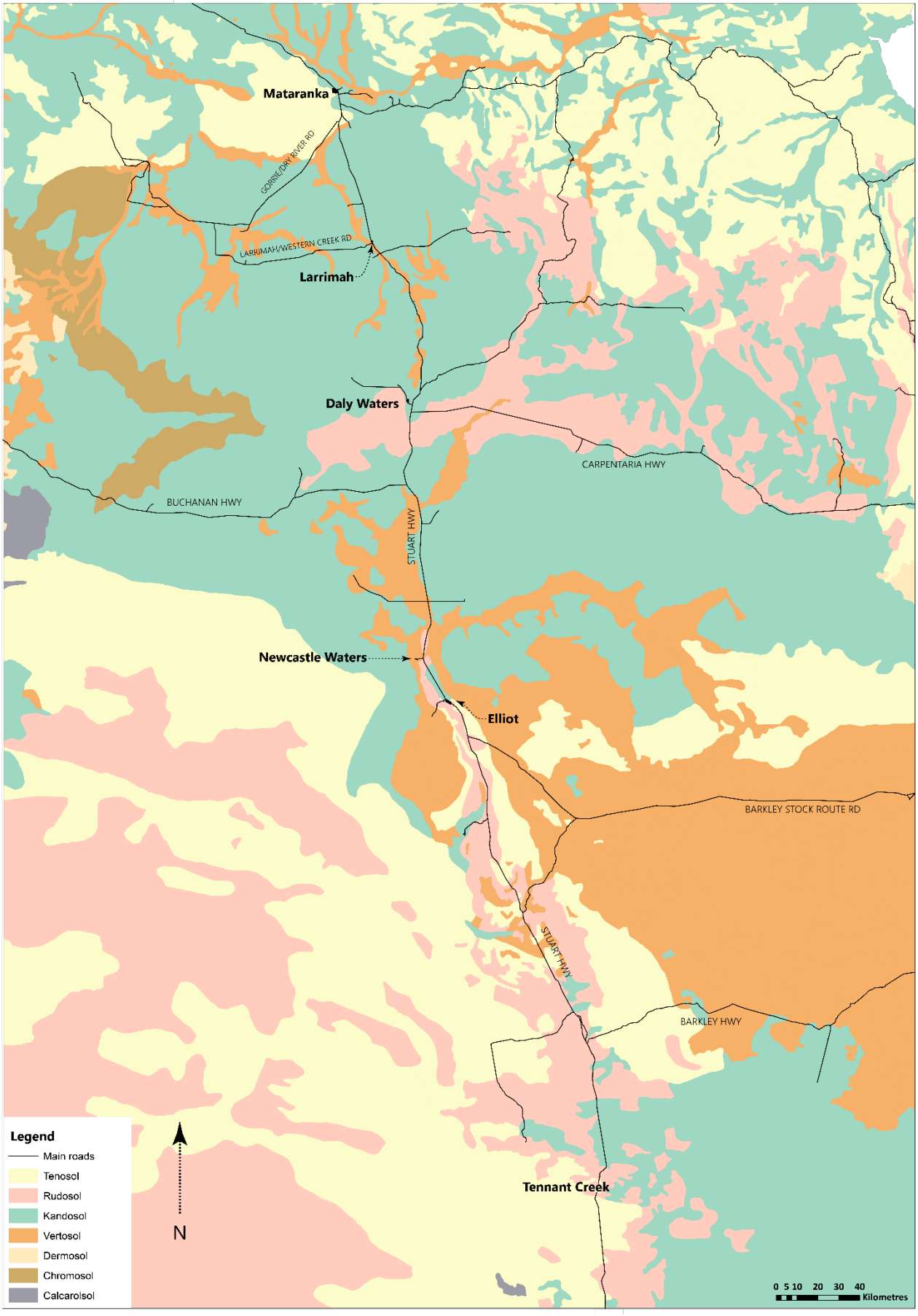

Figure 7: Soil map of the Beetaloo Basin

Modified from NT Government.

1.2.2 Aquifers

The major aquifers of the region are collectively referred to as the Cambrian Limestone Aquifers (CLA) (Figure 8). It is important to note that majority of aquifers of the region are shallow (10-50m), though some deeper aquifers do occur (for instance, in the Bukalara sandstone) (Figure 8). Regardless, these aquifers are vertically separate from the much deeper Kyalla and Velkerri shales which are the targets for onshore gas activities. The commonly used agricultural aquifers of the region are the Tindall, Gum Ridge and Anthony Lagoon aquifers. The latter is a disconnected aquifer, while the Tindall and Gum Ridge aquifers are part of the major regional aquifer and are connected hydrogeologically. Previous GISERA projects (W16)[4] have demonstrated that the predominant water type in the region are calcium (Ca)-sulphate (SO_4)-bicarbonate (HCO_3) types. GISERA W16 indicates this is fairly typical of aquifers associated with limestone and dolomitic aquifers.

[4] GISERA W16 report can be found at the GISERA website www.gisera.csiro.au or downloaded here

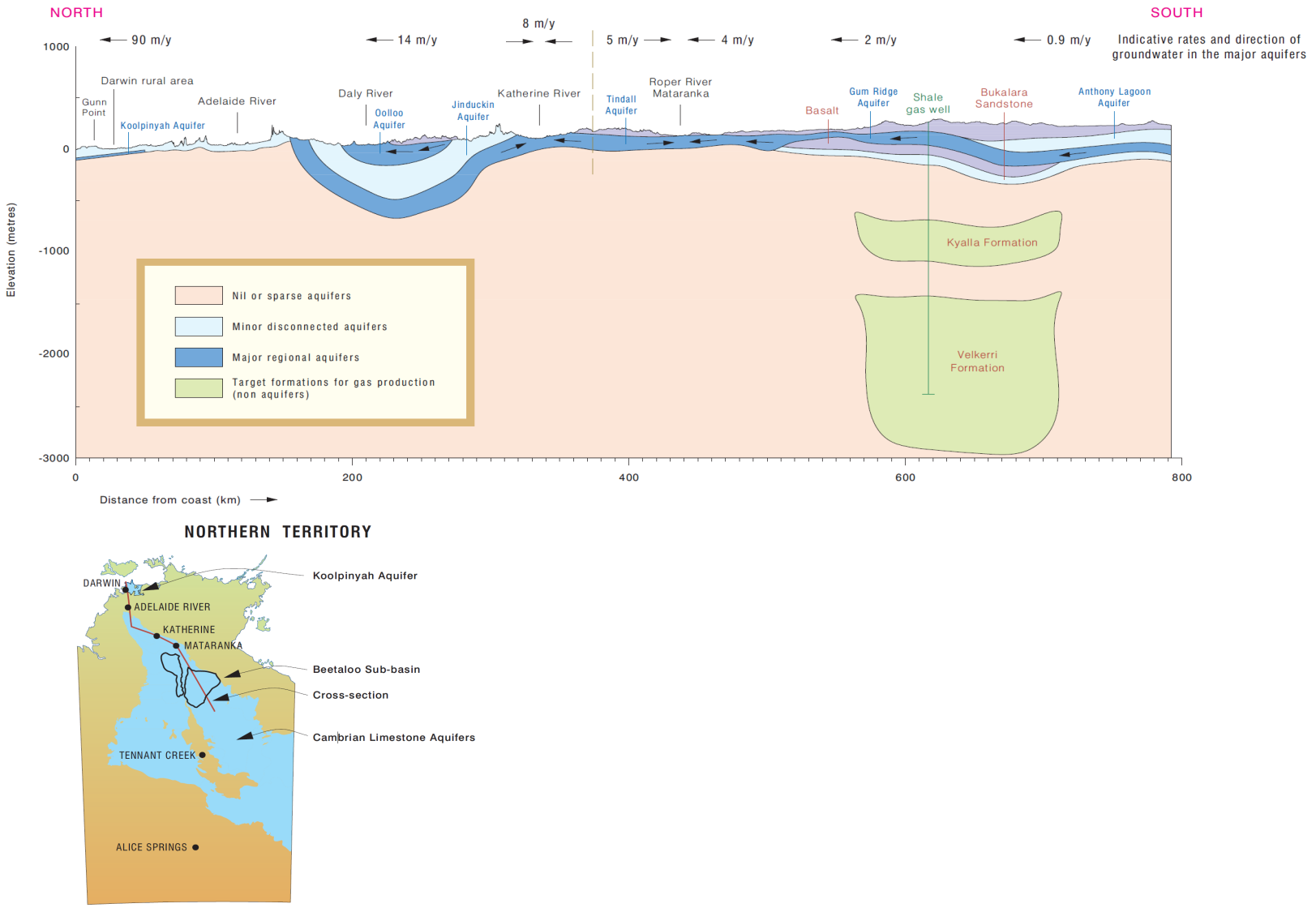

Figure 8: Major aquifers from Gunn Point to the Beetaloo Sub-Basin

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1.2.3 Microbiology and microbiological methods

The methods used in this report mirror those in GISERA W15. A detailed guide to the taxonomies used and biological systematics is available in that report (Download here).

In brief, however, this report follows modern microbiological taxonomic conventions.

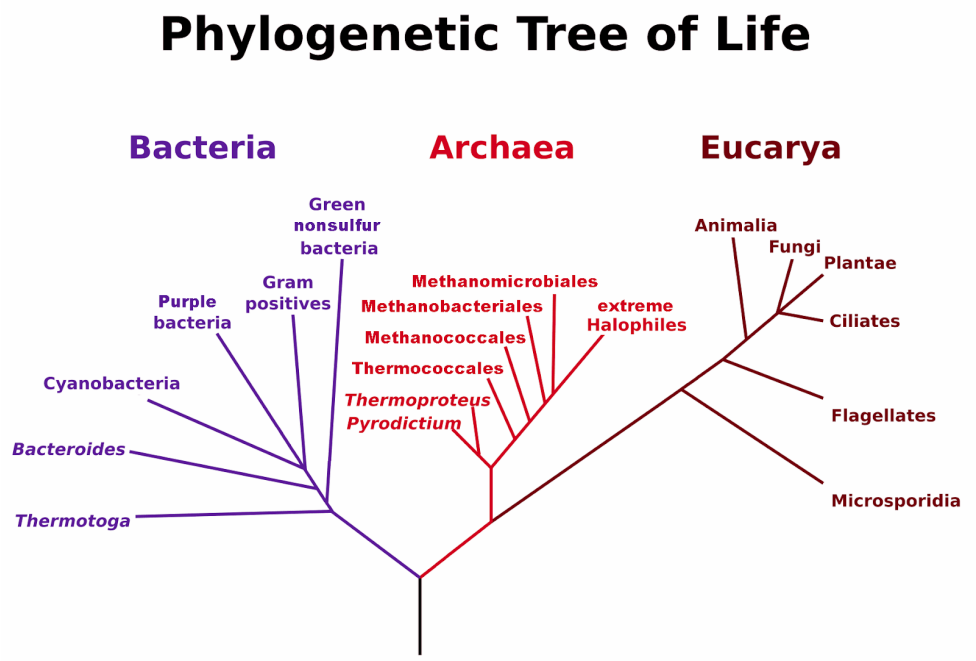

Figure 9: Phylogenetic tree of life

(Woese et al., 1990)

These conventions use the three-domain system of life which divides all living organisms into either Archaea, Bacteria or Eukarya (Figure 9). The first two domains (Archaea, Bacteria) include only prokaryotic life. That is, lifeforms that have no membrane bound organelles. For example, human cells hold their DNA within a membrane-bound nucleus, in comparison inside bacterial or archaeal cells, the DNA floats within the cytoplasm without any specific containment. While both the Archaea and Bacteria are superficially similar and both prokaryotic, these groups contain very significant differences in their genetic and biochemical composition. In contrast to the two prokaryotic domains, Eukarya contains most of the lifeforms that non-scientists would recognise as living (e.g. plants, fungi and animals).

This report focuses on groups of microbes that have major roles in degradation of organic compound i.e. Bacteria, Archaea and one group within the Eukarya (the Fungi). These organisms underpin biogeochemical cycles on earth.

The methods used to assay these organisms all use the polymerase chain reaction (PCR) and Illumina DNA sequencing. In brief, a portion of the 16S rDNA or the internal transcribed spacer (ITS; located between 18S and 5.8S structural rDNAs) are used as taxonomic markers for the Bacteria/Archaea and Fungi, respectively. The sequences of these markers are then compared to suitable databases and identities of detected organisms determined.

1.3 Aims

The aims of this project are:

1. Establish microbial community baselines in aquifer waters and soil samples of sites proximal to prospective unconventional gas activities in the Northern Territory (using bores previously sampled for GISERA water project - Baseline monitoring of groundwater properties in the Beetaloo sub-Basin (project W.16).
2. Understand the microbial degradation of a range of chemicals likely to be used in unconventional gas activities, in both the five major soil types of the region and in relevant aquifer environments.

2 Methods

2.1 Soil and water sample collection

All soil samples represent topsoil samples taken in the top 10cm of the soil. This location was deemed to be the location exposed during a putative spill event. In general soils were collected from locations with no covering debris, though on occasion, leaves or sticks were cleared from the soil surface before collection. Locations of all soils are shown below in Table 1 - Table 6. These tables indicate the sampling locations as shown in Figure 10.

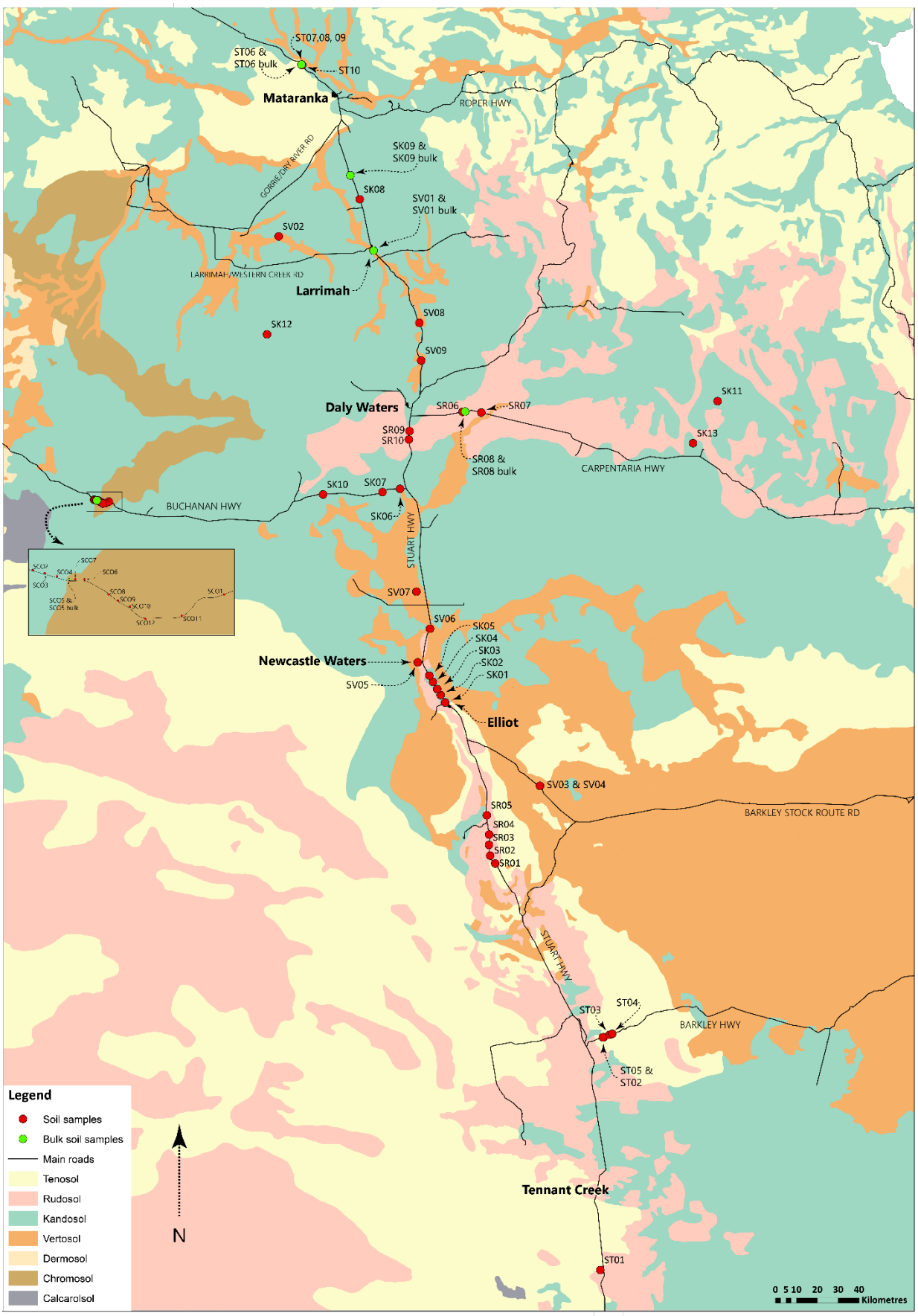

Figure 10: Map of soil sampling locations

Locations of soil samples (red) and bulk soil samples (green) obtained during W17.
SC -chromosols, SK - kandosols, SR - rudosols, ST - tenosols, SV - vertosols.
Major roads and towns are indicated. Soil types in the region are indicated in various colours

Table 1: Chromosol soil sample locations

Sample	Latitude	Longitude
Chromosol 01	-16.677	132.085
Chromosol 02	-16.669	132.022
Chromosol 03	-16.670	132.026
Chromosol 04	-16.671	132.030
Chromosol 05	-16.672	132.034
Chromosol 06	-16.672	132.039
Chromosol 07	-16.672	132.036
Chromosol 08	-16.677	132.047
Chromosol 09	-16.679	132.050
Chromosol 10	-16.681	132.054
Chromosol 11	-16.684	132.071
Chromosol 12	-16.685	132.059

Table 2: Kandosol soil sample locations

Sample	Latitude	Longitude
Kandosol 01	-17.543	133.534
Kandosol 02	-17.511	133.515
Kandosol 03	-17.485	133.500
Kandosol 04	-17.454	133.482
Kandosol 05	-17.427	133.466
Kandosol 06	-16.622	133.340
Kandosol 07	-16.636	133.264
Kandosol 08	-15.374	133.166
Kandosol 09	-15.271	133.126
Kandosol 10	-16.646	133.008
Kandosol 11	-16.244	134.708
Kandosol 12	-15.956	132.767
Kandosol 13	-16.425	134.602

Table 3: Rudosol soil sample locations

Sample	Latitude	Longitude
Rudosol 01	-18.237	133.750
Rudosol 02	-18.203	133.728
Rudosol 03	-18.157	133.723
Rudosol 04	-18.112	133.724
Rudosol 05	-18.029	133.714
Rudosol 06	-16.290	133.609
Rudosol 07	-16.293	133.690
Rudosol 08	-16.289	133.620
Rudosol 09	-16.373	133.381
Rudosol 10	-16.409	133.378

Table 4: Tenosol soil sample locations

Sample	Latitude	Longitude
Tenosol 01	-19.989	134.202
Tenosol 02	-18.986	134.217
Tenosol 03	-18.975	134.242
Tenosol 04	-18.971	134.254
Tenosol 05	-18.986	134.213
Tenosol 06	-14.793	132.915
Tenosol 07	-14.793	132.915
Tenosol 08	-14.793	132.916
Tenosol 09	-14.794	132.916
Tenosol 10	-14.796	132.918

Table 5: Vertosol soil sample locations

Sample	Latitude	Longitude
Vertosol 01	-15.595	133.226
Vertosol 02	-15.534	132.817
Vertosol 03	-17.902	133.943
Vertosol 04	-17.902	133.943
Vertosol 05	-17.370	133.416
Vertosol 06	-17.225	133.469
Vertosol 07	-17.065	133.410
Vertosol 08	-15.906	133.423
Vertosol 09	-16.069	133.431

Table 6: Bulk soil sample locations

Sample	Latitude	Longitude
Chromosol 5 bulk	-16.672	132.034
Kandosol 9 bulk	-15.271	133.126
Rudosol 8 bulk	-16.289	133.620
Tenosol 6 bulk	-14.793	132.915
Vertosol 1 bulk	-15.595	133.226

In total, 38 aquifer samples were collected from the locations shown in Table 7 and in Figure 11. Sampling only occurred from water bores with headworks using existing taps or fitted outlets on the pump to collect water samples. The locations of these samples are shown in Table 7. Where possible water bores were selected from the list of previously sampled bores in GISERA W16 project to provide baseline microbial community data. In some instances, however, collection from previously sampled bores was not possible and new bores were sampled to obtain representative samples for the present study. Table 8 lists the bores sampled in projects W16 and W17. Water bore report information were obtained from The Department of Environment, Parks and Water Security, Northern Territory Government[5], including EC, pH, bottom hole depth, standing water depth and aquifer formation (Table 14).

[5] Water bore reports downloaded from "Know your bore" website (https://nrmaps.nt.gov.au/knowyourbore_desktop.html)

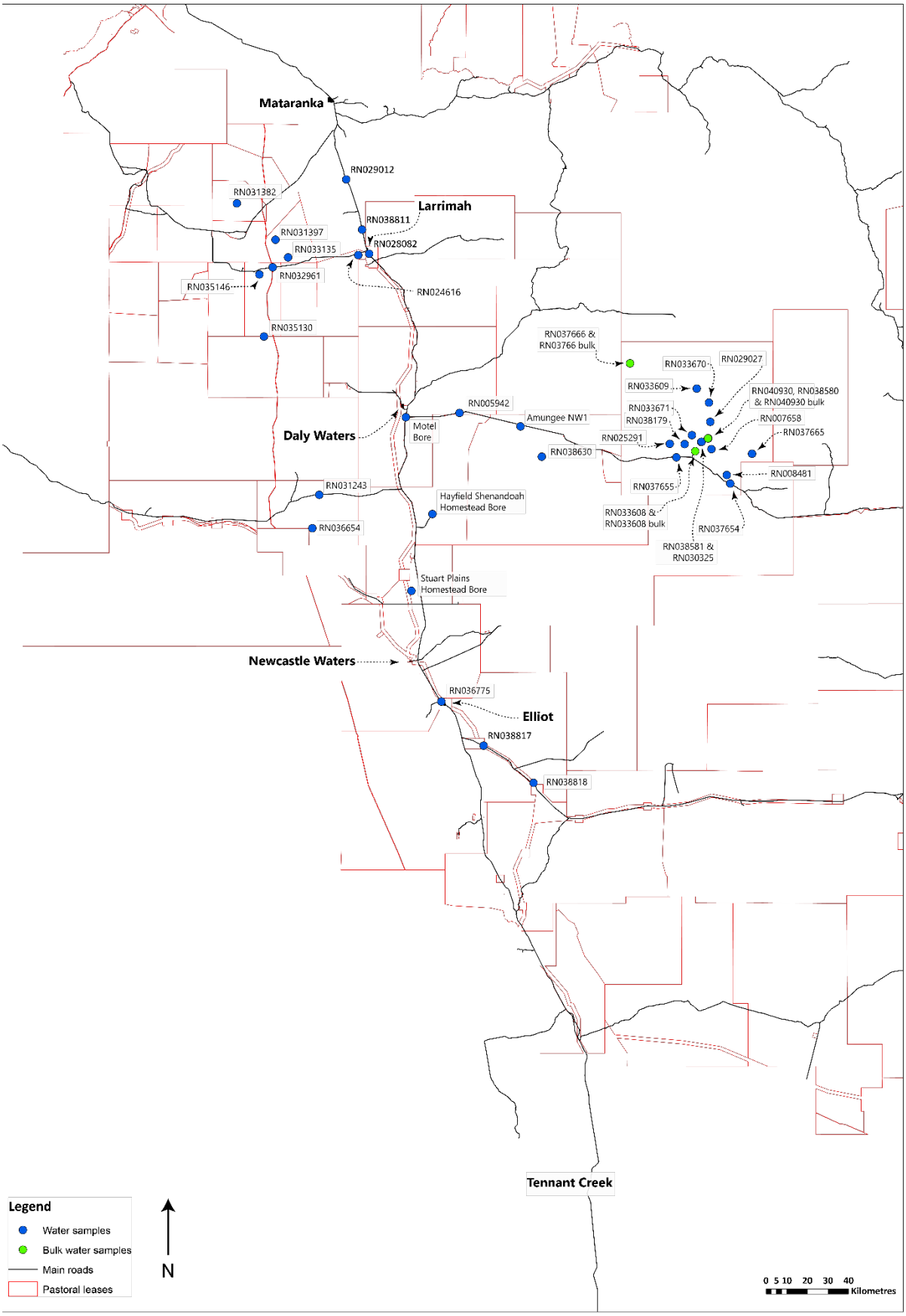

Figure 11: Map of water sampling locations

Locations of water samples (blue) and bulk water samples (green) obtained during W17. Red lines indicate pastoral lease boundaries.

Table 7: Aquifer water sample locations

Sample	Latitude	Longitude	Sample	Latitude	Longitude
Amungee NW1	-16.348	133.886	**RN033670**	-16.244	134.708
Hayfield Shenandoah Homestead Bore	-16.730	133.502	**RN033671**	-16.386	134.634
Motel Bore	-16.308	133.386	**RN035130**	-15.956	132.767
RN005942	-16.289	133.620	**RN035146**	-15.685	132.747
RN007658	-16.447	134.719	**RN036654**	-16.792	132.978
RN008481	-16.560	134.786	**RN036775**	-17.548	133.541
RN024616	-15.601	133.178	**RN037654**	-16.597	134.801
RN025291	-16.424	134.537	**RN037655**	-16.483	134.566
RN028082	-15.595	133.226	**RN037665**	-16.467	134.896
RN029012	-15.271	133.126	**RN037666***	-16.073	134.364
RN029027	-16.328	134.714	**RN038179**	-16.425	134.602
RN030325	-16.416	134.674	**RN038580**	-16.400	134.705
RN031243	-16.646	133.008	**RN038581**	-16.415	134.676
RN031382	-15.375	132.649	**RN038630**	-16.480	133.979
RN031397	-15.534	132.817	**RN038811**	-15.490	133.195
RN032961	-15.654	132.805	**RN038817**	-17.740	133.725
RN033135	-15.611	132.872	**RN038818**	-17.902	133.943
RN033608*	-16.456	134.648	**RN040930***	-16.400	134.704
RN033609	-16.183	134.655	**Stuart Plains Homestead Bore**	-17.065	133.410

*Bulk water and anoxic water samples were also collected at these locations

Table 8: List of Northern Territory water bores sampled in GISERA W16 and W17 projects

Bore	Project	Bore	Project
RN035130	W16, W17	**RN040930**	W17 only
RN033135	W16, W17	**RN036654**	W16, W17
RN031382	W16, W17	**RN029012**	W16, W17
RN032961	W16, W17	**RN037654**	W16, W17
RN035146	W16, W17	**Stuart Plains Homestead**	W16, W17
RN031397	W16, W17	**RN038817**	W16, W17
RN031243	W16, W17	**RN038581**	W17 only
RN005942	W16, W17	**RN033670**	W16, W17
RN036775	W17 only	**RN033609**	W16, W17
RN037665	W16, W17	**RN029027**	W16, W17
Heyfield/Shenandoah	W17 only	**Motel bore**	W17 only
RN037666	W16, W17	**RN038818**	W16, W17
RN038630	W16, W17	**Kalala S-1 Water Bore**	W16 only
RN038179	W16, W17	**RN005764**	W16 only
Amungee NW1	W16, W17	**RN029013**	W16 only
RN007658	W16, W17	**RN032231**	W16 only
RN033608	W16, W17	**RN032233**	W16 only
RN025291	W16, W17	**RN034660**	W16 only
RN037655	W16, W17	**RN036920**	W16 only
RN030325	W17 only	**RN037673**	W16 only
RN008481	W16, W17	**RN037932**	W16 only
RN038580	W16, W17	**RN038159**	W16 only
RN028082	W16, W17	**RN038175**	W16 only
RN024616	W16, W17	**RN038810**	W16 only
RN033671	W16, W17	**RN039080**	W16 only
RN038811	W16, W17	**Shenandoah 1 WB1**	W16 only

2.2 DNA preservation in the field

Both water and soil samples were collected in a preservation solution (DESS; Yoder et al., 2006) to preserve microbial DNA for various molecular analyses. This solution kills microbes in the sample without lysing the cells and protects the DNA from degradation. Additionally, use of a preservation solution simplified sample collection allowing samples to be stored at room temperature, shipped by freight or carried. To achieve this, this solution uses 20% dimethyl sulphoxide (DMSO) and 0.25 M disodium ethylenediaminetetraacetic acid (EDTA) saturated with sodium chloride (NaCl) (Seutin et al., 1991; Yoder et al., 2006). The former assists in carrying the preservative materials across cell membranes, while the latter two components cause microbial growth to cease and stabilises DNA.

2.2.1 Soil

Soils were sampled using sanitised spatulas in a Falcon tube (approximately 50g). Field sanitisation was undertaken by rinsing and wiping with methylated spirits. Soil samples were taken at pre-suggested locations throughout the sampling campaign to include a wide location of each of the five predominant soil types. The surface litter was removed prior to collection being undertaken and soil was sampled no deeper than 20cm.

2.2.2 Water

Three hundred millilitre water samples were collected using prepared Schott bottles of preservation solution (Yoder et al., 2006). Water was allowed to purge to ensure samples were collected from the aquifers and pH readings were taken initially and every 15 mins until the pH stabilised. Samples were sealed in Schott bottles with silicone rubber seals. Three samples were collected at each location (Figure 11).

2.3 Bulk Soils

Soils were sampled using sanitised spades into a sanitised polypropylene bucket (approximately 12kg). Field sanitisation was undertaken by rinsing and wiping with methylated spirits. Soil samples were taken from five predominant soil types (Figure 10).

The surface litter was removed prior to collection. Samples were sealed and transported back to laboratories in North Ryde, NSW.

2.4 Bulk Water

Bulk waters were collected from selected bores (Figure 11) after the appropriate purge time as described for the preserved DNA water samples above and stored in 25 L polypropylene drums for transport.

2.5 Anoxic Water

A smaller volume of ~1 L water was anoxically collected (under a CO_2 atmosphere, given these are limestone aquifers, CO_2 was thought to the be the major gas present) from selected bores (RN033608, RN037666, and RN040930). Water was bubbled vigorously with CO_2 prior to being sealed in Schott bottles with silicone rubber seals. Sealed bottles were transported inside eskies back to the CSIRO laboratory at North Ryde, NSW. On receipt at North Ryde, the bottles were vented inside an anaerobic chamber where the atmosphere comprised ~95% argon, 1-2% hydrogen and the balance nitrogen.

2.6 Chemicals used in this project

This project examined a range of chemicals used in onshore gas activities (Figure 1 - Figure 5). These chemicals were sourced from chemical suppliers as shown in Table 8. The chemicals used in this project were chosen after confirming with gas industry representatives that these chemicals were in use by industry.

Table 9: Chemicals used in this project

Chemicals	Abbreviation	CAS #	Supplier/Concentration Notes
2-aminoethanol	AE	141-43-5	Sigma-Aldrich, 98%
c12 alcohol ethoxylate	AL	68439-45-2	Oleum, 100%
2-butoxyethanol	BE	111-76-2	Sigma-Aldrich, 99%
bronopol	BR	52-51-7	Sigma-Aldrich, 98%
benzisothiazolinone	BZ	2634-33-5	Sigma-Aldrich, 97%
diethylene glycol ethyl ether	DG	111-90-0	Sigma-Aldrich 99%
d-limonene	DL	138-86-3	Sigma-Aldrich, 90%
diesel fuel	DS	68476-34-6	Commercial service station
eicosane	EC	112-95-8	Fluka, 97%
ethylene glycol	EG	107-21-1	Sigma-Aldrich, 99%
2-ethylhexanol	EH	104-76-7	Sigma-Aldrich, 99.6%
glutaraldehyde	GA	111-30-8	Sigma-Aldrich, 50%
glyoxal	GO	107-22-2	Sigma-Aldrich, 40%
hydrotreated light petroleum distillate	HP	64742-47-8	Sigma-Aldrich, 99%
hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine	HT	4719-04-4	Combi-Blocks, 75%
isopropanol	IP	67-63-0	BDH, 100%
methylchloroisothiazolinone	MC	26172-55-4	Combi-Blocks, 68%
methylisothiazolinone	MI	2682-20-4	Sigma-Aldrich, 95%
methanol	MT	67-56-1	Ajax, 99.8%
naphthalene	NP	91-20-3	BDH, 100%
o-cresol	OC	95-48-7	Sigma-Aldrich, 99%
polyacrylamide	PA	9003-05-8	Sigma-Aldrich, 100%, non-ionic water soluble
polyoxypropylene diamine	PD	9046-10-0	Sigma-Aldrich, avg Mn 400
propylene glycol	PG	57-55-6	Sigma-Aldrich, 100%
pristane	PR	1921-70-6	Sigma-Aldrich, 98%
triethanolamine	TE	102-71-6	BDH, 99%

CAS #- Chemical abstract society identifying numbers

2.7 Soil physicochemistry

The soil chemistry was measured by Australian Laboratory Services (ALS) Environmental. The schedule of tests and their method references are shown in Appendix A. Organic matter content was assessed by a loss on ignition method. Briefly, ~10g of oven dry soil (70 ^{o}C for 8 hrs) was weighed on a four decimal place balance (Mettler Toledo AL104 high precision balance) prior to loss on ignition in a muffle furnace at 400 ^{o}C for ~10 hours. The proportion of weight loss after treatment in the muffle furnace was calculated and expressed as the percentage organic matter in the soil. A conversion factor of 0.5 was used to convert soil organic matter to organic carbon content. This conversion factor was shown to accurately estimate organic carbon content in almost all soil types (Pribyl, 2010). Loss on ignition was conducted on two representative subsamples of each soil type.

Initial baseline measurements of chemicals of interest were performed on all soil types. Briefly, ~250mL of soil was flooded with 750mL sterile RO water and left to soak for three days. The recovered water was decanted and subsequently sampled for chemical analysis by ALS. All analyses were performed in triplicate.

2.8 Aquifer water chemistry

Characterisation of the chemical properties of the aquifer water samples was undertaken by ALS Environmental. The schedule of tests and their method references are shown in Appendix A. Initial characteristics of bores, at time of drilling, were obtained from the Department of Environment, Parks and Water Security, Northern Territory Government[6].

Initial baseline measurements of chemicals of interest were performed on three bulk water samples, namely RN033608, RN037666 and RN40930. All chemical analyses were done in triplicate by ALS.

[6] Know Your Bore: https://nrmaps.nt.gov.au/knowyourbore_desktop.html

2.9 Establishment of microcosm experiments

2.9.1 Soil microcosm experiments

On return to the North Ryde laboratories, the bulk soil samples of the five predominant soil types were coarsely sieved through a 6mm mesh, prior to extensive mixing. It was stored in the dark prior to use in experiments.

In order to establish microbial degradation of chemicals of interest (Table 9). Each 50mL vessel contained the following: ~10mL fresh soil, 2mL sterile reverse osmosis water (RO water). Compounds of interest were mostly added as solutions (100µl) to achieve concentrations of the compounds as shown in Table 10. Five chemicals varied from this procedure as follows: polyacrylamide (200µL of 5% polyacrylamide solution); pristane (11µL neat pristane); eicosane (11µL of neat eicosane that had been heated to 70^{o}C to liquify); D-limonene (0.2µL of neat D-limonene); naphthalene (added as a solid at ~2.75mg ± 0.25; n=10). All vessels were established in replicates of ten. Seven of these replicates were used for microcosm experiments, while three were available for chemical analyses.

Table 10: Initial concentrations of chemicals used in microcosm experiments

Chemicals	Abbreviations	Initial concentration (mg/L) microcosm experiments
2-aminoethanol	AE	1300
c12 alcohol ethoxylate	AL	500
2-butoxyethanol	BE	150
2-butoxyethanol	BE	150
bronopol	BR	70
benzisothiazolinone	BZ	10
diethylene glycol ethyl ether	DG	10
d-limonene	DL	20
diesel fuel	DS	Neat
eicosane	EC	1100
ethylene glycol	EG	1400
2-ethylhexanol	EH	250
glutaraldehyde	GA	180
glyoxal	GO	2300
hydrotreated light petroleum distillate	HP	13
hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine	HT	1600
isopropanol	IP	1000
methylchloroisothiazolinone	MC	10
methylisothiazolinone	MI	10
methanol	MT	1200
naphthalene	NP	80
o-cresol	OC	10
polyacrylamide	PA	1200
polyoxypropylene diamine	PD	1000
propylene glycol	PG	600
pristane	PR	1100
triethanolamine	TE	1700

For incubation, vessels were stored in air-tight, transparent polypropylene outer containers(Figure 12). To maintain humidity inside vessels at conditions like those observed in Daly Waters, Northern Territory, open beakers containing a saturated salt solution (NaBr) were included in all closed outer containers (Figure 12). Saturated NaBr solutions maintained the humidity at ~55% at the temperature ranges experienced inside the outer container (20-22 ^{o}C). Vessels were incubated for approximately 34 days with a day/night cycle (~12hrs light/12hrs day) provided by the dedicated lights.

Figure 12: Microcosm incubation (with loose lids) stored in polypropylene containers with open vessels of a saturated salt (NaBr) for humidity control.

2.9.2 Aquifer microcosm experiments

All aquifer microcosms were established in 50mL glass serum vials under an anoxic atmosphere that comprised ~95% argon, 1-2% hydrogen and the balance nitrogen. Three aquifer water samples were used for establishment of aquifer microcosms: RN033608, RN037666 and RN040930. Each vessel contained 10.0mL of filter-sterile aquifer water. This was inoculated with ~2.4mL of aquifer water collected under CO_2 atmosphere (see section 2.5) and compounds of interest were mostly added as solutions (100µl) to achieve concentrations of the compounds as shown in Table 10. Five chemicals varied from this procedure as follows: polyacrylamide (200µL of 5% polyacrylamide solution); pristane (11µL neat pristane); eicosane (11µL of neat eicosane that had been heated to 70^{o}C to liquify); D-limonene (0.2µL of neat D-limonene); naphthalene (added as a solid at ~2.75mg ± 0.25;

n=10). All vessels were established in replicates of ten. Vessels were incubated for approximately 90 days, at 20^{o}C in the dark.

2.10 Establishment of storage control/zero time control

Zero time controls for both soil and aquifer microcosms were set up without the addition of any chemicals, and immediately harvested (see sections 1.1and 2.13) to determine the composition of the microbial communities at the start of the experiments. Zero time controls were set up in replicates of ten.

Storage controls for both soil and aquifer microcosms were set up without the addition of any chemicals, to investigate and compare the changes in the microbial community composition resulting from the experimental procedure. Storage controls were set up in replicates of ten and were incubated with soil and aquifer experimental microcosms (see sections 2.9.1 and 2.9.2, respectively).

2.11 Establishment of single compound soil mimicking assays

Carbon-free soil mimicking medium (SMM) was used to model the five major soil types collected (Chromosol, Kandosol, Rudosol, Tenosol and Vertosol). While it is well accepted that most soil microbes grow poorly in pure culture, the aim of this task was to determine the existence of organisms capable of degradation of a given compound. Any resultant data are therefore likely a minimum set of degrading organisms capable of catabolising a given compound.

The SMM medium recipe varied between the soil types to match pH, total nitrogen and total phosphorus (see Table 11 for details). The pH of each SMM medium was adjusted appropriately for each soil type prior to the addition of 0.007g $FeCl_2.4H_2O$. SMM broth was autoclaved at 121°C for 30 mins with 115kPa pressure and allowed to cool before use.

All assays were established in sterile 70mL specimen jars. Each vessel contained 9.0mL of SMM and was inoculated with 1.0mL of prepared soil inoculum. Soil inoculum was prepared by adding 1g of each soil type to 100mL of the corresponding SMM. A tenfold serial dilution

of soil inoculums to a dilution factor of 10^8 was performed and dilutions of 10^6, 10^7 and 10^8 were used as inoculum to determine organisms capable of catabolising chemicals of interest. Chemicals of interest were mostly added as solutions (100µl) to achieve concentrations of the compounds as shown in Table 10. Five chemicals varied from this procedure as follows: polyacrylamide (200µL of 5% polyacrylamide solution); pristane (11µL neat pristane); eicosane (11µL of neat eicosane that had been heated to 70°C to liquify); D-limonene (0.2µL of neat D-limonene); naphthalene (added as a solid at ~2.75mg ± 0.25; n=10). Vessels were statically incubated for approximately 30 days, at 20°C in the dark.

Table 11: Soil mimicking media for major soil types

	Chromosol-SMM	Kandosol-SMM	Rudosol-SMM	Tenosol-SMM	Vertosol-SMM
NH_4Cl (gL^{-1})	2.06	0.55	2.62	1.04	0.67
KH_2PO_4 (gL^{-1})	1.58	0.44	0.63	0.76	0.61
$MgSO_4.7H_2O$ (gL^{-1})	0.14	0.14	0.14	0.14	0.14
$CaCl_2$ (gL^{-1})	0.05	0.05	0.05	0.05	0.05
NaCl (gL^{-1})	0.025	0.025	0.025	0.025	0.025
$ZnSO_4$ (gL^{-1})	0.003	0.003	0.003	0.003	0.003
Thiamine (µg)	133	133	133	133	133
pH	5.4	6.6	6.6	7.0	6.9

2.12 Harvesting soil microcosms

To harvest the soil microcosms, the vessels were flooded with 30mL sterile RO water and left to soak for two days. The recovered water was filtered through a miracloth to remove soil particulates and subsequently sampled for chemical analysis by ALS.

Initial and final compound measurements

To determine the initial and final compound concentrations measurements soil microcosms were harvested (as described above) immediately and at the end of the incubation period.

DNA recovery

After the incubation period, prior to harvesting the soil microcosms, 100mg of soil was subsampled and added directly into 96-well plates for extraction using the ZymoBIOMICS 96 DNA Kit (see section 2.15; Zymo Research).

2.13 Harvesting aquifer microcosms

Initial and final compound measurements

To determine the initial and final compound concentration measurements, aquifer microcosms were sampled immediately and at the end of the incubation period for analytical analysis by ALS.

DNA recovery

After the incubation period, the aquifer microcosms were vacuum filtered to capture all microorganisms onto a sterile 25mm PVDF disc (0.1µm pore size) using a sampling manifold (Millipore). The disc was sliced into small pieces using a sterile scalpel blade and added directly into 96-well plates for extraction using the ZymoBIOMICS 96 DNA Kit (see section 2.15; Zymo Research).

2.14 Harvesting single compound soil mimicking assays

The single compound soil mimicking assays were visually inspected after the incubation period and the highest dilution factor assays showing growth were harvested by vacuum filtration to capture all microorganisms onto a sterile 25mm PVDF disc (0.1µm pore size) using a sampling manifold (Millipore). The disc was sliced into small pieces using a sterile scalpel blade and added directly into 96-well plates for extraction using the ZymoBIOMICS 96 DNA Kit (see section 2.15; Zymo Research).

2.15 DNA extraction

All DNA extractions from samples used a 96-well plate format and the ZymoBIOMICS 96 DNA Kit. Soil microcosm DNA were extracted directly from 100mg soil samples (see section 2.12). Aquifer microcosm DNA and single compound soil mimicking assay DNA were extracted from filter disks (see sections 2.13 and 2.14 ,respectively). DNA extractions were carried out following the manufacturer's instructions[7] using the recommended lysis protocol for bead-beating with the Mini-BeadBeater-96 (Biospec).

2.16 DNA quantification

DNA quantification was conducted with the use of Quaint-it^{TM} PicoGreen dsDNA Reagent and Kits – an ultrasensitive fluorescent nucleic acid stain for detection of small amounts of double-stranded DNA (dsDNA) in solution. The kit contained a stock DNA solution (λ) of concentration 100µg mL^{-1} (in Tris EDTA buffer), and PicoGreen reagent. Quantification then called for the addition of PicoGreen reagent to extracted samples and subsequent measurement with a plate reader – POLARstar Omega (BMG LABTECH), with excitation at 385nm and emission 520nm, gain 1000, and 10 flashes per well.

For all DNA samples, the PicoGreen reagent was diluted 200-fold. Also included in the Quaint-it^{TM} PicoGreen Kit was a λ DNA standard (100 µg mL^{-1}) which was diluted to make

[7] ZymoBIOMICS 96 DNA kit protocol chrome-extension://oemmndcbldboiebfnladdacbdfmadadm/https://files.zymoresearch.com/protocols/_d4303_d4307_d4309_zymobiomics_96_dna_kit.pdf

5mL of a 4µg mL^{-1}solution. The 4µg mL^{-1} λ solution was then used to make appropriate standards.

All wells used had a total volume of 100µL. For standards, 50µL of standard solution was used. For all samples, 2µL was used, with 48µL of water. Fifty µL of diluted PicoGreen reagent was added to all standards and samples prior to measurement within 5 minutes of addition.

Standard curves were determined for every plate run using the means of each standard. Unknown concentrations were derived from the standard curve associated with that plate.

2.17 Preparation for DNA sequencing

For 16S and ITS PCR product sequencing, Macrogen[8] requires DNA to be in the range 5-10ng μL^{-1}. After quantification, all samples with a concentration outside the range of 3-20ng μL^{-1} were adjusted to comply with the needs of PCR analysis. For those DNA extractions with concentrations lower than detection limit (< 25pg mL^{-1}), the DNA extracts were evaporated in an Eppendorf Concentrator *Plus* for 2.5 hours at 45°C, appropriate volumes of sterile RO water were then added to the remaining DNA. In order to resuspend dry DNA, samples were shaken gently at 50°C and 300rpm for 5 minutes.

2.18 Sequencing control sample

In order to ascertain the level of 'bleed' between samples (i.e. cross-contamination of samples), a single sample was included with its own unique barcode. This sample consisted of an artificial chimeric amplicon (as shown in Figure 13), so as to be distinct from other amplified 16S sequences. The presence of this amplicon with alternative barcodes would identify the amount of pre-PCR cross contamination of the samples.

[8] Macrogen https://dna.macrogen.com/

Figure 13: A schematic of the artificial chimeric amplicon used in the present study

The 16S primers are shown by the red (515F) primer, the purple (806rb) primer. Spacers are shown in cyan. The intervening sequence (black) is a partial fungal ITS sequence from the ericoid mycorrhizal species *Cairneyella variabilis*. This sequence is normally not amplified by PCR for 16S and as such represents an ideal control.

2.19 DNA sequencing

All DNA in this project was sequenced by Macrogen (https://dna.macrogen.com/). PCRs were conducted on extracted DNA using the following 16S or ITS primers (Apprill et al., 2015; Caporaso et al., 2012, 2011; White et al., 1990) (Figure 14).

For Bacteria and Archaea 16S rDNA

515F (Parada; GTG**Y**CAGCMGCCGCGGTAA) & 806R (Apprill; GGACTAC**N**VGGGTWTCTAAT)

For Fungi: Internal Transcribed Spacer (ITS)

ITS: ITS1F (CTTGGTCATTTAGAGGAAGTAA) & ITS2R (GCTGCGTTCTTCATCGATGC)

Figure 14: PCR primers used in the present study

Sequencing was conducted on an Illumina miSEQ DNA sequencer. Both sequence types were requested for their 250bp paired-end service, however, the sequencing provider, Macrogen, in error, completed the ITS sequencing using 150bp paired-end sequencing. This resulted in some non-typical analyses for the ITS data shown under bioinformatics.

2.20 Bioinformatics

Both 16S and ITS amplicon datasets were processed using the Greenfield Hybrid Amplicon Pipeline (GHAP)[9]. Analyses for 16S followed the typical workflow. In brief, data was demultiplexed, subjected to quality control prior to merging, OTU clustering and mapping reads back to the resultant OTU table.

2.21 Statistical analyses and plotting

Ordinations of soil and aquifer communities subject to chemical treatments

The OTU table generated for all treatments in soil and water was subject to ordination using non-metric multidimensional scaling (nMDS) (Hammer and Harper, 2007; Harper, 1999; Kruskal, 1964). Data were normalised and imported into the Past3 statistical package (Hammer et al., 2001) for nMDS analyses using the Bray-Curtis dissimilarity index (Beals, 1984; Bray and Curtis, 1957). The resultant data were plotted in Python 3.7.3 using the Matplotlib module (Hunter, 2007). All chemical data were analysed using principal components analyses (PCA) in the R statistical package (R Core Team, 2017) using the standard library. The resultant data were plotted in R or in Python 3.7.3 using the Matplotlib module (Hunter, 2007).Identification of both sensitive and catabolising taxa was undertaken using Simper (Clarke, 1993).

2.22 Simpson's Index

Simpson's Index (1-D) was calculated for the soil, water and sole-carbon source trials (Simpson, 1949). The formula used to calculate Simpson's Index is shown below:

$$D = \frac{\Sigma n(n-1)}{N(N-1)}$$

where n = is the number of individuals of a particular taxon, and N is the total number of organisms. The index includes both species richness (the number of species) and species

[9] GHAP is available at https://doi.org/10.4225/08/59f98560eba25

evenness. Using the 1-D form of the index means that treatments/environments with values closer to one are more biodiverse, and treatments /environments with values closer to zero are less biodiverse.

3 Results and Discussion

3.1 Soil and aquifer physicochemistry

3.1.1 Soil chemistry

It should be noted here that the soils collected for this study were identified using their location on a soil map sourced from the Northern Territory government. Soils were not chosen based on their appearance or other characteristics (such as local topology). Throughout this study, samples were named for the soil type they were collected from, using the soil map as a guide. It seems likely, given the remoteness of the area, that the soil types shown on the map in Figure 7 were likely extrapolated from agricultural sampling using topological and other data. Such an approach means that there has been no independent confirmation that a given soil type occurs at a given site. Given the vast area that the Beetaloo Basin encompassed, these soil type descriptions are likely not reliable.

This is further supported by the physical appearance of the soils which were all, with the exception of the tenosol, iron-rich, highly sandy soils. Thus, while the soil samples in this report are referred to by their soil type names (e.g. kandosol, tenosol etc), the designations of these soil types are likely unreliable. This is borne out in analyses of soil microbiology (for an expanded discussion, see section 3.2.1) where samples that are geographically proximal to each other are more similar microbiologically (regardless of 'soil type' status).

Large bulk soil samples (20kg) were obtained for the five dominant soil types in the sampling area, which were chromosol, kandosol, rudosol, tenosol and vertosol (Figure 15). Given the large size of these samples and the acknowledged heterogeneity of soils, these samples were individually sieved and mixed prior to three distinct, but representative, samples being taken. These were designated as C, K, R, T or V (for chromosol, kandosol, rudosol, tenosol and vertosol, respectively) with a replicate number (see Table 12). These samples were then subject to soil chemistry characterisation at a NATA accredited laboratory (ALS), while soil organic matter content was determined inhouse by the loss on ignition method.

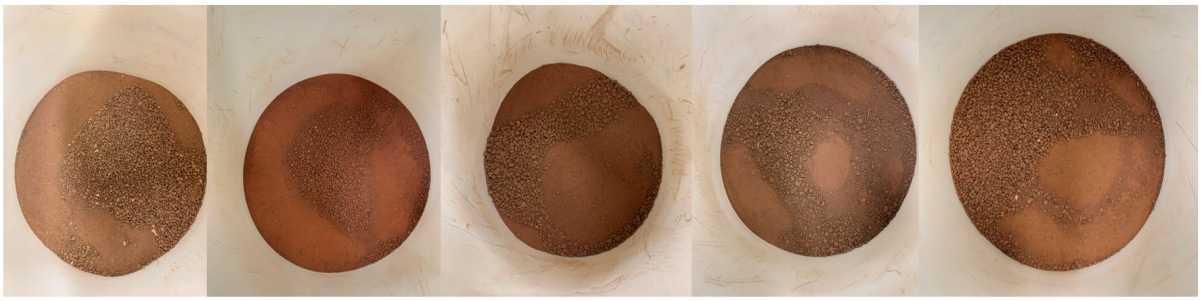

Figure 15: Bulk soils after sieving and mixing.

From left to right: Chromosol, kandosol, rudosol, tenosol and vertosol.

Chemically, the soils were broadly similar. All were acidic to neutral in pH (ranging from 5.1 to 7.0), contained relatively few soluble cations/anions and in turn had low conductivity (Table 12). There were, however, some marked differences. For instance, the chromosols examined had higher iron and phosphate content. Conversely, the tenosol examined was neutral in pH and had markedly lower iron content (Table 12). All soils sampled were low in soil organic carbon content, ranging from 0.46 to 1.73% (Table 13), as would be expected from arid region soils (Jobbágy and Jackson, 2000; Klemmedson, 1989).

Taking the chemistry data as a whole, principal components analyses revealed three broad groupings with the chromosol and rudosol distinct from each other and the remaining three soils which generally grouped together (Figure 16). The main drivers for these differences were the higher pH and (in general) lower metal content of the kandosol, tenosol and vertosol soils. There are many differences, however, the following are worth noting. The iron content varied across the five soil types by almost an order of magnitude. For example, the tenosol had an average iron content of 7,263 mg kg^{-1}, while the chromosol had an average iron content of 55,233 mg kg^{-1}. For manganese, significant differences were also observed, for example the chromosol had just 3 mg kg^{-1} and the manganese concentration was 180X higher in the rudosols ($\bar{x}$ = 546 mg kg^{-1}). The analysis of the variables that drive differences between these soils is shown in Figure 17. Those variables coloured red/orange contribute more to the differences between the soil types.

Soil data for the area are readily available for physical aspects of the soils, noting the caveats described above regarding soil types. It is noteworthy, however, that while pastoralists and other agriculturalists of the region have detailed soil chemistry data, there

are relatively little data published on the soils of this region. Some data are, however, available from the Australian Microbiome initiative. Sampling undertaken by this consortium in the Beetaloo Basin region was mainly undertaken to the east of the Sturt Highway, particularly to the north and south of Arnold. Regardless, data from the Australian Microbiome initiative demonstrate that most soils of the region are acidic to neutral in pH ($\bar{x}$ = 6.7 ± 0.8). Some outliers included moderately acidic soils (~pH 5.5) observed to the south of Arnold, and a single alkaline soil sample to the north of Arnold (> pH 8). Conductivity of the soils determined by the Australian Microbiome Initiative[10] were broadly low having a mean EC of ~26 µS cm^{-1}. All of these observations are consistent with the data presented here. Similarly, a study of soils surrounding termite mounds in the Northern Territory from areas proximal to that of the current study, showed broadly similar results for pH (Lee and Wood, 1971). While the termite soil survey did not directly measure soil EC, low soluble salt concentrations were reported that would be consistent with the low EC values reported here (Lee and Wood, 1971). Lee and Wood (1971) also reported low soil organic carbon contents, like those reported here (Table 13).

[10] Australian Microbiome: https://www.australianmicrobiome.com/

Table 12: Soil chemistry of the major soil types used in the present study

Analyte (mg kg^{-1}, exceptions marked with *)	C1	C2	C3	K1	K2	K3	R1	R2	R3	T1	T2	T3	V1	V2	V3
pH Value*	5.6	5.1	5.6	6.6	6.6	6.7	6.6	6.6	6.6	7	7	7	6.9	7	6.9
Electrical Conductivity @ 25°C**	41	45	48	30	22	26	53	72	56	21	23	18	33	34	45
Moisture Content***	1.6	1.5	1.6	<1.0	<1.0	<1.0	1.7	1.6	2.4	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Silica	569	505	565	1110	853	1110	1070	1000	1100	788	843	893	618	662	726
Chloride	<100	<100	<100	<200	<200	<200	<200	<200	<200	<200	<200	<200	<100	<100	<100
Calcium	<10	<10	<10	<10	<10	<10	20	20	20	<10	<10	<10	<10	<10	<10
Magnesium	<10	<10	<10	<10	<10	<10	10	10	10	<10	<10	<10	<10	<10	<10
Sodium	20	20	20	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Potassium	10	10	10	30	20	30	40	40	40	30	30	30	20	20	20
Aluminium	5310	5980	6140	4350	4300	4740	10300	9230	8290	2490	2740	3030	4520	4120	4290
Barium	90	110	110	20	20	20	150	140	140	30	30	40	30	20	20
Chromium	61	59	55	59	70	82	71	64	61	8	13	10	59	49	53
Cobalt	<2	<2	<2	2	2	<2	16	15	16	5	6	7	6	5	5
Copper	8	10	11	<5	<5	<5	14	13	13	<5	<5	<5	<5	<5	<5
Iron	56400	57000	52300	30300	30700	40200	34900	32300	31800	5940	7880	7970	25600	23200	23800
Lead	9	9	9	6	6	7	18	18	18	<5	<5	<5	7	6	6
Manganese	20	22	21	109	103	110	546	539	553	340	420	463	279	223	227
Nickel	3	3	3	<2	<2	<2	6	6	6	2	3	3	<2	<2	<2
Strontium	15	18	19	2	2	2	25	24	24	<2	2	3	3	2	2
Vanadium	134	134	130	61	64	76	110	102	97	16	22	22	80	72	73
Uranium	1	1.1	1.1	0.4	0.4	0.4	1.4	1.4	1.4	0.1	0.2	0.2	0.5	0.4	0.4
Fluoride	140	180	180	<40	<40	<40	<40	<40	<40	<40	<40	<40	70	40	50
Nitrite + Nitrate as N (Sol.)	8.7	10.2	10.2	1.8	2.1	2.1	20.8	21.1	20.8	2.8	3.1	3.4	5.2	5.3	7.4
Total Nitrogen as N	550	440	630	140	170	120	720	660	680	290	260	270	180	160	190
Total Phosphorus as P	409	285	383	102	100	96	147	137	144	311	110	101	130	144	141
Reactive Phosphorus as P	<1.0	0.5	<1.0	<1.0	<1.0	1.2	<2.0	<2.0	1	<2.0	<2.0	1.1	0.6	0.7	0.8

Chromosol (C), kandosol (K), rudosol (R), tenosol (T) and vertosol (V) bulk (20kg) samples. Each 20kg sample was sieved and mixed prior to being sampled three times to reduce and measure variation within the sample. *pH units, ** µSiemens / cm, *** %

Table 13: Organic matter and carbon content of soil types

Soil	Soil organic matter (%)	Soil organic carbon (%)
Chromosol	2.63	1.31
Kandosol	0.92	0.46
Rudosol	3.45	1.73
Tenosol	1.09	0.54
Vertosol	1.44	0.72

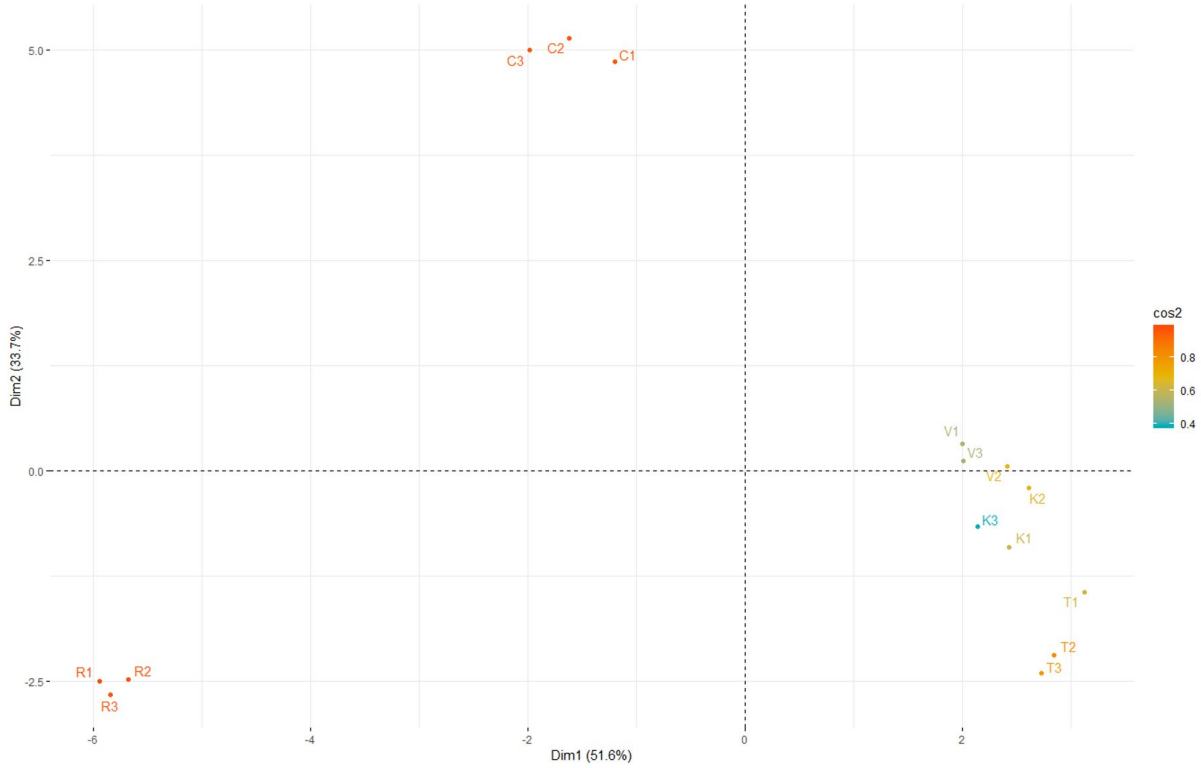

Figure 16: Principal components analysis of soil chemistry data by soil type

Chromosol (C), kandosol (K), rudosol (R), tenosol (T) and vertosol (V)

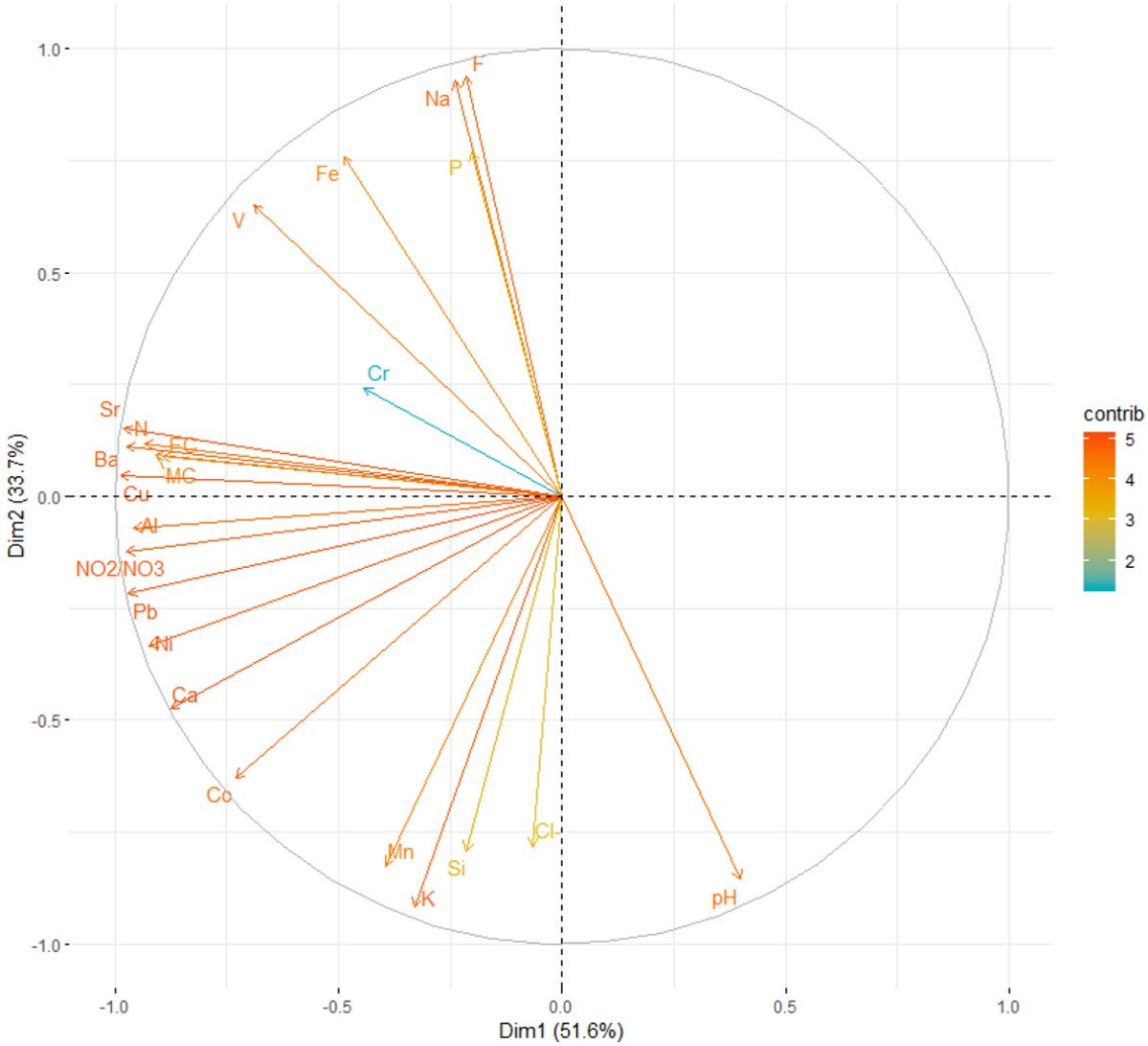

Figure 17: Principal components variable correlation plot of soil chemistry

3.1.2 Aquifer chemistry

Bore completion reports for all 38 bores were examined for initial characteristics (EC, pH, bottom hole depth and standing water level) at time of drilling (Table 14). Table 14 also lists the aquifer formation that the bore interacts with.

Table 14: Water bore report information and aquifer formation details

Bores	Formation	Bottom Hole Depth (m)	Standing Water Level (m)	EC (µS/cm)	pH
RN035130	Tindall Limestone	93	63	-	-
RN033135	Tindall Limestone	52.5	31.2	704	8.34
RN031382	Tindall Limestone	80.6	57.1	747	7.63
RN032961	Tindall Limestone	120	46.4	588	7.7
RN035146	Tindall Limestone	69	46.5	-	-
RN031397	Tindall Limestone	229.1	36.65	2340	8.38
RN031243	Montijinni Limestone	156	103	-	-
RN005942	Gum Ridge Formation	101.4	85.4	900	7.9
RN036775	Unknown	105	59.8	926	7.99
RN037665	Gum Ridge Formation	132	90.5	-	6.7
Heyfield/Shenandoah	Gum Ridge Formation	-	-	-	-
RN037666	Gum Ridge Formation	140	81	-	6.7
RN038630	Anthony Lagoon Beds	135	95	-	8.08
RN038179	Gum Ridge Formation	155	107	-	6.7
Amungee NW1	Gum Ridge Formation	-	-	-	-
RN007658	Gum Ridge Formation	94.5	79.2	1850	7.7
RN033608	Gum Ridge Formation	156	120	1046	-
RN025291	Gum Ridge Formation	150	103	1010	7.8
RN037655	Gum Ridge Formation	155	126		6.77
RN030325	Unknown	101	88.6	-	-
RN008481	Gum Ridge Formation	117	84	1170	7.7
RN038580	Gum Ridge Formation	120	59.9	1640	-
RN028082	Tindall Limestone	203.2	45		8
RN024616	Tindall Limestone	-	-	-	-
RN033671	Gum Ridge Formation	114	75.4	1093	-
RN038811	Tindall Limestone	243.66	47.2	1544	6.96
RN040930	Unknown	182	57.6	1654	-
RN036654	Montijinni Limestone	106	74.5	-	6.49
RN029012	Tindall Limestone	121.8	41.6	1730	6.7
RN037654	Gum Ridge Formation	155	114.6	-	6.5
Stuart Plains Homestead	Anthony Lagoon Beds	-	-	-	-
RN038817	Gum Ridge Formation	203	43	1504	8.79
RN038581	Unknown	114	73.4	1572	-
RN033670	Gum Ridge Formation	60	36.4	1124	-
RN033609	Gum Ridge Formation	48	28	1459	-
RN029027	Gum Ridge Formation	98	65	1645	8
Motel bore	Unknown	-	-	-	-
RN038818	Gum Ridge Formation	285.9	49.15	1771	8.67

In total, detailed water chemistry was collected for 38 bores sampled in this present study (Table 15). In the broadest terms, waters from the region were similar, all were neutral to slightly alkaline, and had low salinity (measured by EC) with samples ranging from 650-2140 µS cm^{-1}.

There were, however, some significant differences between the samples. Specifically, a portion of the waters from the northwest area of the Beetaloo region were generally slightly lower in pH and had lower electrical conductivity (EC), with a notable exception being RN029012. For example, one of the samples from the northwestern area, RN031382 had a pH of 7.2 and an EC of 827 µS cm^{-1}, in comparison, samples from the southeastern region (south of Elliot; RN038817 and RN038818) had pHs of 7.75 and 7.89, along with ECs of 1490 µS cm^{-1} and 1770 µS cm^{-1}, respectively.

These differences are not surprising as the boreholes sampled in this study intersect different formations (see Figure 8) with different geological chemistries. Overlaying formation data onto the water chemistry provides some interesting insights into the differing subsurface chemistry of the Beetaloo Basin region. All samples have modest levels of magnesium (consistently 0.2 in the Ca:Mg:Na/K ratio) with the other two groups (calcium and the sodium/potassium group) varying from 0.8 down to 0.2 depending on the sample (Figure 18). Further examination of the cation data with the formation data demonstrates that a subset of bores (RN033135, RN035130, RN032961, RN031382, RN035146) that intersect the Tindall formation are associated with significantly higher calcium levels (Ca >0.6 in the Ca:Mg:Na/K ratio). Interestingly, a second group of nearby bores within the Tindall formation (RN024616, RN028082, RN038811, RN029012, RN031397) had significantly lower calcium levels (all <0.4 Ca in the Ca:Mg:Na/K ratio). The reasons for this split in cation composition for the samples from the Tindall Limestone are unknown. It may be related to differing recharge rates at the local scale, differences within the formation with respect to the geochemistry of the limestone or the product of different aquifers mixing within a single bore.

The Tindall Limestone aside, those bores that intersect the Gum Ridge formation have a range of calcium and sodium/potassium ratios. Some bores within this formation are quite rich in sodium/potassium. For example, RN031397 and RN038818 all have

sodium/potassium ratio components over 0.6 (in the Ca:Mg:Na/K ratio), while others such as RN037665 have quite low sodium and potassium (Figure 18).

The major anion data demonstrate similar patterns, with the Tindall Limestone bores again being split into two distinct subgroups (Figure 19). Indeed, high bicarbonate content (>0.85 HCO_3 in the HCO_3:Cl:SO_4 ratio) was observed in bores RN035130, RN033135, RN031382, RN032961, RN035146, RN031397. Except for RN031397, these are the same bores that had elevated Ca cation ratios. Except for the single sample from the Montijinni Limestone, the remaining samples cluster together with no discernible pattern in the major anion concentrations by formation (Figure 19). The single bore that intersects the Montijinni Limestone (RN031243) has quite high bicarbonate content and is similar in major anionic composition to the high bicarbonate samples from the Tindall Limestone. This pattern for RN031243 is also echoed in the cationic composition, with this bore being more similar to the high calcium Tindall boreholes than the other samples in this study.

The two chemically distinct groups from the Tindall formation seen here (Figure 18, Figure 19 and Figure 21) were also reported in a previous GISERA survey of aquifers (W16[11]).

[11] GISERA W16 report can be found at the GISERA website www.gisera.csiro.au or downloaded here

Table 15: Water chemistry of the aquifers sampled in this study

Analyte (mg L^{-1}, exceptions marked with *)	Amungee NW1	Heyfield/Shenandoah	Motel bore	RN005942	RN007658	RN008481	RN024616	RN025291	RN028082	RN029012	RN029027	RN030325
pH*	7.51	7.61	7.92	7.5	7.43	7.43	7.33	7.35	7.46	7.41	8.03	7.51
EC**	1140	1100	2140	1110	1290	1280	1620	1240	1620	1790	1710	1260
Total Alk.	350	329	383	358	373	377	434	372	453	442	339	366
SO_4	144	70	247	109	149	167	153	167	151	186	258	164
Cl^-	104	129	378	67	123	121	210	115	219	247	221	115
Ca	117	88	125	121	130	125	145	128	141	138	107	130
Mg	50	58	65	46	57	55	56	56	57	60	72	55
Na	61	62	302	60	81	80	152	76	157	193	186	77
K	10	16	35	10	12	11	16	11	18	29	30	12
Ba	0.106	0.063	0.051	0.059	0.049	0.053	0.078	0.054	0.074	0.053	0.036	0.051
Li	0.052	0.043	0.048	0.045	0.059	0.051	0.029	0.052	0.03	0.042	0.24	0.051
Sr	0.677	0.307	0.908	0.532	0.737	0.771	0.392	0.728	0.417	0.82	0.966	0.747
Zn	0.009	0.005	0.062	0.03	0.017	0.006	0.057	0.005	0.053	0.018	0.005	0.078
B	0.15	0.18	0.38	0.15	0.16	0.17	0.2	0.17	0.22	0.32	0.24	0.16
F^-	0.5	0.4	0.5	0.4	0.7	0.7	0.2	0.7	0.3	0.5	1.2	0.7
NO_2/NO_3	0.01	0.79	2.06	0.01	0.01	0.01	0.24	0.01	0.58	1	0.01	0.01
Total N	0.1	0.8	2.2	0.1	0.1	0.1	0.2	0.1	0.6	1	0.1	0.1
DOC	1	2	1	1	1	1	3	1	1	2	1	1

*pH units, ** µSiemens / cm, *DOC - dissolved organic carbon, Total Alk - Total Alkalinity

Table 15 cont.

Analyte (mg L^{-1}, exceptions marked with *)	RN031243	RN031382	RN031397	RN032961	RN033135	RN033608	RN033609	RN033670	RN033671	RN035130	RN035146	RN036654
pH*	7.36	7.21	7.8	7.32	7.37	7.35	7.48	7.46	7.41	7.53	7.38	7.53
EC**	872	827	650	840	671	1260	1580	1600	1290	713	885	1550
Total Alk.	389	445	275	409	350	371	370	343	351	383	424	380
SO_4	27	2	5	2	1	157	347	299	176	1	7	153
Cl^-	37	19	35	30	14	119	152	150	119	7	30	214
Ca	106	126	44	132	129	124	158	161	132	108	124	112
Mg	42	35	8	33	16	54	67	70	57	29	43	56
Na	32	17	96	19	10	77	108	109	77	9	20	162
K	8	2	2	2	1	11	17	17	12	4	3	20
Ba	0.087	0.021	0.568	0.018	0.177	0.057	0.028	0.027	0.047	0.042	0.031	0.067
Li	0.018	0.016	0.054	0.017	0.01	0.055	0.104	0.097	0.06	0.018	0.016	0.034
Sr	0.196	0.137	0.192	0.118	0.16	0.745	0.755	0.801	0.767	0.074	0.096	0.466
Zn	0.005	0.005	0.005	0.005	0.005	0.005	0.006	0.005	0.005	0.018	0.005	0.005
B	0.07	0.05	0.79	0.06	0.05	0.18	0.2	0.2	0.17	0.05	0.07	0.24
F^-	0.2	0.2	0.4	0.2	0.5	0.7	0.6	0.6	0.7	0.2	0.2	0.2
NO_2/NO_3	0.54	0.24	0.01	0.32	0.61	0.01	0.01	0.01	0.01	0.06	0.87	2.52
Total N	0.5	0.2	0.1	0.3	0.6	0.1	0.1	0.1	0.1	0.1	0.9	2.7
DOC	2	3	6	3	3	1	7	1	1	2	4	2

*pH units, ** µSiemens / cm, *DOC - dissolved organic carbon, Total Alk - Total Alkalinity

Table 15 cont.

Analyte (mg L^{-1}, exceptions marked with *)	RN036775	RN037654	RN037655	RN037665	RN037666	RN038179	RN038580	RN038581	RN038630	RN038811	RN038817	RN038818	RN040930	Stuart Plains Homestead
pH*	7.55	7.45	7.58	7.4	7.66	7.42	7.5	7.52	7.86	7.5	7.75	7.89	7.44	7.61
EC**	1170	1440	1230	1260	940	1140	1320	1570	1160	1740	1490	1770	1310	1480
Total Alk.	362	340	367	399	263	318	374	367	371	444	328	311	345	345
SO_4	44	179	164	167	1	108	182	184	146	173	121	269	208	143
Cl^-	140	158	115	62	167	114	114	222	106	242	230	264	116	217
Ca	101	116	124	145	64	111	137	135	120	143	98	104	137	103
Mg	46	65	55	62	29	48	58	62	51	61	57	52	59	59
Na	87	110	75	52	97	73	75	128	67	186	151	243	75	147
K	24	22	11	12	11	10	12	20	11	24	27	28	12	23
Ba	0.162	0.042	0.057	0.041	0.833	0.071	0.042	0.063	0.054	0.058	0.048	0.034	0.043	0.062
Li	0.017	0.18	0.051	0.072	0.071	0.054	0.063	0.063	0.052	0.036	0.035	0.042	0.065	0.043
Sr	0.986	0.848	0.725	0.673	0.408	0.654	0.767	0.858	0.719	0.589	1.14	1.47	0.749	0.956
Zn	0.005	0.005	0.005	0.114	0.318	0.382	0.005	0.017	0.029	0.035	0.005	0.024	0.025	0.005
B	0.28	0.21	0.16	0.14	0.28	0.16	0.17	0.17	0.16	0.27	0.32	0.54	0.16	0.33
F^-	1	0.8	0.7	0.6	0.5	0.6	0.7	0.7	0.5	0.3	1	1.7	0.7	0.8
NO_2/NO_3	2.77	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.82	2.66	0.01	0.01	2.37
Total N	3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.8	2.8	0.1	0.1	2.6
DOC	4	1	1	1	1	1	1	1	1	2	1	1	1	4

*pH units, ** µSiemens / cm, *DOC - dissolved organic carbon, Total Alk - Total Alkalinity

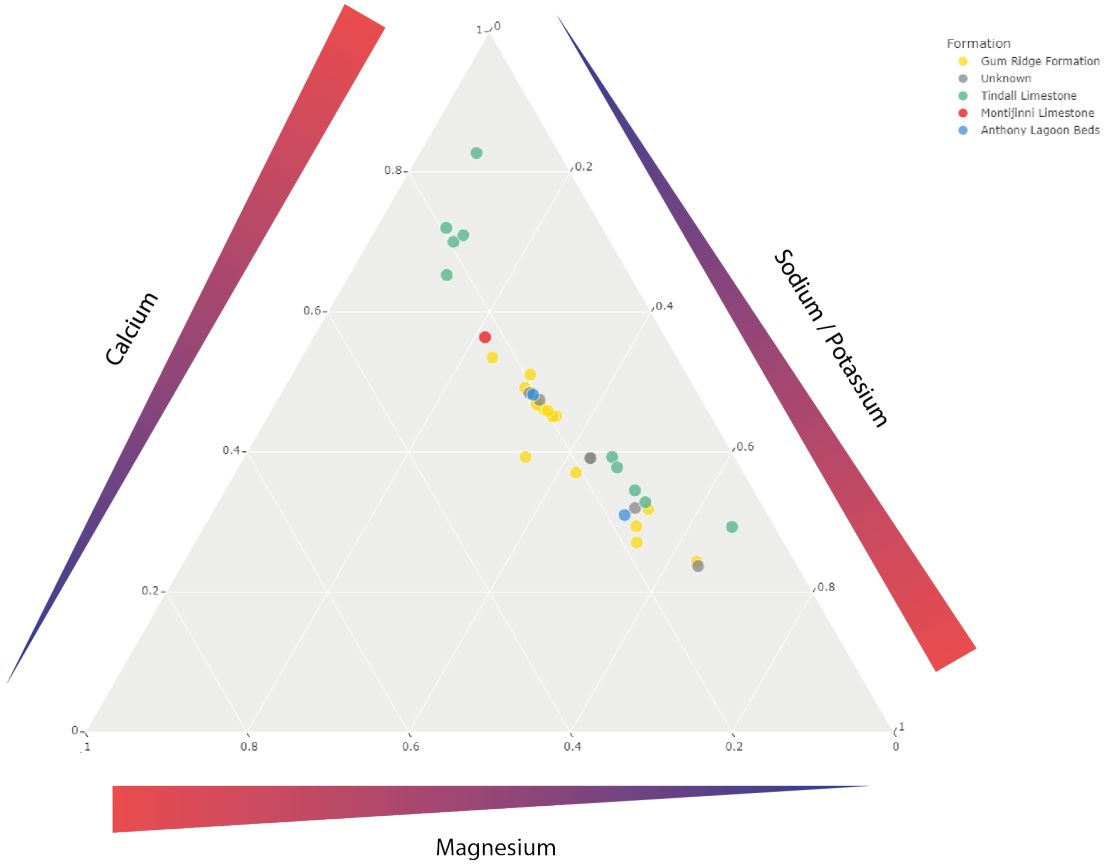

Figure 18: Ternary plot showing the ratio relationship between the major cations (calcium, magnesium and a combined sodium / potassium group) with respect to formation

The bars show increasing components within the ratio.

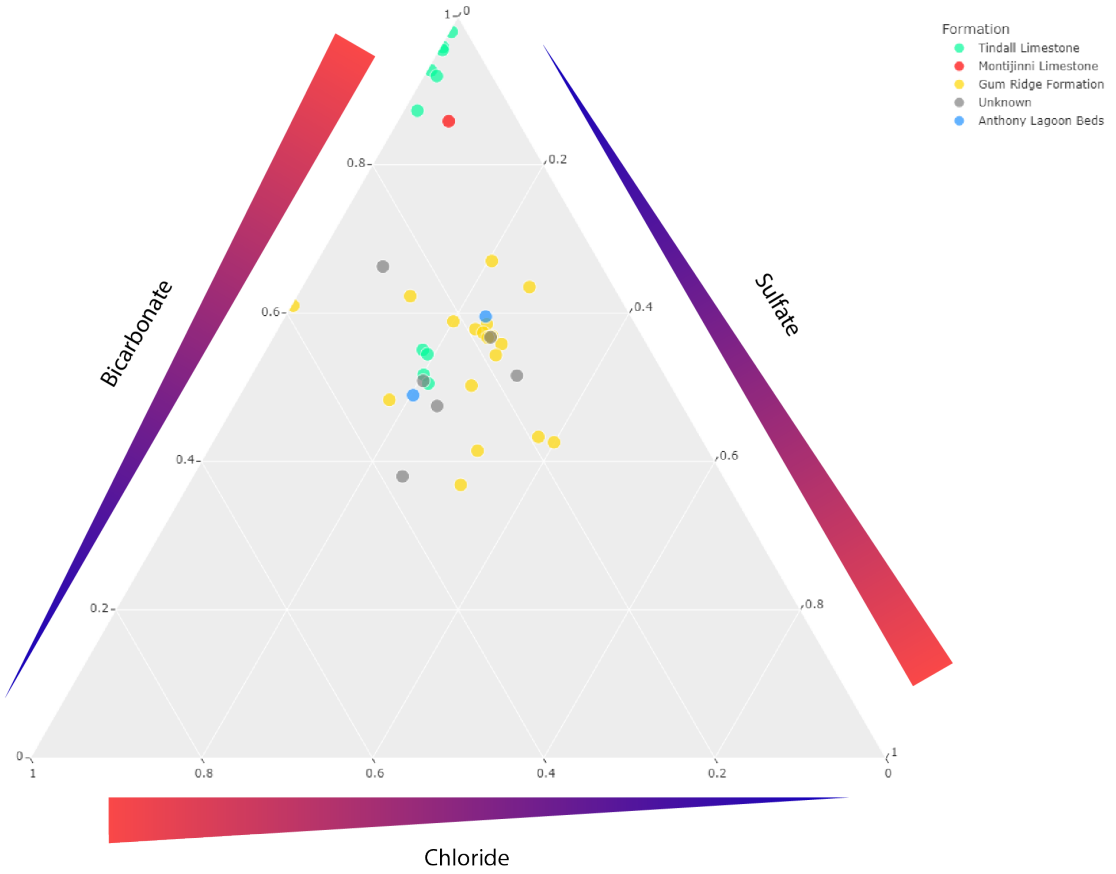

Figure 19: Ternary plot showing the ratio relationship between the major anions (bicarbonate, chloride and sulfate) with respect to formation

The bars show increasing components within the ratio.

Major cations and anions aside, PCAs were conducted to compare the water chemistry from the 38 bores in its entirety (Figure 20 and Figure 21). Trends in the PCA broadly reflect those observed in the major cation and anion analyses of the water chemistry. For example, samples from the Tindall Limestone still appear to be composed of two, distinct groups. The PCA of the entire water chemistry data highlighted another difference of RN031397 from the remaining samples from the Tindall Limestone. This sample, along with a small number of other samples had barium concentrations an order of magnitude higher than most bores examined in this study. Other drivers of modest differences in chemistry included magnesium, sulfate, strontium, potassium, chlorine and sodium (see Figure 22) which were higher in samples such as RN029012 and RN037654.

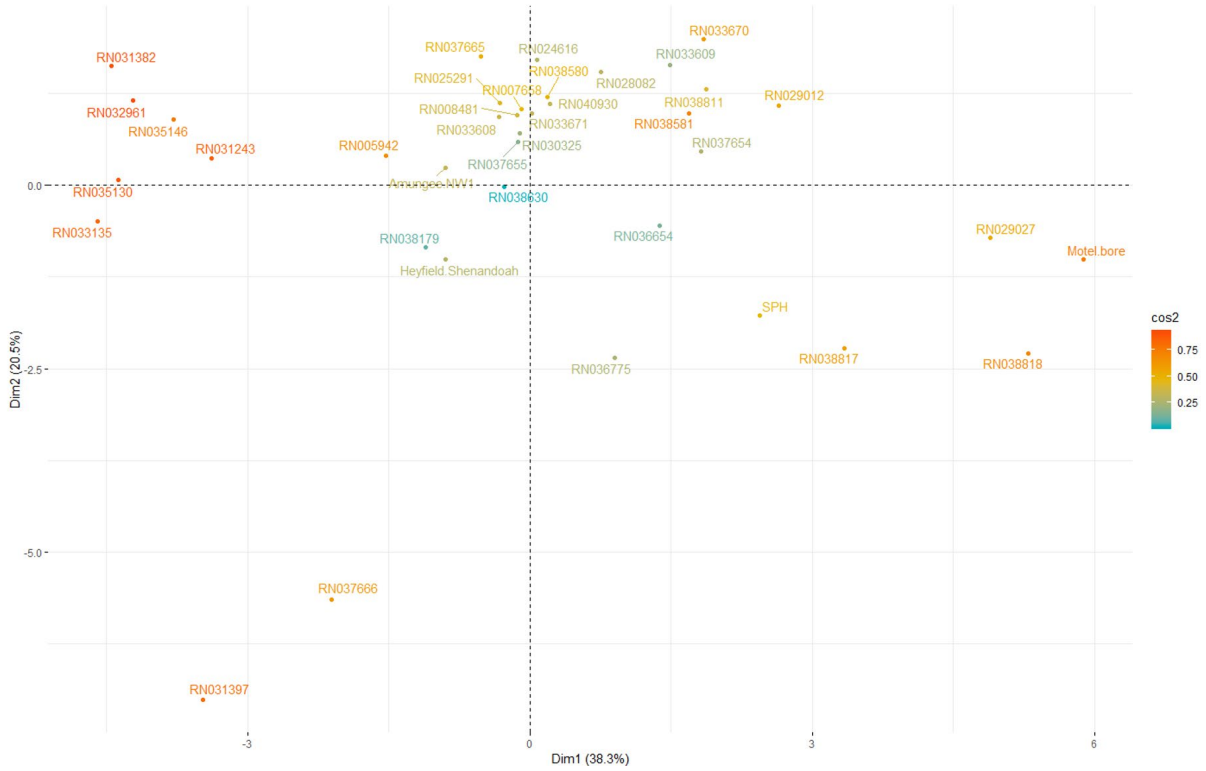

Figure 20: Principal components analysis of water chemistry data by water bore

SPH- Stuart Plains Homestead

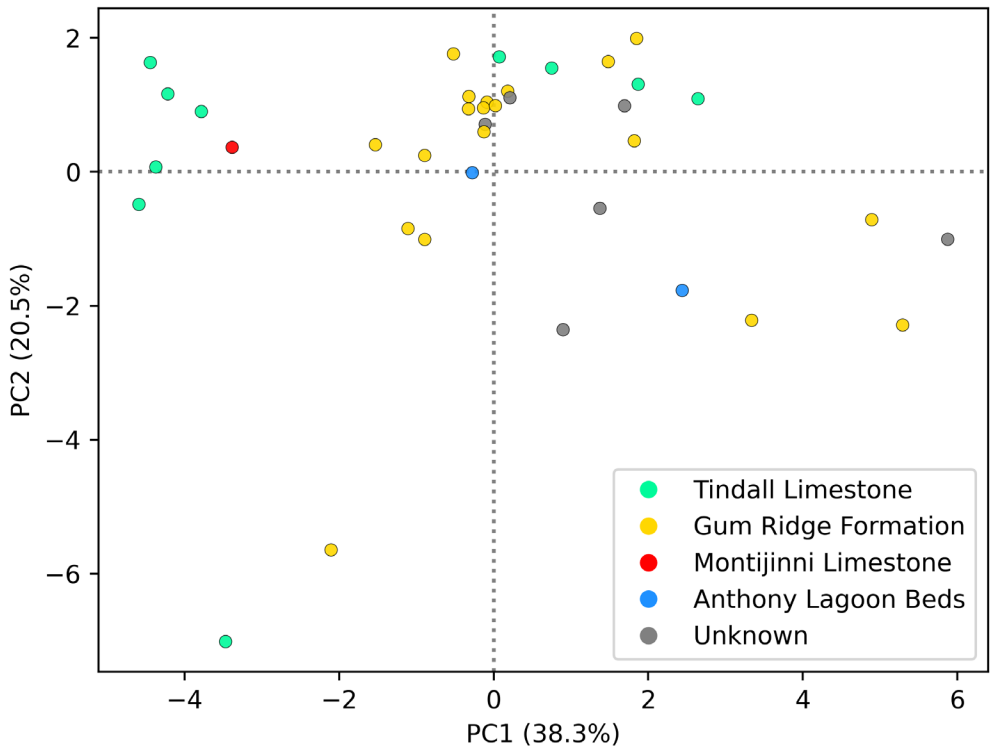

Figure 21: Principal components analysis of water chemistry data by formation

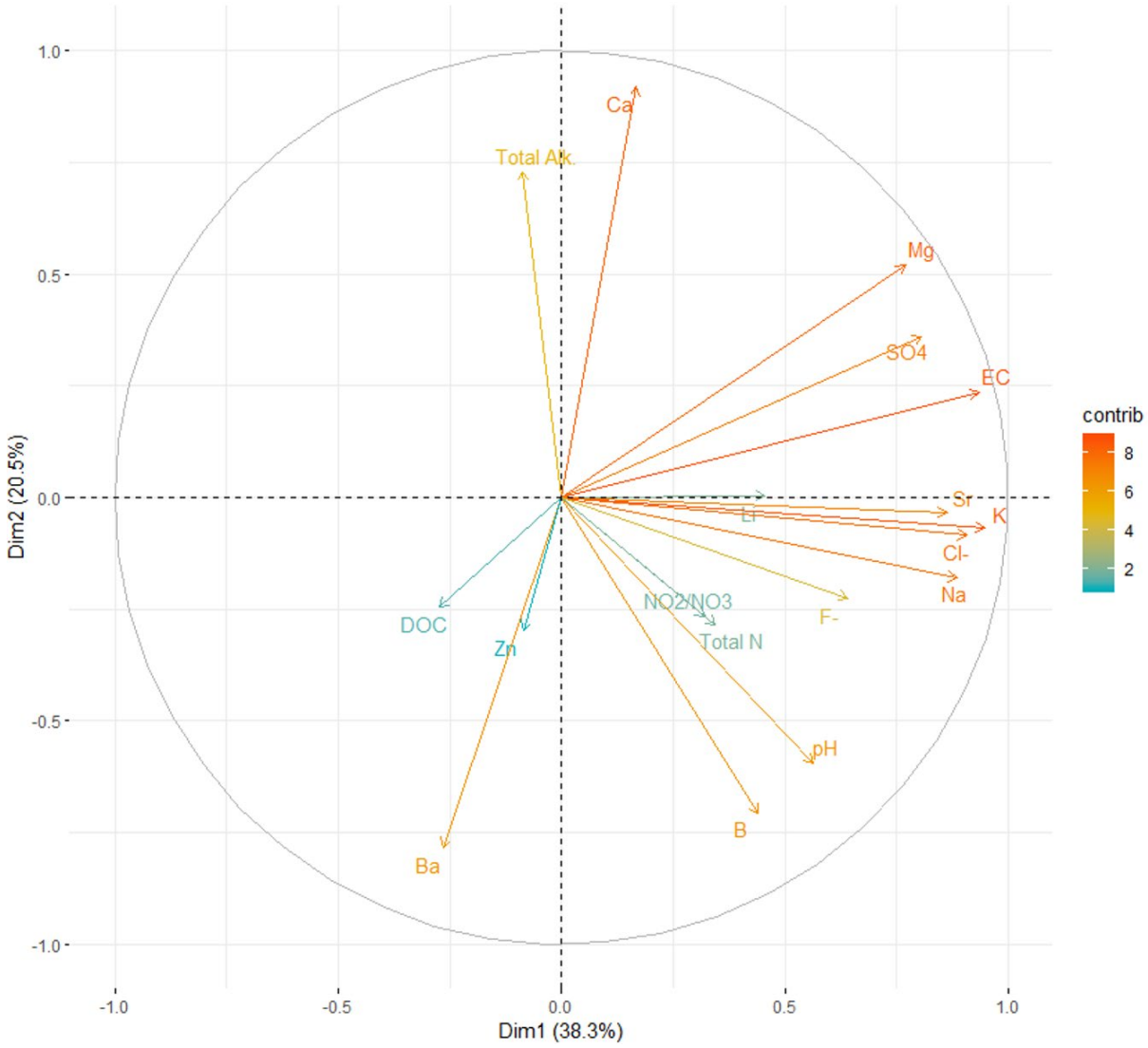

Figure 22: Principal components variable correlation plot of water chemistry

3.2 Microbiology

3.2.1 Soil baselines

Prokaryotic microbiomes

Soil type (e.g. kandosol, tenosol) was not a predictor for prokaryotic diversity of soils in the present study (Figure 23). Instead, it is clear that soil prokaryotic microbiomes are more similar between proximal collection sites and less similar at greater distance. For instance, samples from 'tenosol' ST07, ST08, ST09 and ST10 have similar microbiomes to each other (Figure 23) but are distinct from the other tenosol samples examined in the present study. This is true also for other geographically proximal soil samples (e.g. SV03, SV04 and SV05). This issue with soil type is discussed at length in Section 3.1.1 for soil chemistry and is mirrored by the microbiome data available for these soil samples.

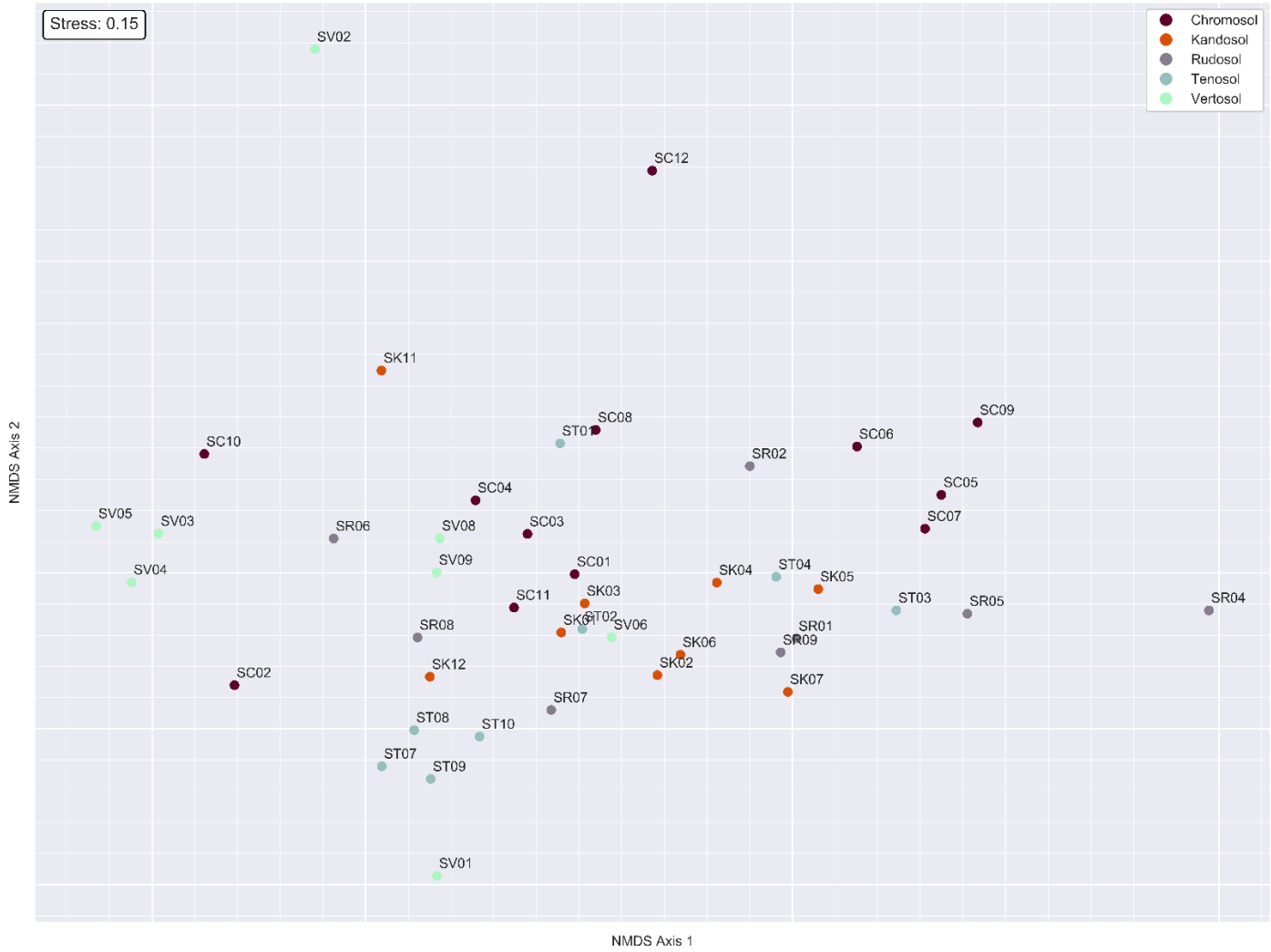

Figure 23: NMDS showing soil bacterial communities from the major soil types

Taxonomic information about observed OTUs

In total, 3372 bacterial and archaeal 16S rDNA OTUs were detected from the soil samples examined in this study. Of these, 1246 OTUs were present at more than 0.1% relative abundance. This tail of rare taxa are not considered further in this report and downstream work (ordinations and summaries) focus on the more abundant 1246 OTUs. Of the 1246 OTUs observed, the majority were bacterial with just 23 archaeal OTUs observed in two phyla (the Euryarchaeota and Thaumarchaeota). In total, 20 bacterial phyla were observed with the most abundant phyla being the Actinobacteria (252 OTUs), Proteobacteria (250 OTUs), Acidobacteria (134 OTUs), Firmicutes (107 OTUs) and the Chloroflexi (103 OTUs). These five phyla represented 23.9, 23.7, 12.7, 10.1 and 9.7% of all OTUs, respectively (Figure 24; Supplementary data 1). These data were broadly consistent with observations from samples from the Australian Microbiome dataset which found these same phyla dominated samples from the region, though the percentages observed in the Australian Microbiome dataset differ from those described here. Indeed, in the Australian Microbiome dataset the Chloroflexi (23.85%) were the most frequently observed phylum, followed by Actinobacteria (19.85%), Acidobacteria (17.43%), Proteobacteria (15.6%) and Firmicutes (6.43%) ("Australian Microbiome consortium," 2020). As previously noted, the samples from the Australian Microbiome dataset are spatially not completely aligned with the sampling in this study and are typically further east (north and south of Arnold) and almost exclusively east of the Stuart Highway. These geographic differences may account for the proportional differences. Alternatively, they may be related to the proximity to rainfalls events or similar phenomena that impact the soil microbiomes in the region.

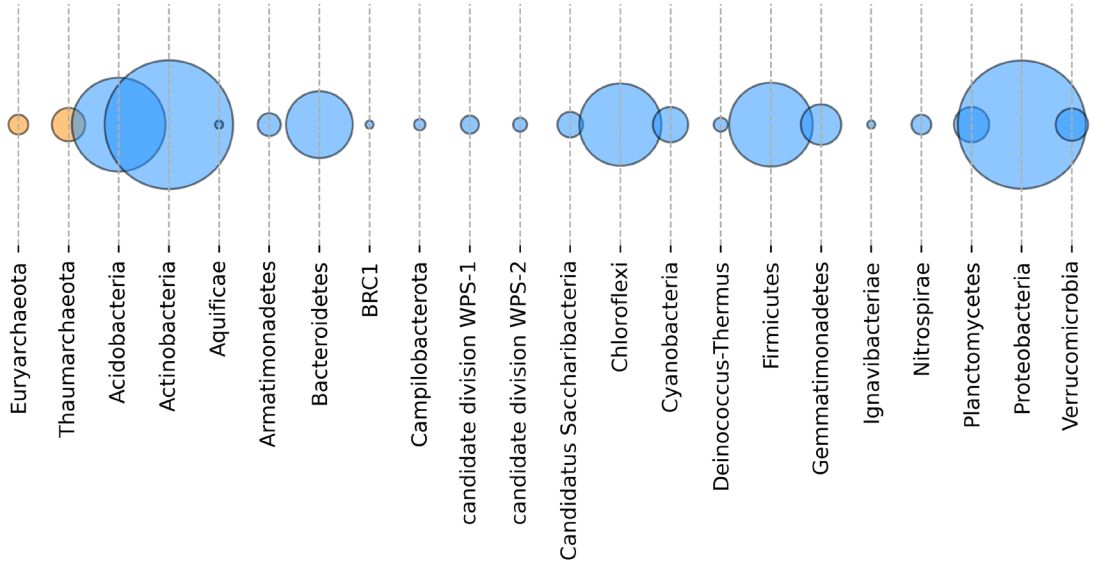

Figure 24: Relative abundance of phyla observed across the soil samples

The bubble size represents the relative abundance of each phylum

Interestingly, all 23 archaeal OTUs belong to three genera: *Haladaptatus*, *Methanomassiliicoccus* or *Nitrosphaera*. The first of these, *Haladaptatus* is an extremophile organism that grows in salt concentrations approaching saturation. Its presence in one of the chromosol samples is intriguing, however, the taxon is not widespread or abundant. The chromosol soil is not particularly high in salt concentrations. It is noteworthy, however, that water films on soil particles as they dry can have significantly elevated salt concentrations and it may be that this taxon grows in these films as they dry, exploiting resources available in these films when other organisms are unable to grow. Culture work would be required to confirm such speculation. Similarly, the methanogenic *Methanomassiliicoccus* taxa are not very abundant, they are, however, widespread occurring in a large number of the soil samples obtained. They are also the only methanogens detected in these soils. In these oxic habitats, they are presumably living inside anoxic clay peds or other anoxic microhabitats in the surface soils. In contrast, the *Nitrososphaera* species detected in the soils of the Beetaloo region are among the most common and widespread microbes. *Nitrososphaera*, like most Thaumarchaeota, are aerobic, ammonia-oxidising, CO_2-fixing organisms. It may be that this autotrophic life strategy provides these organisms with an advantage in the carbon-depleted soils of the Beetaloo Basin region.

From the phylum Actinobacteria, the most abundant order was Gaiellales (Figure 25). This order is relatively recently described (Albuquerque et al., 2011) and contains just one described species *Gaiella occulta*. This order has been observed abundantly in many edaphically more challenging environments including saline, high pH soils and weathered serpentine rocks (Hezbri et al., 2015; Khilyas et al., 2019; Peng et al., 2017). Indeed, *Gaiella* strains were detected frequently in most soils of the Beetaloo region and their presence here likely reflects the challenging soil conditions in the region. Other orders with similar more extreme environmental tolerance were also detected, including the Geodermatophilales and the Rubrobacterales (Figure 25). The former order is known for organisms from arid and UV-irradiated habitats (Gtari et al., 2012; Montero-Calasanz et al., 2013). For example, OTU_113 in the present study was detected in most soils in the Beetaloo region, this taxon is conspecific with the UV-resistant bacterium *Geodermatophilus tzadiensis* described from Saharan desert soils in Chad (Montero-Calasanz et al., 2013). Similarly, members of the Rubrobacterales are known for their resistance to desiccation. For example, members of the Rubrobacterales are known from arid desert soils and sites with UV exposure (Bachar et al., 2012; Crits-Christoph et al., 2013; Vardeh et al., 2018). Taken together, these observations point to a microbiome whose actinobacterial cohort is shaped in response to the arid and UV irradiated nature of the environment in the region.

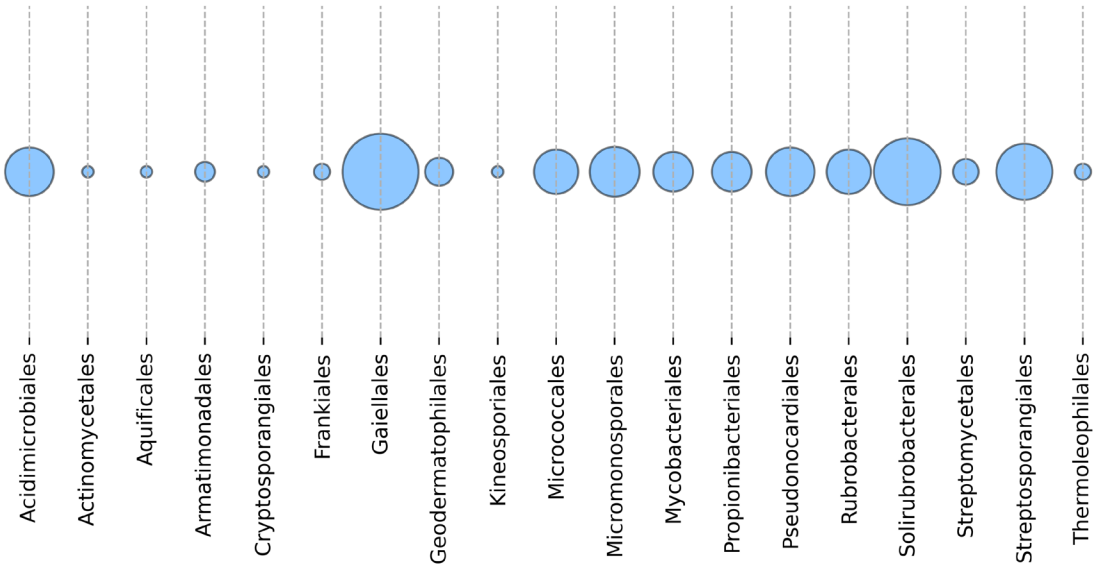

Figure 25: Relative abundance of the actinobacterial orders among the 252 actinobacterial OTUs in soils

The bubble size represents the relative abundance of each order

The proteobacterial assemblages observed in soils of the Beetaloo region include many orders who have members involved in nitrogen fixation (for example, the Rhizobiales and Rhodospirallales; Figure 26). Some of these taxa are likely associated with plant roots, while others may be free living organisms. As for the Actinobacteria, the hot, arid, UV-irradiated nature of the soils of the Beetaloo region is reflected in the adaptions of some taxa within the Proteobacteria. For example, among the most common Rhizobiales OTUs, OTU315 is most closely related to the halophilic taxon *Amorphus orientalis*, originally described from a salt mine in Yunnan, south-west China (Wang et al., 2010). Similarly, the Rhizobiales taxon OTU_220 was detected in many of the soils of the Beetaloo region and was originally described from hot springs where it grows from 40-47°C (Madigan et al., 2019). Indeed, similar patterns occur among the order Rhodospirallales. For example, of the more common Rhodospirallales detected in the soils of the Beetaloo region (OTU_422) was most closely related to *Siccirubricoccus deserti*, a desert soil taxon originally described from Saudia Arabia (Yang et al., 2017).

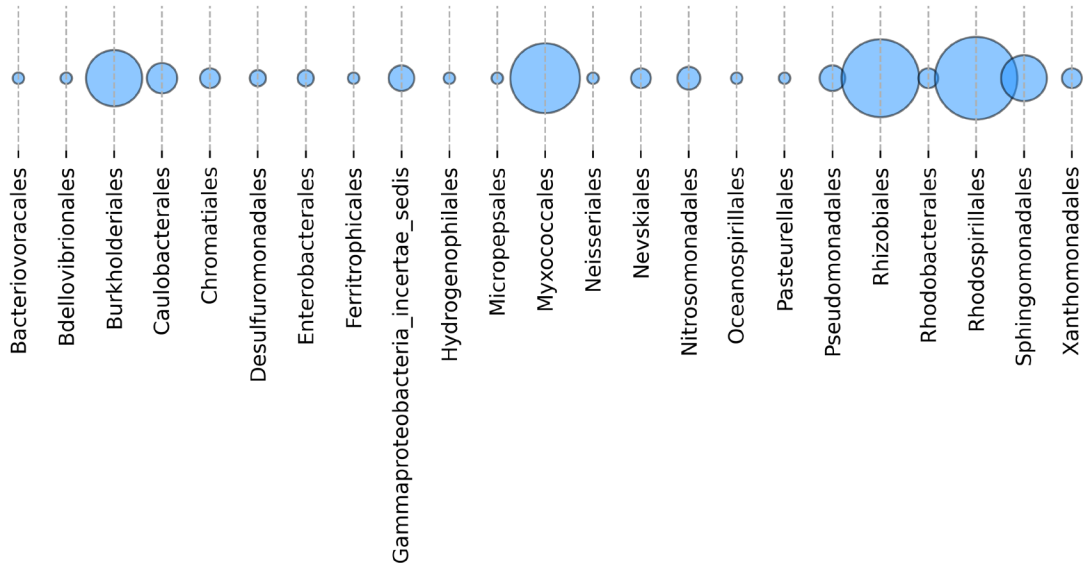

Figure 26: Relative abundance of the proteobacterial orders among the 250 proteobacterial OTUs in soils

The bubble size represents the relative abundance of each order.

The Acidobacteria were among the most commonly detected phyla in soil samples obtained from the Beetaloo region and consisted of a range of genera (Figure 27). This group,

however, is not well understood taxonomically, and relatively few taxa from within this group have been examined in detail in terms of their physiology. The most abundant single OTU from this group is *Arenimicrobium luteum* which occurs in almost all soil samples from the Beetaloo region. This taxon was originally isolated from Namibian, semiarid, savanna soils (Wüst et al., 2016). This again, further, highlights that the microbiology of the soils reflects the abiotic characteristics of the environment (i.e. the surface soils of this region are a challenging place for life).

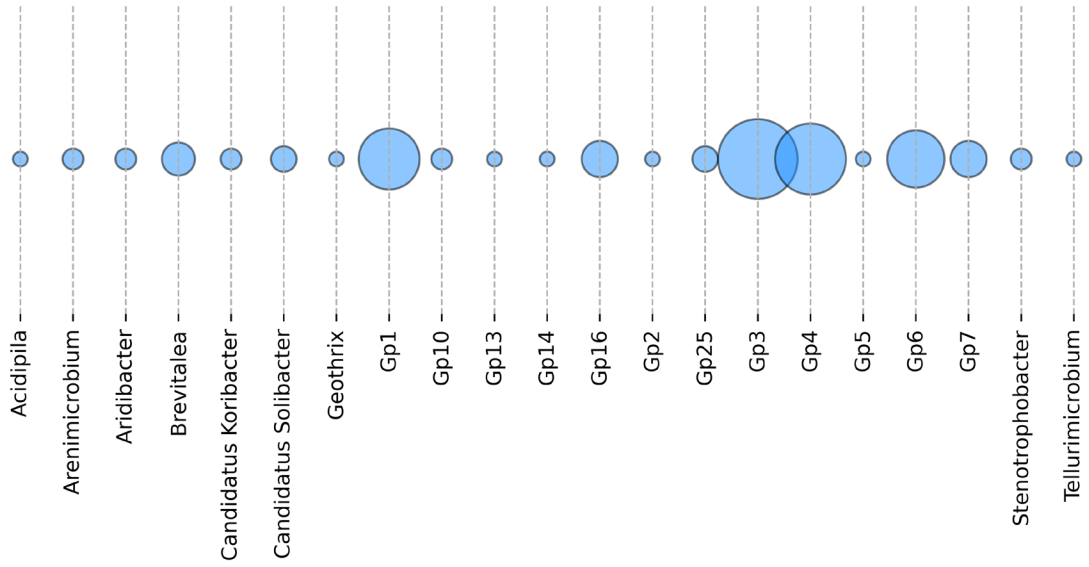

Figure 27: Relative abundance of the acidobacterial genera among the 134 acidobacterial OTUs in soils

Genera, as opposed to orders, are shown for this phylum due to the lack of taxonomic resolution in this phylum. The bubble size represents the relative abundance of each order

Interestingly, the most abundant phylum in the soils of the Beetaloo region was a Firmicute. OTU_1 is widespread in the region and is conspecific with *Neobacillus bataviensis* from the Bacillceae family (Figure 28). This taxon was originally isolated from soil in the Netherlands (Heyrman et al., 2004; Patel and Gupta, 2020), and so is not, on the face of it particularly adapted for hot, dry climates. Many bacilli, including this taxon, however, are prolific producers of resistant spores (Hong et al., 2009). These spores can settle in many environments (soils particularly) and their presence there does not necessarily indicate growth 'in habitat' of these taxa. Another very abundant and ubiquitous taxon from the Beetaloo region was OTU_8, a member of the Paenibacillaceae 2 family (Figure 28). Like OTU_1, this taxon has some of the highest abundances observed in this study. This taxon is

likely very closely related to *Oxalophagus oxalicus* (Collins et al., 1994; Dehning and Schink, 1989), an organism for which oxalate appears to be its main source of food. Tropical grasses in this area are likely particularly rich in oxalate (McKenzie and Schultz, 1983), which becomes incorporated into soils during plant degradation. Based on its abundance and closest relatives, it seems likely this taxon is involved in their degradation in the Beetaloo region.

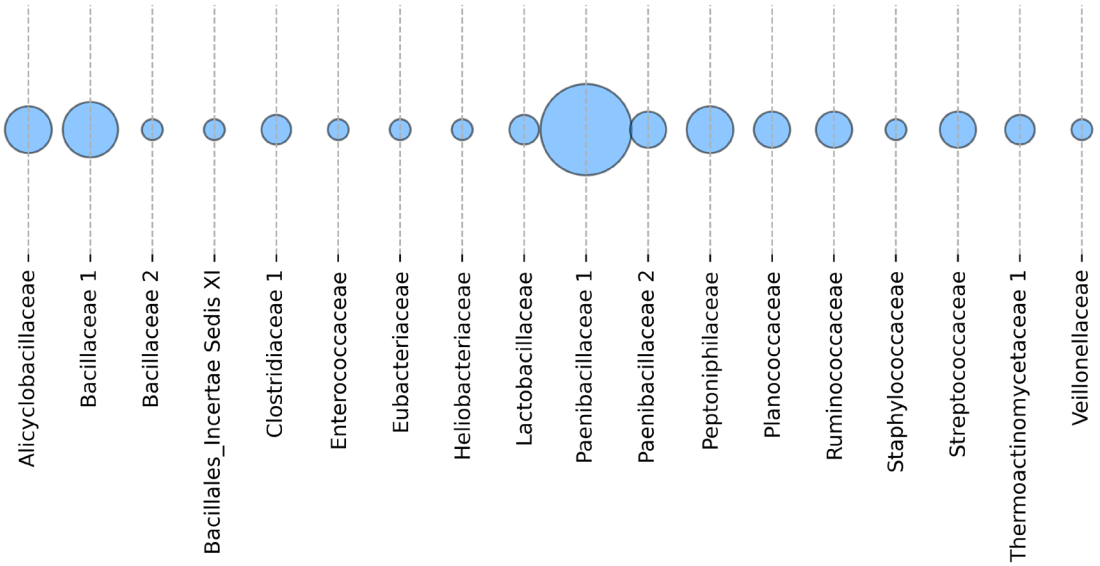

Figure 28: Relative abundance of Firmicutes families in the soil samples

Taxonomy with the Firmicutes remains poor above family level, as such family rather than order is shown here. The bubble size represents the relative abundance of each family

Interestingly, numerous Chloroflexi were detected with affinities to *Ktedonbacter, Kallotenue* and *Dictyobacter* (Figure 29), however, none of the taxa detected in the Beetaloo are closely related to these known species within these genera, indeed, some of the detected OTUs may be novel and simply have these taxa as their closest known relatives (Table 16 and Table 17). This distance between known species and what was observed for the Chloroflexi in soils makes assignation of function to these taxa unreliable. Data from the Australian microbiome project show a similar, poorly classified group of numerous Chloroflexi in the soils of the region ("Australian Microbiome consortium," 2020).

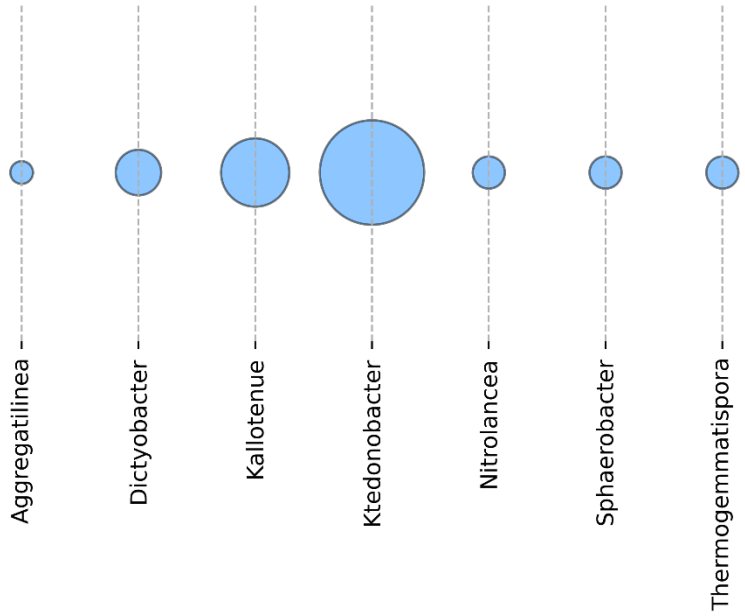

Figure 29: Relative abundance of the genera of Chloroflexi in the soil samples

It is noteworthy that the samples included many novel lineages of Ktedonobacterales and Ktedonobacteria that are not shown in this figure due to their taxonomic ambiguity. The bubble size represents the relative abundance of each genus

The most abundant taxa observed in the present study are shown in Table 16 with the classifications for the sample taxa shown in Table 17. The results suggested that 'soil type' is not a good predictor of the observed microbiology (Figure 30). For example, vertosol samples appear to be divided into two groups that differ considerably from each other; SV03, SV04 and SV05 in one group and SV08 and SV09 in another. It seems likely that local geography (i.e. how proximal the samples are from one another) is more important than the soil type *per se*, understanding that soil type as used in this report refers to an inferred soil type from a soil map rather than the actual soil type (see section 3.1.1).

The most abundant microbe detected across the soil samples was OTU_241. This OTU is poorly classified and may be a member of the Chloroflexi phylum, though it may also belong to a sister group within the FCB superphylum. Regardless, OTU_241 only has a handful of close matches on the GenBank nucleotide database (Elliott et al., 2014; Fonseca-García et al., 2016). The first (99.6% identity over the length of the sequence) was to KU541384, a bacterial sequence obtained from cacti soil in Mexican deserts (Fonseca-García et al., 2016), the second was KJ662359, a sequence detected in soil crusts in the Kalahari Desert (Elliott et

al., 2014). This further adds to the weight of earlier discussion about this environment selecting for microbes adapted for aridity and heat tolerance.

Table 16: The top 50 most abundant soil taxa and their closest matches on Genbank with their percentage identity (over ~290bp)

OTU	Closest match	% ID
OTU_241	•	0
OTU_37	*Nitrososphaera viennensis* EN76 (NR 134097)	96.4
OTU_1	*Neobacillus bataviensis* IDA1115 (NR 036766)	100
OTU_81	*Nitrososphaera viennensis* EN76 (NR 134097)	94.9
OTU_315	*Amorphus orientalis* YIM D10 (NR 104491)	94.5
OTU_187	*Flavisolibacter ginsengiterrae* Gsoil 492 (NR 041499)	94.5
OTU_354	*Micromonospora soli* SL3-70 (NR 146360)	100
OTU_156	unidentified bacterium soil clone (X64382)	97.2
OTU_400	•	0
OTU_113	*Geodermatophilus tzadiensis* CF5/2 (NR 108878)	99.2
OTU_255	*Crossiella equi* NRRL B-24104 (NR 025088)	97.2
OTU_218	*Arenimicrobium luteum* Ac 12 G8 (NR 151986)	100
OTU_154	•	0
OTU_702	*Luteitalea pratensis* HEG -6 39 (NR 156918)	96.8
OTU_314	uncultured soil bacterium (AY289368)	98
OTU_101	*Pseudonocardia acaciae* DSM 45401 GMKU095 (NR 044580)	99.6
OTU_115	*Gaiella occulta* F2-233 (NR 118138)	95.7
OTU_406	*Solirubrobacter phytolaccae* GTGR-8 (NR 133858)	95.3
OTU_1643	•	0
OTU_181	*Parviterribacter kavangonensis* D16/0/H6 (NR 148601)	94.9
OTU_152	*Rubrobacter spartanus* HPK2-2 (NR 158052)	96.8
OTU_65	*Bacillus circulans* NBRC 13626 (NR 112632)	99.2
OTU_327	*Deferrisoma camini* S3R1 (NR 118216)	85.8
OTU_7366	*Agrostis stolonifera* (EF115543)	98.4
OTU_146	*Gaiella occulta* F2-233 (NR 118138)	94.5
OTU_307	*Frankia elaeagni* BMG5.12 (NR 153676)	98
OTU_1541	*Rubrobacter naiadicus* RG-3 (NR 125704)	94.9
OTU_515	*Rubrobacter spartanus* HPK2-2 (NR 158052)	95.7
OTU_112	•	0
OTU_244	*Desulfonatronum thioautotrophicum* ASO4-1 (NR 116693)	87.4
OTU_660	*Caldalkalibacillus uzonensis* JW/WZ-YB58 (NR 043653)	85.5
OTU_495	*Hamadaea flava* YIM C0533 (NR 148827)	98.4
OTU_242	*Sphingomonas limnosediminicola* 03SUJ6 (NR 157773)	99.2
OTU_72	*Thermogemmatispora carboxidivorans* PM5 (NR 133881)	85.4
OTU_801	*Actinophytocola burenkhanensis* MN08-A0203 (NR 113018)	99.6
OTU_71	uncultured bacterium DA101 (Y07576)	100
OTU_398	*Nitrososphaera viennensis* EN76 (NR 134097)	96.8
OTU_304	*Solirubrobacter phytolaccae* GTGR-8 (NR 133858)	92.9
OTU_288	*Solirubrobacter ginsenosidimutans* BXN5-15 (NR 108192)	96.4
OTU_262	*Streptomyces lincolnensis* NBRC 13054 (NR 041104)	100
OTU_97	*Sphingomonas limnosediminicola* 03SUJ6 (NR 157773)	97.6
OTU_329	uncultured bacterium (AY555784)	93.3
OTU_3171	*Arenimicrobium luteum* Ac 12 G8 (NR 151986)	96.8
OTU_335	•	0
OTU_1317	•	0
OTU_787	*Geodermatophilus dictyosporus* G-5 (NR 134737)	99.6
OTU_818	*Xanthobacter viscosus* 7d; VKM B-2253 (NR 025173)	96.5
OTU_324	*Conexibacter woesei* ID131577; DSM 14684; JCM 11494 (NR 028979)	94.5
OTU_144	*Thermoleophilum album* HS-5; ATCC 35263 (NR 025543)	94.9
OTU_130	•	0

Table 17: Ribosomal Database Classifier predicted taxonomic information and probability for the top 50 most abundant soil OTUs

OTU	Domain	Phylum	Class	Order	Family	Genus	Probability
OTU_241	Bacteria	Chloroflexi	Chloroflexia	Kallotenuales	Kallotenuaceae	*Kallotenue*	0.6
OTU_37	Archaea	Thaumarchaeota		Nitrososphaerales	Nitrososphaeraceae	*Nitrososphaera*	1
OTU_1	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	*Neobacillus*	1
OTU_81	Archaea	Thaumarchaeota		Nitrososphaerales	Nitrososphaeraceae	*Nitrososphaera*	1
OTU_315	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales			0.72
OTU_187	Bacteria	Bacteroidetes	Chitinophagia	Chitinophagales	Chitinophagaceae	*Flavisolibacter*	0.85
OTU_354	Bacteria	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae	*Micromonospora*	0.99
OTU_156	Bacteria	Verrucomicrobia	Spartobacteria			Spartobacteria genera *incertae sedis*	1
OTU_400	Bacteria						0.97
OTU_113	Bacteria	Actinobacteria	Actinobacteria	Geodermatophilales	Geodermatophilaceae	*Geodermatophilus*	0.95
OTU_255	Bacteria	Actinobacteria	Actinobacteria				1
OTU_218	Bacteria	Acidobacteria	Blastocatellia	Blastocatellales	Arenimicrobiaceae	*Arenimicrobium*	0.99
OTU_154	Bacteria						0.95
OTU_702	Bacteria	Acidobacteria	Acidobacteria Gp6			Gp6	0.95
OTU_314	Bacteria	Acidobacteria	Acidobacteria Gp4			Gp4	0.98
OTU_101	Bacteria	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	*Pseudonocardia*	0.86
OTU_115	Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	*Gaiella*	0.99
OTU_406	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	*Solirubrobacter*	0.71
OTU_1643	Bacteria						0.84
OTU_181	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Baekduiaceae	*Baekduia*	0.84
OTU_152	Bacteria	Actinobacteria	Rubrobacteria	Rubrobacterales	Rubrobacteraceae	*Rubrobacter*	1
OTU_65	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	*Bacillus*	0.57
OTU_327	Bacteria	Proteobacteria					0.63
OTU_7366	Bacteria	Cyanobacteria/ Chloroplast	Chloroplast		Chloroplast	*Streptophyta*	1
OTU_146	Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	*Gaiella*	0.99
OTU_307	Bacteria	Actinobacteria	Actinobacteria				1
OTU_1541	Bacteria	Actinobacteria	Rubrobacteria	Rubrobacterales	Rubrobacteraceae	*Rubrobacter*	0.91
OTU_515	Bacteria	Actinobacteria	Rubrobacteria	Rubrobacterales	Rubrobacteraceae	*Rubrobacter*	0.98
OTU_112	Bacteria						1
OTU_244	Bacteria						1
OTU_660	Bacteria	Firmicutes					0.56
OTU_495	Bacteria	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae		0.99
OTU_242	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	0.98
OTU_72	Bacteria	Chloroflexi	Ktedonobacteria	Thermogemmatisporales	Thermogemmatisporaceae	*Thermogemmatispora*	0.59
OTU_801	Bacteria	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	*Actinophytocola*	0.55
OTU_71	Bacteria	Verrucomicrobia	Spartobacteria			Spartobacteria genera *incertae sedis*	1
OTU_398	Archaea	Thaumarchaeota		Nitrososphaerales	Nitrososphaeraceae	*Nitrososphaera*	1
OTU_304	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales			0.71
OTU_288	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	*Solirubrobacter*	0.94
OTU_262	Bacteria	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	*Streptomyces*	0.98
OTU_97	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	0.97
OTU_329	Bacteria	Acidobacteria	Acidobacteria Gp3			Gp3	0.89
OTU_3171	Bacteria	Acidobacteria	Blastocatellia	Blastocatellales	Arenimicrobiaceae	*Arenimicrobium*	0.95
OTU_335	Bacteria	Chloroflexi					0.5
OTU_1317	Bacteria						0.89
OTU_787	Bacteria	Actinobacteria	Actinobacteria	Geodermatophilales	Geodermatophilaceae	*Geodermatophilus*	0.66
OTU_818	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales			1
OTU_324	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales			0.93
OTU_144	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Baekduiaceae	*Baekduia*	0.73
OTU_130	Bacteria	Chloroflexi	Ktedonobacteria	Ktedonobacterales	Ktedonobacteraceae	*Ktedonobacter*	0.77

16S training set used for classifications

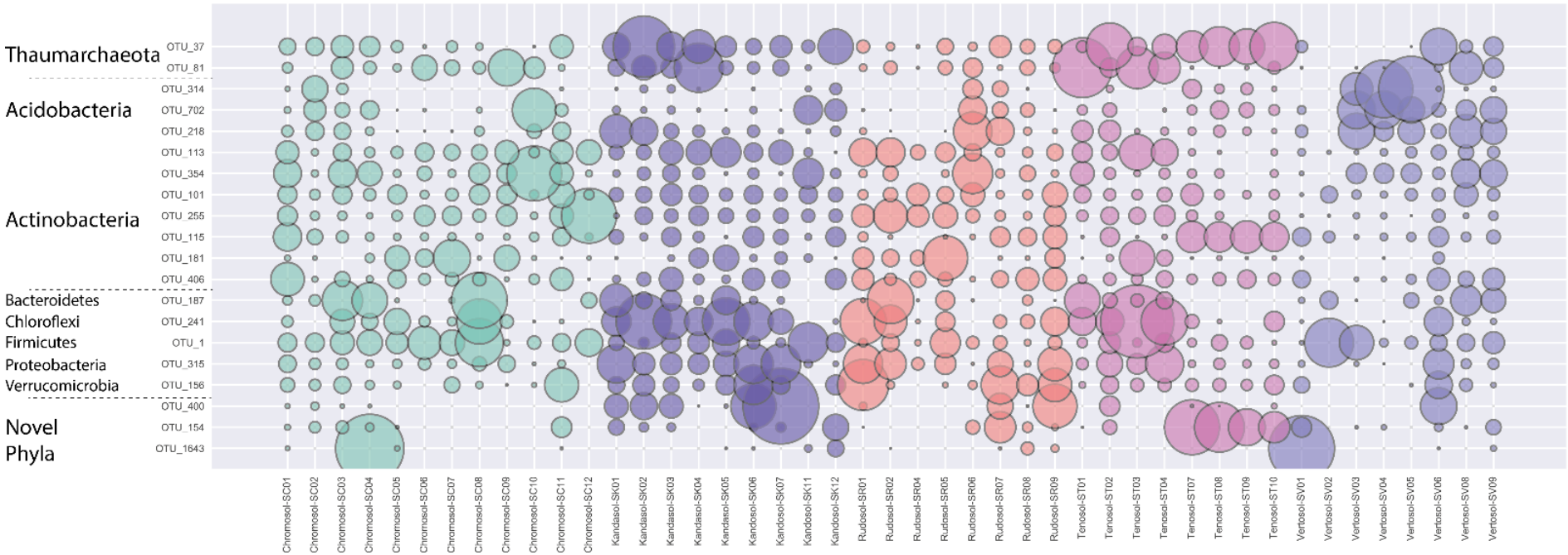

Figure 30: Top 20 most abundant bacterial OTUs by soil type

From left to right, chromosol (green), kandosol (purple), rudosol (salmon), tenosol (pink), vertosol (violet). The bubble size represents the relative abundance of each OTU

Ranking soil prokaryote taxa by their ubiquity rather than their abundance yields a similar cohort of taxa (Table 18 and Table 19). *Neobacillus bataviensis* is the most widespread taxon in soils of the Beetaloo region, as discussed earlier though its role in these soils remains unclear. Among the other taxa detected there are organisms that are putative plant root endophytes (for example OTUs: 16, 37, 101, 202 and 304), thermophilic (e.g. OTU_144, OTU_1541) or UV tolerant taxa (e.g. OTU_113).

Table 18: The top 50 most frequently observed soil taxa and their closest matches on GenBank with their percentage identity (over ~290bp)

OTU	Frequency	Closest match	% ID
OTU_1	45	*Neobacillus bataviensis* IDA1115 (NR 036766)	100
OTU_101	45	*Pseudonocardia acaciae* DSM 45401 GMKU095 (NR 044580)	99.6
OTU_242	45	*Sphingomonas limnosediminicola* 03SUJ6 (NR 157773)	99.2
OTU_304	45	*Solirubrobacter phytolaccae* GTGR-8 (NR 133858)	92.9
OTU_144	45	*Thermoleophilum album* HS-5; ATCC 35263 (NR 025543)	94.9
OTU_45	45	*Streptomyces spinoverrucosus* NBRC 14228 (NR 041159)	100
OTU_113	44	*Geodermatophilus tzadiensis* CF5/2 (NR 108878)	99.2
OTU_255	44	*Crossiella equi* NRRL B-24104 (NR 025088)	97.2
OTU_115	44	*Gaiella occulta* F2-233 (NR 118138)	95.7
OTU_307	44	*Frankia elaeagni* BMG5.12 (NR 153676)	98
OTU_288	44	*Solirubrobacter ginsenosidimutans* BXN5-15 (NR 108192)	96.4
OTU_97	44	*Sphingomonas limnosediminicola* 03SUJ6 (NR 157773)	97.6
OTU_15	44	*Mycolicibacterium phocaicum* N4 (NR 043237)	100
OTU_406	43	*Solirubrobacter phytolaccae* GTGR-8 (NR 133858)	95.3
OTU_495	43	*Hamadaea flava* YIM C0533 (NR 148827)	98.4
OTU_4221	43	*Spirilliplanes yamanashiensis* YU 127-1; IFO 15828 (NR 025872)	97.6
OTU_2016	43	*Singulisphaera rosea* S26 (NR 116969)	94.1
OTU_315	42	*Amorphus orientalis* YIM D10 (NR 104491)	94.5
OTU_354	42	*Micromonospora soli* SL3-70 (NR 146360)	100
OTU_801	42	*Actinophytocola burenkhanensis* MN08-A0203 (NR 113018)	99.6
OTU_90	42	*Hyalangium minutum* DSM 14724 (NR 043949)	100
OTU_167	42	*Gaiella occulta* F2-233 (NR 118138)	90.9
OTU_39	42	*Amycolatopsis circi* S1.3 (NR 117914)	100
OTU_226	42	*Methylobacterium iners* 5317S-33 (NR 044129)	97.2
OTU_109	42	*Conexibacter stalactiti* YC2-25 (NR 157993)	97.6
OTU_1860	42	*Angustibacter speluncae* YC2-20 (NR 158006)	97.6
OTU_430	42	*Blastococcus capsensis* BMG 804 (NR 152659)	97.6
OTU_401	42	*Solirubrobacter soli* Gsoil 355 (NR 041365)	98.8
OTU_411	42	*Aciditerrimonas ferrireducens* JCM 15389 IC-180 (NR 112972)	93.7
OTU_218	41	*Arenimicrobium luteum* Ac 12 G8 (NR 151986)	100
OTU_1541	41	*Rubrobacter naiadicus* RG-3 (NR 125704)	94.9
OTU_787	41	*Geodermatophilus dictyosporus* G-5 (NR 134737)	99.6
OTU_818	41	*Xanthobacter viscosus* 7d; VKM B-2253 (NR 025173)	96.5
OTU_16	41	*Bradyrhizobium valentinum* LmjM3 (NR 125638)	100
OTU_585	41	uncultured Acidobacteria bacterium (EF457308)	99.2
OTU_313	41	*Thermoleophilum minutum* ORS 571 (NR 036932)	92.1
OTU_609	41	*Pseudonocardia zijingensis* 6330; JCM 11117 (NR 028805)	99.6
OTU_369	41	*Aciditerrimonas ferrireducens* JCM 15389 IC-180 (NR 112972)	92.1
OTU_220	41	*Blastochloris tepida* GI (NR 169404)	97.2
OTU_1133	41	*Labedella gwakjiensis* KSW2-17 (NR 043900)	99.6
OTU_933	41	*	0
OTU_59	41	*Phenylobacterium zucineum* HLK1 (NR 115292)	100
OTU_1627	41	*Conexibacter woesei* ID131577; DSM 14684; JCM 11494 (NR 028979)	95.7
OTU_1644	41	*Amycolatopsis dongchuanensis* YIM 75904 (NR 109504)	100
OTU_202	41	*Solirubrobacter phytolaccae* GTGR-8 (NR 133858)	96.8
OTU_37	40	*Nitrososphaera viennensis* EN76 (NR 134097)	96.4
OTU_81	40	*Nitrososphaera viennensis* EN76 (NR 134097)	94.9
OTU_146	40	*Gaiella occulta* F2-233 (NR 118138)	94.5
OTU_168	40	*Gaiella occulta* F2-233 (NR 118138)	92.5
OTU_155	40	*Micromonospora marina* JSM1-1 (NR 112537)	99.2

Table 19: Ribosomal Database Classifier predicted taxonomic information and probability for the top 50 most frequent soil OTUs

OTU	Domain	Phylum	Class	Order	Family	Genus	Probability
OTU_1	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	*Neobacillus*	1
OTU_101	Bacteria	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	*Pseudonocardia*	0.86
OTU_242	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	0.98
OTU_304	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales			0.71
OTU_144	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Baekduiaceae	*Baekduia*	0.73
OTU_45	Bacteria	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	*Streptomyces*	1
OTU_113	Bacteria	Actinobacteria	Actinobacteria	Geodermatophilales	Geodermatophilaceae	*Geodermatophilus*	0.95
OTU_255	Bacteria	Actinobacteria	Actinobacteria				1
OTU_115	Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	*Gaiella*	0.99
OTU_307	Bacteria	Actinobacteria	Actinobacteria				1
OTU_288	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	*Solirubrobacter*	0.94
OTU_97	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	0.97
OTU_15	Bacteria	Actinobacteria	Actinobacteria	Mycobacteriales	Mycobacteriaceae	*Mycobacterium*	1
OTU_406	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	*Solirubrobacter*	0.71
OTU_495	Bacteria	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae		0.99
OTU_4221	Bacteria	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae	*Asanoa*	0.61
OTU_2016	Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Isosphaeraceae	*Aquisphaera*	0.65
OTU_315	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales			0.72
OTU_354	Bacteria	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae	*Micromonospora*	0.99
OTU_801	Bacteria	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	*Actinophytocola*	0.55
OTU_90	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Cystobacteraceae	*Archangium*	0.8
OTU_167	Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	*Gaiella*	0.87
OTU_39	Bacteria	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	*Amycolatopsis*	0.91
OTU_226	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae		0.7
OTU_109	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales			1
OTU_1860	Bacteria	Actinobacteria	Actinobacteria				1
OTU_430	Bacteria	Actinobacteria	Actinobacteria	Geodermatophilales	Geodermatophilaceae		1
OTU_401	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	*Solirubrobacter*	1
OTU_411	Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Acidimicrobiales incertae sedis	*Aciditerrimonas*	0.7
OTU_218	Bacteria	Acidobacteria	Blastocatellia	Blastocatellales	Arenimicrobiaceae	*Arenimicrobium*	0.99
OTU_1541	Bacteria	Actinobacteria	Rubrobacteria	Rubrobacterales	Rubrobacteraceae	*Rubrobacter*	0.91
OTU_787	Bacteria	Actinobacteria	Actinobacteria	Geodermatophilales	Geodermatophilaceae	*Geodermatophilus*	0.66
OTU_818	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales			1
OTU_16	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Bradyrhizobium*	1
OTU_585	Bacteria	Acidobacteria	Acidobacteria Gp6			Gp6	1
OTU_313	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Parviterribacteraceae	*Parviterribacter*	0.6
OTU_609	Bacteria	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	*Pseudonocardia*	0.98
OTU_369	Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Acidimicrobiales incertae sedis	*Aciditerrimonas*	0.53
OTU_220	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Blastochloridaceae	*Blastochloris*	0.64
OTU_1133	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Amnibacterium*	0.67
OTU_933	Bacteria						1
OTU_59	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Phenylobacterium*	1
OTU_1627	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Conexibacteraceae	*Conexibacter*	0.54
OTU_1644	Bacteria	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	*Amycolatopsis*	0.83
OTU_202	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	*Solirubrobacter*	0.91
OTU_37	Archaea	Thaumarchaeota		Nitrososphaerales	Nitrososphaeraceae	*Nitrososphaera*	1
OTU_81	Archaea	Thaumarchaeota		Nitrososphaerales	Nitrososphaeraceae	*Nitrososphaera*	1
OTU_146	Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	*Gaiella*	0.99
OTU_168	Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	*Gaiella*	0.83
OTU_155	Bacteria	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae		0.99

16S training set used for classification

Fungal microbiomes

As for prokaryotic microbiomes, soil type (e.g. kandosol, tenosol) was not a predictor for fungal microbiome diversity of soils (Figure 31). Some clustering by geographic location is evident in the samples (Figure 10) but this is less pronounced than it was for prokaryotes. This is likely due to the generally poor fungal PCR amplifications that occurred in soil DNA extracts in the present study. Given that many of these poor fungal PCRs yielded good quality prokaryote data it seems likely that this is due to a low fungal biomass in the arid soils of the Beetaloo regions. Possible reasons for this are discussed below. It should be noted that the issue with soil type is discussed in detail in Section 3.1.1.

Taxonomic information about observed OTUs

In general, fungal PCRs in these soils did not always result in successful amplification. It seems likely that the factors that contribute to the success of arid-tolerant, thermophilic, UV-tolerant prokaryotes in these soils may create edaphic conditions that are challenging for many fungi. Regardless, in total ~254 fungal OTUs were detected from the soils of the Beetaloo region. It is also noteworthy, that using OTUs for fungi may significantly underestimate the fungal diversity present in the region as the genetic region used for fungal characterisation, the internal transcribed spacer (ITS) region is considerably less conserved than the 16S rDNA region used for prokaryotes. The most abundant phylum detected was the Ascomycota with 143 OTUs (56% of all OTUs), followed by the Basidiomycota with 49 OTUs (19% of all OTUs) (Figure 32; Supplementary data 2). In addition, representatives of the Chytridmycota, Glomeromycota and Zygomycota were also detected with seven, seven and two OTUs detected for these groups, respectively. The Chytridiomycota, Glomeromycota comprise ~3% each of all OTUs described here, while the Zygomycota are represented by just 0.8% of all observations.

This is broadly consistent with observations from the Australian Microbiome which showed fungal communities that have ~42% Ascomycota, ~28% Basidiomycota, ~13% Glomeromycota and just 0.85% and 0.5% for the Zygomycota (called Mortierellomycota in the Australian Microbiome data) and Chytridiomycota, respectively (“Australian Microbiome consortium,” 2020). Of note between the two datasets are the higher proportion of Glomeromycota observed in the Australian Microbiome data and the higher proportion of chytrids observed in the present study. The reasons for these differences are unclear and

may be due to the slightly different regions being sampled or to rainfall events proximal to the time of sampling which presumably have profound effects on the fungal microbiomes of these arid soils.

The Ascomycota were identified as the dominant fungi from the surface soils of the Beetaloo region and this is consistent with soil surveys from other arid grasslands and shrublands (Porras-Alfaro et al., 2017; Porras-Alfaro and Bayman, 2011). For the ascomycetes, FOTUs that were classified to known genera are shown in Figure 33. It is important, however, to note that while these genera were detected most ascomyceteous fungi from the region were from unknown genera and are not included in Figure 33. Indeed, 36 of the 143 ascomycetes detected (~25%) could not be classified to a known genus. This wealth of novel taxa applies also to the most abundantly detected fungal taxon in the Beetaloo region, FOTU_3 is the taxon with the greatest abundance across the soil samples in the present study (Table 20). This taxon does not map to any known described taxa (Table 20 and Table 21), it may possibly be a member of the Sordariales and comparison of its DNA sequence to the Genbank nucleotide database yields only one related taxon (MK226167[12]) which was isolated from the roots of *Eulophia speciosa*, a South African orchid .

Of the remaining taxa, many are typical, mycelial, soil fungi and are saprobic, opportunistic plant pathogens or plant root endophytes that are important in both carbon and nitrogen cycling, and plant interactions.

[12] https://www.ncbi.nlm.nih.gov/nuccore/MK226167

Figure 31: NMDS showing soil fungal communities from the major soil types

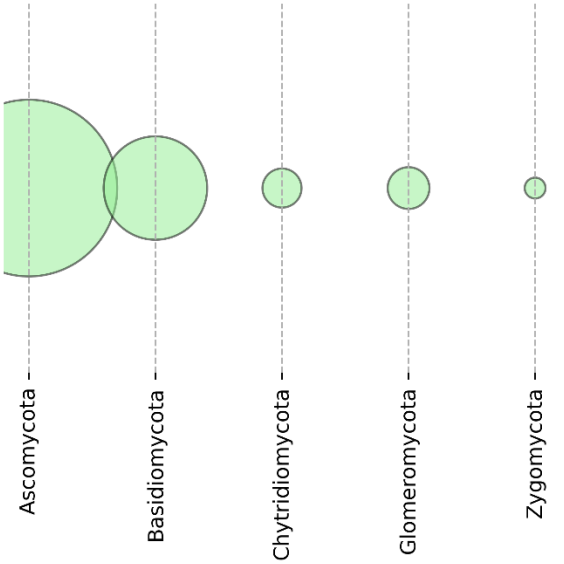

Figure 32: Fungal phyla detected in the soils of the Beetaloo region and their relative abundance

The bubble size represents the relative abundance of each phylum

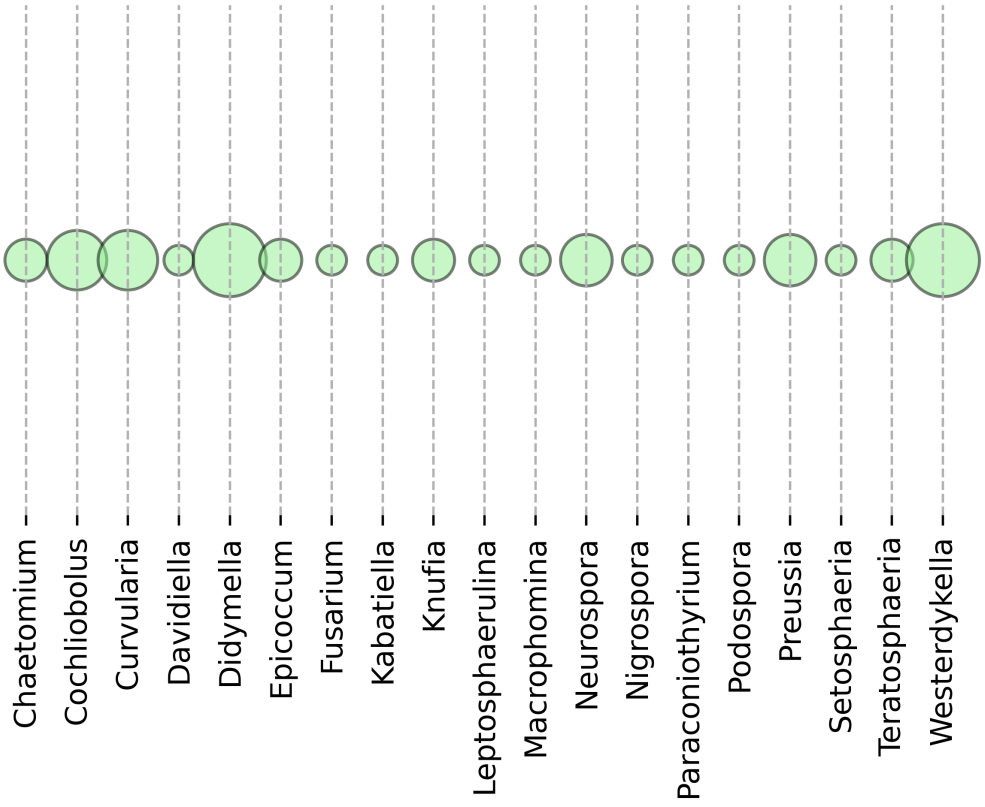

Figure 33: Ascomycete genera detected in the soils of the Beetaloo region and their relative abundance

The bubble size represents the relative abundance of each genus

Table 20: The top 50 most abundant soil fungal taxa and their closest matches

OTU	Closest Match (Warcup)	% ID (Warcup)	Closest Match (UNITE)	% ID (UNITE)
FOTU_3	*	0	*Sordariales* sp|SH195274.06FU	0.65
FOTU_13	*Cryptococcus podzolicus* (HF558650)	90.6	*Cryptococcus* sp|SH197002.06FU	1
FOTU_5	*Cochliobolus eragrostidis* (JN943411)	100	*Pleosporaceae* sp|SH224806.06FU	1
FOTU_87	*	0	*Sordariales* sp|SH234961.06FU	0.17
FOTU_33	*	0	*Helotiales* sp|SH234185.06FU	0.01
FOTU_97	*	0	*Pulchromyces fimicola*|SH227367.06FU	0.16
FOTU_15	*	0	*Phymatotrichopsis omnivora*|SH207548.06FU	0.03
FOTU_108	*	0	*Sordariomycetes* sp|SH223067.06FU	0.72
FOTU_134	*	0	*Dothideomycetes* sp|SH232444.06FU	0.98
FOTU_56	*	0	*Ceramothyrium carniolicum*|SH238238.06FU	0.23
FOTU_10	*Leptosphaerulina australis* (GU237829)	96.1	*Phoma brasiliensis*|SH202145.06FU	0.66
FOTU_11	*	0	*Montagnulaceae* sp|SH239377.06FU	0.89
FOTU_874	*Tomentella radiosa* (UDB016790)	88.8	*Thelephora* sp|SH199036.06FU	0.92
FOTU_365	*	0	*Sordariomycetes* sp|SH223067.06FU	0.81
FOTU_9	*	0	*Lachnum* sp FC 2211|SH197896.06FU	0.03
FOTU_58	*	0	*Naumovozyma* sp QMW 2012|SH236489.06FU	0.01
FOTU_275	*	0	*Glomeraceae* sp|SH204479.06FU	0.41
FOTU_194	*	0	*Glomeraceae* sp|SH204486.06FU	0.05
FOTU_1532	*	0	*Trichocladium asperum*|SH234966.06FU	0.19
FOTU_210	*	0	*Lophiostoma* sp|SH217127.06FU	0.91
FOTU_426	*Mycocalicium victoriae* (AY128701)	88.6	*Capnodiales* sp|SH238980.06FU	0.28
FOTU_50	*	0	Fungal sp|SH238459.06FU	0.08
FOTU_290	*	0	*Retroconis fusiformis*|SH234970.06FU	0.11
FOTU_350	*Rhodosporidium toruloides* (AB073265)	100	*Sporidiobolales* sp|SH228923.06FU	1
FOTU_157	*	0	*Knufia perforans*|SH209750.06FU	0.48
FOTU_212	*Neurospora crassa* (GU327635)	96.9	*Sordariaceae* sp|SH213056.06FU	1
FOTU_226	*	0	*Glomeraceae* sp|SH204529.06FU	0.22
FOTU_32	*	0	*Pleosporales* sp|SH232445.06FU	0.57
FOTU_576	*Tomentella pilosa* (UDB003324)	92	*Thelephoraceae* sp|SH202556.06FU	0.6
FOTU_1285	*	0	*Lachnum* sp FC 2211|SH197896.06FU	0.04
FOTU_227	*	0	*Geminibasidium hirsutum*|SH204178.06FU	0.51
FOTU_165	*	0	*Hypocreales* sp|SH223288.06FU	0.38
FOTU_129	*	0	*Rhodotorula lamellibrachiae*|SH227552.06FU	0.09
FOTU_277	*	0	*Capnodiales* sp|SH213804.06FU	0.79
FOTU_188	*	0	*Meliniomyces* sp|SH215088.06FU	0.11
FOTU_113	*	0	*Sordariales* sp|SH224745.06FU	0.74
FOTU_48	*Phoma multirostrata* (FJ427034)	98.3	*Phoma brasiliensis*|SH202145.06FU	0.62
FOTU_1	*Westerdykella ornata* (AY943045)	98.9	*Westerdykella ornata*|SH190499.06FU	0.97
FOTU_208	*	0	*Geastrum floriforme*|SH213979.06FU	0.21
FOTU_44	*	0	*Sordariales* sp|SH224745.06FU	0.56
FOTU_74	*	0	*Sordariales* sp|SH224745.06FU	0.45
FOTU_53	*Monosporascus cannonballus* (AB097106)	85	*Sordariomycetes* sp|SH200621.06FU	0.62
FOTU_22	*	0	*Retroconis fusiformis*|SH234970.06FU	0.14
FOTU_335	*	0	*Sporormiella* sp|SH190508.06FU	0.06
FOTU_7	*	0	*Pleosporales* sp|SH232712.06FU	0.69
FOTU_96	*Curvularia inaequalis* (AM924157)	95	*Cochliobolus lunatus*|SH224802.06FU	1
FOTU_84	*	0	*Sordariomycetes* sp|SH223067.06FU	0.78
FOTU_565	*	0	*Diversisporaceae* sp|SH200219.06FU	0.03
FOTU_329	*Peyronellaea pomorum* (FJ839850)	93.9	*Phoma* sp P17E3|SH202157.06FU	0.5
FOTU_49	*Cochliobolus lunatus* (JQ388927)	95.5	*Cochliobolus lunatus*|SH224836.06FU	0.89

Ribosomal Database Classifier using both the Warcup (Deshpande et al., 2016) and UNITE (Abarenkov et al., 2010) training sets

Table 21: Ribosomal Database Classifier predicted taxonomic information and probability for the top 50 most abundant soil fungal OTUs

OTU	Superdivision	Division	Class	Order	Family	Genus	Species	Probability
FOTU_3	Fungi	Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae			0.55
FOTU_13	Fungi	Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus	*Cryptococcus podzolicus*	0.54
FOTU_5	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Cochliobolus	*Cochliobolus eragrostidis*	0.71
FOTU_87	Fungi	Ascomycota	Sordariomycetes	Sordariales				0.77
FOTU_33	Fungi							1
FOTU_97	Fungi	Basidiomycota						0.52
FOTU_15	Fungi							1
FOTU_108	Fungi	Ascomycota	Sordariomycetes	Sordariales				0.7
FOTU_134	Fungi	Ascomycota						0.5
FOTU_56	Fungi	Ascomycota						0.9
FOTU_10	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella		0.65
FOTU_11	Fungi	Ascomycota	Dothideomycetes	Pleosporales				0.95
FOTU_874	Fungi	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	Tomentella		0.84
FOTU_365	Fungi	Ascomycota	Sordariomycetes	Sordariales				0.63
FOTU_9	Fungi							1
FOTU_58	Fungi							1
FOTU_275	Fungi	Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus		0.73
FOTU_194	Fungi	Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus		0.52
FOTU_1532	Fungi	Ascomycota	Sordariomycetes					0.61
FOTU_210	Fungi	Ascomycota	Dothideomycetes					0.5
FOTU_426	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Teratosphaeriaceae	Teratosphaeria		0.81
FOTU_50	Fungi	Ascomycota	Dothideomycetes					0.52
FOTU_290	Fungi	Ascomycota	Sordariomycetes	Sordariales				0.63
FOTU_350	Fungi	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolales Incertae sedis	Rhodosporidium	*Rhodosporidium toruloides*	1
FOTU_157	Fungi	Ascomycota						0.84
FOTU_212	Fungi	Ascomycota	Sordariomycetes	Sordariales	Sordariaceae	Neurospora	*Neurospora sitophila*	0.55
FOTU_226	Fungi	Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus		0.64
FOTU_32	Fungi	Ascomycota						0.5
FOTU_576	Fungi	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	Tomentella	*Tomentella pilosa*	0.53
FOTU_1285	Fungi	Ascomycota						0.71
FOTU_227	Fungi	Basidiomycota	Agaricomycetes					0.62
FOTU_165	Fungi	Ascomycota	Sordariomycetes					0.74
FOTU_129	Fungi							1
FOTU_277	Fungi	Ascomycota						0.86
FOTU_188	Fungi	Ascomycota						0.62
FOTU_113	Fungi	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae			0.69
FOTU_48	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella		0.79
FOTU_1	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Westerdykella	*Westerdykella ornata*	1
FOTU_208	Fungi	Basidiomycota	Agaricomycetes	Geastrales	Geastraceae	Geastrum	*Geastrum schmidelii*	0.5
FOTU_44	Fungi	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae			0.7
FOTU_74	Fungi	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae			0.69
FOTU_53	Fungi	Ascomycota	Sordariomycetes	Xylariales				0.89
FOTU_22	Fungi	Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae			0.54
FOTU_335	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae			0.52
FOTU_7	Fungi	Ascomycota						0.64
FOTU_96	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Curvularia		0.59
FOTU_84	Fungi	Ascomycota	Sordariomycetes	Sordariales				0.52
FOTU_565	Fungi	Basidiomycota						0.62
FOTU_329	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella		0.69
FOTU_49	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Curvularia		0.53

Warcup training set (Deshpande et al., 2016) used for classification

Table 22: The top 50 most frequently observed soil fungal taxa and their closest matches

OTU	Frequency	Closest Match (Warcup)	% ID (Warcup)	Closest Match (UNITE)	% ID (UNITE)
FOTU_5	21	*Cochliobolus eragrostidis* (JN943411)	100	*Pleosporaceae* sp|SH224806.06FU	1
FOTU_11	20	*	0	*Montagnulaceae* sp|SH239377.06FU	0.89
FOTU_33	17	*	0	*Helotiales* sp|SH234185.06FU	0.01
FOTU_3	16	*	0	*Sordariales* sp|SH195274.06FU	0.65
FOTU_97	15	*	0	*Pulchromyces fimicola*|SH227367.06FU	0.16
FOTU_108	14	*	0	*Sordariomycetes* sp|SH223067.06FU	0.72
FOTU_58	14	*	0	*Naumovozyma* sp QMW 2012|SH236489.06FU	0.01
FOTU_9	13	*	0	*Lachnum* sp FC 2211|SH197896.06FU	0.03
FOTU_50	13	*	0	Fungal sp|SH238459.06FU	0.08
FOTU_15	12	*	0	*Phymatotrichopsis omnivora*|SH207548.06FU	0.03
FOTU_74	12	*	0	*Sordariales* sp|SH224745.06FU	0.45
FOTU_53	11	*Monosporascus cannonballus* (AB097106)	85	*Sordariomycetes* sp|SH200621.06FU	0.62
FOTU_98	11	*	0	*Ascomycota* sp|SH221644.06FU	0.56
FOTU_10	9	*Leptosphaerulina australis* (GU237829)	96.1	*Phoma brasiliensis*|SH202145.06FU	0.66
FOTU_13	9	*Cryptococcus podzolicus* (HF558650)	90.6	*Cryptococcus* sp|SH197002.06FU	1
FOTU_44	9	*	0	*Sordariales* sp|SH224745.06FU	0.56
FOTU_1285	9	*	0	*Lachnum* sp FC 2211|SH197896.06FU	0.04
FOTU_166	9	*Aureobasidium pullulans* (FJ150879)	100	*Aureobasidium pullulans*|SH206629.06FU	0.44
FOTU_1119	9	*Asordaria tenerifae* (AY681172)	98.6	*Sordariaceae* sp|SH213057.06FU	0.78
FOTU_1	8	*Westerdykella ornata* (AY943045)	98.9	*Westerdykella ornata*|SH190499.06FU	0.97
FOTU_22	8	*	0	*Retroconis fusiformis*|SH234970.06FU	0.14
FOTU_28	8	*	0	Fungal sp|SH241018.06FU	0.25
FOTU_188	8	*	0	*Meliniomyces* sp|SH215088.06FU	0.11
FOTU_194	8	*	0	*Glomeraceae* sp|SH204486.06FU	0.05
FOTU_211	8	*	0	*Malasseziales* sp|SH219325.06FU	1
FOTU_477	8	*	0	*Glomeromycota* sp|SH239990.06FU	0.25
FOTU_576	8	*Tomentella pilosa* (UDB003324)	92	*Thelephoraceae* sp|SH202556.06FU	0.6
FOTU_32	7	*	0	*Pleosporales* sp|SH232445.06FU	0.57
FOTU_87	7	*	0	*Sordariales* sp|SH234961.06FU	0.17
FOTU_60	7	*	0	*Mortierella ambigua*|SH232483.06FU	0.28
FOTU_190	7	*Sarcinomyces petricola* (FJ489613)	85.8	*Chaetothyriales* sp|SH209756.06FU	0.53
FOTU_157	7	*	0	*Knufia perforans*|SH209750.06FU	0.48
FOTU_276	7	*Cladosporium rectoides* (JN033474)	100	*Davidiella tassiana*|SH196750.06FU	1
FOTU_295	7	*	0	*Cladoriella eucalypti*|SH233933.06FU	0.15
FOTU_618	7	*	0	*Peziza* sp|SH235922.06FU	0.11
FOTU_546	7	*	0	*Glomeraceae* sp|SH204529.06FU	0.1
FOTU_2	6	*	0	*Retroconis fusiformis*|SH234970.06FU	0.22
FOTU_7	6	*	0	*Pleosporales* sp|SH232712.06FU	0.69
FOTU_25	6	*	0	Fungal sp|SH238459.06FU	0.1
FOTU_37	6	*Cochliobolus lunatus* (JQ388927)	100	*Pleosporaceae* sp|SH224799.06FU	1
FOTU_84	6	*	0	*Sordariomycetes* sp|SH223067.06FU	0.78
FOTU_49	6	*Cochliobolus lunatus* (JQ388927)	95.5	*Cochliobolus lunatus*|SH224836.06FU	0.89
FOTU_1055	6	*	0	*Lachnum* sp FC 2211|SH197896.06FU	0.05
FOTU_1180	6	*	0	*Montagnulaceae* sp|SH239377.06FU	0.92
FOTU_113	6	*	0	*Sordariales* sp|SH224745.06FU	0.74
FOTU_100	6	*Macrophomina phaseolina* (FJ395234)	98.6	*Macrophomina phaseolina*|SH235476.06FU	1
FOTU_165	6	*	0	*Hypocreales* sp|SH223288.06FU	0.38
FOTU_290	6	*	0	*Retroconis fusiformis*|SH234970.06FU	0.11
FOTU_226	6	*	0	*Glomeraceae* sp|SH204529.06FU	0.22

Ribosomal Database Classifier using both the Warcup (Deshpande et al., 2016) and UNITE (Abarenkov et al., 2010) training sets

Table 23: Ribosomal Database Classifier predicted taxonomic information and probability for the top 50 most frequently observed soil fungal OTUs

OTU	Superdivision	Division	Class	Order	Family	Genus	Species	Probability
FOTU_5	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Cochliobolus	*Cochliobolus eragrostidis*	0.71
FOTU_11	Fungi	Ascomycota	Dothideomycetes	Pleosporales				0.95
FOTU_33	Fungi							1
FOTU_3	Fungi	Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae			0.55
FOTU_97	Fungi	Basidiomycota						0.52
FOTU_108	Fungi	Ascomycota	Sordariomycetes	Sordariales				0.7
FOTU_58	Fungi							1
FOTU_9	Fungi							1
FOTU_50	Fungi	Ascomycota	Dothideomycetes					0.52
FOTU_15	Fungi							1
FOTU_74	Fungi	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae			0.69
FOTU_53	Fungi	Ascomycota	Sordariomycetes	Xylariales				0.89
FOTU_98	Fungi	Ascomycota	Sordariomycetes					0.88
FOTU_10	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella		0.65
FOTU_13	Fungi	Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus	*Cryptococcus podzolicus*	0.54
FOTU_44	Fungi	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae			0.7
FOTU_1285	Fungi	Ascomycota						0.71
FOTU_166	Fungi	Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	Kabatiella	*Aureobasidium pullulans*	0.6
FOTU_318	Fungi	Basidiomycota	Ustilaginomycotina Incertae sedis	Malasseziales	Malasseziales Incertae sedis	Malassezia	*Malassezia restricta*	1
FOTU_1119	Fungi	Ascomycota	Sordariomycetes	Sordariales	Sordariaceae	Neurospora	*Asordaria tenerifae*	0.55
FOTU_1	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Westerdykella	*Westerdykella ornata*	1
FOTU_22	Fungi	Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae			0.54
FOTU_28	Fungi	Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae			0.57
FOTU_188	Fungi	Ascomycota						0.62
FOTU_194	Fungi	Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus		0.52
FOTU_211	Fungi	Basidiomycota						0.66
FOTU_477	Fungi							1
FOTU_576	Fungi	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	Tomentella	*Tomentella pilosa*	0.53
FOTU_32	Fungi	Ascomycota						0.5
FOTU_87	Fungi	Ascomycota	Sordariomycetes	Sordariales				0.77
FOTU_60	Fungi	Zygomycota	Mucoromycotina Incertae sedis	Mortierellales	Mortierellaceae	Mortierella	*Mortierella wolfii*	0.58
FOTU_190	Fungi	Ascomycota	Pezizomycotina Incertae sedis	Pezizomycotina Incertae sedis	Pezizomycotina Incertae sedis	Knufia	*Sarcinomyces petricola*	0.73
FOTU_157	Fungi	Ascomycota						0.84
FOTU_276	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Davidiella		1
FOTU_295	Fungi	Ascomycota						0.85
FOTU_618	Fungi	Ascomycota	Pezizomycetes	Pezizales	Pezizaceae			0.57
FOTU_546	Fungi	Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus		0.75
FOTU_2	Fungi	Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae			0.5
FOTU_7	Fungi	Ascomycota						0.64
FOTU_25	Fungi	Ascomycota	Dothideomycetes	Capnodiales				0.63
FOTU_37	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Cochliobolus	*Cochliobolus lunatus*	0.69
FOTU_84	Fungi	Ascomycota	Sordariomycetes	Sordariales				0.52
FOTU_49	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Curvularia		0.53
FOTU_1055	Fungi							1
FOTU_1180	Fungi	Ascomycota	Dothideomycetes	Pleosporales				0.87
FOTU_113	Fungi	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae			0.69
FOTU_100	Fungi	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Macrophomina	*Macrophomina phaseolina*	0.99
FOTU_165	Fungi	Ascomycota	Sordariomycetes					0.74
FOTU_290	Fungi	Ascomycota	Sordariomycetes	Sordariales				0.63
FOTU_226	Fungi	Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus		0.64

Warcup training set (Deshpande et al., 2016) used for classification

Compared to the ascomycete lineages observed, the basidiomycete communities observed were generally evenly abundant across known genera (Figure 34). The basidiomycete genera observed had a greater range of life strategies than those of the ascomycete lineages observed. These included some taxa with yeast morphologies. For example, a novel *Cryptococcus* species (FOTU_13) was detected in chromosols, kandosols, rudosols and tenosols of the Beetaloo region. This taxon was among the most abundant fungi observed from the Beetaloo region soils (Table 20). Interestingly, Australian Microbiome[13] data for the same region indicates the same genus is very well represented from their samples of the region highlighting the high abundance of this taxon across time in the Northern Territory. Similarly, several other basidiomycete yeasts were also observed including a *Rhodosporidium* species and several smut species (*Ustilago* spp.) that are likely plant pathogens, which include yeast and quasi-hyphal modes to their lifecycle (for examples see: Cabrera-Ponce et al., 2012). In addition, several wood-rot species were also observed including the polypores taxa such as a Ganoderma species (FOTU_502), a probable *Phanerochaete* species (FOTU_173) a *Trametes* species (FOTU_353) and several *Panus* species (FOTUs 63 & 235; including one that likely is misclassified in by RDP as a *Neolentinus* species). These taxa are likely involved in the degradation of woody components in the soils of the region.

[13] Australian Microbiome: https://www.australianmicrobiome.com/

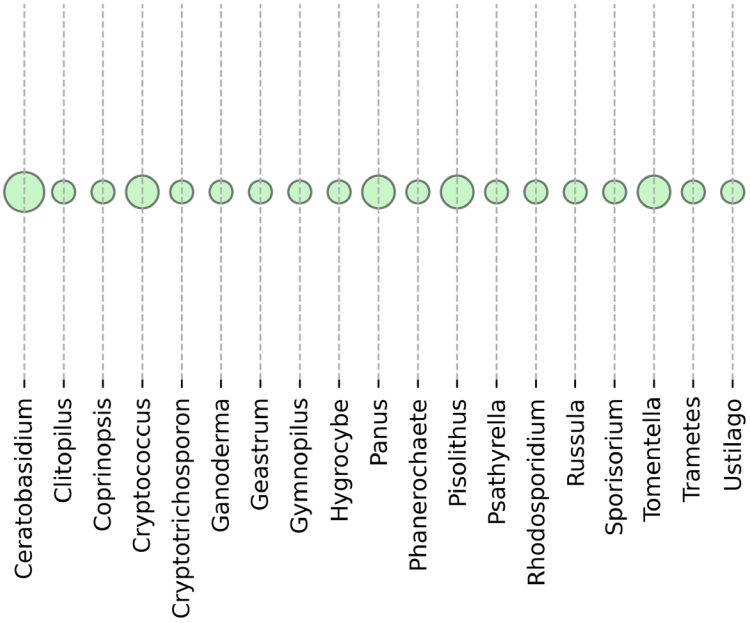

Figure 34: Basidiomycete genera detected in the soils of the Beetaloo region and their relative abundance

The bubble size represents the relative abundance of each genus

In addition to wood-rot species, a number of ectomycorrhizal species were also detected. These taxa form symbiotic associations with plants and facilitate nitrogen and phosphate uptake from organic matter in soils (Smith and Read, 2008) and potentially aid in water uptake by trees (Lehto and Zwiazek, 2011). For example, the basidiomycete data for the region included several *Pisolithus* species. *Pisolithus* have been shown to occur Australian wide, including the Northern Territory (Lebel et al., 2018) and their identification in these soils (probably in association with *Eucalyptus* or *Acacia* species) is unsurprising. Similarly, *Tomentella* and *Russula* species are both ectomycorrhizal genera (Jakucs and Erős-Honti, 2008; Miller and Buyck, 2002) and likely form these associations with trees and shrubs in the Beetaloo region. Of these, data from the current study suggest the most abundant ectomycorrhizal fungi of the region appear to be *Tomentella* species with taxa from this genus being observed in the top 50 most abundant and frequently observed data presented here (Table 20 and Table 22).

Along with ectomycorrhizal taxa, a number of common saprobic genera (organisms that consume decaying organic matter) were also observed in the soils of the Beetaloo region including members of the *Coprinopsis* and *Psathyrella* genera. Both genera typically produce numerous, ephemeral and rather fragile fruiting bodies when growing on organic matter.

The latter was common in the Australian Microbiome dataset, though the former was not detected. This may be due to taxonomic inconsistencies in this family rather than a real change in the presence of these taxa. This is supported by the Atlas of Living Australia which has records of this both *Coprinopsis* and *Coprinellus* for this region ("Atlas of Living Australia – Open access to Australia's biodiversity data,").

Interestingly, the most ubiquitous (and third most abundant) taxon observed in soils of the Beetaloo region was *Cochliobolus eragrostidis* (Table 22 and Table 23). Fungi often have two names, one for the sexual state (known as the teleomorph) and one for the asexual state known as the anamorph. The anamorphic name for *Cochliobolus eragrostidis* is *Curvularia eragrostidis*. *Curvularia* species are well known plant foliar pathogens that produce leaf spot diseases in a range of hosts. In the Beetaloo region cropping is uncommon and most areas feature pastural lands. It thus seems reasonable to assume that this fungus is a fungal pathogen on native grasses. Support for such speculation may be found in a study of northern Australian wild, native rice species which appear to host a range of *Curvularia* species as pathogens (Khemmuk et al., 2016). The presence of this taxon in this region over time can be confirmed through interrogation of the Australian Microbiome dataset ("Australian Microbiome consortium," 2020), which indicates frequent detection of this taxon in the area.

In addition to the two large fungal phyla, representatives of the Glomeromycota were also detected. This phylum contains fungi that are obligate plant symbionts and form beneficial relationships with plants known as arbuscular mycorrhiza (AM). The formation of AM is critical for plant success, particularly in environments where nutrients are bound in plant inaccessible forms. In addition to providing nutritional advantages, plants forming AM have improved water access and resistance to a range of abiotic and biotic stressors (Andrew Smith and Smith, 2011). Almost all grasses form AM, and so their presence here in this study is not surprising. Indeed, despite representing a very small component of the overall microbiome, AM fungi were overrepresented in terms of abundance and ubiquity (Table 20 and Table 22). For example, FOTUs 194, 226, 275 and 546 were all among the most abundant or ubiquitous microbes observed in this study. All of these fungi appeared to have affinities to the genus *Rhizophagus*. This genus was erected to house a well-known but misclassified species '*Glomus intraradices'*. This taxon, now known as *Rhizophagus*

irregularis has a global distribution and has been found in arid, desert and savannah biomes previously (Morton et al., 2004).

The remaining two phyla observed in the present study were from the Chytridiomycota and the Zygomycota. For the former, several *Spizellomyces* species were detected along with a putative *Rhizophlyctis* species. These chytrids are common, often cellulolytic, saprobes in some Australian soils (Letcher et al., 2004), and presumably have a cosmopolitan distribution across the Australian continent though little work has been done on these fungi. Similarly, the Zygomycota -while better studied than chytrids- have been the subject of considerably less research focus than the Asco- or Basidiomycota. This study reports very few zygomycetes in the soils of the Beetaloo region, indeed, only two OTUs were detected (FOTUs 42 & 60) both with some affinities to the zygomycete genus *Mortieriella* were detected in the soils of the Beetaloo region. There is scant literature on their abundance in arid soils, though some studies suggest the phylum does not do well in arid conditions (Richardson, 2009).

3.2.2 Aquifer baselines

Prior to discussing its microbial taxonomic aspects, it is worth noting that, in general, aquifers have low estimated cell density for bacteria and that has been shown to range from 10^2 and 10^6 cells per ml of water (Griebler and Lueders, 2009; Whitman et al., 1998). It is expected that the aquifers of the Beetaloo Region would be similarly low in cell numbers. Furthermore, bacteria and other prokaryotes in aquifers have been frequently demonstrated to preferentially attach to sediment particles, rock surfaces and detritus (Alfreider et al., 1997; Ekendahl et al., 1994; Griebler et al., 2002; Hazen et al., 1991; Pedersen, 1997). The dissolved organic carbon content of these aquifers was very low (ranging from a high of 7 mg L^{-1} down to 1 mg L^{-1}; see Table 15). This absence of dissolved organic carbon presumably creates a situation where microbial (and other) life in these aquifers is based on autotrophy (fixing carbon from inorganic forms). Additionally, some of these aquifers feature largely anaerobic taxa while others include aerobes and microaerophilic taxa, indicating that the oxygen status in these bores differs considerably.

Interestingly, the microbiomes observed in the present study mirror the chemistry data to some extent. In brief, the chemistry data revealed that water from bores that intersect the Tindall Limestone was divided into two groups (see section 3.1.2). These same divisions can be observed here. The NMDS ordination shows that the microbiomes from the bores that intersect the Tindall Limestone divide into these same two groups (Figure 35). The microbiome data clearly separate the higher calcium-associated group (RN033135, RN035130, RN032961, RN031382, RN035146) from the lower calcium-association group (RN024616, RN028082, RN029012, RN031397) though RN038811, one of the lower-calcium associated taxa clusters microbially with the higher calcium group. Like the water chemistry, the remaining samples show diverse microbiomes not necessarily associated with the formation that the bore intersects. This further highlights the pattern observed in the chemical data and likely indicates that formation is not predictive, nor has a direct influence on the microbiome that is found in a particular well. As such, local factors such as recharge rate, mixing of water from other aquifers or hydrogeological discontinuities would be more influential to shape microbiomes in particular bores.

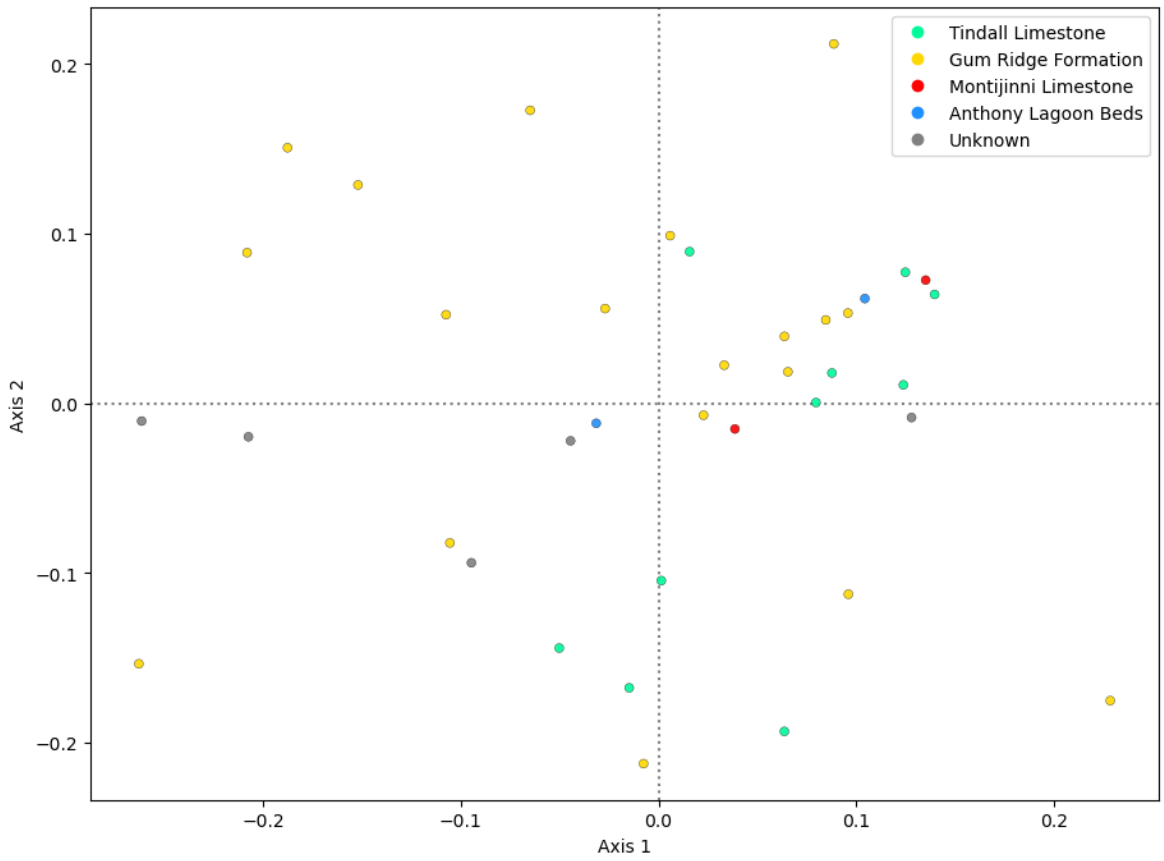

Figure 35: NMDS of microbiome data from the 38 bores examined in the present study, coloured by intersecting formation

In total, 1452 OTUs were detected in the 38 sampled aquifers. Most of the OTUs were bacterial, however, some archaeal OTUs were detected (Figure 36; Supplementary Material 2). These archaeal OTUs comprised 19 Euryarchaeota, 18 Thaumarchaeota, five Pacearchaeota and five Woesarchaeota. The latter two phyla are relatively recently described with DNA from organisms within these phyla being recovered from an aquifer in Colorado, USA indicating that these taxa are common, albeit low abundance, members of subsurface microbiomes associated with aquifers (Castelle et al., 2015). The Pacearchaeota and the Woesarchaeota have both been implicated in saccharolytic and fermentative life strategies, while the former may have genes that enable it to grow autotrophically from carbon dioxide in the aquifers (Castelle et al., 2015).

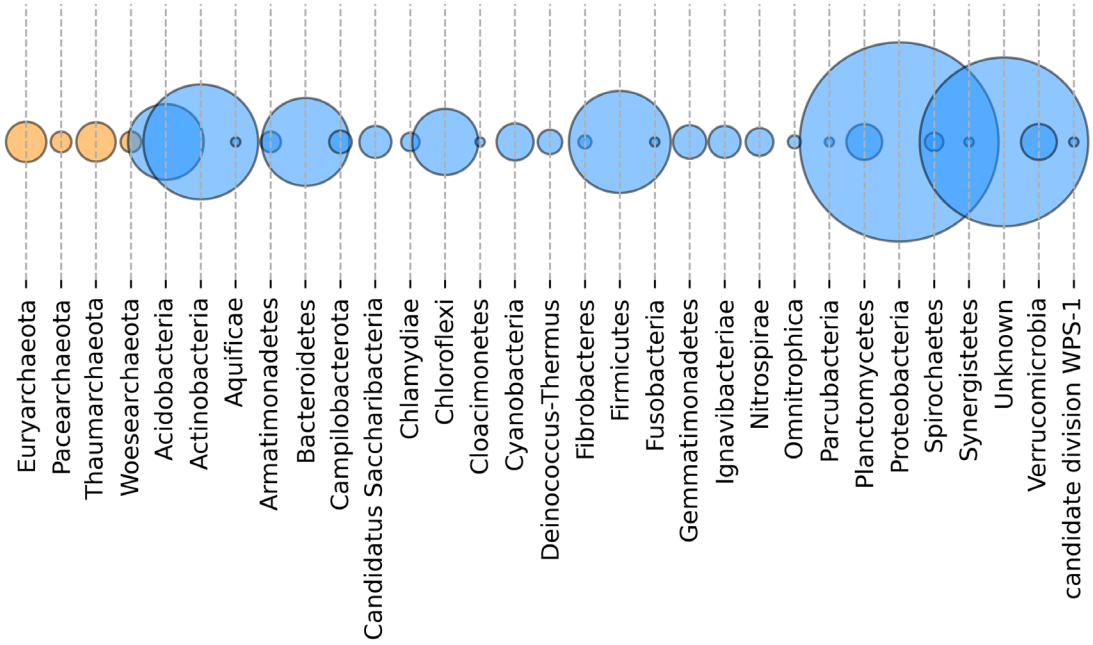

Figure 36: Relative abundance of the various phyla from the 1452 OTUs detected in the 38 aquifers examined

Bubble size represents relative abundance. Archaeal phyla are shown here in orange, bacterial phyla in blue

Of the more abundant archaeal phyla, all Thaumarchaeota detected were ammonia-oxidising, CO_2-fixing autotrophs allied loosely to – but not belonging to- either the genera

Nitrososphaeria or *Nitrosopumilus*. In contrast, two groups of euryarchaeotes were detected. The first of these, methanogens, included a number of methanogen genera notably *Methanobacterium, Methanocalculus, Methanosarcina, Methanospirillum* and *Methanomassiliicoccus*. It is noteworthy, that while relatively few Euryarchaeota and Thaumarchaeota OTUs were detected, this lineage was among the most abundant taxa observed in the aquifers (Table 24 and Table 25). For example, among the top 50 most abundant taxa detected in the 38 aquifers, two methanogens (OTUs 25 and 78) and two thaumarcheote were detected (OTUs 164 and 519). The abundance of these taxa emphasises the organic carbon deficit in these environments; both thaumarchaetes can likely fix CO_2 to biomass. Methanogens, in contrast, include a range of auto- and heterotrophic species, the former of which fix carbon using the Wood-Ljungdahl pathway (Berg, 2011; Borrel et al., 2016; Chen et al., 2019). Interestingly, neither *Methanocalculus pumilus* nor *Methanobacterium palustre* (the two species most closely related to OTUs 25 and 78, respectively) were able to fix CO_2 (Mori et al., 2000; Zellner et al., 1989). It may be that OTUs 25 and 78 are capable of carbon fixation via the Wood-Ljungdahl pathway in the aquifers of the Northern Territory, or they instead are using simple carboxylic acids derived (originally) from autotrophs in this environment.

Table 24: The top 50 most abundant aquifer taxa and their closest matches on Genbank with their percentage identity (over ~290bp)

OTU	Match	% ID
OTU_3	*Rectinema cohabitans* HM (NR 156915)	100
OTU_87	*Acinetobacter lwoffii* DSM 2403 (NR 026209)	100
OTU_69	*Thermodesulfovibrio yellowstonii* YP87 (NR 041318)	86.2
OTU_74	*Pandoraea thiooxydans* ATSB16 (NR 116008)	97.2
OTU_89	*Cupriavidus gilardii* CIP 105966 (NR 116146)	98
OTU_48	uncultured Acidobacteriaceae bacterium (AY225643)	86.2
OTU_78	*Methanocalculus pumilus* MHT-1 (NR 028148)	99.6
OTU_62	*Thermodesulfovibrio yellowstonii* YP87 (NR 041318)	85.8
OTU_4	*Sulfurisoma sediminicola* BSN1 (NR 125471)	99.2
OTU_127	*Phenylobacterium koreense* Slu-01 (NR 041016)	96.4
OTU_26	*Melioribacter roseus* P3M-2 (NR 118349)	94.1
OTU_120	*Thiohalophilus thiocyanatoxydans* HRhD 2 (NR 043875)	95.7
OTU_47	*Ottowia beijingensis* GCS-AN-3 (NR 133803)	99.6
OTU_172	*Lawsonella clevelandensis* X1036 (NR 151867)	100
OTU_122	*Magnetospirillum moscoviense* BB-1 (NR 149243)	100
OTU_44	*Melioribacter roseus* P3M-2 (NR 118349)	92.5
OTU_117	*Thermodesulfovibrio hydrogeniphilus* DSM 18151 Hbr5 (NR 044075)	85.8
OTU_121	*Desulfuromonas acetexigens* 2873 (NR 044770)	96.8
OTU_66	*Melioribacter roseus* P3M-2 (NR 118349)	94.9
OTU_4755	*Knoellia sinensis* HKI 0119 (NR 028931)	98.8
OTU_193	*Blastomonas natatoria* DSM 3183 (NR 040824)	100
OTU_111	*Denitratisoma oestradiolicum* AcBE2-1 (NR 043249)	98
OTU_856	*Zoogloea resiniphila* DhA-35; ATCC 700687 (NR 027188)	100
OTU_413	*Sulfuricurvum kujiense* DSM 16994 YK-1 (NR 112144)	100
OTU_274	*Pseudomonas chlororaphis* subsp. *aurantiaca* NCIB 10068 (NR 043935)	100
OTU_108	*Thermodesulfovibrio hydrogeniphilus* DSM 18151 Hbr5 (NR 044075)	86.2
OTU_25	*Methanobacterium palustre* F (NR 114485)	98
OTU_164	*Nitrosopumilus ureiphilus* PS0 (NR 159208)	92.1
OTU_216	*	0
OTU_153	*Desulfatitalea tepidiphila* S28bF (NR 113315)	88.9
OTU_151	*	0
OTU_33	*Sphingomonas alpina* S8-3 (NR 117230)	100
OTU_222	*Halomonas rifensis* HK31 (NR 117775)	100
OTU_94	*Moraxella osloensis* A1920 (NR 104936)	100
OTU_99	*Sulfurifustis variabilis* skN76 (NR 137347)	91.3
OTU_149	*Brevundimonas viscosa* F3 (NR 117900)	100
OTU_297	*Thermanaerovibrio velox* Z-9701 (NR 104765)	87
OTU_162	*Pelomonas aquatica* CCUG 52575 (NR 042614)	100
OTU_281	*Brevundimonas terrae* KSL-145 (NR 043726)	99.6
OTU_267	*Dietzia papillomatosis* N 1280 (NR 116687)	100
OTU_232	*Ferriphaselus amnicola* OYT1 (NR 114334)	97.2
OTU_295	*Syntrophobacter sulfatireducens* TB8106 (NR 043073)	90.2
OTU_236	*Aeromicrobium marinum* T2; DSM 15272 (NR 025681)	100
OTU_523	*	0
OTU_227	*	0
OTU_519	*Nitrososphaera viennensis* EN76 (NR 134097)	88.9
OTU_310	*	0
OTU_192	*Thermodesulfovibrio aggregans* TGE-P1 (NR 040795)	86.2
OTU_199	*Lactobacillus iners* DSM 13335; CCUG 28746 (NR 036982)	100
OTU_289	*	0

Table 25: Ribosomal Database Classifier predicted taxonomic information and probability for the top 50 most abundant aquifer OTUs

OTU	Domain	Phylum	Class	Order	Family	Genus	Confidence
OTU_3	Bacteria	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Rectinema	1
OTU_87	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	1
OTU_69	Bacteria						0.99
OTU_74	Bacteria	Proteobacteria	Betaproteobacteria			1	
OTU_89	Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Gallionellaceae	Sideroxydans	0.6
OTU_48	Bacteria	Firmicutes	Clostridia				0.55
OTU_78	Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiales_incertae_sedis	Methanocalculus	1
OTU_62	Bacteria						0.99
OTU_4	Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Sterolibacteriaceae	Sulfurisoma	0.73
OTU_127	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium	1
OTU_26	Bacteria	Ignavibacteriae	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	Melioribacter	1
OTU_120	Bacteria	Proteobacteria	Gammaproteobacteria	Chromatiales	Thioalkalispiraceae	Thiohalophilus	0.56
OTU_47	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	1	
OTU_172	Bacteria	Actinobacteria	Actinobacteria	Mycobacteriales	Lawsonellaceae	Lawsonella	1
OTU_122	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Magnetospirillum	1
OTU_44	Bacteria	Ignavibacteriae	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	Melioribacter	0.6
OTU_117	Bacteria						1
OTU_121	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Desulfuromonadaceae	Desulfuromonas	0.9
OTU_66	Bacteria	Ignavibacteriae	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	Melioribacter	0.99
OTU_4755	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Oryzihumus	0.76
OTU_193	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Blastomonas	1
OTU_111	Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Sterolibacteriaceae	0.5	
OTU_856	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Zoogloeaceae	Zoogloea	1
OTU_413	Bacteria	Campilobacterota	Campylobacteria	Campylobacterales	Thiovulaceae	Sulfuricurvum	1
OTU_274	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1
OTU_108	Bacteria						1
OTU_25	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium	1
OTU_164	Archaea	Thaumarchaeota	Nitrosopumilales	Nitrosopumilaceae	Nitrosopumilus	1	
OTU_216	Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	Vampirovibrio	0.54
OTU_153	Bacteria	Proteobacteria	Deltaproteobacteria		0.62		
OTU_151	Bacteria	Firmicutes	Clostridia				0.53
OTU_33	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	1
OTU_222	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	1
OTU_94	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	1
OTU_99	Bacteria	Proteobacteria	Gammaproteobacteria		0.91		
OTU_149	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	1
OTU_297	Bacteria						1
OTU_162	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.96
OTU_281	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	1
OTU_267	Bacteria	Actinobacteria	Actinobacteria	Mycobacteriales	Dietziaceae	Dietzia	0.99
OTU_232	Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales		0.54	
OTU_295	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfobacterales		0.54	
OTU_236	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Aeromicrobium	1
OTU_523	Bacteria						0.98
OTU_227	Bacteria						0.99
OTU_519	Archaea	Thaumarchaeota	Nitrososphaerales	Nitrososphaeraceae	Nitrososphaera	0.72	
OTU_310	Bacteria	Bacteroidetes				0.82	
OTU_192	Bacteria						0.99
OTU_199	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.92
OTU_289	Bacteria						1

16S training set used for classification

Table 26: The top 50 most frequently observed aquifer taxa and their closest matches on GenBank with their percentage identity (over ~290bp)

OTU	Match	% ID
OTU_87	*Acinetobacter lwoffii* DSM 2403 (NR 026209)	100
OTU_127	*Phenylobacterium koreense* Slu-01 (NR 041016)	96.4
OTU_47	*Ottowia beijingensis* GCS-AN-3 (NR 133803)	99.6
OTU_33	*Sphingomonas alpina* S8-3 (NR 117230)	100
OTU_89	*Cupriavidus gilardii* CIP 105966 (NR 116146)	98
OTU_193	*Blastomonas natatoria* DSM 3183 (NR 040824)	100
OTU_94	*Moraxella osloensis* A1920 (NR 104936)	100
OTU_149	*Brevundimonas viscosa* F3 (NR 117900)	100
OTU_121	*Desulfuromonas acetexigens* 2873 (NR 044770)	96.8
OTU_4755	*Knoellia sinensis* HKI 0119 (NR 028931)	98.8
OTU_274	*Pseudomonas chlororaphis* subsp. *aurantiaca* NCIB 10068 (NR 043935)	100
OTU_216	*	0
OTU_162	*Pelomonas aquatica* CCUG 52575 (NR 042614)	100
OTU_43	*Pseudorhodoferax caeni* SB1 (NR 042216)	100
OTU_44	*Melioribacter roseus* P3M-2 (NR 118349)	92.5
OTU_385	*Labilithrix luteola* B00002 (NR 126182)	94.1
OTU_74	*Pandoraea thiooxydans* ATSB16 (NR 116008)	97.2
OTU_172	*Lawsonella clevelandensis* X1036 (NR 151867)	100
OTU_245	*Massilia niabensis* 5420S-26 (NR 044571)	100
OTU_16	*Bradyrhizobium valentinum* LmjM3 (NR 125638)	100
OTU_455	*Aliidongia dinghuensis* 7M-Z19 (NR 156087)	88.5
OTU_999	uncultured Acidobacteria bacterium (EF457461)	87.6
OTU_3	*Rectinema cohabitans* HM (NR 156915)	100
OTU_78	*Methanocalculus pumilus* MHT-1 (NR 028148)	99.6
OTU_394	uncultured Acidobacteria bacterium (EF457454)	97.2
OTU_497	*Acidovorax defluvii* BSB411; DSM 12644 (NR 026506)	100
OTU_234	*Acetobacterium psammolithicum* CN-E (NR 114509)	100
OTU_437	*Desulfohalovibrio alkalitolerans* RT2 (NR 043069)	99.2
OTU_84	*Annwoodia aquaesulis* ATCC 43788 (NR 044793)	96.4
OTU_658	*Nevskia lacus* Seoho-38 (NR 164939)	98.4
OTU_21	*Massilia pinisoli* T33 (NR 152009)	99.6
OTU_4	*Sulfurisoma sediminicola* BSN1 (NR 125471)	99.2
OTU_26	*Melioribacter roseus* P3M-2 (NR 118349)	94.1
OTU_111	*Denitratisoma oestradiolicum* AcBE2-1 (NR 043249)	98
OTU_164	*Nitrosopumilus ureiphilus* PS0 (NR 159208)	92.1
OTU_267	*Dietzia papillomatosis* N 1280 (NR 116687)	100
OTU_58	*Microbacterium imperiale* DSM 20530 (NR 026161)	100
OTU_389	*Rhodococcus cerastii* C5 (NR 117103)	100
OTU_363	*Enterobacter kobei* CIP 105566 (NR 028993)	100
OTU_323	*Lactobacillus acidophilus* BCRC10695 (NR 043182)	100
OTU_762	*Chryseobacterium xinjiangense* TSBY-67 (NR 131771)	100
OTU_1224	*Geothermobacter ehrlichii* SS015 (NR 042754)	91.3
OTU_28	*Azoarcus olearius* DQS-4 (NR 108183)	100
OTU_887	*Desulfonatronum lacustre* DSM 10312 DSM 10312 (NR 041848)	89.3
OTU_609	*Pseudonocardia zijingensis* 6330; JCM 11117 (NR 028805)	99.6
OTU_222	*Halomonas rifensis* HK31 (NR 117775)	100
OTU_99	*Sulfurifustis variabilis* skN76 (NR 137347)	91.3
OTU_281	*Brevundimonas terrae* KSL-145 (NR 043726)	99.6
OTU_436	*Bellilinea caldifistulae* GOMI-1 (NR 041354)	85.4
OUT_5967	*Sulfurisoma sediminicola* BSN1 (NR 125471)	97.2

Table 27: Ribosomal Database Classifier predicted taxonomic information and probability for the top 50 most frequently observed aquifer OTUs

OTU	Domain	Phylum	Class	Order	Family	Genus	Confidence
OTU_87	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	1
OTU_127	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium	1
OTU_47	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		1
OTU_33	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	1
OTU_89	Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Gallionellaceae	Sideroxydans	0.6
OTU_193	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Blastomonas	1
OTU_94	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	1
OTU_149	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	1
OTU_121	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Desulfuromonadaceae	Desulfuromonas	0.9
OTU_4755	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Oryzihumus	0.76
OTU_274	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1
OTU_216	Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	Vampirovibrio	0.54
OTU_162	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.96
OTU_43	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pseudorhodoferax	0.75
OTU_44	Bacteria	Ignavibacteriae	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	Melioribacter	0.6
OTU_385	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Labilitrichaceae	Labilithrix	0.78
OTU_74	Bacteria	Proteobacteria	Betaproteobacteria				1
OTU_172	Bacteria	Actinobacteria	Actinobacteria	Mycobacteriales	Lawsonellaceae	Lawsonella	1
OTU_245	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Massilia	1
OTU_16	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium	1
OTU_455	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae		0.6
OTU_999	Bacteria	Firmicutes	Clostridia	Clostridiales			0.54
OTU_3	Bacteria	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Rectinema	1
OTU_78	Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiales_incertae_sedis	Methanocalculus	1
OTU_394	Bacteria	Acidobacteria	Acidobacteria_Gp16		Gp16		1
OTU_497	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	0.96
OTU_234	Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Acetobacterium	1
OTU_437	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfohalovibrio	1
OTU_84	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales			0.63
OTU_658	Bacteria	Proteobacteria	Gammaproteobacteria	Nevskiales	Nevskiaceae	Nevskia	1
OTU_21	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Massilia	0.99
OTU_4	Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Sterolibacteriaceae	Sulfurisoma	0.73
OTU_26	Bacteria	Ignavibacteriae	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	Melioribacter	1
OTU_111	Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Sterolibacteriaceae		0.5
OTU_164	Archaea	Thaumarchaeota		Nitrosopumilales	Nitrosopumilaceae	Nitrosopumilus	1
OTU_267	Bacteria	Actinobacteria	Actinobacteria	Mycobacteriales	Dietziaceae	Dietzia	0.99
OTU_58	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Microbacterium	0.96
OTU_389	Bacteria	Actinobacteria	Actinobacteria	Mycobacteriales	Nocardiaceae	Rhodococcus	1
OTU_363	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Klebsiella	0.98
OTU_323	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	1
OTU_762	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Weeksellaceae	Chryseobacterium	1
OTU_1224	Bacteria	Proteobacteria	Deltaproteobacteria				0.75
OTU_28	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Zoogloeaceae	Thauera	0.51
OTU_887	Bacteria	Proteobacteria					0.61
OTU_609	Bacteria	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	0.98
OTU_222	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	1
OTU_99	Bacteria	Proteobacteria	Gammaproteobacteria				0.91
OTU_281	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	1
OTU_436	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae		0.79
OTU_5967	Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Sterolibacteriaceae		0.61

16S training set used for classification

Archaea aside, the Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes phyla together make up more than 50% of all OTUs detected, with 462, 154, 121 and 90 OTUs belonging to these phyla, respectively. It is thus unsurprising that among the most abundant OTUs detected in the reservoir Proteobacteria are commonplace. Indeed, almost a third of all OTUs observed were from the Proteobacteria. Among these taxa, a betaproteobacterial OTU (OTU_4) which is probably conspecific with *Sulfurisoma sediminicola* was very abundant in many of the bores. *Sulfurisoma sediminicola* is a facultative anaerobic, autotroph that oxidises a range of electron donors (thiosulfate, sulfur, hydrogen) for energy, using nitrate as an electron acceptor. The abundance of this taxon also supports the case for organic carbon-limited environment in the bores of the Beetaloo region. Numerous other Proteobacteria were also detected including numerous Rhodospirallales, Rhizobiales, Burkholderiales and Myxococcales (Figure 37). Among these, one OTU (OTU_127) is among the most abundant and ubiquitous taxa observed in the present study (Table 24 - Table 27). This OTU is probably a novel Phenylobacterium species, but it was most closely related to *P. koreense,* an isolate obtained from waste water in Daejeon, South Korea (Aslam et al., 2005) and a second species *Phenylobacterium lituiforme* isolated from subsurface aquifers near Longreach, Queensland (Kanso and Patel, 2004). The presence of *Phenylobacterium* species in aquifers in western Queensland, and their common detection here suggests that they may be a common subsurface genus in Australian aquifer environments. The role these taxa play in these environments is less clear, however, the genus is known for its degradation of unusual, complex aromatic compounds including the aromatic amino acid phenylalanine l-phenylalanine and the aromatic herbicides chloridazon, antipyrin and pyramidon (Eberspächer and Lingens, 2006). Their widespread distribution in this habitat may indicate their use of dissolved aromatic compounds in the aquifers. If this is the case, the origin of these compounds warrants further investigation.

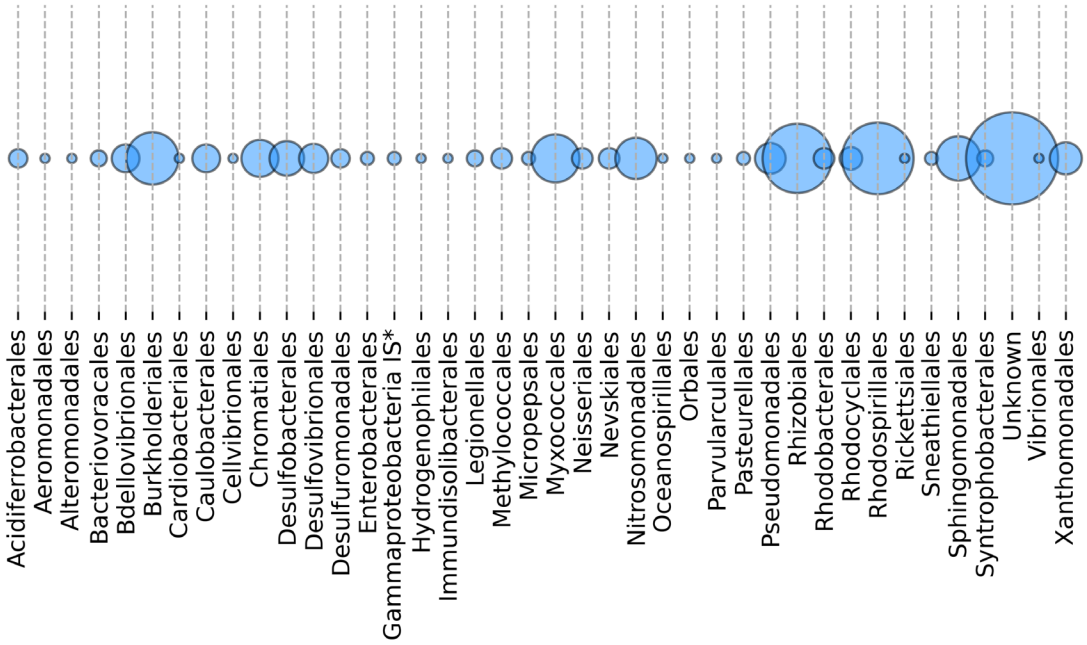

Figure 37: Relative abundance of proteobacterial orders from the from the 462 proteobacterial OTUs detected in the 38 aquifers examined

Bubble size represents relative abundance

Actinobacteria comprise over 10% of all OTUs detected in the aquifers and include some of the most abundant and ubiquitous taxa in these environments (Table 24 and Table 26). Interestingly, the actinobacterial lineages observed in the 38 water samples do not follow the same patterns of autotrophy observed in some of the other lineages, indeed, some of these isolates have no known 'natural reservoir' having been isolated from a diverse array of potential habitats and some are known from skin, suggesting some taxa may be contaminants during sampling. The most abundant and ubiquitous actinobacterium observed was OTU_172 (Table 24 and Table 26), a taxon that is probably conspecific with *Lawsonella clevelandensis* (Table 24 and Table 26), a medically interesting taxon that was originally isolated from human abscesses (Bell et al., 2016) but has also been observed in a range of habitats. While there are reports of this taxon in deep CO_2-saturated miocene sediments (Liu et al., 2020), there are also common reports from human nasal microbiomes (Selway et al., 2020) and it is unclear whether this taxon is a contaminant obtained during collection, DNA extraction or DNA sequencing or whether it represents a naturally occurring organism in these aquifers. Similarly, other actinobacterial OTUs also have closest relatives with medical relevance. For example, OTU_267 is conspecific with *Dietzia papillomatosis*, a

taxon also described from human skin (Jones et al., 2008) and is a possible contaminant of these data. Conversely, other abundant actinobacterial taxa were closely related to taxa from caves (OTU_4755) (Groth et al., 2002), leaf surfaces (OTU_389) (Kämpfer et al., 2013) or marine waters (OTU_236)(Bruns et al., 2003).

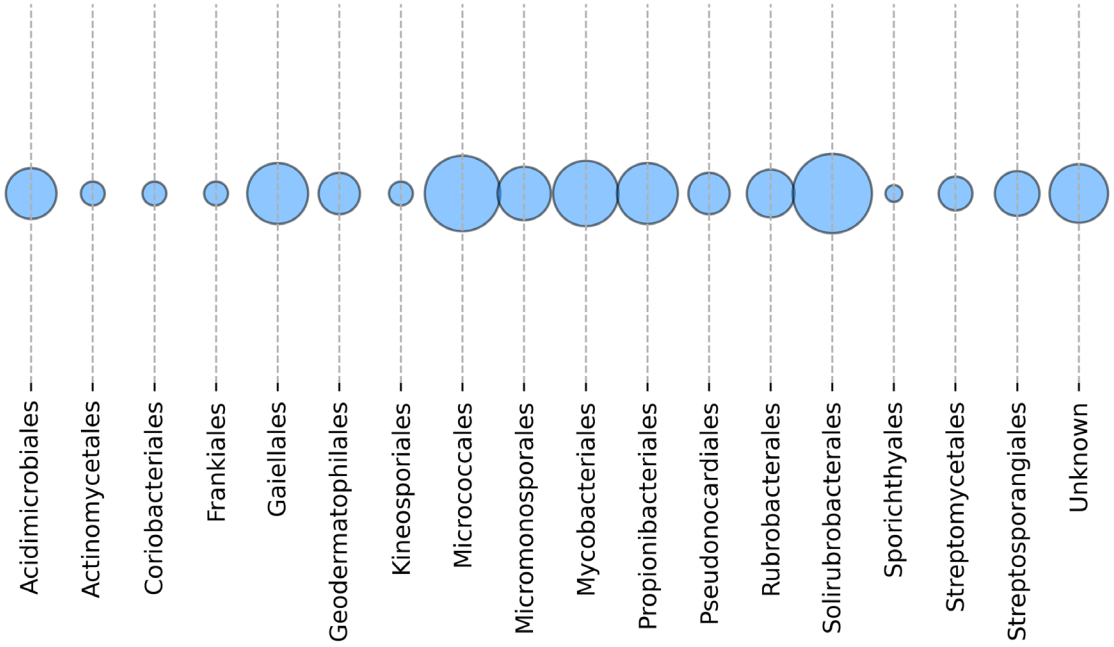

Figure 38: Relative abundance of actinobacterial orders from the from the 154 proteobacterial OTUs detected in the 38 aquifers examined

Bubble size represents relative abundance

While a comparatively large number of Firmicutes OTUs were detected (8% of all OTUs), most of these taxa were rare and they are not frequently among the more commonly observed taxa in the aquifers. For example, the most abundant Firmicutes taxon detected was OTU_48, this taxon is poorly taxonomically resolved in the analyses presented here (Table 24 and Table 26). There were also a relatively abundant number of OTUs belonging to unknown Firmicute families (Figure 39). Further, BLAST comparison reveals this taxon has no close relatives, indeed, its closest relatives share only 87-89% 16S DNA identity which make extrapolation of inferences about this taxon questionable. Similarly, OTU_151, another comparatively abundant Firmicutes taxon is poorly taxonomically resolved in the present study. BLAST comparisons of this taxon reveal it does have close identity with undescribed

DNA sequences (AM778018, HM066593) retrieved from groundwater habitats in Portugal and the USA, respectively (Gray and Engel, 2013; Tiago and Veríssimo, 2013).

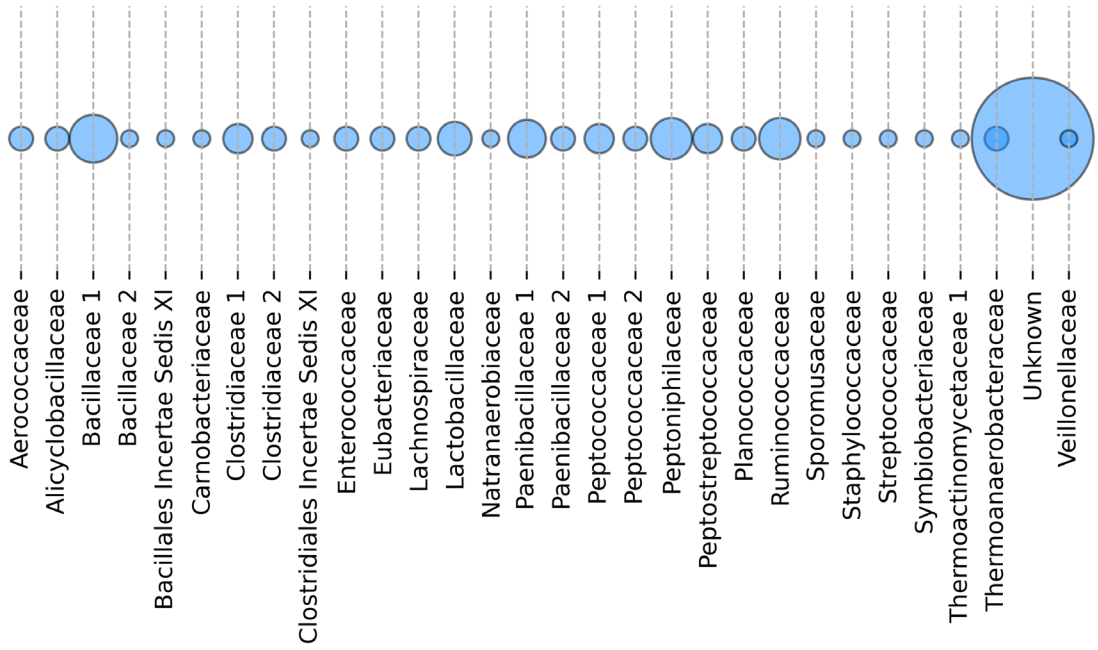

Figure 39: Relative abundance of Firmicutes families from the from the 121 proteobacterial OTUs detected in the 38 aquifers examined

Bubble size represents relative abundance

Among the 90 Bacteroidetes families detected, the largest number of OTUs belonged to the Chitinophagaceae, a group of bacteria well known for their degradation of plant and fungal polymers (Figure 40). It is unclear whether these taxa are engaged in the degradation of complex organic carbon in the subsurface or whether they are engaged in other processes in this environment. Bacteroidetes are rarely among the more abundant or ubiquitous taxa observed in the bores and where they are, their roles in this environment are unclear. For example, OTU_762 was a *Chryseobacterium* species, with a close match to *C. xinjiangense* (Zhao et al., 2011), which was originally isolated and described from the permafrost within a cirque glacier in the Tianshan Mountains in northwestern China (Bai et al., 2006), its presence here likely indicates the taxon is very widespread and inhabits environments that vary markedly from its original isolation.

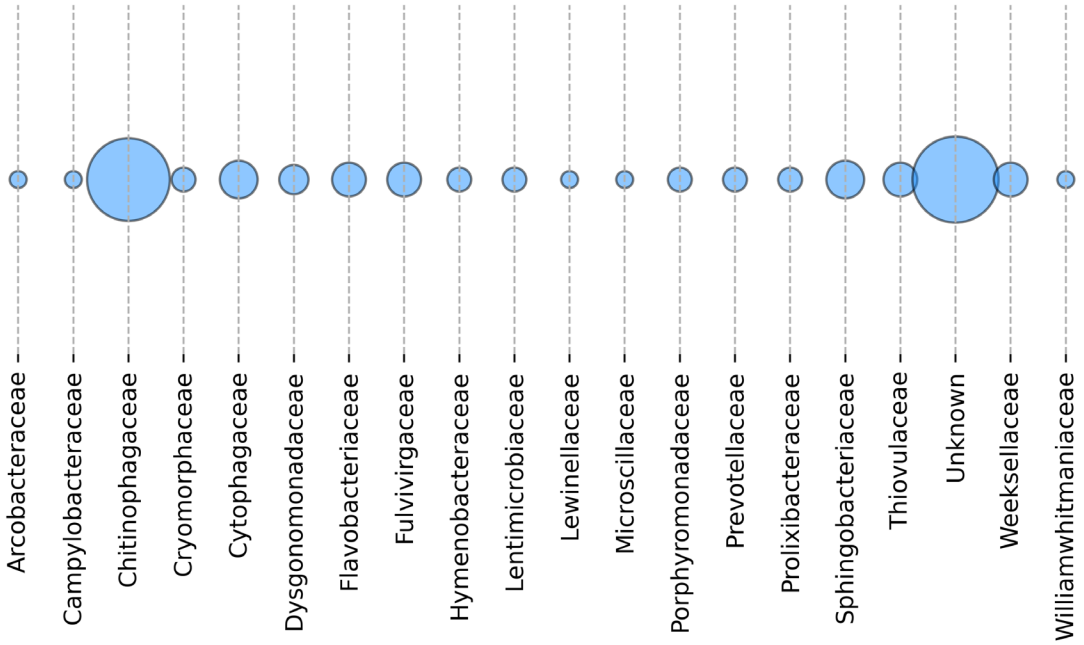

Figure 40: Relative abundance of Bacteroidetes families from the from the 90 Bacteroidetes OTUs detected in the 38 aquifers examined

Bubble size represents relative abundance

It should be noted that there are a number of OTUs that are very abundant but are not from phyla that are common in the dataset. For example, OTU_69 and OTU_62 are both novel organisms whose closest match is to *Thermodesulfovibrio yellowstonii* YP87 (NR 041318), these matches were poor (Match % 86.2 and 85.8, respectively) and so limited inferences can be drawn from these classifications. BLAST searching, however, reveals numerous environmental sequences that are closely related to OTUs_69 and 62. Interestingly, all of the top matches for these two OTUs are to other subsurface microbes. For instance, its closest match is to KF836333[14] a sequence obtained from a deep, fractured rock aquifer in the Mojave Desert, USA. Other close matches were to KC604624, a sequence from groundwater (Flynn et al., 2013), FR683079 from mine tailings (Huang et al., 2011)and HM991583 another aquifer associated sequence[15]. Taken together with data from OTUs like OTU_48 (discussed above), this suggests that there may be prokaryotic taxa present in the aquifers of the Beetaloo Region that are subsurface, aquifer specialists.

[14] https://www.ncbi.nlm.nih.gov/nucleotide/KF836333.1

[15] https://www.ncbi.nlm.nih.gov/nucleotide/HM991583.1

3.3 Degradation of chemicals by microbes from soils and aquifers of the Beetaloo Basin region

The degradation of two groups of chemicals, the glycols (ethylene glycol and propylene glycol) and the alcohols (butoxyethanol, isopropanol, methanol) were chemically assayed from soils and aquifers in the present study using NATA accredited, third-party facilities. This was conducted due to the availability of NATA accredited tests for these chemicals.

Key findings

- Microbes from soil were able to degrade all five chemicals.
- In soil, chemicals were frequently undetectable after less than one month due to degradation by microbes.
- In aquifers, microbes were able to degrade all chemicals to some extent, however, this degradation was not completed during the time period examined (3 months) for any chemical/aquifer combination.
- The extent of chemical degradation varied between different aquifers and different soils.

3.3.1 Biodegradation by soil microbiomes

Alcohols

In broad terms all alcohols were significantly degraded in the soils of the Beetaloo Region, though this varied for individual alcohols with some showing some remnant concentrations after the four weeks incubation. At high concentrations, alcohols are regularly used as disinfectants as they readily kill bacteria and fungi. They achieve this activity through alterations to the cell membrane that effect both its fluidity and the protein transporters bound in the membrane. The length of the alcohol also impacts its toxicity with longer chained alcohols more toxic than shorter chain alcohols (Jia et al., 2010).

For 2-butoxyethanol, this alcohol was undetectable in samples after four weeks incubation with all soils indicating its rapid degradation (Figure 41). It should be noted that it is unclear how long this degradative process took, it may be that it was degraded in a considerably shorter timeframe than the month long incubation. Indeed, the literature suggest its degradation time may be considerably shorter (Woiski et al., 2020). Work by Woiski et al., 2020, demonstrated that butoxyethanol was degraded to 2-butoxyacetic acid, which was subject to ester cleave resulting in glyoxylate/glyoxalic acid and n-butanol. Soil bacteria are readily able to use all of these substrates as sole sources of carbon (Woiski et al., 2020). The literature has relatively scant information on fungal use of this substrate, and while it likely is degraded by fungi, work to confirm this would be useful. Regardless, it is clear that this compound is readily degraded by the soil microbes within the Beetaloo region without the addition of other exogenous nutrients (like nitrogen or phosphorus).

In contrast to the soils, degradation of 2-butoxyethanol was more limited in the aquifer environment (Figure 44). Nevertheless, significant degradation did occur in the three months incubation period, particularly in RN033608, and RN040930. The literature is fairly sparse on anaerobic degradation of 2-butoxyethanol. There is some research in the area, notably a Masters thesis by Rhiner (2014) demonstrates that anaerobic pathways for 2-butoxyethanol degradation exist, even under highly-reduced conditions (Rhiner, 2014).

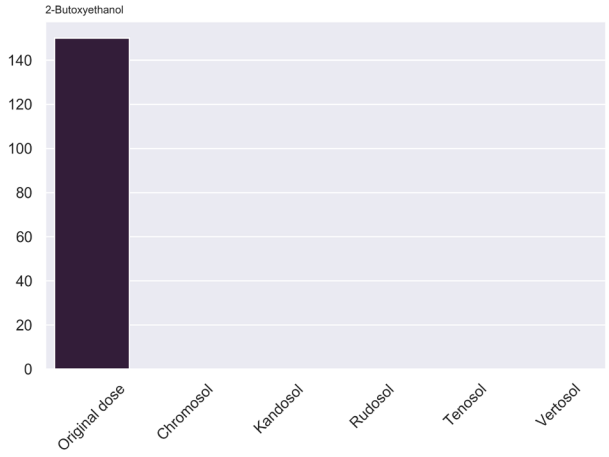

Figure 41: Butoxyethanol biodegradation in Northern Territory soil types

2-butoxyethanol concentration in mg L^{-1}

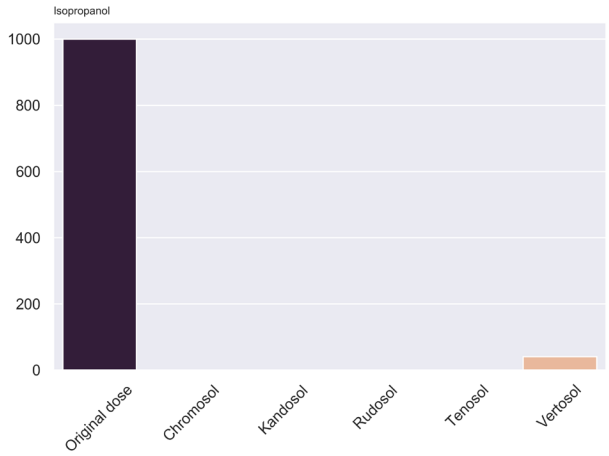

Figure 42: Isopropanol biodegradation in Northern Territory soil types

Isopropanol concentration in concentration in mg L^{-1}

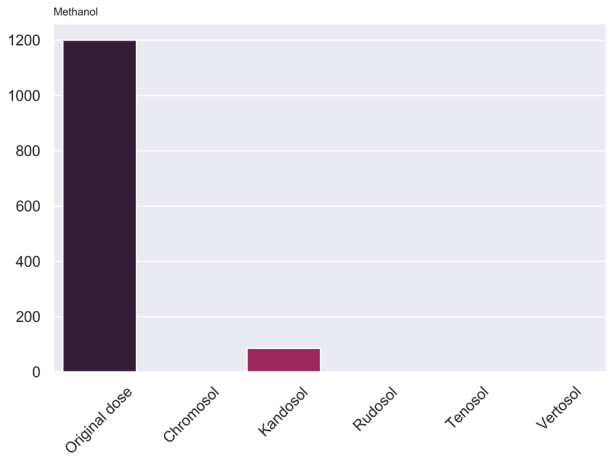

Figure 43: Methanol biodegradation in Northern Territory soil types

Methanol concentration in concentration in mg L^{-1}

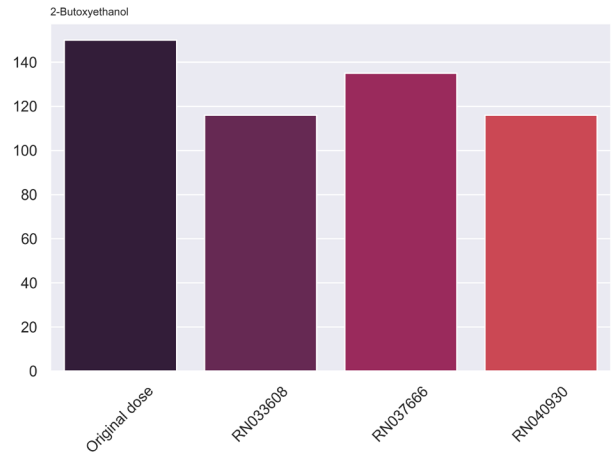

Figure 44: Butoxyethanol biodegradation in Northern Territory aquifers

2-butoxyethanol concentration in mg L^{-1}

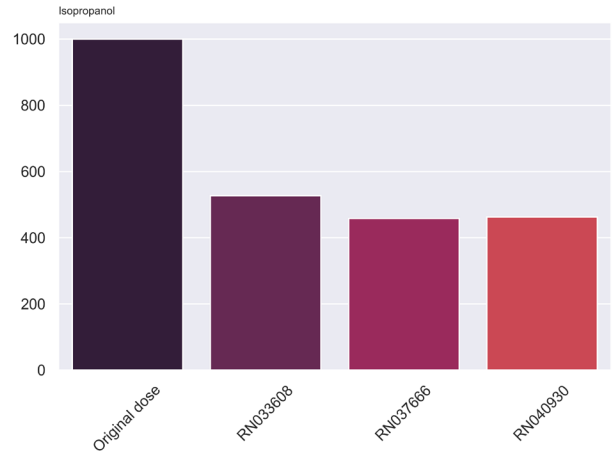

Figure 45: Isopropanol biodegradation in Northern Territory aquifers

Isopropanol concentration in concentration in mg L^{-1}

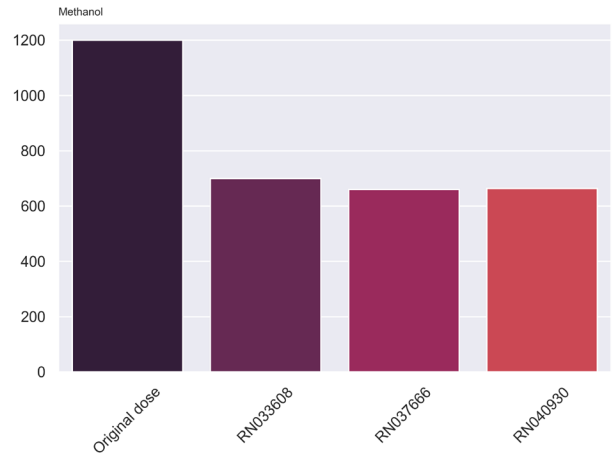

Figure 46: Methanol biodegradation in Northern Territory aquifers

Methanol concentration in concentration in mg L^{-1}

Microbiomes from the chromosol, kandosol, rudosol and tenosol soils were able to completely degrade isopropanol in one month (Figure 42). In contrast to 2-butoxyethanol, some residual isopropanol was observed in one of the five soils (vertosol) tested after one month of incubation, though even in the vertosol the vast majority of the 1000mg of isopropanol had degraded after the incubation. Many microbes will tolerate significantly high concentrations of isopropanol, for example, *Sphingobacterium mizutae* was able to tolerate isopropanol concentrations ~30X higher and retain growth and the ability to degrade this substrate (Mohammad et al., 2006). A major intermediate of isopropanol degradation is the ketone acetone. Acetone is in turn rapidly degraded, for instance, by a strain of *Paracoccus denitrificans*, which was able to degrade isopropanol to acetone and then subsequently degrade all the acetone in 216 and 240 hours, respectively (Geng et al., 2015). Fungal catabolism of isopropanol is less clear and may proceed via alternative pathways. Regardless, it is clear that isopropanol is readily degraded, mostly to undetectable levels in less than a month in the soils of the Beetaloo region.

In comparison to soils, degradation of isopropanol within aquifers was more modest. Under anoxic conditions after three month incubations ~50% of the isopropanol had been degraded in aquifer samples (Figure 45). Anoxic isopropanol degradation is poorly studied, there are, however, reports of its degradation under anoxic conditions. For example, a study by Vermorel and colleagues (2017) demonstrated that a batch reactor was able to readily consume quantities of isopropanol two orders of magnitude higher than described here (Vermorel et al., 2017). These observations and data presented here indicate microbial degradation of these compounds does occur, though the mechanisms and taxa responsible largely remain to be identified.

The pattern for methanol mirrors that in isopropanol, almost complete degradation in all soils (except kandosol) in a time period under one month and the partial degradation (~50% loss) in three months for all aquifer samples (Figure 43 and Figure 46). As is often the case, the microbiology of methane production is better understood in oxic compared to anoxic settings. In oxic soils, a range of facultative- and obligate-methanol utilising bacteria are known to occur (Kolb, 2009). For the most part, the facultative prokaryotic methylotrophs can also use other multicarbon compounds, while the obligate prokaryotic taxa (such as methanotrophs) can only use single carbon compounds like methanol or methane. Furthermore, some yeasts can also use methanol (Ito et al., 2007; Lee and Komagata, 1980).

In anoxic settings the situation is not as well studied, though numerous lineages are known to use methanol under these settings. Most obvious amongst these taxa are the methylotrophic methanogens which make methane from compounds like methanol and methylated sulfur compounds. In addition, *Moorella* and *Thermotoga* species have both been shown to use methanol as an electron donor with a range of electron acceptors (Balk et al., 2003, 2002), the former of which has relatives that occur in the aquifer systems of the Beetaloo region.

Glycols

Of the five soils test, only the kandosol and vertosol failed to degrade all of the ethylene glycol added in the one month time course of the experiment (Figure 47). Of the initial dose of ~1400mg of ethylene glycol only 200mg was detectable in the kandosol and less than 100mg was detected in the vertosol, indicating that even in these two treatments most of the ethylene glycol was degraded. In contrast to soils, relatively little ethylene glycol was degraded during the three month time course in the aquifer experiment (Figure 49). Bacterial degradation of ethylene glycol (regardless of aerobic or anaerobic metabolism) results in the production of ethanol and acetic acid (or acetate, pH depending), both of these components are readily used by a vast number of microbial lineages (Keller et al., 2019; Mrklas et al., 2004; Salaspuro, 1997). In oxic settings the former is generally converted to acetate via acetaldehyde, while the latter can be directly used as a carbon source by many microbes. That microbes can access ethylene glycol anaerobically but did not do so in the aquifer microcosm experiments is likely a reflection of the low nutrient status of the aquifer waters. That is, without an adequate source of nitrogen and phosphorus catabolism can be significantly retarded. Evidence of the rapid degradation under better nutrient conditions can be found in many studies, for example, Carnegie and Ramsey, demonstrated very rapid degradation of this compound in willow (*Salix nigra*) and poplar (*Populus balsamifera*) rhizosphere soils under anoxic, nitrate-reducing conditions (Carnegie and Ramsay, 2009).

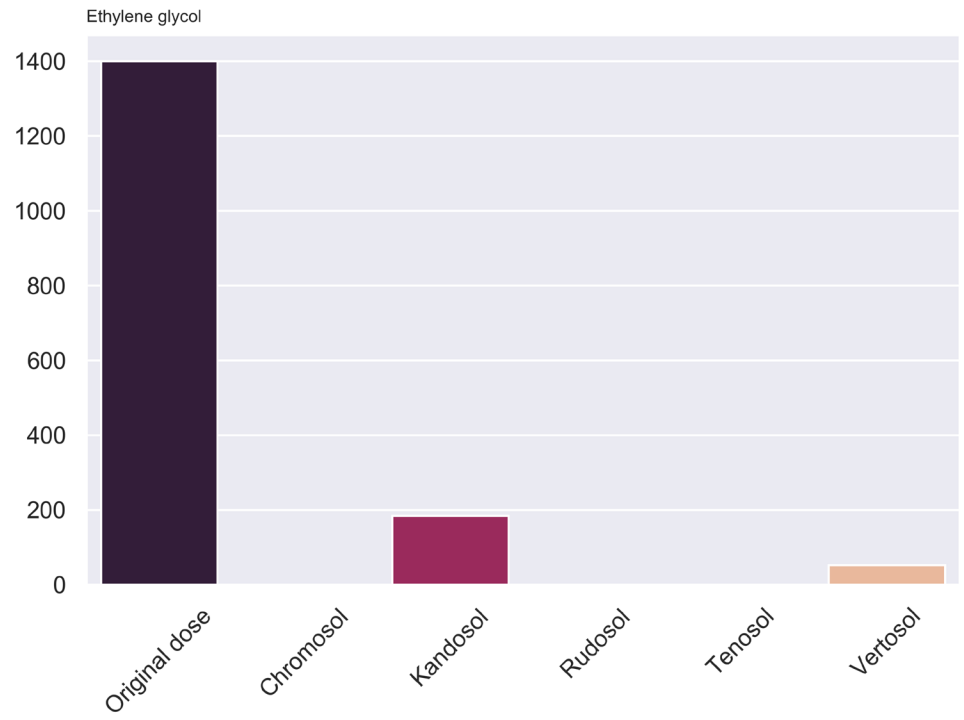

Figure 47: Ethylene glycol biodegradation in Northern Territory soil types

Ethylene glycol concentration in mg L^{-1}

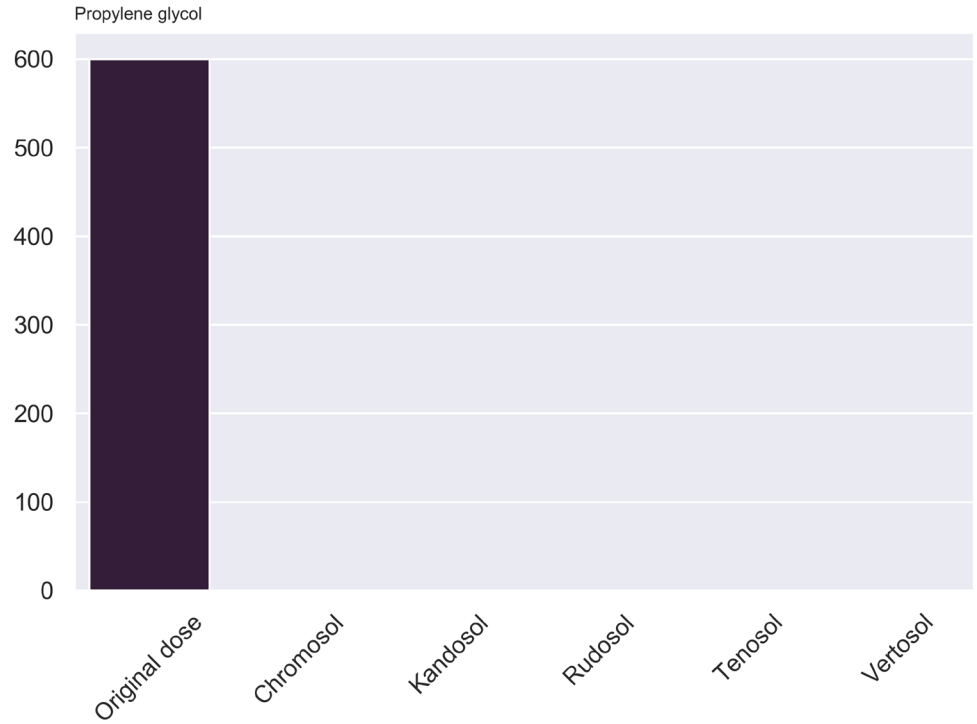

Figure 48: Propylene glycol biodegradation in Northern Territory soil types

Propylene glycol concentration in mg L^{-1}

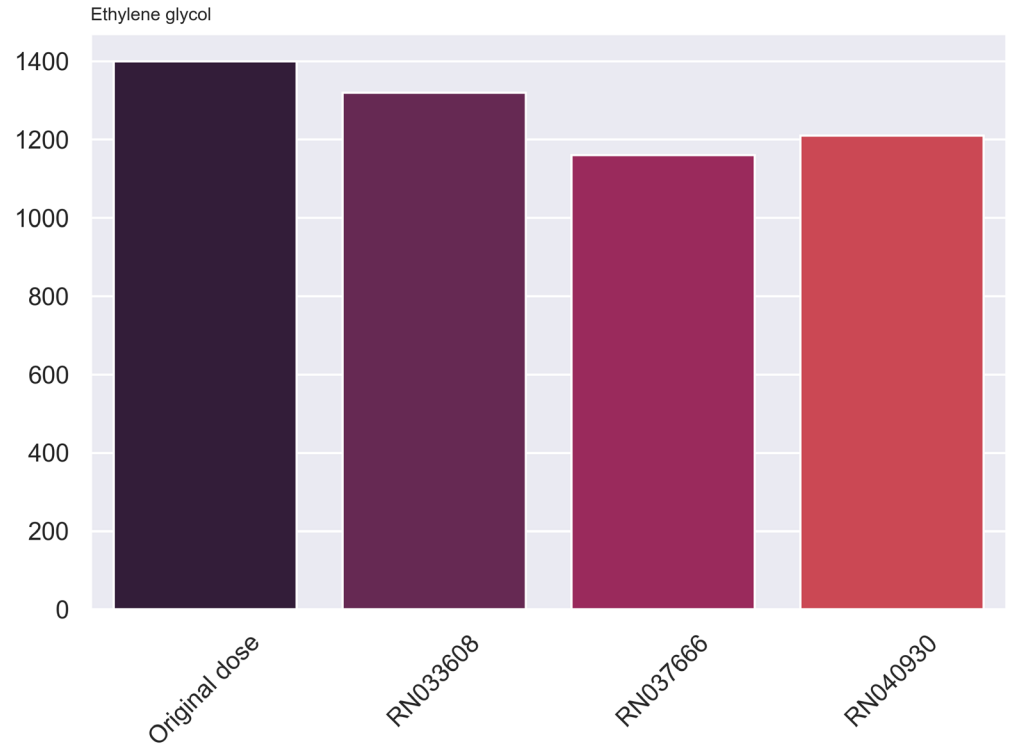

Figure 49: Ethylene glycol biodegradation in Northern Territory aquifers

Ethylene glycol concentration in mg L^{-1}

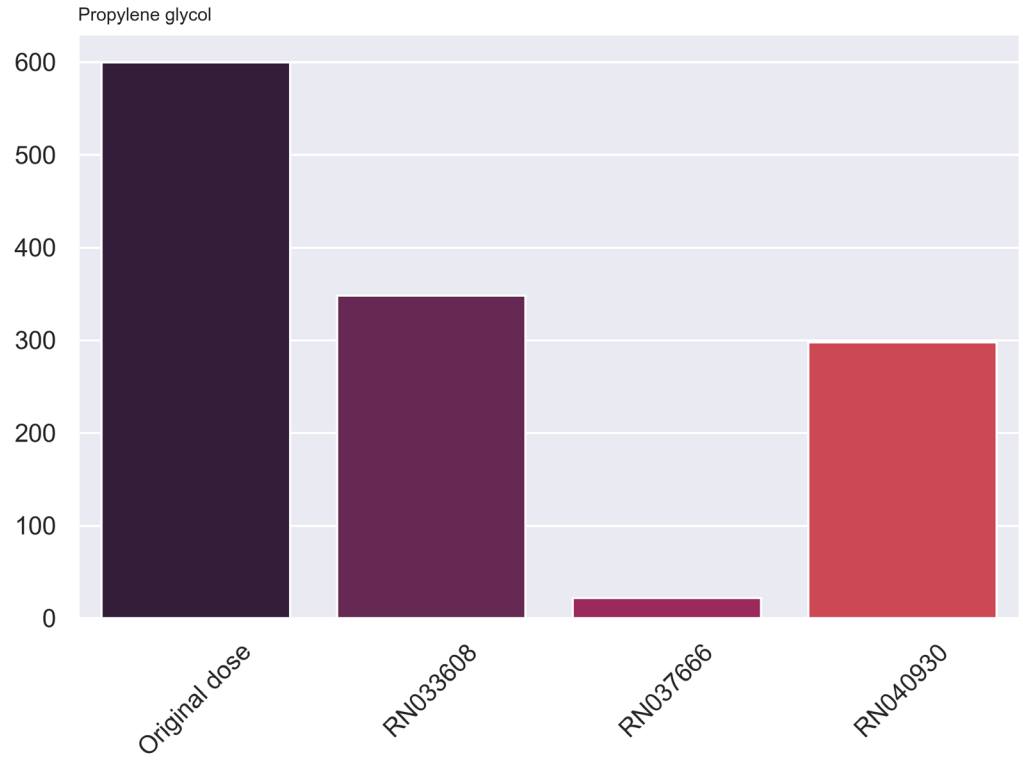

Figure 50: Propylene glycol biodegradation in Northern Territory aquifers

Propylene glycol concentration in mg L^{-1}

Interestingly, despite the chemical similarity between ethylene and propylene glycol, the latter compound is much more easily degraded by microbes of the Beetaloo region in both soil and aquifer environments (Figure 48 and Figure 50). Indeed, no soils had any detectable propylene glycol after one month, and ~50% of the propylene glycol was degraded in the aquifer microcosms RN033608 and RN049030 after a three month time course. In contrast, in RN037666 almost of all of the propylene glycol had degraded in the same time period. In a parallel to ethylene glycol degradation, degradation produces the carboxylic acid and a corresponding primary alcohol. For propylene glycol, degradation produces propionate (propionic acid at lower pH) and n-propanol (Veltman et al., 1998).

It is evident from the data that degradation of alcohols and glycols in soils are considerably more rapid than in aquifer settings. This may have less to do with the oxygen status of these environments and instead may be related to the lower cell number in aquifers, or the nutrient status of these environments. For the latter, it should be noted here, that no nutrients except the chemical itself were added to soils or aquifers, modelling the ingress of the sole chemical into the soil or aquifer environment. It is very likely, however, that addition of small amounts of either nitrate or phosphate would result in relatively rapid consumption of these compounds in the aquifer as numerous taxa occur in these water that are genetically capable of their degradation. Regardless, it is important to note that aquifer environments have slower catabolic processes than the soils. Indeed, for soils, many reports in the literature suggest catabolism rates for chemicals tested here that are much more rapid than one month. In this experiment, it is not possible to say whether degradation occurred in some shorter timeframe as no intermediate time points were assayed.

3.4 Effects of chemicals on the microbial community Northern Territory soils

Prior to a discussion of the effects of chemicals on microbial communities found in Northern Territory soils, it is important to understand the two controls used in these experiments. The first, called the storage control, is soil that was subject to the addition of the same amount of water as in all the treatments, however, no chemicals were added to the water. The storage control was then incubated as per all other samples in this experiment and harvested at the same time, under the same conditions. The storage control provides an indication of how soil microbial communities change as a result of moistening and incubation for approximately 34 days, without the addition of any chemicals. In contrast, the zero time control is simply soil that during the establishment of this experiment was moistened, no chemicals added, and then immediately frozen to preserve the soil as it was at "time zero" in the experiment (prior to incubation). These zero time controls were defrosted at the completion of the experiment and processed in the same fashion as all other experiments. The zero time control provides an indication of the microbial community present in the soils, at the start of the experiment.

Furthermore, it should be noted that due to technical/experimental issues towards the conclusion of the soil microcosm experiments, that were beyond the control of the research team, several chemical treatments from the tenosol experiments (2-butoxyethanol; d-limonene; diesel fuel; eicosane; ethylene glycol; isopropanol; methanol; naphthalene; o-cresol; polyacrylamide; and propylene glycol) and all the rudosol chemical treatment experiments failed, and will not be reported.

A key result of this experiment is that moistening the soil causes a marked shift in the microbiomes in these soils. This is unsurprising given the climatic conditions from which the samples come. In the semi-arid region around Daly Waters, rainfall is limited to the summer months and only very meagre rainfall occurs May through September. The microbiome thus responds fairly dramatically to this change. It is also worth noting that these soils are fairly depleted in organic carbon (Table 13), so the addition of organic carbon (in the form of the chemicals of interest) further shifts the microbiome through growth of organisms capable of its degradation and the deaths of sensitive microbes.

3.4.1 Response of soil prokaryotic microbiomes to chemicals

For prokaryotes, most of the chemicals tested caused observable, predictable and statistically significant shifts ($p < 0.05$) in the microbiome (Table 28). All chemicals caused significant changes in at least one soil. Some chemicals, like diethylene glycol ethyl ether or methylchloroisothiazolinone did not cause any significant change in two of the four soils (chromosol and vertosol, and chromosol and tenosol, respectively). Further, the chromosol microbiome was unaffected by the addition of methylisothiazolinone, naphthalene, 2-methylphenol, polyacrylamide or polyoxypropylene diamine and several other chemicals (C12 ethoxylated alcohol, d-limonene, hydrotreated light petroleum distillate, methanol and triethanolamine) were close to the p-value above which results were not significant. Conversely, this same soil microbiome was markedly altered ($p<0.001$) by the addition of 2-aminoethanol, 2-butoxyethanol, diesel, ethylene glycol, glutaraldehyde, glyoxal, propylene glycol and pristane indicating that specific chemicals can cause significant changes in a soil microbiome that is unaltered by the addition of other chemicals (Table 28). The chromosol soil represents something of an outlier as the remainder of the microbiomes of the bulk soils of the region were significantly altered by the addition of chemicals.

In addition to changes caused by the addition of chemicals, one other important phenomenon can be observed. Some chemicals appear to cause a specific change in the microbial community that precludes the normal shift during a wetting event. This effect was particularly pronounced for biocides, notably bronopol (which caused this phenomenon in all but one soil), but also the biocides benzisothizolinone and hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine. All of these chemicals are used as biocides but some may have a bacteriostatic mode of action at the community level and this may be the cause of this phenomenon. Interestingly, other biocides, e.g. glutaraldehyde, methylchloro- and methylisothiazolinone did not cause the same effect, possibly due to the known bacteriocidal effects of these particular biocides (Fraise et al., 2012; Morley et al., 2005; Silva et al., 2020).

It is importantly to note, however, that aside from the biocide-specific effects described above, the reasons underpinning these other changes are less clear from these data.

Table 28: PERMANOVA results for prokaryotic microbiome changes relative to the storage control in soils

Chemical	Chromosol	Kandosol	Tenosol	Vertosol
2-aminoethanol	***	**	***	**
C12 ethoxylated alcohol	*	**	***	**
2-butoxyethanol	***	**	-	***
bronopol	***†	**†	**	***†
benzisothiazolinone	**†	**	*	***
diethylene glycol ethyl ether	ND	**	*	ND
d-limonene	*	*	-	*
diesel	***	**	-	***
eicosane (linear-C20)	**	ND	**	**
ethylene glycol	***	**	-	**
2-ethylhexanol	**	**	-	**
glutaraldehyde	***	**	**	***
glyoxal	***	**	***	***
hydrotreated light petroleum distillate	*	**	*	*
hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine	***†	**	**	**
isopropanol	*	*	-	***
methylchloroisothiazolinone	ND	**	ND	**
methylisothiazolinone	ND	**	**	**
methanol	*	**	-	**
naphthalene	ND	**	-	**
2-methylphenol (o-cresol)	ND	**	-	***
polyacrylamide	ND	*	-	***
polyoxypropylene diamine	ND	**	-	**
propylene glycol	***	**	-	**
pristane (C15)	***	*	***	***
triethanolamine	*	**	**	***

ND = no significant difference, * 0.05 – 0.01, ** 0.009-0.001, *** < 0.001. Daggers (†) indicate those samples that while significantly different to the storage control, were highly correlated (r >= 0.9) to the zero time control

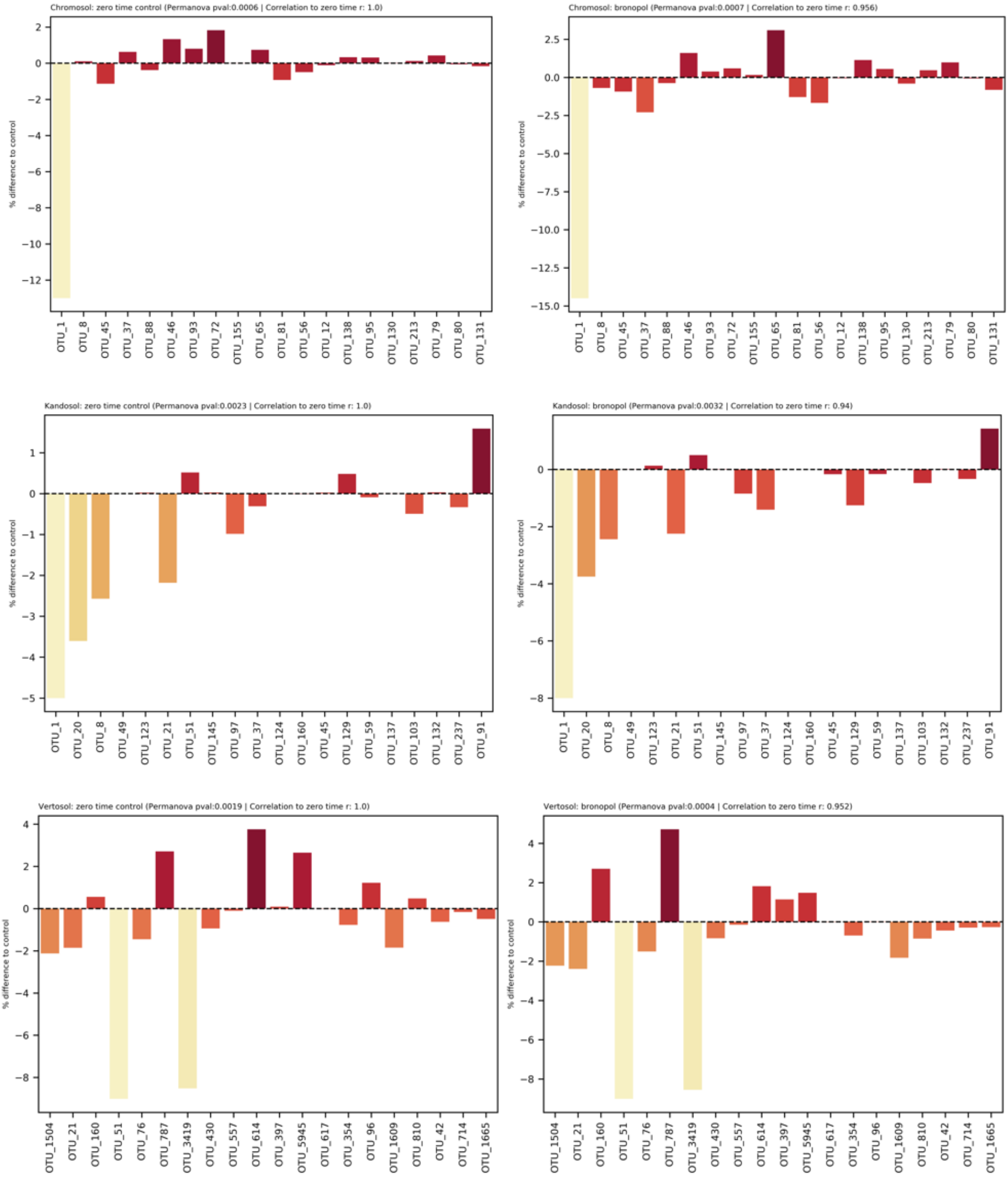

Figure 51: The abundance of the twenty most changed prokaryotic taxa (relative to the storage control) for the zero time control (left) and bronopol (right) highlighting the correlation between bronopol and the zero time control.

Top row: Chromosol, Middle row: Kandosol, Bottom row: Vertosol

Some insight into the drivers of the observed differences in soil microbiome changes can be seen from the SIMPER analyses which showed the dissimilarity between treatments relative to the storage control. Overall, these data indicate that significant changes are mostly associated with the growth of a small number of taxa though some sensitive taxa can also be observed (Figure 52 - Figure 55). Exploring the chromosol data as an example, it is evident that OTU_1 responded negatively (i.e. in decreases in relative abundance) to a range of chemical additions. For instance, its relative abundance decreased when glyoxal or ethylene glycol was added to the soil. This same taxon, however, declined in relative abundance simply with storage (note its negative change in the zero time control). In contrast, in the chromosol soil, OTU_93 responds negatively to a range of additions but did not decline on storage. OTU_93 is an example of a taxon that appears to be impacted widely, in contrast, OTU_37 appears to decrease in relative abundance in response to the addition of only a select few chemicals (for example glyoxal, 2-ethylhexanol and aminoethanol).

Conversely, OTU_1 (*Neobacillus batievensis*) responded very positively to the addition of chemicals such as ethylhexanol , glutaraldehyde or naphthalene and may be able to degrade these chemicals as a source of carbon. Indeed, in the tenosol (and to a lesser extent in the kandosol) (Figure 54 and Figure 53, respectively) this same OTU increased very significantly in relative abundance when ethylhexanol was added to the soil microcosms, providing more evidence that OTU_1 was involved in the degradation of ethylhexanol. Similarly, OTU_8, *Oxalophagus oxalicus*, increased in relative abundance on the addition of propylene glycol. This taxon is known to degrade oxylates, and other clostridial species can ferment ethylene glycol (Gaston and Stadtman, 1963) so this seems a probable candidate for the degradation of this chemical in these soils. Similarly, OTU_155, a taxon related to *Micromonospora marina*, increased markedly when glyoxal was added to the chromosol. *Micromonospora* species have genes with affinities to glyoxal oxidases (Gaskell et al., 1995), and its growth here may indicate this taxon is able to access carbon in this aldehyde. Other putative catabolists were also identified including OTU_88, an *Actinomadura* species that increased in abundance when the terpenoid alkane pristane was added to soil or OTU_65 (an unknown Acidobacteria) which, like OTU_155, increased when glyoxal was present.

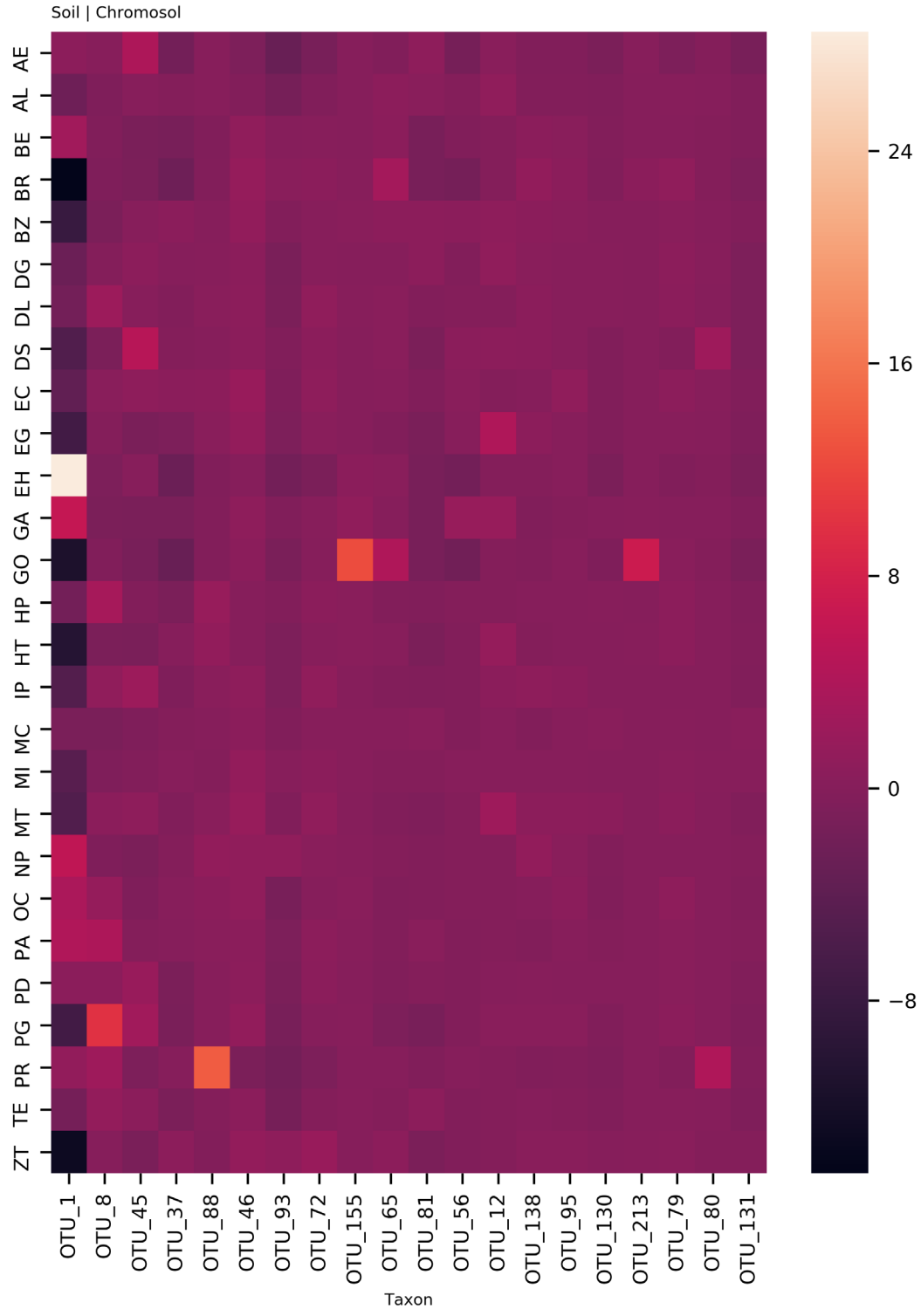

Figure 52: SIMPER analysis of the top 20 prokaryotic taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in Northern Territory chromosol soil experiments

AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BE: 2-butoxyethanol; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; DL: d-limonene; DS: diesel fuel; EC: eicosane; EG: ethylene glycol; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; IP: isopropanol; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; MT: methanol; NP: naphthalene; OC: o-cresol; PA: polyacrylamide; PD: polyoxypropylene diamine; PG: propylene glycol; PR: pristane; TE: triethanolamine; ZT: zero time control

Scale bar indicates percentage change relative to the storage control

In broad terms, similar patterns were evident in the kandosol (Figure 53), with a few taxa being sensitive to the addition of the vast majority of the chemicals (for example, OTU_37 and OTU_129), and a number of taxa showing positive responses to particular chemicals. One clear example of the latter is OTU_49 which increased markedly when methanol was added to the microcosm experiments. OTU_49 is a strain of *Novimethylophilus kurashikiensis* that has been previously shown to grow well on methanol as a sole source of carbon (Lv et al., 2018), and its response here is presumably a result of this taxon degrading this chemical. Intriguingly, OTU_8 increased in the kandosol when hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine, a biocide, was added to this treatment. This same OTU from the chromosol did not increase when this compound was added. It may be that OTU_8 contains multiple, closely-related species with different resistances (or catabolic potentials) for degrading this complex biocide. Intriguingly, two taxa increased in abundance when 2-aminoethanol was supplied to the soil OTUs 123 and 51. The former is a relative of *Brevibacillus fluminis*, a taxon known to degrade complex polyaromatic compounds (Choi et al., 2010), while the latter is another *Brevibacillus* species, *B. agri*. Taken together, it suggests that *Brevibacillus* species may be involved in the degradation of 2-aminoethanol in this soil.

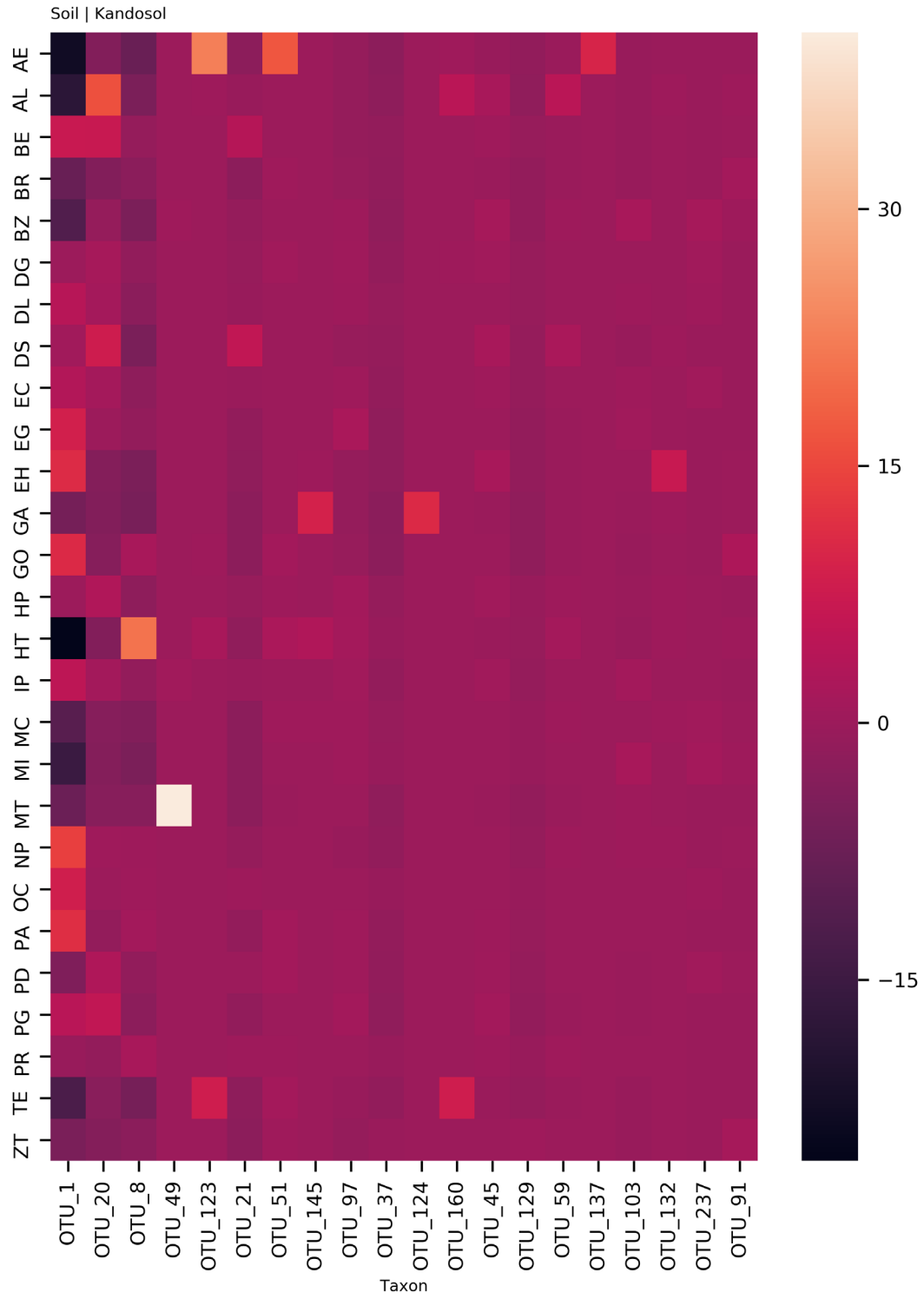

Figure 53: SIMPER analysis of the top 20 prokaryotic taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in Northern Territory kandosol soil experiments

AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BE: 2-butoxyethanol; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; DL: d-limonene; DS: diesel fuel; EC: eicosane; EG: ethylene glycol; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; IP: isopropanol; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; MT: methanol; NP: naphthalene; OC: o-cresol; PA: polyacrylamide; PD: polyoxypropylene diamine; PG: propylene glycol; PR: pristane; TE: triethanolamine; ZT: zero time control

Scale bar indicates percentage change relative to the storage control

The tenosol data were similar to those from the chromosol and kandosol, although no universally sensitive taxa were detected (Figure 54). The *Oxalophagus* species (OTU_8) that may be involved in the degradation of hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine in the kandosol, was also detected here at elevated levels when hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine was added to soils. Similarly, both *Brevibacillus* species OTU_51 and OTU_123 that increased in abundance when 2-aminoethanol was added to the kandosol were also observed to increase when this chemical was added to this soils, further evidence that they are likely involved in the degradation of this chemical. Furthermore, two other taxa (OTUs 142 and 349), *Flavisobacter* and *Sphingomonas* species, respectively, were also elevated on 2-aminoethanol. The latter of these genera has been demonstrated to grow on 2-aminoethanol (Lee and Jeon, 2017), suggesting that it may also be involved in its degradation.

Interestingly the vertosol data are somewhat different (Figure 55). Numerous taxa appear to be intolerant of the experimental conditions (decline in all treatments compared to the storage control) though these are not sensitive to particular chemicals it is more of a global sensitivity to the experimental conditions used.

Further taxa that increase in relative abundance were observed, these included a taxon (OTU_1504) conspecific with *Clostridium vulturis* on methanol, glutaraldehyde and benzisothiazolinone, OTU_21, a poorly resolved *Acidobacteria*, which increased in abundance on triethanolamine, naphthalene and ethylene glycol and OTU_160 (*Azospirillum largimobile*) which increased in relative abundance on ethylhexanol. Presumably their growth on these chemical additions is evidence of their catabolism of these substrates, however, few data exist on these taxa and their roles here remain subject to further confirmation. OTU_430 which followed a similar pattern on diesel and ethyoxylated alcohols is a probable *Blastococcus* species which increased in relative abundance on ethoxylated alcohols, again the lack of knowledge about this taxon makes extrapolation of its role in the treatments difficult, on balance, as above it seems likely that this taxon is involved in catabolism of ethoxylated alcohols in this soil. Interestingly, while *Brevibacillus* species were commonly observed in other soils in response to addition of 2-aminoethanol, here OTUs 397 and 617 become more relatively abundant in this treatment. These are both poorly resolved species from a taxonomic perspective with the former being an *Acidobacteria* species and

the latter being a member of a putatively novel bacterial phylum. Regardless, their increases here are suggestive of these taxa playing roles in biodegradation of this chemical.

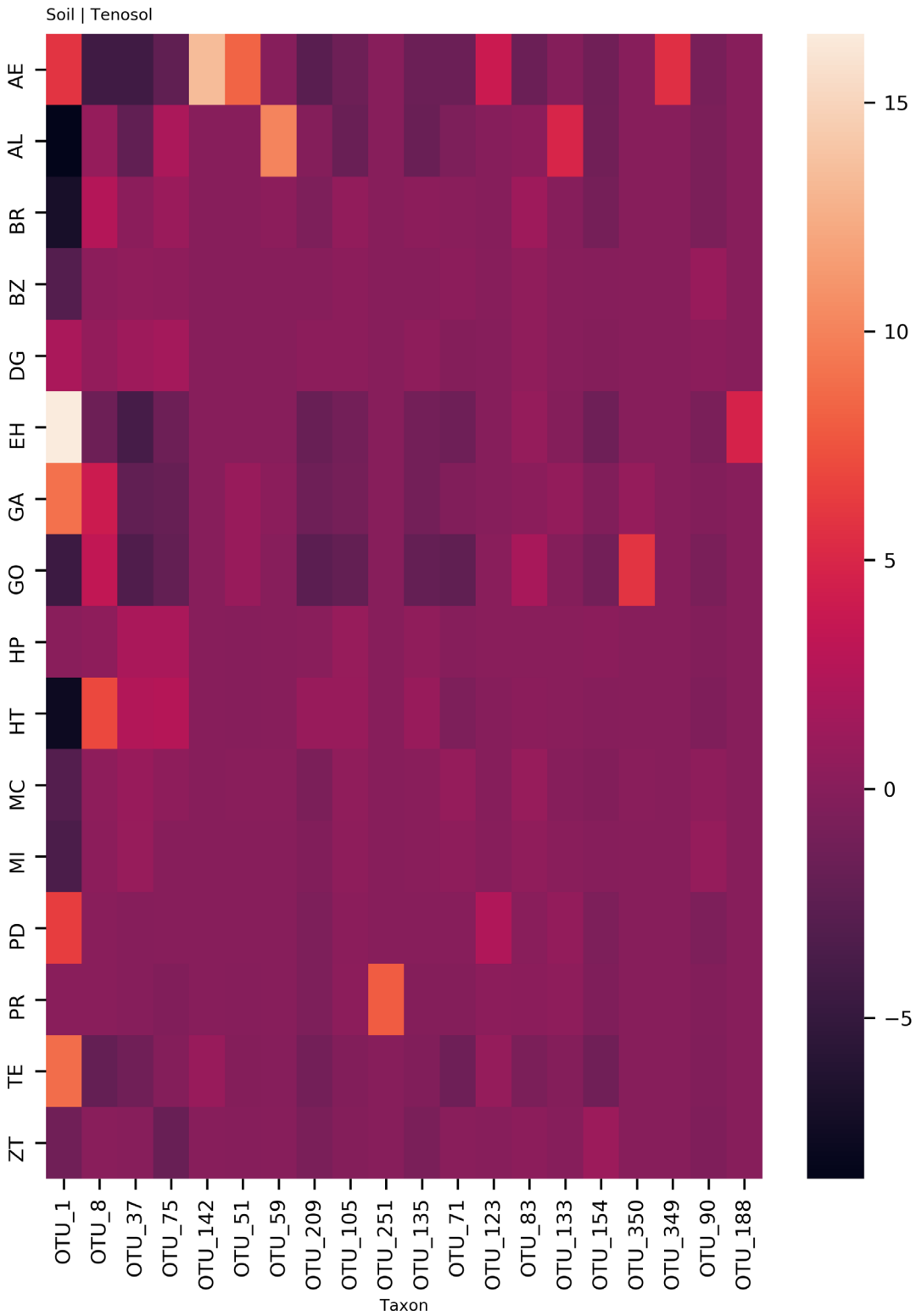

Figure 54: SIMPER analysis of the top 20 prokaryotic taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in Northern Territory tenosol soil experiments

AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; PD: polyoxypropylene diamine; PR: pristane; TE: triethanolamine; ZT: zero time control

Scale bar indicates percentage change relative to the storage control

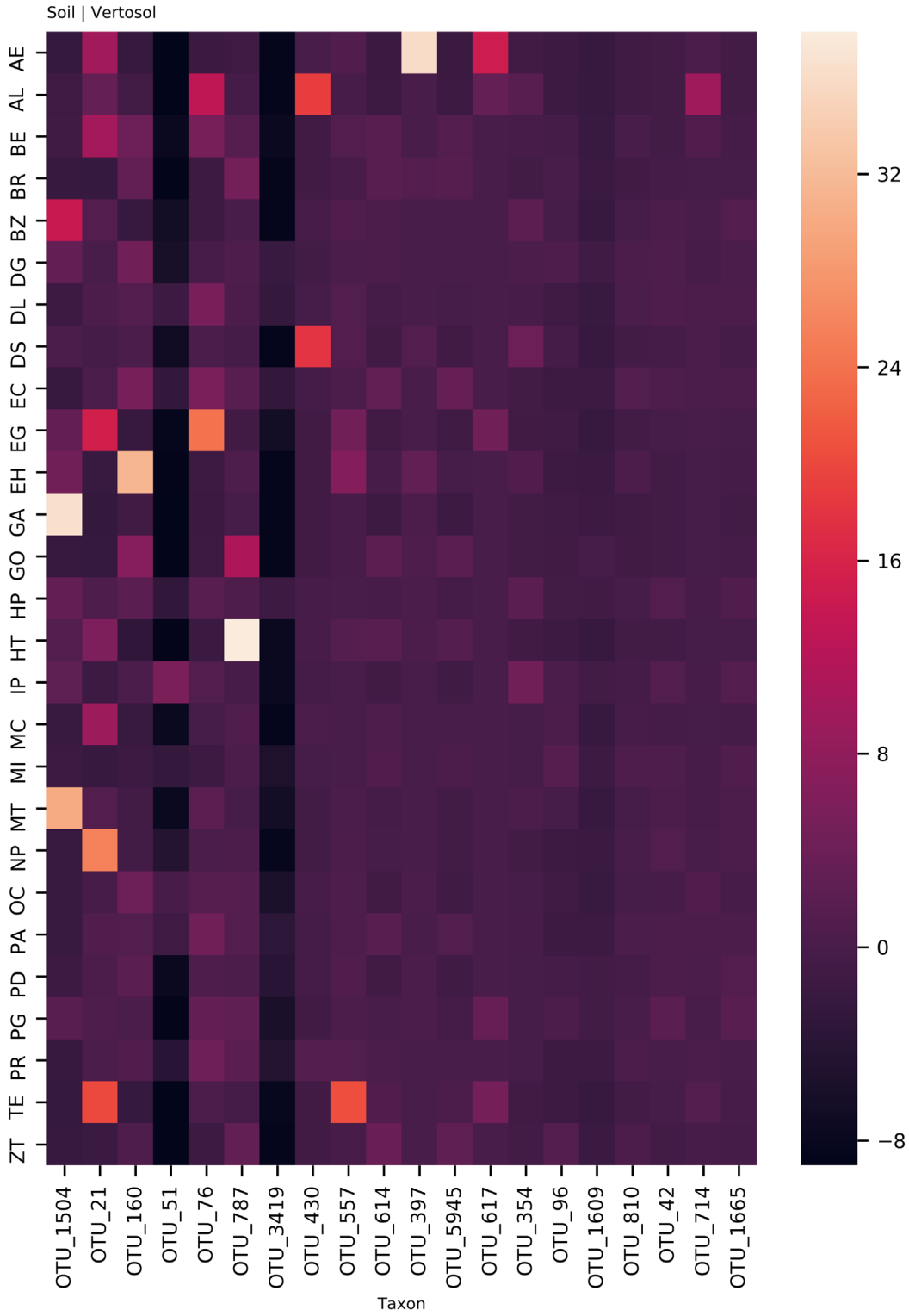

Figure 55: SIMPER analysis of the top 20 prokaryotic taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in Northern Territory vertosol soil experiments

AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BE: 2-butoxyethanol; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; DL: d-limonene; DS: diesel fuel; EC: eicosane; EG: ethylene glycol; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; IP: isopropanol; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; MT: methanol; NP: naphthalene; OC: o-cresol; PA: polyacrylamide; PD: polyoxypropylene diamine; PG: propylene glycol; PR: pristane; TE: triethanolamine; ZT: zero time control

Scale bar indicates percentage change relative to the storage control

3.4.2 Response of soil fungal microbiomes to chemicals

In comparison to the prokaryotic communities, in general fungal communities seem less perturbated by the addition of chemicals to the soil (Table 29). This effect did vary by soil type, for instance in kandosol soil and vertosol soil, 14 and 16 of the chemicals, respectively, had no effect greater than wetting the soil (difference to storage control $p>0.05$) (Table 29). Intriguingly, the chromosol fungal microbiomes were more frequently and significantly altered by the addition of chemicals (Table 29). Of the chemicals added, only isopropanol and methylchloroisothiazolinone did not alter the fungal microbiome in this soil. There were also very few treatments that correlated positively with the zero time control (Table 29), suggesting a fungistatic effect for these chemicals is rarer than that observed for prokaryotes.

Table 29: PERMANOVA results for fungal microbiome changes relative to the storage control in soils

Chemical	Chromosol	Kandosol	Tenosol	Vertosol
2-aminoethanol	**	ND	**	ND
C12 ethoxylated alcohol	***	**	*	ND
2-butoxyethanol	***	***	-	*†
bronopol	**	**	*	ND
benzisothiazolinone	**	**	*	**
diethylene glycol ethyl ether	*	ND	ND	ND
d-limonene	***	ND	-	ND
diesel	***	*	-	ND
eicosane (linear-C20)	***	ND	-	ND
ethylene glycol	**	ND	-	**
2-ethylhexanol	**	ND	*	*†
glutaraldehyde	***	***	**	**
glyoxal	**	**	**	**
hydrotreated light petroleum distillate	*	ND	ND	ND
hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine	***	***	ND	*
isopropanol	ND	ND	-	ND
methylchloroisothiazolinone	ND	*	*	ND
methylisothiazolinone	*	**	**	*
methanol	*	ND	-	ND
naphthalene	***	ND	-	ND
2-methylphenol (o-cresol)	*	ND	-	ND
polyacrylamide	*	ND	-	*
polyoxypropylene diamine	*	ND	***	ND
propylene glycol	***	ND	-	ND
pristane (C15)	**	**	ND	ND
triethanolamine	**	*	*†	**

ND = no significant difference, * 0.05 – 0.01, ** 0.009-0.001, *** < 0.001. Daggers (†) indicate those samples that while significantly different to the storage control, were highly correlated (*r* >= 0.9) to the zero time control

For the chromosol, no taxa were detected that reacted negatively to most chemicals (Figure 56). FOTU_32 and FOTU_162 appear to negatively respond to many chemicals, however, they were also significantly reduced in the zero time control, suggesting these taxa do not thrive during the storage/incubation phase of the experiment.

There were, however, numerous taxa that increased in abundance in response to certain chemicals. For example, FOTU_2 increased significantly in response to the biocides methylisothiazolinone, methylchloroisothiazolinone, hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine, bronopol and glutaraldehyde along with ethoxylated alcohols. This taxon increased in two soils (chromosol and vertosol) and while the two soils likely have different strains of this fungus (Figure 56 and Figure 59), it would seem likely that this taxon was involved in degradation of these chemicals. This taxon is poorly taxonomically resolved, its closest match is to LT611336, a taxon isolated from roots (Raghavendra et al., 2017) from a range of sites around Australia, including northern Australia. Similar patterns were observed for other taxa including FOTU_11 which increased significantly in abundance in kandosol and vertosol when glyoxal was added to the soil (Figure 57 and Figure 59). This taxon also has somewhat poor taxonomic resolution, but BLAST comparisons revealed it may be related to the genus *Paraconiothyrium* (see KP235791[16] for an example of a closely matching accession). This genus has been shown to have members that have genes for degrading complex, recalcitrant organic matter (for example, see Zeiner et al., 2021), and their presence here in two soils, increasing in relative abundance on the same substrate likely indicate catabolism of this chemical. Other taxa that increased when certain chemicals were supplied included a strain of *Westerdykella ornata* (FOTU_1) which increased when either butoxyethanol or 2-ethylhexanol was present in both the chromosol and vertosol. Its presence on just these substrates, in multiple soils, likely indicates the taxon is able to catabolise these compounds. Interestingly, FOTU_5 was observed to increase when various isothiazolinone biocides (methylisothiazolinone and benzisothiazolinone) were added. For methylisothiazolinone it has been established that fungi from two different classes, Eurotiomycetes and the Sordariomycetes, are able to grow in very high concentrations of this biocide (up to 40g L^{-1}; Gomes et al., 2018). Fungal degradation of methylisothiazolinone has been documented from a range of ascomycetes including *Trichoderma, Fusarium* and

[16] https://www.ncbi.nlm.nih.gov/nuccore/KP235791

Aspergillus species. That this taxon, a putative *Curvularia* (teleomorph *Cochliobolus*) species, on two different soils (kandosol and vertosol, see Figure 57 and Figure 59) increased in relative abundance on these selected substrates, and the widespread nature of degradative pathways for degrading this compound likely indicates this taxon is able to degrade these substrates in the natural environment. One further taxon that was detected on the same substrate at multiple sites was FOTU_42. This taxon was a poorly resolved zygomycete, with some affinities to the Mortierellales, it was comparatively rare in the soil baselines and in the controls but increased markedly in abundance when eicosane is added to the soils. This taxon was observed, increasing in relative abundance in the eicosane treatments of the chromosol and kandosol soils (Figure 56 and Figure 57). Fungi from the genus *Mortierella* have been previously implicated as increasing in abundance in petroleum contaminated soils (Galitskaya et al., 2021) and their presence here likely indicates this taxon degrades aliphatic and aromatic hydrocarbons.

Some additional FOTUs from individual soils also warrant discussion. FOTU_7 from the chromosol soil increased its abundance markedly when propylene glycol was supplied to the soil (Figure 56). This taxon, however, also increased substantially on pristane, glyoxal, diesel and in the bronopol treatments (Figure 56), suggesting it has broad catabolic capabilities for hydrocarbons (pristane and diesel), aldehydes and xenobiotics like bronopol. This taxon is poorly taxonomically resolved, but BLAST comparison reveals its ITS region is ~97% identical to a strain of *Torula deospora* isolate in Mozambique in 1956 (Vu et al., 2019). At 97% identity, FOTU_7 is presumably a *Torula* species, though further work would be required to identify this taxon further. One similarly taxonomically vague OTU was FOTU_68 from the kandosol soil (Figure 57) which increased markedly in response to the addition of pristane. This taxon was most closely related to LT610256[17], a sequence obtained from a fungus isolated from plant roots in Australia and, interestingly, was part of the same study as the taxon most closely related to FOTU_2 (LT611336;Raghavendra et al., 2017). That these two OTUs were identified in the work of Raghavendra and co-workers and match comparatively few other sequenced taxa may suggest both these taxa are endemic to Australia.

[17] https://www.ncbi.nlm.nih.gov/nuccore/LT610256

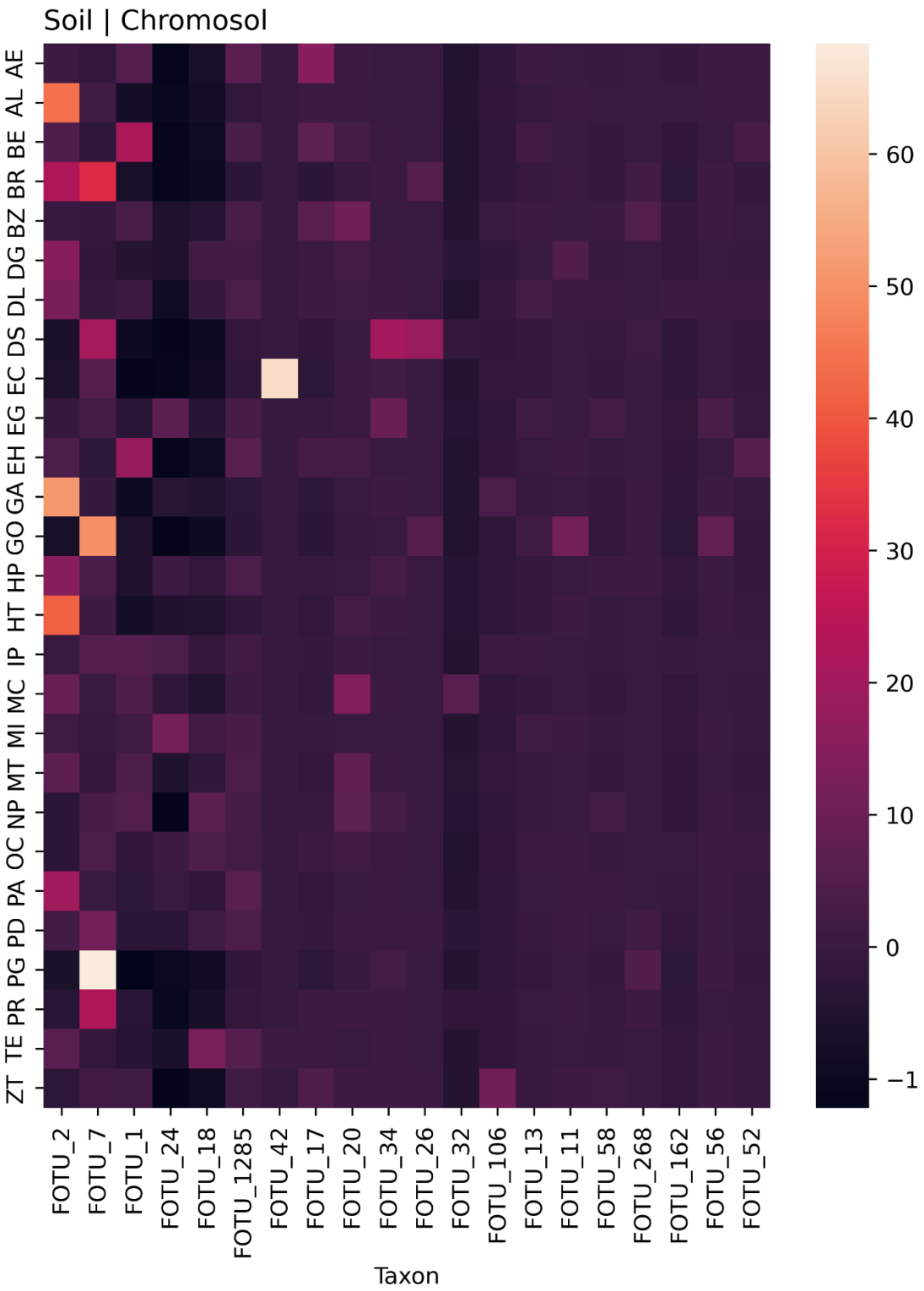

Figure 56: SIMPER analysis of the top 20 fungal taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in Northern Territory chromosol soil experiments

AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BE: 2-butoxyethanol; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; DL: d-limonene; DS: diesel fuel; EC: eicosane; EG: ethylene glycol; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; IP: isopropanol; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; MT: methanol; NP: naphthalene; OC: o-cresol; PA: polyacrylamide; PD: polyoxypropylene diamine; PG: propylene glycol; PR: pristane; TE: triethanolamine; ZT: zero time control

Scale bar indicates percentage change relative to the storage control

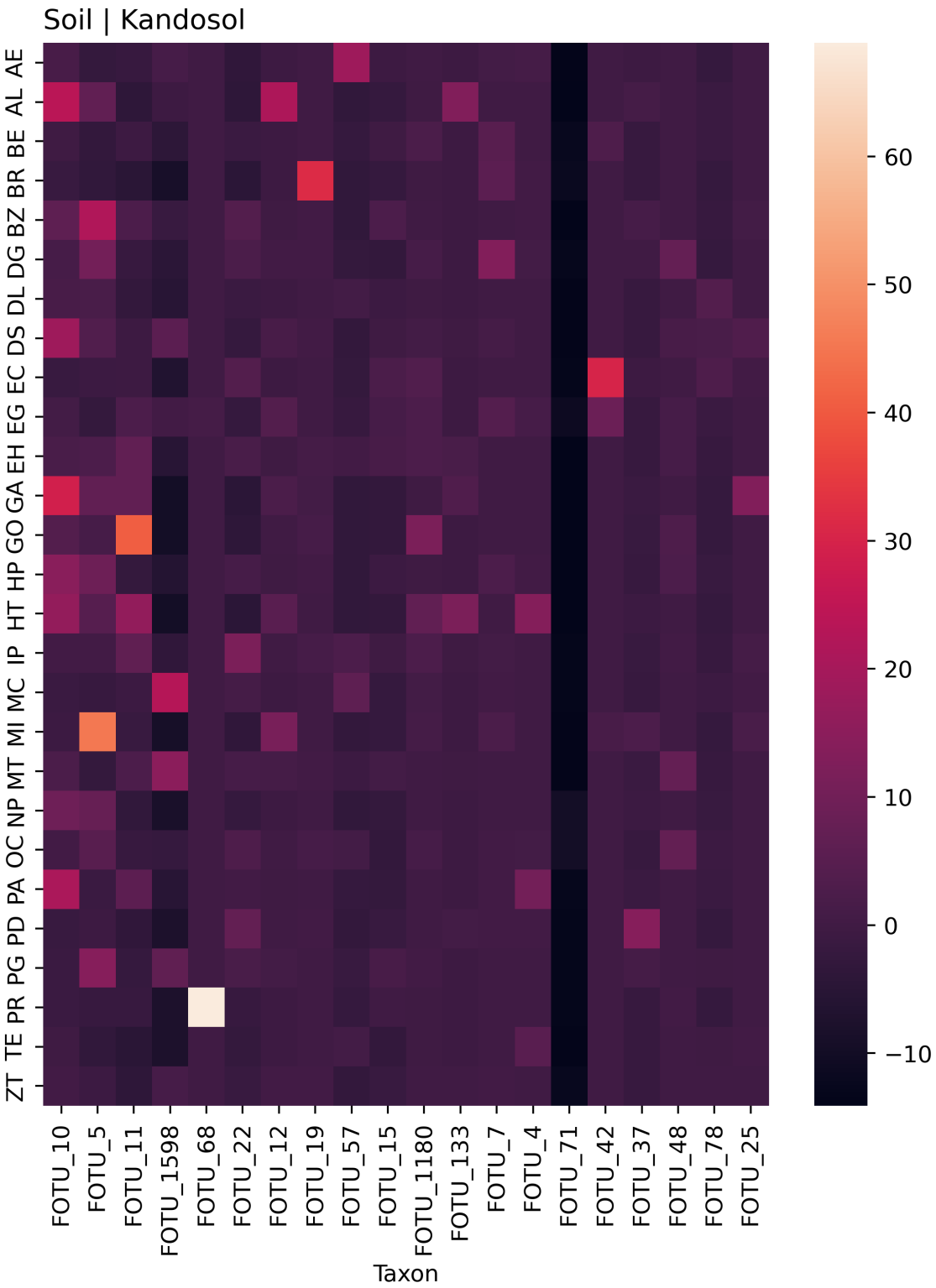

Figure 57: SIMPER analysis of the top 20 fungal taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in Northern Territory kandosol soil experiments

AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BE: 2-butoxyethanol; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; DL: d-limonene; DS: diesel fuel; EC: eicosane; EG: ethylene glycol; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; IP: isopropanol; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; MT: methanol; NP: naphthalene; OC: o-cresol; PA: polyacrylamide; PD: polyoxypropylene diamine; PG: propylene glycol; PR: pristane; TE: triethanolamine; ZT: zero time control

Scale bar indicates percentage change relative to the storage control

In the tenosol, one fungal OTU (FOTU_38) appeared to increase significantly in abundance in response to bronopol (Figure 58). This taxon is very poorly taxonomically characterised, with all matches <85% ITS identity by BLAST comparison, so inferences about its relatives are absent. Nevertheless, it is noteworthy that bronopol is fairly rapidly hydrolysed in soils to form a range of degradation products including: 2-bromoethanol, 2-bromo-2-nitroethanol, bromonitromethane, formaldehyde, nitromethane and tri(hydroxymethyl)nitromethane, each with their toxicity profiles (Cui et al., 2011). In soils, fungi also act on bronopol, for example, two species of a common soil genus *Fusarium* (*F. solani* and *F. oxysporum*) were able tolerate high concentrations of bronopol (1250 mg L^{-1}) and used bronopol as a sole source of nitrogen (Thomas and Moss, 1990), after which fungi grew with much faster rates, suggesting fungi can remove the nitro group from the compound and whatever breakdown products persist resulted in a loss of biocidal activity. For comparison, this study examined relatively low concentrations of bronopol (~70 mg L^{-1}). Further work on these breakdown products would clarify the fate of these compounds in soils of the Northern Territory.

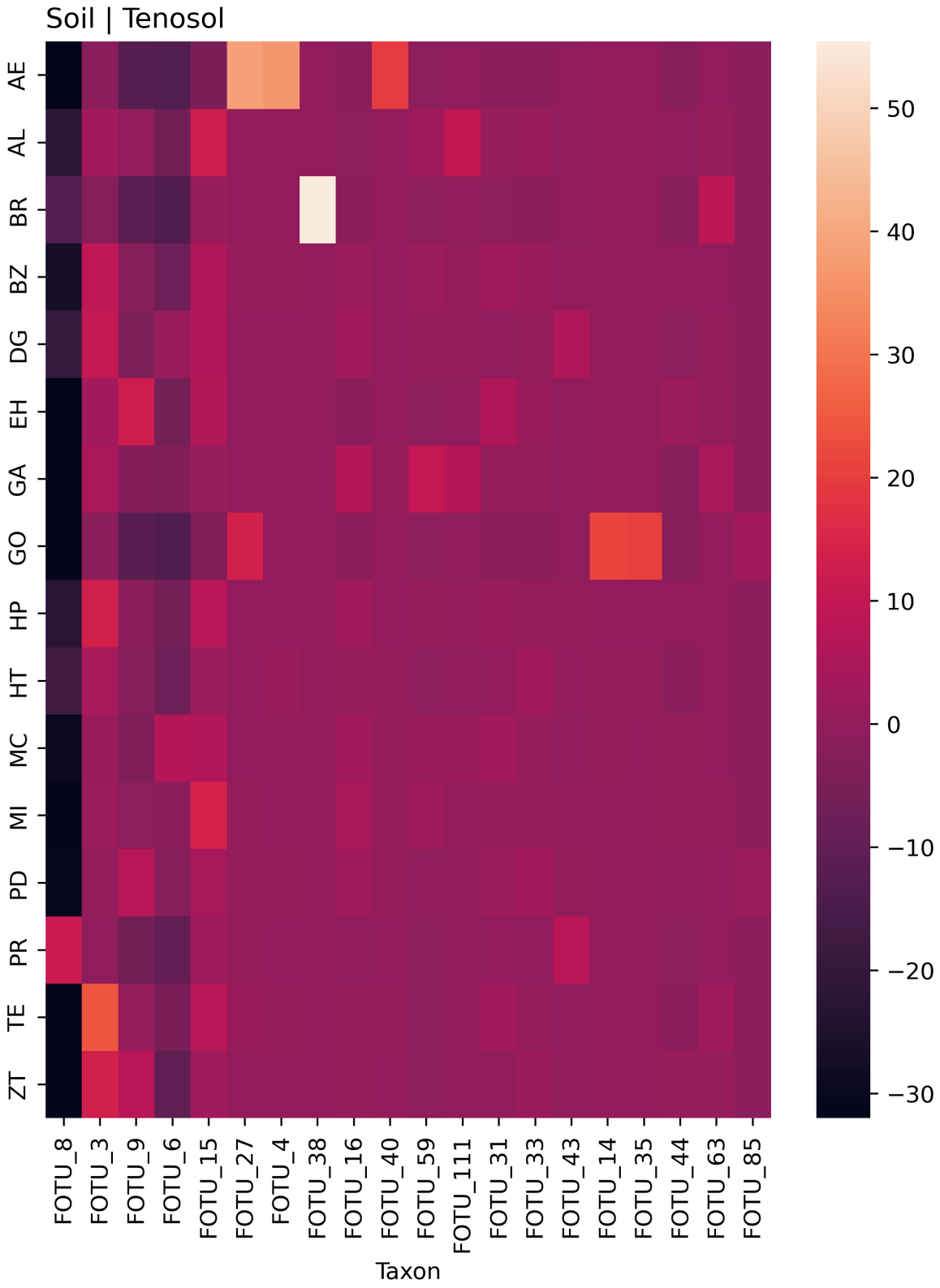

Figure 58: SIMPER analysis of the top 20 fungal taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in Northern Territory tenosol soil experiments

AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; PD: polyoxypropylene diamine; PR: pristane; TE: triethanolamine; ZT: zero time control

Scale bar indicates percentage change relative to the storage control

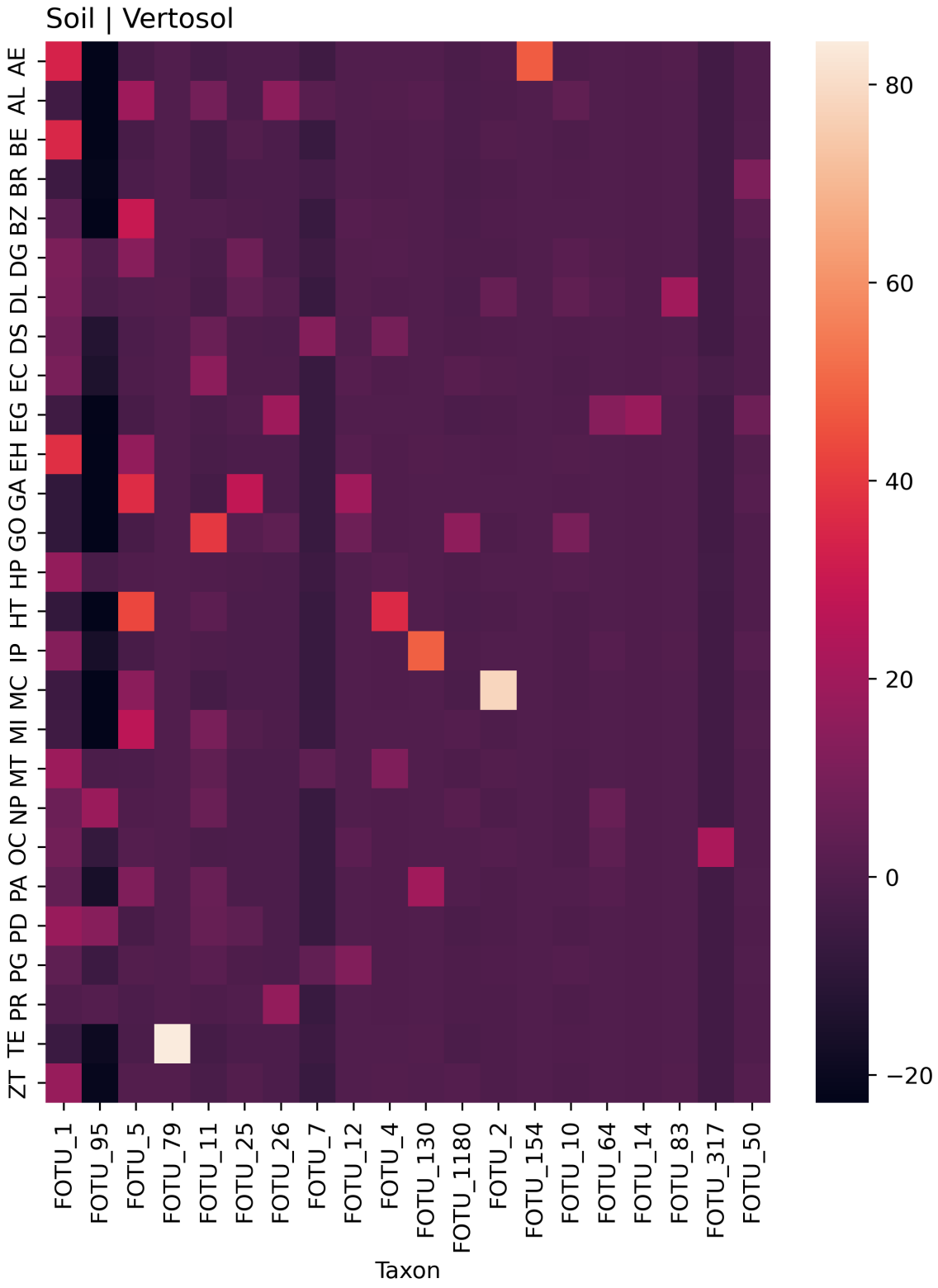

Figure 59: SIMPER analysis of the top 20 fungal taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in Northern Territory vertosol soil experiments

AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BE: 2-butoxyethanol; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; DL: d-limonene; DS: diesel fuel; EC: eicosane; EG: ethylene glycol; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; IP: isopropanol; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; MT: methanol; NP: naphthalene; OC: o-cresol; PA: polyacrylamide; PD: polyoxypropylene diamine; PG: propylene glycol; PR: pristane; TE: triethanolamine; ZT: zero time control

Scale bar indicates percentage change relative to the storage control

3.4.3 Response of aquifer microbiomes to chemicals

For the aquifer microcosm experiments, most chemicals caused a statistically significant shift in the microbial community composition and all chemicals caused a significant change in at least one of the aquifer samples tested (Table 30). All chemicals had a significant effect on the microbiome of RN040930 (Table 30). Conversely, a third of the chemical additions (ethoxylated alcohol, diethylene glycol ethyl ether, hydrotreated light petroleum, isopropanol, methylchloroisothiazolinone, methanol, polyacrylamide, polyoxypropylene diamine and propylene glycol) had no significant effect on the microbiome of RN033608 (Table 30).The chemical hydrotreated light petroleum distillate only caused a significant change in the microbiome of RN040930. Data from SIMPER analyses of the aquifer microbiome responses reveals a similar picture to that seen in the Northern Territory soils, though different taxa respond in the different environment of the aquifer. Just as occurred in soils, some sensitive taxa were identified from the aquifers that responded negatively (being reduced in relative abundance). The most marked of these was OTU_66 in the RN033608 aquifer experiments (Figure 60), which was significantly reduced in relative abundance when the majority of chemicals were present in the microcosms. OTU_66 is a poorly resolved member of the *Ignavibacteria* and thus finding the reasons for its sensitivity is difficult. Nevertheless, this taxon may be a useful marker for any contamination events in these aquifers.

Numerous taxa increased in abundance in response to the presence of certain chemicals. In RN033608, numerous taxa increased in response to chemical addition, these included: OTU_2 (benzisothiazolinone, diesel and d-limonene), OTU_3 (2-aminoethanol), OTU_13 (naphthalene), OTU_4 (glutaraldehyde) and OTU_60 (methylisothiazolinone) (Figure 60). Of these, OTU_3 also responded to the addition of 2-aminoethanol in RN037666. This taxon is a strictly anaerobic spirochaete from the genus *Rectinema*. This taxon was isolated and characterised relatively recently from a 20 year old, highly enriched (it largely contained just the *Rectinema* and a deltaproteobacterium) naphthalene-degrading culture (Koelschbach et al., 2017). The researchers who examined this taxon concluded it was not involved directly in naphthalene degradation, but instead worked in syntrophy with a naphthalene-degrading deltaproteobacterium. Degradation of 2-aminoethanol is facilitated by a relatively simple set of genes (for a review see: Kaval and Garsin, 2018) and these genes are reasonably

widespread among bacteria. While it is always possible, that OTU_3 is degrading downstream catabolic products of 2-aminoethanol, its relative abundance in two of the aquifers increasing on only this substrate likely suggests it is involved in 2-aminoethanol catabolism (Figure 60 and Figure 61).

Table 30: PERMANOVA results for prokaryotic microbiome changes relative to the storage control in aquifers

Chemical	RN033608	RN037666	RN040930
2-aminoethanol	**†	**	*
C12 ethoxylated alcohol	ND	**	**
2-butoxyethanol	*	*	*
bronopol	*	**	**
benzisothiazolinone	**	**	**
diethylene glycol ethyl ether	ND	***	***
d-limonene	**	**†	**
diesel	**	**	**
eicosane (linear-C20)	**	**	**
ethylene glycol	**	***	**
2-ethylhexanol	*	ND	**
glutaraldehyde	*	**†	**
glyoxal	**	**†	*
hydrotreated light petroleum distillate	ND	ND	**
hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine	**	**	**
isopropanol	ND	*	**
methylchloroisothiazolinone	ND	**	*
methylisothiazolinone	**	**	**
methanol	ND	**	**
naphthalene	*	**	**
2-methylphenol (o-cresol)	**	**	**
polyacrylamide	ND	**†	***
polyoxypropylene diamine	ND	**†	***
propylene glycol	ND	*	**
pristane (C15)	*	**	**
triethanolamine	**	**	**

ND = no significant difference, * 0.05 – 0.01, ** 0.009-0.001, *** < 0.001. Daggers (†) indicate those samples that while significantly different to the storage control, were highly correlated (*r* >= 0.9) to the zero time control

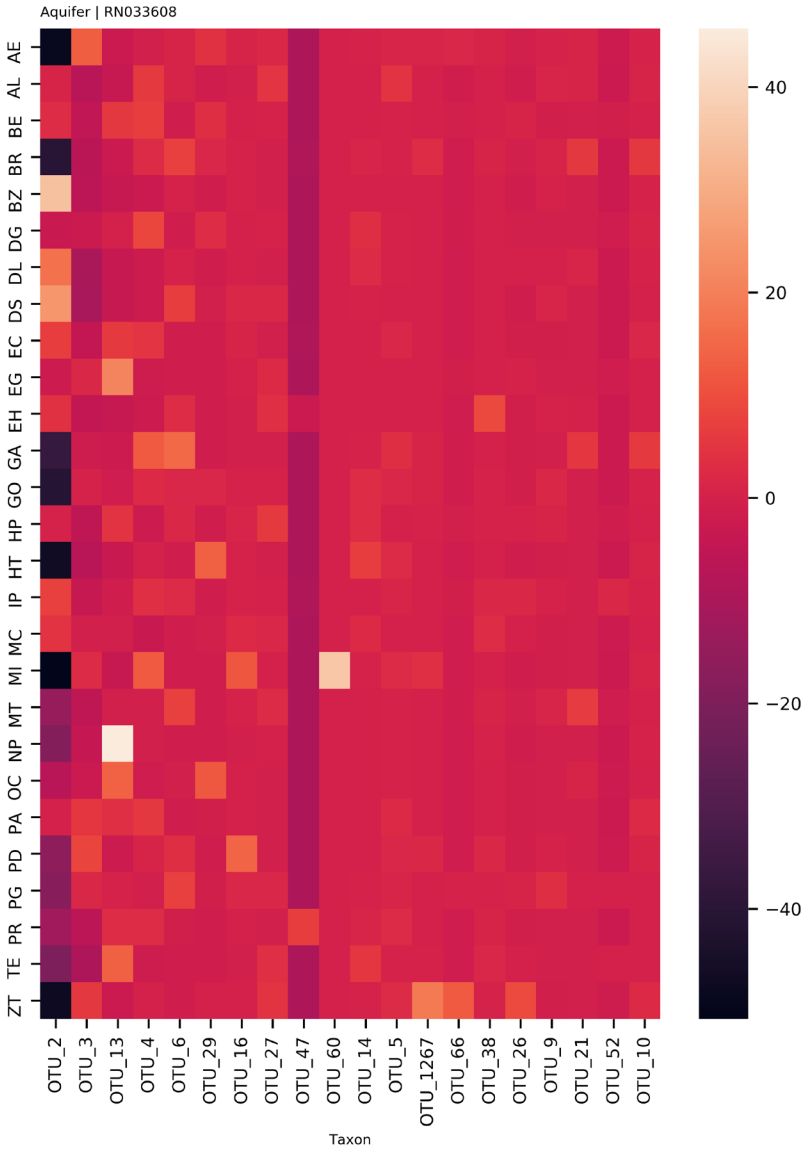

Figure 60: SIMPER analysis of the top 20 prokaryotic taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in Northern Territory aquifer RN033608 experiments

AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BE: 2-butoxyethanol; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; DL: d-limonene; DS: diesel fuel; EC: eicosane; EG: ethylene glycol; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; IP: isopropanol; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; MT: methanol; NP: naphthalene; OC: o-cresol; PA: polyacrylamide; PD: polyoxypropylene diamine; PG: propylene glycol; PR: pristane; TE: triethanolamine; ZT: zero time control

Scale bar indicates percentage change relative to the storage control

For RN037666, aside from OTU_3 there were several taxa that increased in abundance including OTU_7 (benzisothiazolinone), OTU_5 (bronopol), OTU_10 (triethanolamine and butoxyethanol) and OTU_58 (methylisothiazolinone) (Figure 61). Interestingly, OTU_7

occurred at elevated relative abundance in both RN033666 and RN040930 when isothiazolinone biocides (benzisothiazolinone and methylchloroisothiazolinone) were present (Figure 61 and Figure 62). This taxon is a species of *Pedomicrobium*, most closely related to *Pedomicrobium manganicum*. *Pedomicrobium* species can oxidise manganese ions to insoluble manganese oxide. The enzymes they use for this process are multicopper oxidases which are known to have activities against a range of aromatic xenobiotics such as 2,6-xylenol (2,6-dimethylphenol) and ρ-phenylenediamine (Bonaccorsi Di Patti et al., 2000; Sanchez-Amat et al., 2001), which are structurally similar to isothiazolinones. Further evidence of probable degradation of isothiazolinones can be found in OTU_57 which was found in RN040930 only (Figure 62). This taxon is conspecific with *Xanthobacter tagetidis,* which was originally described from soil, and is known to degrade a range of structurally similar thiophenes (Padden et al., 1997). Taken together with data from OTU_7, this and other data presented here likely indicate which taxa catabolise these substrates in the aquifers of the Beetaloo region.

Interestingly, OTU_58 increased in relative abundance in RN037666 when bronopol was supplied to this aquifer sample (Figure 61). OTU_58 is conspecific with *Microbacterium imperiale*, an actinobacterium with some known activity against brominated aromatic compounds. For example, Pasquarelli and co-workers in 2015 examined an enzyme system that detoxifies and partially degrades the brominated aromatic compound bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) to less toxic intermediates (Pasquarelli et al., 2015). Its presence here may indicate that this taxon has activity against other brominated compounds included bronopol, further work is required to clarify its activity against this substrate.

In RN040930 several OTUs were shown to increase with chemical addition (Figure 62), these comprised: OTU_76 (on bronopol, glutaraldehyde and 2-ethylhexanol), OTU_28 (on polyacrylamide), OTU_11 (on 2-ethylhexanol and naphthalene), OTU_40 (on polypropylene diamine) and OTU_19 (on polyacrylamide). Of these taxa, OTU_11 is a probably *Sphingorhabdus* species, a genus previous implicated in the degradation of a range of aliphatic and aromatic compounds (Kertesz et al., 2019; Woo et al., 2021). This same taxon also occurs in RN037666 where it is at increased relative abundance in the triethanolamine, naphthalene, 2-ethylhexanol, diesel and diethylene glycol ethyl ether treatments (Figure

61). Taken together this is probable evidence that the taxon is involved in degrading a range of aliphatic and aromatic compounds within the aquifers of the Beetaloo region.

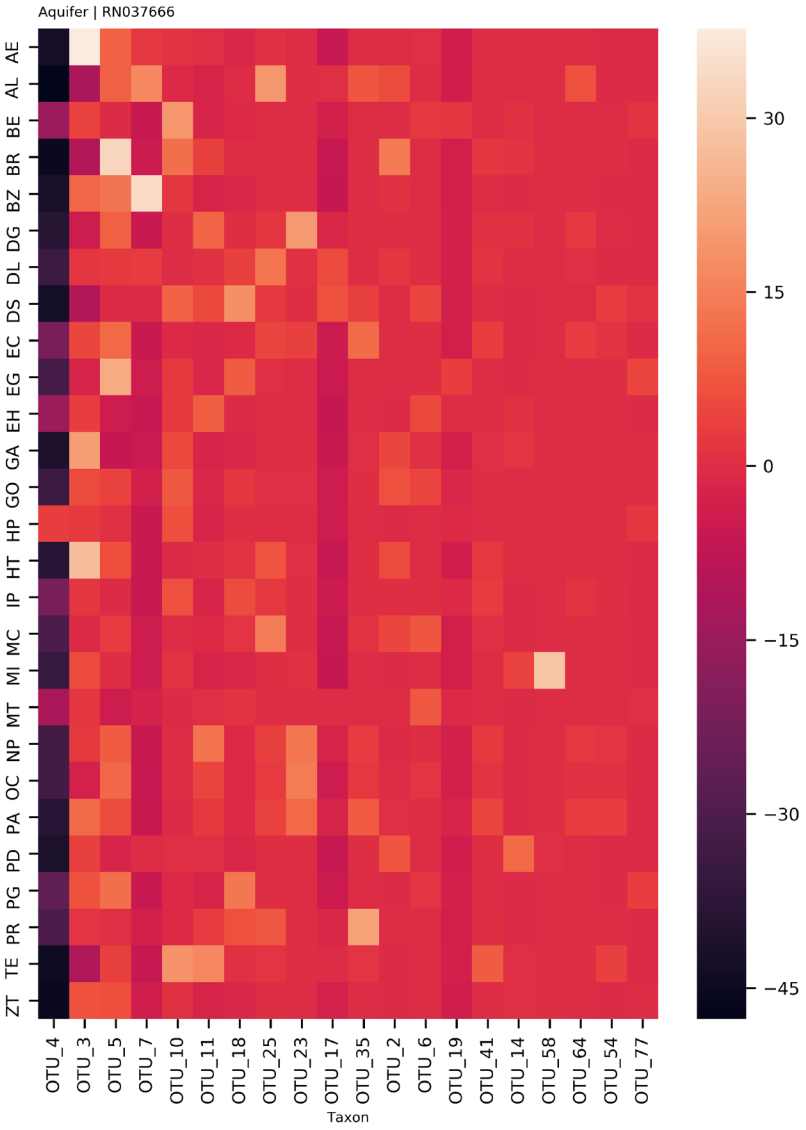

Figure 61: SIMPER analysis of the top 20 prokaryotic taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in Northern Territory aquifer RN037666 experiments

AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BE: 2-butoxyethanol; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; DL: d-limonene; DS: diesel fuel; EC: eicosane; EG: ethylene glycol; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; IP: isopropanol; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; MT: methanol; NP: naphthalene; OC: o-cresol; PA: polyacrylamide; PD: polyoxypropylene diamine; PG: propylene glycol; PR: pristane; TE: triethanolamine; ZT: zero time control

Scale bar indicates percentage change relative to the storage control

Interestingly, two taxa in RN040930 were at elevated relative abundance when polyacrylamide was added to the soils (Figure 62). The degradation of polyacrylamide is not completely understood, but under oxic conditions, the compound is rapidly deaminated leaving polyacrylates (Xiong et al., 2018). These polyacrylates are subsequently degraded, however, the genetic and enzymatic underpinnings of this process are not well understood. Under anoxic conditions, there are reports that acrylamide, the more toxic monomer may be released during degradation (Nyyssölä and Ahlgren, 2019). Neither OTU_19 nor OTU_28, which both increase in relative abundance when polyacrylamide is added to aquifer samples, are known polyacrylamide degraders, being an *Ignavibacterium* and a *Azoarcus* species, respectively, though this ability appears to be widespread in the bacterial domain. For instance, bacteria that are known to be able to deaminate polyacrylamide from multiple phyla: Actinobacteria, Firmicutes and Proteobacteria and include diverse linages within these phyla (Xiong et al., 2018). Similarly, a range of taxa have been shown to use the polyacrylate backbone as a sole source of carbon including various Firmicutes and Proteobacteria (Xiong et al., 2018). That such a diverse array of taxa can access polyacrylates as a source is suggestive of their inability to persist in these aquifers. Further work would be required to ascertain whether OTU_19 and OTU_28 observed here are using the polyacrylamide as simply a nitrogen source or are benefitting from nitrogen and carbon from this compound.

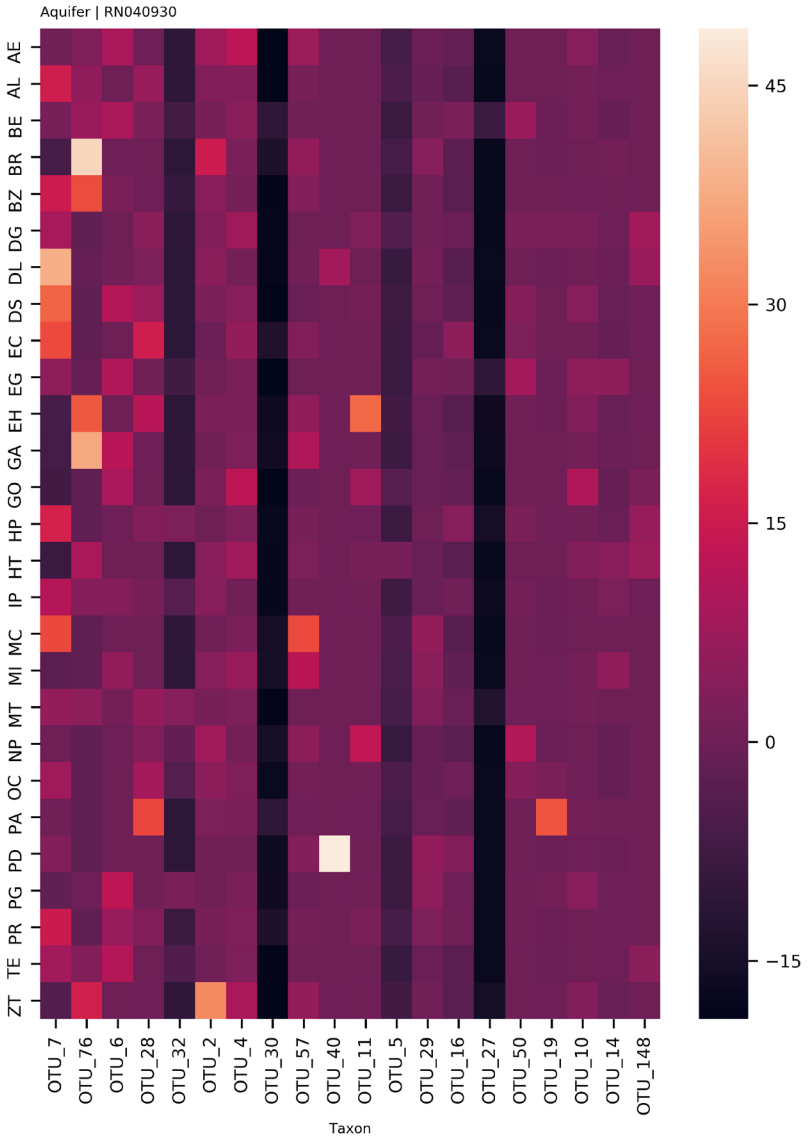

Figure 62: SIMPER analysis of the top 20 prokaryotic taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in Northern Territory aquifer RN040930 experiments

AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BE: 2-butoxyethanol; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; DL: d-limonene; DS: diesel fuel; EC: eicosane; EG: ethylene glycol; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; IP: isopropanol; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; MT: methanol; NP: naphthalene; OC: o-cresol; PA: polyacrylamide; PD: polyoxypropylene diamine; PG: propylene glycol; PR: pristane; TE: triethanolamine; ZT: zero time control

Scale bar indicates percentage change relative to the storage control

3.5 Growth on chemicals as sole sources of carbon by soil microbes

Sole carbon source trials refer to experiments in which only one source of carbon is supplied to the microbial community. For the Norther Territory aquifer microcosm experiments, the addition of the chemicals of interest closely mirrored a sole carbon source trial as organic carbon was very low in the aquifer samples. All aquifer samples had less than 7ppm of dissolved organic carbon (see data in section 3.1.2). Indeed, the average organic carbon content of the aquifers was 1.9 (±1.4) with a median organic carbon content of 1ppm from the 38 aquifer samples. In contrast, in soils significantly more carbon was available and as such, it was considered prudent to undertake sole carbon trials, to find taxa capable of growing when a single chemical was the only source of carbon in the medium. To prevent carry over of organic carbon from soils used as an inoculum, the samples were diluted between 10^{-5} and 10^{-8} with the only carbon source being the chemical added and growth was detected at these very low dilutions. Below are a series of heatmaps which describe those taxa detected from soil when the chemical being examined is the sole source of carbon available (Figure 63- Figure 87). In general, the method employed would disadvantage fungi so these taxa are not reported though a few fungal organisms were observed in some soil samples.

For each chemical it should be noted that as cultures were continuously diluted only those OTUs with sufficient growth on the substrate would be detected. As such, these data represent those taxa which grow most rapidly on the chemical provided. This may differ from the situation in soil as here only the chemical is provided with no other carbon sources, thus taxa that are able to degrade the compound and do so competitively in soil may not necessarily be represented in these data. Further, many taxa are difficult to grow in enrichment cultures and some putative catabolists of chemicals may not be identified in this approach as they resist growth in culture or are slow growing and evade detection. Regardless, these data provide a useful snap shot of microorganisms capable of degrading particularly chemicals as a sole source of carbon, some of which are present in more than one soil. This too warrants some explanation, not all species occur in all soils, though there is some overlap between the soils. It is noteworthy that an OTU represents a cluster of similar closely-related species, united by their shared 16S identity (above 97% identity) as

such, a single OTU may be present in more than one soil and behave similarly or differently in these trials.

Aminoethanol

For aminoethanol, 15 abundant OTUs (>5% of the enrichment culture) were identified. Of the five soils tested, three soils yielded successful aminoethanol degrading taxa (tenosol, kandosol and chromosol; Figure 63). From these soils. one taxon, OTU_9 grew on aminoethanol as a sole source of carbon in all three soils. OTU_9 was conspecific with *Methylorubrum* (formerly *Methylobacterium*) *populi*. This taxon was originally isolated from poplar (*Populus deltoides* × nigra DN34) roots, and little mention is made of its catabolism besides it being a facultative methanotroph. The presence of three species or strains of this taxon from the chromosol, tenosol and kandosol clearly indicate this taxon can degrade aminoethanol in culture. It's important to note here that only this taxon was observed to grow from all three soils. One other taxon, OTU_15, was observed in two of the three soils that successfully generated cultures (tenosol and chromosol; Figure 63). OTU_15 is conspecific with a strain of *Mycolicibacterium phocaicum* known from marine environments. In the literature, this species has been described degrading complex, recalcitrant xenobiotics including the environmental pollutant di-(2-ethylhexyl) phthalate (Ren et al., 2021). As such, its ability to grow on aminoethanol, a much simpler compound, is fairly unsurprisingly though it would appear to be the first description of this ability in this taxon.

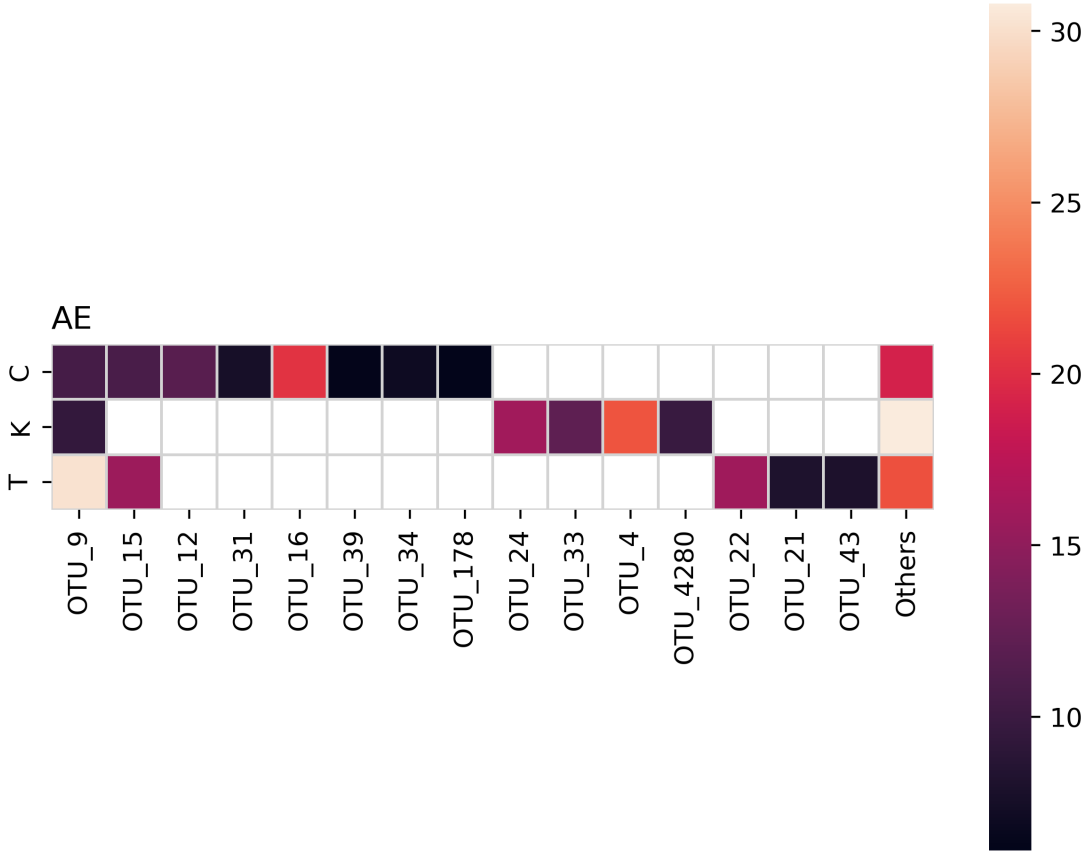

Figure 63: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using 2-aminoethanol as a sole carbon source

AE: 2-aminoethanol; C- chromosol soil; K- kandosol soil; T- tenosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Ethoxylated alcohols

For ethyoxylated alcohols, six abundant OTUs (>5% of the enrichment culture) were identified. Of the five soils tested, all soils yielded successful aminoethanol degrading taxa (Figure 64). These included OTU_15, which was identified from all soils on this substrate, which is described in detail above. Two other taxa occurred in multiple soils, OTU_21 which was detected in three of the five soils (chromosol, kandosol and rudosol), and OTU_36 which was detected in all soils except the tenosol. OTU_21 was a strain of *Massilia pinisoli*, isolated from pine soil (Altankhuu and Kim, 2016), while OTU_36 was conspecific with *Sediminibacterium* (*Vibrionimonas*) *magnilacihabitans* (Albert et al., 2014). Neither have

been reported to catabolise ethyoxylated alcohols, though their detection here likely indicates these taxa can readily degrade ethoxylated alcohols.

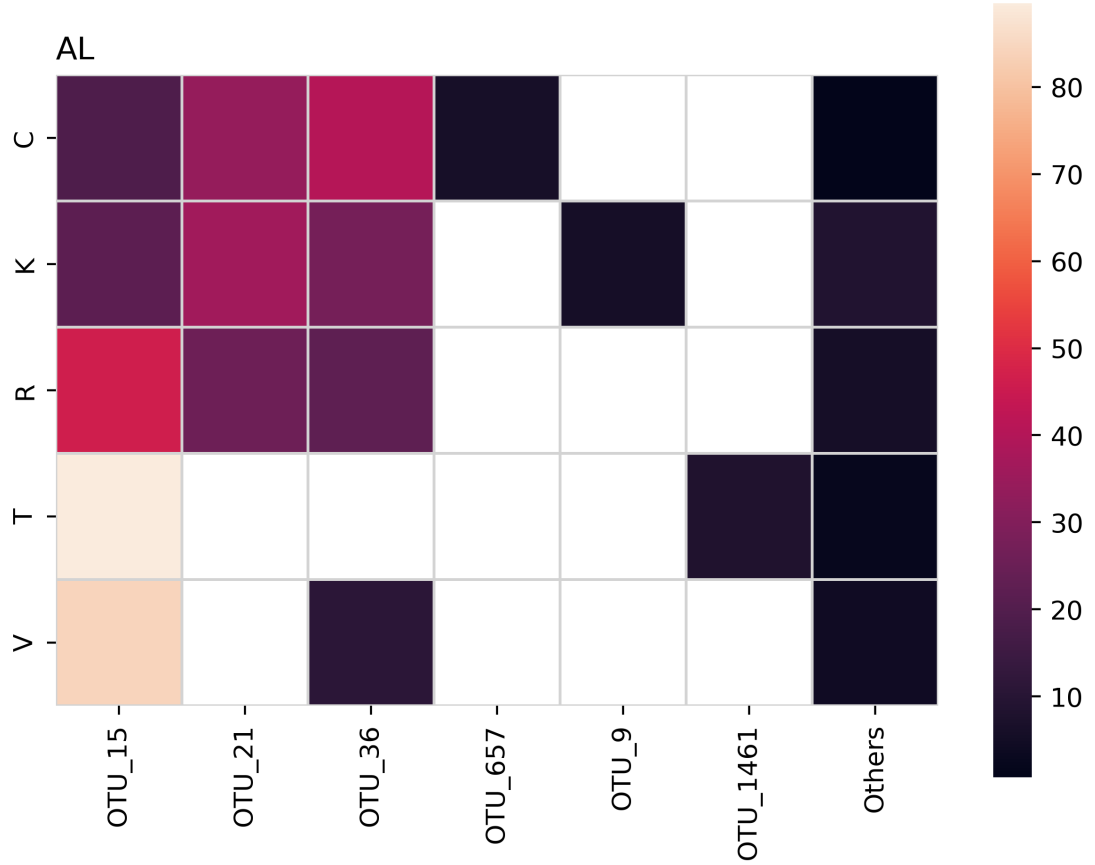

Figure 64: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using ethoxylate alcohol as a sole carbon source

AL: C12 alcohol ethoxylate; C- chromosol soil; K- kandosol soil; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Butoxyethanol

For the 2-butoxyethanol (BE) treatment, six abundant OTUs (>5% of the enrichment culture) were identified. Of the five soils tested, four soils, all except rudosol, yielded successful butoxyethanol degrading taxa (Figure 65). OTU_9, described above under aminoethanol, is present in these soils, being particularly abundant in the kandosol, tenosol and vertosol. Indeed, in the vertosol, the enrichment was virtually a pure culture of OTU_9. Other taxa

also increased markedly including OTU_55 (on chromosol and kandosol) and OTU_33 (on kandosol and tenosol). The former is a species of *Mesorhizobium*, closely related to *M. thiogangeticum*, while the latter is strain of *Sphingomonas alpina*. Little data is available on butoxyethanol degradation by the former, for the latter, however, numerous *Sphingomonas* species have been implicated in the degradation of ethylene glycol ethers (Kawai, 2001) which presumably form part of the oxic degradative pathway of 2-butoxyethanol (Woiski et al., 2020).

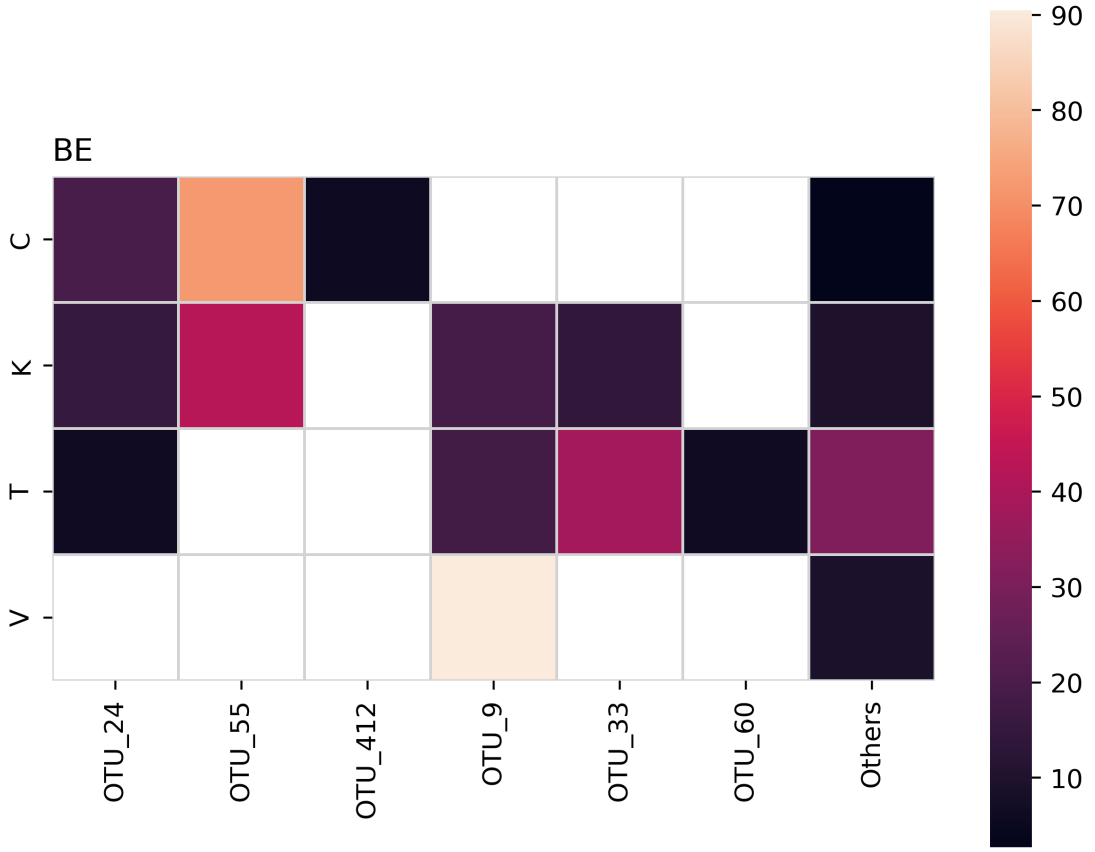

Figure 65: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using 2-butoxyethanol as a sole carbon source

BE: 2-butoxyethanol; C- chromosol soil; K- kandosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Bronopol

When bronopol was the sole source of carbon 11 OTUs increased significantly (>5%) in abundance (Figure 66). Interestingly, two taxa that have been repeatedly observed in other

chemical treatments (OTU_9 and OTU_15) were also observed growing on bronopol as a sole source of carbon (see section on aminoethanol). Similarly, OTU_55 (see section on benzisothiazolinone) also was able to grow on bronopol as sole source of carbon. None of these taxa have been reported to grow in the presence of bronopol, though work on the biodegradation of this biocide is somewhat limited.

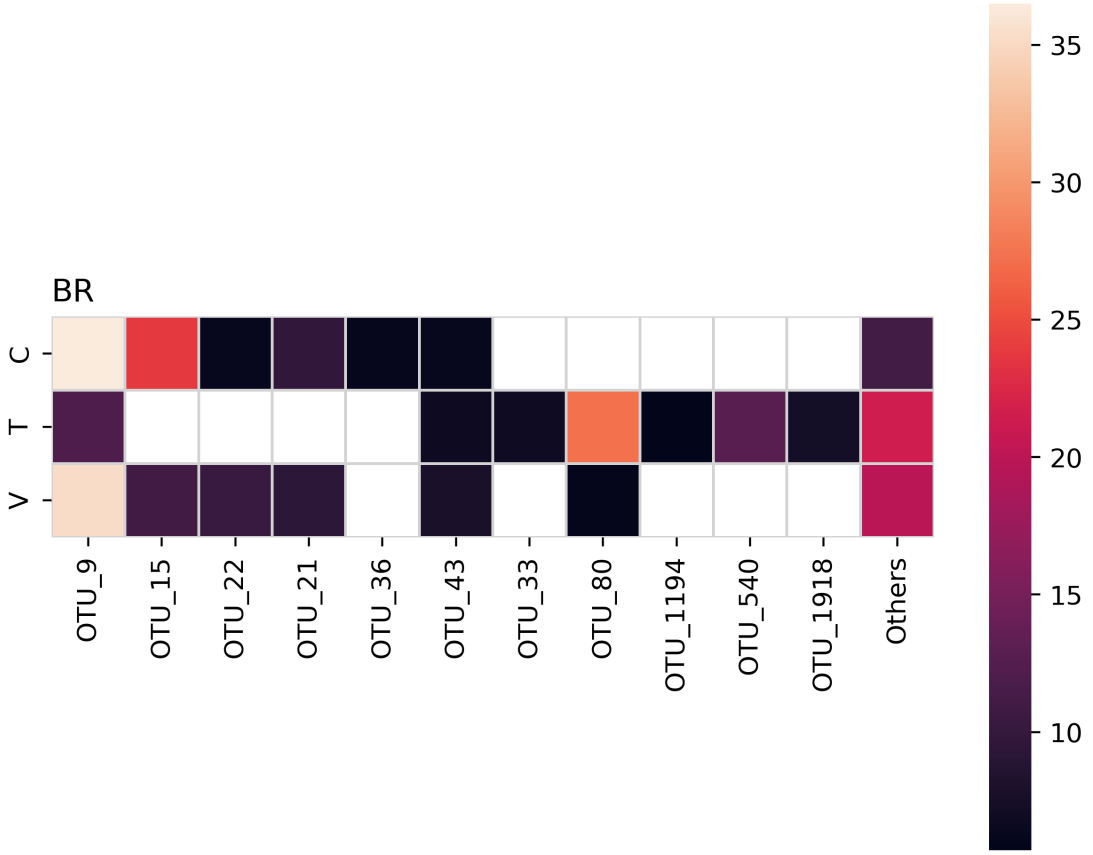

Figure 66: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using bronopol as a sole carbon source

BR: bronopol; C- chromosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Benzisothiazolinone

Like bronopol, benzisothiazolinone also facilitated the growth of OTUs 9 and 15, however, 12 other taxa were also able to grow on benzisothiazolinone as a sole source of carbon (Figure 67). These included OTU_22 and OTU_31 from the tenosol and chromosol, or the rudosol and chromosol respectively. The former is a strain of *Sinomonas humi*, while the

latter is a strain of *Dyella flava*. Members of the *Sinomonas* genus have been previous implicated in the degradation of polyphenols (Kumano et al., 2016), while *Dyella* is known to catabolise a range of complex substrates including the antimicrobial triclosan, biphenyl and 17β-estradiol (LI et al., 2009; Wang et al., 2014, 2018). These data demonstrate that these strains are also able to use benzisothiazolinone as a sole source of carbon for growth.

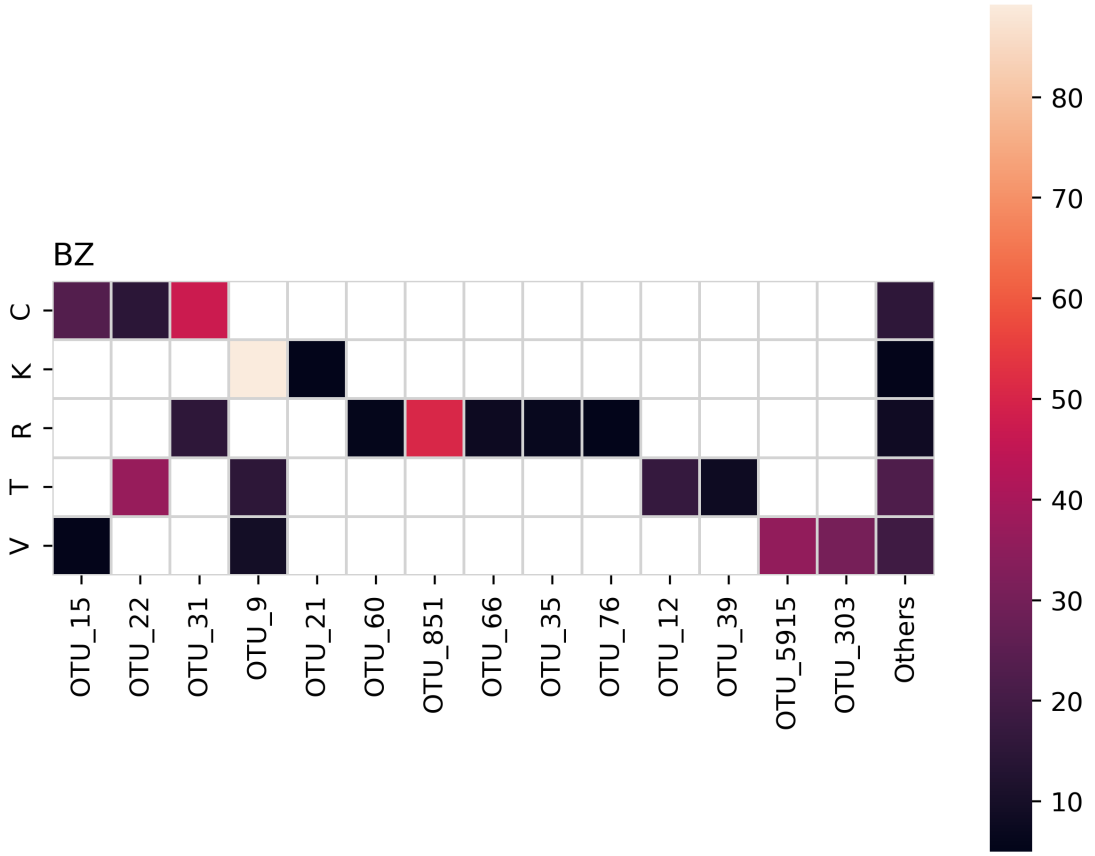

Figure 67: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using benzisothiazolinone as a sole carbon source

BZ: benzisothiazolinone; C- chromosol soil; K- kandosol soil; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

d-limonene

Of the seven abundant taxa that used d-limonene as a sole carbon source, most notable were OTU_34, OTU_60 and another example of a compound that serves as sole source of carbon for OTU_9. OTU_34 is an *Arthrobacter* species, and is a probably a strain of *A. livingstonensis* (Figure 68). *Arthrobacter* species are known for their degradation of various

recalcitrant agrochemicals and pollutants (Gobbetti and Rizzello, 2014; Mawang et al., 2021). Similarly, OTU_60 is another *Actinobacteria*, in this case a strain of *Nocardioides pakistanensis*. Along with their relatives in *Arthrobacter*, *Nocardioides* species are well known degrader of complex organic compounds and pollutants. For example, *Nocardioides* have been demonstrated to be involved in the degradation of carbendazim, centachlorophenol, hexachlorobenzene and pentachloronitrobenzene (Pandey et al., 2010; Takagi et al., 2009). That they also grow on d-limonene should not be surprising as it is a comparatively simple compound.

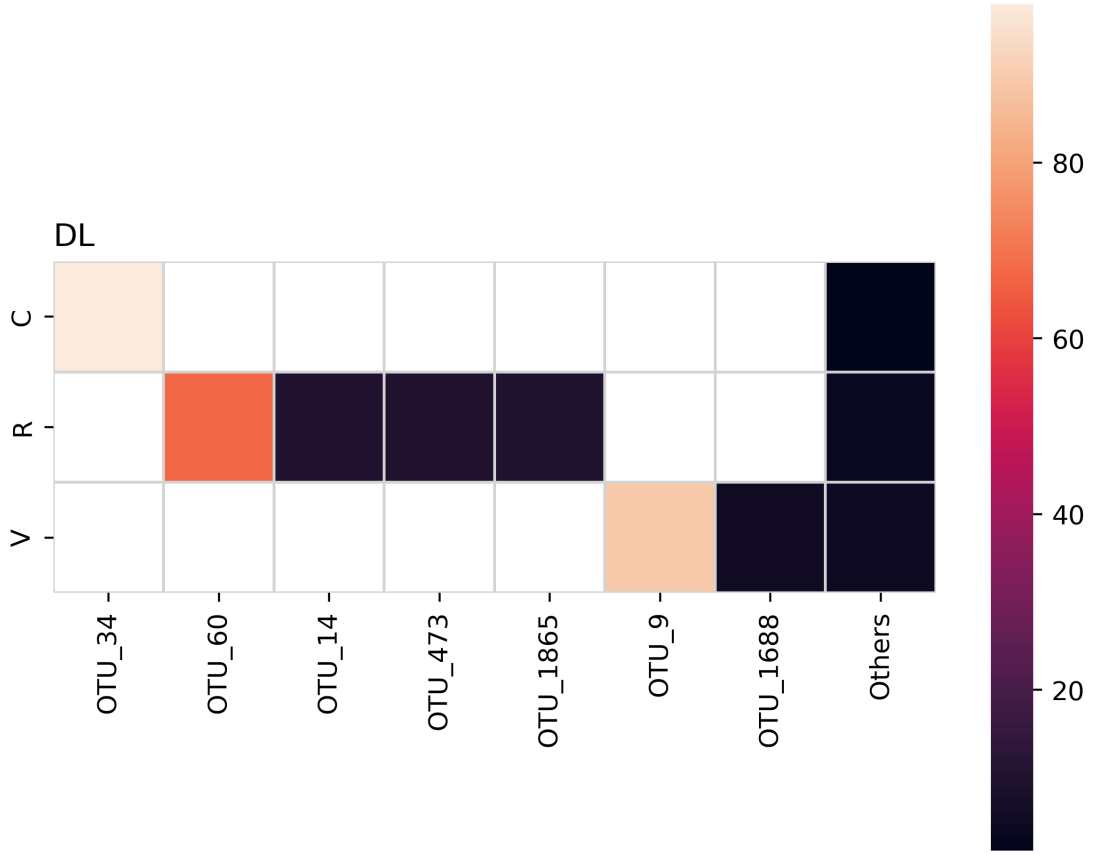

Figure 68: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using d-limonene as a sole carbon source

DL: d-limonene; C- chromosol soil; R- rudosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Diethylene glycol ethyl ether

When diethylene glycol ethyl ether was the sole source of carbon, 10 common OTUs were detected (at >5% of the amplicon pool) (Figure 69). Most of the OTUs that are able to use diethylene glycol ethyl ether as a sole source of carbon have been discussed previously. The main OTUs increasing being OTUs 9 and 15 (see aminoethanol), OTU_22 (see benzisothiazolinone) and OTU_34 (see d-limonene).

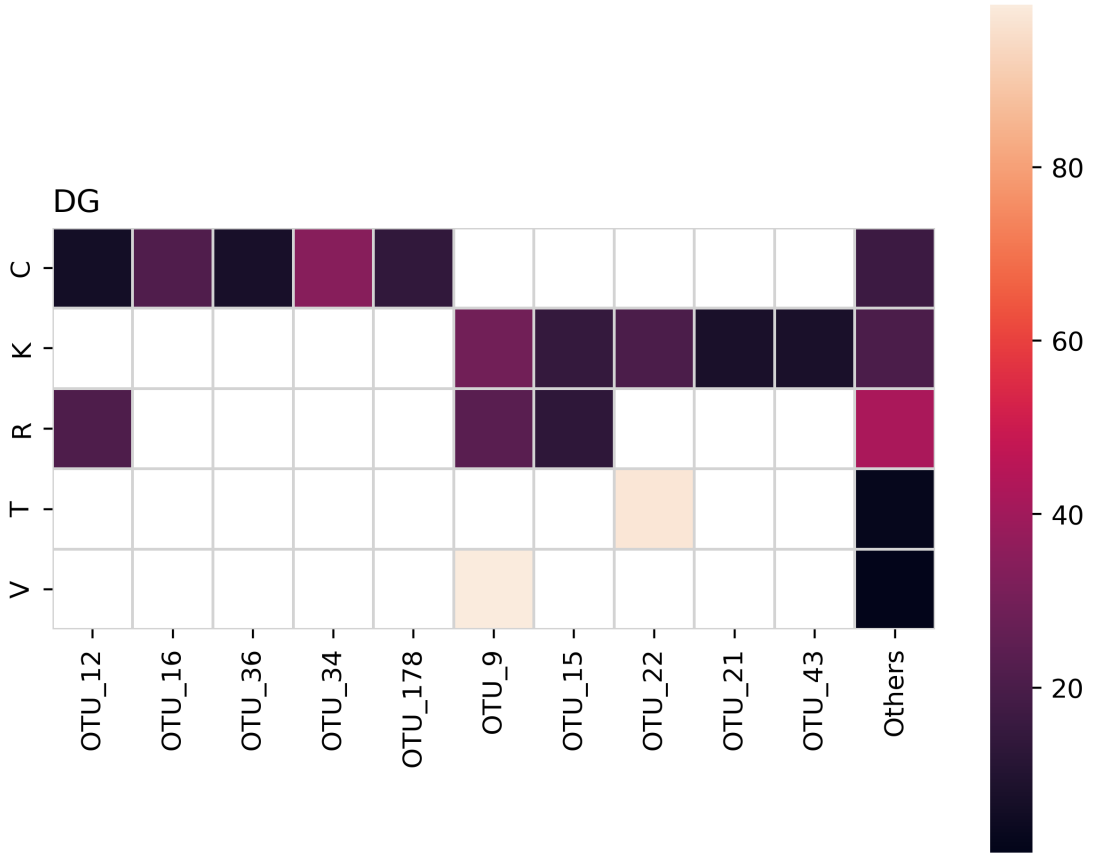

Figure 69: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using diethylene glycol ethyl ether as a sole carbon source

DG: diethylene glycol ethyl ether; C- chromosol soil; K- kandosol soil; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Diesel

When diesel was the sole source of carbon, 11 common OTUs were detected (at >5% of the amplicon pool) (Figure 70). Several OTUs discussed previous can be observed including OTU_9, OTU_15 (see aminoethanol), OTU_22 (see benzisothiazolinone) and OTU_60 (see d-

limonene). In addition, OTU_17, a strain of *Ferrovibrio denitificans*, was observed use diesel as a sole source of carbon. *Ferrovibrio* species have been previously observed increasing in relative abundance in soils contaminated with lubricant oils in a railway marshalling yard (Bodor et al., 2021).

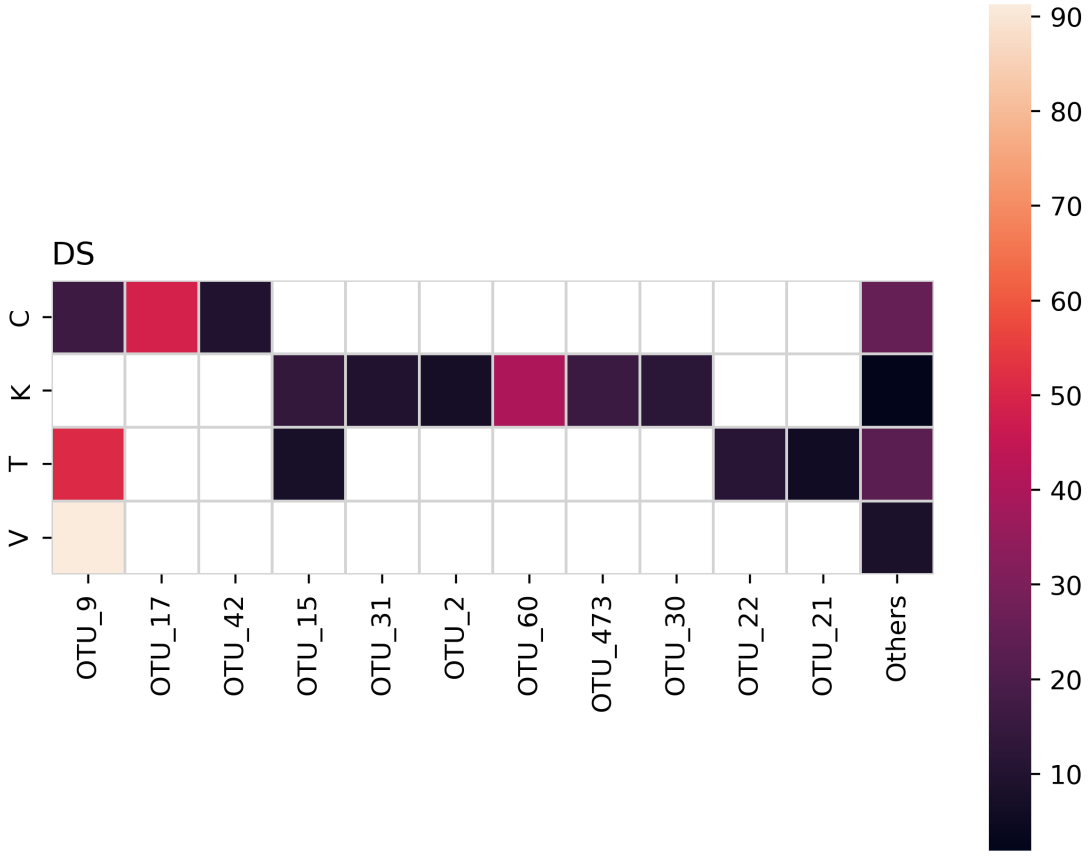

Figure 70: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using diesel as a sole carbon source

DS- diesel; C- chromosol soil; K- kandosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Eicosane

Unfortunately the eicosane cultures appeared to contaminated with common skin and air microbes and have thus been excluded from analysis.

Ethylene glycol

Eleven OTUs were able to grow on ethylene glycol as a sole source of carbon (Figure 71). These included numerous taxa observed on other substrates; including OTUs 9 and 15 (see aminoethanol), OTU_21 (see ethoxylated alcohols), OTU_22 (see benzisothiazolinone) and OTUs 33 and 55 (see butoxyethanol). In addition, some other taxa were also observed when ethylene glycol was the sole source of carbon, notable OTUs 24 and 86. The former is a strain of *Agrobacterium fabrum*, while the latter is another *Methylobacterium* species, this time *M. nodulans. Methylobacterium* are frequently involved in degradation of xenobiotic chemicals (Li et al., 2020), however, less is known about *Agrobacterium* species which are most commonly known for their roles in plant disease (Nester, 2015)a. That aside, there are reports that some *Agrobacterium* species can grow on ethylene glycol as a sole source of carbon (McVicker et al., 1998).

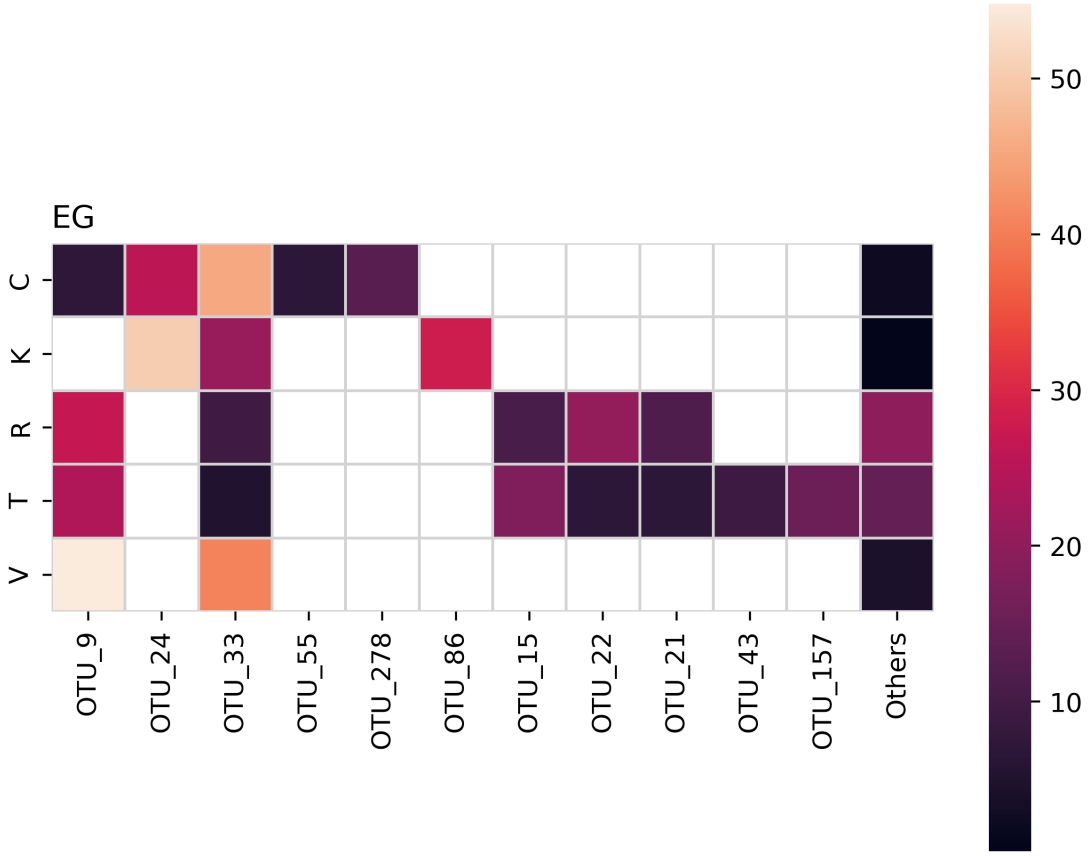

Figure 71: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using ethylene glycol as a sole carbon source

EG- ethylene glycol; C- chromosol soil; K- kandosol soil; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

2-ethylhexanol

When ethylhexanol was the sole source of carbon, 12 common OTUs were detected (at >5% of the amplicon pool) (Figure 72). Almost all of these OTUs have been discussed previously including OTU_9, OTU_15 (see aminoethanol), OTU_21 (see ethyoxylated alcohols) OTU_22 (see benzisothiazolinone) and OTU_60 (see d-limonene), but their presence here indicates they can also use ethylhexanol as a sole source of carbon.

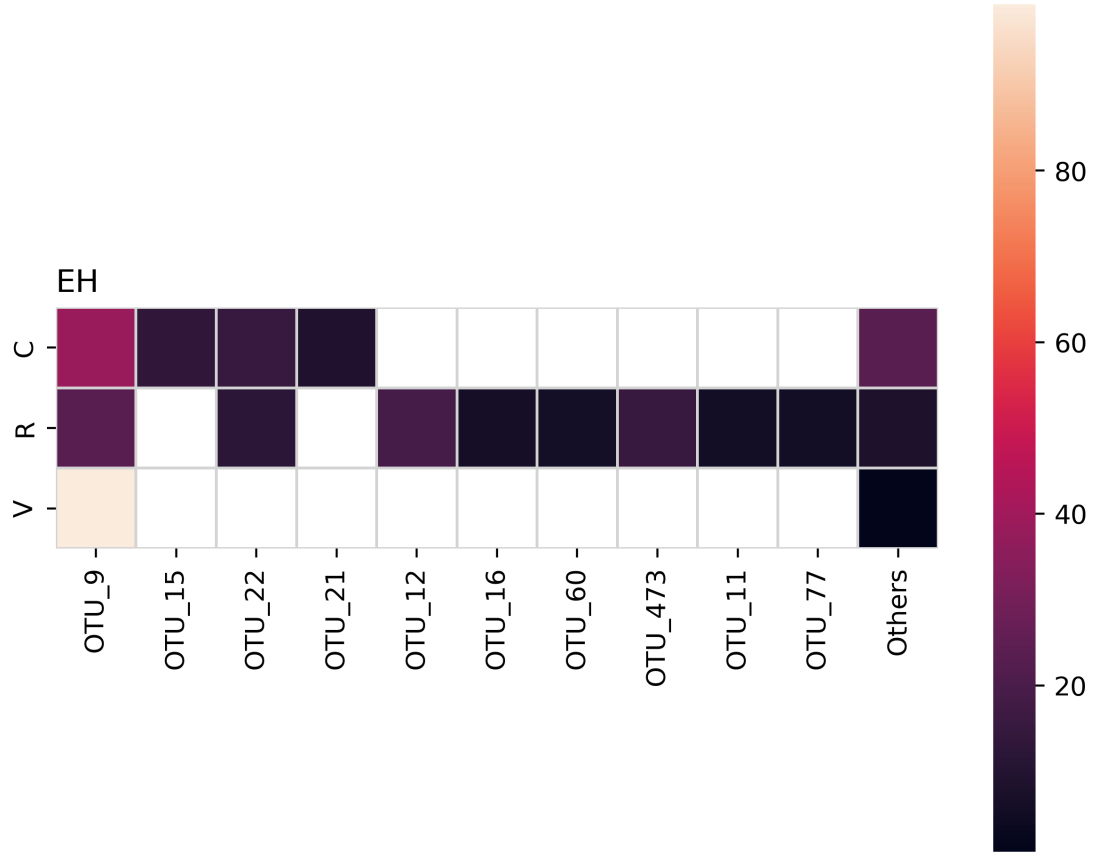

Figure 72: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using ethylhexanolas a sole carbon source

EH- ethylhexanol; C- chromosol soil; R- rudosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Glutaraldehyde

When glutaraldehyde was the sole source of carbon, 10 common OTUs were detected (at >5% of the amplicon pool) (Figure 73). Almost all of these OTUs have been discussed previously including OTU_9, OTU_15 (see aminoethanol), OTU_21 (see ethyoxylated alcohols) but their presence here indicates they can also use glutaraldehyde as a sole source of carbon. In addition, OTU_80 was also observed at high abundance in this treatment (from the vertosol soil). OTU_80 is a strain of *Nocardia africana*. The genus *Nocardia* is known for its diverse array of catabolic activities (Luo et al., 2014a, 2014b; Yang et al., 2019) and its presence here indicates it too can use glutaraldehyde as a sole carbon source.

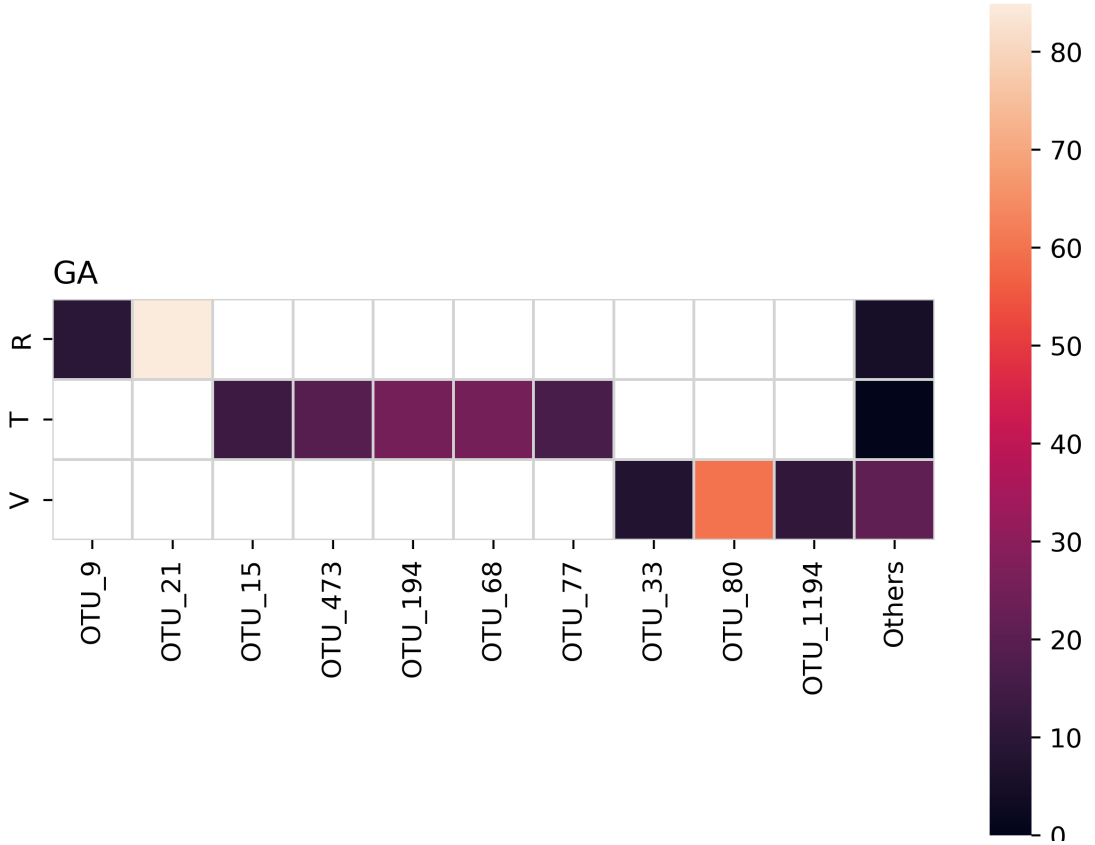

Figure 73: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using glutaraldehyde as a sole carbon source

GA- glutaraldehyde; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Glyoxal

When glyoxal was the sole source of carbon, 9 common OTUs were detected (at >5% of the amplicon pool) (Figure 74). Almost all of these OTUs have been discussed previously including OTU_9, OTU_15 (see aminoethanol), OTU_21 (see ethyoxylated alcohols), OTU_24 (see ethylene glycol), OTUs 33 and 55 (see butoxyethanol) but their presence here indicates they can also use glyoxal as a sole source of carbon.

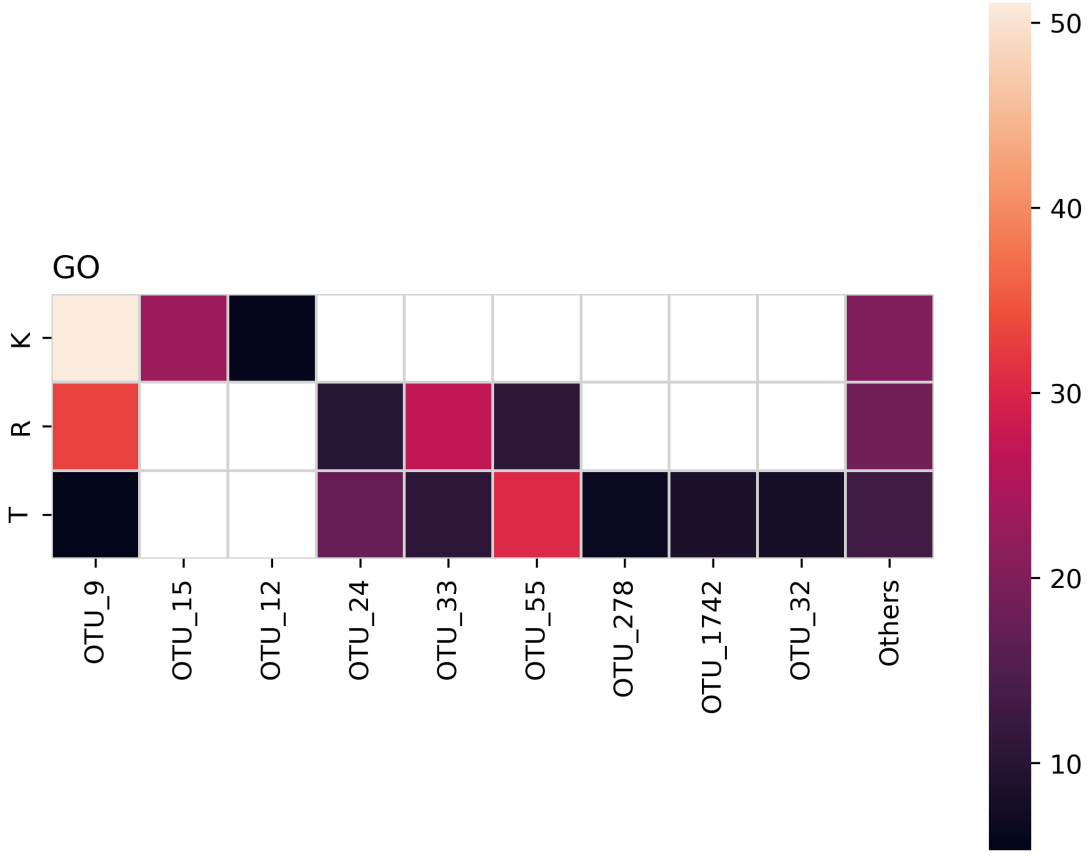

Figure 74: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using glyoxal as a sole carbon source

GO- glyoxal; K- kandosol soil; R- rudosol soil; T- tenosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Hydrotreated light petroleum distillate

When hydrotreated light petroleum distillate was the sole source of carbon, 8 common OTUs were detected (at >5% of the amplicon pool) (Figure 75). A number of these OTUs

have been observed and discussed previously including OTU_9, OTU_15 (see aminoethanol) and OTU_31 (see benzisothiazolinone). In addition to these taxa, OTU_43, a strain of *Pseudorhodoferax caeni*, was observed. This taxon was originally isolated from an industrial area in Marschacht, Germany and was isolated using thioether 3,3'-thiodipropionic acid as the sole source of carbon (Bruland et al., 2009) and its presence here indicates it can also use hydrotreated light petroleum distillate as a sole source of carbon.

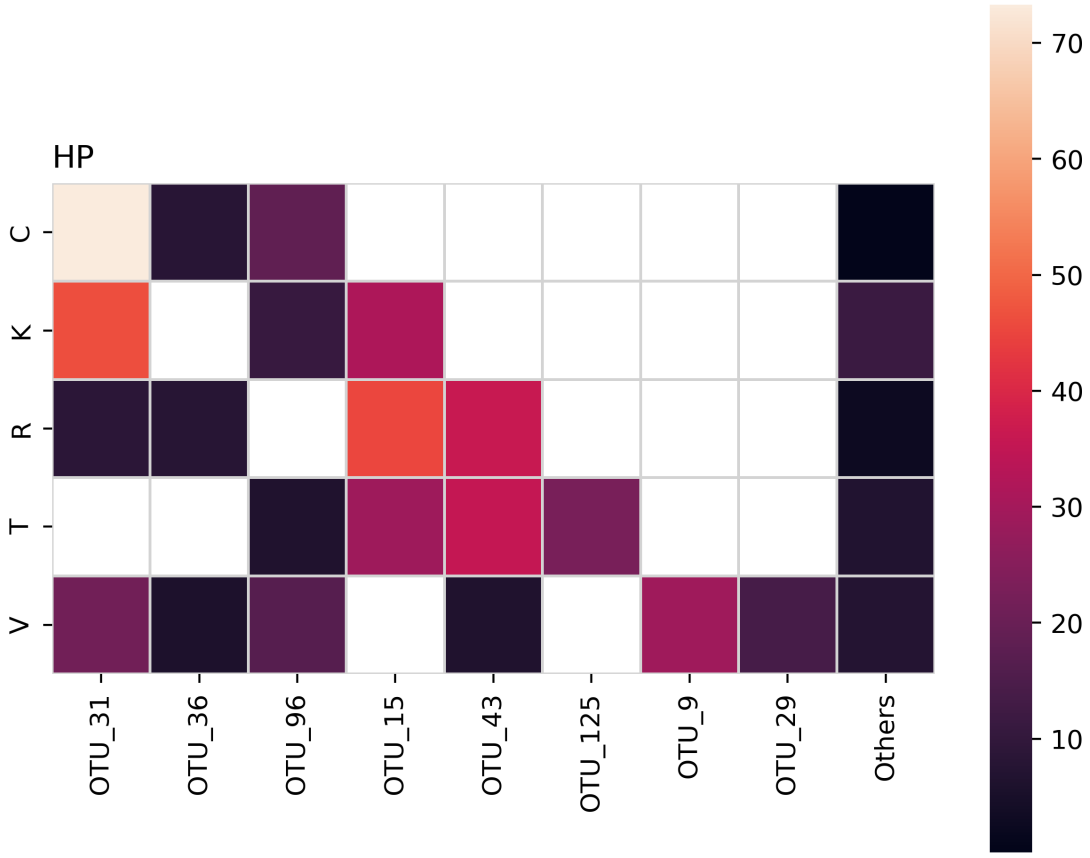

Figure 75: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using hydrotreated light petroleum distillate as a sole carbon source

HP-hydrotreated light petroleum distillate; C- chromosol soil; K- kandosol soil; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine

When hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine was the sole source of carbon, 8 common OTUs were detected (at >5% of the amplicon pool) (Figure 76). Many of these

OTUs have been observed and discussed previously including OTU_9, OTU_15 (see aminoethanol), OTU_21 (see ethoxylated alcohols) and OTU_22 (see benzisothiazolinone). Additionally, OTU_4 is able to grow on this chemical, this taxon is a strain of *Sulfurisoma sediminicola*. This taxon has little information on its catabolic potential outside of its abilities to utilise simple compounds and sulfur species, as such, it is not known whether this is an unusual finding. Nevertheless, its growth here in an enrichment from the vertosol where it is the main organism present at high densities suggests that it can access carbon from this biocide.

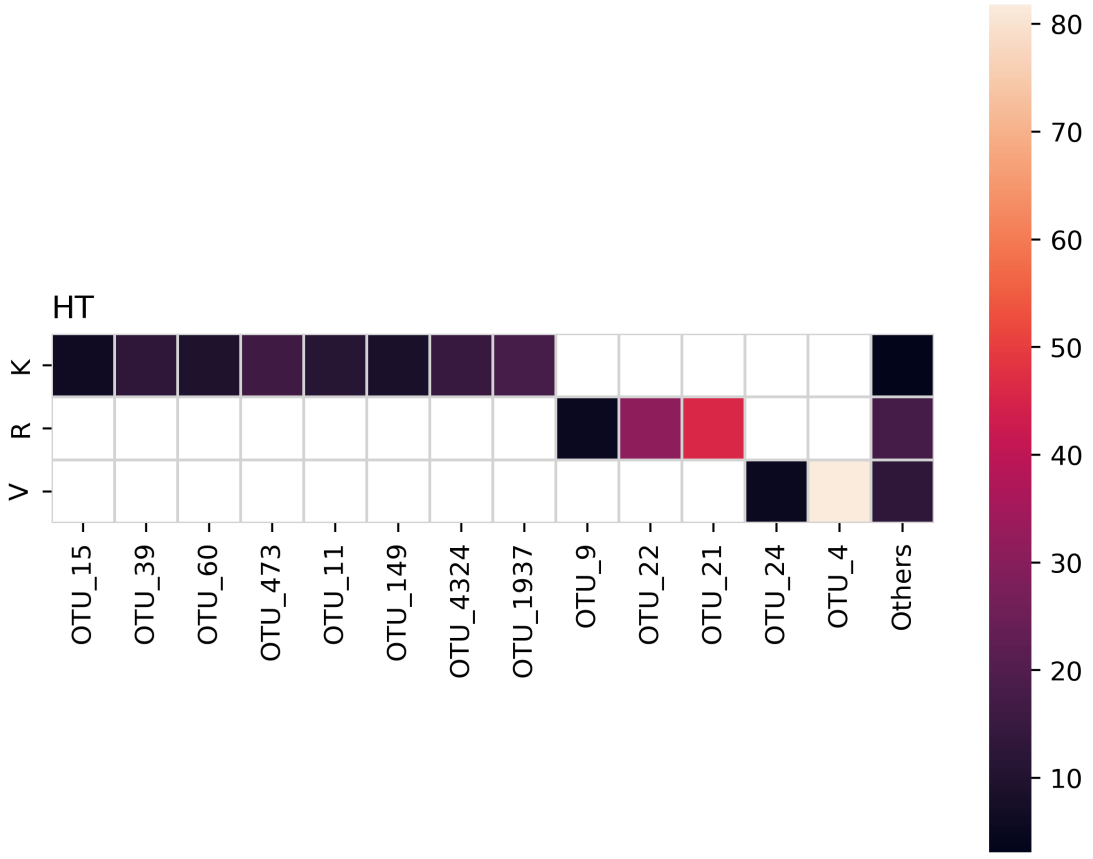

Figure 76: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine as a sole carbon source

HT- hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine; K- kandosol soil; R- rudosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Isopropanol

Almost all of the taxa detected growing on isopropanol have been discussed previously (Figure 77). Growth on isopropanol as a sole source of carbon is reasonably widespread in the bacterial domain and has been the subject of various studies for the last 70 years, for example, in work from the 1950s on isopropanol degradation, *Rhodopseudomonas gelatinosa* was able to rapidly degrade isopropanol to acetone, CO_2 and biomass (Siegel and Kamen, 1950). In more recent work, isopropanol was shown to be biodegraded below detection limits in oxic conditions with synthetic hydraulic fracturing fluids (Kekacs et al., 2015).

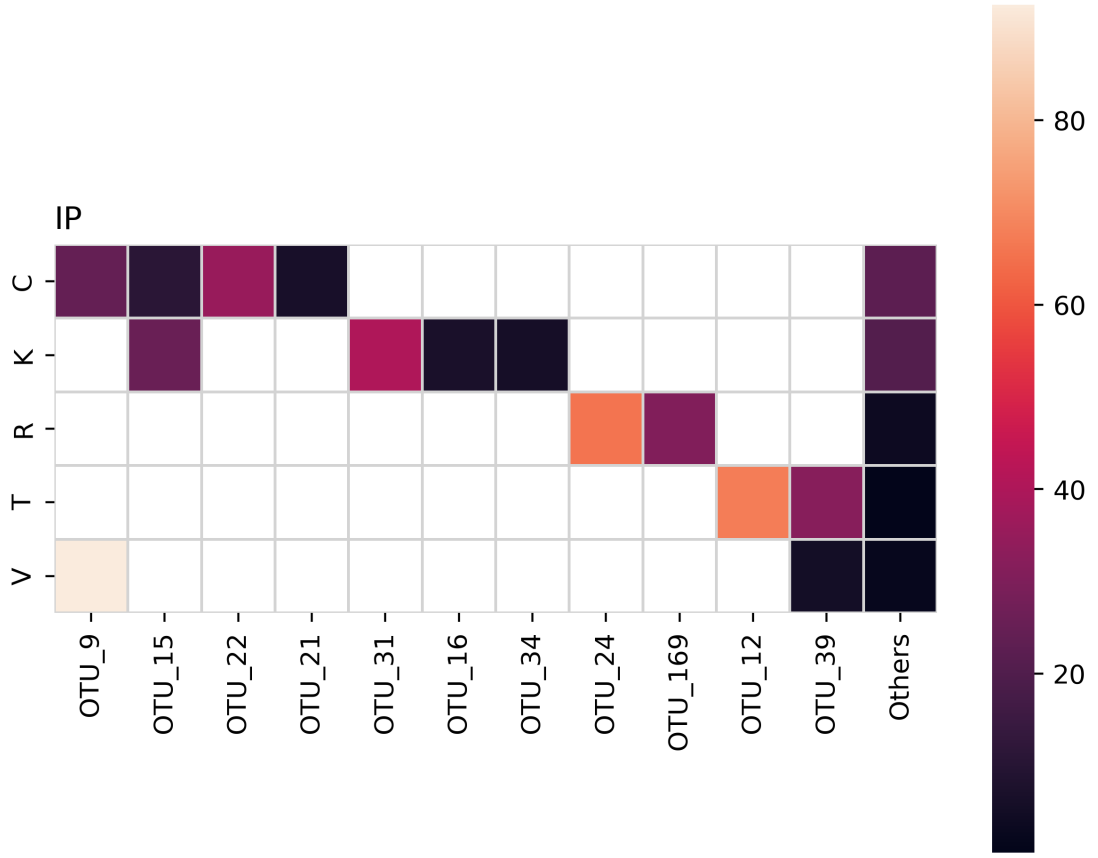

Figure 77: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using isopropanol as a sole carbon source

IP- isopropanol; C- chromosol soil; K- kandosol soil; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Methylisothiazolinone and methylchloroisothiazolinone

These two chemicals are broadly similar in terms of which taxa were able to utilise them as a sole source of carbon (Figure 78 and Figure 79). Both featured OTUs 9, 15, 21, 22, with fewer taxa able to grow on methylchloroisothiazolinone as a sole source of carbon compared with methylisothiazolinone. These taxa have for the most part been observed growing on other chemicals as sole sources of carbon. One taxon that was observed on methylisothiazolinone (OTU_39) is conspecific with *Amycolatopsis circi*. This genus is known for its catabolism of challenging substrates, for example, *A. tucumanensis* has been shown to degrade a range of aliphatic, aromatic hydrocarbons and other strains can degrade recalcitrant bioplastics (Bourguignon et al., 2016; Decorosi et al., 2019). Their presence here indicates that *Amycolatopsis circi* can also degrade methylisothiazolinone.

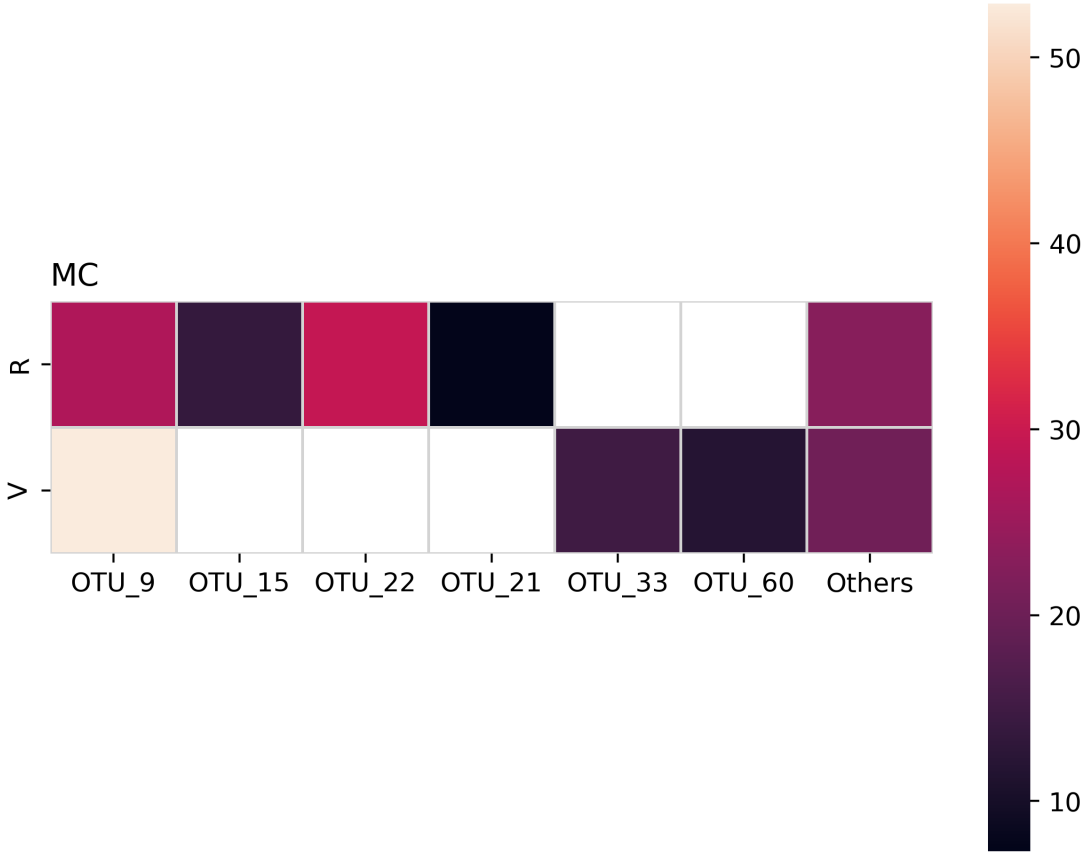

Figure 78: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using methylchloroisothiazolinone as a sole carbon source

MC- methylchloroisothiazolinone; R- rudosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

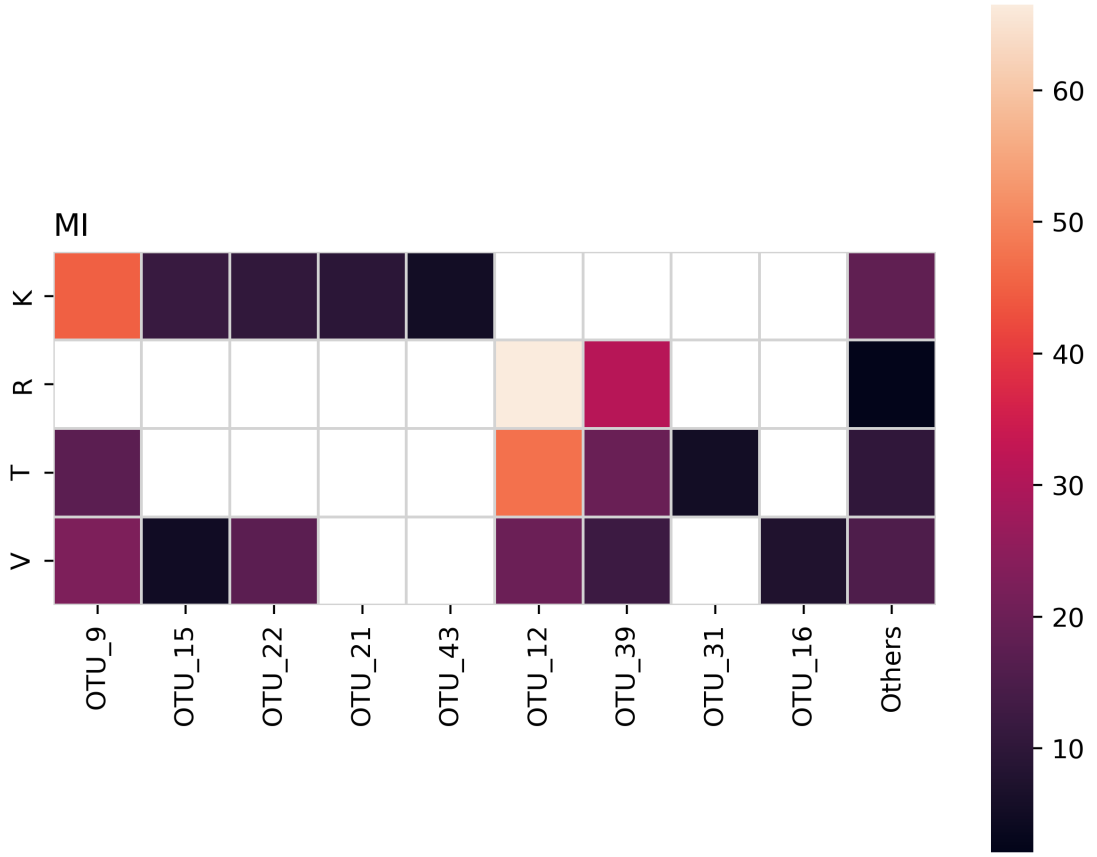

Figure 79: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using methylisothiazolinone as a sole carbon source

MI- methylisothiazolinone; K- kandosol soil; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Methanol

Almost all of the taxa detected growing on methanol have been discussed previously (Figure 80). Growth methanol as a sole source of carbon is not universal in the bacterial domain but numerous diverse lineages can grow on methanol, including a range of methanogens, methylotrophic bacteria and acetogens (Kolb, 2009; Kremp et al., 2018; Tani et al., 1972). The culture conditions used in this study likely exclude most of the strict anaerobes (as vials were incubated with a headspace of atmospheric air). Regardless, numerous taxa shown here are able to grow using methanol as a sole source of carbon.

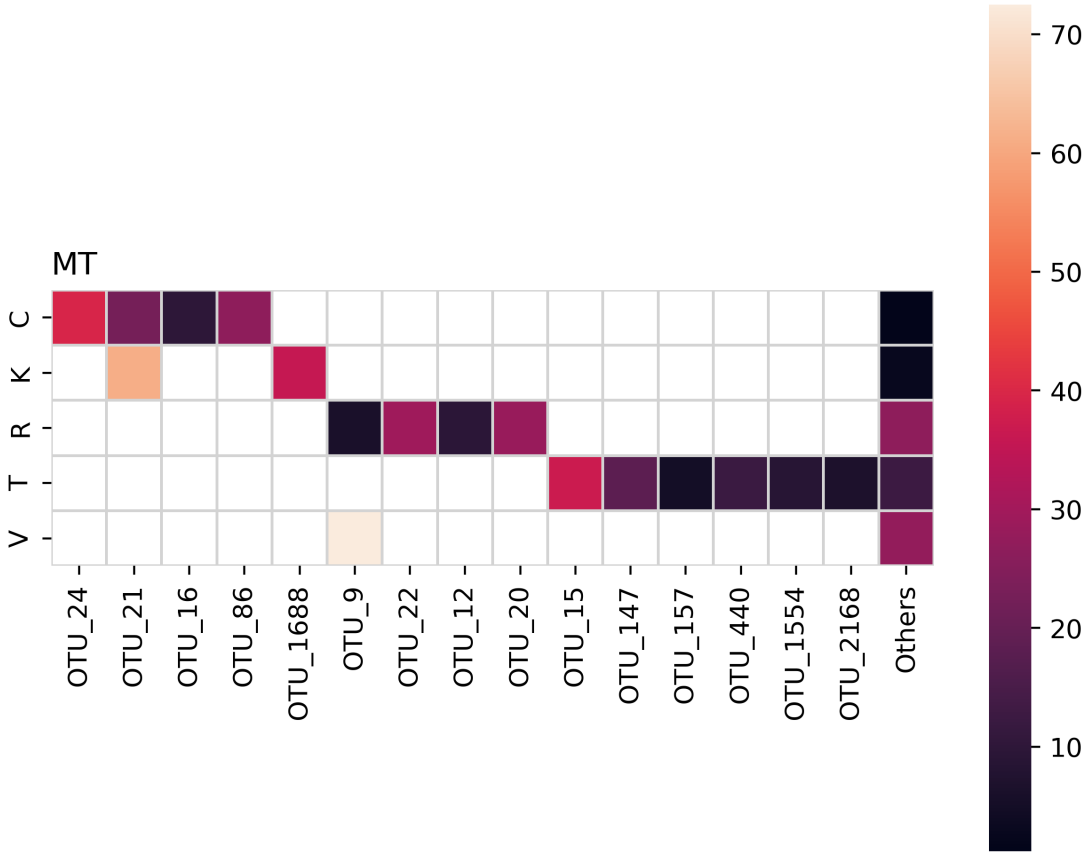

Figure 80: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using methanol as a sole carbon source

MT- methanol; C- chromosol soil; K- kandosol soil; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Naphthalene

Almost all of the taxa that grew on naphthalene as a sole source of carbon were observed in other treatments (Figure 81). These included: OTU_9, OTU_15 (see aminoethanol), OTU_21 (see ethoxylated alcohols) and OTU_22 (see benzisothiazolinone). OTU_12 is a relative of OTU_9 (another *Methylorubrum* species, *M. phyllostachyos*). This taxon is the most abundant taxon in the enrichment from tenosol. In parallel, OTU_9 which is *M. populi* (see discussion for aminoethanol) dominates the consortium for vertosol. It should be noted that OTU_9 occurs in the tenosol sample, so its absence in this enrichment on naphthalene is intriguing. It may be that OTU_12 degrades naphthalene more efficiently or in some other way outcompetes OTU_9 for this chemical.

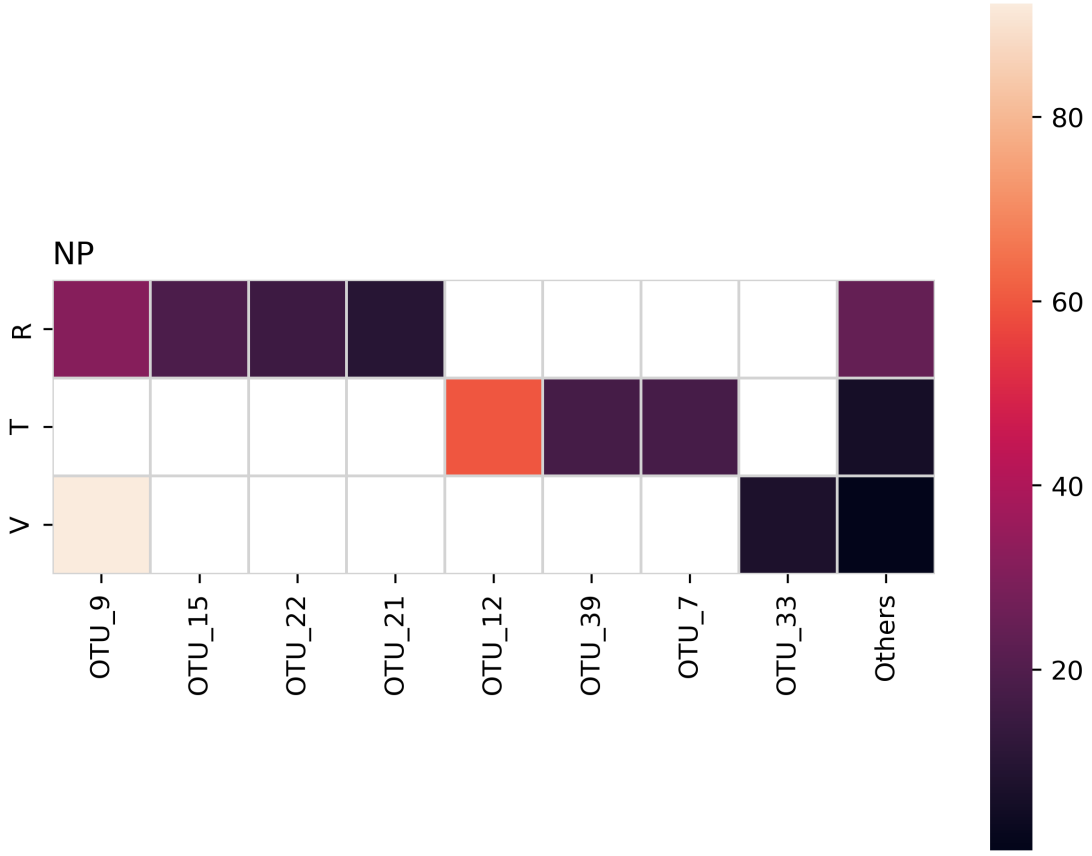

Figure 81: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using naphthalene as a sole carbon source

NP- naphthalene; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

o-cresol

While many of the same species are observed on o-cresol as other chemicals (OTUs 9, 15, 21, 22, 33 and 60 for instance) this chemical not only has some unusual catabolists, but also has one of the greatest numbers of taxa able to use it as a sole source of carbon (Figure 82). Across the four soils, 14 OTUs were detected (at >5% of the amplicon pool), which is the second largest number detected in the sole carbon source assays (after polyoxypropylene diamine). Numerous taxa were observed on this chemical but not many other chemicals. These included OTUs 11, 16 and 1879, which were strains of *Sphingorhabdus buctiana*, *Bradyrhizobium valentinum* and *Limnobacter thiooxidans*, respectively. All three of these genera have been shown to possess members that can degrade aromatic compounds and

their presence here extends that capability to these three taxa (Chen et al., 2016; Jeong et al., 2016; Okubo et al., 2012).

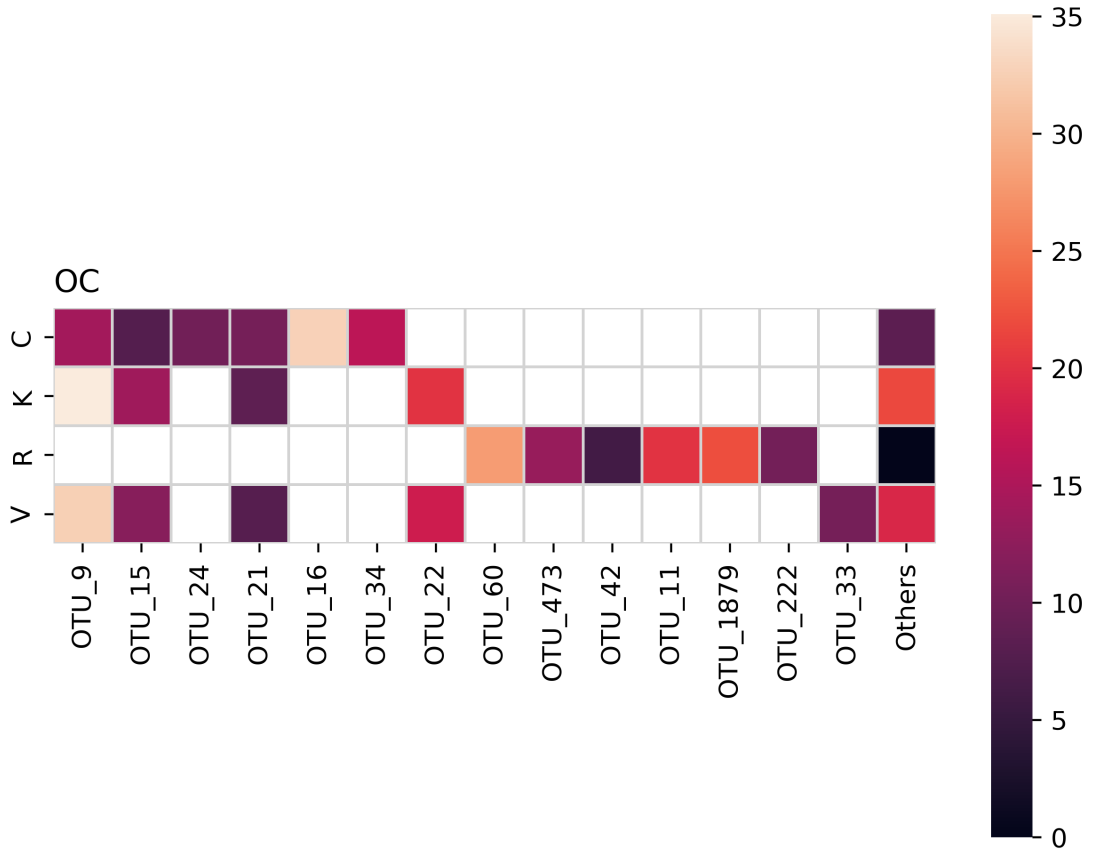

Figure 82: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using o-cresol as a sole carbon source

OC- o-cresol; C- chromosol soil; K- kandosol soil; R- rudosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Polyacrylamide

As discussed in section 3.4.3 (aquifer SIMPER microbiomes) polyacrylamide degradation proceeds in oxic settings normally via the rapid deamination of polyacrylamide, and there are suggestions that subsequent reactions that attack the remnant polyacrylate backbone occur more slowly – though data are lacking for this claim. Regardless, it is noteworthy that here, polyacrylamide served as a carbon source (i.e. the polyacrylate backbone was being degraded) for microbes from four of the soils in this experiment (Figure 83). It may also have

served as a source of nitrogen, however, the media also contained a nitrogen source. The taxa that undertake these degradations are not well understood. Here, OTU_20 is of note as it comprises a significant portion of all the enrichment cultures from multiple soils when polyacrylamide was the sole source of carbon. This taxon is a *Glaciimonas* species, a genus that is poorly characterised and unfortunately little data is available on its catabolic potential. Its growth here, however, likely indicates it readily accesses carbon from polyacrylamide.

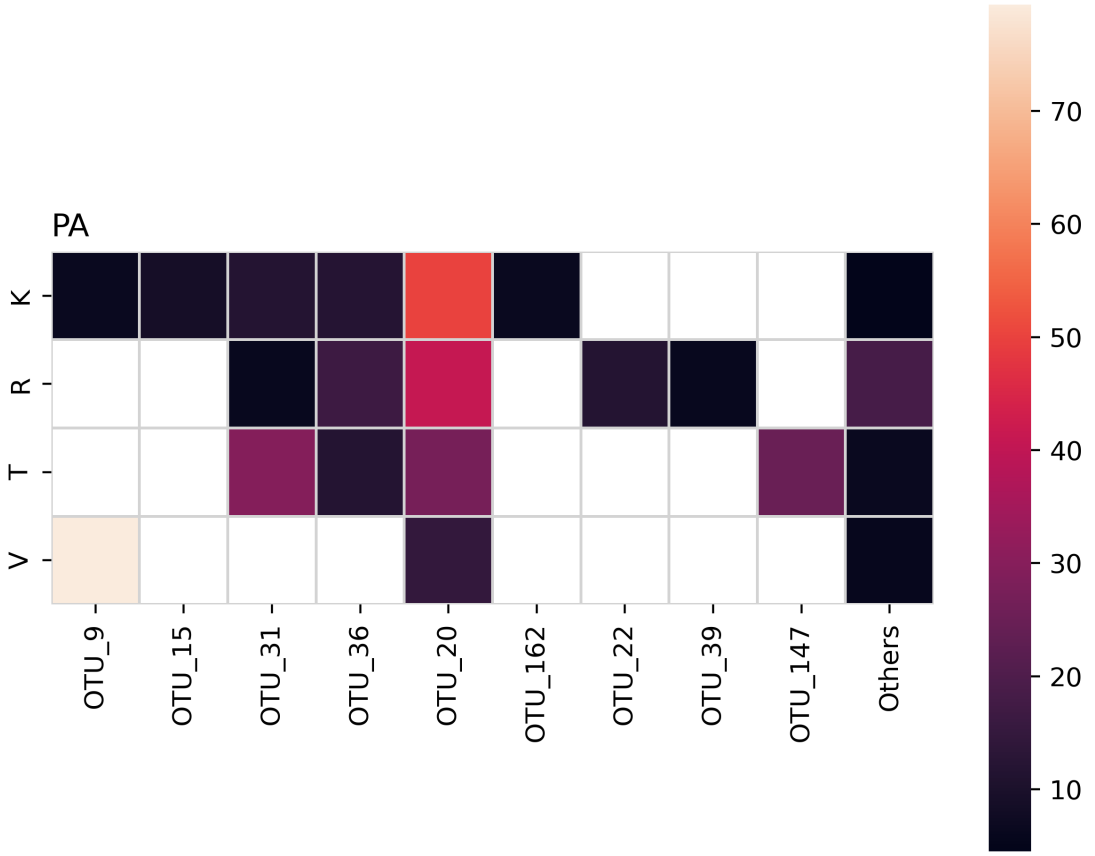

Figure 83: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using polyacrylamide as a sole carbon source

PG- polyacrylamide; K- kandosol soil; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Polyoxypropylene diamine

Using this chemical as a sole source of carbon for microbes resulted in the greatest number of OTUs with more than 5% of the amplicon pools for their respective soils (Figure 84). Indeed, 15 OTUs were detected across the soils when this chemical was the sole source of carbon. The OTUs in question have largely been described for previous chemicals and included OTU_9, 15, 21 and 22 among others. No new OTUs were observed at particularly high frequency for this chemical. Regardless, these data demonstrate that a range of taxa can degrade polyoxypropylene diamine and use carbon in this compound for biomass.

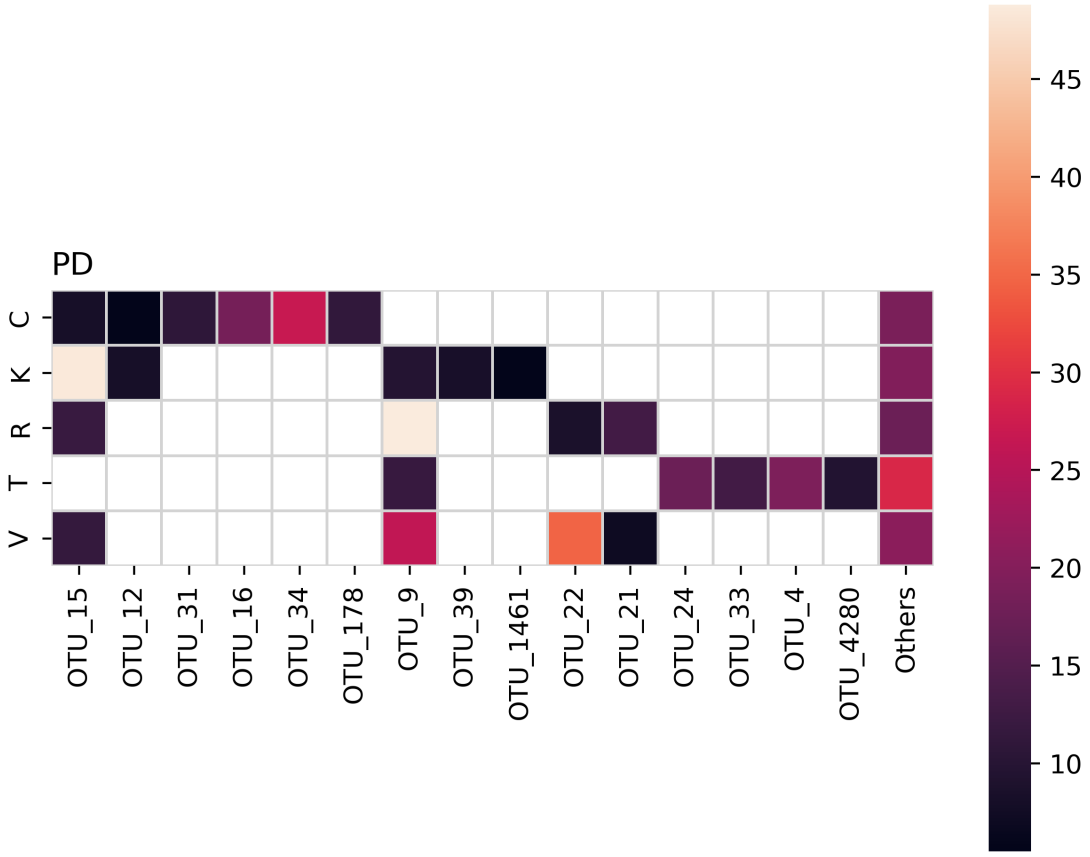

Figure 84: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using polyoxypropylene diamine as a sole carbon source

PD- Polyoxypropylene diamine; C- chromosol soil; K- kandosol soil; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Propylene glycol

Thirteen OTUs were able to grow on propylene glycol as a sole source of carbon (Figure 85). These included numerous taxa observed on other substrates; including OTUs 9 and 15 (see aminoethanol), OTU_21 (see ethoxylated alcohols), OTU_22 (see benzisothiazolinone) and OTUs 33 and 55 (see butoxyethanol). In addition, one of the other taxa (OTU_86) observed on ethylene glycol was observed here, suggesting this *Methylobacterium* species is able to degrade multiple glycols.

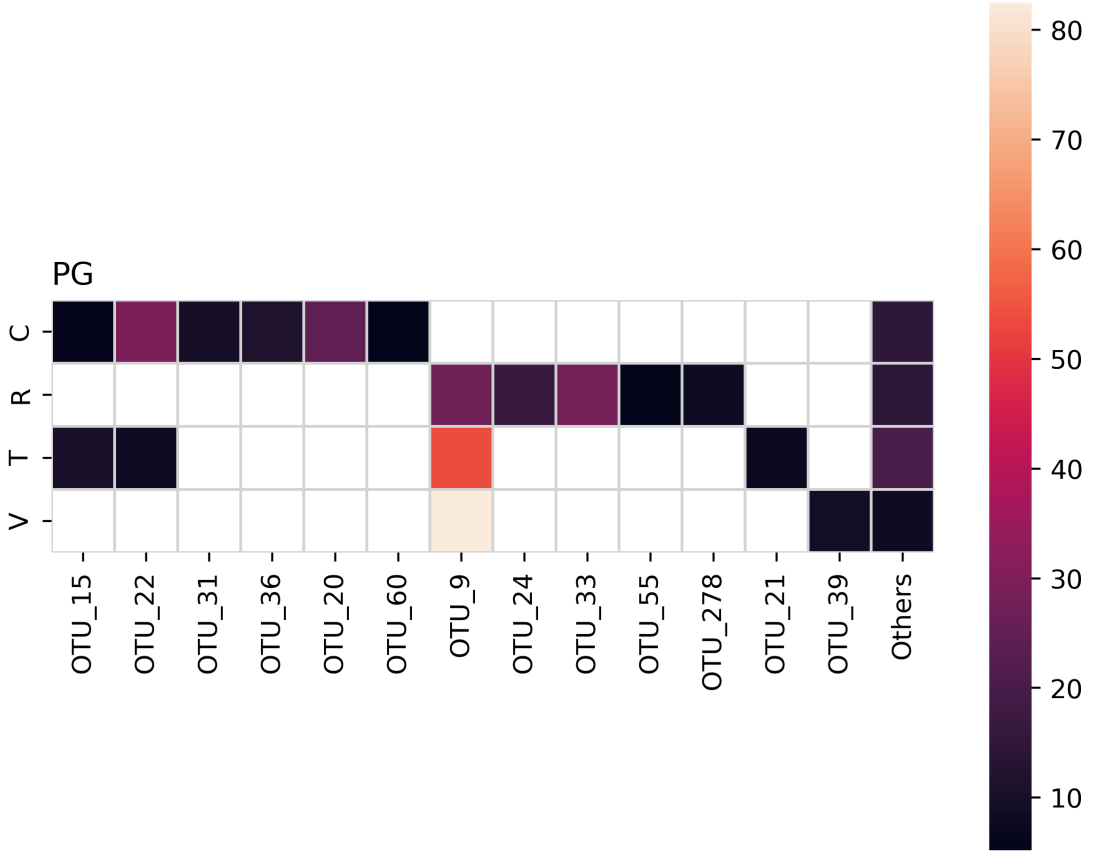

Figure 85: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using propylene glycol as a sole carbon source

PG- propylene glycol; C- chromosol soil; K- kandosol soil; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Pristane

When pristane was the sole source of carbon, 11 common OTUs were detected (at >5% of the amplicon pool) (Figure 86). Almost all of these OTUs have been discussed previously

including OTU_9, OTU_15 (see aminoethanol), OTU_16 (see o-cresol). OTU_21 (see ethyoxylated alcohols), OTU_22 (see benzisothiazolinone) and OTU_60 (see d-limonene), but their presence here indicates they can also use pristane as a sole source of carbon. One taxon was detected in the tenosol sample on pristane (OTU_106), but not in other chemical treatments. This taxon was an *Aquabacterium* species, a genus known for its degradation of complex hydrocarbons (Bondici et al., 2014).

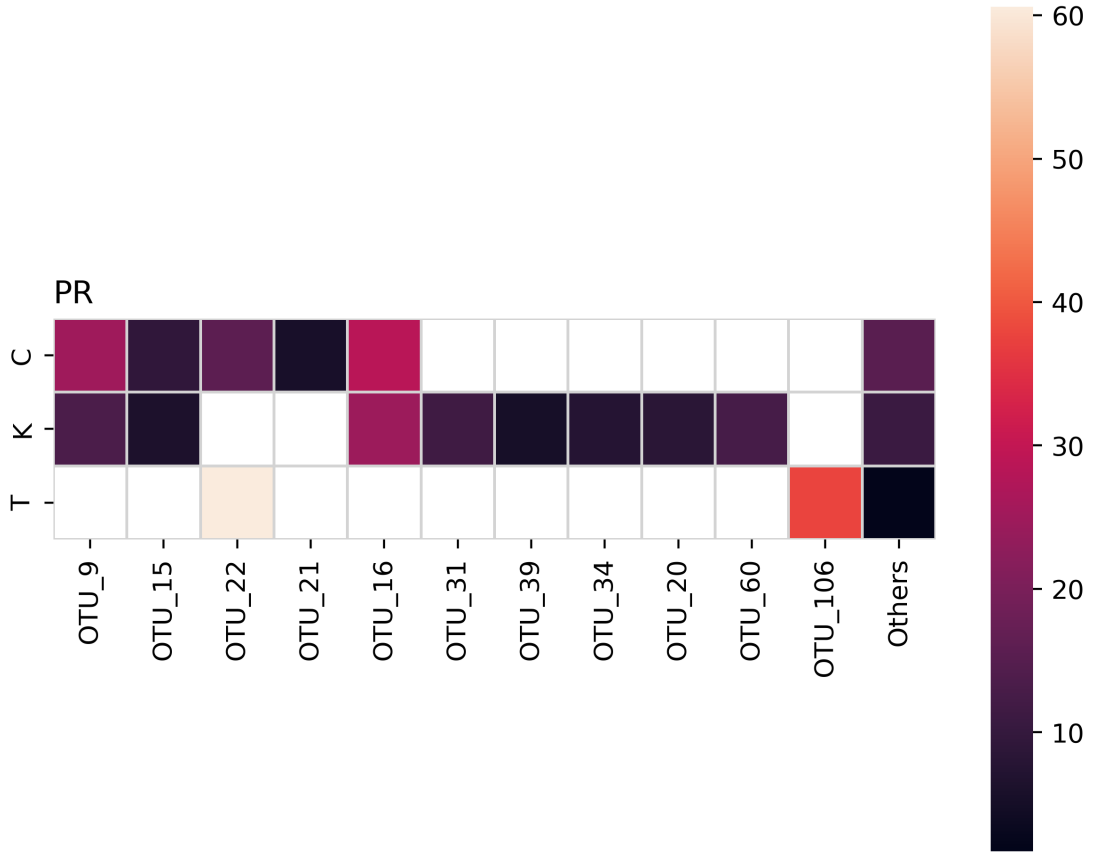

Figure 86: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using pristane as a sole carbon source

PR- pristane; C- chromosol soil; K- kandosol soil; T- tenosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

Triethanolamine

When triethanolamine was the sole source of carbon, 16 common OTUs were detected (at >5% of the amplicon pool) (Figure 87). Almost all of these OTUs have been discussed previously including OTU_9, OTU_15 (see aminoethanol), OTU_16 (see o-cresol). OTU_22

(see benzisothiazolinone), and OTU_31 (see o-cresol), but their presence here indicates they can also use triethanolamine as a sole source of carbon.

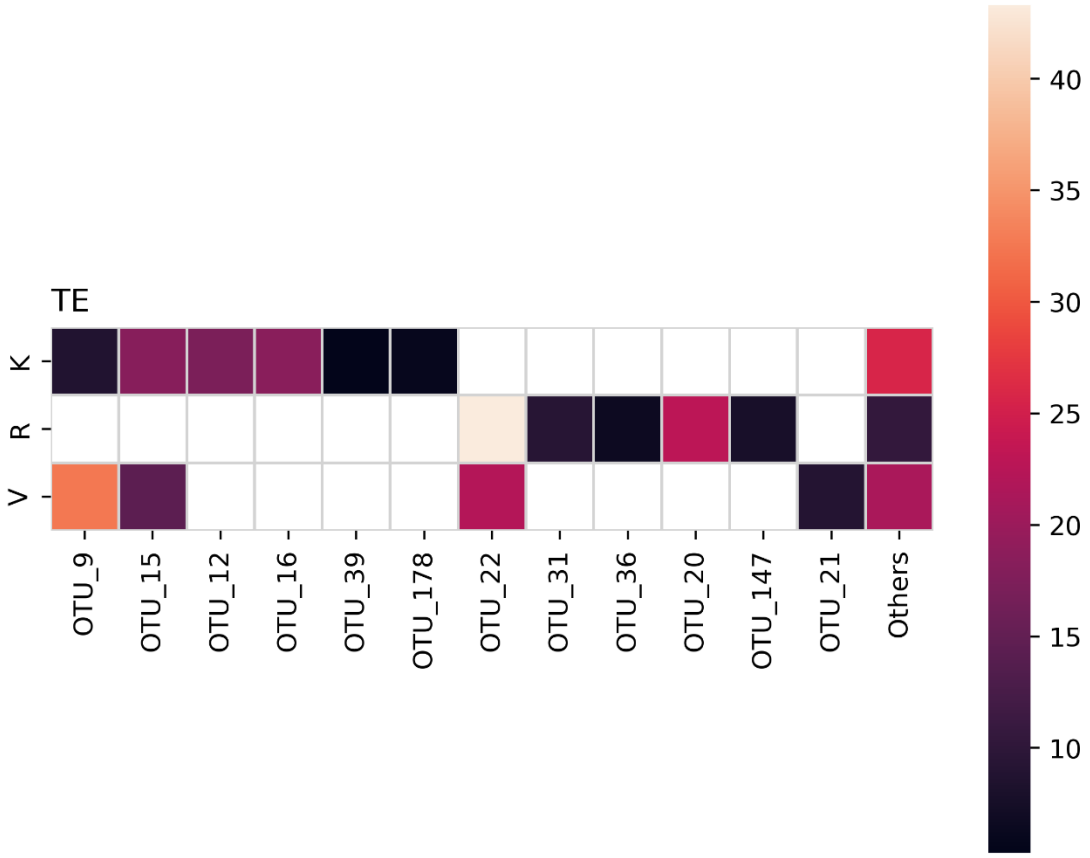

Figure 87: Heatmap of the most abundant prokaryotic taxa identified from Northern Territory soils using triethanolamine as a sole carbon source

TE- triethanolamine; C- chromosol soil; K- kandosol soil; R- rudosol soil; T- tenosol soil; V- vertosol soil

Scale bar indicates relative abundance (percentage of amplicon pool). Others represents the sum of OTUs that comprise less than 5% of the amplicon pool

The sole carbon experiments described here illustrate that numerous microbial catabolists of chemicals exist in the soils of the Beetaloo region. In particular, this series of experiments reveals a small cohort of OTUs that can degrade multiple chemicals (Figure 88).

Most notable of these are OTUs 9, 15, 21 and 22 with this group of four OTUs degrading a large number of chemicals as a sole source of carbon (Table 31 and Figure 88). Of these taxa, only OTU_21 was also observed to significantly interact with these chemicals in the soil microcosms in response to chemicals in two of the four soils (Figure 53 and Figure 55).

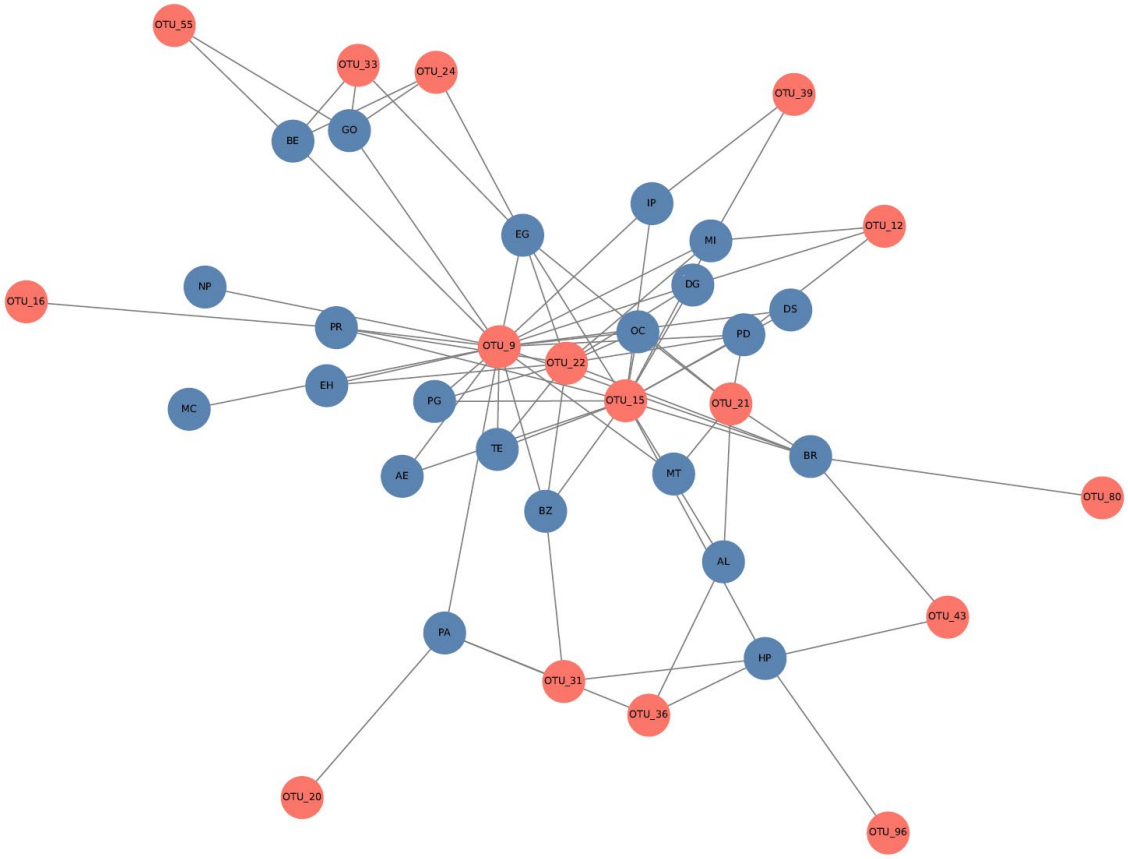

Figure 88: Network plot showing the relationships between OTUs and the chemicals they can use as a sole source of carbon

Lines between chemicals (in blue) and OTUs (in red) indicate that OTU degrade that chemical. Numbers in circles are OTUs. AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BE: 2-butoxyethanol; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; DL: d-limonene; DS: diesel fuel; EC: eicosane; EG: ethylene glycol; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; IP: isopropanol; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; MT: methanol; NP: naphthalene; OC: o-cresol; PA: polyacrylamide; PD: polyoxypropylene diamine; PG: propylene glycol; PR: pristane; TE: triethanolamine

There are, however, significant differences between the soil microbial response study and this sole carbon experiment. Most notably the sole carbon experiment requires that a species (a) grows readily in culture and (b) can use the chemical as a sole source of carbon (in the presence of exogenous nitrogen and phosphorus).

Many soil microbes do not readily grow in culture, and as such these sole carbon trials demonstrate the minimum number of taxa present in these soil that can use these chemicals as sole sources of carbon. This culture-reliant difference is reflected in the taxonomic resolution between the taxa identified in the two studies. In the soil microcosm experiments, taxa that increased were more often from poorly taxonomically defined

lineages, while in the sole carbon studies, more of the taxa were conspecific with known strains (i.e. they have been cultured previously by other researchers). Additionally, in the soil (as compared to the sole carbon source trials), different microbes may play different roles in chemical degradation, for example, some species may be able to deaminate polyacrylamide and use this polyacrylamide-N as a nitrogen source and be using alternative sources of carbon in the soil for growth (i.e. they may be unable to attack the polyacrylate backbone of polyacrylamide). Such taxa would be detected in the SIMPER (as they benefit from the available nitrogen) but would be absent here in the sole carbon trials where only those taxa that can use the compound as a source of carbon are able to thrive. Further, in the soil microcosms no additional nutrients were added other than the chemical in question. This means in practice that microbes faced with using an exogenous chemical as a newly available carbon source must source their nitrogen and phosphorus (and other minor/trace nutrients) from (mostly more complex forms) within the soil itself. This is strikingly different to the sole carbon source trials where nitrogen and phosphorus are supplied as soluble ammonium and phosphate, respectively.

In addition, OTUs 12, 24, 31, 33, 36 and 39 also degrade up to three different chemicals as a sole source of carbon (Table 32). Of these taxa, OTU_12 was also detected in the soil microcosm experiments. It is noteworthy that many of the lineages detected in sole carbon experiments are those that have been previously ascribed the catabolism of complex carbon, for example, many of the *Actinobacteria* detected (OTUs 15, 22 and 39) are from lineages known to be involved in degrading recalcitrant compounds or pollutants (Bourguignon et al., 2016; Decorosi et al., 2019; Kumano et al., 2016; Ren et al., 2021). Similarly, taxa in the *Methylobacteriaceae* while facultatively methylotrophic are also known to engage in a range of complex catabolic processes against a diverse range of often recalcitrant chemicals (Kelly et al., 2014).

Taken together, these data demonstrate that soils of the Beetaloo Region of the Northern Territory contain numerous species that are capable of degrading almost all chemicals tested at industrially relevant concentrations.

Table 31: OTUs that degrade the most chemicals as a sole source of carbon

OTU	Domain	Phylum	Class	Order	Family	Genus	Closest match	% ID
OTU_9	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	*Methylorubrum*	Methylorubrum populi BJ001(NR_029082)	100
OTU_15	Bacteria	Actinobacteria	Actinobacteria	Mycobacteriales	Mycobacteriaceae	*Mycobacterium*	Mycolicibacterium phocaicum N4 (NR_043237)	100
OTU_21	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Massilia*	Massilia pinisoli T33 (NR_152009)	99.6
OTU_22	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	*Sinomonas*	Sinomonas humi MUSC 117 (NR_134809)	100

Table 32: OTUs that degrade up to three different chemicals as a sole source of carbon

OTU	Domain	Phylum	Class	Order	Family	Genus	Closest match	% ID
OTU_12	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	*Methylobacterium*	*Methylobacterium phyllostachyos* BL47 (NR_108242)	100
OTU_24	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*	*Agrobacterium fabrum* C58 (NR_074266)	100
OTU_31	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Rhodanobacteraceae	*Dyella*	*Dyella flava* DHOC52 (NR_157998)	98.8
OTU_33	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	*Sphingomonas alpina* S8-3_(NR_117230)	100
OTU_36	Bacteria	Bacteroidetes	Chitinophagia	Chitinophagales	Chitinophagaceae	*Sediminibacterium*	*Sediminibacterium magnilacihabitans* MU-2 (NR_133888)	100
OTU_39	Bacteria	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	*Amycolatopsis*	*Amycolatopsis circi* S1.3 (NR_117914)	100

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