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Microbial degradation of chemical compounds used in onshore gas production in the SE of South Australia

GISERA W.15

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We thank Glenn Toogood and Simon Mooney of Beach Energy for their assistance with sampling in the Penola region of South Australia.

We acknowledge the Traditional Custodians whose ancestral lands we live and work upon and we pay our respects to their Elders past and present. We acknowledge and respect the deep spiritual connection and the relationship that Aboriginal and Torres Strait Islander people have to Country.

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

Executive summary

The primary objective of this report is to provide information on the microbial degradation of chemicals used in gas production from conventional reservoirs in the onshore Otway Basin, South Australia. With various projects investigating the impacts of conventional gas production within south east South Australia already underway or completed, this report is designed to provide further scientific insights for stakeholders about the biodegradation of chemicals used in gas exploration and production activities.

Key findings

- All chemical compounds examined by commercially available accredited tests (2-butoxyethanol, 2-ethylhexanol, acetic acid, 2-aminoethanol, ethanol, ethylene glycol, isopropanol, methanol and propylene glycol) were undetectable in soil after 34 days of incubations.
- In aquifer microcosms, commercially available accredited tests measured the partial degradation of methanol, ethylene and propylene glycol and 2-aminoethanol. Ethanol, isopropanol, acetic acid, 2-butoxyethanol and 2-ethylhexanol did not degrade during the 34 days of incubation.
- With one exception, all chemical compounds tested as sole carbon sources (2-aminoethanol, 2-ethylhexanol, benzisothiazolinone, bronopol, c12 alcohol ethoxylate, diethylene glycol ethyl ether, d-limonene, d-limonene x10, eicosane, glutaraldehyde, glyoxal, hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine, methylchloroisothiazolinone, methylisothiazolinone, naphthalene, naphthalene x10, o-cresol, o-cresol x10, polyacrylamide, polyoxypropylene diamine, pristane, triethanolamine, xanthan gum) had observable fungal and bacterial growth. On bronopol, only fungal growth was observed. Taxa that were significantly increased in abundance, compared to controls, were observed on all chemicals, except for c12 alcohol ethoxylate and glutaraldehyde in fungi and 2-aminoethanol, bronopol, o-cresol (at x10 concentration) in bacteria. For a small number of these chemicals (hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine, glutaraldehyde,

benziosthiazolinone, methylchloroisothiazolinone, 2-ethylhexanol and o-cresol), contradictory results were observed in liquid culture trials (data not shown). For this select group of chemicals further work to determine their biodegradation potential may be valuable.

- Effects of tested chemicals (2-aminoethanol, 2-butoxyethanol, 2-ethylhexanol, o-cresol, acetic acid, d-limonene, ethanol, ethylene glycol, isopropanol, methanol, naphthalene and propylene glycol) on microbial community structure in soil microcosms differed between the prokaryotic and fungal communities. Briefly, the prokaryotic community structure was largely unaltered by exposure to tested chemicals, with the exception of 2-aminoethanol and ethylene glycol. In contrast, the fungal community structure was altered by many of the chemicals tested, most markedly, 2-butoxyethanol and 2-aminoethanol. For fungal communities, intra-treatment variation on individual chemicals was significant.
- Putative indicator prokaryotic taxa were identified for 2-aminoethanol, 2-butoxyethanol, acetic acid and ethylene glycol in soil microcosms.
- Putative indicator fungal taxa were identified for 2-aminoethanol, 2-butoxyethanol, ethylene glycol, isopropanol and o-cresol in soil microcosms.
- Effects of tested chemicals (2-aminoethanol, 2-ethylhexanol, acetic acid, benzisothiazolinone, bronopol, c12 alcohol ethoxylate, diethylene glycol ethyl ether, d-limonene, d-limonene x10, eicosane, glutaraldehyde, glyoxal, hexahydro-1,3,5-tris (2-hydroxyethyl)-sym-triazine, methylchloroisothiazolinone, methylisothiazolinone, naphthalene, naphthalene x10, o-cresol, o-cresol x10, polyacrylamide, polyoxypropylene diamine, pristane, triethanolamine, xanthan gum) on prokaryotic microbial community structure in aquifer microcosms were marked. All treatments, with the exception of diethylene glycol ethyl ether had significant and replicable effects on microbial community structure.
- Putative indicator prokaryotic taxa were identified for 2-ethylhexanol, acetic acid, benzisothiazolinone, naphthalene, o-cresol, triethanolamine and xanthum gum in aquifer microcosms.

Overall, most chemicals examined in the present study either appear to be completely degraded in soil or are able to be used as sole-carbon sources for microbial growth by taxa from Penola soils. While soil bacterial communities are largely unaltered by chemical additions, soil fungal

communities are altered more by chemical additions. Soil bacteria are responsible for a range of geochemical processes and their resilience to chemical additions presumably indicates these ecosystem services undertaken by bacteria are largely unaffected. In contrast, fungal communities support a range of soil functions (e.g. carbon cycling and translocation, support of plant growth through mycorrhizal interactions) and alterations in their structure, resulting from chemical additions, have unknown implications to ecosystem services provided by affected fungi.

In the Penola Tertiary Limestone Aquifer, some chemicals were significantly degraded, while others were not degraded in experiments. Unlike soils, the aquifer examined is limited in important macronutrients (carbon, nitrogen and phosphorus) and an absence of degradation may be due to nitrogen or phosphorus limitation rather than the inability of microbes present in the aquifer to degrade these chemicals. As a result of lower microbial population sizes and less macronutrients, aquifer prokaryotic communities were proportionally more affected by the addition of the various chemicals examined. Further work is required to understand the ecosystem services supported by these microbial communities.

1 Introduction

1.1 Project goals and summary

As Australia progressively shifts towards a renewable-based energy supply, natural gas is expected to play a significant role as a transitional energy source. Australian onshore gas operations require the use of a range of chemical products for various exploration and production activities. The risks associated with these chemicals have been the focus of numerous reviews, which identified potential environmental and human health impacts[1]. While the risks of these chemicals have largely been identified, less is known regarding their migration and degradation in relevant edaphic and subsurface environments. This project was commissioned with a view to understand the degradation potential of various chemical compounds used during onshore gas production and to identify organisms involved in these degradations. In parallel, the project sought to identify potential indicator taxa whose increased growth or loss may be an early indicator of environmental disturbance. Particularly, this project examined soil and aquifer microbial communities from the onshore Otway Basin in SE of South Australia as they likely inhabit zones of disturbance in the event of a chemical spill. This study will also provide information on (a) microbes involved in degradation and (b) changes in microbial community structure (i.e. composition and abundance of microorganisms). The latter data may be useful for environmental monitoring and similar insights have been previously used in ecosystem health assessments as indicators of disturbance and change.

1.2 Chemicals associated with conventional onshore gas activities

Community concerns regarding the chemicals used in onshore gas exploration, production and reservoir stimulation are mainly centred on potential contamination risks to soil and aquifers, particularly in agriculturally important areas. As part of Project W15, an extensive literature review was conducted to investigate the types of chemicals used in onshore gas activities in Australia and

[1] (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)

to provide estimates of chemical concentrations used, potential biodegradation pathways and associated policy frameworks (Schinteie et al., 2019).

While the chemicals used in onshore gas activities typically comprise only a small fraction of drilling and stimulation fluids, they perform important functions in the exploration and production processes. At various stages of onshore gas operations, water, proppants, and chemical additives are mixed to produce drilling and reservoir stimulation fluids. Estimates of water use for such fluids can vary and depend on multiple factors including well type, number of wells, number of stimulation stages and designs. A range of naturally occurring abiotic and biotic processes have the capacity to attenuate such chemical compounds in the environment. It is therefore important to identify such processes when assessing potential contamination impacts in areas where onshore gas activities are about to be undertaken.

Table 1-1 lists the chemicals examined in this report and their role in onshore gas activities. These roles include:

a. Biocide - used to control microbial growth and souring problems within reservoirs, by destroying or inhibiting the growth of microorganisms.
b. Buffer - used to control pH in fluids.
c. Corrosion inhibitor - generally adsorb onto pipework steel and prevents corrosion.
d. Epoxy resins - used in pipework for protective coatings.
e. Friction reducer - used to reduce water friction pressure within pipes during high rate pumping.
f. Surfactants - used to modify emulsion surface or interfacial tensions.
g. Viscosity management - used to control gelation of fluids and assists in carrying chemicals and proppants.

Table 1-1: Onshore gas production chemical compounds and their uses.

Chemicals	Additive role in onshore gas activities
2-aminoethanol	Viscosity management/ drilling additive
2-butoxyethanol	Surfactant
2-ethylhexanol	Surfactant
acetic acid	Buffer, stabiliser, solvent
benzisothiazolinone	Biocide
bronopol	Biocide
c12 alcohol ethoxylate	Surfactant
diethylene glycol ethyl ether	Solvent
d-limonene	Surfactant
eicosane	Surfactant
ethanol	Surfactant
ethylene glycol	Viscosity management
glutaraldehyde	Biocide
glyoxal	Viscosity management/ crosslinker
hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine	Biocide
isopropanol	Surfactant
methanol	Surfactant
methylchloroisothiazolinone	Biocide
methylisothiazolinone	Biocide
naphthalene	Corrosion inhibitor
o-cresol	Biocide
polyacrylamide	Friction reducer
polyoxypropylene diamine	Pipework/Epoxy resins/Hardener
pristane	Surfactant
propylene glycol	Viscosity management
triethanolamine	Viscosity management
xanthan gum	Viscosity management

1.3 Classification of microorganisms and taxonomic schemes

This report uses modern taxonomic descriptors to classify microorganisms, including bacteria, fungi and archaea. To assist with the interpretation of results described in this report, a brief introduction to microbial taxonomy is provided below.

Microbial taxonomy is the science of naming, defining and classifying groups of microorganisms based on their shared morphological and molecular (mainly DNA-based) similarities with reference to their evolutionary relationships. Microbial taxonomy can be thought of as a filing system to organise microorganisms based on evolutionary relationships and to provide labels to assist with understanding the complex inter-relationships between microorganisms. This filing system used for microorganisms is called a taxonomic scheme and was first conceived by Carl Linnaeus in the 1730s to classify plants and animals. A taxonomic hierarchy is used, whereby microorganisms are organised into progressively more inclusive groups, or taxa, based on observable similarities or evolutionary relationships. The highest taxonomic rank is “domain”, with the lowest rank being the “species”.

Presently, the three-domain system suggested by Carl Woese in 1977 (Woese and Fox, 1977) is the accepted classification system for all living organisms. The three domains of this system are the Bacteria, the Archaea and the Eukaryota (Figure 1-1). Animals and plants belong to the domain Eukaryota, as do fungi. Bacteria and Archaea belong to their own domains, and despite morphological similarities, are not related. Indeed, Archaea are more closely related to Eukaryota than they are to the Bacteria (Figure 1-1).

In the taxonomic scheme, there are seven taxonomic ranks, which are domain, phylum (termed ‘division’ for plants and fungi), class, order, family, genus and species. By convention, a binomial naming system or nomenclature, using the genus and species names, is used to name microorganisms. For example, the bacterium *Pseudomonas aeruginosa* belongs to the domain Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, family Psudomonadaceae, genus *Pseudomonas* and species *aeruginosa* (Figure 1-2). Similarly, humans are *Homo sapiens*, belong to the species *sapiens*, the genus *Homo*, the family Hominidae, the order Primates, the class Mammalia, the phylum Chordata and the domain Eukarya. Figure 1-2 gives other examples of lineages and their place in the taxonomic scheme.

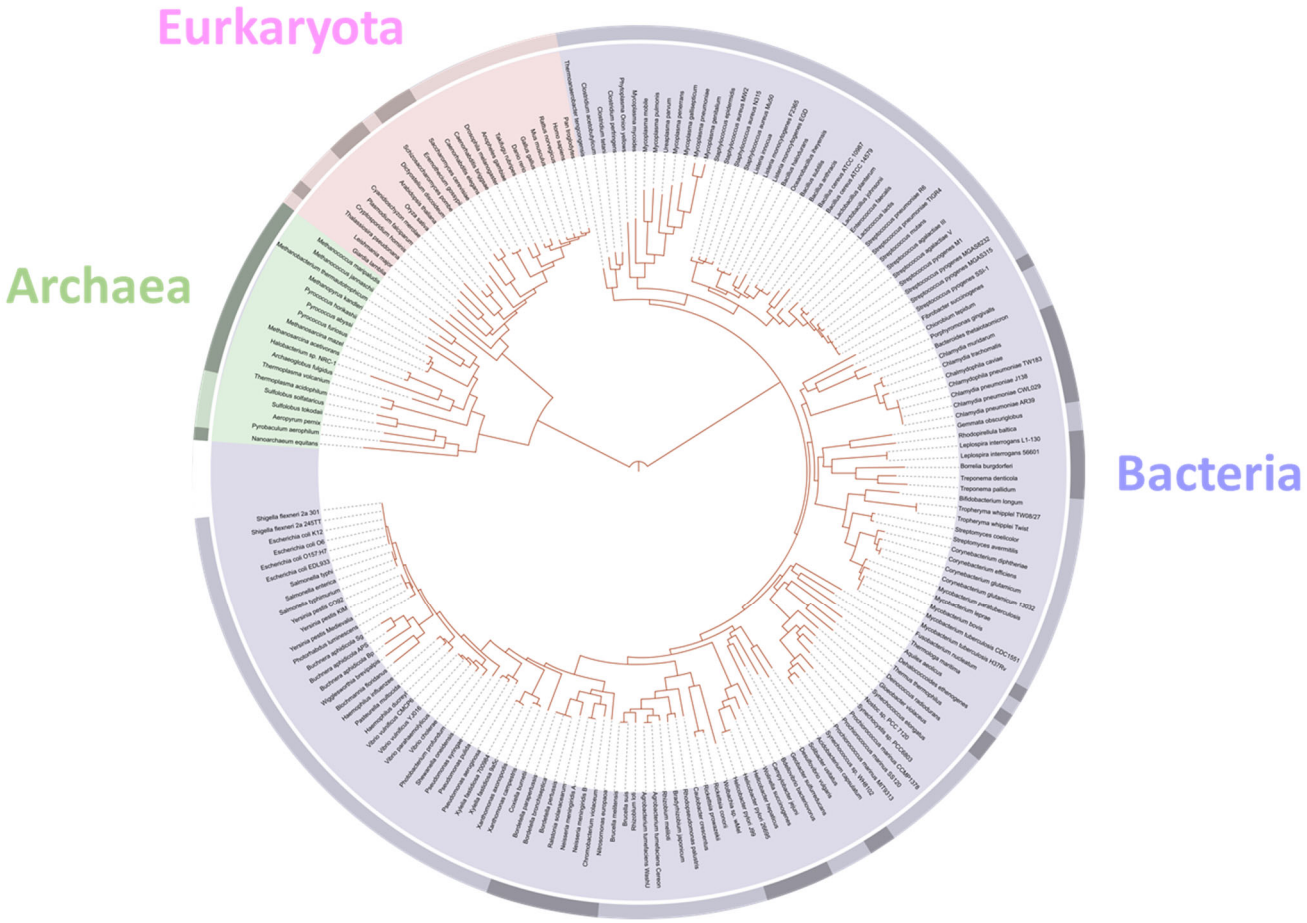


Figure 1-1: A phylogenetic tree of life.

The three domains of life are shown. Archaea in green, Bacteria in purple and Eukaryota in pink.

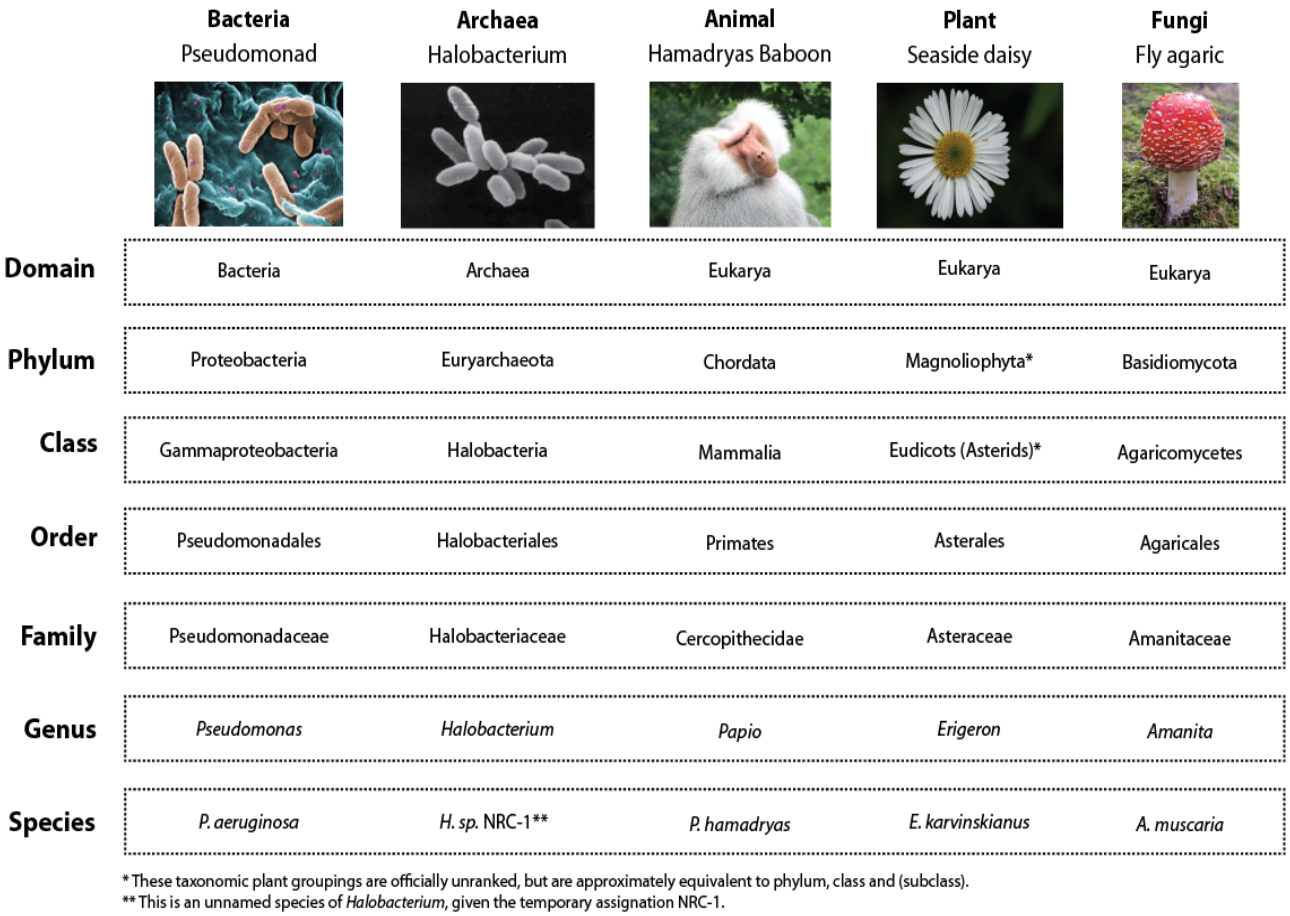


Figure 1-2: Examples of taxonomic lineages for groups from the three domains of life.

The advent of DNA-based approaches and technologies to identify and classify microbes resulted in a more systematic and analytical understanding of taxonomy. DNA sequencing analyses of microbial communities relies on the presence of a particular taxonomic marker gene that is found in the genomes of all DNA-based lifeforms. For bacteria and archaea the 16S ribosomal RNA sequence region (16S rRNA) is typically used, and for fungi the Internally Transcribed Spacer gene region (ITS) is commonly used (Figure 1-3). Both the ITS and the 16S regions are part of the genomes that are associated with the ribosome (a non-membrane bound organelle required for RNA translation to protein).

DNA sequencing of environmental samples can be used to identify, classify and quantify microorganisms in complex biological samples. Bioinformatic analysis of DNA sequencing data identifies closely related microorganisms and clusters them into Operational Taxonomic Units (OTUs). OTUs can be roughly equated to a microbial genus (for 16S) or a microbial species (for ITS) and can be used for describing microbial diversity. OTUs are defined by DNA sequence similarity clustering, and the currently accepted similarity threshold used is 97% (Schmidt et al., 2014).

2014). In this report, bacterial and archaeal OTUs, referred to in this report as prokaryotic OTUs (pOTUs), have been assigned numbers derived from analyses of 16S RNA sequences and fungal OTUs, referred to in this report as fungal OTUs (fOTUs), have been separately assigned using ITS sequences.

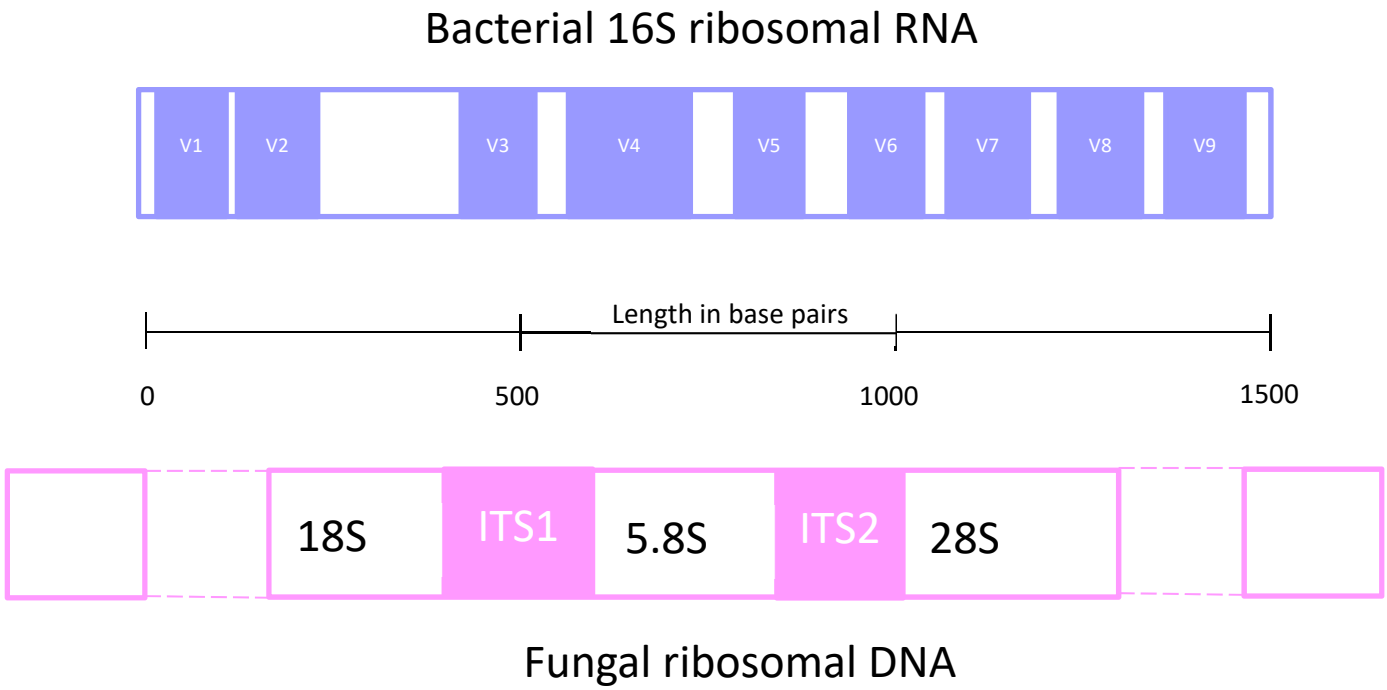


Figure 1-3: Schematic representation of bacterial and fungal ribosomal gene regions. Variable regions of ribosomal genes are labelled (V1-9 in bacteria and ITS1 and ITS2 in fungi).

Note: In this project the bacterial V4 region and the fungal ITS1 region were used for DNA sequencing analysis to identify, classify and quantify bacteria/archaea and fungi, respectively.

1.4 Soil microbiology

Soils provide a habitat for a diverse range of microorganisms, including bacteria, archaea and fungi and it has been estimated that in a gram of soil there are billions of microbial cells (Rossello-Mora and Amann, 2001; Whitman et al., 1998). Indeed, one gram of soil contains more microbial cells than there are humans on planet Earth. Bacteria are the most abundant and diverse in these microbial communities, however, fungi contribute the largest part of the total microbial biomass in soils (Montgomery et al., 2000; Pepper, 2019). Estimates of microbial diversity range from between 2,000 to 18,000 types of organisms in a gram of soil (Sandaa et al., 1999; Torsvik et al.,

1996, 1990; Vigdis and Lise, 2002). Only a small fraction of these species has been identified, classified and characterised.

Soil microbial communities are an essential component of biodegradative processes and decomposition and represent a primary pathway for the chemical contaminants from onshore gas activities to be removed from the environment. Soil microbial communities can change rapidly in response to changes in soil properties, including physical and chemical characteristics (soil type, moisture, temperature, pH, electrical conductivity, salinity, compaction, organic matter content), as well as nutrient availability (carbon substrates). Some taxa in microbial communities are very sensitive to changes in soil environment, while others are extremely adaptive and able to withstand extreme changes. These shifts in microbial function relate to stress responses and changes in metabolism. Changes in microbial community structure are an indicator of the complex biogeochemistry involved in the biodegradation of onshore gas-related chemicals and are an adaptation of microbial communities towards taxa best suited for biodegradation of these compounds. Biodegradation of chemicals in soils is a complex process that depends on the nature and on the concentration of the chemical present.

1.5 Aquifer microbiology

While aquifer or groundwater ecosystems offer vast and complex habitats for microbial communities, such environments are less species rich and offer less physicochemical niches compared to soils. Physicochemically, aquifers represent a diverse array of environments, ranging from shallow alluvial sediment-rich aquifers to groundwater of various chemistries flowing through rock strata. In the latter, bacteria and archaea are the main microorganisms found in anoxic aquifer systems (Griebler and Lueders, 2009) and these communities are active and important in biogeochemical processes and in the degradation of chemical contaminants (Cho and Kim, 2000; K. P. Feris et al., 2004; Kevin P. Feris et al., 2004; Franzmann et al., 2002; Haack et al., 2004; Johnson et al., 2004); while in the former, stygofauna, fungi and other microbial eukaryotes can also be found (Madsen and Ghiorse, 1993). Estimates of cell density of bacteria in groundwater vary by the type of aquifer and can span several orders of magnitude between 10^2 and 10^6 cells per ml of water (Griebler and Lueders, 2009; Whitman et al., 1998).

Shallow aquifers behave more similarly to soil ecosystems, but in the present study we are dealing with limestone aquifers. Within the limestone aquifer, cell carbon can only be derived from either

ingress of exogenous carbon with meteoric water flows, or autotrophic carbon fixation via CO_2. The latter requires chemical energy via oxidation of substrates (Gold, 1992; Kotelnikova and Pedersen, 1997; Saini et al., 2011; Stevens, 1997; Stevens and McKinley, 1995).

Aside from alluvial aquifers, these ecosystems are generally low in organic carbon and relatively depleted in biologically important nutrients. All microorganisms in this environment whether autotrophs or heterotrophs, are adapted to nutrient-poor ground water (Ghiorse and Wilson, 1988; Madsen and Ghiorse, 1993).

In these environments, the addition of organic chemical compounds is likely to cause profound shifts in microbial community structure, favouring microorganisms capable of catabolising these compounds and disadvantaging microbes for which these compounds are either inaccessible or toxic.

1.6 Soils and aquifers of the Penola region, South Australia

The Penola land system consist of two main soil types, being shallow red loam on limestone (Petrocalcic red-brown dermosol) and shallow loam over red-brown clay on calcrete (Petrocalcic red chromosol-kandosol). These two soil types make up more than 50% of the soils of the Penola region, with a variety of minor soils. All soils are well drained and have low organic matter (*Penola Land System*, n.d.).

The Tertiary Limestone Aquifer is the principal source of water in the Penola area, and is used extensively for domestic, agricultural (irrigation, stock) and industrial purposes. This aquifer is a shallow subsurface aquifer (less than 500m, subsurface) that is mostly in the Gamibier Limestone and Dilwyn Formation layers (Figure 1-4). In comparison, the hydrocarbon targets for energy exploration are more than 1km deeper and span the Katnook to Sawpit Sandstone layers (Figure 1-4).

In most areas the aquifer is only moderately brackish having an electrical conductivity (EC) less than 3000µS/cm and, given its limestone origins it is also moderately alkaline (*The Tertiary Limestone Aquifer*, 2014).

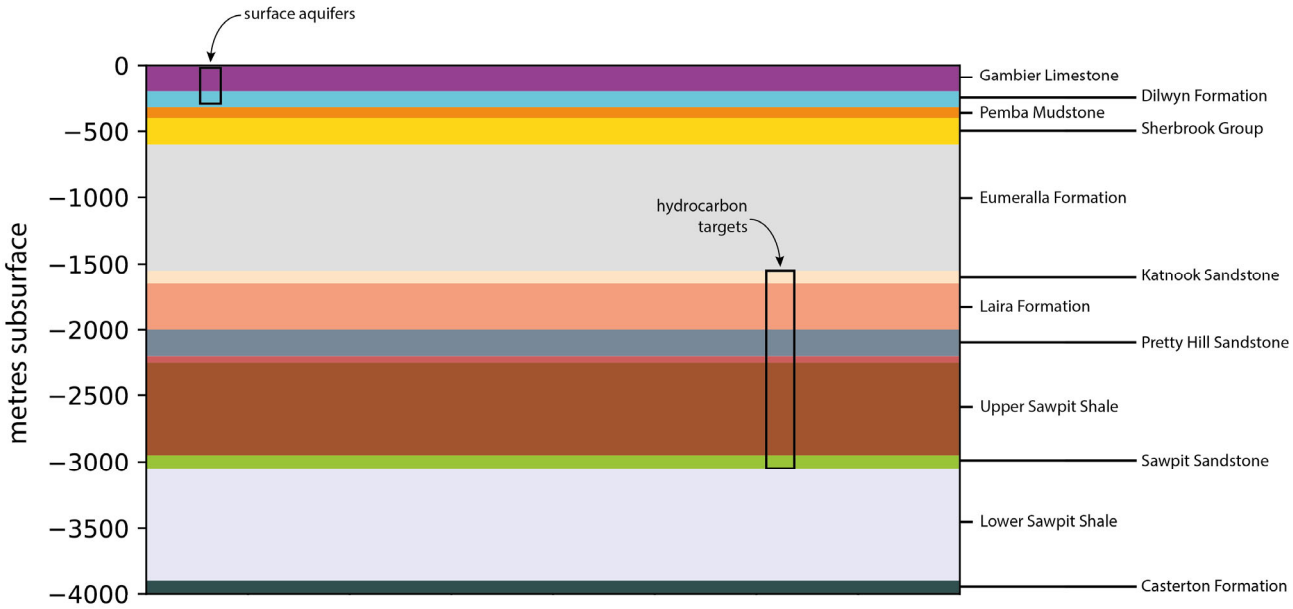


Figure 1-4: Indicative cross-section in the onshore Otway Basin, showing major stratigraphy and location of hydrocarbon targets.[2]

Information used to construct the figure was sourced from Beach Energy

1.7 Experimental design

Prior to experimental work, a literature and policy review (Schinteie et al., 2019) was conducted to ascertain prior knowledge of the degradation of the compounds of interest and their breakdown mechanisms (Task 1).

Soil and Tertiary Limestone Aquifer samples were collected from the Penola region of South Australia (Figure 1-5) under oxic and anoxic conditions, respectively (Task 2). These samples were used to setup replicated microcosms (both anoxic and oxic) to measure soil or aquifer degradation of individual chemicals using either analytical techniques (Task 3) or microbial growth assays (Task 4; see Figure 1-6). Where available, degradation of chemicals were determined with existing commercially analytical methods, otherwise sole carbon source growth trials were conducted on solid media and in anoxic water samples (Figure 1-6). Growth assays provide evidence that

[2] (*Assessment of Beach Energy ' s proposed onshore Otway Basin petroleum production & processing activities*, 2019)

microbes can grow on the chemical compounds as a sole source of carbon, however the rates of degradation and the residual compounds of degradation cannot be ascertained from the growth assay.

Figure 1-5: Geographic location of sampling. Copyright ©Google Maps 2019.

A: South Australia; B: Detailed map of South Australia; Red pin indicates the location of Penola.

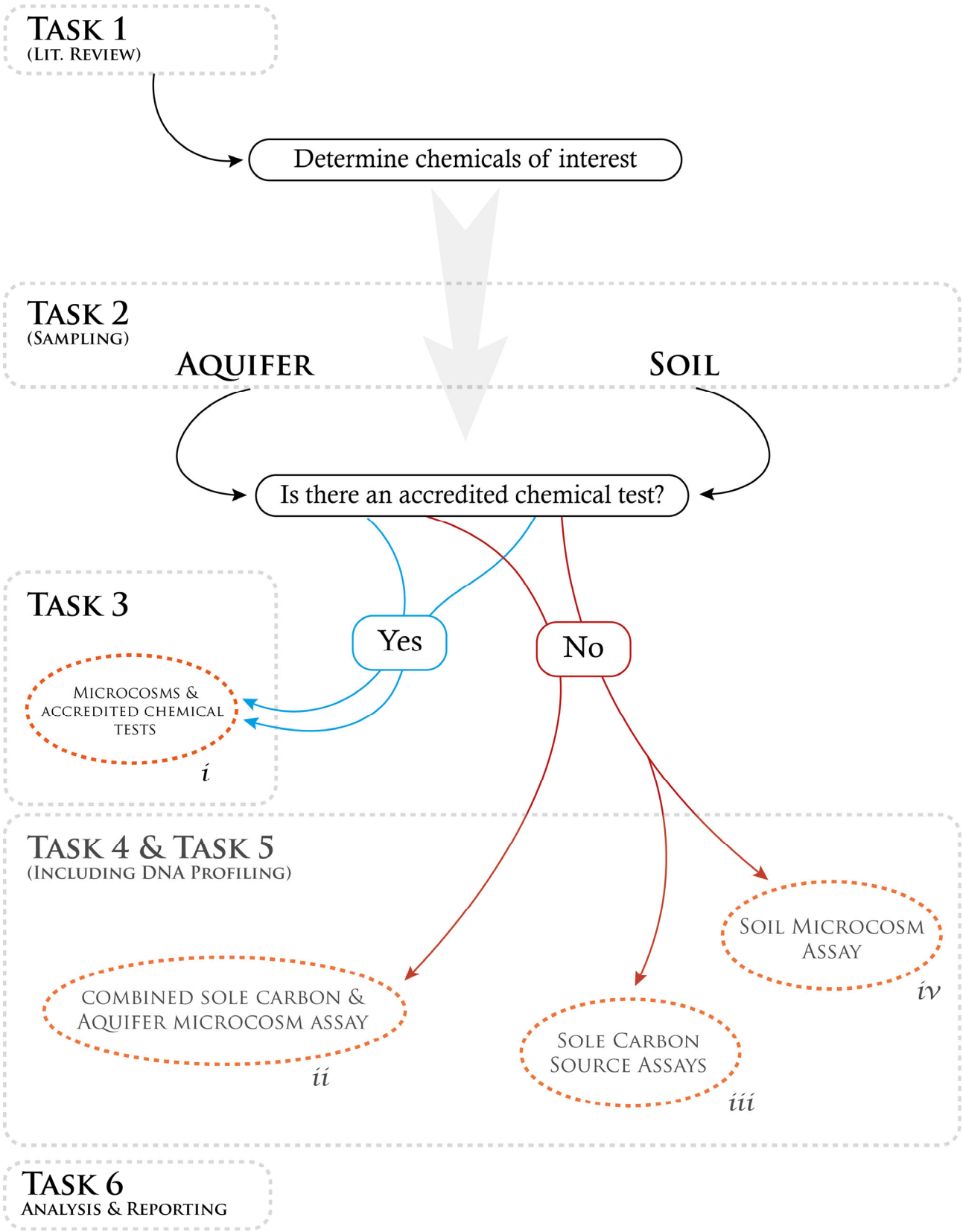


Figure 1-6: Schematic of workflow for onshore gas production chemical compound testing.

List of chemicals examined by activity:

i – 2-butoxyethanol, 2-ethylhexanol, acetic acid, 2-aminoethanol, ethanol, ethylene glycol, isopropanol, methanol and propylene glycol

ii – 2-aminoethanol, 2-butoxyethanol, 2-ethylhexanol, acetic acid, d-limonene, ethanol, ethylene glycol, isopropanol, methanol, naphthalene, o-cresol, propylene glycol. Naphthalene, o-cresol and d-limonene were also tested at 10x concentrations.

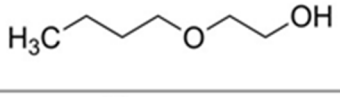
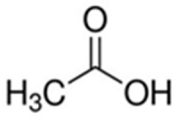
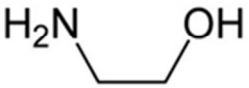
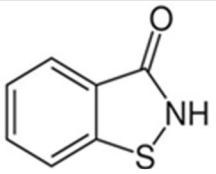
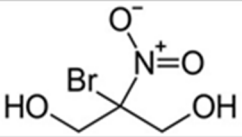
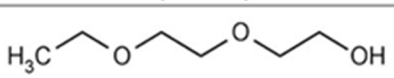
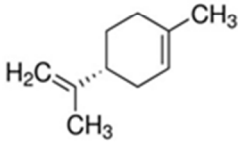
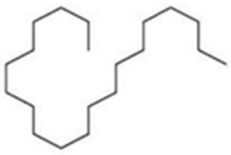
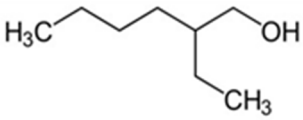
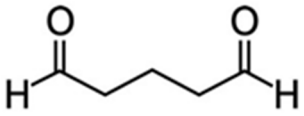
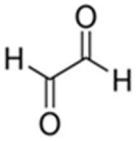
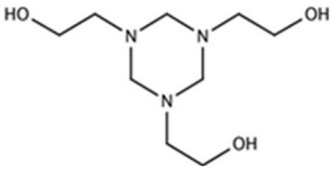
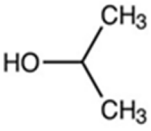
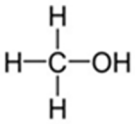
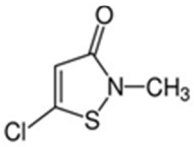
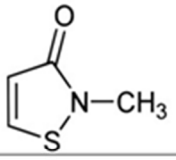
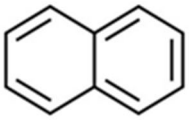
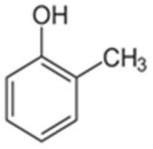
iii – 2-aminoethanol, 2-ethylhexanol, benzisothiazolinone, bronopol, c12 alcohol ethoxylate, diethylene glycol ethyl ether, d-limonene, eicosane, glutaraldehyde, glyoxal, hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine, methylchloroisothiazolinone, methylisothiazolinone, naphthalene, o-cresol, polyacrylamide, polyoxypropylene diamine, pristane, triethanolamine, xanthan gum. Naphthalene, o-cresol and d-limonene were also tested at 10x concentrations.

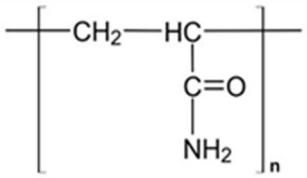
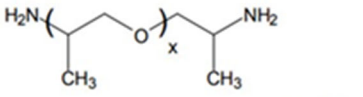
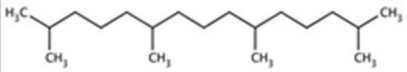
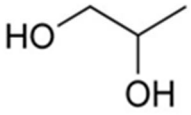
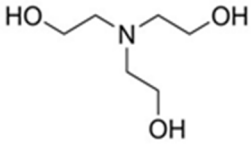
iv – 2-aminoethanol, 2-butoxyethanol, 2-ethylhexanol, o-cresol, acetic acid, d-limonene, ethanol, ethylene glycol, isopropanol, methanol, naphthalene and propylene glycol

1.8 Chemicals used in this study

The following chemical compounds were investigated: 2-aminoethanol; 2-butoxyethanol; 2-ethylhexanol; acetic acid; benzisothiazolinone; bronopol; c12 alcohol ethoxylate; diethylene glycol ethyl ether; d-limonene; eicosane; ethanol; ethylene glycol; glutaraldehyde; glyoxal; hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine; isopropanol; methanol; methylchloroisothiazolinone; methylisothiazolinone; naphthalene; o-cresol; polyacrylamide; polyoxypropylene diamine; pristane; propylene glycol; triethanolamine and xanthan gum.

The structures of all chemicals used in this project are shown in Figure 1-7.

2-butoxyethanol	
acetic acid	
2-aminoethanol	
c12 alcohol ethoxylate	
benzisothiazolinone	
bronopol	
diethylene glycol ethyl ether	
d-limonene	
eicosane	
ethanol	
ethylene glycol	
2-ethylhexanol	
glutaraldehyde	
glyoxal	
hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine	
isopropanol	
methanol	
Methylchloroisothiaz-olinone	
methylisothiazolinone	
naphthalene	
o-cresol	

polyacrylamide	
polyoxypropylene diamine	
pristane	
propylene glycol	
triethanolamine	

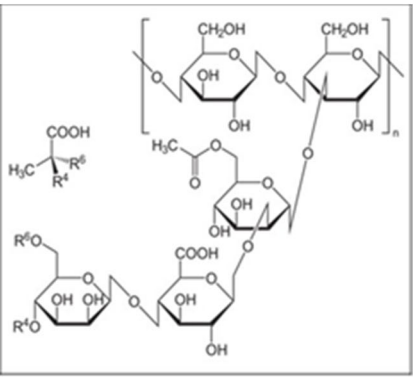
xanthan gum	

Figure 1-7: Structures of chemicals used in this project

2 Aims

The aim of this project is to better understand the impacts and residual risk of chemical compounds used in onshore gas activities to the natural environment over time.

Specifically, this project seeks to understand whether compounds used in the production of onshore gas are degraded by microbes in relevant southeast South Australian soils and subsurface aquifers, under oxic and anoxic conditions, respectively. In addition, the project seeks to examine the impact of these compounds on microbial communities. This will provide additional information on (a) those microbes involved in degradation, and (b) changes in microbial consortia. The latter data may be useful for environmental monitoring and has previously been applied elsewhere in ecosystem health assessments as indicators of disturbance and change.

3 Methods

3.1 Task 1

A literature and policy review was conducted at the commencement of the project (Schinteie et al., 2019) to ascertain prior knowledge of the degradation of the compounds of interest and their breakdown mechanisms.

3.2 Task 2

3.2.1 Samples used in this project

Soil

Soil was sampled using sanitised spades into a sanitised polypropylene box. Field sanitisation was undertaken by washing in water, followed by rinsing with 70% ethanol. A soil sample was taken from land ~1km south of the Katnook Gas Plant (Beach Energy). The land was directly adjacent to grazing properties at -37.464448 S, 140.781994 E (see Figure 3-1). Upon collection the surface litter was removed prior to collection being undertaken. The sample was stored in the dark and transported by road back to laboratories in North Ryde, NSW.

Bulk Water

The water used in this project was sourced from the primary aquifer (Tertiary Limestone Aquifer) used for agriculture in the region. Bulk collections were collected at the Katnook Gas Plant at -37.454111 S, 140.784397 E (Figure 3-1) from an irrigation hose and stored in 20L polypropylene drums for transport.

Anoxic Water

A smaller volume of ~3L water was anoxically collected (under an argon atmosphere) at the same location as the bulk water collection (see Figure 3-1). Water was bubbled vigorously with argon prior to being sealed in Schott bottles with silicone rubber seals. Sealed bottles were transported inside eskies 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.

Figure 3-1: Sampling locations in Penola, South Australia. Copyright ©Google Maps 2019.

Chemicals used in this project

Chemicals used in this project were sourced from chemical suppliers as shown in Table 3-1. Of those compounds described in the literature review as part of the W.15 project (Task 1) (Schinteie et al., 2019), all were included in the present study except for Pigment Red 5 which was unable to

be sourced. C12 alcohol ethoxylate was used to represent alcohols, C6-12, ethoxylated. Pristane (branched C15) and eicosane (linear C20) were used to represent alkanes, C12-26 branched and linear compound, respectively.

Table 3-1: Sources and concentrations of chemicals used in this project

Chemicals	Abbreviations	CAS #	Supplier/Concentration Notes
2-aminoethanol	AE	141-43-5	Sigma-Aldrich, 98%
2-butoxyethanol	BE	111-76-2	Sigma-Aldrich, 99%
2-ethylhexanol	EH	104-76-7	Sigma-Aldrich, 99.6%
acetic acid	AA	64-19-7	Sigma Aldrich, 100%
benzisothiazolinone	BZ	2634-33-5	Sigma-Aldrich, 97%
bronopol	BR	52-51-7	Sigma-Aldrich, 98%
c12 alcohol ethoxylate	eAL	68439-45-2	Oleum, 100%
diethylene glycol ethyl ether	DG	111-90-0	Sigma-Aldrich 99%
d-limonene	DL	138-86-3	Sigma-Aldrich, 90%
d-limonene x10	DLx	138-86-3	Sigma-Aldrich, 90%
eicosane	EC	112-95-8	Fluka, 97%
ethanol	ET	64-17-5	Sigma-Aldrich, 200 Proof Absolute
ethylene glycol	EG	107-21-1	Sigma-Aldrich, 99%
glutaraldehyde	GL	111-30-8	Sigma-Aldrich, 50%
glyoxal	GO	107-22-2	Sigma-Aldrich, 40%
hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine	HHT	4719-04-4	Combi-Blocks, 75%
isopropanol	IP	67-63-0	BDH, 100%
methanol		67-56-1	Ajax, 99.8%
methylchloroisothiazolinone	MCT	26172-55-4	Combi-Blocks, 68%
methylisothiazolinone	MT	2682-20-4	Sigma-Aldrich, 95%

naphthalene	NP	91-20-3	BDH, 100%
naphthalene x10	NPx	91-20-3	BDH, 100%
o-cresol	OC	95-48-7	Sigma-Aldrich, 99%
o-cresol x10	OCx	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
pristane	PR	1921-70-6	Sigma-Aldrich, 98%
propylene glycol	PG	57-55-6	Sigma-Aldrich, 100%
triethanolamine	TE	102-71-6	BDH, 99%
xanthan gum	XG	11138-66-2	Sigma-Aldrich, 100%

CAS #- Chemical abstract society identifying numbers

3.3 Task 3, 4 and 5

3.3.1 Soil preparation

At the North Ryde laboratories, the soil was coarsely sieved through a 6mm mesh, prior to extensive mixing. It was stored in the dark prior to use in experiments in Task 3 and Task 4.

3.3.2 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, ~3g of oven-dried soil (80^{o}C | 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. Loss on ignition was conducted on four representative subsamples.

3.3.3 Aquifer water chemistry

Water chemistry on the bulk water sample was undertaken by ALS Environmental. The schedule of tests and their method references are shown in Appendix A.

3.3.4 Establishment of soil microcosms

In order to establish microbial degradation of 2-aminoethanol, 2-butoxyethanol, 2-ethylhexanol, o-cresol, acetic acid, d-limonene, ethanol, ethylene glycol, isopropanol, methanol, naphthalene and propylene glycol, soil microcosms were setup in 250mL, transparent, polypropylene vessels. Each vessel contained the following: 50mL soil (59.3g ± 0.43, n= 10), 20mL sterile reverse osmosis water (RO water) and the compound of interest. Concentrations of the compounds were determined from the literature review (Task 1) (Schinteie et al., 2019) and are shown in

Table 3-2. All vessels were established in triplicate. Vessels were setup for compound recovery analyses to determine initial compound concentrations. For incubation, vessels were stored in air-tight, transparent polypropylene outer containers (Figure 3-2). In order to maintain humidity inside vessels at conditions similar to those observed in Penola, South Australia, open beakers containing a saturated salt solution (NH_4NO_3) were included in all closed outer containers (Figure 3-2). Saturated NH_4NO_3 solutions maintained the humidity at ~65-70% at the temperature ranges experienced inside the outer container (20-22 ^oC). Vessels were incubated for 34 days with a day/night cycle (~12hrs light/12hrs day) provided by the laboratory lights.

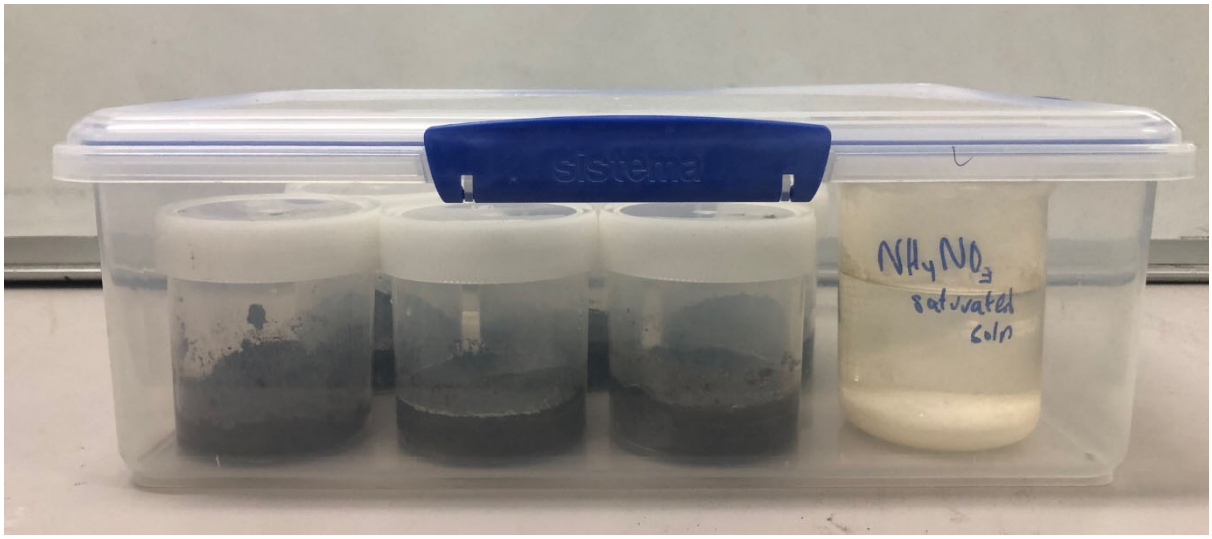


Figure 3-2: Showing microcosms (with loose lids) stored in snap-lock, polypropylene containers with open vessels of a saturated NH_4NO_3 for humidity.

Table 3-2: Initial concentrations of chemicals used in soil experiments

Chemicals	Abbreviations	Initial concentration (mg/L) in soil experiments
2-aminoethanol	AE	2600
2-butoxyethanol	BE	300
2-ethylhexanol	EH	500
acetic acid	AA	600
d-limonene	DL	40
d-limonene x10	DLx	400
ethanol	ET	2000
ethylene glycol	EG	2800
isopropanol	IP	2000
methanol		2400
naphthalene	NP	160
naphthalene x10	NPx	1600
o-cresol	OC	20
o-cresol x10	OCx	200
propylene glycol	PG	1200

3.3.5 Establishment of aquifer microcosms

All aquifer microcosms were established in 165mL glass serum vials under an anoxic atmosphere that comprised ~95% argon, 1-2% hydrogen and the balance nitrogen. Each vessel contained 100mL of filter-sterile aquifer water. This was inoculated with 1mL (1%) of aquifer water collected under an argon atmosphere (see section 3.2.1) and 1mL of each compound from Table 3-3. Vessels were incubated for approximately 34-36 days, at 18 ^{o}C.

Table 3-3: Initial concentrations of chemicals used in aquifer water experiments

Chemicals	Abbreviations	Initial concentration (mg/L) in aquifer water experiments
2-aminoethanol	AE	1300
2-butoxyethanol	BE	150
2-ethylhexanol	EH	250
acetic acid	AA	300
d-limonene	DL	20
d-limonene x10	DLx	200
ethanol	ET	1000
ethylene glycol	EG	1400
isopropanol	IP	1000
methanol		1200
naphthalene	NP	80
naphthalene x10	NPx	800
o-cresol	OC	10
o-cresol x10	OCx	100
propylene glycol	PG	600

3.3.6 Establishment of storage control

Storage controls were set up to investigate and compare the degradation of compounds under aerobic conditions with no added microbial activity. All storage controls were set up using the polypropylene vessels (see section 3.3.4). Each vessel contained 100mL sterile RO water and the compound of interest. Concentrations of the compounds were as shown in Table 3-3 (see section 3.3.5). Vessels were setup for compound recovery analyses to determine initial compound concentrations. Vessels were incubated in the dark for 33 days at room temperature.

3.3.7 Establishment of sole carbon source soil mimicking agar assay

As single chemical catabolism cannot be readily measured in a complex, carbon-rich matrix such as soil, a carbon-free soil mimicking medium (SMM) was used to model the soil. 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 medium was (in gL^{-1}): 3.51g $(NH_4)_2HPO_4$, 0.51g KH_2PO_4, 0.14g $MgSO_4.7H_2O$, 0.05g $CaCl_2$, 0.025g NaCl, 0.003g $ZnSO_4$ along with 133µg of thiamine. The pH was adjusted to 5.8 prior to the addition of 0.007g $FeCl_2.4H_2O$. SMM agar was autoclaved at 121°C for 30 mins with 115kPa pressure, prior to cooling to 50°C and pouring into 90mm diameter sterile polystyrene petri-dishes (Techno Plas, Adelaide, Australia). The concentrations of chemicals used to establish single compound degradation assays is shown in Table 3-4.

Table 3-4: Amounts of chemical used to establish sole carbon source degradation assays.

Chemicals	Abbreviations	Amount of chemical used (µg)
2-aminoethanol	AE	1300
2-ethylhexanol	EH	250
benzisothiazolinone	BZ	10
bronopol	BR	70
c12 alcohol ethoxylate	eAL	500
diethylene glycol ethyl ether	DG	10
d-limonene	DL	20
d-limonene x10	DLx	200
eicosane	EC	1100
glutaraldehyde	GA	180
glyoxal	GO	2300
hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine	HHT	2400
methylchloroisothiazolinone	MCT	10
methylisothiazolinone	MT	10
naphthalene	NP	80
naphthalene x10	NPx	800
o-cresol	OC	10
o-cresol x10	OCx	100
polyacrylamide	PA	1200
polyoxypropylene diamine	PD	1000
pristane	PR	1100
triethanolamine	TE	1700
xanthan gum	XG	250

3.3.8 Harvesting soil microcosms

To harvest the soil microcosms, the vessels were flooded with 150mL sterile reverse osmosis (RO) water and left to soak for two days. The recovered water was filtered through a cloth to remove soil particulates and subsequently sampled for chemical analysis by Australian Laboratory Services (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, 250mg of soil was subsampled and added directly into PowerSoil DNA extraction Kit (see section 3.3.11).

3.3.9 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 0.1µm pore size PVDF disc. Half the disc was sliced into small pieces using a sterile scalpel blade and added directly into the PowerSoil DNA extraction Kit (see section 3.3.11).

3.3.10 Harvesting storage control

Initial and final compound measurements

As described in above in harvesting aquifer microcosms (see section 3.3.9).

3.3.11 DNA extraction

Soil and water extractions

All DNA was extracted using the PowerSoil Kit (formerly MO BIO, now Qiagen). Following the manufacturer's instructions with minor modifications. Briefly, these modifications were:

1) an additional 70°C incubation for 10 minutes with shaking (800 rpm) after the addition of Solution 1;

2) bead beating was performed in a FASTPREP Bead Beater for 40 seconds at 6 m/s;

3) an additional centrifuge step was performed to remove residual ethanol from the PowerSoil spin filters.

Single compound soil mimicking agar extractions

Petri-dishes were scraped using a new, sterile scalpel blade. Scraped material of ~250mg was placed into 2mL sterile microcentrifuge tubes. DNA extraction was carried out by a rapid thermolysis method (Zhang et al., 2010). Briefly, the method utilises both physical and chemical extraction processes using a chemical lysis buffer and incubation at 85°C, prior to centrifugation to remove non-DNA components of cells.

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 (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 sample.

For all DNA samples, the PicoGreen reagent was diluted 200-fold. Also included in the Quaint-it™ PicoGreen Kit was a λ DNA standard (100 µg/mL) which was diluted to make 5mL of a 4µg/mL solution. The 4µg/mL λ solution was then used to make appropriate standards according to Table 3-5.

Table 3-5: DNA quantitation standards were made using the following volumes.

Volume H_2O (µL)	Volume 4µg/mL λ Stock (µL)	Concentration (ng/mL)
0	1000	1000
200	800	800
500	500	500
900	100	100
990	10	10
999	1	1
1000	0	Blank

Each DNA concentration measurement used a total volume of 100µL. For standards, 50µL of standard solution was used. For all samples, 2µL was used, with 48µL of water. Diluted PicoGreen reagent (50µL) 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.

3.3.12 Preparation for DNA sequencing

For 16S and ITS polymerase chain reactions (PCRs), the Ramiciotti Centre for Genome Analyses requires DNA to be in the range 5-10ng/µL. After quantification, all samples with a concentration outside the range of 3-20ng/µL were adjusted to comply with the needs of PCR analysis. For those DNA extractions with concentrations too low, 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.

3.3.13 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 3-3), 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.

ACGTGTGCCAGCAGCCGCGGTAACATTATAGAGTTCTGCCCTCTAGGGTA
GACCTCCCACCCTTGAATCTCAAACCTTTGTTGCTTTGGCAGCTGGCCTT
CGGGCTGTTATAGCTGCCAGAGGACCAAAACTCTGTGTTCAGTGATGTCT
GAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGATTAGATACCCGAGTAGTCCTGCA

Figure 3-3: 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.*

3.3.14 DNA sequencing

All DNA in this project was sequenced by the Ramiciotti Centre for Genomic Analyses (UNSW, Kensington, NSW). PCRs were conducted on extracted DNA using the following 16S or ITS primers (Figure 3-4).

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 3-4: 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, in error, the ITS sequencing was completed using 150bp paired-end sequencing. This resulted in some non-typical analyses for the ITS data shown under bioinformatics.

3.3.15 Bioinformatics

Both 16S and ITS amplicon datasets were processed using the Greenfield Hybrid Amplicon Pipeline (GHAP | https://doi.org/10.4225/08/59f98560eba25). 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.

The short-read length for ITS sequences provided by the Ramiciotti Centre for Genome Analyses meant that only unmerged 150bp reads were used for analyses.

3.3.16 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 both principal components and detrended correspondence analyses (PCA and DCA, respectively) (Hill and Gauch, 1980). While PCA is typically used less in ecological datasets as it is commonly represented by sparse data matrices, DCA overcomes some of these issues, but significance tests are not available. For this reason, both types of ordinations are included in the current report. In order to undertake both PCA and DCA, data were normalised and imported into the statistical software package R (ver. 1.1.456). For DCA, data from the OTU table, taken by chemical treatment, were then subjected to the decorana function in the Vegan package (https://cran.r-project.org, https://github.com/vegandevs/vegan). The resultant data were plotted in Python 3.7.3 using the Matplotlib module (Hunter, 2007). Principal components analyses were undertaken using the standard library in R.

Identification of responding taxa was also undertaken using R. For individual taxa, one-way analysis of variance (ANOVA) were conducted, and where significant differences, using Tukeys HSD post hoc contrasts (Tukey, 1949), were observed, significantly responding taxa were identified.

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 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.

3.3.17 Network analyses

Undirected network analyses were conducted in Python 3.7.3 using the networkx module (Hagberg et al., 2012). Plots were modified in matplotlib as described above.

4 Results

4.1 Soil and aquifer physicochemistry

Table 4-1 shows the inorganic soil chemistry results of various elements and compounds of interests. All measurements were conducted in triplicates. The average pH, electrical conductivity and moisture content of the soil was 5.8 pH Unit, 40 µS/cm and 6.7%, respectively. The main metals detected were aluminium and iron with barium, manganese and strontium reported at lower levels. The average concentration of total nitrogen was 737 ± 43 mg/kg and total phosphorus was 117 ± 44 mg/kg.

The total organic carbon content of the soil was 6.2% ± 1.2%.

Table 4-1: Results of inorganic soil chemistry in triplicate measured by Australian Laboratory Services (ALS).

	Analyte grouping/Analyte	Soil (mg/kg)[1,2,3]
	pH	5.8 ± 0.2[1]
	Electrical Conductivity @ 25Â°C	40 ± 6[2]
	Moisture Content	6.7 ± 0.3[3]
	Sulfate as SO4 $^{2-}$	<10
	Sulfur as S	<10
	Silica	238 ± 25
	Chloride	20
	Calcium	<10
	Magnesium	<10
	Sodium	13 ± 3
	Potassium	20
Total Metals	Aluminium	893 ± 303
	Antimony	<5
	Arsenic	<5
	Barium	23 ± 3
	Beryllium	<1
	Boron	<50
	Cadmium	<1
	Chromium	<2
	Cobalt	<2
	Copper	<5
	Iron	597 ± 92
	Lead	<5
	Manganese	8
	Molybdenum	<2
	Nickel	<2
	Selenium	<5
	Silver	<2
	Strontium	5
	Tin	<5
	Vanadium	<5
	Zinc	<5

Uranium	<0.1
Mercury (recoverable)	<0.1
Total Fluoride	45 ± 5
Ammonia as N	<20
Nitrite as N (Sol.)	<0.1
Nitrate as N (Sol.)	0.7[#]
Nitrite + Nitrate as N (Sol.)	0.7[#]
Total Kjeldahl Nitrogen as N	737 ± 43
Total Nitrogen as N	737 ± 43
Total Phosphorus as P	117 ± 44
Reactive Phosphorus as P	0.8

[1]pH Unit [2] µS/cm [3] % [#]only one result greater than reporting limit within triplicate

All organic compounds analysed were below the reporting limits of the analytical methods, Appendix A.

Table 4-2 shows the compounds that were analysed by ALS to determine their pre-existing concentrations in the soil. The results showed that all compounds were below the reporting limits of the respective analytical methods except for acetic acid at 545 ± 0.038 µg/L and naphthalene which showed one result within the triplicate greater than the reporting limit.

Table 4-2: Results of compounds of interest to determine pre-existing concentrations in soil flood, measured in triplicate by Australian Laboratory Services (ALS).

Analyte grouping/Analyte	Soil (mg/L)
o-cresol	<0.001
naphthalene	0.0034[#]
methanol	<1
2-butoxyethanol	<2
propylene glycol	<2
ethylene glycol	<2
ethanol	<0.05
isopropanol	<0.05
acetic acid	0.545 ± 0.038
2-aminoethanol	<0.001
2-ethylhexanol	<2
limonene	<0.2

[#]*only one result greater than reporting limit within triplicate*

Table 4-3 shows the inorganic aquifer chemistry results in triplicates. The average pH and electrical conductivity measurements of the aquifer was 7.74 pH Units and 1410 µS/cm, respectively. The main dissolved metal detected was strontium with barium, copper, manganese and zinc reported at lower levels. The main total metals detected were strontium and bromine with barium, copper, lithium, manganese and uranium reported at lower levels. The average concentration of total nitrogen was 0.3 mg/L with total phosphorus not detected. Total anions and cations for the aquifer averaged at 14.8 meg/L and 14.1 meg/L, respectively.

The total organic carbon content of the aquifer water was 5.5mg / L (0.00055%).

Table 4-3: Results of inorganic aquifer bulk water chemistry measured by Australian Laboratory Services (ALS).

	Analyte grouping/Analyte	Aquifer (mg/L)[1,2,3]
	pH Value	7.74 ± 0.03[1]
	Electrical Conductivity @ 25Â°C	1410 ± 6[2]
	Hydroxide Alkalinity as CaCO3	<1
	Carbonate Alkalinity as CaCO3	<1
	Bicarbonate Alkalinity as CaCO3	351 ± 2
	Total Alkalinity as CaCO3	351 ± 2
	Sulfur as S	10
	Silicon as SiO2	12.4
	Sulfate as SO4 - Turbidimetric	48
	Chloride	242 ± 2
	Calcium	104
	Magnesium	16
	Sodium	174 ± 1
	Potassium	2
Dissolved Metals	Aluminium	<0.01
	Antimony	<0.001
	Arsenic	<0.001
	Beryllium	<0.001
	Barium	0.018
	Cadmium	<0.0001
	Chromium	<0.001
	Cobalt	<0.001
	Copper	0.002
	Lead	<0.001

	Manganese	0.02
	Molybdenum	<0.001
	Nickel	<0.001
	Selenium	<0.01
	Silver	<0.001
	Strontium	0.308 ± 0.003
	Tin	<0.001
	Uranium	0.001[#]
	Vanadium	<0.01
	Zinc	0.019[#]
	Boron	<0.05
	Iron	<0.05
Total Metals	Aluminium	0.01[#]
	Antimony	<0.001
	Arsenic	<0.001
	Beryllium	<0.001
	Barium	0.022 ± 0.001
	Cadmium	<0.0001
	Chromium	<0.001
	Cobalt	<0.001
	Copper	0.002
	Lead	<0.001
	Lithium	0.002
	Manganese	0.021
	Molybdenum	<0.001

	Nickel	<0.001
	Selenium	<0.01
	Silver	<0.001
	Strontium	0.331 ± 0.003
	Tin	<0.001
	Uranium	0.001
	Vanadium	<0.01
	Zinc	<0.005
	Boron	<0.05
	Bromine	0.37 ± 0.02
	Mercury (dissolved)	<0.0001
	Mercury (recoverable)	<0.0001
	Ferrous Iron	<0.05
	Total Fluoride	0.2 ± 0.1
	Ammonia as N	0.01[#]
	Nitrite as N	0.01
	Nitrate as N	0.27 ± 0.06
	Nitrite + Nitrate as N	0.28 ± 0.06
	Total Kjeldahl Nitrogen as N	<0.1
	Total Nitrogen as N	0.3 ± 0.1
	Total Phosphorus as P	<0.01
	Reactive Phosphorus as P	<0.01
	Total Anions	14.9 ± 0.1[3]
	Total Cations	14.1 ± 0.1[3]
	Ionic Balance	2.52 ± 0.13[4]

[1]pH Unit [2] µS/cm [3]meg/L [4] % [#]only one result greater than reporting limit within triplicate

All organic compounds analysed were below the reporting limits of the analytical methods (Appendix A).

Table 4-4 shows the compounds that were analysed by ALS to determine their pre-existing concentrations in the aquifer. The results showed that all compounds were below the reporting limits of the respective analytical methods.

Table 4-4: Results of the compounds of interest to determine pre-existing concentrations in aquifer water, measured in triplicate by Australian Laboratory Services (ALS).

Analyte grouping/Analyte	Aquifer (mg/L)
o-cresol	<0.001
naphthalene	<0.001
methanol	<1
2-Butoxyethanol	<2
propylene glycol	<2
ethylene glycol	<2
ethanol	<0.05
isopropanol	<0.05
acetic acid	<0.05
2-aminoethanol	<0.001
2-ethylhexanol	<2
limonene	<0.2

4.2 Measured degradation of chemical compounds

In Task 3, the ability of microbes to degrade 2-butoxyethanol, 2-ethylhexanol, acetic acid, 2-aminoethanol, ethanol, ethylene glycol, isopropanol, d-limonene, methanol and propylene glycol were assessed in soil and aquifer microcosms.

Plots have been made for each compound that were analysed by ALS (Figure 4-2). Each compound shows results for the three treatments: storage controls, aquifer- and soil microcosms. Figure 4-1 is an example of how to interpret the results from the compound concentration experiments. It displays the results from acetic acid that was analysed by ALS using standard methods. The results presented for the soil microcosm have been calculated based on 50mL of soil. The green data points illustrate initial concentration measurements that were conducted in triplicates; overlapping values are indicated in a darker colouration. The red data points show the final concentrations in each treatment after 34-36 days of incubation. These measurements were also conducted in triplicates with overlapping values indicated by a darker colouration. The dashed orange line represents the initial known compound dose added, which provides information about the recovery efficiency of each compound. The blue asterisk (*) describes treatment means that have a significant difference, at the 95% confidence level, between their initial and final measured concentrations. Values plotted as ‘N.D’ were reported as not detected. This means that compound concentrations were lower than the reporting limit of the analytical method.

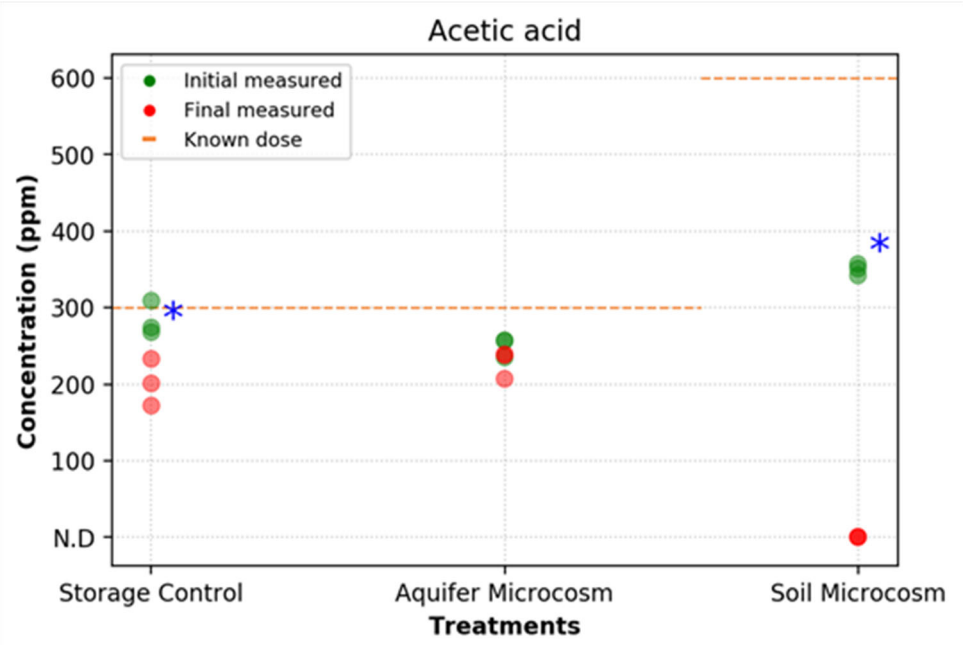


Figure 4-1: Compound concentration measurements in various treatments over time (34-36 days of incubation) for acetic acid. See full description in caption of Figure 4-2.

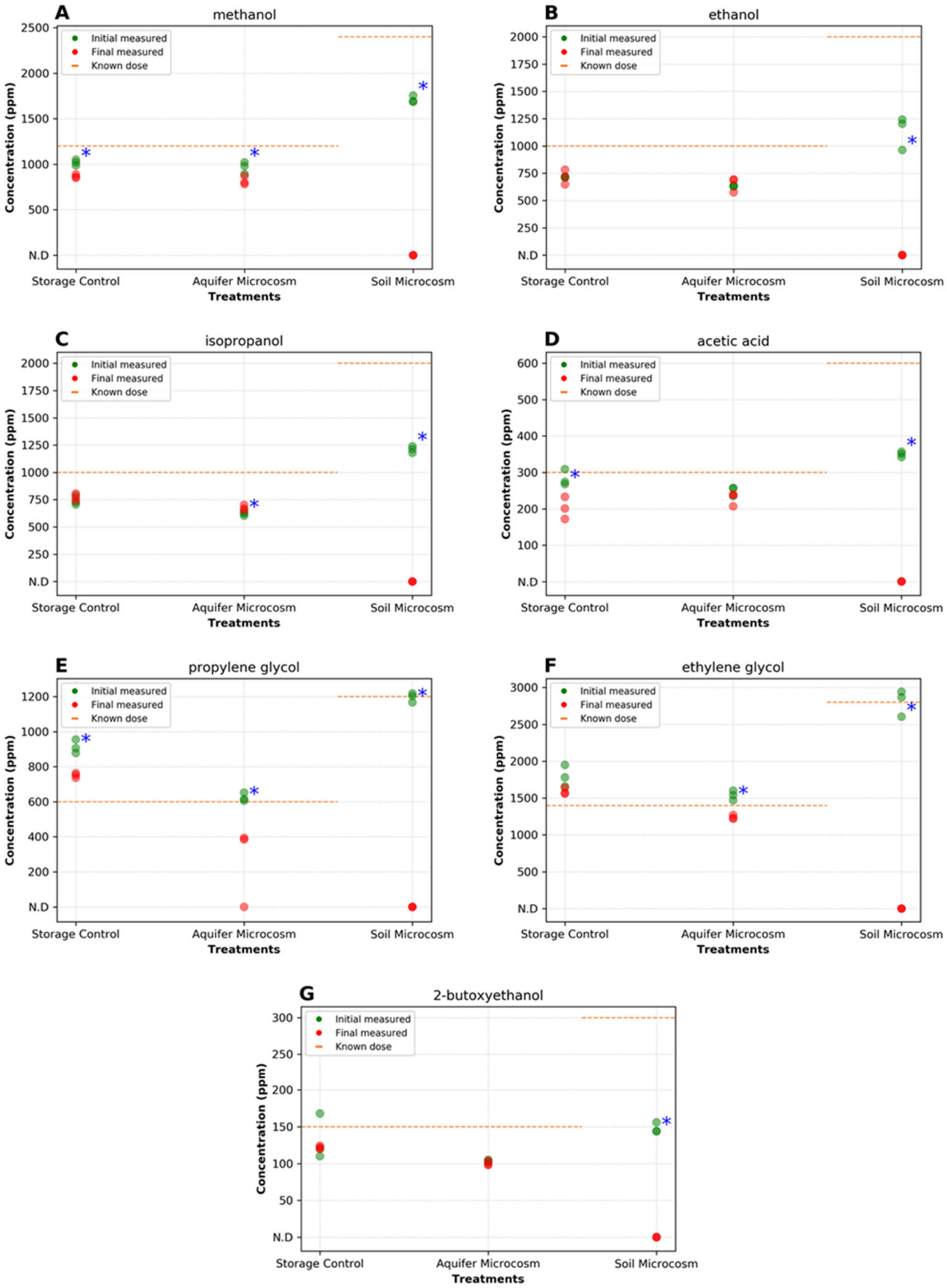


Figure 4-2: Compound concentration measurements in various treatments over time (34-36 days of incubation).

A) methanol, B) ethanol, C) isopropanol, D) acetic acid, E) propylene glycol, F) ethylene glycol, and G) 2-butoxyethanol. Compound was mixed with RO water in 'storage control', sampled aquifer water from SA in 'aquifer microcosm', and

*sampled soil from SA in 'soil microcosm'. Initial measured compound doses at the beginning of each experiment are represented in green, while final measured doses are represented in red. Each measurement was conducted in triplicates, with overlapping values resulting in darker colouration in the graph. Dashed orange line represents the initial known compound dose added. * = initial measured value is statistically different to the final measured value using t-test at the 95% confidence level. 'N.D' represents compound not detected. All compound concentrations were measured by ALS.*

Storage control treatments across all the compounds in Figure 4-2 showed statistically significant decreases between the initial and final concentrations for methanol, acetic acid and propylene glycol. Methanol, isopropanol, propylene glycol and ethylene glycol showed statistically significant decreases between the initial and final concentrations within the aquifer microcosm treatments whilst ethanol, acetic acid and 2-butoxyethanol did not show statistical differences. The initial concentrations for the storage control and aquifer microcosm treatments were usually lower than the known dose concentrations. Exceptions to this trend were for propylene glycol and ethylene glycol, where the initial concentrations were higher or at the same concentration as the known dose. All compounds in Figure 4-2 showed statistically significant decreases between the initial and final concentrations within the soil microcosm treatments. The final concentration of all compounds in the soil microcosm treatment were reported below the detection limit of each analytical method. All compounds except for propylene glycol and ethylene glycol showed much lower initial concentrations to the known dose concentrations for the soil microcosm treatments.

The compounds 2-aminoethanol and 2-ethylhexanol displayed anomalous results (Figure 4-3), with the initial concentrations usually being lower than the known dose concentrations. The initial concentration values for 2-aminoethanol in the aquifer microcosm treatment was an exception as it showed around double the concentration of the known dose. Storage control and soil microcosm treatments for both compounds showed statistically significantly lower values between the initial and final concentrations. However, the initial and final concentrations for the aquifer microcosm treatment of 2-ethylhexanol did not show statistically significant differences and the initial concentrations were much lower than the known dose concentration (Figure 4-3 B). The compounds 2-aminoethanol and 2-ethylhexanol were added to the single compound degradation assays.

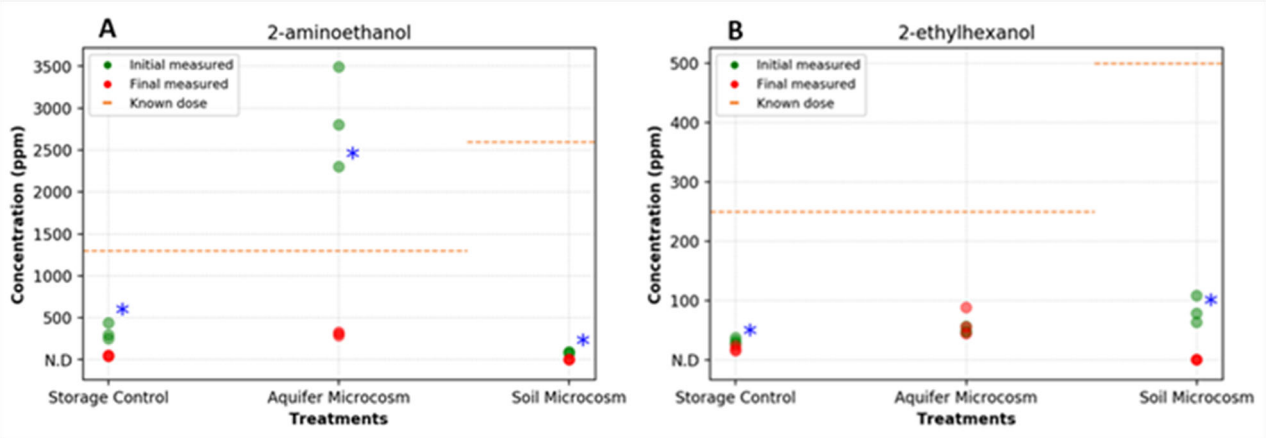


Figure 4-3: Anomalous compound concentration measurements in various treatments over time (34-36 days of incubation).

*A) 2-aminoethanol, and B) 2-ethylhexanol. Compound was mixed with RO water in 'storage control', sampled aquifer water from SA in 'aquifer microcosm', and sampled soil from SA in 'soil microcosm'. Initial measured compound doses at the beginning of each experiment are represented in green, while final measured doses are represented in red. Each measurement was conducted in triplicates, with overlapping values resulting in darker colouration in the graph. Dashed orange line represents the initial known compound dose added. * = initial measured value is statistically different to the final measured value using t-test at the 95% confidence level. 'N.D' represents compound not detected. All compound concentrations were measured by ALS.*

The compounds o-cresol, naphthalene and d-limonene displayed anomalous results (Table 4-5), with the initial concentrations being lower than the known dose concentrations of 10 ppm, 80 ppm and 20 ppm respectively for the storage control and aquifer microcosm treatments and 20 ppm, 160 ppm and 20 ppm respectively for the soil microcosm treatment. D-limonene and o-cresol were not detected for each of the treatments. Naphthalene was detected in the storage control (0.289 ± 0.079 mg/L) and aquifer microcosm (0.149 ± 0.011 mg/L) treatments but much lower than the known dose concentration of 80 ppm. Naphthalene was not detected in the soil microcosm treatment. Due to the anomalous initial concentrations, the final concentrations were not analysed for o-cresol, naphthalene and d-limonene. These compounds were added to the single compound degradation assays.

Table 4-5: Anomalous initial measured compound concentrations in various treatments at the beginning of each experiment.

Compound	Treatments		
	Storage Control	**Aquifer microcosm**	**Soil microcosm**
Naphthalene (mg/L)	0.289 ± 0.079	0.149 ± 0.011	<0.001
d-limonene (mg/L)	<0.2	<0.2	<0.2
o-cresol (mg/L)	<0.001	<0.001	<0.001

Each measurement was conducted in triplicates. 'N.D.' represents compound not detected, detection limit of analysis is shown in brackets. All compound concentrations were measured by ALS.

4.3 Soil mimicking agar single compound degradation trials

Microbial degradation was assessed for soil microbes using carbon-free SMM agar onto which chemicals shown in Table 3-4 were applied. All plates had observed visible growth with a range of bacterial and fungal colonies present on most treatments and in the control (Figure 4-4). Colony morphology was highly variable and many microcolonies were also present. Fungal colonies were represented by both sporulating and non-sporulating forms.

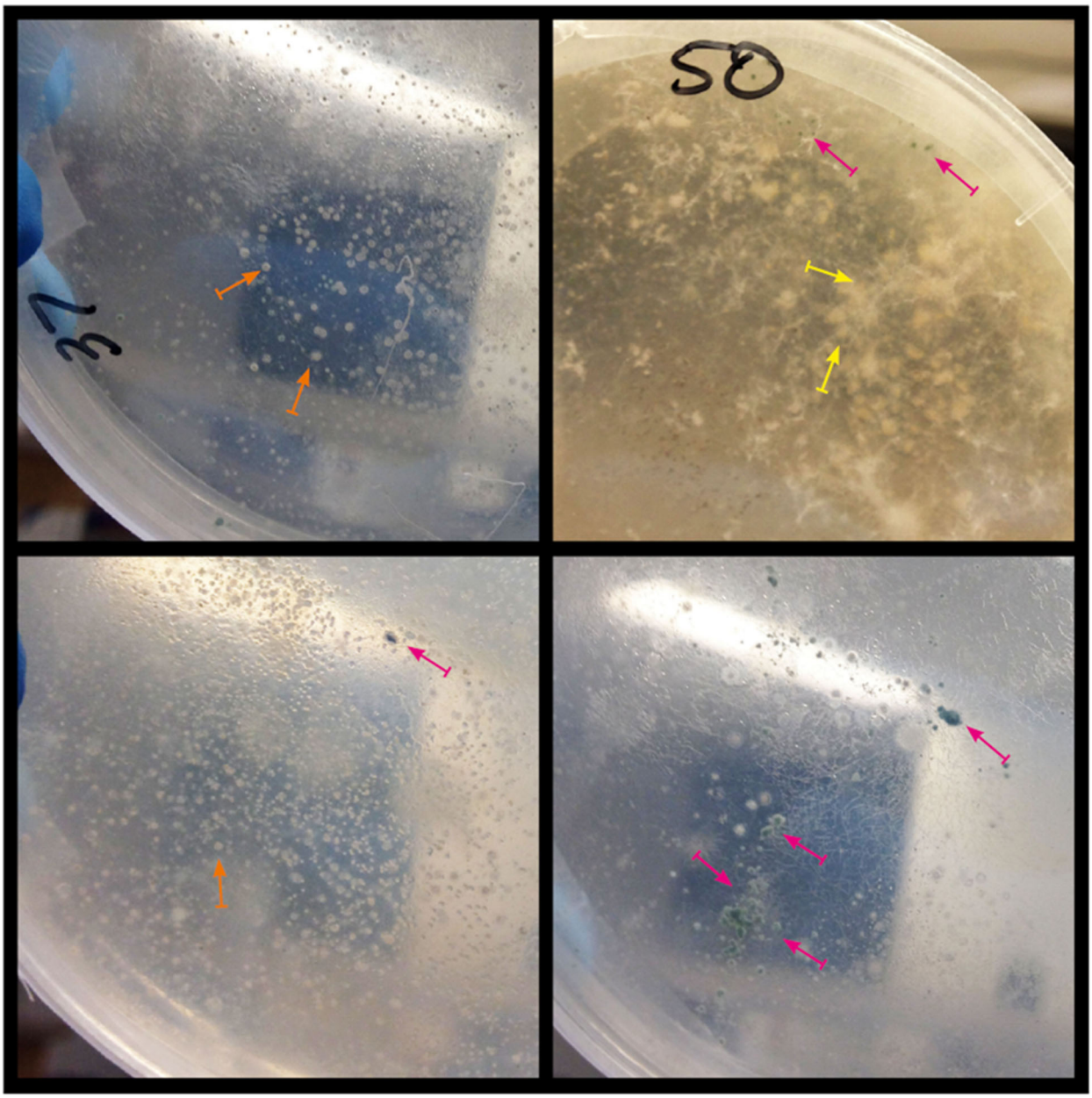


Figure 4-4: Photos of microbial growth on plates with target chemicals.

Top left: untreated, Top right: pristane, Bottom left: naphthalene, Bottom right: xanthan gum. Magenta arrows show green conidia on small fungal colonies, yellow arrows show thick white unevenly margined colonies on pristane, orange arrows show some example bacterial colonies.

Since colony morphology is largely noninformative, DNA was extracted and molecular methods applied to profile microbial communities growing on SMM agar.

In addition to experiments conducted on the SMM agar, a liquid culture experiment was performed outside of the work program described in this study. These series of experiments used the same soil inoculum from the Penola region and minimised carbon carryover from soils through multiple subculturing passages. In these liquid culture experiments, minimal to no growth were

observed on hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine, glutaraldehyde, 2-ethylhexanol, o-cresol, benzisothiazolinone and methylchloroisothiazolinone. Observable biomass (bacterial and/or fungal) was produced on methylisothizolinone, bronopol, butoxyethanol, polyacrylamide, ethylene glycol, polypropylene diamine, 2-aminoethanol and diethyl glycol ethyl ether. Bacterial growth and either solubilisation or emulsification was observed for naphthalene and d-limonene, respectively. Very heavy microbial growth was observed for glyoxal, polypropylene glycol, isopropanol, ethanol, acetic acid and xanthan gum. Lastly, the surfactant C12 alcohol ethyoxylate was cleared and moderate microbial growth was observed.

Rather than comparing extracted DNA concentration, DNA concentration was converted to a theoretical cell number (assuming an average genome size of 4Mbp) as it is more intuitive. The greatest DNA yields were recovered from the eicosane treated SMM plates, while the least DNA was extracted from the bronopol treated SMM plates. ANOVA analyses demonstrated that significant differences were observed in the quantity of DNA extracted by treatment ($p<0.000001$). Of particular note, eicosane yielded significantly more DNA than the untreated control ($p<0.003$). All other treatments were not significantly different from the carbon-free controls (Figure 4-5). It is noteworthy that these DNA extractions comprise all DNA recovered from the scraped agar including both bacteria and fungi.

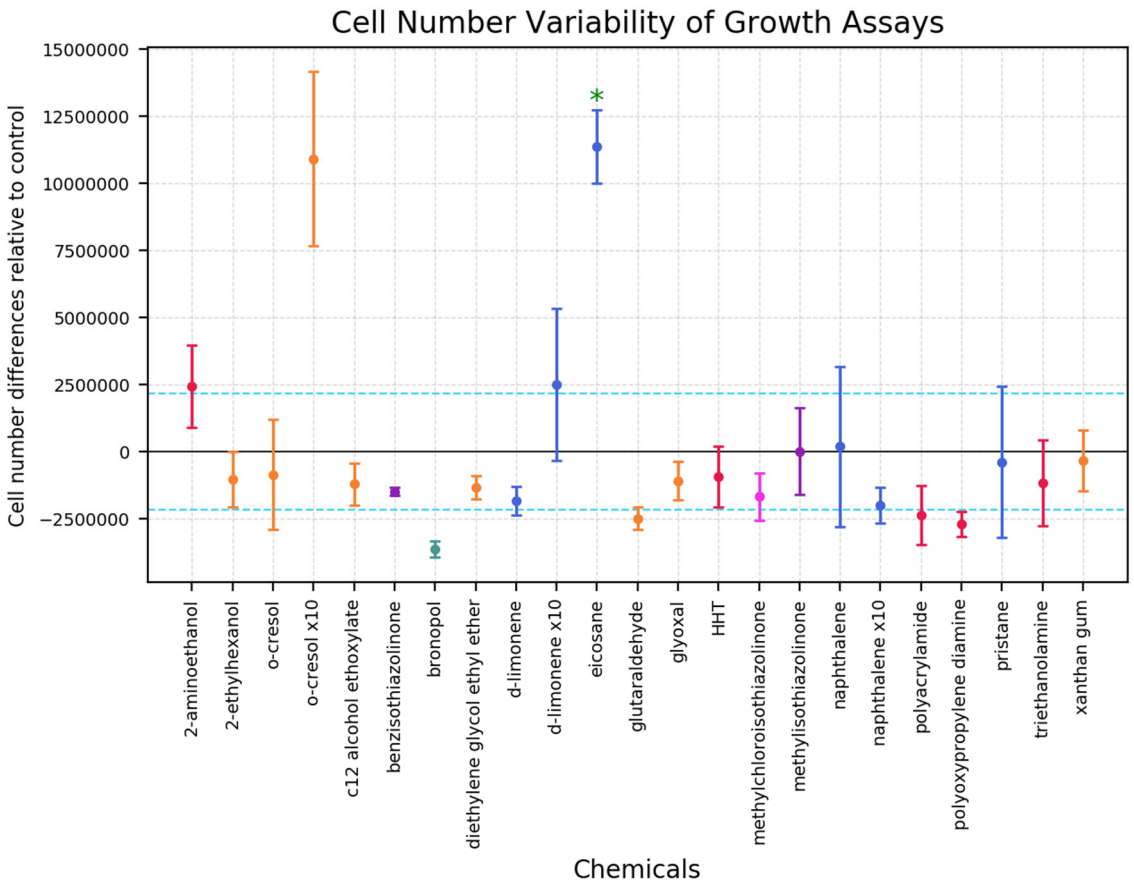


Figure 4-5: Cell number variability from SMM agar plates amended with a range of compounds.

*Colours represent the presence of certain elements in each chemical. Chemicals containing C, H only (blue), O (orange), N and O (red), N, O and Br (teal), N, O and S (purple), N, O, S and Cl (magenta). Cell number was calculated from DNA concentration assuming an average genome size of 4Mbp. Error bars show the standard error. * = cell number is statistically different to the control using t-test at the 95% confidence level. The untreated control with no chemical amendment had a calculated cell number of ~6 x 10^6 cells per plate. The dotted blue lines indicate the standard error of the untreated control.*

4.4 Identification of fungal taxa that degrade target chemicals

In order to identify organisms that catabolise compounds, a mathematical method was utilised where the proportion of each taxon's abundance was compared to the control; any taxon with a percentage change (increase or decrease) of 1000% was retailed to generate a table of taxa significantly affected by the chemicals tested. These data were then collated for all the chemicals tested and OTUs were analysed using network analyses. Of these OTUs only those present in > 40% of replicates for a given treatment were included.

In total, 130 fungal OTUs (fOTUs) were associated with catabolism of chemicals. Network analyses clearly demonstrated that groups of some fOTUs were uniquely involved with the degradation of individual compounds. The 51 fOTUs that grow in the presence of just one chemical treatment make up ~39% of the 130 catabolic taxa. These groups can be identified on the network diagram as 'fans' or single points (green) radiating out of individual chemical nodes (red). For example, glyoxal (Figure 4-6, Red GO) 13 taxa are shown to increase in abundance significantly only in its presence.

Notably, some OTUs are also more abundant under several treatments. For example, fOTU_18, was increased under eleven different chemical treatments (2-aminoethanol (AE), bronopol (BR), diethylene glycol ethyl ether (DG), 2-ethylhexanol (EH), methylchloroisothiazolinone (MCT), methylisothiazolinone (MT), naphthalene (NP), napthalene x10 (NPx), polyacrylamide (PA), pristane (PR), xanthan gum (XG); Figure 4-6). Similarly, fOTU_38 was able to increase in abundance under nine treatments including: 2-aminoethanol (AE), d-limonene (DL), d-limonene x10 (DLx), glyoxal (GO), hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine (HHT), naphthalene (NP), pristane (PR), triethanolamine (TE) and xanthan gum (XG).

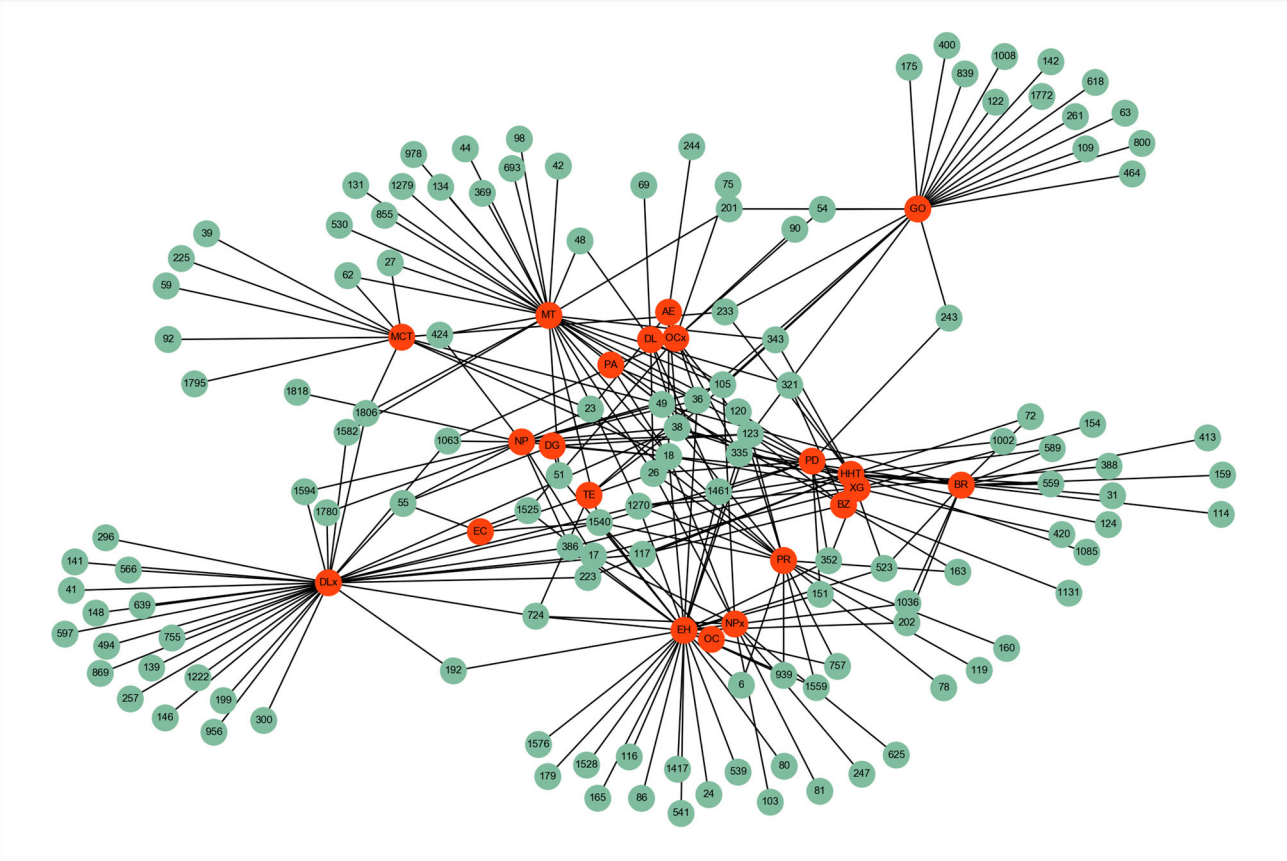


Figure 4-6: Undirected network analyses of fungal OTUs (green) and their growth in the presence of particular chemicals (red).

Lines between chemicals and OTUs indicate that OTU degrades that compound. Numbers in circles are fungal OTUs. 2-aminoethanol (AE), 2-ethylhexanol (EH), benzisothiazolinone (BZ), bronopol (BR), diethylene glycol ethyl ether (DG), d-limonene (DL), d-limonene x10 (DLx), eicosane (EC), glyoxal (GO), hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine (HHT), methylchloroisothiazolinone (MCT), methylisothiazolinone (MT), naphthalene (NP), naphthalene x10 (NPx), o-cresol (OC), o-cresol x10 (OCx), polyacrylamide (PA), polyoxypropylene diamine (PD), pristane (PR), triethanolamine (TE), xanthan gum (XG).

Putative fungal catabolisers of chemicals were most common in the 2-ethylhexanol, d-limonene x10 and methylisothiazolinone treatments, all of which had ~30 distinct fungal taxa associated with the treatment. Table 4-6 lists the top 5 fungal taxa that responded positively for each individual treatment. Where there were less than five fungal taxa that responded positively, all the top responders are listed.

Table 4-6: Top 5 putative fungal catabolisers of chemical treatments in sole carbon experiments.

Chemical treatment	OTU	Phylogeny	Closest match	ID*
AE	fOTU_23	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Nectria; Fusarium oxysporum f sp psidii	*Neocosmospora_vasinfecta* (JQ954881)	97
	fOTU_38	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Neonectria	*Ilyonectria_estremocensis* (JF735320)	100
	fOTU_244	Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetes_Incertae sedis; Polyporales; Fomitopsidaceae; Oligoporus; Oligoporus rennyi	*Oligoporus_rennyi* (AJ006668)	99
	fOTU_18	Fungi	Unknown	0
	fOTU_36	Fungi	Unknown	0
BZ	fOTU_123	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales; Saccharomycetales_Incertae sedis; Candida; Candida coipomoensis	*Candida_coipomoensis* (AJ606466)	100
	fOTU_1131	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Clavicipitaceae; Metacordyceps;	*Metacordyceps_chlamydosporia* (JQ433953)	100
	fOTU_321	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales	Unknown	0
	fOTU_49	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreales_Incertae sedis; Acremonium; Acremonium cavaraeanum	*Acremonium_cavaraeanum* (JF912333)	92
	fOTU_163	Fungi	Unknown	0
BR	fOTU_335	Fungi; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetidae; Capnodiales; Mycosphaerellaceae; Davidiella;	*Cladosporium_pseudocladosporioides* (JN033472)	100
	fOTU_523	Fungi; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Pleosporales; Didymosphaeriaceae; Asteromella; Asteromella pistaciarum	*Asteromella_pistaciarum* (FR681903)	87

	fOTU_202	Fungi; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Pleosporales; Pleosporaceae; Alternaria; Alternaria alternata	*Alternaria*_sp_MY_2011 (JN038447)	100
	fOTU_559	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Sordariomycetidae; Sordariales; Chaetomiaceae; Chaetomium; Chaetomium globosum	*Chaetomium_subaffine* (JN209929)	100
	fOTU_18	Fungi	Unknown	0
DG	fOTU_123	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales; Saccharomycetales_Incertae sedis; Candida; Candida coipomoensis	*Candida_coipomoensis* (AJ606466)	100
	fOTU_18	Fungi	Unknown	0
	fOTU_55	Fungi	Unknown	0
	fOTU_51	Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Chaetothyriomycetidae; Chaetothyriales; Herpotrichiellaceae; Cladophialophora; Cladophialophora chaetospira	*Cladophialophora_chaetospira* (EU137333)	94
DL	fOTU_26	Fungi; Zygomycota; Mucoromycotina; Mucoromycotina_Incertae sedis; Mucoromycotina_Incertae sedis; Mortierellales; Mortierellaceae; Mortierella; Mortierella elongata	*Mortierella_elongata* (AB542099)	100
	fOTU_38	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Neonectria;	*Ilyonectria_estremocensis* (JF735320)	100
	fOTU_48	Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Chaetothyriomycetidae; Chaetothyriales; Herpotrichiellaceae; Cladophialophora; Cladophialophora chaetospira	*Cladophialophora_chaetospira* (EU137333)	100
	fOTU_1461	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreaceae; Hypocrea; Trichoderma evansii	*Trichoderma_evansii* (AF414320)	95
	fOTU_105	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Sordariomycetidae; Coniochaetales; Coniochaetaceae; Lecythophora; Lecythophora sp olrim15	*Lecythophora*_sp_olrim15 (AY781228)	87

DLx	fOTU_1270	Fungi; Basidiomycota; Agaricomycotina; Tremellomycetes; Tremellomycetidae; Tremellales; Tremellaceae; Cryptococcus; Cryptococcus laurentii	*Cryptococcus_laurentii* (JN626994)	98
	fOTU_192	Fungi; Basidiomycota; Pucciniomycotina; Microbotryomycetes; Microbotryomycetes_Incertae sedis; Sporidiobolales; Sporidiobolales_Incertae sedis; Rhodotorula; Rhodotorula cresolica	*Rhodotorula_cresolica* (AB038114)	94
	fOTU_639	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales; Saccharomycetales_Incertae sedis; Candida; Candida subhashii	*Candida_subhashii* (EU836707)	96
	fOTU_38	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Neonectria	*Ilyonectria_estremocensis* (JF735320)	100
	fOTU_117	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae	*Fusarium_merismoides* (AB586998)	96
EC	fOTU_26	Fungi; Zygomycota; Mucoromycotina; Mucoromycotina_Incertae sedis; Mucoromycotina_Incertae sedis; Mortierellales; Mortierellaceae; Mortierella; Mortierella elongata	*Mortierella_elongata* (AB542099)	100
	fOTU_1540	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreaceae; Hypocrea; Trichoderma evansii	*Trichoderma_evansii* (EU856295)	98
	fOTU_55	Fungi	Unknown	0
EH	fOTU_24	Fungi; Zygomycota; Mucoromycotina; Mucoromycotina_Incertae sedis; Mucoromycotina_Incertae sedis; Mortierellales; Mortierellaceae; Mortierella;	Unknown	0
	fOTU_192	Fungi; Basidiomycota; Pucciniomycotina; Microbotryomycetes; Microbotryomycetes_Incertae sedis; Sporidiobolales; Sporidiobolales_Incertae sedis; Rhodotorula; Rhodotorula cresolica	*Rhodotorula_cresolica* (AB038114)	94
	fOTU_541	Fungi; Basidiomycota	Unknown	0
	fOTU_1270	Fungi; Basidiomycota; Agaricomycotina; Tremellomycetes; Tremellomycetidae; Tremellales; Tremellaceae; Cryptococcus; Cryptococcus laurentii	*Cryptococcus_laurentii* (JN626994)	98

	fOTU_386	Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Eurotiales; Trichocomaceae; Penicillium	*Eupenicillium_brefeldianum* (AF033435)	96
GO	fOTU_122	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Gibberella; Fusarium avenaceum	*Fusarium_avenaceum* (AY147282)	95
	fOTU_123	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales; Saccharomycetales_Incertae sedis; Candida; Candida coipomoensis	*Candida_coipomoensis* (AJ606466)	100
	fOTU_175	Fungi; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Pleosporales	Unknown	0
	fOTU_54	Fungi; Ascomycota; Pezizomycotina; Pezizomycetes; Pezizomycetidae; Pezizales; Pyronemataceae; Wilcoxina; Wilcoxina mikolae	*Wilcoxina_mikolae* (JQ310818)	100
	fOTU_1772	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales; Saccharomycetales_Incertae sedis; Candida; Candida sp ATCC MYA_4651	*Candida*_sp_ATCC_MYA_4651 (HQ652049)	95
HHT	fOTU_38	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Neonectria	*Ilyonectria_estremocensis* (JF735320)	100
	fOTU_386	Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Eurotiales; Trichocomaceae; Penicillium	*Eupenicillium_brefeldianum* (AF033435)	96
	fOTU_1270	Fungi; Basidiomycota; Agaricomycotina; Tremellomycetes; Tremellomycetidae; Tremellales; Tremellaceae; Cryptococcus; Cryptococcus laurentii	*Cryptococcus_laurentii* (JN626994)	98
	fOTU_117	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae	*Fusarium_merismoides* (AB586998)	96
	fOTU_559	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Sordariomycetidae; Sordariales; Chaetomiaceae; Chaetomium; Chaetomium globosum	*Chaetomium_subaffine* (JN209929)	100
MCT	fOTU_27	Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Agaricales; Strophariaceae; Pholiota; Pholiota multicingulata	*Pholiota_multicingulata* (HQ533029)	100

	fOTU_18	Fungi	Unknown	0
	fOTU_39	Fungi; Ascomycota; Pezizomycotina	Unknown	0
	fOTU_49	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreales_Incertae sedis; Acremonium; Acremonium cavaraeanum	*Acremonium_cavaraeanum* (JF912333)	92
	fOTU_92	Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Chaetothyriomycetidae; Chaetothyriales; Herpotrichiellaceae; Cladophialophora; Cladophialophora chaetospira	*Cladophialophora_chaetospira* (EU137333)	92
MT	fOTU_1270	Fungi; Basidiomycota; Agaricomycotina; Tremellomycetes; Tremellomycetidae; Tremellales; Tremellaceae; Cryptococcus; Cryptococcus laurentii	*Cryptococcus_laurentii* (JN626994)	98
	fOTU_26	Fungi; Zygomycota; Mucoromycotina; Mucoromycotina_Incertae sedis; Mucoromycotina_Incertae sedis; Mortierellales; Mortierellaceae; Mortierella; Mortierella elongata	*Mortierella_elongata* (AB542099)	100
	fOTU_201	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Sordariomycetidae; Sordariales; Chaetomiaceae; Humicola; Humicola nigrescens	*Humicola_nigrescens* (AB625592)	99
	fOTU_424	Fungi; Basidiomycota; Basidiomycota_Incertae sedis; Wallemiomycetes; Wallemiomycetes_Incertae sedis; Wallemiales; Wallemiaceae; Wallemia; Wallemia muriae	*Wallemia_muriae* (AY302529)	97
	fOTU_855	Fungi; Ascomycota	Unknown	0
NP	fOTU_23	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Nectria; Fusarium oxysporum f sp psidii	*Neocosmospora_vasinfecta* (JQ954881)	97
	fOTU_123	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales; Saccharomycetales_Incertae sedis; Candida; Candida coipomoensis	*Candida_coipomoensis* (AJ606466)	100
	fOTU_38	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Neonectria	*Ilyonectria_estremocensis* (JF735320)	100
	fOTU_18	Fungi	Unknown	0

	fOTU_424	Fungi; Basidiomycota; Basidiomycota_Incertae sedis; Wallemiomycetes; Wallemiomycetes_Incertae sedis; Wallemiales; Wallemiaceae; Wallemia; Wallemia muriae	*Wallemia_muriae* (AY302529)	97
NPx	fOTU_26	Fungi; Zygomycota; Mucoromycotina; Mucoromycotina_Incertae sedis; Mucoromycotina_Incertae sedis; Mortierellales; Mortierellaceae; Mortierella; Mortierella elongata	*Mortierella_elongata* (AB542099)	100
	fOTU_625	Fungi	Unknown	0
	fOTU_18	Fungi	Unknown	0
	fOTU_724	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreaceae; Hypocrea; Hypocrea caerulescens	*Cosmospora_butyri* (JQ070093)	86
	fOTU_105	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Sordariomycetidae; Coniochaetales; Coniochaetaceae; Lecythophora; Lecythophora sp olrim15	*Lecythophora*_sp_olrim15 (AY781228)	87
OC	fOTU_1540	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreaceae; Hypocrea; Trichoderma evansii	*Trichoderma_evansii* (EU856295)	98
OCx	fOTU_123	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales; Saccharomycetales_Incertae sedis; Candida; Candida coipomoensis	*Candida_coipomoensis* (AJ606466)	100
	fOTU_90	Fungi; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Pleosporales; Montagnulaceae; Paraconiothyrium; Paraconiothyrium sporulosum	*Paraconiothyrium_sporulosum* (EU821483)	94
	fOTU_75	Fungi	Unknown	0
	fOTU_335	Fungi; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetidae; Capnodiales; Mycosphaerellaceae; Davidiella	*Cladosporium_pseudocladosporioides* (JN033472)	100
	fOTU_1525	Fungi	Unknown	0
PA	fOTU_18	Fungi	Unknown	0

	fOTU_49	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreales_Incertae sedis; Acremonium; Acremonium cavaraeanum	*Acremonium_cavaraeanum* (JF912333)	92
	fOTU_36	Fungi	Unknown	0
PD	fOTU_26	Fungi; Zygomycota; Mucoromycotina; Mucoromycotina_Incertae sedis; Mucoromycotina_Incertae sedis; Mortierellales; Mortierellaceae; Mortierella; Mortierella elongata	*Mortierella_elongata* (AB542099)	100
	fOTU_1002	Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Eurotiales; Trichocomaceae; Penicillium; Penicillium skrjabinii	*Penicillium_skrjabinii* (GU981576)	99
	fOTU_1461	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreaceae; Hypocrea; Trichoderma evansii	*Trichoderma_evansii* (AF414320)	95
	fOTU_36	Fungi	Unknown	0
	fOTU_352	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales; Helotiaceae; Leptodontidium; Leptodontidium elatius	*Leptodontidium_elatius* (AY781230)	95
PR	fOTU_26	Fungi; Zygomycota; Mucoromycotina; Mucoromycotina_Incertae sedis; Mucoromycotina_Incertae sedis; Mortierellales; Mortierellaceae; Mortierella; Mortierella elongata	*Mortierella_elongata* (AB542099)	100
	fOTU_123	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales; Saccharomycetales_Incertae sedis; Candida; Candida coipomoensis	*Candida_coipomoensis* (AJ606466)	100
	fOTU_1270	Fungi; Basidiomycota; Agaricomycotina; Tremellomycetes; Tremellomycetidae; Tremellales; Tremellaceae; Cryptococcus; Cryptococcus laurentii	*Cryptococcus_laurentii* (JN626994)	98
	fOTU_38	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Neonectria	*Ilyonectria_estremocensis* (JF735320)	100
	fOTU_757	Fungi; Zygomycota; Mucoromycotina; Mucoromycotina_Incertae sedis; Mucoromycotina_Incertae sedis; Mortierellales; Mortierellaceae; Mortierella;	Unknown	0

TE	fOTU_38	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Neonectria	*Ilyonectria_estremocensis* (JF735320)	100
	fOTU_724	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreaceae; Hypocrea; Hypocrea caerulescens	*Cosmospora_butyri* (JQ070093)	86
	fOTU_36	Fungi	Unknown	0
XG	fOTU_123	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales; Saccharomycetales_Incertae sedis; Candida; Candida coipomoensis	*Candida_coipomoensis* (AJ606466)	100
	fOTU_1270	Fungi; Basidiomycota; Agaricomycotina; Tremellomycetes; Tremellomycetidae; Tremellales; Tremellaceae; Cryptococcus; Cryptococcus laurentii	*Cryptococcus_laurentii* (JN626994)	98
	fOTU_38	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Neonectria	*Ilyonectria_estremocensis* (JF735320)	100
	fOTU_388	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales	Unknown	0
	fOTU_420	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales; Trichomonascaceae; Blastobotrys; Blastobotrys mokoenaii	*Blastobotrys_mokoenaii* (DQ898171)	91

** - Identity score. 2-aminoethanol (AE), 2-ethylhexanol (EH), benzisothiazolinone (BZ), bronopol (BR), diethylene glycol ethyl ether (DG), d-limonene (DL), d-limonene x10 (DLx), eicosane (EC), glyoxal (GO), hexahydro-1,3,5-tris (2-hydroxyethyl)-sym-triazine (HHT), methylchloroisothiazolinone (MCT), methylisothiazolinone (MT), naphthalene (NP), naphthalene x10 (NPx), o-cresol (OC), o-cresol x10 (OCx), polyacrylamide (PA), polyoxypropylene diamine (PD), pristane (PR), triethanolamine (TE), xanthan gum (XG).*

By comparing fungi with prokaryotes, it is apparent that growth on some chemicals was primarily fungal (Figure 4-7). For 2-ethylhexanol, bronopol, d-limonene x10, glyoxal, methylchloroisothiazolinone and methylisothiazolinone, naphthalene and o-cresol x10, fungal taxa that increased in abundance were more common than prokaryotes.

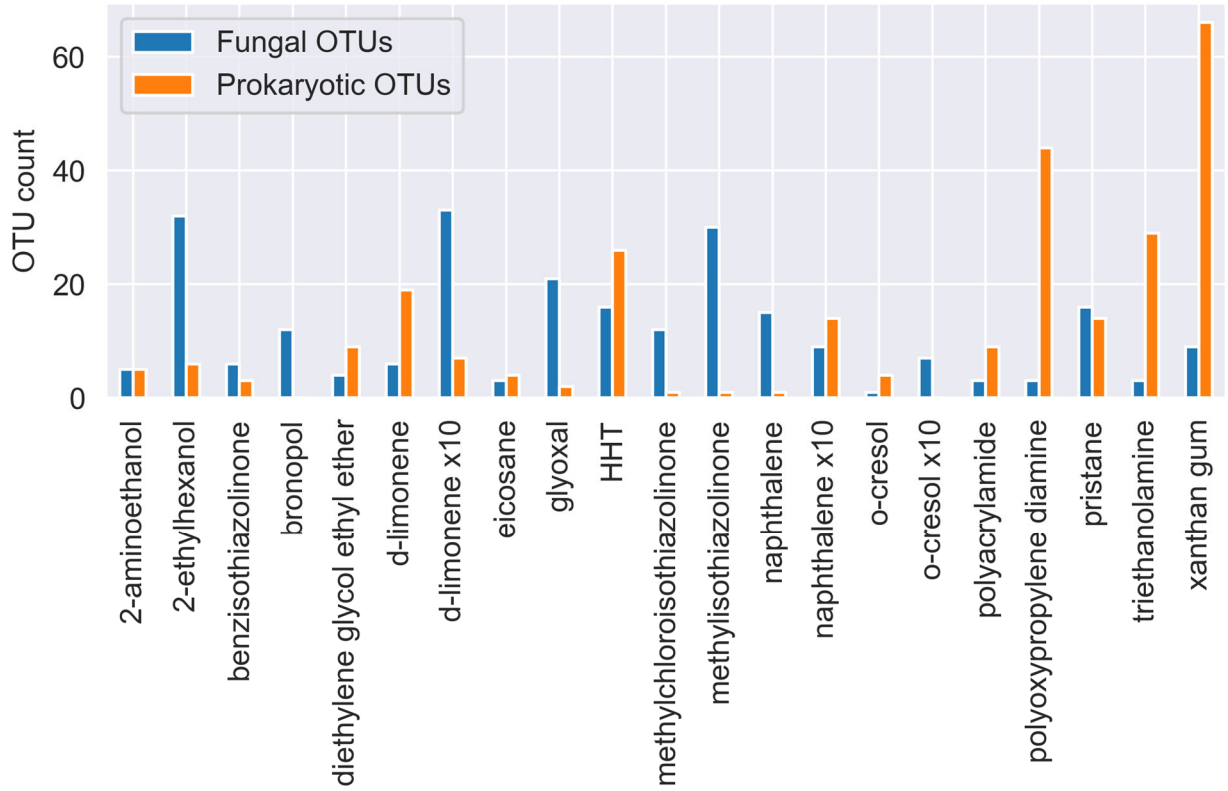


Figure 4-7: Bar plot of the number of fungi and prokaryotes that increased in abundance under various chemical treatments.

Most fungi that responded to chemical treatments were Ascomycetes, though both Basidiomycetes and Zygomycetes were also detected (Figure 4-8). Over 15% of fungal taxa detected could not be assigned to a known phylum.

Taxonomically, it is apparent that there are clear patterns across the treatments. Both pristane and 2-ethylhexanol, for example, have unusually high numbers of responding Zygomycota species (mostly *Mortieriella* species). Of the Basidiomycota, methylchloroisothiazoline and methylisothiazoline both have Agaricales taxa enriched under these treatments (Figure 4-9). Agaricales for both of these treatments appear to be *Pholiota* species and possibly *Pholiota multicingulata*. Unlike the two above examples, some fungal groups appear to be widespread. For

example, the ascomycete order Hypocreales, represents a significant pool of taxa in all treatments except bronopol, diethylene glycol ethyl ether and o-cresol x10 (Figure 4-9 and Appendix B).

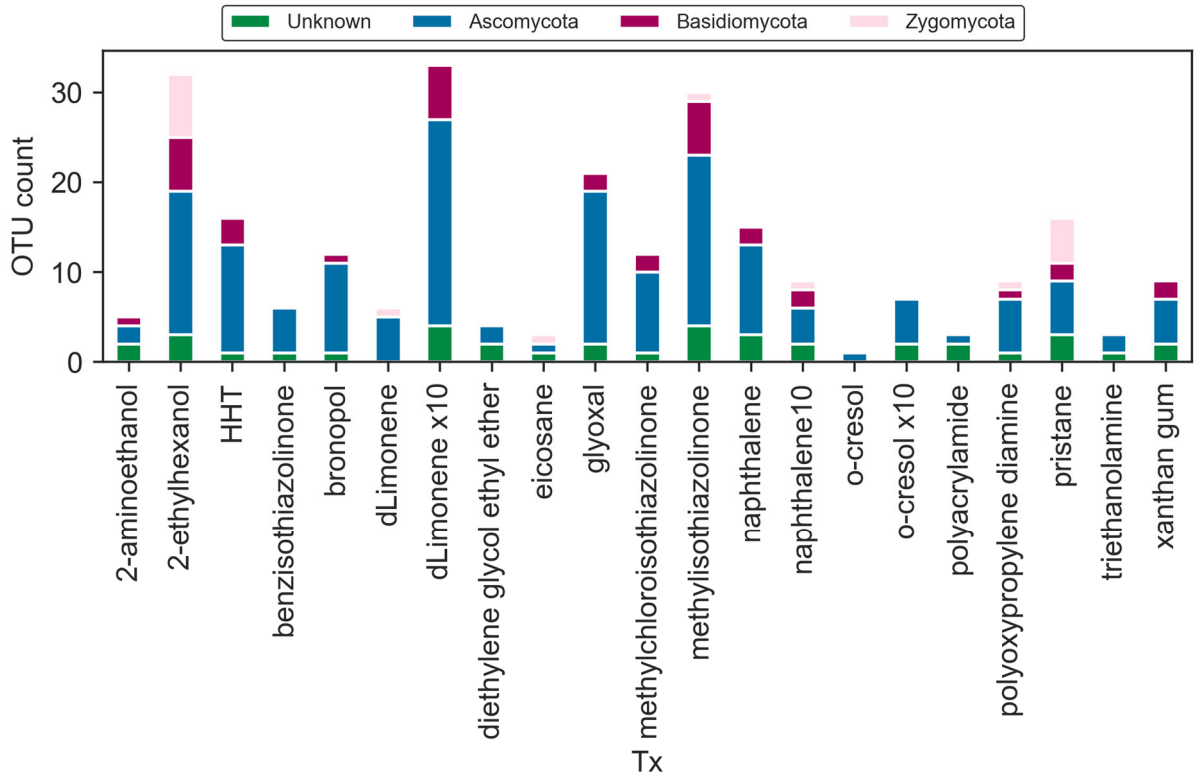


Figure 4-8: Stacked bar plot representing the counts of fungi from each phylum that responded to individual treatments.

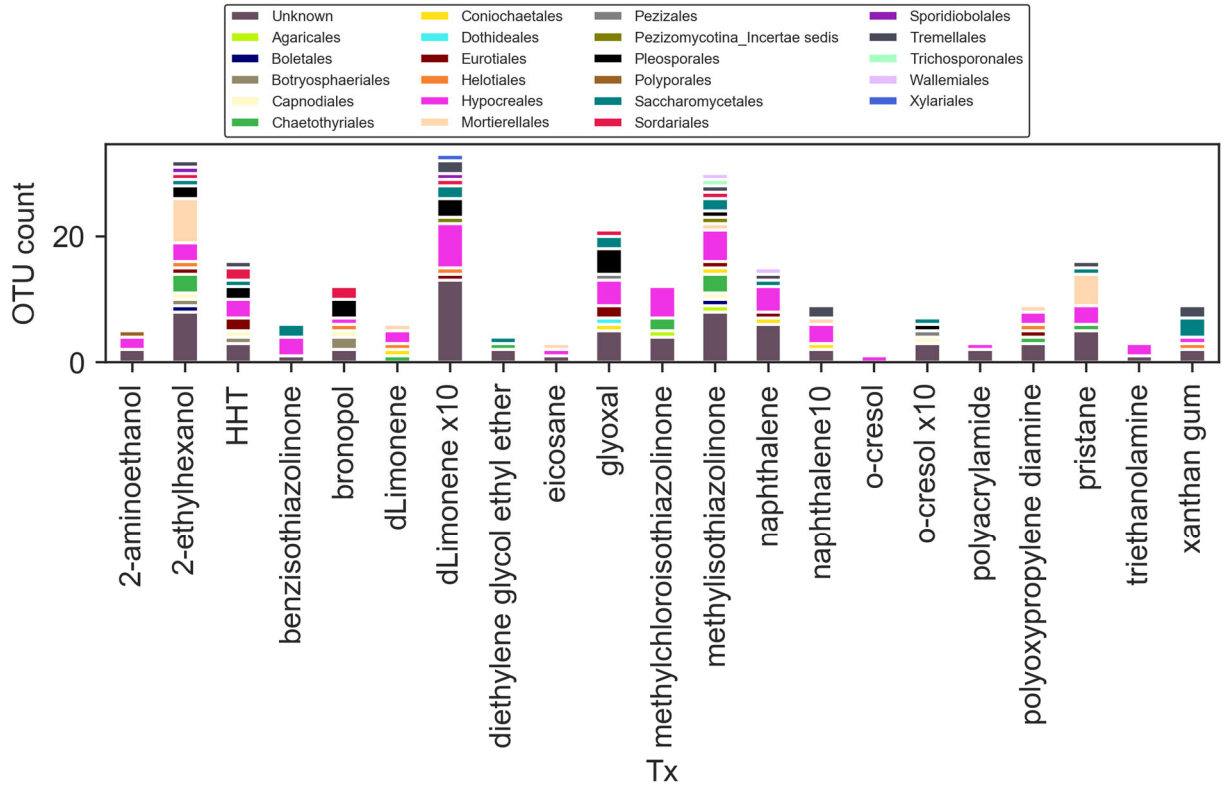


Figure 4-9: Stacked bar plot representing the counts of fungi from each order that responded to individual treatments.

Interestingly, mean fungal species richness was 97.5 (±5.5) on untreated plates without a carbon source (Figure 4-10). This represents the number of fungi that were able to survive on the medium without an exogenous source of carbon during the course of the trial. It is notably lower than the number of species of fungi estimated to be inoculated on the plates (~1500 species, based on OTU tables found in Appendix B).

Across the experiment, fungal species richness was highest on the control plates without a carbon source (Figure 4-10). All treatments resulted in lower species richness for fungi, though only bronopol (31 species), d-limonene (50.8 species ± 4.5), methylchloroisothiazoline (23 species ± 3), naphthalene x10 (41.5 species ± 9), o-cresol (47.0 species ± 13.7) and o-cresol x10 (47.3 species ± 8.4) were significantly lower ($p<0.05$).

It is noteworthy that while DNA could be extracted from glutaraldehyde and c12 alcohol ethoxylate treated plates, neither treatment produced a fungal PCR amplicon. Examination of each of the three glutaraldehyde and c12 alcohol ethoxylate treated petri dishes showed distinct hyphal-like organisms and small patches of sporulation, so the reason for the lack of PCR product is unknown.

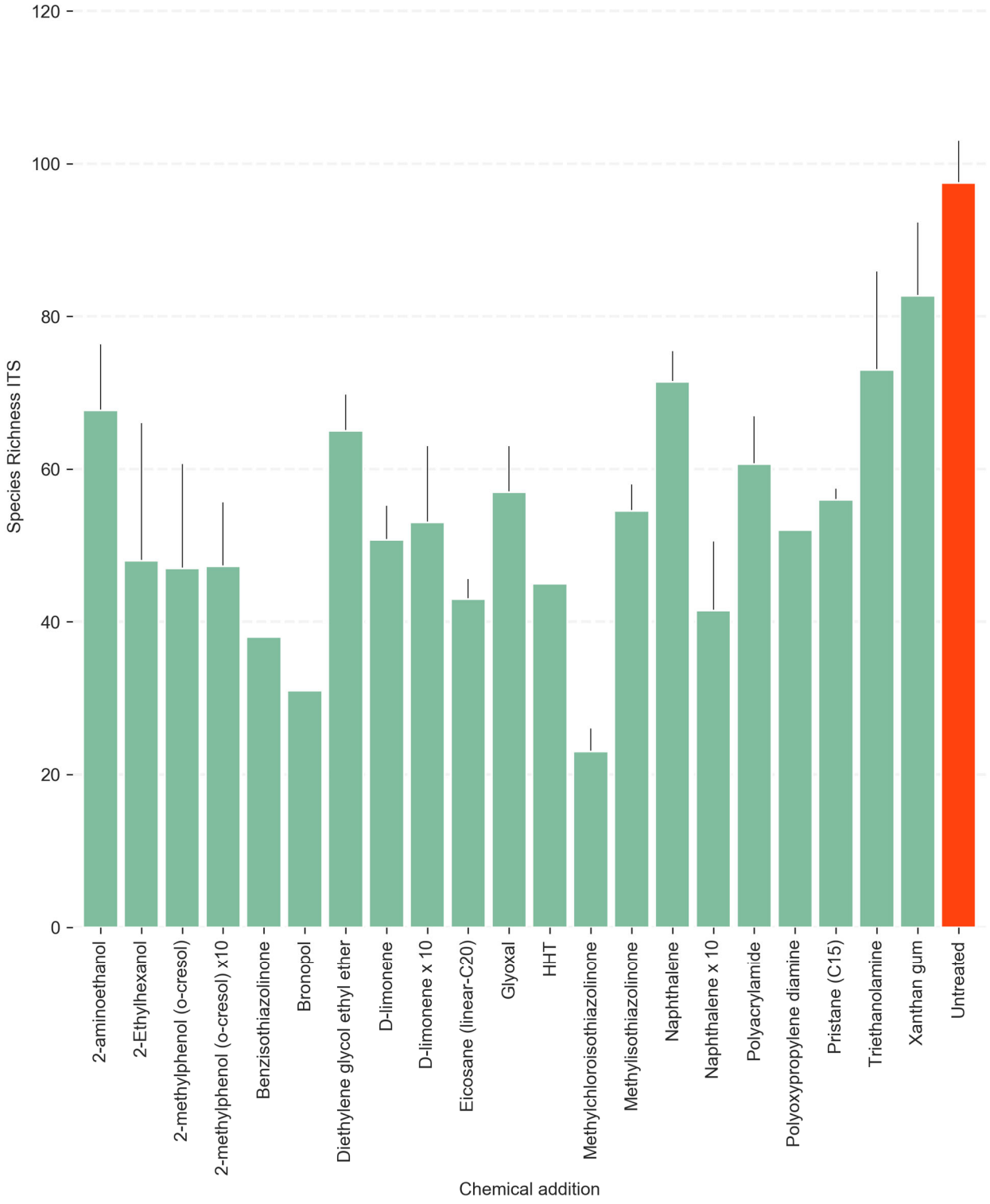


Figure 4-10: Fungal species richness on petri-dishes containing SMM agar amended with a range of compounds.

Mean Simpson's Index (1-D) for fungi was 0.73 (±0.04) on untreated plates without a carbon source (Figure 4-11). While analysis of variance revealed differences between treatments ($p<0.02$), the differences are modest and the relatively low replicate numbers meant that insufficient statistical power was present to identify significantly different treatments. While the biodiversity analyses are inconclusive, it seems probable that some of these treatments represent significant losses in biodiversity and may mirror statistical differences observed in the species richness.

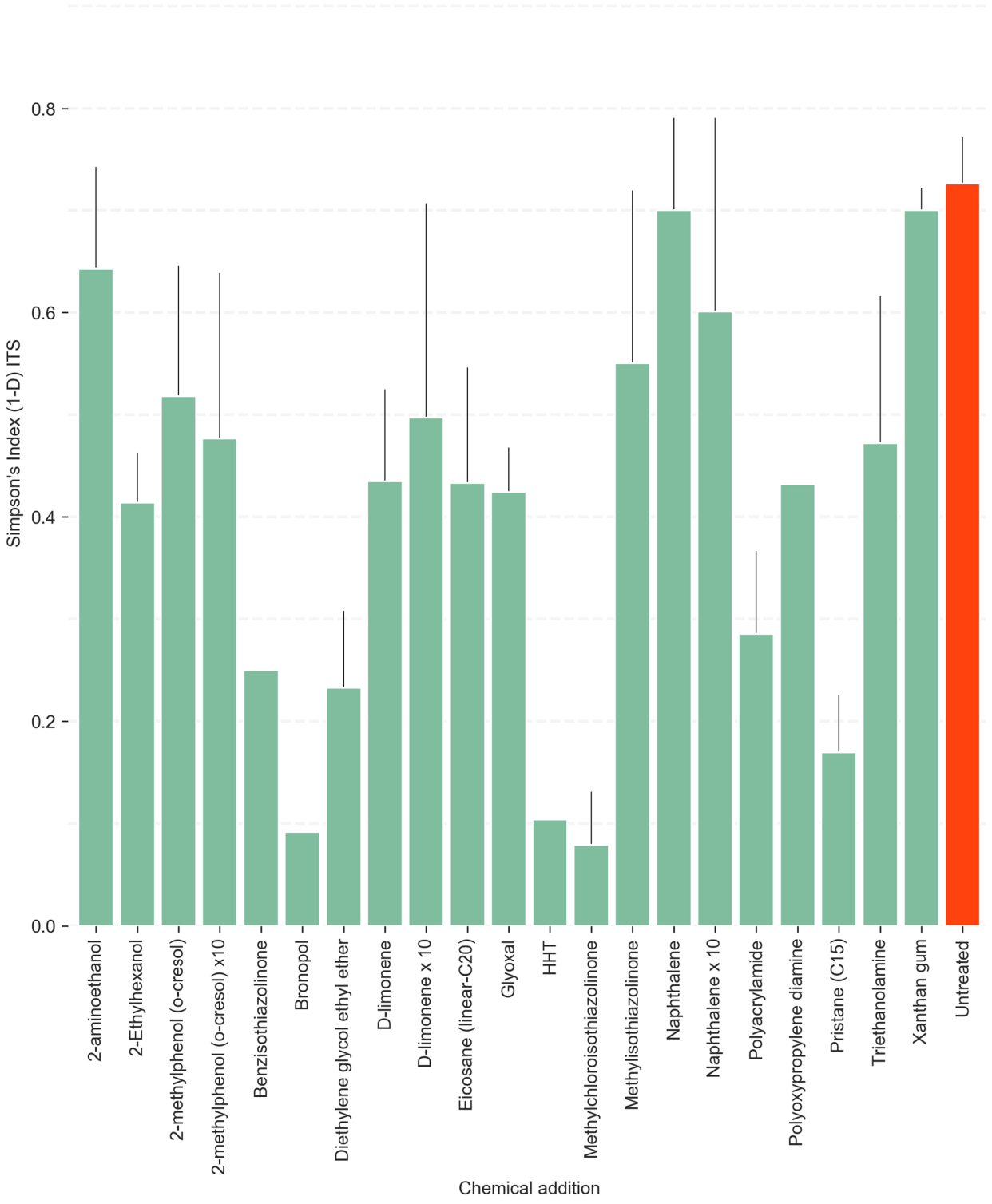


Figure 4-11: Fungal biodiversity (Simpson's 1-D) on petri-dishes containing SMM agar amended with a range of compounds.

4.5 Identification of prokaryotic taxa that degrade target chemicals

Three hundred and ninety-three (393) prokaryotic taxa were found to increase under treatments with chemicals used in onshore gas activities. Of these, 193 were increased in every replicate of a treatment. Because of the large number of responding taxa for prokaryotes compared to fungi, only these 193 taxa are analysed further. While some archaea were detected on the plates used to assay growth on target chemicals, all responding taxa were bacterial.

As for the fungal network map, OTUs uniquely involved in the degradation of single compounds are shown as fans or points radiating out from a single red point (Figure 4-12). Methylchloroisothiazoline (MCT) was unique in that just a single bacterial OTU (pOTU_190) showed increased growth in its presence and this OTU was not involved in the degradation of any other chemicals. This treatment forms somewhat of an island in the network map (Figure 4-12).

Some OTUs were involved in the degradation of multiple compounds. Most numerous of these were pOTU_3159 which grew at increased abundance under o-cresol (OC), diethylene glycol ethyl ether (DG), d-limonene (DL), d-limonene x10 (DLx), eicosane (EC), naphthalene (NP), naphthalene x10 (NPx), polyacrylamide (PA), polyoxypropylene diamine (PD) , pristane (PR) and triethanolamine (TE), along with pOTU_201 which grew at increased abundance in the presence of 2-ethylhexanol (EH), o-cresol (OC), diethylene glycol ethyl ether (DG), d-limonene (DL), d-limonene x10 (DLx), naphthalene x10 (NPx), polyacrylamide (PA), polyoxypropylene diamine (PD), triethanolamine (TE) and xanthan gum. pOTU_3159 was likely a *Phenylobacterium* spp., while pOTU_201 was a probable *Paenibacillus* species.

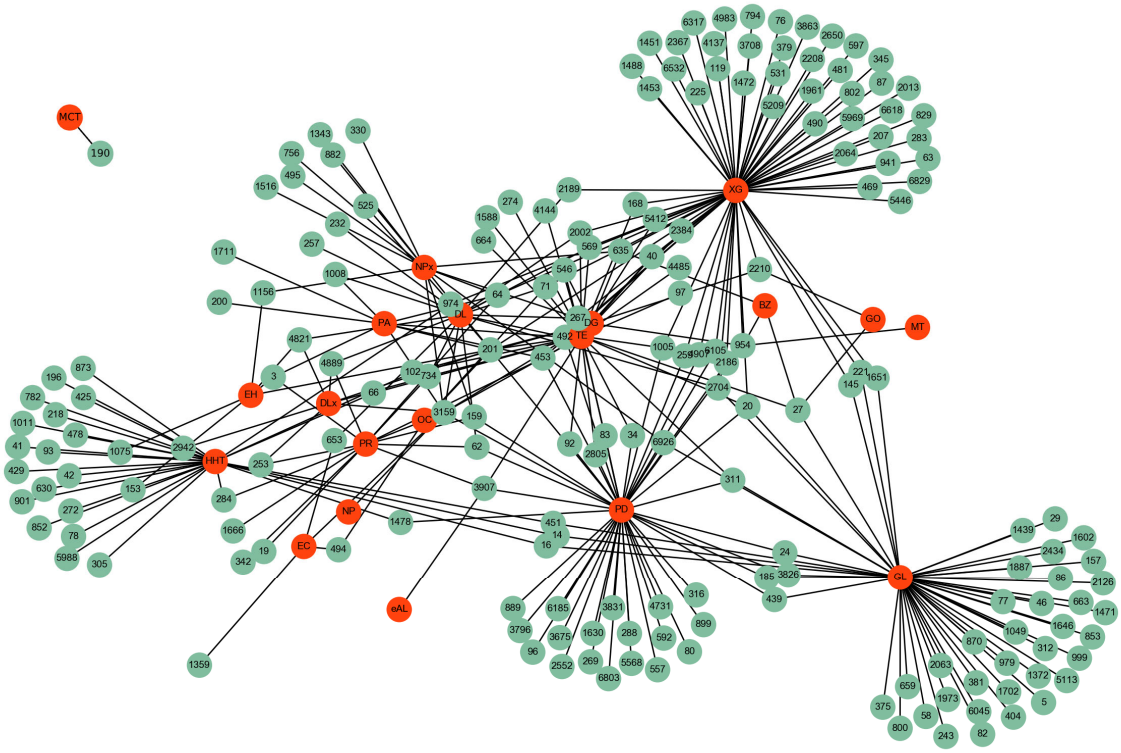


Figure 4-12: Undirected network analyses of prokaryotic OTUs (green) and their growth in the presence of particular chemicals (red).

Lines between chemicals and OTUs indicate that OTU degrades that compound. Numbers in circles are fungal OTUs. 2-ethylhexanol (EH), Benzisothiazolinone (BZ), c12 alcohol ethoxylate (eAL), diethylene glycol ethyl ether (DG), d-limonene (DL), d-limonene x10 (DLx), eicosane (EC), glutaraldehyde (GL), glyoxal (GO), hexahydro-1,3,5-tris (2-hydroxyethyl)-sym-triazine (HHT), methylchloroisothiazolinone (MCT), methylisothiazolinone (MT), naphthalene (NP), naphthalene x10 (NPx), o-cresol (OC), polyacrylamide (PA), polyoxypropylene diamine (PD), pristane (PR), triethanolamine (TE), xanthan gum (XG).

Putative bacterial catabolisers of chemicals were most common in the xanthan gum, glutaraldehyde and polyoxypropylene diamine treatments, which had 66, 49 and 44 distinct, bacterial taxa positively associated with the treatment. Table 4-7 lists the top 5 bacterial taxa that responded positively for each individual treatment. Where there were less than five bacterial taxa that responded positively, all the top responders are listed.

Table 4-7: Top 5 putative prokaryotic catabolisers of chemical treatments in sole carbon experiments.

Chemical treatment	OTU	Phylogeny	Closest match	ID*
eAL	pOTU_3907	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia	*Burkholderia_ferrariae_* (T)_FeGl01_ (DQ514537)	97.6
BZ	pOTU_635	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae	*Rhodopila_globiformis_* (T)_DSM161_ (D86513)	95.3
	pOTU_954	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae	*Rhodopila_globiformis_* (T)_DSM161_ (D86513)	94.9
	pOTU_27	Bacteria; Acidobacteria; Acidobacteria_Gp1; Gp1	uncultured_bacterium_FW29_ (AF523990)	98.8
DG	pOTU_954	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae	*Rhodopila_globiformis_* (T)_DSM161_ (D86513)	94.9
	pOTU_635	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae	*Rhodopila_globiformis_* (T)_DSM161_ (D86513)	95.3
	pOTU_492	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae; Flavisolibacter	*Flavisolibacter_ginsengisoli_* (T)_Gsoil_643_ (AB267477)	94.9
	pOTU_3159	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Phenylobacterium	*Phenylobacterium_falsum_* (T)_type_strain:_AC-49_ (AJ717391)	96.4
	pOTU_546	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Sphingobacteriaceae; Mucilaginibacter	*Mucilaginibacter_puniceus*_strain_WS71_ (NR_152668.1)	99.2
DL	pOTU_232	Bacteria	Unknown	0
	pOTU_2002	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Sphingobacteriaceae; Mucilaginibacter	*Mucilaginibacter_boryungensis*_BDR-9_ (HM061614)	99.6
	pOTU_71	Bacteria	Unknown	0
	pOTU_3159	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Phenylobacterium	*Phenylobacterium_falsum_* (T)_type_strain:_AC-49_ (AJ717391)	96.4

	pOTU_653	Bacteria	Unknown	0
DLx	pOTU_3159	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Phenylobacterium	*Phenylobacterium_falsum_* (T)_type_strain:_AC-49_ (AJ717391)	96.4
	pOTU_4889	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Massilia	*Massilia_namucuonensis*_333-1-0411_ (JF799985)	98.8
	pOTU_4821	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Oxalicibacterium	*Herminiimonas_aquatilis_* (T)_type_strain:_CCUG_36956_ (AM085762)	96.4
	pOTU_253	Bacteria; Firmicutes; Bacilli; Bacillales; Paenibacillaceae 1; Brevibacillus	*Brevibacillus_centrosporus*_strain_NBRC_15540_ (NR_113768.1)	100
	pOTU_734	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales	*Alkalilimnicola_ehrlichii_* (T)_MLHE-1_ (AF406554)	93.3
EC	pOTU_3159	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Phenylobacterium	*Phenylobacterium_falsum_* (T)_type_strain:_AC-49_ (AJ717391)	96.4
	pOTU_494	Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides	*Nocardioides_hankookensis_* (T)_DS-30_ (EF555584)	96.8
	pOTU_653	Bacteria	Unknown	0
	pOTU_1359	Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus	*Rhodococcus_antrifimi*_strain_D7-21_ (NR_145614.1)	100
EH	pOTU_1075	Bacteria; Proteobacteria; Alphaproteobacteria	*Lyticum_flagellatum*_strain_299_ (NR_125566.1)	87.7
	pOTU_4821	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Oxalicibacterium	*Herminiimonas_aquatilis_* (T)_type_strain:_CCUG_36956_ (AM085762)	96.4

	pOTU_1156	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae; Dongia	*Dongia*_soli_strain_D78_ (NR_146690.1)	96.8
	pOTU_201	Bacteria; Firmicutes; Bacilli; Bacillales; Paenibacillaceae 1; Paenibacillus	*Paenibacillus_rhizoryzae*_strain_1ZS3-5_ (NR_137245.1)	98.8
	pOTU_3	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Massilia	*Massilia*_sp._NS9_ (HG798294)	100
GL	pOTU_185	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Reyranella	*Reyranella*_soli_KIS14-15_ (JX260424)	96
	pOTU_2126	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobulbaceae; Desulfobulbus	*Desulfobulbus_elongatus*_ (T)_DSM_2908_ (X95180)	98.8
	pOTU_1702	Bacteria; Verrucomicrobia; Spartobacteria; Spartobacteria_genera_incertae_sedis	unidentified_bacterium_ (T)_soil_clone,member_ov_a_novel_phylum;_MC31_ (X64380)	93.7
	pOTU_1439	Bacteria	Unknown	0
	pOTU_6045		Unknown	0
GO	pOTU_27	Bacteria; Acidobacteria; Acidobacteria_Gp1; Gp1	uncultured_bacterium_FW29_ (AF523990)	98.8
	pOTU_2210	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae	*Rhodovastum_atsumiense*_strain_G2-11_ (NR_112776.1)	94.9
HHT	pOTU_630	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae	*Azospirillum_amazonense*_DSM_2787_ (GU256437)	94.1
	pOTU_478	Bacteria; Actinobacteria; Actinobacteria; Rubrobacteridae; Solirubrobacterales	*Conexibacter_arvalis*_KV-962_ (AB597950)	90.5
	pOTU_93	Bacteria; Verrucomicrobia; Spartobacteria; Spartobacteria_genera_incertae_sedis	unidentified_bacterium_ (T)_soil_clone,novel_line_of_descented;_MC17_ (X64381)	94.1
	pOTU_425	Bacteria; Acidobacteria; Acidobacteria_Gp1; Gp1	uncultured_Acidobacteria_bacterium_BGC.0078_ (EF457414)	98.4
	pOTU_102	Bacteria; Acidobacteria; Acidobacteria_Gp1; Gp1	uncultured_Acidobacteria_bacterium_BGA.0027_ (EF457379)	96.4

MCT	pOTU_190	Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Leifsonia	*Leifsonia_shinshuensis_* (T)_DB102;_JCM10591_ (DQ232614)	100
MT	pOTU_954	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae;	*Rhodopila_globiformis_* (T)_DSM161_ (D86513)	94.9
NP	pOTU_3159	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Phenylobacterium	*Phenylobacterium_falsum_* (T)_type_strain:_AC-49_ (AJ717391)	96.4
NPx	pOTU_1516	Bacteria	Unknown	0
	pOTU_1343	Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Cellulomonadaceae; Cellulomonas	*Cellulomonas_oligotrophica*_Kc5_ (AB602499)	100
	pOTU_3159	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Phenylobacterium	*Phenylobacterium_falsum_* (T)_type_strain:_AC-49_ (AJ717391)	96.4
	pOTU_6926	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	*Geobacter_lovleyi*_strain_SZ_ (NR_074979.1)	98
	pOTU_635	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae	*Rhodopila_globiformis_* (T)_DSM161_ (D86513)	95.3
OC	pOTU_71	Bacteria	Unknown	0
	pOTU_3159	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Phenylobacterium	*Phenylobacterium_falsum_* (T)_type_strain:_AC-49_ (AJ717391)	96.4
	pOTU_494	Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides	*Nocardioides_hankookensis_* (T)_DS-30_ (EF555584)	96.8
	pOTU_201	Bacteria; Firmicutes; Bacilli; Bacillales; Paenibacillaceae 1; Paenibacillus	*Paenibacillus_rhizoryzae*_strain_1ZS3-5_ (NR_137245.1)	98.8

PA	pOTU_492	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae; Flavisolibacter	*Flavisolibacter_ginsengisoli_* (T)_Gsoil_643_ (AB267477)	94.9
	pOTU_1008	Bacteria	Unknown	0
	pOTU_3159	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Phenylobacterium	*Phenylobacterium_falsum_* (T)_type_strain:_AC-49_ (AJ717391)	96.4
	pOTU_453	Bacteria; Verrucomicrobia; Subdivision3; Subdivision3_genera_incertae_sedis	uncultured_Verrucomicrobia_bacterium_ (T)_VC12_ (AY211073)	96
	pOTU_1711	Bacteria; Firmicutes; Bacilli; Bacillales; Paenibacillaceae 1; Paenibacillus	*Paenibacillus_marchantiophytorum*_strain_R55_ (NR_148618.1)	97.6
PD	pOTU_453	Bacteria; Verrucomicrobia; Subdivision3; Subdivision3_genera_incertae_sedis	uncultured_Verrucomicrobia_bacterium_ (T)_VC12_ (AY211073)	96
	pOTU_3907	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia	*Burkholderia_ferrariae_* (T)_FeGl01_ (DQ514537)	97.6
	pOTU_92	Bacteria; Acidobacteria; Acidobacteria_Gp1; Granulicella	*Granulicella_sapmiensis_* (T)_S6CTX5A_ (HQ687090)	99.6
	pOTU_83	Bacteria; Acidobacteria; Acidobacteria_Gp1	*Granulicella_pectinivorans_* (T)_type_strain:_TPB6011_ (AM887757)	96.8
	pOTU_6803	Bacteria; Acidobacteria; Acidobacteria_Gp1; Granulicella	*Granulicella_acidiphila*_strain_MCF40_ (NR_148567.1)	96.8
PR	pOTU_253	Bacteria; Firmicutes; Bacilli; Bacillales; Paenibacillaceae 1; Brevibacillus	*Brevibacillus_centrosporus*_strain_NBRC_15540_ (NR_113768.1)	100
	pOTU_3159	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Phenylobacterium	*Phenylobacterium_falsum_* (T)_type_strain:_AC-49_ (AJ717391)	96.4
	pOTU_4889	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Massilia	*Massilia_namucuonensis*_333-1-0411_ (JF799985)	98.8
	pOTU_453	Bacteria; Verrucomicrobia; Subdivision3; Subdivision3_genera_incertae_sedis	uncultured_Verrucomicrobia_bacterium_ (T)_VC12_ (AY211073)	96

	pOTU_3907	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia	*Burkholderia_ferrariae*_ (T)_FeGl01_ (DQ514537)	97.6
TE	pOTU_492	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae; Flavisolibacter	*Flavisolibacter_ginsengisoli*_ (T)_Gsoil_643_ (AB267477)	94.9
	pOTU_453	Bacteria; Verrucomicrobia; Subdivision3; Subdivision3_genera_incertae_sedis	uncultured_Verrucomicrobia_bacterium_ (T)_VC12_ (AY211073)	96
	pOTU_2002	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Sphingobacteriaceae; Mucilaginibacter	*Mucilaginibacter_boryungensis*_BDR-9_ (HM061614)	99.6
	pOTU_40	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas	*Sphingomonas_lutea*_strain_JS5_ (NR_153746.1)	99.6
	pOTU_3907	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia	*Burkholderia_ferrariae*_ (T)_FeGl01_ (DQ514537)	97.6
XG	pOTU_492	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae; Flavisolibacter	*Flavisolibacter_ginsengisoli*_ (T)_Gsoil_643_ (AB267477)	94.9
	pOTU_453	Bacteria; Verrucomicrobia; Subdivision3; Subdivision3_genera_incertae_sedis	uncultured_Verrucomicrobia_bacterium_ (T)_VC12_ (AY211073)	96
	pOTU_954	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae	*Rhodopila_globiformis*_ (T)_DSM161_ (D86513)	94.9
	pOTU_2002	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Sphingobacteriaceae; Mucilaginibacter	*Mucilaginibacter_boryungensis*_BDR-9_ (HM061614)	99.6
	pOTU_71	Bacteria	Unknown	0

** - Identity score. 2-ethylhexanol (EH), Benzisothiazolinone (BZ), c12 alcohol ethoxylate (eAL), diethylene glycol ethyl ether (DG), d-limonene (DL), d-limonene x10 (DLx), eicosane (EC), glutaraldehyde (GL), glyoxal (GO), hexahydro-1,3,5-tris (2-hydroxyethyl)-sym-triazine (HHT), methylchloroisothiazolinone (MCT), methylisothiazolinone (MT), naphthalene (NP), naphthalene x10 (NPx), o-cresol (OC), polyacrylamide (PA), polyoxypropylene diamine (PD), pristane (PR), triethanolamine (TE), xanthan gum (XG).*

Phylogenetically, OTUs that were more abundant under chemical treatments came from 10 bacterial phyla: Acidobacteria, Actinobacteria, Bacteroidetes, Chlamydiae, Cyanobacteria, Firmicutes, Planctomycetes, Proteobacteria, Verrucomicrobia and the candidate division WPS-1 (WPS = Wittenberg polluted soil), along with the archaeal phylum Thaumarchaeota (Figure 4-13). Differences were apparent between treatments, for example, Acidobacteria were common in the HHT, glutaraldehyde, polyoxypropylene glycol, triethanolamine and xanthan gum treatment, but where rare or absent from remaining treatments. Similarly, candidate division WPS-1 taxa were observed in the xanthan gum and glutaraldehyde treatments, but not elsewhere.

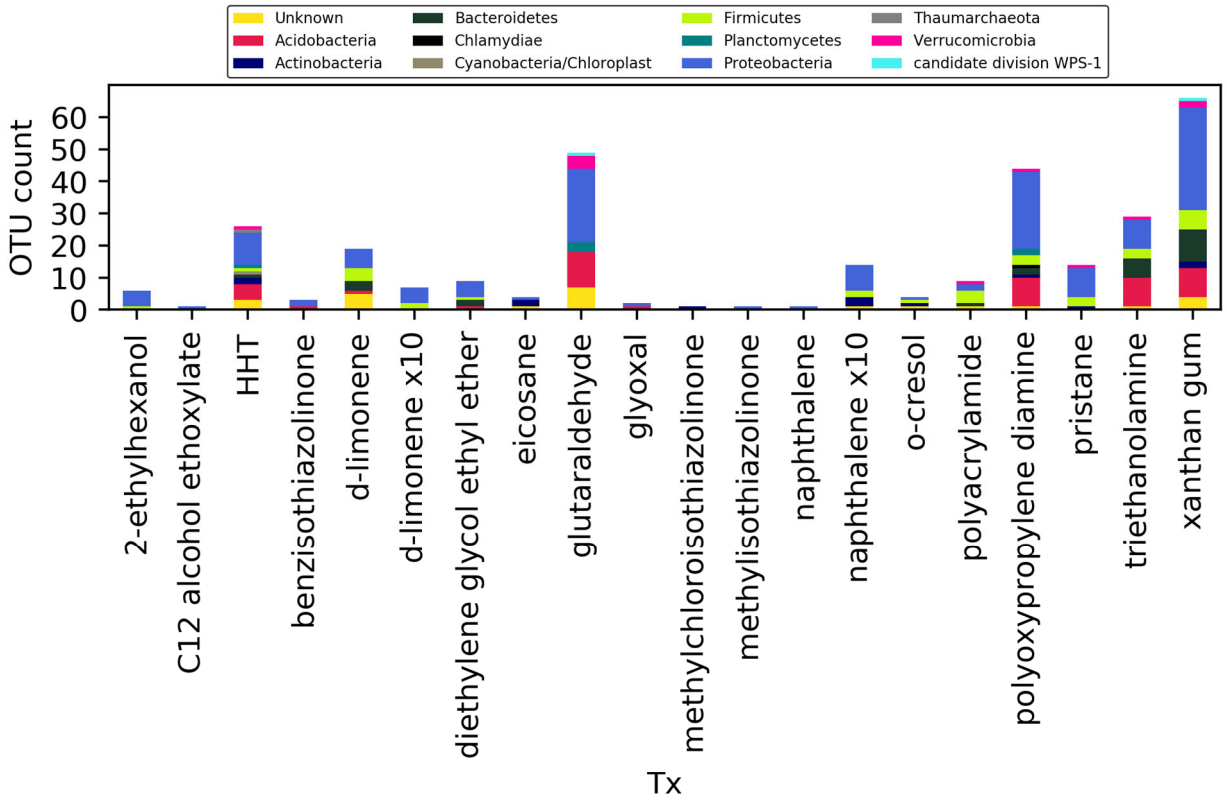


Figure 4-13: Stacked bar plot representing the counts of prokaryotes from each phylum that responded to individual treatments.

Order level phylogenetic differences were also apparent in prokaryotic OTUs that responded to chemical treatments (Figure 4-14). For example, Legionellales are common in the glutaraldehyde treatment but are comparatively rare in other treatments. Similarly, Actinobacteria were common in the eicosane and naphthalene x10 treatments but rare elsewhere.

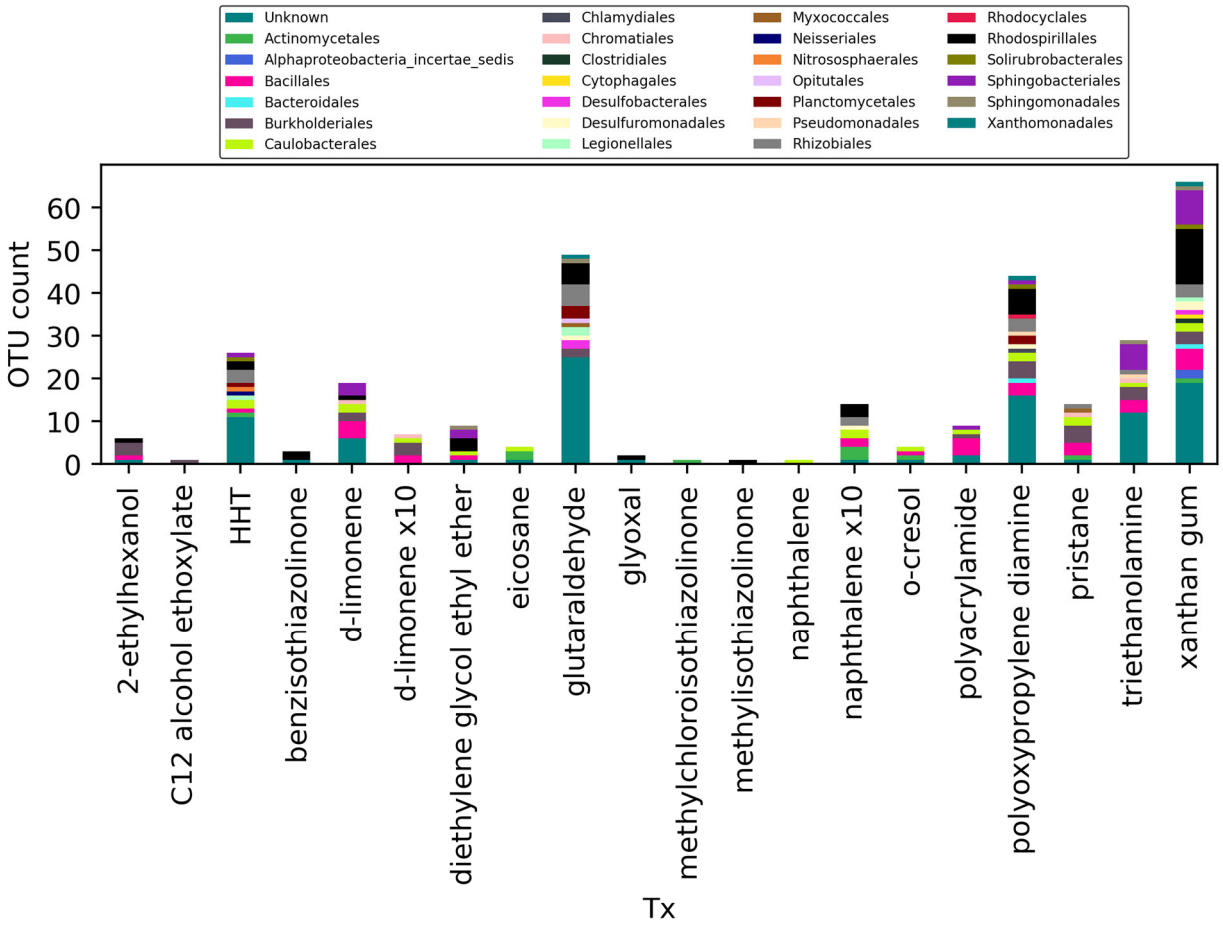


Figure 4-14: Stacked bar plot representing the counts of prokaryotes from each order that responded to individual treatments.

In terms of prokaryotic species richness between treatments, richness ranged from a maximum of 237 (±54.7) taxa in the polyacrylamide treatment to a low of 46 (±5.0) taxa. HHT had fewer taxa (43), but only one replicate of three was measurable for prokaryotes (Figure 4-15).

The untreated plates hosted an average of 123 (±27.2) taxa. Comparing treatments, some significant differences were observed ($p<0.02$). Pairwise comparisons lacked the required statistical power and except for the differences between polyacrylamide and either o-cresol x10 or c12 alcohol ethoxylate (both $p<0.03$) no significant differences were observed.

The significant ANOVA, and casual observation suggests that with more replication, differences between the untreated control and benzisothialzolinone, glyoxal, HHT and polyacrylamide may have been observed. It is also noteworthy that DNA extraction from the bronopol plates failed to yield amplifiable DNA for prokaryotes. Examination of one of the three bronopol-treated petri dishes (plate SC1; Figure 4-16) from which the DNA was extracted reveals an absence of bacterial colonies.

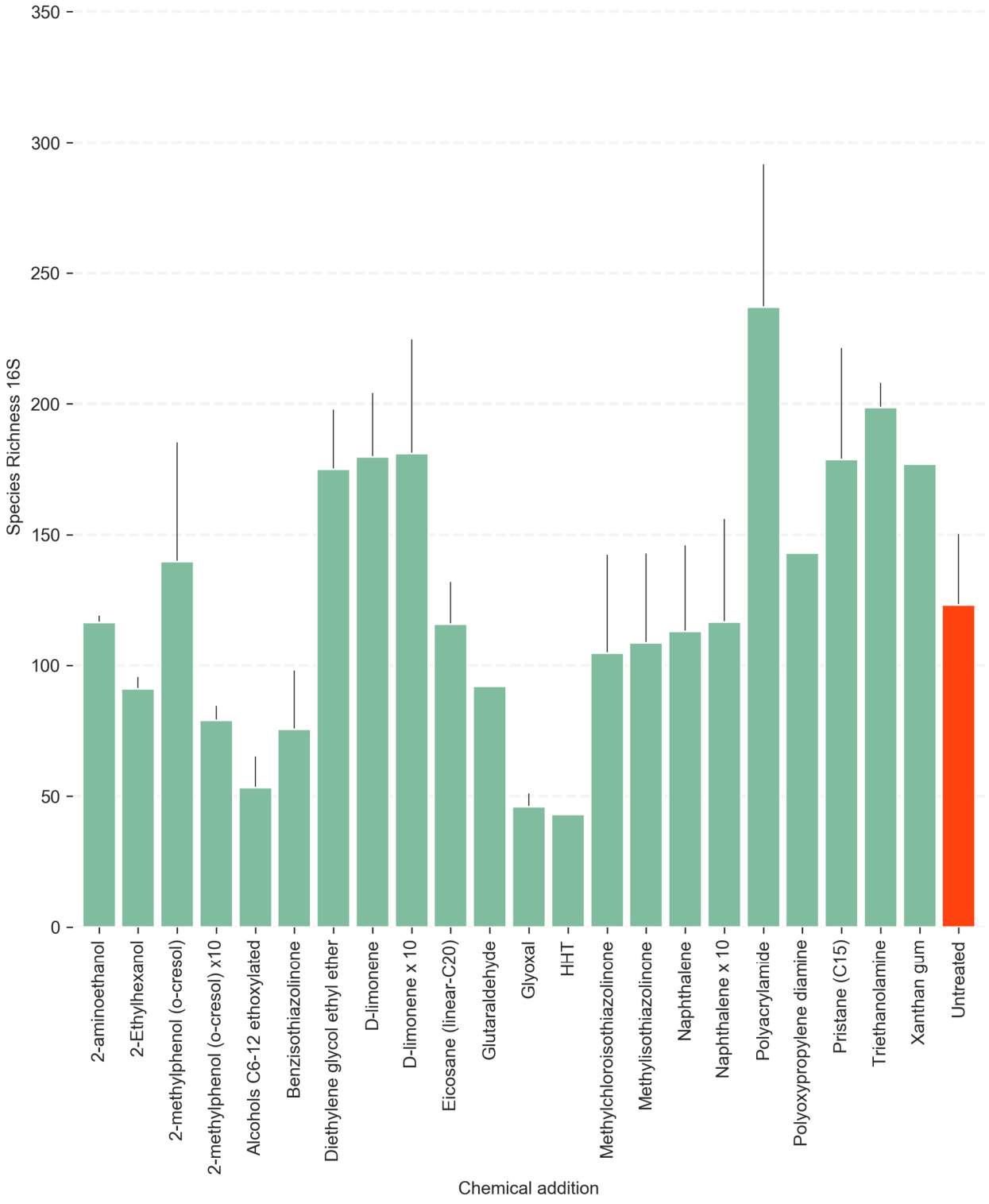


Figure 4-15: Prokaryotic species richness on petri-dishes containing SMM agar amended with a range of compounds.

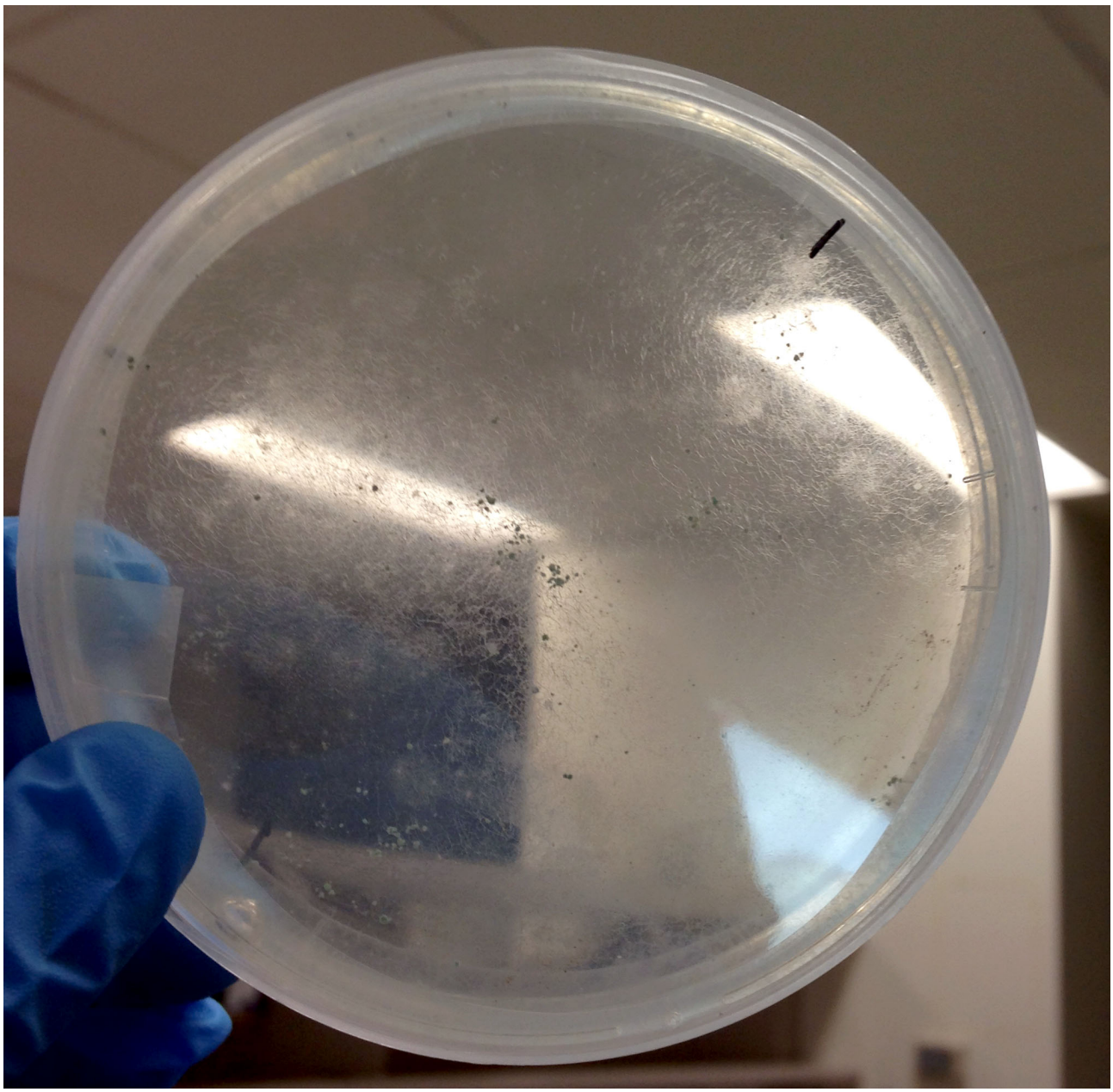


Figure 4-16: Plate SC1 had an absence of bacterial colonies in the presence of bronopol. Many large and small hyphal (fungal) colonies are present.

Prokaryotic biodiversity, as measured by the Simpson's Index, was greatest in the polyacrylamide treatment 0.82 (±0.04) and lowest in the o-cresol x10 treatment 0.52 (±0.04) (Figure 4-17). Similar to the situation for species richness, prokaryotic biodiversity was lowest for HHT treatment (0.35), but only a single replicate was amplified. Comparing the Simpson's Index from the untreated control 0.76 (±0.02) to the treatments, revealed only the o-cresol x10 treatment differed significantly ($p<0.01$).

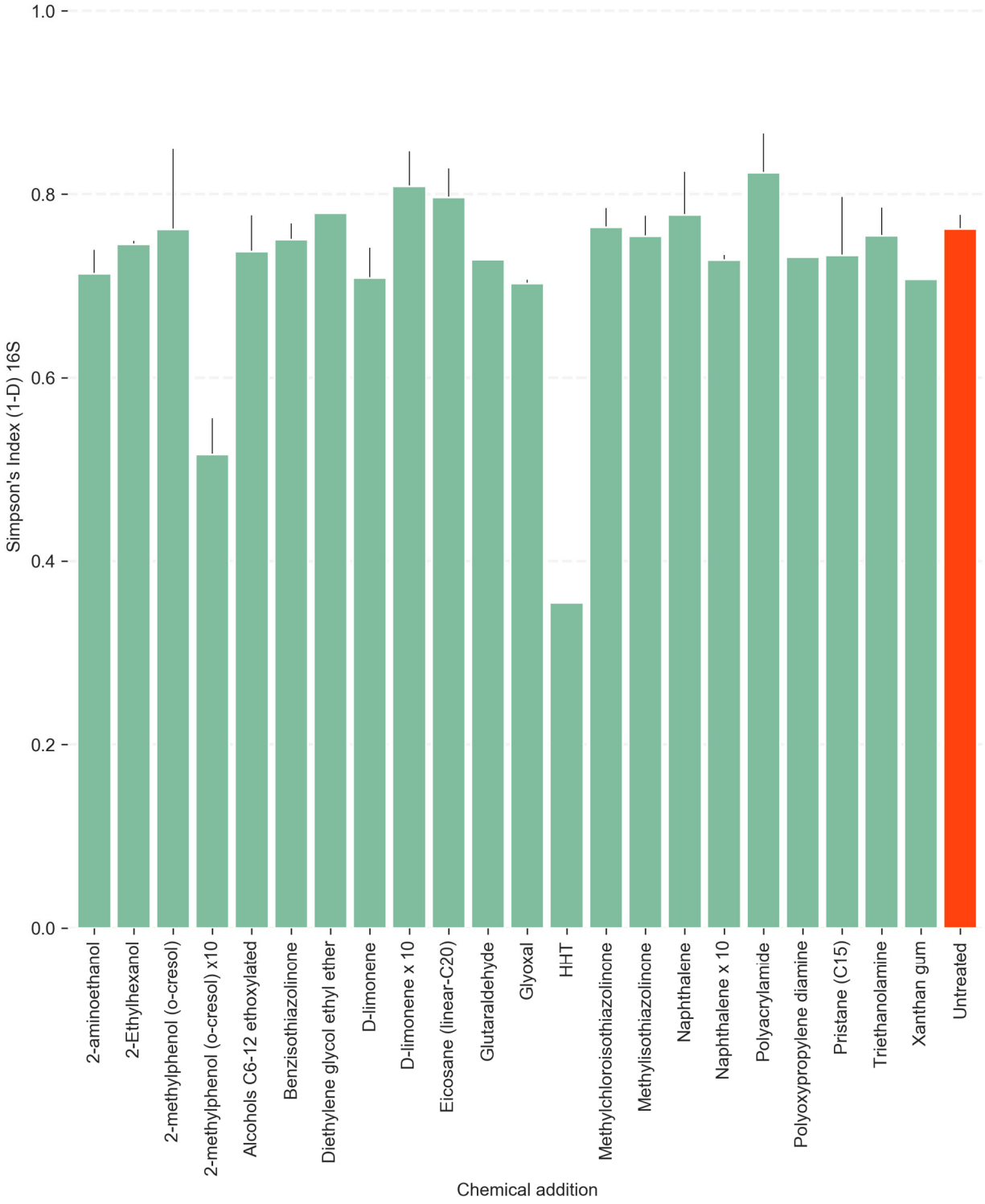


Figure 4-17: Prokaryotic biodiversity (Simpson's 1-D) on petri-dishes containing SMM agar amended with a range of compounds.

4.6 Effect of chemical treatments on soil microbiomes and identification of indicator taxa

DNA yields from the chemical treatments in the soil microcosms, in general, recovered between 5 x 10^6 and 1.3 x 10^7 cells per treatment (Figure 4-18). The exceptions to this were 2-aminoethanol and methanol (~1.3 x 10^7 and ~1.1 x 10^7cell numbers, respectively). It is noteworthy that these DNA extractions include all DNA recovered from the soil microcosms including both bacteria and fungi.

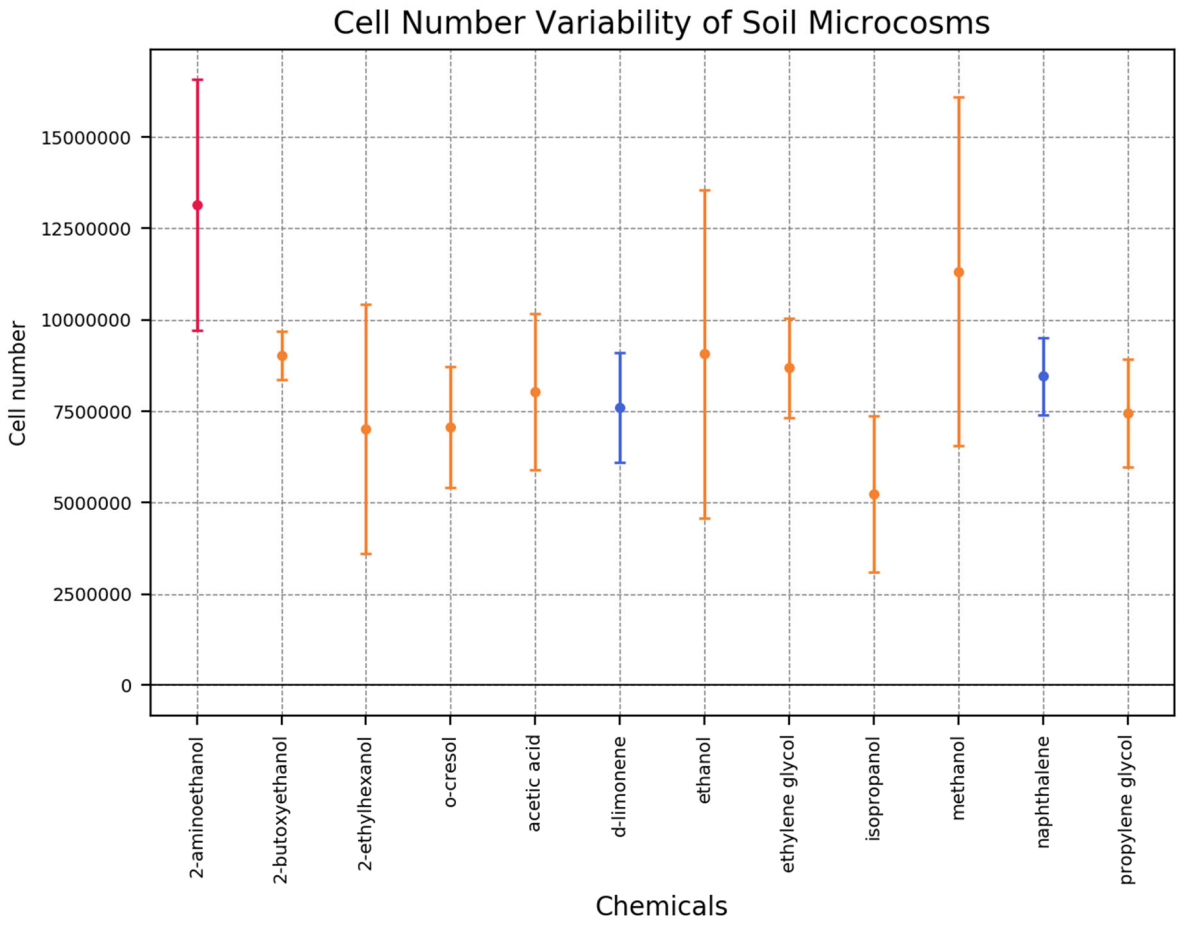


Figure 4-18: Cell number variability of soil microcosms.

Colours represent the presence of certain elements in each chemical. Chemicals containing C, H only (blue), O (orange), N and O (red). Cell number was calculated from DNA concentration assuming an average genome size of 4Mbp. Error bars show the standard error.

4.6.1 Prokaryotes

In total, 6835 prokaryotic taxa were detected in the soil from Penola (Appendix B). Of these, 6760 were bacterial while only eight where archaeal. The archaeal taxa were mostly Thaumarchaeota. In total, 24 phyla were represented in the soil including: Acidobacteria, Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, candidate division BRC1 (Bacterial rice cluster 1), candidate division WPS-1, candidate division WPS-2 (WPS = Wittenberg polluted soil), Candidatus Saccharibacteria, Chlamydiae, Chloroflexi, Cyanobacteria/Chloroplast, Deinococcus-Thermus, Firmicutes, Gemmatimonadetes, Hydrogenedentes, Microgenomates, Nitrospirae, Parcubacteria, Planctomycetes, Proteobacteria, Spirochaetes, Tenericutes and Verrucomicrobia.

Comparisons between chemical treatments in this experiment where somewhat problematic, since the experiment lacked an untreated control due to an oversight during the experimental setup. Nevertheless, several results are clear. Both ordinations (PCA and DCA; Figure 4-19, Figure 4-20 and Figure 4-21, respectively) show that 2-aminoethanol causes a clear shift in microbial community structure with marked increases in populations of pOTU_55, pOTU_25, pOTU_108, pOTU_106 and pOTU_101 (*Pseudomonas, Devosia, Stenotrophomonas, Brevundimonas* & *Niastella* taxa, respectively). To a lesser extent, ethylene glycol was also separated from other treatments through increased abundance of several *Burkholderia* species (pOTU_1, pOTU_2 and pOTU_16). Table 4-12 shows the pOTU responsible for these shifts in microbial community structure that are evident from both the OTU table and the loading scores for the PCA (Table 4-8, Table 4-9, Table 4-10 and Table 4-11). The microbial community shifts due to the chemicals 2-butoxyethanol and acetic acid are second order effects evident only in the loading scores in PC3 and PC4 (Table 4-10 and Table 4-11), which account for a smaller proportion of variance.

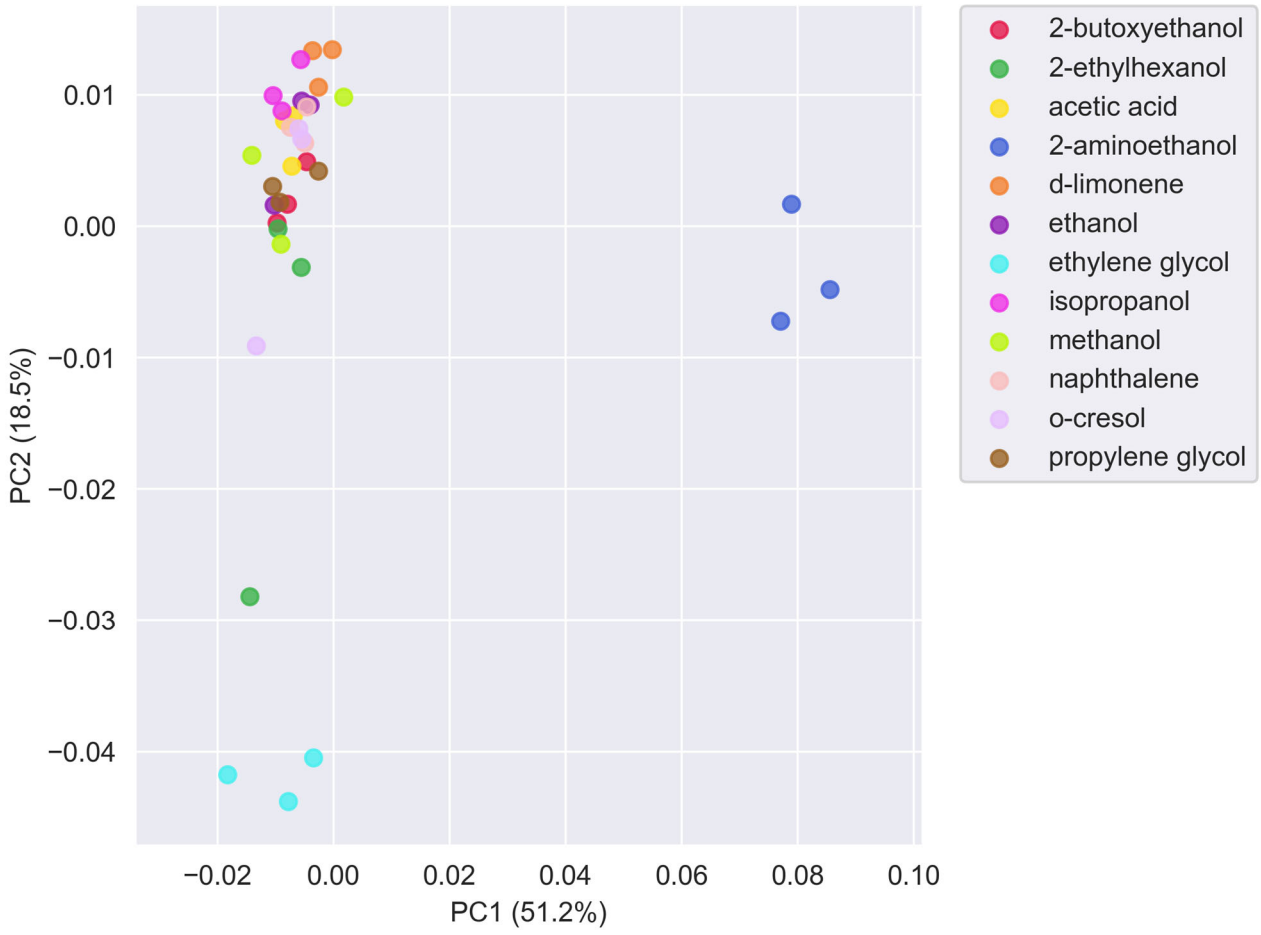


Figure 4-19: Principal components analysis of 16S soil OTUs by treatment (PC1 and PC2).

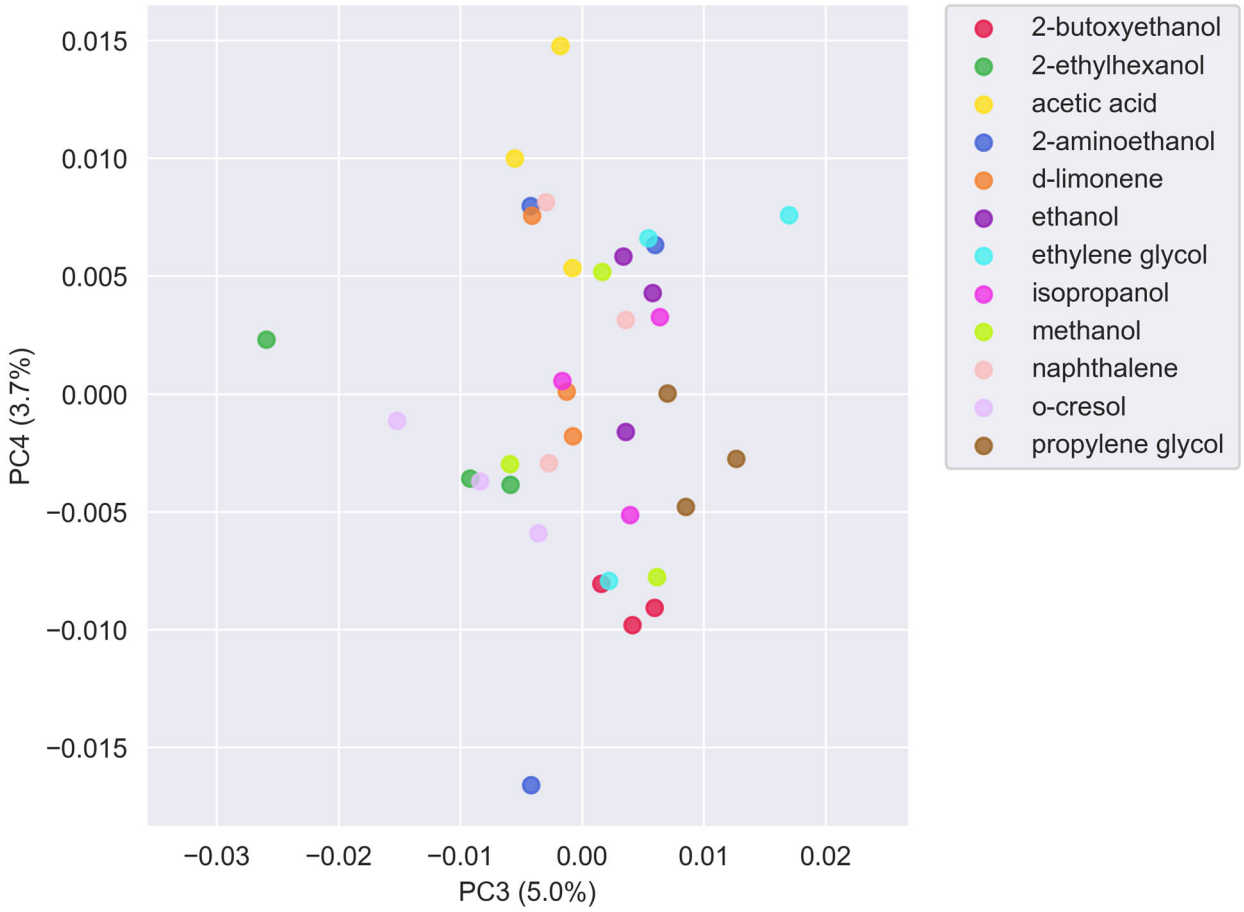


Figure 4-20: Principal components analysis of 16S soil OTUs by treatment (PC3 and PC4).

Table 4-8: Loading scores for the top 10 organisms from the 16S soil PCA for PC1.

OTU	LS †	Phylogeny	Closest match	ID*
Positive loading scores				
pOTU_55	0.379	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	*Pseudomonas putida* ICMP 2758 (NR_114794)	100
pOTU_25	0.305	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Devosia	*Devosia humi* THG-MM1 (NR_147759)	100
pOTU_108	0.203	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas	*Stenotrophomonas tumulicola* T5916-2-1b (NR_148818)	99.6
pOTU_106	0.184	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Brevundimonas	*Brevundimonas bullata* IAM_13153 (D12785)	100
pOTU_101	0.168	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae; Niastella	*Niastella gongjuensis* 5GH22-11 (NR_137250)	99.6
Negative loading scores				
pOTU_1	-0.268	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia	*Burkholderia oxyphila* OX-01 (NR_112887)	100
pOTU_41	-0.263	Bacteria; Acidobacteria; Acidobacteria_Gp1	*Occallatibacter savannae* A2-1c (NR_147737)	99.6
pOTU_16	-0.210	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales	*Rhodoplanes* sp. JA793_tr2 (HG531388)	97.6
pOTU_14	-0.204	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Rhodoplanes	*Rhodoplanes piscinae* JA266 (AM712913)	97.6
pOTU_9	-0.148	Bacteria	Unknown	0

*† - Loading scores; * - Identity score*

Table 4-9: Loading scores for the top 10 organisms from the 16S soil PCA for PC2.

OTU	LS †	Phylogeny	Closest match	ID*
		Positive loading scores		
pOTU_14	0.258	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Rhodoplanes	*Rhodoplanes piscinae* JA266 (AM712913)	97.6
pOTU_16	0.220	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales	*Devosia humi* THG-MM1 (NR_147759)	100
pOTU_9	0.122	Bacteria	Unknown	
pOTU_30	0.070	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae	*Lysobacter_koreensis_* (T)_Dae16_ (AB166878)	96
pOTU_34	0.069	Bacteria; Acidobacteria; Acidobacteria_Gp1; Candidatus Koribacter	*Candidatus_Koribacter_versatilis*_Ellin345_ (CP000360)	99.6
		Negative loading scores		
pOTU_1	-0.742	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia	*Burkholderia oxyphila* OX-01 (NR_112887)	100
pOTU_2	-0.384	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia	*Burkholderia ginsengisoli* NBRC100965 (NR_113964)	100
pOTU_16	-0.168	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia	*Burkholderia glebae* LMG29325 (NR_145597)	100
pOTU_14	-0.168	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Bradyrhizobium	*Rhizobium lupini* USDA3051 (KM114861)	100
pOTU_9	-0.109	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas	*Sphingomonas lutea* JS5 (NR_153746)	99.6

*† - Loading scores; * - Identity score*

Table 4-10: Loading scores for the top 10 organisms from the 16S soil PCA for PC3.

OTU	LS †	Phylogeny	Closest match	ID*
Positive loading scores				
pOTU_2	0.385	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia	*Burkholderia_ginsengisoli*_strain_NBRC_100965_ (NR_113964.1)	100
pOTU_11	0.342	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Bradyrhizobium	*Rhizobium_lupini*_USDA_3051_ (KM114861)	100
pOTU_41	0.278	Bacteria; Acidobacteria; Acidobacteria_Gp1	*Occallatibacter_savannae*_strain_A2-1c_ (NR_147737.1)	99.6
pOTU_20	0.215	Bacteria; Acidobacteria; Acidobacteria_Gp1; Acidipila	*Occallatibacter_riparius*_strain_277_ (NR_147738.1)	97.2
pOTU_47	0.181	Bacteria; Chlamydiae; Chlamydiia; Chlamydiales; Simkaniaceae; Simkania	*Simkania_negevensis*_ (T)_Z_ (U68460)	90.2
pOTU_7	0.174	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia	*Burkholderia_glebae*_strain_LMG_29325_ (NR_145597.1)	100
pOTU_35	0.147	Bacteria	Unknown	0
pOTU_40	0.144	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas	*Sphingomonas_lutea*_strain_JS5_ (NR_153746.1)	99.6
Negative loading scores				
pOTU_1	-0.502	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia	*Burkholderia_oxyphila*_strain_OX-01_ (NR_112887.1)	100
pOTU_37	-0.129	Bacteria; Verrucomicrobia; Opitutae; Opitutales; Opitutaceae; Opitutus	*Opitutus_terrae*_ (T)_PB90-1_ (AJ229235)	95.3

*† - Loading scores; * - Identity score*

Table 4-11: Loading scores for the top 10 organisms from the 16S soil PCA for PC4.

OTU	LS †	Phylogeny	Closest match	ID*
Positive loading scores				
pOTU_9	0.652	Bacteria	Unknown	0
pOTU_85	0.112	Bacteria; Verrucomicrobia; Subdivision3; Subdivision3_genera_incertae_sedis	uncultured_*verrucomicrobium*_DEV114_ (T)_ (AJ401132)	90.1
Negative loading scores				
pOTU_41	-0.372	Bacteria; Acidobacteria; Acidobacteria_Gp1	*Occallatibacter_savannae*_strain_A2-1c_ (NR_147737.1)	99.6
pOTU_108	-0.225	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas	*Stenotrophomonas_tumulicola*_strain_T59 16-2-1b_ (NR_148818.1)	99.6
pOTU_55	-0.224	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	*Pseudomonas_putida*_strain_ICMP_2758_ (NR_114794.1)	100
pOTU_14	-0.214	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Rhodoplanes	*Rhodoplanes_piscinae*_type_strain:_JA266 _ (AM712913)	97.6
pOTU_20	-0.184	Bacteria; Acidobacteria; Acidobacteria_Gp1; Acidipila	*Occallatibacter_riparius*_strain_277_ (NR_147738.1)	97.2
pOTU_101	-0.165	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae; Niastella	*Niastella_gongjuensis*_strain_5GH22-11_ (NR_137250.1)	99.6
pOTU_16	-0.125	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;	*Rhodoplanes*_sp._JA793_tr2_ (HG531388)	97.6
pOTU_366	-0.100	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Devosia	*Devosia_subaequoris*_ (T)_type_strain:_HST3-14_ (AM293857)	98.8

*† - Loading scores; * - Identity score*

Table 4-12: Chemical treatments in soil resulting in large shifts in prokaryotic microbial communities and the indicator taxa.

Chemical treatment	Taxa responsible for changes in microbial community structure
2-aminoethanol	*Burkholderia* taxa (pOTU_1, pOTU_2 and pOTU_16), *Devosia* taxon (pOTU_25), *Pseudomonas* taxon (pOTU_55), *Stenotrophomonas* taxon (pOTU_108),
ethylene glycol	*Burkholderia* taxa (pOTU_1, pOTU_2 and pOTU_16), *Occallatibacter* taxon (pOTU_41), *Rhodoplanes* taxon (pOTU_16),
2-butoxyethanol	*Burkholderia* taxon (pOTU_2), *Occallatibacter* taxon (pOTU_41), *Pseudomonas* taxon (pOTU_55), *Rhizobium* taxon (pOTU_11), *Stenotrophomonas* taxon (pOTU_108)
acetic acid	Unknown taxon (pOTU_9), uncultured *Verrucomicrobium* taxon (pOTU_85)

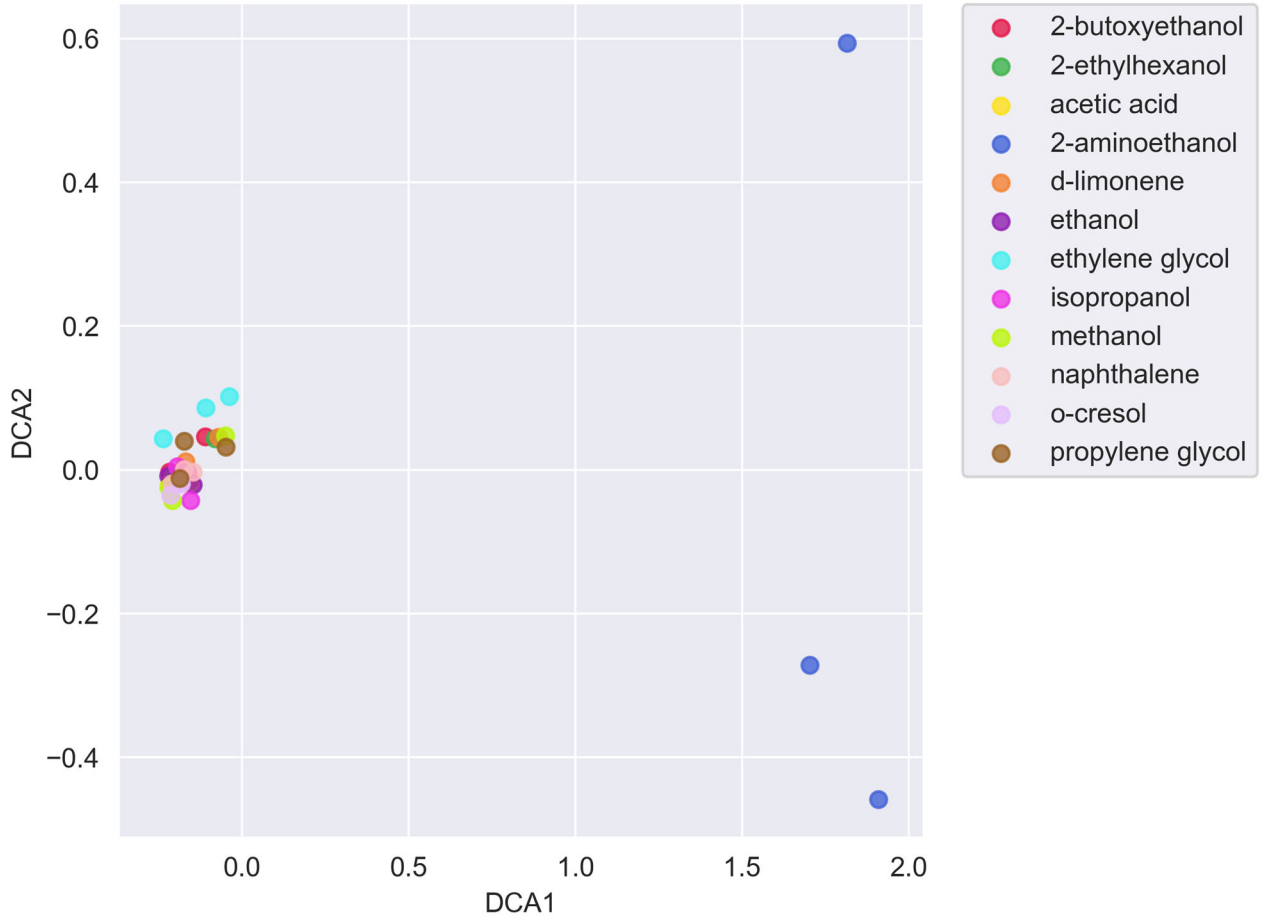


Figure 4-21: Detrended correspondence analysis of 16S soil OTUs by treatment.

It is noteworthy that DCA, a more ecologically meaningful measure, indicated that all but the 2-aminoethanol treatments are more or less similar. That is, in the absence of an untreated control, most chemical treatments appear to either not affect the community or all affect it in the same way. The latter seems unlikely given the diversity of compounds being tested.

Along with differences in structure, differences were also observed in species richness for 16S from soils (Figure 4-22; $p<0.01$). Mean richness across all treatments was 2340 (±28) species/taxa. Notably, the microbial communities in 2-aminoethanol and ethylene glycol had less species richness than most of the other treatments, and significantly so when compared with isopropanol (both $p<0.02$). The 2-aminoethanol treatment was also significantly ($p<0.05$) less rich than the methanol and d-limonene and acetic acid treatments.

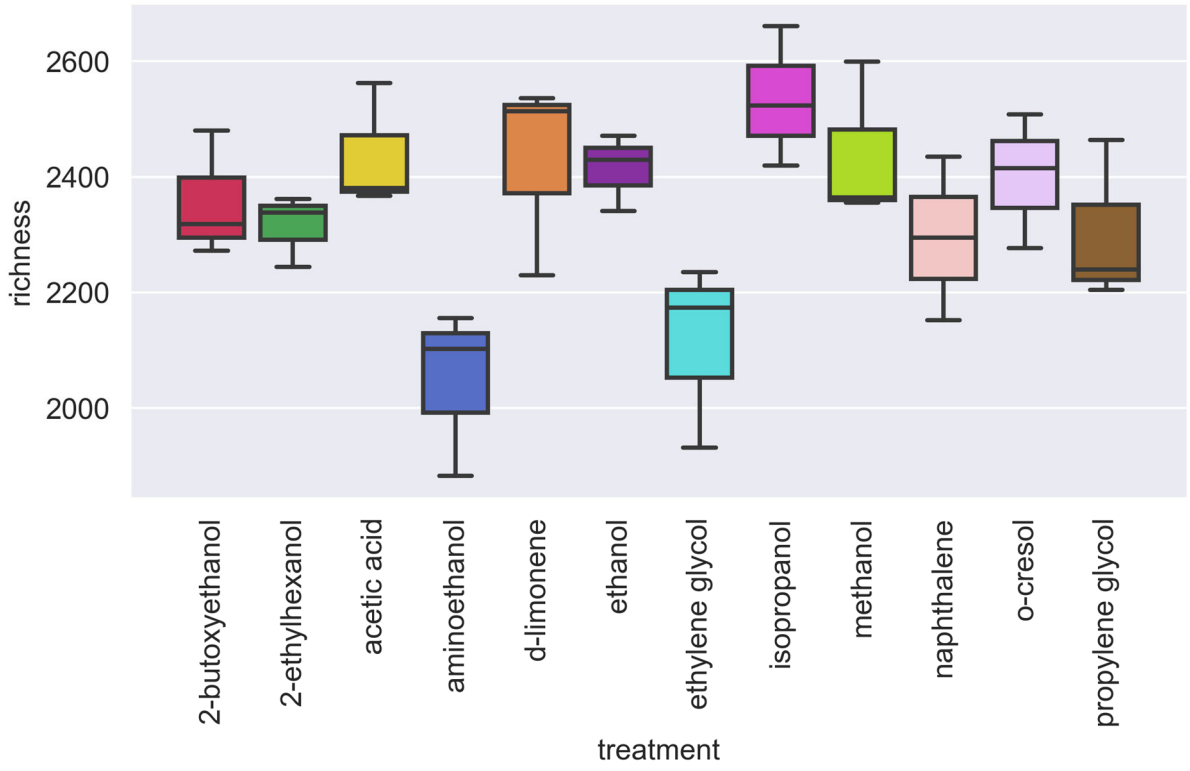


Figure 4-22: Species richness of the 16S soil microbial community by treatment.

In terms of biodiversity, all treatments, except ethylene glycol, produced similar biodiversity indices. Average prokaryote biodiversity was very high (mean Simpsons (1-D) = 0.99), which is typical for soils. Notably, biodiversity measures in the ethylene glycol treatment were significantly lower than d-limonene, naphthalene, ethanol, o-cresol, methanol, isopropanol, acetic acid and propylene glycol (Figure 4-23; $p<0.05$).

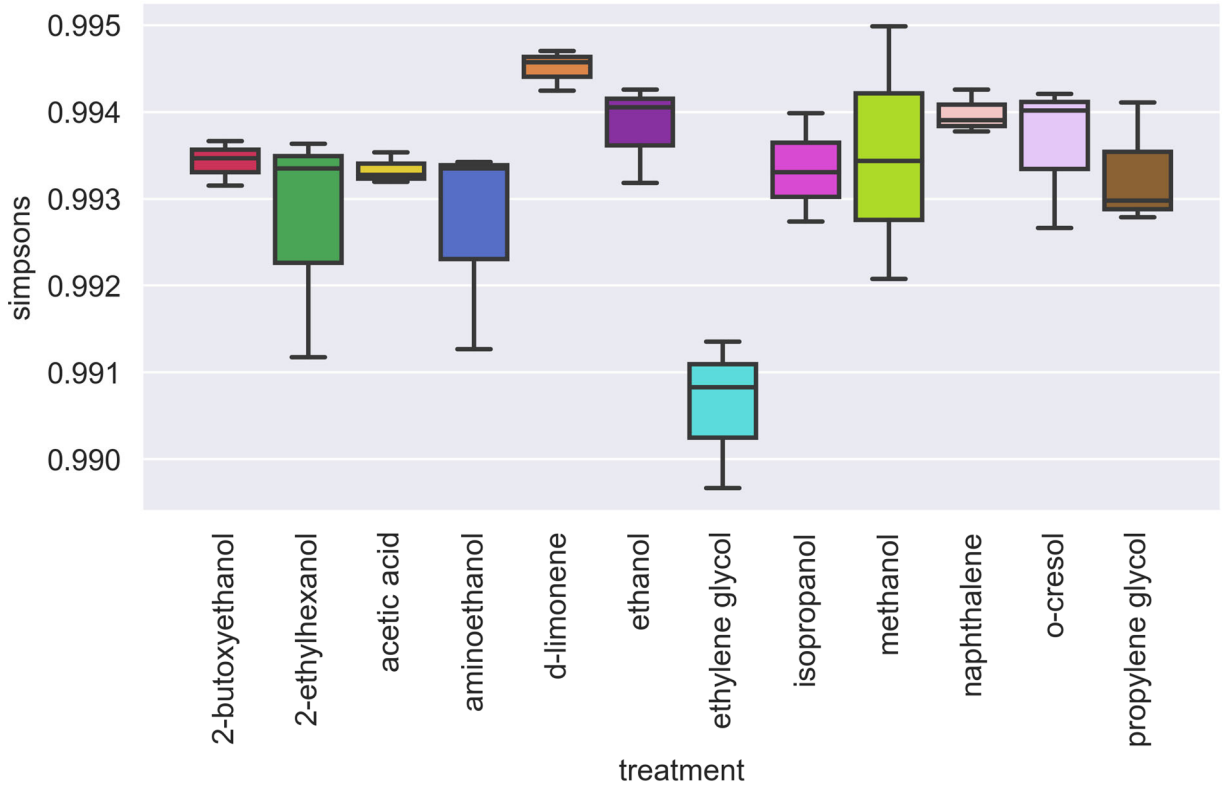


Figure 4-23: Biodiversity (Simpson's Index 1-D) of the 16S soil microbial community by treatment.

4.6.2 Fungi

Soil samples from Penola were determined to host ~1750 species of fungi (Appendix B). The majority (51%) of OTUs were from the Ascomycota, 27% were Basidiomycota while just 36 OTUs (~2%) were from the Zygomycota. Approximately 380 OTUs were detected without known identity to these phyla. Fungal classifiers tend to be biased against chytrids and other zoosporic species and it is probable that many of these OTUs are chytrids or other zoosporic taxa.

Fungal community structure was markedly altered by chemical treatment and those changes were not always consistent across replicates. This observation was most pronounced for the isopropanol, ethylene glycol and o-cresol treatments. Both PCA and DCA show this variability within treatment (Figure 4-24, Figure 4-25 and Figure 4-26). As with the soil 16S data, the absence of an untreated control limits the scope to which the results can be interpreted as it is unclear where the 'untreated' fungal community would plot.

Regardless of the lack of control, it was clear that unlike the prokaryotic communities, most treatments had different impacts on the fungal microbial community. The most consistent and clear trends were that 2-butoxyethanol, and to a lesser extent, 2-aminoethanol, caused different, distinct changes to fungal microbial community structure. These trends were apparent in both the PCA and DCA, though the latter highlights these differences more clearly (Figure 4-24, Figure 4-25 and Figure 4-26).

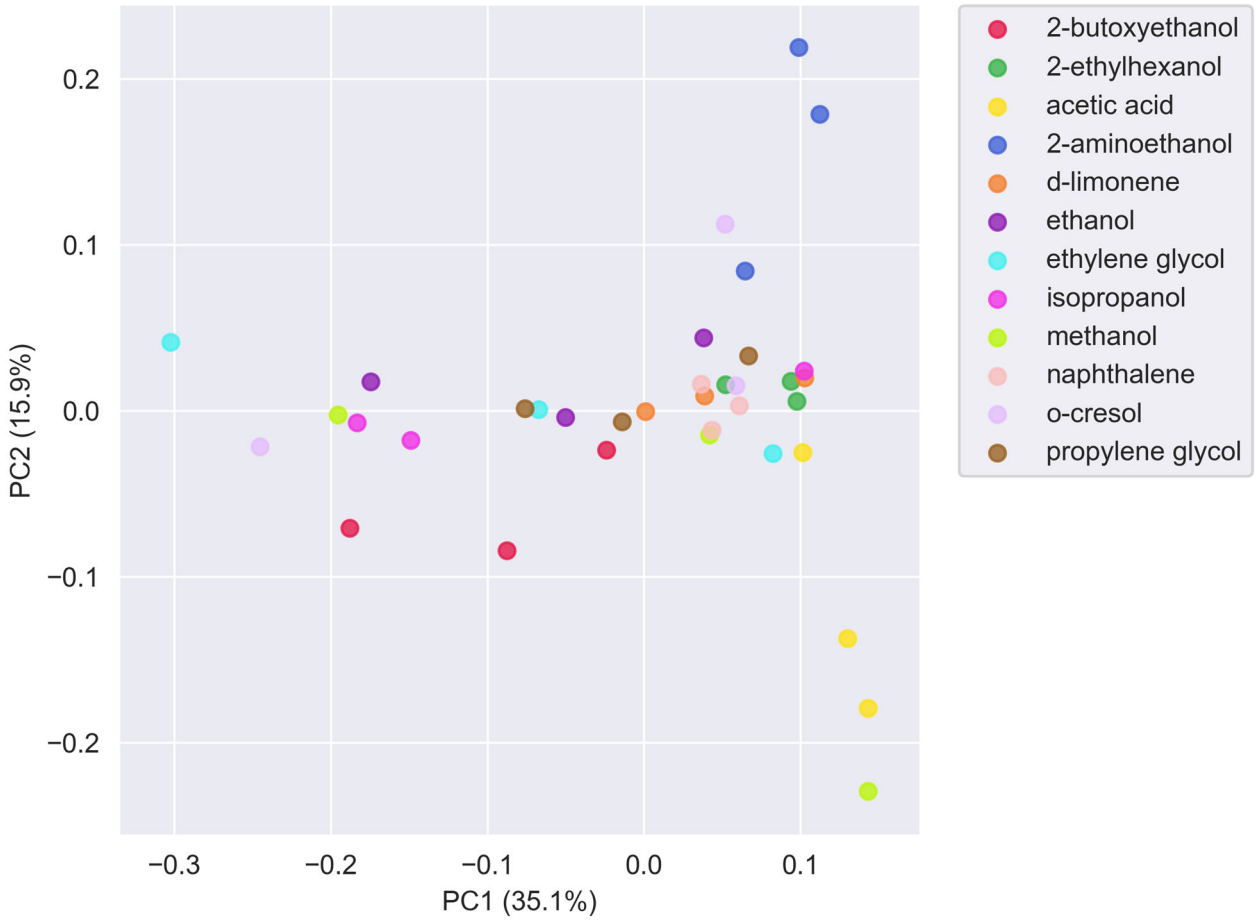


Figure 4-24: Principal components analysis of fungal OTUs by treatment (PC1 and PC2).

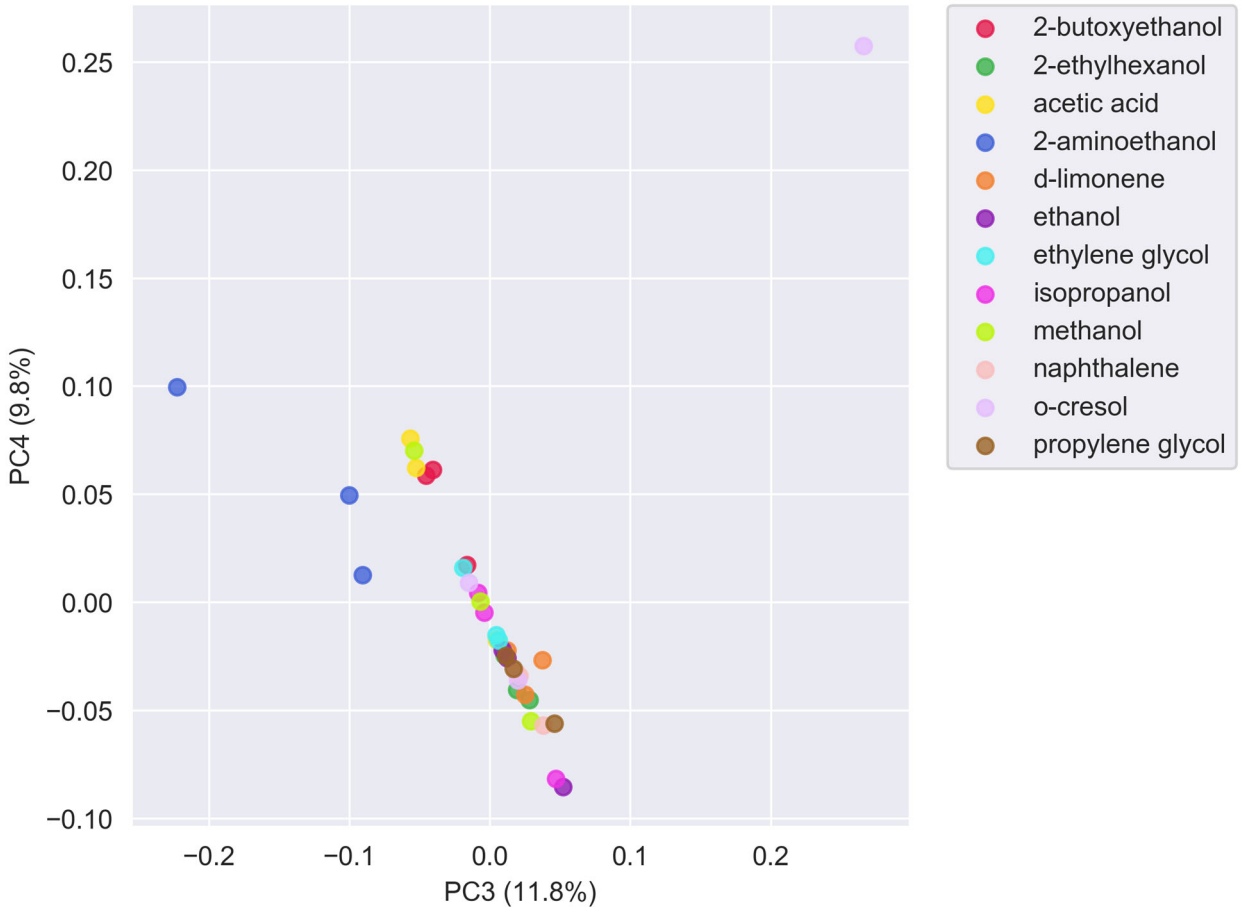


Figure 4-25: Principal components analysis of fungal OTUs by treatment (PC3 and PC4).

The changes in the 2-aminoethanol treatment are driven primarily by increases in abundances of two *Fusarium* species (fOTU 8 and 98), a related genus (*Mariannaea*; fOTU_21), the basidiomycete *Pholiota* (fOTU_27) and a probable Helotiales taxon (fOTU_7; Table 4-14). In contrast, differences in the 2-butoxyethanol treatment were primarily driven by increases in abundance of a putative *Rhizoctonia* species (fOTU_5) and a strain of *Leucogyrophana mollusca (*fOTU_25; Table 4-13).

Table 4-17 shows the fOTU responsible for these shifts in microbial community structure that are evident from both the OTU table and the loading scores for the PCA (Table 4-13, Table 4-14, Table 4-15 and Table 4-16).

Table 4-13: Loading scores for the top 10 organisms from the ITS soil PCA for PC1.

OTU	LS †	Phylogeny	Closest match	ID*
		Positive loading scores		
fOTU_3	0.189	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales	Unknown	-
fOTU_8	0.110	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Gibberella	*Fusarium oxysporum* (EU159118)	100
fOTU_7	0.078	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales	*Leptodontidium elatius* (AY781230)	94
fOTU_21	0.074	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Nectria	*Mariannaea elegans* (JF340240)	100
fOTU_11	0.053	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Sordariomycetidae; Coniochaetales; Coniochaetaceae; Lecythophora	*Lecythophora fasciculata* (GQ377492)	90.1
fOTU_1	0.051	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreaceae; Hypocrea	*Trichoderma evansii* (EU856295)	100
fOTU_14	0.051	*Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales; Vibrisseaceae; Phialocephala*	*Phialocephala fortinii (HQ406812)*	100
fOTU_27	0.045	Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Agaricales; Strophariaceae; Pholiota	*Pholiota multicingulata* (HQ533029)	99.6
		Negative loading scores		
fOTU_5	-0.944	Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetes_Incertae sedis; Cantharellales; Ceratobasidiaceae; Rhizoctonia	*Rhizoctonia* sp. TBR (AF407006)	93.2
fOTU_25	-0.167	Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Boletales; Coniophoraceae; Leucogyrophana	*Leucogyrophana mollusca* (AJ419915)	99.6

*† - Loading scores; * - Identity score*

Table 4-14: Loading scores for the top 10 organisms from the ITS soil PCA for PC2.

OTU	LS †	Phylogeny	Closest match	ID *
		Positive loading scores		
fOTU_8	0.442	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Gibberella	*Fusarium oxysporum* (EU159118)	100
fOTU_21	0.273	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Nectria	*Mariannaea elegans* (JF340240)	100
fOTU_27	0.195	Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Agaricales; Strophariaceae; Pholiota	*Pholiota multicingulata* (HQ533029)	99.6
fOTU_7	0.093	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales	*Leptodontidium elatius* (AY781230)	94
fOTU_98	0.048	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Gibberella	*Fusarium equiseti* (GU291255)	99.4
		Negative loading scores		
fOTU_3	-0.797	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales	Unknown	-
fOTU_13	-0.0794	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales	*Meliniomyces vraolstadiae* (AJ292199)	85.5
fOTU_5	-0.077	Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetes_Incertae sedis; Cantharellales; Ceratobasidiaceae; Rhizoctonia	*Rhizoctonia* sp. TBR (AF407006)	93.2
fOTU_15	-0.059	Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetes_Incertae sedis; Polyporales; Hyphodermataceae; Hyphoderma	*Hyphoderma puberum* (GQ409535)	100
fOTU_30	-0.055	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales; Saccharomycetales_Incertae sedis; Candida	*Candida novakii* (JQ901911)	100

*† - Loading scores; * - Identity score*

Table 4-15: Loading scores for the top 10 organisms from the ITS soil PCA for PC3.

OTU	LS †	Phylogeny	Closest match	ID*
		Positive loading scores		
fOTU_27	0.628	Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Agaricales; Strophariaceae; Pholiota; Pholiota multicingulata	*Pholiota_multicingulata (*HQ533029)	99.6
fOTU_15	0.131	Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetes_Incertae sedis; Polyporales; Hyphodermataceae; Hyphoderma; Hyphoderma puberum	*Hyphoderma_puberum (*GQ409535)	100
fOTU_22	0.115	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales; Helotiaceae; Pezoloma;	*Meliniomyces_bicolor (*HQ157926)	98.9
fOTU_14	0.112	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales; Vibrisseaceae; Phialocephala; Phialocephala fortinii	*Phialocephala_fortinii (*HQ406812)	100
fOTU_20	0.093	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales; Dermateaceae; Mollisia;	*Mollisia_cinerea (*AY259135)	94.4
fOTU_7	0.087	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales; ; ;	*Leptodontidium_elatius (*AY781230)	94
fOTU_10	0.085	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales; Hyaloscyphaceae; Hyaloscypha; Hyaloscypha aureliella	*Hyaloscypha_aureliella (*EU940228)	97.1
		Negative loading scores		
fOTU_8	-0.560	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Gibberella;	*Fusarium_oxysporum (*EU159118)	100
fOTU_21	-0.313	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Nectria; Mariannaea elegans	*Mariannaea_elegans (*JF340240)	100
fOTU_3	-0.270	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales	Unknown	0

*† - Loading scores; * - Identity score*

Table 4-16: Loading scores for the top 10 organisms from the ITS soil PCA for PC4.

OTU	LS †	Phylogeny	Closest match	ID*
		Positive loading scores		
OTU_27	0.719	Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Agaricales; Strophariaceae; Pholiota; Pholiota multicingulata	*Pholiota_multicingulata (*HQ533029)	99.6
OTU_3	0.374	Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetidae; Saccharomycetales	Unknown	0
OTU_8	0.280	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Gibberella	*Fusarium_oxysporum (*EU159118)	100
OTU_21	0.139	Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Nectria; Mariannaea elegans	*Mariannaea_elegans (*JF340240)	100
		Negative loading scores		
OTU_15	-0.251	Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetes_Incertae sedis; Polyporales; Hyphodermataceae; Hyphoderma; Hyphoderma puberum	*Hyphoderma_puberum (*GQ409535)	100
OTU_10	-0.160	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales; Hyaloscyphaceae; Hyaloscypha; Hyaloscypha aureliella	*Hyaloscypha_aureliella (*EU940228)	97.1
OTU_22	-0.158	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales; Helotiaceae; Pezoloma;	*Meliniomyces_bicolor (*HQ157926)	98.9
OTU_7	-0.147	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales	*Leptodontidium_elatius (*AY781230)	94
OTU_14	-0.145	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales; Vibrisseaceae; Phialocephala; Phialocephala fortinii	*Phialocephala_fortinii (*HQ406812)	100
OTU_20	-0.139	Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Leotiomycetidae; Helotiales; Dermateaceae; Mollisia	*Mollisia_cinerea (*AY259135)	94.4

*† - Loading scores; * - Identity score*

Table 4-17: Chemical treatments in soils resulting in large shifts in fungal microbial communities and the indicator taxa.

Chemical treatment*	Taxa responsible for changes in microbial community structure
Isopropanol	*Leucogyrophana* taxon (fOTU_25), *Rhizoctonia* taxon (fOTU_5)
Ethylene glycol	*Leucogyrophana* taxon (fOTU_25), *Rhizoctonia* taxon (fOTU_5)
o-cresol	*Fusarium* taxon (fOTU_8), *Leptodontidium* taxon (fOTU_7), Unknown taxon (fOTU_3)
2-butoxyethanol	*Fusarium* taxon (fOTU_8), *Leucogyrophana* taxon (fOTU_25), *Mariannaea* taxon (fOTU_21), *Meliniomyces* taxon (fOTU_13), *Pholiota* taxon (fOTU_27), *Rhizoctonia* taxon (fOTU_5), Unknown taxon (fOTU_3)
2-aminoethanol	*Fusarium* taxon (fOTU_8), *Leptodontidium* taxon (fOTU_7), *Mariannaea* taxon (fOTU_21), *Pholiota* taxon (fOTU_27), *Pholiota* taxon (fOTU_27), Unknown taxon (fOTU_3)

** Chemical treatments listed had consistent shifts in fungal microbial community structure in at least two of three replicates.*

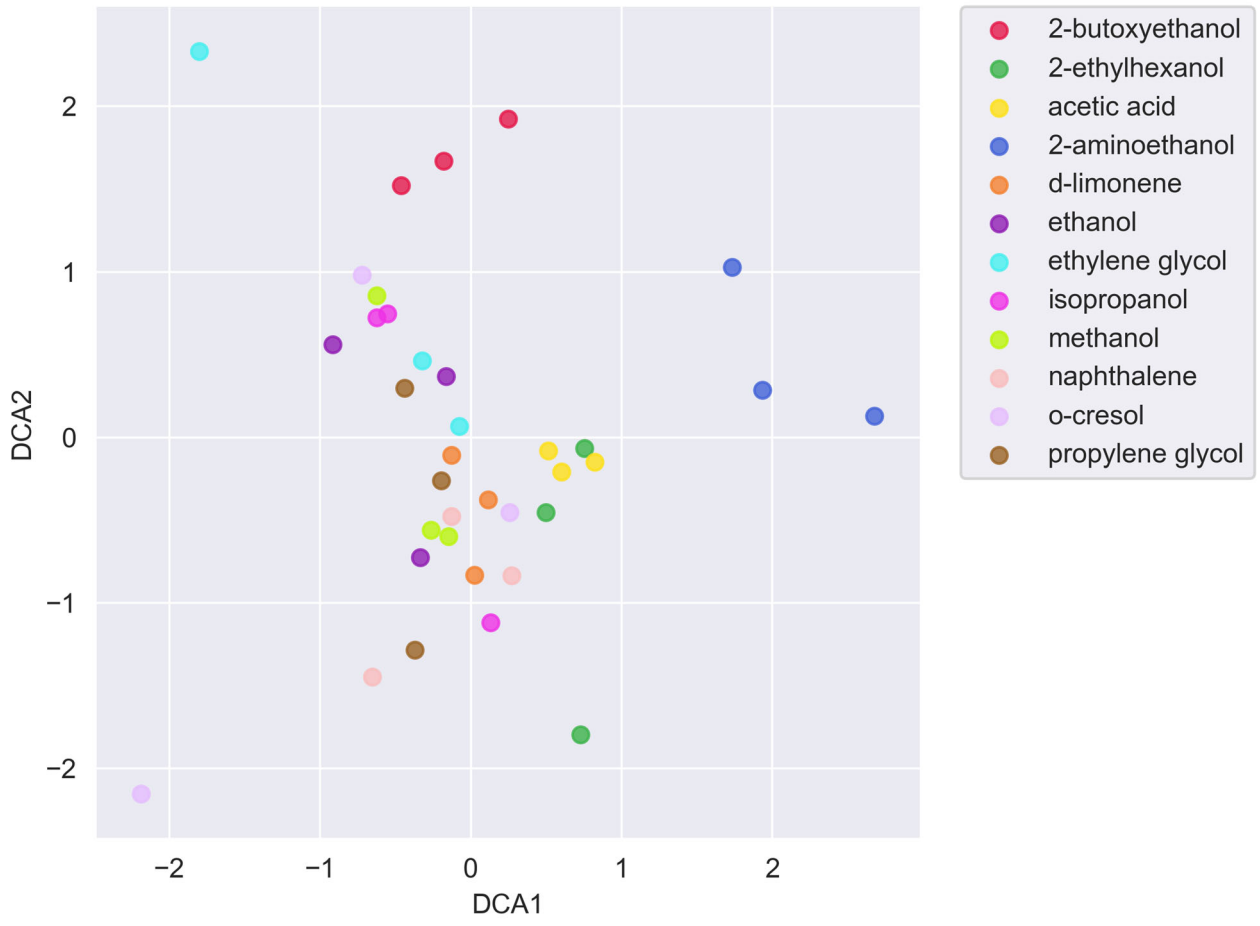


Figure 4-26: Detrended correspondence analysis of soil fungal OTUs by treatment.

Significant differences in richness were observed across the fungal treatments ($p<0.01$). Average species richness was 584 (±13) species across all treatments. Examining these treatments individually it was clear that the 2-aminoethanol treatment was significantly different to ethanol, ethylene and propylene glycol along with d-limonene and isopropanol (all $p<0.05$; Figure 4-27). All treatments were similarly biodiverse (Simpson's 1-D = 0.93 ±0.01) and no significant differences were observed (Figure 4-28).

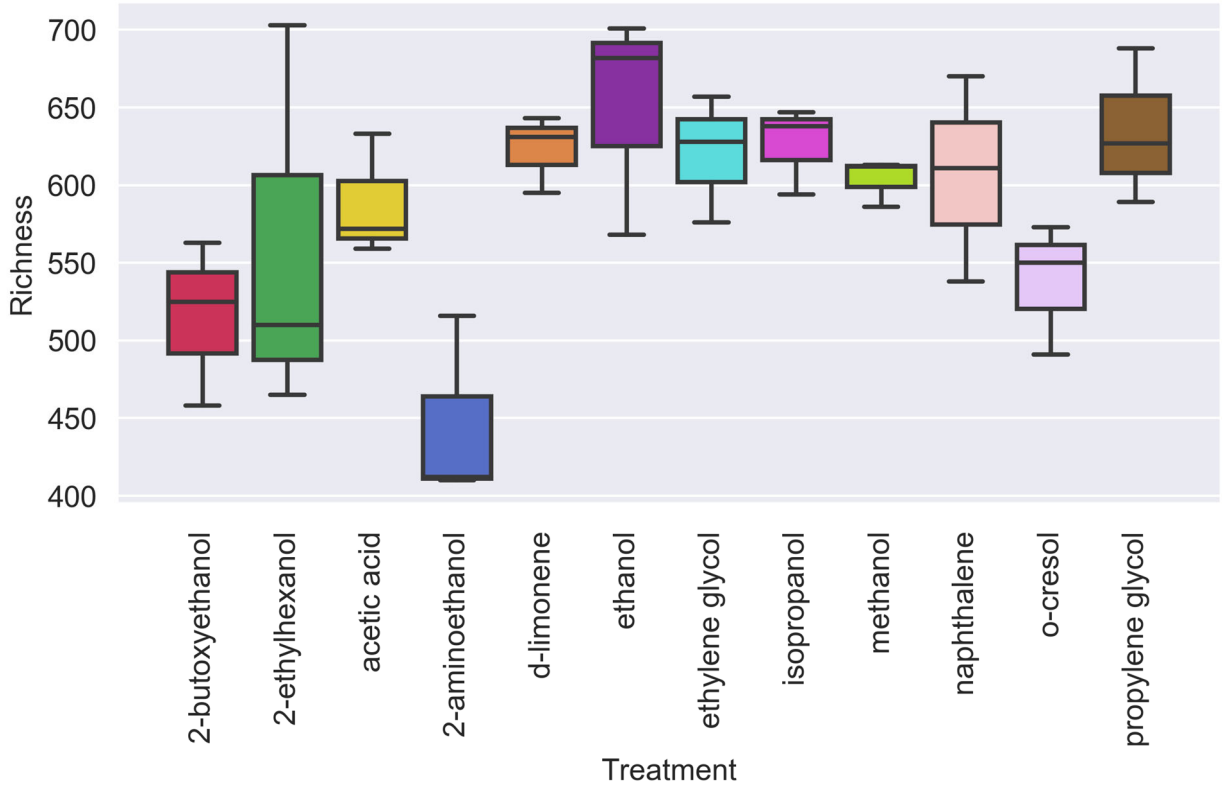


Figure 4-27: Species richness of the soil fungal community by treatment.

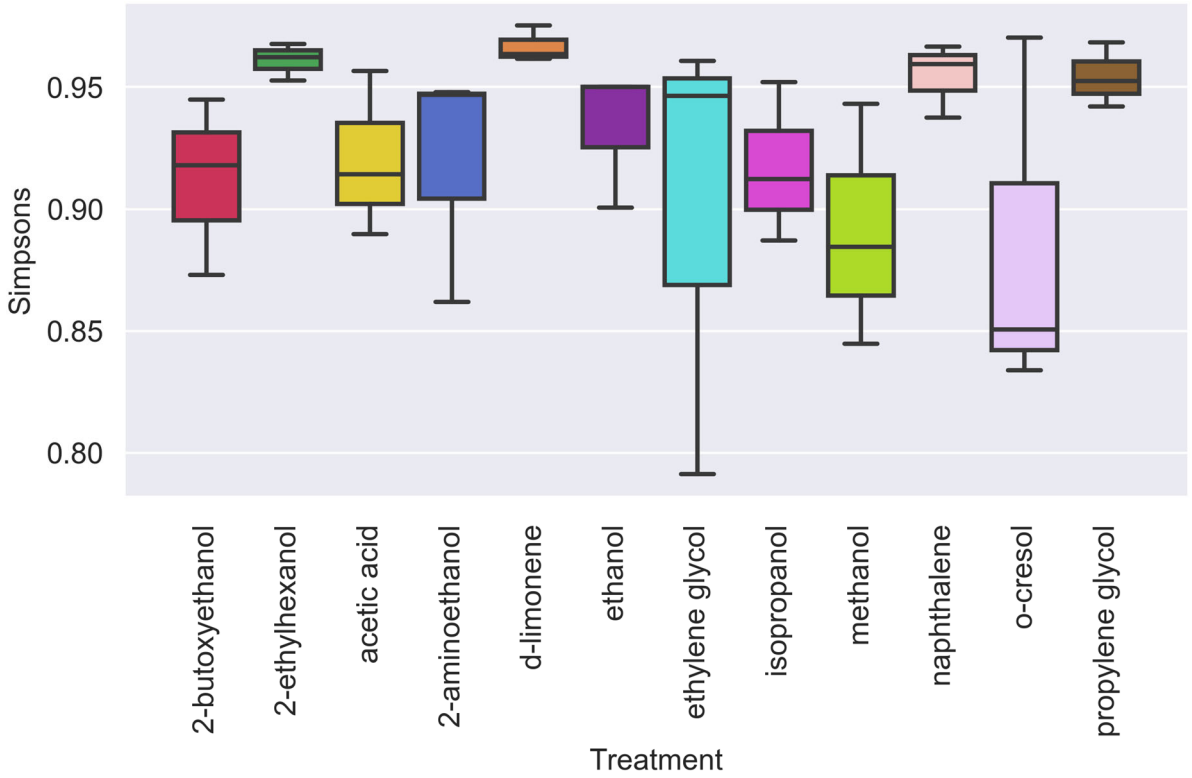


Figure 4-28: Biodiversity (Simpson's Index 1-D) of the soil fungal community by treatment.

4.7 Effect of chemical treatments on aquifer microbiomes and identification of indicator taxa

In total, 5764 prokaryotic taxa were detected in the aquifer water sample collected from Penola (Appendix B). Of these taxa, there were 399 archaeal taxa. In total, 6 archaeal phyla were represented in the aquifer including: Crenarchaeota, Diapherotrites, Euryarchaeota, Pacearchaeota, Thaumarchaeota and Woesearchaeota. For the bacterial taxa, there were a total of 31 phyla including: Acidobacteria, Actinobacteria, Aminicenantes, Armatimonadetes, Bacteroidetes, candidate division BRC1, candidate division WPS-1, candidate division WPS-2, Candidatus Saccharibacteria, Chlamydiae, Chloroflexi, Cyanobacteria, Deferribacteres, Deinococcus-Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Hydrogenedentes, Ignavibacteriae, Latescibacteria, Microgenomates, Nitrospinae, Nitrospirae, Omnitrophica, Parcubacteria, Planctomycetes, Proteobacteria, Spirochaetes, candidate division SR1, Tenericutes and Verrucomicrobia. Several unknown taxa were also detected in both archaeal and bacterial analyses.

In general, a large number of chemical treatments in the aquifer experiments had an effect of shifting the microbial communities away from the untreated controls, as seen in both the PCA and DCA ordinations (Figure 4-30, Figure 4-31 and Figure 4-32, respectively). The PCA ordination showed that the largest microbial community structure shifts were due to benzisothiazolinone, 2-ethylhexanol, o-cresol (x10), naphthalene (standard concentration and x10), and triethanolamine. The DCA ordination also showed these shifts, and additionally highlight microbial community structural shifts due to acetic acid and xanthan gum. Table 4-22 shows the pOTU responsible for these large shifts in microbial community structure that are evident from both the OTU table and the loading scores for the PCA (Table 4-18, Table 4-19, Table 4-20 and Table 4-21).

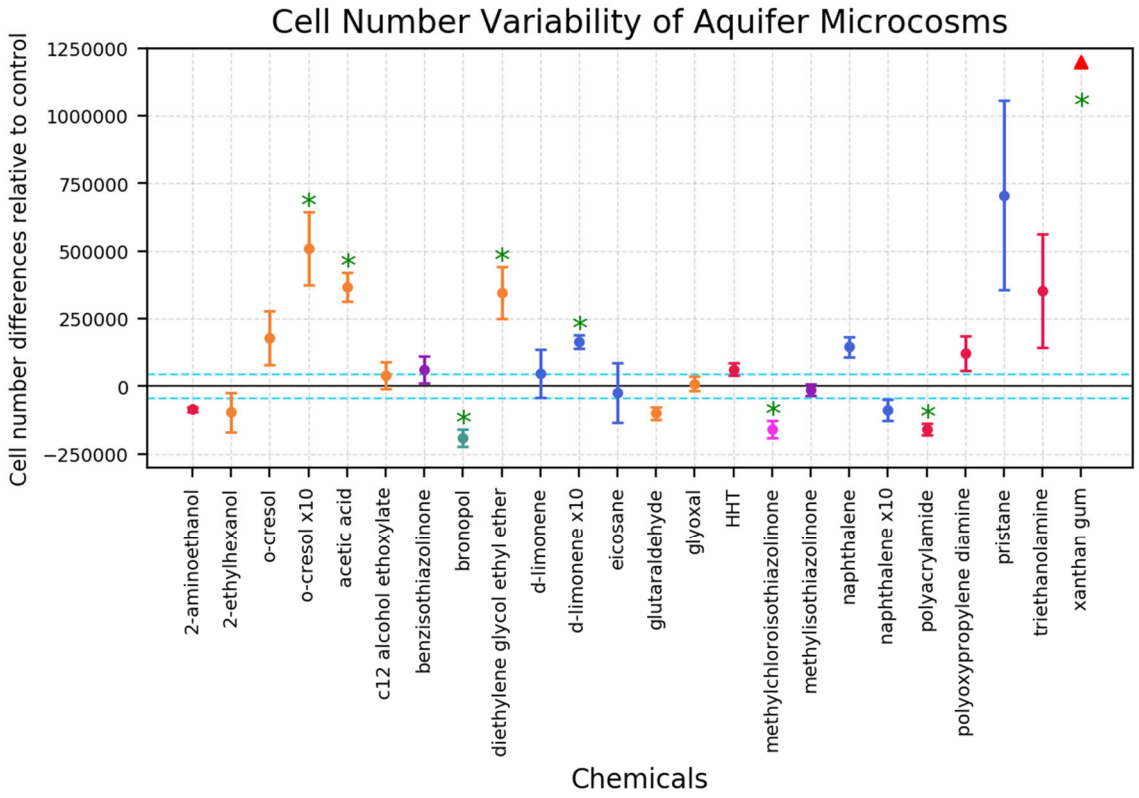


Figure 4-29: Cell number variability of aquifer microcosms relative to the control

*Colours represent the presence of certain elements in each chemical. Chemicals containing C, H only (blue), O (orange), N and O (red), N, O and Br (teal), N, O and S (purple), N, O, S and Cl (magenta). Error bars show the standard error. Dashed blue lines show the standard error of the control. The untreated control with no chemical amendment had a calculated cell number of ~1.3x 10^6 cells treatment vessel. The dotted blue lines indicate the standard error of the untreated control. * = cell number is statistically different to the control using t-test at the 95% confidence level. ^ = cell number (3.4 x 10^6 ± 1.1 x 10^6 cells per treatment vessel) is outside plot range.*

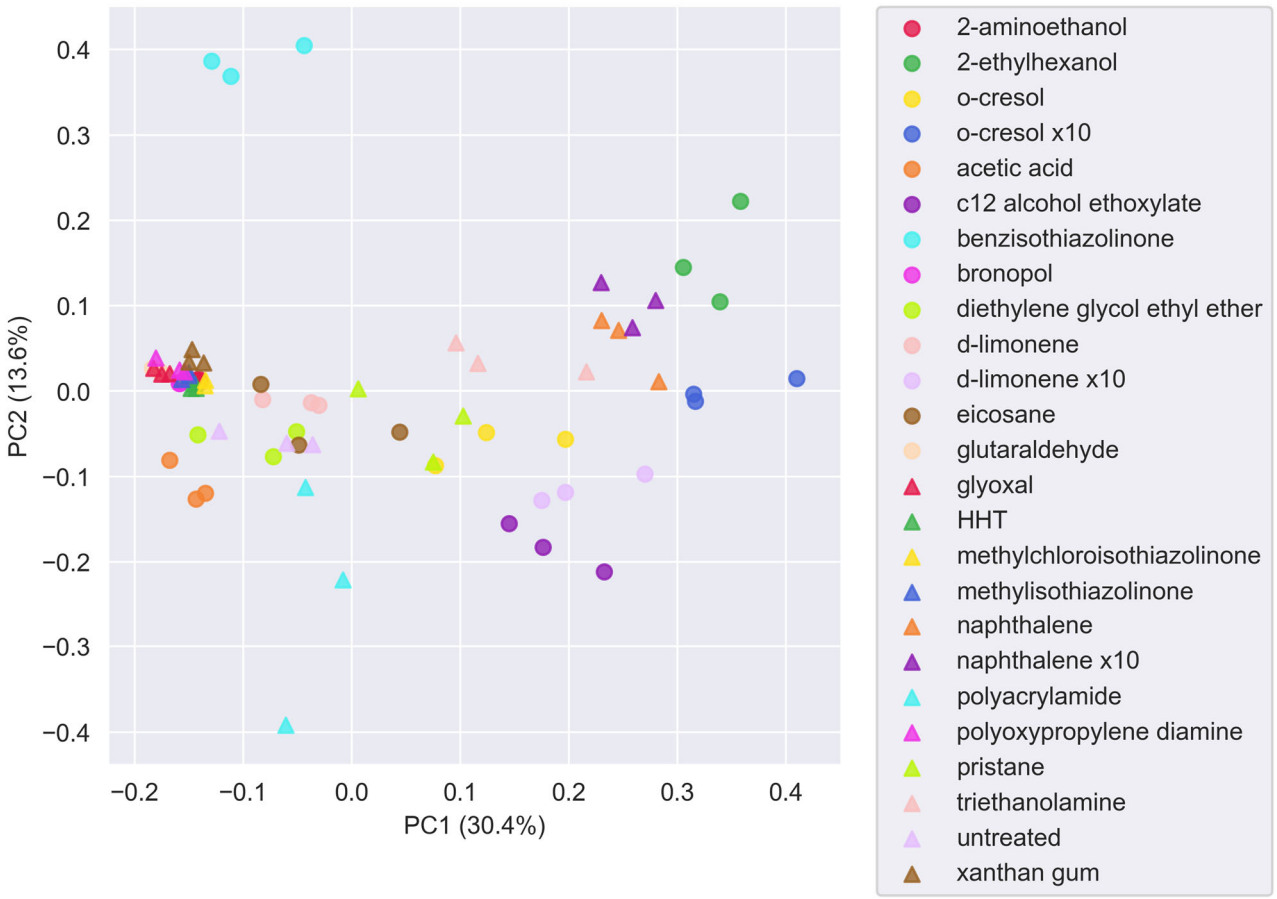


Figure 4-30: Principal components analysis of aquifer 16S OTUs by treatment (PC1 and PC2).

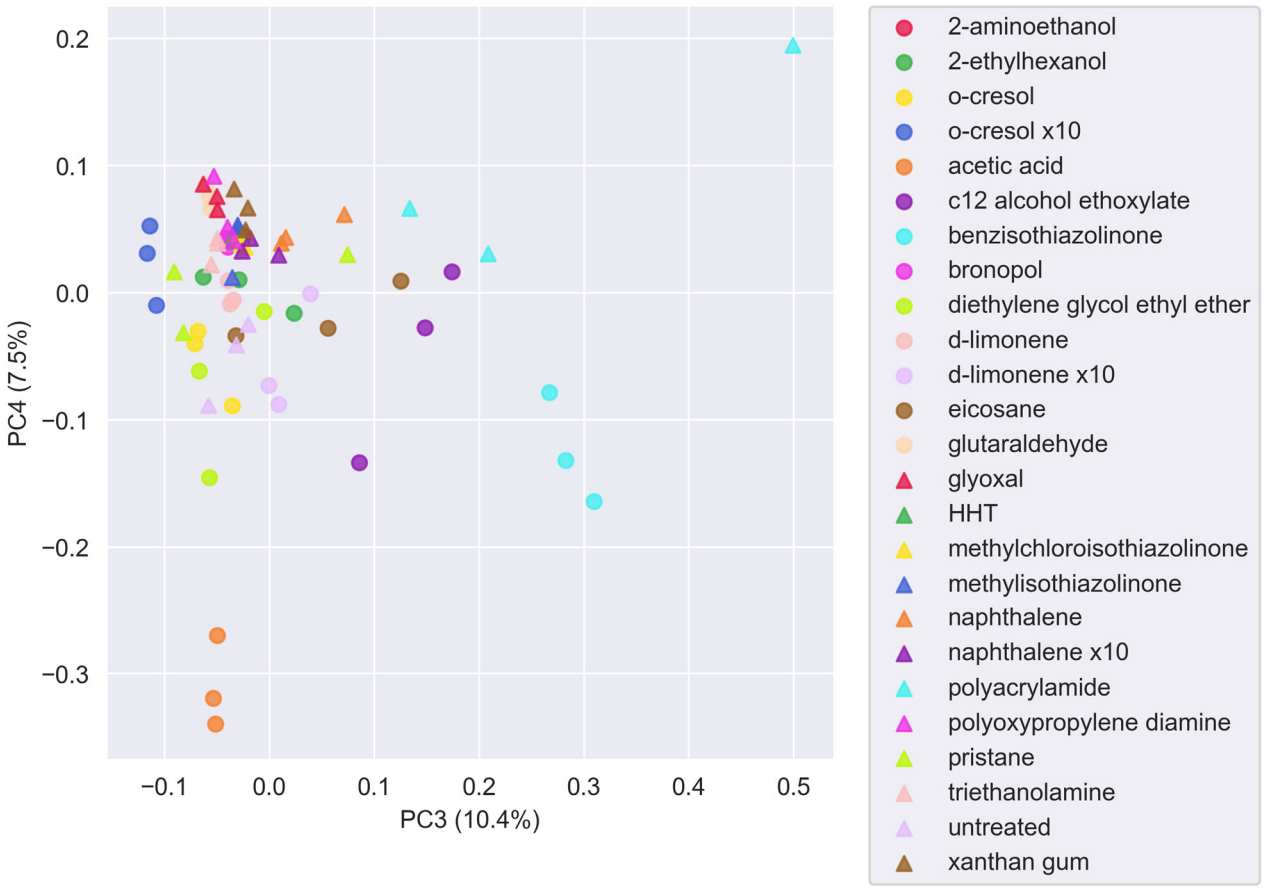


Figure 4-31: Principal components analysis of aquifer 16S OTUs by treatment (PC3 and PC4).

Table 4-18: Loading scores for the top 20 organisms from the aquifer 16S PCA for PC1.

OTU	LS †	Phylogeny	Closest match	ID*
Positive loading scores				
pOTU_1	0.940	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfomicrobiaceae; Desulfomicrobium	*Desulfomicrobium_hypogeium*_strain_CN-A_ (NR_114508.1)	99.2
pOTU_7	0.180	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	*Pseudomonas_linyingensis*_LYBRD3-7_ (HM246142)	100
pOTU_2	0.116	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Vogesella	*Vogesella_fluminis*_Npb-07_ (JN315669)	100
pOTU_13	0.097	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Acidovorax	*Curvibacter_delicatus*_strain_NBRC_14919_ (NR_113696.1)	100
pOTU_15	0.071	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Paludibacter	*Paludibacter_propionicigenes*_strain_WB4_ (NR_074577.1)	98
pOTU_20	0.039	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobulbaceae; Desulfobulbus	*Desulfobulbus_elongatus*_ (T)_DSM_2908_ (X95180)	98.8
Negative loading scores				
pOTU_8	-0.087	Bacteria; Firmicutes; Clostridia; Clostridiales	*Acholeplasma_brassicae*_ (T)_0502_ (AY538163)	85.7
pOTU_16	-0.085	Bacteria	Unknown	0
pOTU_17	-0.076	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	*Pelobacter_propionicus*_DSM_2379_ (CP000482)	99.6
pOTU_4	-0.059	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobulbaceae; Desulfocapsa	*Desulfocapsa_thiozymogenes*_ (T)_DSM_7269_ (X95181)	98
pOTU_24	-0.053	Bacteria; Candidatus Saccharibacteria; Saccharibacteria_genera_incertae_sedis	uncultured_bacterium_SBR1071_ (AF268996)	92.1
pOTU_35	-0.046	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prolixibacteraceae; Sunxiuqinia	*Sunxiuqinia_elliptica*_ (T)_DQHS4_ (GQ200190)	92.9
pOTU_34	-0.046	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	*Pseudomonas_umsongensis*_ (T)_Ps_3-10_ (AF468450)	100
pOTU_31	-0.044	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Bosea	*Bosea*_sp._R-46060_R-46060_=_B1199_ (FR774993)	100
pOTU_11	-0.044	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae; Desulfovibrio	*Desulfovibrio_aerotolerans*_ (T)_DvO5_ (AY746987)	100
pOTU_1211	-0.044	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	*Geobacter_uraniireducens*_strain_Rf4_ (NR_074940.1)	99.2
pOTU_9	-0.040	Bacteria; Proteobacteria; Epsilonproteobacteria; Campylobacterales; Helicobacteraceae; Sulfuricurvum	*Sulfuricurvum_kujiense*_strain_YK-1_ (NR_112144.1)	100
pOTU_39	-0.036	Bacteria; Chloroflexi; Anaerolineae; Anaerolineales; Anaerolineaceae	*Bellilinea_caldifistulae*_ (T)_GOMI-1_ (AB243672)	94.5
pOTU_33	-0.036	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Desulfuromonadaceae; Desulfuromonas	Desulfuromonas_acetoxidans_strain_DSM_684_ (NR_121678.1)	97.6
pOTU_27	-0.035	Bacteria; Verrucomicrobia; Subdivision5; Subdivision5_genera_incertae_sedis	uncultured_eubacterium_WCHA1-33_ (T)_ (AF050557)	98.4

*† - Loading scores; * - Identity score*

Table 4-19: Loading scores for the top 20 organisms from the aquifer 16S PCA for PC2.

OTU	LS †	Phylogeny	Closest match	ID*
Positive loading scores				
pOTU_7	0.557	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae;Pseudomonas	*Pseudomonas linyingensis* LYBRD3-7 (HM246142)	100
pOTU_213	0.428	Bacteria;Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	*Pseudomonas indica* (AF302795)	98.4
pOTU_35	0.033	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prolixibacteraceae; Sunxiuqinia	*Sunxiuqinia elliptica* DQHS4 (GQ200190)	92.9
pOTU_10	0.033	Bacteria	Unknown	0
pOTU_16	0.029	Bacteria	Unknown	0
pOTU_8	0.028	Bacteria; Firmicutes; Clostridia; Clostridiales	*Acholeplasma brassicae* 0502 (AY538163)	85.7
pOTU_61	0.025	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;Pseudomonadaceae; Pseudomonas	*Pseudomonas stutzeri* VKM B-975 (NR 116489.1)	100
pOTU_34	0.024	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	*Pseudomonas umsongensis* Ps 3-10 (AF468450)	100
Negative loading scores				
pOTU_2	-0.579	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Vogesella	*Vogesella fluminis* Npb-07 (JN315669)	100
pOTU_3	-0.349	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae; Desulfovibrio	*Desulfovibrio mexicanus* DSM 13116; Lup1 (AF227984)	99.6
pOTU_6	-0.114	Bacteria; Proteobacteria;Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	*Geobacter lovleyi* SZ (NR 074979.1)	98.4
pOTU_13	-0.093	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;Comamonadaceae; Acidovorax	*Curvibacter delicatus* NBRC 14919 (NR 113696.1)	100
pOTU_11	-0.076	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae; Desulfovibrio	*Desulfovibrio aerotolerans* DvO5 (AY746987)	100
pOTU_9	-0.056	Bacteria; Proteobacteria; Epsilonproteobacteria; Campylobacterales; Helicobacteraceae; Sulfuricurvum	*Sulfuricurvum kujiense* YK-1 (NR 112144.1)	100
pOTU_14	-0.047	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae; Desulfovibrio	*Desulfovibrio arcticus* B15 (DQ296030)	96.8
pOTU_17	-0.045	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	*Pelobacter propionicus* DSM 2379 (CP000482)	99.6
pOTU_43	-0.036	Bacteria; Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Dechloromonas	*Dechloromonas hortensis* MA-1 (AY277621)	100

pOTU_271	-0.035	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	*Geobacter toluenoxydans* TMJ1 (EU711072)	98
pOTU_75	-0.027	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Ralstonia	*Ralstonia insidiosa* AU2944 (AF488779)	100
pOTU_4	-0.021	Bacteria;Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobulbaceae; Desulfocapsa	*Desulfocapsa thiozymogenes* DSM 7269 (X95181)	98

*† - Loading scores; * - Identity score*

Table 4-20: Loading scores for the top 20 organisms from the aquifer 16S PCA for PC3.

OTU	LS †	Phylogeny	Closest match	ID*
Positive loading scores				
pOTU_2	0.730	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Vogesella	*Vogesella_fluminis*_Npb-07_ (JN315669)	100
pOTU_213	0.434	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	*Pseudomonas_indica*_ (T)_ (AF302795)	98.4
pOTU_7	0.406	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	*Pseudomonas_linyingensis*_LYBRD3-7_ (HM246142)	100
pOTU_13	0.064	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Acidovorax	*Curvibacter_delicatus*_strain_NBRC_14919_ (NR_113696.1)	100
pOTU_75	0.044	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Ralstonia	*Ralstonia_insidiosa*_ (T)_AU2944_ (AF488779)	100
Negative loading scores				
pOTU_1	-0.201	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfomicrobiaceae; Desulfomicrobium	*Desulfomicrobium_hypogeium*_strain_CN-A_ (NR_114508.1)	99.2
pOTU_8	-0.151	Bacteria; Firmicutes; Clostridia; Clostridiales; ;	*Acholeplasma_brassicae*_ (T)_0502_ (AY538163)	85.7
pOTU_6	-0.095	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	*Geobacter_lovleyi*_strain_SZ_ (NR_074979.1)	98.4
pOTU_16	-0.063	Bacteria	Unknown	0
pOTU_271	-0.058	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	*Geobacter_toluenoxydans*_TMJ1_ (EU711072)	98
pOTU_11	-0.054	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae; Desulfovibrio	*Desulfovibrio_aerotolerans*_ (T)_DvO5_ (AY746987)	100
pOTU_4	-0.052	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobulbaceae; Desulfocapsa	*Desulfocapsa_thiozymogenes*_ (T)_DSM_7269_ (X95181)	98
pOTU_1211	-0.046	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	*Geobacter_uraniireducens*_strain_Rf4_ (NR_074940.1)	99.2
pOTU_12	-0.045	Bacteria	Unknown	0
pOTU_20	-0.041	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobulbaceae; Desulfobulbus	*Desulfobulbus_elongatus*_ (T)_DSM_2908_ (X95180)	98.8
pOTU_34	-0.040	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	*Pseudomonas_umsongensis*_ (T)_Ps_3-10_ (AF468450)	100
pOTU_24	-0.040	Bacteria; Candidatus Saccharibacteria; ; ; ; Saccharibacteria_genera_incertae_sedis	uncultured_bacterium_SBR1071_ (AF268996)	92.1
pOTU_17	-0.036	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	*Pelobacter_propionicus*_DSM_2379_ (CP000482)	99.6
pOTU_31	-0.033	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Bosea	*Bosea*_sp._R-46060_R-46060_=_B1199_ (FR774993)	100
pOTU_9	-0.031	Bacteria; Proteobacteria; Epsilonproteobacteria; Campylobacterales; Helicobacteraceae; Sulfuricurvum	*Sulfuricurvum_kujiense*_strain_YK-1_ (NR_112144.1)	100

*† - Loading scores; * - Identity score*

Table 4-21: Loading scores for the top 20 organisms from the aquifer 16S PCA for PC4.

OTU	LS †	Phylogeny	Closest match	ID*
Positive loading scores				
pOTU_2	0.228	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Vogesella	*Vogesella_fluminis*_Npb-07_ (JN315669)	100
pOTU_8	0.168	Bacteria; Firmicutes; Clostridia; Clostridiales; ;	*Acholeplasma_brassicae*_ (T)_0502_ (AY538163)	85.7
pOTU_16	0.123	Bacteria	Unknown	0
pOTU_13	0.117	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Acidovorax	*Curvibacter_delicatus*_strain_NBRC_14919_ (NR_113696.1)	100
pOTU_35	0.102	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prolixibacteraceae; Sunxiuqinia	*Sunxiuqinia_elliptica*_ (T)_DQHS4_ (GQ200190)	92.9
pOTU_34	0.097	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	*Pseudomonas_umsongensis*_ (T)_Ps_3-10_ (AF468450)	100
pOTU_24	0.075	Bacteria; Candidatus Saccharibacteria; ; ; ; Saccharibacteria_genera_incertae_sedis	uncultured_bacterium_SBR1071_ (AF268996)	92.1
pOTU_31	0.064	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Bosea	*Bosea*_sp._R-46060_R-46060_=_B1199_ (FR774993)	100
pOTU_27	0.063	Bacteria; Verrucomicrobia; Subdivision5; ; ; Subdivision5_genera_incertae_sedis	uncultured_eubacterium_WCHA1-33_ (T)_ (AF050557)	98.4
pOTU_10	0.056	Bacteria	Unknown	0
pOTU_39	0.048	Bacteria; Chloroflexi; Anaerolineae; Anaerolineales; Anaerolineaceae;	*Bellilinea_caldifistulae*_ (T)_GOMI-1_ (AB243672)	94.5
Negative loading scores				
pOTU_3	-0.720	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae; Desulfovibrio	*Desulfovibrio_mexicanus*_ (T)_DSM_13116;_Lup1_ (AF227984)	99.6
pOTU_213	-0.292	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	*Pseudomonas_indica*_ (T)_ (AF302795)	98.4
pOTU_11	-0.272	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae; Desulfovibrio	*Desulfovibrio_aerotolerans*_ (T)_DvO5_ (AY746987)	100
pOTU_9	-0.253	Bacteria; Proteobacteria; Epsilonproteobacteria; Campylobacterales; Helicobacteraceae; Sulfuricurvum	Sulfuricurvum_kujiense_strain_YK-1_ (NR_112144.1)	100
pOTU_14	-0.186	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae; Desulfovibrio	*Desulfovibrio_arcticus*_B15_ (DQ296030)	96.8
pOTU_6	-0.135	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	*Geobacter_lovleyi*_strain_SZ_ (NR_074979.1)	98.4
pOTU_7	-0.132	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	*Pseudomonas_linyingensis*_LYBRD3-7_ (HM246142)	100
pOTU_53	-0.102	Bacteria; Firmicutes; Clostridia; Clostridiales; Peptococcaceae 1; Desulfosporosinus	*Desulfosporosinus_lacus*_ (T)_type_strain:_STP12_ (AJ582757)	97.6
pOTU_1211	-0.087	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	*Geobacter_uraniireducens*_strain_Rf4_ (NR_074940.1)	99.2

*† - Loading scores; * - Identity score*

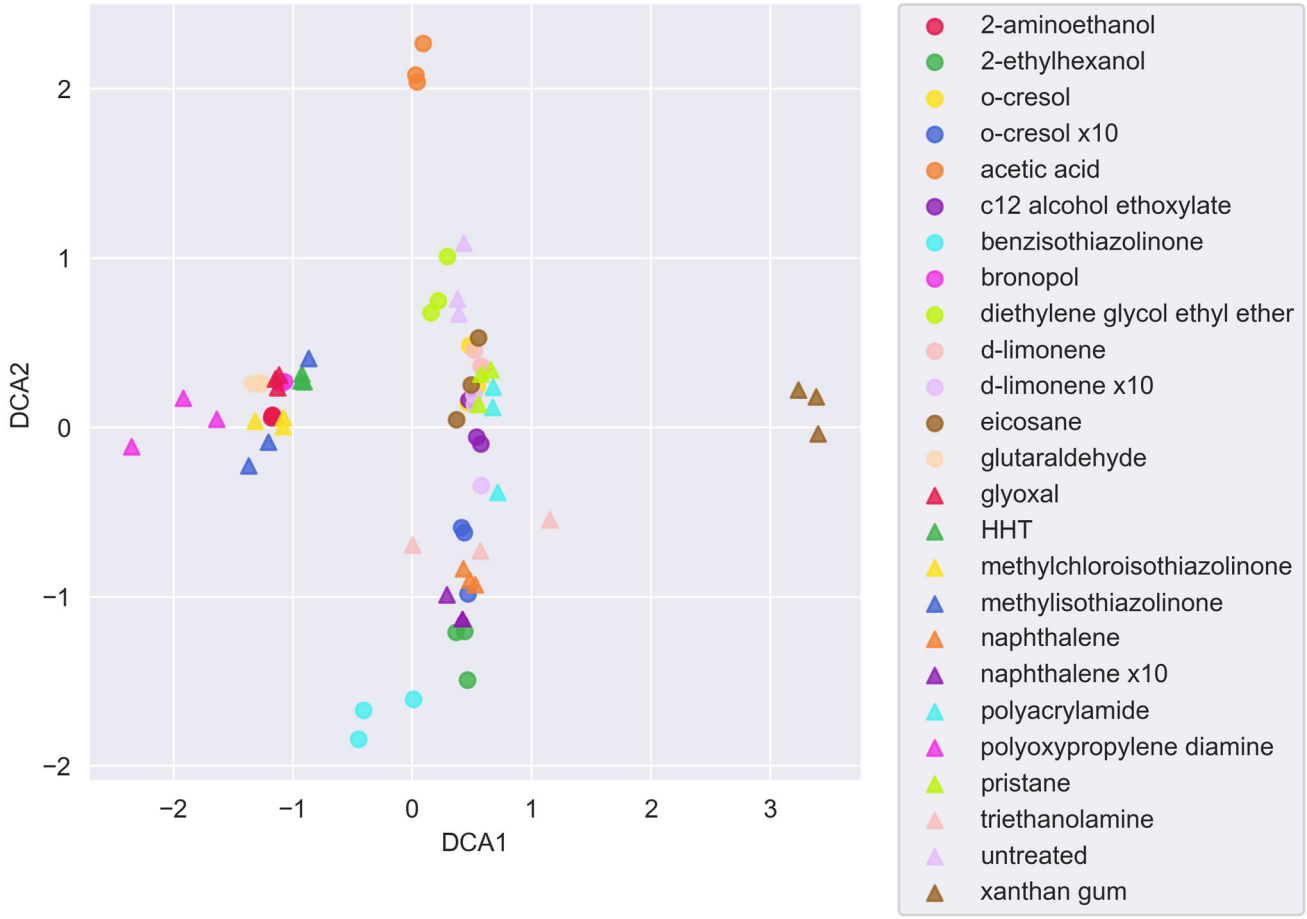


Figure 4-32: Detrended correspondence analysis of aquifer 16S OTUs by treatment.

Table 4-22: Chemical treatments in aquifers resulting in large shifts in prokaryotic microbial communities and the indicator taxa.

Chemical treatment	Taxa responsible for changes in microbial community structure
benzisothiazolinone	*Acholeplasma* taxon (pOTU_8), *Desulfocapsa* taxon (pOTU_4), *Desulfovibrio* taxa (pOTU_3 and pOTU_11), *Pelobacter* taxon (pOTU17), *Pseudomonas* taxa (pOTU_7 and pOTU_213), *Sunxiuqinia* taxon (pOTU_35), *Vogesella* taxon (pOTU_2),
2-ethylhexanol	*Desulfomicrobium* taxa (pOTU_1), *Pseudomonas* taxa (pOTU_7 and pOTU_213), *Sunxiuqinia* taxon (pOTU_35), *Vogesella* taxa (pOTU_2),
o-cresol (x10)	*Acholeplasma* taxon (pOTU_8), *Desulfomicrobium* taxon (pOTU_1), *Geobacter* taxon (pOTU_6), *Pseudomonas* taxon (pOTU_7), *Vogesella* taxon (pOTU_2), Unknown bacterial taxon (pOTU_16)
naphthalene (standard concentration and x10)	*Acholeplasma* taxon (pOTU_8), *Desulfomicrobium* taxon (pOTU_1), *Pseudomonas* taxon (pOTU_7 and pOTU_213), *Sunxiuqinia* taxon (pOTU_35), *Vogesella* taxon (pOTU_2), Unknown bacterial taxon (pOTU_16)
triethanolamine	*Acholeplasma* taxon (pOTU_8), *Desulfomicrobium* taxon (pOTU_1), *Geobacter* taxon (pOTU_6), *Pseudomonas* taxon (pOTU_7), *Vogesella* taxon (pOTU_2)
acetic acid	*Acholeplasma* taxon (pOTU_8), *Desulfocapsa* taxon (pOTU_4), *Desulfomicrobium* taxon (pOTU_1), *Desulfovibrio* taxa (pOTU_3 and pOTU_11), *Geobacter* taxon (pOTU_6), *Pelobacter* taxon (pOTU17), *Pseudomonas* taxon (pOTU_213), *Vogesella* taxon (pOTU_2)
xanthan gum	*Acholeplasma* taxon (pOTU_8), *Desulfocapsa* taxon (pOTU_4), *Desulfovibrio* taxa (pOTU_3 and pOTU_11), *Pelobacter* taxon (pOTU17), *Pseudomonas* taxa (pOTU_7 and pOTU_213), *Sunxiuqinia* taxon (pOTU_35)

5 Discussion

This study examined microbial degradation of chemicals in soil and aquifer samples from the Penola region, in south-east of South Australia. Experiments to ascertain degradation were either through direct measurement of the chemical (i.e. an accredited chemical test for its presence) or through microbial growth on that chemical as a sole carbon source. Additionally, the current study demonstrated changes in the microbial community associated with chemical exposure and identified indicator taxa that appear to respond to the chemical by increasing or decreasing in abundance. It is noteworthy that physicochemical interactions between chemicals of interest and soils and aquifer waters, may also have occurred (discussed in section 5.16).

It is unsurprising, but notable that the soil and aquifer samples were markedly different from each other and the respective responses of these environments and the microbes contained therein were similarly distinct. These two environments have markedly different physical and chemical attributes.

Soil is a complex, heterogeneous mixture of inorganic and organic matter and provides a huge array of niches and physicochemical microhabitats. For example, soil peds are highly oxic environments on the outside and transition to anoxic zones at their centre. Various microhabitats (a low oxygen or microaerophilic zone) occur along this transition, such that within soils oxic and anoxic habitats can occur throughout the soil alongside the general loss of oxic environment by depth. Along with their oxygen status, soils are also marked by the presence of considerable organic matter, as well as inorganic and organic nitrogen and phosphorus. In the current study, key attributes of the dark loam examined was that it had pH of 5.8, low conductivity of ~30 µS/cm, and an average of 62000, 736 and 117 mg/kg of total organic matter, nitrogen and phosphorus, respectively.

In contrast, the aquifer environment examined in this study, at least from the perspective of the planktonic cells, is much more homogenous and is relatively uniform from a physicochemical perspective. The aquifer samples were all anoxic, with a mean pH of 7.7, a moderately low EC (~1400 µS/cm), very limited total nitrogen (around 0.3mg/L) and no detectable phosphorus.

The differences between these two environments are further highlighted in the DNA yield (hypothetical cell numbers from soils and aquifers) which was approximately 1000x greater for the

soil than aquifer samples. Though there is no data to support it here, it seems probable that microbial activity (cell division and growth) is also considerably higher in the soil than in the aquifer; the very low phosphorus level in the aquifer likely limits microbial activity markedly since this element is critical for DNA (its low level impacting replication and gene expression) and formation of new cell membranes (through limiting phospholipids).

For those chemicals that had an accredited chemical test available (2-butoxyethanol, 2-ethylhexanol, acetic acid, 2-aminoethanol, ethanol, ethylene glycol, isopropanol, methanol and propylene glycol), the ability of microbes to degrade these chemicals varied between the soil and the aquifer.

In the soil microcosms, all these chemicals were completely undetectable after 34 days of incubation; indicating the probable rapid biodegradation of these compounds in the soil. For a discussion of the individual groups of chemicals see the sections 5.1 through 5.14. It seems probable that this observation primarily represents microbial catabolism of these chemicals and not adsorption. For a detailed discussion of this distinction see section 5.16.

In the aquifer microcosms, small but significant biodegradations of methanol, ethylene and propylene glycols, and 2-aminoethanol were observed. In contrast, ethanol, isopropanol, acetic acid, 2-butoxyethanol and 2-ethylhexanol did not appear to degrade during the 34-36 days of incubation. Based on the catabolic potential of the large number of microbes resident in the aquifer microcosms, it seems likely that this phenomenon is related to the limiting nature of phosphorus or perhaps nitrogen in the aquifer, and not the inability of microbes to degrade these compounds *per se*. The limiting nature of nitrogen and phosphorus on catabolism are well studied, and examples of nutrient amendment resulting in catabolism of compounds is well established in a variety of fields including the bioremediation of oil or pesticide contamination (Lipthay et al., 2007; Obbard et al., 2004; Singh, 2008; Xu and Obbard, 2003). While it is beyond the scope of the current study, nutrient amendments may be of interest to stakeholders for their ability to remove contaminating compounds from nutrient-limited environments.

In the sole carbon source experiments conducted in the present study on agar, all chemical compounds tested (2-aminoethanol, 2-ethylhexanol, benzisothiazolinone, bronopol, c12 alcohol ethoxylate, diethylene glycol ethyl ether, d-limonene, d-limonene x10, eicosane, glutaraldehyde, glyoxal, hexahydro-1,3,5-tris (2-hydroxyethyl)-sym-triazine, methylchloroisothiazolinone, methylisothiazolinone, naphthalene, naphthalene x10, o-cresol, o-cresol x10, polyacrylamide, polyoxypropylene diamine, pristane, triethanolamine, xanthan gum) had observable fungal and

bacterial growth. It is noteworthy, however, that considerable organic matter (~0.62mg) from the soil was likely carried over with the inoculation of soil microbes. As such, a subtractive approach was used in the present study, removing organisms that could grow on the control plates to observe increasing taxa and counting only organisms that increased consistently within the replicates of a treatment. It is noteworthy that liquid culture experiments conducted outside the work program of the present study, indicate that the majority of chemicals support observable to heavy microbial growth. These experiments, conducted in the absence of any carried over soil carbon, indicate the ready use of these chemicals as sole-carbon sources by a range of bacterial and fungal species. There were, however, a small number of chemicals for which minimal or no growth was observed. Unsurprisingly, these chemicals were mainly biocides.

Across this experiment, a range of different microbes were observed to grow on plates. While all treatments had plates that differed in appearance, bronopol is worth discussing separately. This treatment, even at the very low concentration tested (70µg per plate) appeared to completely inhibit bacterial colony formation and no prokaryotic taxa were observed with the subtractive method described above. For a more detailed discussion of bronopol see section 5.7. Taxa that significantly increased in abundance, compared to controls, were observed on all chemicals, except for c12 alcohol ethoxylate and glutaraldehyde in fungi and 2-aminoethanol, bronopol, o-cresol (at x10 concentration) in prokaryotes. While these increasing taxa are putative catabolisers of these chemicals, it is also worth considering the responses of microbial communities in the more environmentally meaningful microcosms of aquifers and soils.

For soil microcosm experiments, the effect of exposure to 2-aminoethanol, 2-butoxyethanol, 2-ethylhexanol, o-cresol, acetic acid, d-limonene, ethanol, ethylene glycol, isopropanol, methanol, naphthalene and propylene glycol was determined. Microbial community structure responses in soil differed between the prokaryotic and fungal communities. In the main, the prokaryotic community structure was largely unaltered by exposure to the tested chemicals, except for 2-aminoethanol and ethylene glycol. These two treatments caused marked, but distinct shifts in prokaryotic microbial community structure. The former was characterised by increases in *Pseudomonas* and *Devosia* species, while the latter was characterised by marked increases in *Burkholderia* species. In contrast, the fungal community structure was altered by many of the chemicals tested, most markedly, 2-butoxyethanol and 2-aminoethanol. In the former, there were large increases in *Fusarium* and *Mariannaea* species, while the latter was characterised by increases in an unknown ascomycetous yeast and an unknown Heliotiales taxon. It was

noteworthy, that fungal communities, exhibited significantly more intra-treatment variation on individual chemicals, perhaps suggesting greater fungal heterogeneity within soil samples.

In comparison with soils, aquifer microbial community responses were much more consistent between replicates, probably reflecting the homogeneity and chemical distribution within individual microcosms. DNA yields, a proxy for cell number, were greatest (and significantly higher than the control treatment; $p<0.05$) in the o-cresol, acetic acid, diethylene glycol ethyl ether, d-limonene and xanthan gum treatments. Indeed, the xanthan gum treatment, caused a massive increase in cell number with an approximately 35-fold increase. Conversely, bronopol, methylchloroisothiazolinone and polyacrylamide all caused significant declines in DNA yields/cell numbers. All treatments, except for diethylene glycol ethyl ether had significant and replicable effects on microbial community structure.

The most significant prokaryotic microbial community structure changes were observed in benzisothiazolinone, o-cresol and 2-ethylhexanol. For benzisothiazolinone, the putative indicator taxa were two *Pseudomonas* species. For o-cresol and 2 ethyl hexanol, putative indicator taxon belonged to the genus *Desulfomicrobium*.

Interpretation of results from the present study are separated into broad chemical groupings, where applicable (sections 5.1 through 5.15). Common intermediates and shared degradative pathways for chemicals investigated are discussed in section 5.17 and Figure 5-2.

5.1 Acetic Acid

Acetic acid degradation by microbes is both widespread and well established. It is a key compound for the citric acid cycle (CAC) (also known as the Krebs cycle or the tricarboxylic acid cycle or TCA cycle), the major biological series of reactions that in aerobes yields energy and in anaerobes mostly is used for biosynthesis of important cellular compounds. Acetate is carried as the acetyl moiety attached to coenzyme A

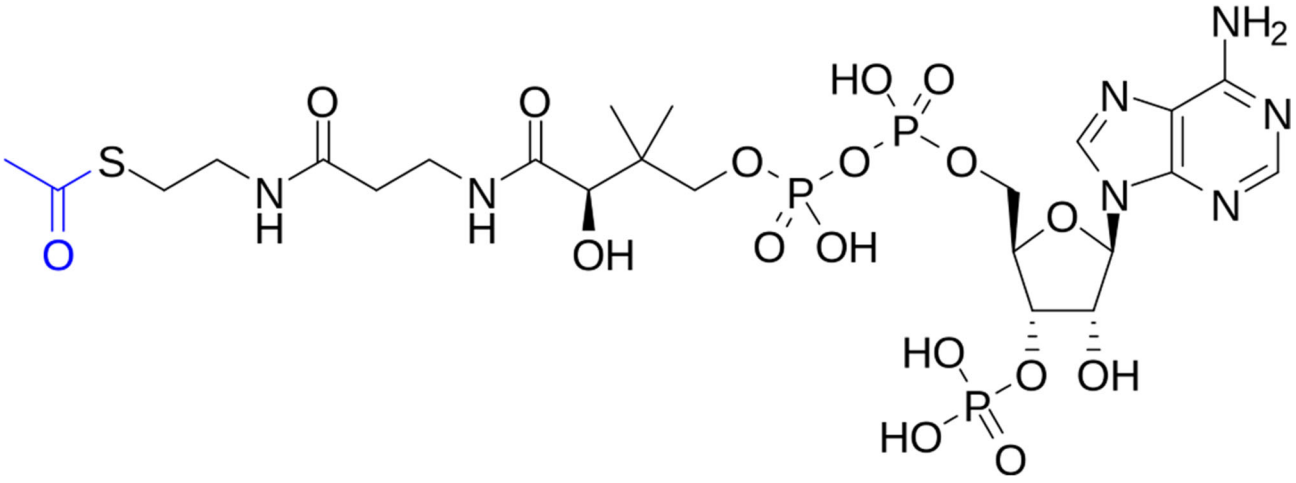


Figure 5-1: Acetyl coenzyme A. The acetyl group is shown in blue, while the reminder of the structure (coenzyme A) is used as a substrate for synthesis and oxidation of fatty acids and pyruvate in the CAC.

In the present study, acetic acid was completely degraded in soil and was not observable after the 34 day duration of the study. In contrast, no significant difference was observed in acetic acid concentration in the aquifer environment after 34 days. Acetate is a common intermediate/by-product in a range of microbial catabolic and anabolic processes, it may be that the acetate measured represents a mixture of biogenically produced acetate and acetate added experimentally.

5.2 Isothiazolinone biocides

Isothiazolinone biocides impact microbes through disruption of central metabolism by binding to dehydrogenases and have a broad spectrum of activity (Williams, 2006). Somewhat surprisingly, all the isothiazolinone biocides (benzisothiazolinone, methylchloroisothiazolinone and methylisothiazolinone) tested in the sole carbon source experiments had little effect on recoverable DNA concentrations (≈ cell number). In the aquifer experiments, however, the methylchloroisothiazolinone significantly reduced recoverable DNA, while benzisothiazolinone and methylisothiazolinone had no effect on DNA recoverable. A small number of mostly fungal catabolisers were identified for all three biocides. Fungi have been previously demonstrated to degrade isothiazolinone biocides, though responses differ between fungal species. For example, *Trichoderma* was partially inhibited by the biocide, while growth of *Aspergillus* and *Fusarium* species were enhanced in the presence of the biocide (Gomes et al., 2018). A range of organic acids, including malonic acid, appear to be the primary degradative products (Gomes et al., 2018).

Comparing the effects of these three biocides on the prokaryotic microbial community, methylchloroisothiazolinone and methylisothiazolinone appear to cause similar shifts in structure, while benzisothiazolinone causes distinct changes. For the latter, increases in *Pseudomonas* species are associated with its degradation.

5.3 Ethanol, isopropanol and methanol

The typical microbial degradation of alcohols proceeds via either an aldehyde or ketone then to a carboxylic acid. Primary alcohols like ethanol are degraded to aldehydes (in the case of ethanol, acetaldehyde) via alcohol dehydrogenases and then subsequently to a carboxylic acid (in this instance, acetic acid) via aldehyde dehydrogenase. These enzymes are common in a range of microbes including many bacteria and fungi in oxic environments. In anoxic environments, energy for this reaction is typically derived from concomitant reduction of compounds like nitrate or sulphate to reduced forms. Alternatively, ethanol can be oxidised using energy derived syntrophies (i.e. interspecies hydrogen transfer, typically to methanogens) (Bryant et al., 1977). Other anaerobic organisms can utilise ethanol and typically produce butyrate or propionate as end-products of its fermentation (Schink et al., 1985).

In the present study, methanol and ethanol were degraded rapidly and were undetected in soil after 34 days. The abundance of organisms with the ability to degrade these compounds in soil likely indicates that it was consumed in a much shorter timeframe. In the aquifer microcosms, both methanol and ethanol persisted for the 34 days of the experiment, though methanol was significantly reduced in concentration. Methanol has some alternative degradation pathways in anoxic environments like aquifers, including its oxidation to methane by methanogenic archaea (Evans et al., 2019; Figure 4). This may account for the significant loss of methanol, compared to the absence of change for ethanol. From the aquifer waters that were tested, various archaea capable of methanogenesis from methanol were found including, a comparatively abundant *Methanomethylovorans* species (pOTU_95), a *Methanolobus* (pOTU_1105) and various putative *Methanomassiliicoccus* species (OTUs: pOTU_1859, pOTU_2736, pOTU_3776 and pOTU_3993).

The absence of ethanol degradation in the aquifer samples in this study is intriguing. As discussed above ethanol is readily biodegraded anaerobically. Its failure to do so here may represent its genuine recalcitrance in the sample of the aquifer studied, or more likely, some artefact of the experimental design. The most abundant organisms in the aquifer were taxa that included species previously reported to degrade ethanol, most notably *Desulfomicrobium* and *Geobacter* species (Dias et al., 2008; Kushkevych, 2014; Viulu et al., 2013). While there is sufficient sulphate present in the aquifer water to support sulphate reduction, other organic compounds present may be more readily degraded through the course of the experiment or that a necessary co-factor was absent in the aquifer water. Alternatively, ethanol detected in these experiments may be the product of anaerobic fermentation. Alcohol as a by-product of fermentation is a well-understood biological phenomenon and numerous fungi and bacteria produce ethanol anoxically. If this latter idea is true, it may be that ethanol is constantly produced as organic matter in the aquifer water is catabolised.

In contrast to primary alcohols, secondary alcohols like isopropanol are degraded to ketones not aldehydes. Isopropanol, the secondary alcohol examined during this study is oxidised to acetone then to pyruvate. Pyruvate subsequently enters the TCA cycle and, in anaerobes, is likely used for biosynthesis of various important compounds for the cell. In the present study, degradation of isopropanol was demonstrated through analytical testing. In soil, over the 34 days of the experiment, isopropanol was completely degraded and could not be detected. In contrast, the aquifer samples showed a significant, but small, increase in isopropanol concentration. This could be artefactual or could be a result of biogenesis of isopropanol by anaerobic bacteria. Numerous

clostridia are known to produce this compound in significant concentrations(Collas et al., 2012; Xin et al., 2017; Zhang et al., 2018). For example, a study by George et al., (1983), showed various clostridia could produce 10mM of isopropanol in culture. Such solventogenic clostridia occur in the aquifer waters of the region and may be responsible for the production of the small amounts of isopropanol observed in this study.

5.4 2-aminoethanol

Chemically, 2-aminoethanol is both an alcohol and an amine. It is a common addition to hydraulic fracturing fluids where it acts as a chemical crosslinker and hydrogen sulphide scavenger. Biologically, it is also prevalent, resulting from the degradation or catabolism of the membrane lipid phosphatidylethanolamine (He et al., 2015; Kaval and Garsin, 2018). This lipid occurs in almost all cell-membranes. Genes for recycling this compound are fairly common, though the majority of work has been conducted on members of the Enterobacteraceae (Kaval and Garsin, 2018). 2-aminoethanol is readily deaminated (the ammonia being then available for assimilatory uses in the cell, e.g. amino acid or DNA biosynthesis) and converted into various common cellular compounds such as acetate, acetaldehyde and ethanol (Kaval and Garsin, 2018).

This study indicated that 2-aminoethanol is readily degraded to undetectable levels in soil microcosms. In the aquifer, there are some anomalous results for 2-aminoethanol. Namely, initial measurements in the aquifer microcosms appear to exceed the dosed concentration. Regardless, the concentration in the aquifer also appears to decline significantly over time indicating the rapid consumption of 2-aminoethanol. Relatively few taxa grew well on this substrate as a sole source of carbon, though significant and rapid growth was observed in liquid cultures where 2-aminoethanol was the only source of carbon.

5.5 Aliphatic-type compounds – eicosane, pristane, 2-ethylhexanol and polyacrylamide

Aliphatic compounds are broken down in soils using various catabolic pathways. The best studied of these are probably the beta-oxidation pathways. This pathway uses ATP to bind coenzyme A (Figure 5-1) to the end of the aliphatic chain. Beta-oxidation is a cycle that includes a complex series of reactions that essentially results in acetyl-CoA (acetate bound to coenzyme A) being cleaved from the aliphatic compound once per cycle. Acetyl-CoA can then enter the citric acid cycle where it is either used for energy generation (resulting in CO_2 production) or is used for biosynthesis of other cellular components.

Numerous other pathways exist including those that use alkane hydroxylases (e.g. toluene monoxygenase) or fumerate addition systems. All these pathways result in similar outcomes; simple products that are used either for energy generation or biosynthesis of cellular components. Degradation of aliphatic compounds are thoroughly reviewed (Abbasian et al., 2015).

Aliphatic degradation is, at least in part, dependant on the length of the aliphatic chain. As aliphatic compounds increase in length their solubility decreases and reactions to degrade these compounds are largely dependent on the co-production of surfactants that allow emulsification of hydrophobic aliphatic compounds (Al-Mallah et al., 1990; Rojo, 2009).

One such long aliphatic compound is eicosane, a straight chain alkane composed of 20 carbon atoms, which is a solid at room temperature, melting at ~37-38°C. Eicosane is degraded in a similar fashion to other aliphatic compounds and its unbranched structure makes the process more straightforward. In the present study, microbial growth in sole carbon assays was highest for eicosane (which had the greatest DNA yield). This is somewhat surprising as, while alkanes are attractive sources of carbon, this study contains other presumably more tractable sources of carbon, such as acetic acid. The main contributors to the extensive growth observed on eicosane appear to be Actinomycetes, which increased significantly in number. Other actinomycetes have been previously shown to degrade eicosane for example, *Corynebacterium* species have been shown to degrade eicosane in axenic culture (Syakti et al., 2004).

The overall changes in the aquifer microbial communities observed in the eicosane treatment were similar to those observed for the branched aliphatic compound pristane and polyacrylamide. This result may have been expected as eicosane and pristane are closely related compounds. For polyacrylamide, microbial degradation is generally initiated by the removal of the nitrogen-

containing amide groups leaving the carbon backbone (Nakamiya and Kinoshita, 1995; Wen et al., 2010). Acinetobacter, Azomonas, Bacillus, Clostridium, Enterobacter and various pseudomonads have been demonstrated to be involved in polyacrylamide degradation (Caulfield et al., 2002; Joshi and Abed, 2017; Ma et al., 2008; Matsuoka et al., 2002; Xiong et al., 2018). While there are suggestions that the carbon backbone may be more recalcitrant (Xiong et al., 2018), results presented here may indicate that the carbon backbone of polyacrylamide is degraded via the same organisms that degrade aliphatic compounds, at least in the aquifer. Additionally, sole carbon trials in liquid culture have readily supported microbial growth from Penola soils in the absence of other carbon sources (data not shown).

Branched aliphatic compounds, like pristane, are generally degraded via similar pathways though they involve more steps than those for unbranched compounds. Pristane is known to be degraded by a range of microbes through either ω-hydroxic acids or α-ω-dioic acids to carboxylic acids (Nhi-Cong et al., 2009). In the present study, numerous fungi and bacteria were associated with the pristane treatment. Growth was readily apparent when pristane was the sole source of carbon. For fungi, zygomycetes were especially abundant on the pristane treatment. These same organisms were also abundant on the 2-ethylhexanol treatment, both compounds are branched and have aliphatic motifs, and their common fungal degraders may indicate similar mechanisms in the zygomycetes for their degradation. Bacterial degraders of pristane appear to be mostly Proteobacteria. Data from previous studies indicate numerous Actinobacteria are capable of pristane degradation (Brzeszcz and Kaszycki, 2018; Nhi-Cong et al., 2010, 2009; Sharma and Pant, 2000), taken together with fungal and prokaryotic data presented here, it suggests that pristane degradation is widespread in the soil microbes of Penola.

In the present study, 2-ethylhexanol was tested by a commercially available accredited test and was completely degraded in soil after 34 days, however, there was no evidence of degradation in the aquifer experiments. The 2-ethylhexanol experiments in the aquifer indicated a large shift in prokaryotic microbial community structure, that resembled the shift caused by the structurally unrelated compound, naphthalene (at the x10 concentration). Within the soil microcosm experiments, 2-ethylhexanol had an inconsistent effect on the prokaryotic microbial community structure, and no effect on fungal microbial community structure. Similar to other alcohols, degradation of 2-ethylhexanol uses alcohol and aldehyde dehydrogenases with the product of these degradations (ethylhexanoic acid) being metabolised through beta-oxidation (Wyatt et al., 1987). Numerous *Pseudomonas* species have been reportedly involved in biodegradation of 2-

ethylhexanol (Wyatt et al., 1987). There is, however, evidence that 2-ethylhexanol can persist in certain environments (Horn et al., 2004).

5.6 Glycols – ethylene, propylene glycols and 2-butoxyethanol

In accredited chemical tests, dosed quantities of glycols and 2-butoxyethanol were undetectable in soil microcosms after 34 days of incubation at room temperature. Both ethylene and propylene glycols were significantly degraded in aquifer microcosms ($p<0.05$), however, the 2-butoxyethanol concentration was unchanged after 34 days of incubation, indicating a lack of degradation of this compound in the aquifer, over the timeframe examined. Neither the glycols nor 2-butoxyethanol altered recoverable DNA (≈ cell number) from sole carbon source experiments, compared to controls. Similarly, in both soil and aquifer microcosm experiments, addition of glycols or 2-butoxyethanol, had no effect on recoverable DNA.

All three compounds altered fungal microbial community structure in the soil microcosms, often with inconsistent results between replicates. These fungal microbial community shifts were due to taxa belonging to the genera *Fusarium*, *Leucogyrophana, Rhizoctonia*, *Mariannaea*, *Meliniomyces*, *Pholiota* and an unknown taxon.

The aerobic degradation pathways for both ethylene glycol and propylene glycol are well characterised (Bolbot and Anthony, 1980; Child and Willetts, 1978; Fincher and Payne, 1962; Gonzalez et al., 1972; Sridhara et al., 1969; Willetts, 1979). Anaerobic degradation of these glycols has also been characterised (Hartmanis and Stadtman, 1986). The degradation of 2-butoxyethanol has also been characterised (Pérez et al., 2016).

In the present study, both ethylene glycol and propylene glycol were readily degraded in both soils and aquifers, which indicated that they likely pose minimal environmental hazards. The inability of the aquifer microbiome to degrade 2-butoxyethanol may be related to the status of other macronutrients in the aquifer (nitrogen and phosphorus; see Recommendations).

5.7 Bronopol

Of all the compounds examined in the single carbon source trials, bronopol had the most marked effect on the microbial community. On plates examined in this study, no bacterial colonies were observed, though numerous fungi appeared to grow on bronopol as a sole source of carbon. Clearly, bronopol is acutely bacteriocidal or bacteriostatic at the concentrations used in this study (70µg across the petri dish surface). This observation is unsurprising as the compound is used as a biocide in the oil and gas industries. Bronopol is alleged to have broad-spectrum activity against a range of microbes including fungi (Oono et al., 2007; Oono and Hatai, 2007), but in the current study significant fungal growth was observed where bronopol was the sole source of carbon. This result may be a dose-dependent and higher concentrations of bronopol may be fungicidal.
This has important implications for bronopol in the aquifer. In this environment there is no air, and no fungi. This is reflected in the results of this study, where bronopol treatments in the aquifer microcosms had significantly fewer ($p<0.05$) 'cells' (DNA) than the control. In water, bronopol is known to rapidly hydrolyse to a range of intermediates (2-bromo-2-nitroethanol, bromonitromethane, tri (hydroxymethyl)nitromethane, nitromethane, 2-bromoethanol and formaldehyde) (Dokulilova et al., 2017), some of which (2-bromo-2-nitroethanol and bromonitromethane) (Cui et al., 2011), are potentially more recalcitrant to degradation and may persist in the environment. In the sole carbon source assays, addition of bronopol to plates mimicking soil caused an increase in growth of Botryosphaeriales and Sordariales. This observation has since been confirmed in liquid cultures which show the marked growth of hyphae on bronopol as a sole source of carbon (data not shown). Given the low concentration at which it is active, the absence of potential catabolisers in the aquifer, and the potential for bronopol and its degradation products to persist in the environment, it would appear that bronopol presents a greater risk than many other compounds examined in the present study.

5.8 c12 alcohol ethoxylate

The sole carbon source experiments revealed that c12 alcohol ethoxylate had no significant increase in fungal taxa abundance observed, however, this may have been due to an unknown reason for failure of PCR amplification in the sole carbon source experiments. Visible hyphal-like growth and sporulation were observed on plates, indicating that fungi may have a role in catabolism of c12 alcohol ethoxylate. The prokaryotic microbial community analysis of the sole carbon source experiments indicated a potential bacterial cataboliser from the genus *Burkholderia* (pOTU3907), which is supported by previous literature reporting *Burkholderia* species as being able to grow on various ethers (Hur et al., 1997). Within aquifer microcosm experiments there was no change in the overall DNA extracted (≈ cell numbers), however, there were marked changes in prokaryotic microbial community structure, most probably associated with catabolism. Numerous previous studies have demonstrated that relatively high doses of linear alcohol ethoxylate are readily biodegraded under anoxic conditions (Balson and Felix, 1995; Kravetz et al., 1991; Motteran et al., 2014; Scott and Jones, 2000; White, 1993). Taken together, this suggests that this group of compounds likely pose minimal environmental risks.

5.9 Diethylene glycol ethyl ether

In the present study, the sole carbon source experiments revealed that diethylene glycol ethyl ether increased the growth of a comparatively small number of bacteria and fungi, indicating that its catabolism is probably limited to a relatively small number of taxa. In general, it appeared to have limited or no effects on species richness or biodiversity at the concentration levels tested. This may suggest that diethylene glycol ethyl ether is relatively biologically neutral and does not interact much with microbes in soil. Further evidence of this lack of interaction is present in the aquifer microcosm experiments. Addition of diethylene glycol ethyl ether did not appear to alter aquifer microbial communities, which were quite sensitive to a range of other compounds. The toxicity of this compound appears to be relatively low, with lethal doses (LD50) in rats being ~10g /

kg of body mass and aquatic toxicity being present only at 3-9g/L in water fleas and minnows, respectively[3].

There is relatively little information on the biodegradation of diethylene glycol ethyl ether in the environment, though a few studies report biodegradation rates of 48-87% in 20 days in non-acclimated cultures (Price et al., 1974; Staples et al., 1998; *The Glycol Ethers Handbook*, 1981).

5.10 Polyoxypropylene diamine

In the sole carbon source experiments polyoxypropylene diamine had very little effect on DNA recovery (≈ cell number). Similarly, in the aquifer experiments, DNA recovery was unchanged. Most potential catabolisers appear to be prokaryotic, however, several fungal catabolisers were identified. Additionally, in sole carbon liquid assays (data not shown), heavy prokaryotic growth was observed in a soil inoculum, after only two weeks of incubation at room temperature. There is very little research on the degradation of this compound available in the literature, however, degradation of polyoxypropylene diamine likely proceeds to the production of urea and hydroxybenzoic acid.

Given this paucity of research, further work to characterise its biodegradation would be valuable.

5.11 Triethanolamine

Triethanolamine has been demonstrated to be degraded by the anaerobic acetogenic bacterium *Acetobacterium LuTria* 3 which uses the compound as a sole source of carbon and energy (Frings et al., 1994). This organism uses a triethanolamine lyase, which results in the production of acetaldehyde and diethanolamine (Wyman, 1999). It was therefore not surprising, to note that most organisms which increased in response to triethanolamine were prokaryotes. From the literature it would seem that fungi can grow either on triethanolamine as a source of carbon or that growth is altered by its presence (Fattakhova et al., 1991). One study of a range of common

[3] Diethylene Glycol Monoethyl Ether Safety Data Sheet available through Sigma-Aldrich ("Diethylene Glycol Monoethyl Ether Safety Data Sheet," n.d.)

soil fungi found some species of fungi (*Saccharomyces*, *Trichoderma* and *Rhizopus*) were stimulated by triethanolamine, while others (*Alternaria, Penicillium* and *Botrytis*) were inhibited by its presence (Garabadzhiu et al., 2011). In the present study, triethanolamine altered microbial communities in the aquifer microcosms and enhanced the growth of mostly prokaryotes and some fungi in the sole carbon utilisation assays. In terms of total DNA (≈ cell number), triethanolamine did not alter the recovery of DNA from sole carbon source assays (Figure 4-5) and more DNA was recovered from aquifer microcosms when triethanolamine was present (Figure 4-29). The latter likely indicates the growth of taxa capable of degradation.

5.12 Xanthan gum

Xanthan gum is a natural polymeric compound produced by *Xanthomonas campestris* (Jansson et al., 1975; Sworn, 2009; Whistler and BeMiller, 1993). Structurally, xanthan gum is a polymer of the hexose sugars glucose and mannose and the sugar acid, glucuronic acid. It is used in onshore gas activities for viscosity management and is one of the least toxic compounds examined in the present study. It can be degraded by various bacteria with xanthanases (Ashraf et al., 2017; Cadmus et al., 1982), and is partially attacked by fungal cellulases (Cantrell and Duval-pérez, 2017). In the present study, xanthan gum mainly enriched bacteria when it was the sole source of carbon. Indeed, network analyses revealed that 39 OTUs were only enriched when xanthan gum was the sole source of carbon. In the aquifer microcosms, xanthan gum caused a unique and marked change in community structure. This was probably a result of increases in taxa known to degrade xanthan gum. This increase is evidence in the amount of DNA recovered (≈$3.5x10^6$ cells / mL) in the xanthan gum treatment, representing almost 35-fold more than the control.

5.13 Glyoxal, glutaraldehyde and Hexahydro-1,3,5-tris (2-hydroxyethyl)-sym-triazine

Microbially, glutaraldehyde is degraded via one of two known pathways both via the carboxylic acid glutarate (glutaric acid). In the anaerobic pathway, glutaraldehyde is first converted to 1,5-pentanediol, while this step is omitted in the aerobic pathway. Glutarate is subsequently degraded via oxidation and eventually enters the TCA cycle (in those organisms that possess this pathway), where its carbon is recycled into common cellular components or CO_2. Similarly, glyoxal is rapidly degraded to the α–hydroxy acid glycolic acid, which in turn enters to the TCA cycle (in those organisms that possess this pathway) and carbon is recycled into common cellular components or CO_2.

In the present study, sole carbon source assays indicated that both bacteria and fungi were able to grow on glyoxal as a source of carbon with fungi outnumbering bacteria by ~10-fold. The bacteria identified were a putative *Rhodovastum* species and novel Acidobacteria taxon identical with Genbank accession (AF523990). *Rhodovastum* have been previously observed in microbial communities associated with hydraulic fracturing fluids (Ulrich et al., 2018) and has also been isolated from methane-rich paddy fields (Okamura et al., 2009) and methane-emitting fen soils (Hunger et al., 2011). The novel Acidobacteria taxon was detected in soil impacted by reject coal (Brofft et al., 2002). The fungi involved in increased growth in the presence of glyoxal were mostly ascomycetes. Ascomycetes are known to degrade a range of aldehydes via aldehyde dehydrogenases that convert aldehydes to their carboxylic acid equivalents (Datta et al., 2017). These have been demonstrated extensively in yeasts; indeed numerous *Candida* species were detected in the Penola soil used in this study, and they may be involved in glyoxal degradation.

In terms of community level effects, no marked changes in species richness or biodiversity were observed with the addition of glyoxal. In contrast, glyoxal did alter the microbial community structure. Ordinations suggest that glyoxal altered aquifer microbiomes in a similar way as glutaraldehyde. This is probably unsurprising as both compounds are aldehydes and have similar chemical properties. Both are involved in cross-linking other compounds and form adducts with various compounds including biologically important molecules such as proteins or DNA.

It is also noteworthy that chemically, the reactivity of compounds like glyoxal likely means they have very short half-lives in the environment, as it would bind to many biological or abiotic chemicals in the environment. Based on these observations, it seems unlikely that this compound

poses a significant risk to the soil environment, though its effect on aquifer communities may be worth further study.

Interestingly, the biocide hexahydro-1,3,5-tris (2-hydroxyethyl)-sym-triazine appears to have similar impacts on the microbial community to glyoxal and glutaraldehyde. This is likely due to the mechanism of action for his biocide, which is reliant on the production of formaldehyde.

5.14 Aromatic compounds – naphthalene and o-cresol

Microbes encounter a range of aromatic compounds in the environment. These often derive from plant alkaloids, aromatic amino acids or lignin-like compounds. Given the diversity of these compounds, microbes employ numerous strategies to alter these compounds using a range of accessory enzymes, funnelling this array of compounds to a central intermediate which is then processed through a common pathway. In aerobes this pathway frequently coalesces on catechol, while for anaerobes it is centred on benzoyl-coA (a benzoate-like compound joined to co-enzyme A, see section 5.1) (Feng et al., 1999; Spormann et al., 1997).

Naphthalene degradation is well-understood for a few key taxa that have been studied in detail, this is particularly true for aerobes (Annweiler et al., 2000; Eaton and Chapman, 1992; Torok et al., 1995). Aerobically, a key central intermediate appears to be salicylate. Anaerobically, there are numerous reports of the complete oxidation of naphthalene (Galushko et al., 1999; Rockne et al., 2000), and though the pathway is less well characterised, some key genes and intermediates have been identified (Kleemann and Meckenstock, 2011; Kümmel et al., 2015).

In the present study, naphthalene did not affect fungal or prokaryotic richness in sole carbon source experiments. In these experiments, most of the taxa that increased in abundance were fungi. Fungi have been previously shown to degrade a range of aromatic compounds including naphthalene (Govarthanan et al., 2017), and their abundance here provides further evidence of their importance in soil environments. In the soil and aquifer microcosms, it appeared to cause similar shifts in prokaryote community structure to o-cresol, a monoaromatic compound. Somewhat surprisingly, this similarity did not extend to fungal communities which suggests naphthalene and o-cresol are degraded by different fungi in the soils of south-eastern South Australia. Work to isolate bacteria and fungi involved in naphthalene degradation in soils of south-eastern South Australia has revealed that in enrichment liquid cultures grown with naphthalene as

a sole source of carbon, naphthalene is solubilised and degraded rapidly by what appear to be bacteria and fungi (data not shown).

O-cresol degradation has been comparatively well-studied as the chemical is an intermediate of aerobic toluene degradation in *Burkholderia* species (Johnson et al., 2006). The compound can also be degraded anaerobically (Schink et al., 2000). In the present study, o-cresol increased the growth of a small number of bacteria and fungi, presumably involved in its catabolism. While high concentrations of o-cresol are toxic, the levels tested here do not appear to adversely impact microbial community structure or cell number. There did not appear to be any toxicity associated with o-cresol at the concentrations tested, certainly DNA recovery was not discernibly different from other treatments in soil and markedly higher in the aquifer microcosms.

5.15 d-limonene

d-Limonene is a monocyclic terpene that is naturally relatively abundant, particularly in the oils from many citrus fruits. While d-limonene has some antimicrobial properties against both fungi and bacteria (Bevilacqua et al., 2010; Bridges, 1987; Chee et al., 2009), numerous other microbes have been demonstrated to grow on this chemical as a sole source of carbon (Bicas and Pastore, 2007).

In the present study, d-limonene was tested at two concentrations, with similar but slightly different results. In the sole carbon assays, the majority of catabolisers for d-limonene were bacterial at the lower concentration, although at the higher concentration, more fungi appeared to be involved with its catabolism. Regardless, it is clear that both groups can potentially contribute to its catabolism. In soil microcosms, the addition of d-limonene appeared to have modest but measurable effects on soil microbiome structure and recovered DNA (≈cell number). In contrast, in the aquifer microcosms, greater yields of DNA (greater cell numbers) were observed for d-limonene at the higher concentration compared to controls. Changes to the aquifer microbiome structure were modest for d-limonene and resembled those caused by other aliphatic compounds tested during this study (eicosane and pristane).

While d-limonene does appear to impact microbial community structure, organisms that appear to catabolise this compound are common in the Penola soil studied. It thus appears to represent a minimal risk to the environment.

5.16 Adsorption vs microbial degradation

One challenge in the present study is disentangling physicochemical processes: solubility, volatilisation, adsorption and abiotic degradation from microbial biodegradation. The results from the chemical degradation of compounds (Section 3.3) indicate that in many cases the initial measured concentrations (taken 48 hours after dosing) were markedly below the dosed amount. This phenomenon was greatest in the soil microcosms. While it is tempting to assign most of this loss to adsorption, many soil microbes are fast-growing and can potentially respond to new sources of carbon rapidly. This is particularly true for chemicals whose degradative pathways occur across a diverse range of taxa. Examples from tested chemicals include: methanol, ethanol and acetic acid. As such, early losses of chemicals are likely to be a combination of these two factors, the extent to which one or the other factor contributes likely varies by chemical.

For those chemicals examined by accredited chemical tests (mostly short-chained alcohols, glycols and organic acids; Section 4.2, also see Figure 4-2) most would appear to have high mobility in soil with relatively low adsorption. Adsorption in soils occurs through a range of mechanisms including ion exchanges, interactions with metal cations, polar interactions, charge transfers and Van der Waals dispersion forces/hydrophobic effect (Calvet, 1989) with soils rich in organic matter frequently having greater adsorptive capacity (Thompson and Way, 1850; Way, 1850). In the present study, the comparatively low total organic carbon of the South Australian soil likely contributes to the mobility of these compounds. The most likely scenario therefore, for those compounds at least, is degradation via microbial catabolism.

Other chemicals in this study may behave quite differently. For example, naphthalene concentrations may decrease via a variety of different processes including: adsorption, volatilization and biodegradation. In soil, the non-polar characteristics of naphthalene gives it a stronger affinity to the soil surface rather than the water phase. This affinity to soil is also dependent on soil type, for example, naphthalene is known to have stronger adsorption to clay than to sandy soils (Osagie and Owabor, 2015). In both soil and aquifer microcosms volatilisation is also likely to contribute to the loss of naphthalene.

Taken across the scope of this report, these examples demonstrate that losses of chemicals demonstrated here likely have multiple sources and while microbial growth was demonstrated on virtually all the compounds examined, it is not the sole mechanism of loss.

5.17 Conceptual model

Data presented in the current study has been analysed to date on a by-chemical basis. Here a model has been developed from insights gained during the project and from the literature. The conceptual model (Figure 5-2) highlights that many chemicals share common intermediates. Exploring some examples, acetaldehyde is a common breakdown product of ethanol, ethylene glycol, 2-aminoethanol and triethanolamine. Similarly, formate is a central intermediate in the catabolism of malonic acid (a breakdown product of the isothiazolinone biocides), it is also a key metabolite in the degradation of formaldehyde, methanol and a number of other compounds. The conceptual model provides a useful framework for understanding the potential fates of chemicals in the soils and, to a lesser extent, aquifers of the Penola area of South Australia. In the conceptual model most of the pathways are shown with blue arrows, indicating oxic pathways. Undoubtedly, there are many anoxic equivalents to many of these pathways, though the paucity of studies of anaerobes compared with aerobes limits our understanding in this area. It is also noteworthy that the conceptual model does not describe central metabolism in detail, instead it emphasises key intermediates that feed into central metabolism.

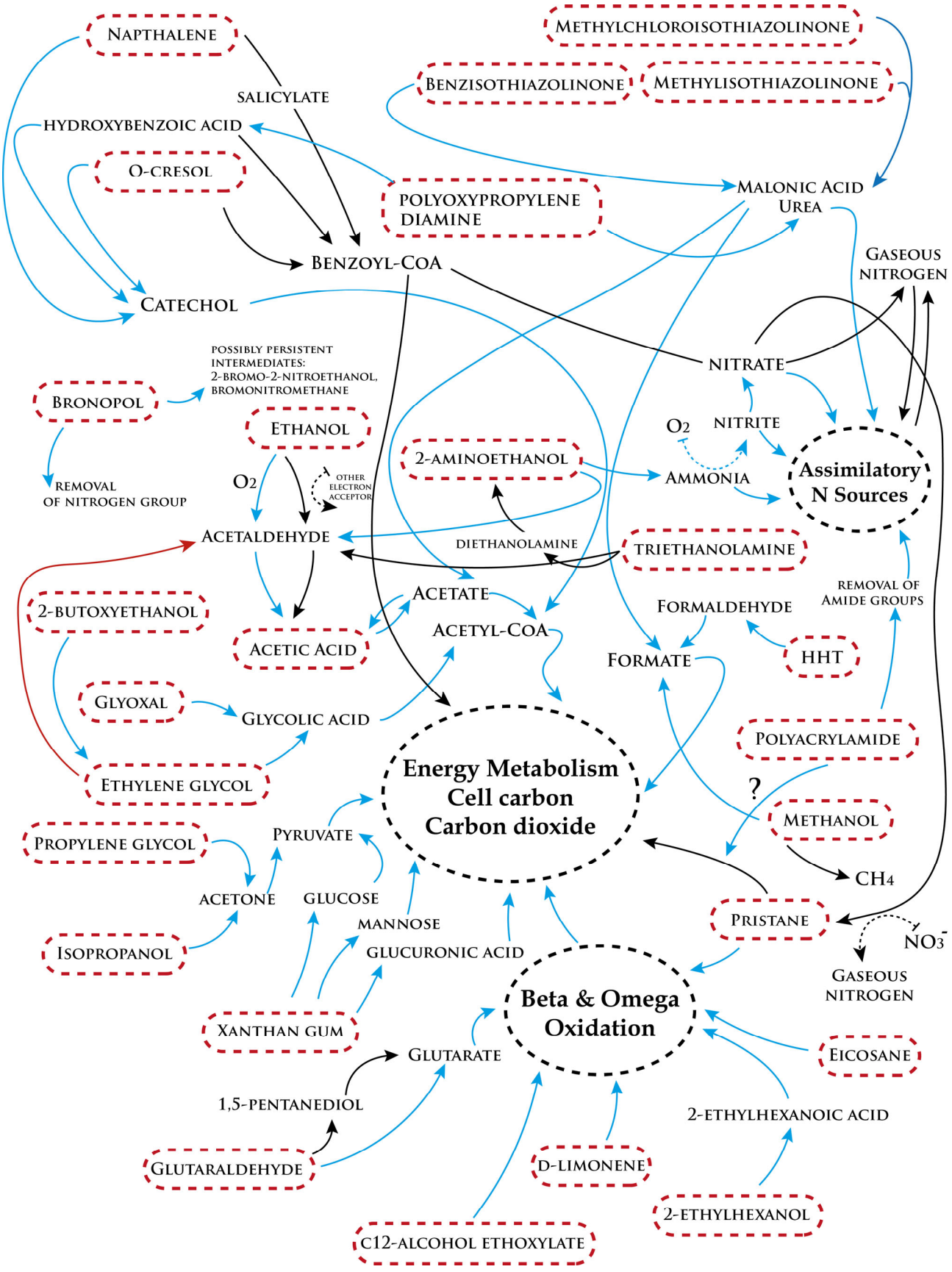


Figure 5-2: Conceptual model of chemical catabolism in soils and aquifers of south east South Australia.

Black arrows indicate anaerobic pathways of degradation. Blue arrows indicate aerobic pathways of degradation. Red arrows indicate microaerophilic pathways of degradation.

6 Recommendations

- While the current study has demonstrated structural changes to microbial communities in soils and aquifers, it is unknown whether these changes have an impact on ecosystem services, i.e. carbon, nitrogen or other geochemical cycling.
- The greater impact of tested chemical compounds on fungal communities may have implications on plant productivity, translocation of nutrients and soil structure.
- The current study tested a single concentration of each individual chemical, it is not known how microbial communities are affected by combinations of chemicals and whether any synergistic interactions occur. From the perspective of ecosystem services, the threshold values (i.e. the concentration at which ecosystem services are impacted) of chemicals is not known. In particular, the threshold values for the biocides would be valuable in order to develop regulations and guidelines for their use.
- Bronopol had a marked impact on bacterial growth in sole carbon source experiments at a concentration of 70µg per plate, indicating that it has a high potential residual risk of toxicity. Bronopol is used as a biocide and is clearly effective, however, spills of this compound may have greater risk than other biocides given its effectiveness at low concentrations. Additionally, degradation products of bronopol include a number of microbially recalcitrant intermediates. Fungi from the Penola soil are able to grow on bronopol as a sole source of carbon, but further work to characterise its degradation products from this catabolism would be valuable.
- Further examination of the absence of degradation for ethanol, isopropanol, acetic acid, 2-butoxyethanol and 2-ethylhexanol in aquifer microcosms would be of value. Microbes with the catabolic potential to degrade these compounds are present in the aquifer. Are other nutrient deficiencies preventing the biodegradation of these compounds?
- The present study took an experimental approach whereby each chemical was tested either using accredited chemical analytical tests, sole carbon source experiments, or whole microbial community microcosm experiments. In hindsight, this approach while providing information on individual compounds did not provide a holistic system view of the compound's interactions with soil and aquifer microbiomes. It is recommended for future

studies that all compounds of interest be analysed using all available experimental approaches.

Appendix A Chemical analyses

Chemical analyses of soils, aquifer waters and soil microcosms were undertaken by Australian Laboratory Services (ALS) Environmental and are described below.

A.1 Soil chemistry analyses

Apx Table A.1 shows the chemical analyses performed on soil samples.

Apx Table A.1: Schedule of tests and method reference for soil chemistry measured by Australian Laboratory Services (ALS).

Parameter	Technique/Method Reference
1:5 Leach	In house
Soluble Major Anions: Sulfur, Silica	In house (APHA 3120)
pH (1:5)	Rayment and Lyons 4A1 (mod) and APHA 4500-H+ B
Electrical Conductivity (1:5)	Rayment and Lyons 3A1 and APHA 2510 B
15 Metals (NEPM 2013 Suite - incl. Digestion): As, Ba, Be, C, Cd, Cr, Co, Cu, Pb, Mn, Ni, Se, V, Zn. Hg	USEPA 6010, APHA 3112 Hg - B, In house
Additional Metals by ICP-AES: Al, Sb, Fe, Mo, Ag, Sr, Sn	USEPA 6010
Total Metals by ICP-MS: U	USEPA 6020
Major Cations (Ca, Mg, Na, K)	USEPA 6010, In house
Major Anions (Cl, SO4)	APHA 4500-Cl- E, In house, In house (APHA 3120)
TRH/BTEXN/PAH + Phenols	USEPA 8015A, USEPA 8270D, USEPA 8260B, In house
Total Fluoride	In house
NH3, NO2, NO3, NOX, TKN, TN, TP, RP	APHA 4500-NH3 B/G/H, In house, APHA 4500-NO3 F, Thermo Scientific Method D08727 and NEMI (National Environmental Method Index) Method ID: 9171, APHA 4500-NO3 B, APHA 4500-P F, APHA 4500-Norg D, APHA 4500-Norg/NO3, APHA 4500-P B&H

Results of additional compounds tested as a part of the testing suites for the compounds of interest are shown in Apx Table A.2.

Apx Table A.2: Results of additional compounds tested to determine pre-existing concentrations in soil flood, measured in triplicate by ALS.

Analyte grouping/Analyte	Soil (µg/L)
2-Ethoxyethyl acetate	<2000
Diethylene glycol monobutyl ether	<2000
Diethylene glycol	<2000
Triethylene glycol	<2000
n-Propanol	<50
Isobutanol	<50
n-Butanol	<50
Formic Acid	449 ± 11
Propionic Acid	<50
Isobutyric Acid	<50
n-Butyric Acid	<50
Isovaleric Acid	<55
Valeric Acid	<55
Isocaproic Acid	<60
Caproic Acid	<60
Heptanoic Acid	<70
Diethanolamine	<1
Methyl diethanolamine (MDEA)	<1
Amyl Alcohol	<2000
Cyclohexanol	<2000
Iso-Amyl Alcohol	<2000
Phenol	<1.0
2-Chlorophenol	<1.0
3- & 4-Methylphenol	<2.0
2-Nitrophenol	<1.0
2.4-Dimethylphenol	<1.0
2.4-Dichlorophenol	<1.0
2.6-Dichlorophenol	<1.0

4-Chloro-3-methylphenol	<1.0
2.4.6-Trichlorophenol	<1.0
2.4.5-Trichlorophenol	<1.0
Pentachlorophenol	<2.0
Acenaphthylene	<1.0
Acenaphthene	<1.0
Fluorene	<1.0
Phenanthrene	<1.0
Anthracene	<1.0
Fluoranthene	<1.0
Pyrene	<1.0
Benz (a)anthracene	<1.0
Chrysene	<1.0
Benzo (b+j)fluoranthene	<1.0
Benzo (k)fluoranthene	<1.0
Benzo (a)pyrene	<0.5
Indeno (1.2.3.cd)pyrene	<1.0
Dibenz (a.h)anthracene	<1.0

Apx Table A.3 shows the results of organic soil analyses. The results showed all organic compounds analysed were below the detection limits of the analytical methods.

Apx Table A.3: Results of organic soil chemistry in triplicate measured by ALS.

Analyte grouping/Analyte	Soil (mg/kg)
Phenol	<0.5
2-Chlorophenol	<0.5
2-Methylphenol	<0.5
3- & 4-Methylphenol	<1
2-Nitrophenol	<0.5
2.4-Dimethylphenol	<0.5
2.4-Dichlorophenol	<0.5
2.6-Dichlorophenol	<0.5
4-Chloro-3-methylphenol	<0.5
2.4.6-Trichlorophenol	<0.5
2.4.5-Trichlorophenol	<0.5
Pentachlorophenol	<2
Naphthalene	<0.5
Acenaphthylene	<0.5
Acenaphthene	<0.5
Fluorene	<0.5
Phenanthrene	<0.5
Anthracene	<0.5
Fluoranthene	<0.5
Pyrene	<0.5
Benz (a)anthracene	<0.5

Chrysene	<0.5
Benzo (b+j)fluoranthene	<0.5
Benzo (k)fluoranthene	<0.5
Benzo (a)pyrene	<0.5
Indeno (1.2.3.cd)pyrene	<0.5
Dibenz (a.h)anthracene	<0.5
Benzo (g.h.i)perylene	<0.5
C6 - C9 Fraction	<10
C10 - C14 Fraction	<50
C15 - C28 Fraction	<100
C29 - C36 Fraction	<100
C6 - C10 Fraction	<10
>C10 - C16 Fraction	<50
>C16 - C34 Fraction	<100
>C34 - C40 Fraction	<100
Benzene	<0.2
Toluene	<0.5
Ethylbenzene	<0.5
meta- & para-Xylene	<0.5
ortho-Xylene	<0.5
Naphthalene	<1

A.2 Aquifer chemistry analyses

Apx Table A.4 shows the chemical analyses performed on aquifer water samples.

Apx Table A.4: Schedule of tests and method reference for aquifer water chemistry measured by Australian Laboratory Services (ALS).

Parameter	Technique/Method Reference
Unfiltered Metals by ICPMS: Br	USEPA 6020
PAH/Phenols (SIM)	USEPA 8270D
Total Major Anions (including digestion): Sulfur, Silica (Slicon as SiO2)	APHA 3120
pH (PCT)	APHA 4500-H+ B
Electrical Conductivity (PCT)	APHA 2510 B
Ferrous Iron	APHA 3500-Fe B
15 Dissolved Metals (NEPM): As, Ba, Be, C, Cd, Cr, Co, Cu, Pb, Mn, Ni, Se, V, Zn. Hg	USEPA 6020, APHA 3112- Hg B
Additional Dissolved Metals: Al, Sb, Fe, Mo, Ag, Sr, Sn, U	USEPA 6020
15 Total Metals (NEPM): As, Ba, Be, C, Cd, Cr, Co, Cu, Pb, Mn, Ni, Se, V, Zn. Hg	USEPA 6020, APHA 3112- Hg B
Additional Total Metals: Al, Sb, Fe, Mo, Ag, Sr, Sn, U	USEPA 6020
Alcohols by Static Headspace GCMS	In house (GCMS)
Methanol in Waters	In house (GCFID)
TRH/BTEXN/PAH/Phenols	USEPA 8015A, USEPA 8270D, USEPA 8260B
Glycols analysis in water by GCMS	In house (GCMS)

Total Nitrogen + NO2 + NO3 + NH3 + Total P + Reactive P	APHA 4500-NH3 G, APHA 4500-NO3 F, APHA 4500-NO2 B, APHA 4500-P F, APHA 4500-Norg D, APHA 4500-Norg/NO3, APHA 4500-P B&F
Miscellaneous Alcohols/Solvents (Allyl, Amyl, Cyclohexanol, 2-Ethylhexanol and Limonene) (Non-NATA)**	In house/ HS/GC/FID
Ethanolamines by LCMSMS	LC/MS/MS
Ca, Mg, Na, K, Cl, SO4, Alkalinity & Fluoride	APHA 2320 B, USEPA 6010, APHA 4500-Cl G, APHA 4500-F C, APHA 1030 F, APHA 4500-SO4
Volatile Organic Acids	In house, HS/GC/MS

Results of additional compounds tested as a part of the testing suites for the compounds of interest are shown in Apx Table A.5.

Apx Table A.5: Results of additional compounds tested to determine pre-existing concentrations in the aquifer measured in triplicate by ALS.

Analyte grouping/Analyte	Aquifer (µg/L)
2-Ethoxyethyl acetate	<2000
Diethylene glycol monobutyl ether	<2000
Diethylene glycol	<2000
Triethylene glycol	<2000
n-Propanol	<50
Isobutanol	<50
n-Butanol	<50
Formic Acid	<50
Propionic Acid	<50
Isobutyric Acid	<50
n-Butyric Acid	<50
Isovaleric Acid	<55
Valeric Acid	<55
Isocaproic Acid	<60
Caproic Acid	<60
Heptanoic Acid	<70
Diethanolamine	<1
Methyl diethanolamine (MDEA)	<1
Amyl Alcohol	<2000
Cyclohexanol	<2000
Iso-Amyl Alcohol	<2000

Apx Table A.6 shows the results of the organic aquifer water. The results showed all organic compounds analysed were below the detection limits of the analytical methods.

Apx Table A.6: Results of organic chemistry in triplicate measured by Australian Laboratory Services (ALS).

Analyte grouping/Analyte	Aquifer (µg/L)
Phenol	<1.0
2-Chlorophenol	<1.0
2-Methylphenol	<1.0
3- & 4-Methylphenol	<2.0
2-Nitrophenol	<1.0
2.4-Dimethylphenol	<1.0
2.4-Dichlorophenol	<1.0
2.6-Dichlorophenol	<1.0
4-Chloro-3-methylphenol	<1.0
2.4.6-Trichlorophenol	<1.0
2.4.5-Trichlorophenol	<1.0
Pentachlorophenol	<2.0
Naphthalene	<1.0
Acenaphthylene	<1.0
Acenaphthene	<1.0
Fluorene	<1.0
Phenanthrene	<1.0
Anthracene	<1.0
Fluoranthene	<1.0
Pyrene	<1.0
Benz (a)anthracene	<1.0

Chrysene	<1.0
Benzo (b+j)fluoranthene	<1.0
Benzo (k)fluoranthene	<1.0
Benzo (a)pyrene	<0.5
Indeno (1.2.3.cd)pyrene	<1.0
Dibenz (a.h)anthracene	<1.0
Benzo (g.h.i)perylene	<1.0
C6 - C9 Fraction	<20
C10 - C14 Fraction	<50
C15 - C28 Fraction	<100
C29 - C36 Fraction	<50
C6 - C10 Fraction	<20
>C10 - C16 Fraction	<100
>C16 - C34 Fraction	<100
>C34 - C40 Fraction	<100
Benzene	<1
Toluene	<2
Ethylbenzene	<2
meta- & para-Xylene	<2
ortho-Xylene	<2
Naphthalene	<5

Appendix B Sequencing data

Apx Table A.6 summarises the DNA sequencing information from GISERA W15 and provides links to the OTU tables used for analyses of microbial communities from aquifers, soils and sole carbon experiments.

Apx Table B.6: Sequencing information summary for GISERA W15 and links to OTU tables.

Sequencing pool	Total number of reads	OTU table link
Aquifer: 16S	4037681	https://cloudstor.aarnet.edu.au/plus/s/Ilu1fUNHFXvy5bN
Soils: 16S	1681907	https://cloudstor.aarnet.edu.au/plus/s/TbIVZraQGYBhvjj
Soils: ITS	2738418	https://cloudstor.aarnet.edu.au/plus/s/AqLKD5BUCGKSpyp
Sole Carbon: 16S	1792926	https://cloudstor.aarnet.edu.au/plus/s/Mfhgh8d0aa5Fli6
Sole Carbon: ITS	1763089	https://cloudstor.aarnet.edu.au/plus/s/6QqJW0R1XBSNBJK
Whole of project	~12 Million	

Glossary

Terms	Meaning
16S rRNA	16S ribosomal RNA genes used in taxonomy and molecular phylogeny of bacteria.
aquifer	An underground geological formation containing water.
ATP	Adenosine triphosphate. This is a common cellular energy carrying molecule.
axenic culture	In microbiology, is used to grow a single species or strain of a microorganism, free of any other contaminating organisms.
biocide	A substance that controls microbial growth by destroying or inhibiting the activities of microorganisms.
bp	A base pair (bp) is a unit used for measuring the length of DNA fragments. It represents the two nucleotides that are bonded together on opposing strands of a double-stranded DNA molecule.
catabolism	A process used by living organisms to breakdown complex molecules into simpler ones, and in the process producing energy.
eukaryotic	Refers to organisms with membrane bound organelles. Animals, plants, fungi and protists.
genome	The entire genetic material of an organism.
groundwater	Water occurring below the Earth's surface in aquifers.
heterogeneous	A mixture that is not uniform in its composition.
homogeneous	A mixture that is uniform in its composition.
ITS	Internally Transcribed Spacer region between genes encoding ribosomal RNA, and is commonly used in taxonomy and molecular phylogeny of fungi.
microbes/microorganisms	Microscopic organisms including bacteria, archaea, fungi and protozoans.
OTU	Operational Taxonomic Unit are groups or clusters of closely related microorganisms, as determined by DNA sequencing

	analyses. In this report, pOTUs refers to prokaryotic OTUs, and fOTUs refers to fungal OTUs.
PCR	Polymerase Chain Reaction is a laboratory technique used to amplify or copy a segment of DNA.
ped	A natural aggregate in soil.
prokaryotic	Refers to organisms without membrane bound organelles. Bacteria and archaea.
RO water	Reverse osmosis water.
TCA cycle	Tricarboxylic acid cycle is a set of chemical reactions used by aerobic organisms to generate energy.

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