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Microbial degradation of onshore gas activity chemicals and fluids in aquifers of the Limestone Coast, South Australia

GISERA W.22

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

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Executive summary

This project had three broad research goals in the Limestone Coast region of South Australia:

1) to provide geochemical and microbial baseline data for aquifers of the region.

2) to determine if microbes could degrade common chemicals used in onshore gas activities and measure the impacts of these chemicals on microbial communities and identify putative indicator taxa for environmental health monitoring.

3) to determine the genomic capability of microbes in the Tertiary Limestone Aquifer (TLA) to degrade chemicals.

1) Baseline chemistry and microbiology

Groundwater samples were obtained from the TLA at 21 sites in early 2021 in Hydrogeological Provinces 1 and 2 under a range of land-use types. These samples were used to provide baseline data for both water chemistry and microbial communities and were essential for understanding chemical biodegradation, nutrient cycling, and for environmental health monitoring.

In terms of water chemistry, all samples were moderately alkaline to neutral in pH with relatively low concentrations of dissolved salts. Water chemistry broadly mirrors known hydrogeological provinces and zones, with marked separation between Hydrogeological Provinces 1 and 2, and moderate distinctions between hydrogeological zones within these provinces.

In terms of microbiological baselining, 25,698 OTUs (species) were detected across the 21 sites sampled. Numerous common aquifer-dwelling phyla were detected, dominated by Proteobacteria and Firmicutes, however, the diversity of aquifers of the TLA appears to be somewhat unusual in that there was a high abundance of uncommon phyla (e.g., Pacearchaeota, Woesearchaeota, Parcubacteria, and Microgenomates), along with almost 50% of OTUs being novel and not readily classifiable by modern methods.

2) Chemical degradation and microbial community responses

In the current study, concentrations of chemicals tested reflect the maximum concentration used by the gas industry in the well/bore. It is noteworthy that scenarios of concentrated chemical spill

events (e.g., during transportation of concentrated chemicals to gas industry sites) are not considered here.

The sampled aquifer microbes were able to partially biodegrade the alcohols, butoxyethanol, isopropanol, methanol, and propylene glycol. In contrast, no biodegradation of ethylene glycol was observed across all aquifer samples tested.

In most microbial degradation experiments, the addition of chemicals caused a statistically significant shift ($p < 0.05$) in the microbial community profile. In broad terms, from the chemicals measured, the biocides and the miscellaneous group of chemicals had the largest effects on aquifer microbiomes. The alcohols, amines and hydrocarbons had variable effects on the aquifer microbiomes. In contrast, the glycols had limited effects on microbiomes.

Most changes to microbial communities were driven by the growth of species capable of degrading the added chemicals. Few species from the aquifer microbiomes were identified as sensitive (i.e., reduced in relative abundance) to the concentrations of chemicals examined.

These results are consistent with previous GISERA projects (W15 and W17) and suggest that degradation of chemicals by indigenous microbial communities may offer an additional defence against chemical contamination of the local environment.

3) Metagenomes

In this study, ten samples underwent metagenomic analyses, which included experimental treatments from four aquifer sites and six chemical additions. The volume of assembled data varied between samples, ranging from 72 megabase pairs (Mbp) to 474 Mbp, and the binning of metagenomic data recovered a mixture of complete, incomplete, and chimeric (mixed) genomes.

For the six chemical additions (ethylene glycol, isopropanol, polyacrylamide, methylisothiazolinone, diesel, and hydrotreated light petroleum distillate) analysed, complete or near complete gene pathways for degradation of these chemicals were identified in the metagenomic data.

Options for future work and limitations of the current study

1) The microbial and chemical baselining in this study produced a large dataset for the TLA and provides an important benchmark to measure potential gas industry related disturbances against. However, further collections to extend sampling periodicity would be beneficial to gauge stability of the baseline data. This would be particularly useful for temporal or hydrogeological (recharge or drawdown) changes, and useful for creating an environmental monitoring network.
2) It would be valuable to integrate data from the present study with existing knowledge related to chemical pathways, abiotic degradation, and chemical mobility to create better models of environmental risk.
3) The current study only chemically quantified the degradation of a subset of chemicals; those with existing NATA-accredited methods. For the other chemicals, if they remain of concern, development of tests, and direct quantitation of chemical degradation, may be of value to understand chemical half-lives in the subsurface.
4) In this study, chemicals were tested individually and not in combination. In the well/bore, however, chemicals are used as mixtures and these combinations may change the rates of biodegradation and impacts on microbial communities. Work to quantify these chemicals as collective stressors would be valuable.
5) Understanding the potential impacts of surface-spill events of concentrated chemicals would be of value, particularly with reference to aquifer contamination pathways. Additionally, understanding the effects of increasing chemical concentrations would be useful to identify ecological tipping points for chemicals.

1 Introduction

The Limestone Coast, located in south-eastern South Australia, is characterised by rich geological, ecological, and agricultural significance, typified by its distinct karst landscapes and limestone formations. The region comprises the City of Mount Gambier along the District Councils of Grant, Kingston, Robe, Tatiara and Naracoorte Lucindale, and the Wattle Range Council. From a land use perspective, the area is particularly rich hosting a significant viticulture industry and a diverse array of agricultural enterprises.

Much of this activity is underpinned by groundwater sourced from Tertiary Limestone Aquifer (TLA) (Barnett et al., 2020; Walker et al., 2021). This water serves multiple purposes including irrigation, stock watering, individual and municipal domestic usage as in the case of Mount Gambier. The water in the aquifer is generally fresh[1], with salinities less than 3000 µS cm^{-1}. Recharge varies by location, with Hydrogeological Provinces 1 and 2 being principally supplied through vertical recharge with some lateral groundwater flow, while Province 3 is confined and receives little if any vertical recharge[1] (Figure 1). This study exclusively examined waters from Hydrogeological Provinces 1 and 2.

Hydrogeological Provinces 1 and 2 have been environmentally impacted by excessive usage, irrigation, recycling, and vegetation clearance causing localised increases in salinity, as well as declining groundwater levels [1,2]. Therefore, heightened community concerns about this important resource exist. Globally, groundwater resource management is a growing area of concern due to depletion, salinisation, and pollutant contamination (Foster and Chilton, 2003).

[1] Department for Environment and Water, Government of South Australia. https://cdn.environment.sa.gov.au/environment/docs/groundwaters-agreement-tertiary-limestone-aquifer-fact.pdf

[2] Department of Water, Government of South Australia. https://www.waterconnect.sa.gov.au/Content/Publications/DEW/Lower_Limestone_Coast_PWA_GSR_2011.pdf

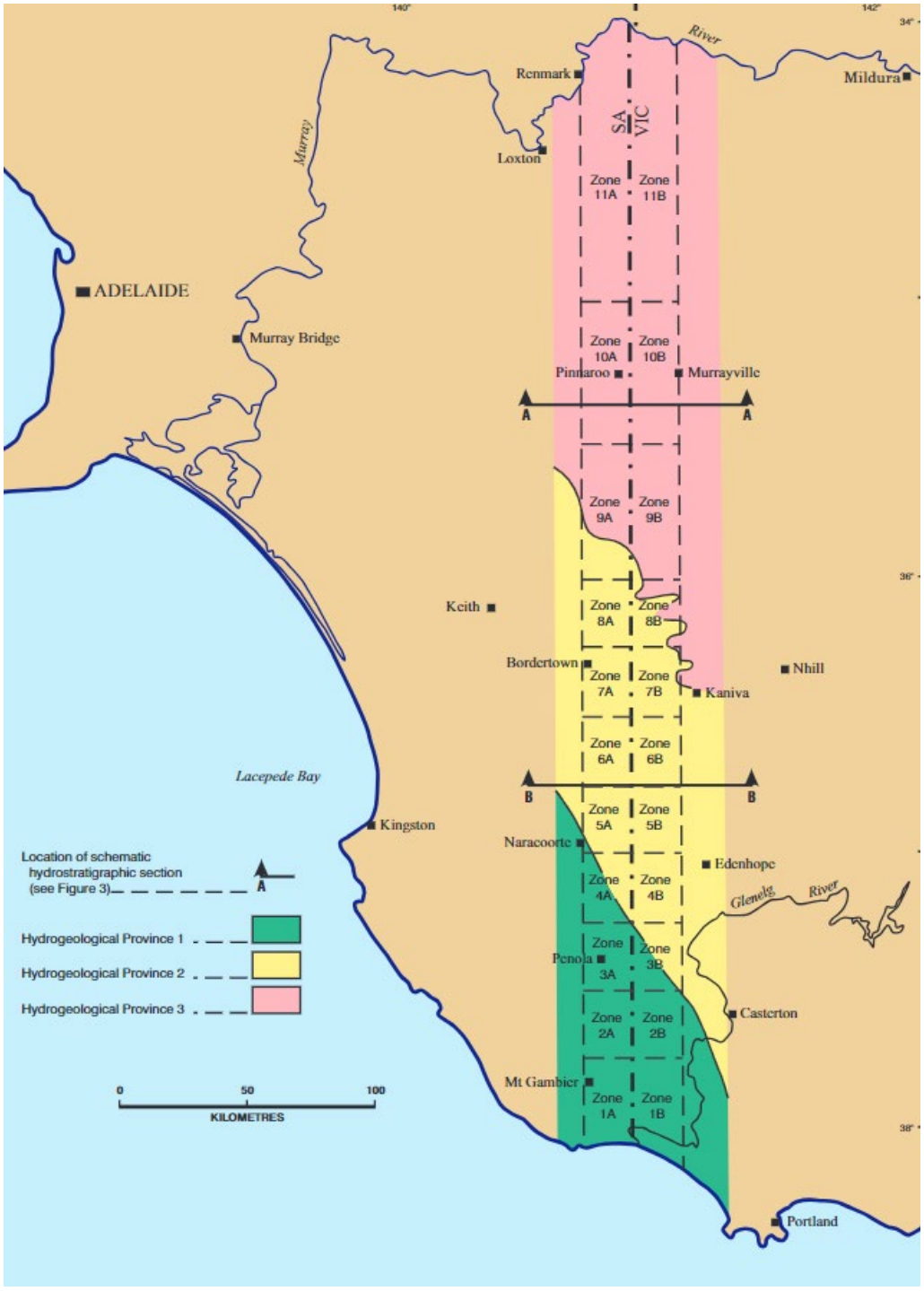

Figure 1: Hydrogeological provinces of the TLA.

Reproduced from the Department for Environment and Water, Government of South Australia.

1.1 Degradation of chemicals in aquifers and the impacts of these chemicals on microbial communities.

Australia's progress to a low carbon future will include the use of natural gas as a transitional energy source to meet both residential and industrial energy requirements. Natural gas resources found in Australia also have the potential to position the country as a major global supplier of natural gas to assist in supplying cleaner energy as the world moves towards a low emissions future. Natural gas production, however, has potential environmental impacts, particularly to water resources, due to potential leaks and spills of chemical additives used during exploration, development, and production. The environmental and human health risks associated with these chemicals have been identified[3][4], however, there is less known about the migration and biodegradation of these chemicals in edaphic and groundwater environments.

This project was commissioned as a follow-up project to GISERA W.15 (Microbial degradation of chemical compounds used in onshore gas production in the SE of South Australia), which demonstrated that for a single, widespread soil type (dark sandy loam) from the Limestone Coast region of South Australia, limited impacts were observed on the soil microbial communities. Furthermore, soil microbes were shown to rapidly degrade most chemicals tested. In contrast, a single sample of microbially rich water from the TLA after 30 days incubation did not degrade many of the tested chemicals. It was not clear whether this observation was due to insufficient incubation times, nutrient limitation, or some innate quality of the single sample tested. This uncertainty led to the commissioning of this broader project which aims to examine chemical behaviour in water with microbes from numerous aquifer samples from the region.

This project is part of a series of linked GISERA projects (GISERA W15, W17, W22, W26, and W27) seeking to holistically characterise aquifers, putative chemical migration, and biodegradation of chemicals associated with onshore gas activities. In particular, this project focussed on chemicals used in onshore gas activities that have been previously identified to pose an environmental or human health risk.

[3] GISERA Health 2 final report: https://gisera.csiro.au/wp-content/uploads/2023/04/GISERA-Health-2-Synthesis-Final.pdf

[4] GISERA Health 2 extension final report: https://gisera.csiro.au/wp-content/uploads/2023/04/Health2_Short_Technical_Brief_final.pdf

The chemicals used in onshore gas activities are of concern to communities in and around the Limestone Coast region due to the potential contamination risks to soil and aquifers, particularly in agriculturally important areas and near populated areas[5].

It should be noted that across most of the Limestone Coast region there is a 10-year moratorium on hydraulic fracturing[6]. As such, the chemicals tested here are used in other onshore gas activities, mostly drilling, workovers or in surface facilities. Fluids used in onshore gas activities for exploration and production, typically contain less than 2% of chemical additives (Conrad et al., 2020) with the remainder being water. In terms of their purpose, most of the chemicals fall into a small number of groups (Box 1).

[5] GISERA final report: Understanding natural gas impacts and opportunities on agriculture in the South East of South Australia (https://gisera.csiro.au/wp-content/uploads/2019/12/Gas-impacts-and-opportunities-on-agriculture-in-south-east-South-Aust-final-report.pdf)

[6] Department of Energy and Mining, Government of South Australia. https://www.energymining.sa.gov.au/industry/energy-resources/regulation/policies-and-guidelines/policies/10_year_moratorium_on_fracture_stimulation_in_limestone_coast_region_sa

Box 1. Chemicals used in onshore gas activities have a range of putative roles.

Biocides - used to control microbial growth and souring problems within reservoirs by eliminating or inhibiting the growth of microorganisms.

Buffers - used to control pH in fluids.

Corrosion inhibitors - generally adsorb onto pipework steel and prevents corrosion.

Epoxy resins - used in pipework for protective coatings.

Friction reducers - used to reduce water friction pressure within pipes during high-rate pumping.

Surfactants - used to modify emulsion surface or interfacial tensions.

Viscosity management - used to control gelation of fluids and assists in carrying chemicals.

The chemical additives used are not unique to the gas industry and are employed in other industries and found in many domestic products. 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 for contamination in areas where onshore gas activities are about to be undertaken.

Several reviews of contamination pathways and their adverse effects have demonstrated that the highest risks associated with onshore gas activities were losses from surface facilities (typically leakage of water holding ponds) or from vehicle related events (e.g., trucks carrying chemicals or fluids involved in accidents)[7][8]. Such events cause acute contamination of soils and surface waters, but can, under certain conditions, also make their way into groundwater. Acute damage caused by these events (e.g., vegetation loss) is most commonly due to the high salinity of the water involved in the spill, rather than the chemical additives per se and microbial activity can do little to mitigate the impacts of saline water on surface environments. That said, microbial activity in soils is high and GISERA W15 and other projects indicate that most chemicals are rapidly degraded in soils. Assuming a pathway through soils, for an aquifer contamination event to occur, chemicals contained in the spilled water must move through the soil horizons and the deeper subsurface to

[7] https://www.europarl.europa.eu/thinktank/en/document/IPOL-ENVI_ET(2011)464425

[8] https://www.dcceew.gov.au/sites/default/files/documents/technical-report-number-06-lit-rev-identification-potential-pathways.pdf

reach the aquifer. Through this path, the chemicals would be subject to both microbial activity and abiotic processes like adsorption. It is noteworthy, however, that aquifers that are unconfined (such as the TLA in Hydrogeological Provinces 1 and 2) are more prone to contamination via this pathway, confined aquifers in contrast are often “trapped” beneath impermeable rocks, mudstones, or shales and similar. These rocks typically serve as a barrier to prevent aquifer contamination.

The work described here explored the ability of microbes originating from a range of groundwater samples collected from the Limestone Coast to degrade a range of chemicals (Table 1) putatively used by the onshore gas industry.

Table 1: Chemicals examined in this study.

Chemicals	Additive role in onshore gas activities
2-aminoethanol	Viscosity management/ drilling additive
2-butoxyethanol	Surfactant
2-ethylhexanol	Surfactant
benzisothiazolinone	Biocide
bronopol	Biocide
c12 alcohol ethoxylate	Surfactant
diesel fuel	Fuel
diethylene glycol ethyl ether	Solvent
d-limonene	Surfactant
eicosane	Surfactant
hydrotreated light petroleum distillate	Carrier fluid
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

1.2 Baselining microbial communities for ecosystem health monitoring

Increasingly, environmental DNA (eDNA) is being used as a key tool in monitoring ecosystem health (Bunce and Freeth, 2022). Essentially, this approach requires obtaining environmental samples such as soil, water, and plant tissue, extracting the DNA contained therein and using the

polymerase chain reaction (PCR) to amplify the DNA from within that sample. This is typically done on conserved genes (i.e., genes that have undergone limited evolutionary changes over time) such as ribosomal DNA, mitochondrial DNA, or cytochrome oxidase) as it allows for easy comparisons between different biological entities or taxa (e.g., species, genera, order etc.). The products of these PCRs, called amplicons, are then subjected to DNA sequencing and analysis using bioinformatics. Typically, this analysis produces what is called an OTU table or ASV table. OTU here stands for Operational Taxonomic Unit, ASV stands for Amplicon Sequence Variant. There is some debate about whether OTUs or ASVs are better for determining taxa present and quantifying reads assigned, the former being a method that groups similar DNA sequences, while each ASV is a sequence variant. In practice, however, both have their uses. In this study, OTUs, rather than ASVs are used (Chiarello et al., 2022).

For all intents and purposes, OTUs can be thought of broadly as "species" though they represent higher level taxonomic groups in practice. Nevertheless, readers should mentally equate an OTU with a species of microbe. In this study, the only microbes being examined are prokaryotes: bacteria and archaea. No other microbes, for example fungi or protists, are considered here as they typically do not occur in anoxic environments like aquifers.

OTU tables show the number (or proportion) of microbes of a given species at a given site. An example of a mock OTU table is shown below (Table 2), here, there are four "species" and their abundance (counts) from five aquifers. From these mock data, one can see that aquifer 1 and aquifer 5 have similar species, in approximately the same proportions. This likely indicates these two aquifers are connected. As well as demonstrating this connection, these data can also be used as a "pre-industry" baseline, as microbial diversity and composition will change in response to changes in the environment.

Table 2: A mock 'OTU table' showing counts of DNA amplicons belonging to each OTU/'species'.

	Aquifer 1	Aquifer 2	Aquifer 3	Aquifer 4	Aquifer 5
OTU6 (*Curvibacter fontanus*)	85	0	0	10	100
OTU3 (*Pseudomonas aeruginosa*)	88	172	3	98	110
OTU7 (*Hydrogenophaga taeniospiralis*)	0	5	65	65	0
OTU5 (*Sideroxydans lithotrophicus*)	67	0	10	0	88

1.3 Chemicals concentrations used in the present study.

The Swiss physician Paracelsus (1493-1541) is often paraphrased as saying “the dose makes the poison”(Grandjean, 2016). This statement is central to all toxicological studies and is best exemplified by micronutrients in plants and animals. For example, while in plants boron is an essential nutrient (helping to stabilise their cell walls), above a certain concentration, however, this element becomes toxic. The same principal applies to the chemicals used in this study. For example, the chemical triethanolamine (Figure 2) is undoubtedly toxic to microbes at moderately high concentrations, but at lower concentrations, however, this compound can be used as a sole carbon source for growth.

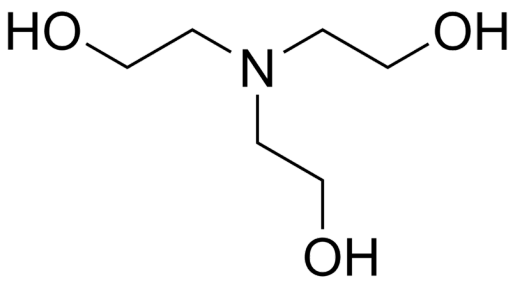

Figure 2: Chemical structure of triethanolamine.

One example from the literature, for instance, demonstrates that a bacterium in culture was able to grow solely on triethanolamine as a source of carbon, while a yeast (*Rhodotorula mucilaginosa*) was able to grow on that chemical as a sole source of carbon and nitrogen at concentrations of 9mM (1342 mg/L)(Fattakhova et al., 1991). Triethanolamine in aqueous solution is basic and a high pH does have antimicrobial activity against some microbes. For example, *Photobacterium phosphoreum* is intolerant of triethanolamine concentrations at ~525mg/L[9]. This highlights that different organisms are tolerant to different concentrations of a single compound.

It is also worth noting that choosing a concentration to test in this study is a somewhat complex task. As a general principal, this study has chosen to test concentrations used in the well bore. This concentration is substantially less than the concentration in the constituent products, but substantially higher than the concentration that would be in the reservoirs with which the bores

[9] Triethanolamine MSDS: https://www.dcfinechemicals.com/catalogo/Hojas%20de%20seguridad%20(EN)/117270-SDS-EN.pdf

intersect. As previously mentioned, concentrated spill events of chemicals e.g., during transportation of concentrated chemicals to gas industry sites, is not considered here. To address these concentrated spill events, different methods of testing would be required.

1.4 Aims

This study aimed to:

1) Determine if degradation of chemicals used in common onshore gas activities occurred.
2) Determine the impacts of these chemicals on the microbial communities.
3) Determine which species positively respond to chemicals (likely “consumers” of that chemical) and which species respond negatively (likely “indicator/sensitive taxa” to the addition of that chemical.
4) Determine microbial community baselines for various regions of the Tertiary Limestone Aquifer.

2 Methods

2.1 Sampling campaign

Task 1 involved obtaining geographic and hydrogeological maps of the Limestone Coast region, along with consultation with the South Australian Departments of Energy and Mining (DEM), and Environment and Water (DEW) to guide sample collection. Sampling locations were selected to include the various land uses of interest (viticulture, animal grazing, grain growing, fruit and tree nut farms, and vegetable farms) and known hydrogeological and physicochemical heterogeneity across the TLA. Landowners of properties in sampling areas were contacted to ask if they would be willing to share water samples for the project. Priority was given to agriculturally important sites and to also obtain broad coverage of the region such that aquifer heterogeneity and microbial variation (and variation in their catabolism if it exists) across the TLA could be captured. The sampling campaign logistics were planned and provisioned in a safe and environmentally sensitive manner.

The list of chemicals examined in this study were based on those examined in GISERA W15, with diesel and hydrotreated light petroleum distillate added (Table 1). Prior to commencement, all chemicals to be examined were confirmed with industry representatives as still being relevant to their proposed operations in the Limestone Coast region of South Australia.

Task 2 was the sampling campaign, conducted in February 2021, in the Limestone Coast region of South Australia where triplicate microbially preserved aquifer samples were collected from the 21 sites identified by Task 1. A total of 63 samples were collected and used for the microbial community baselining (Task 3). Additionally, five biological (live and anoxic) and bulk aquifer samples were collected for Tasks 4 and 5 to measure chemical degradation and microbial indicators of degradation. These five biological and bulk water samples aimed to cover the land use types (orchards, pasture, small seeds/grains and vegetables) and heterogeneity in the TLA.

2.1.1 Aquifer water samples

Aquifer water samples were collected in a preservation solution containing dimethyl sulphoxide (DMSO), disodium EDTA, and saturated NaCl (abbreviated to DESS) to preserve microbial DNA for

various molecular analyses (Seutin et al., 1991; Yoder et al., 2006). This solution kills microbes in the sample without lysing their cell walls and thereby protecting the DNA from degradation. Additionally, use of DESS simplified collection allowing samples to be stored at room temperature, shipped by freight, or carried.

Three hundred millilitre water samples were collected using prepared Schott bottles of DESS (200mL). Prior to sampling, water was allowed to purge from the bore (minimum time of 5 minutes) to ensure samples were collected directly from the aquifers and not stagnant water in the pipelines. Samples were sealed in Schott bottles with silicone rubber seals. Three samples were collected at each location.

2.1.2 Live microbial water samples

Water samples were taken from selected sites for microbial chemical degradation growth trials. The live microbial samples were collected under a carbon dioxide atmosphere to preserve the anoxic environment found in the aquifer and prevent overgrowth of organisms that flourish in oxic conditions. A volume of ~ 1L of aquifer water were anoxically collected by bubbling vigorously with carbon dioxide 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, the bottles were vented inside an anaerobic chamber where the atmosphere comprised ~95% argon, 1-2% hydrogen and the balance nitrogen.

2.1.3 Bulk water samples

Bulk samples were collected at the same sites as anoxic water samples and stored in 10L polypropylene containers for transport. The bulk samples were used for media preparation in Tasks 4 and 5.

2.2 Baselining microbial communities

2.2.1 DNA extraction

Each aquifer water sample was vacuum filtered through a sterile 25mm Millipore Durapore® PVDF disc (0.1µm pore size) using a sampling manifold (Millipore) to collect the microbial cells. The disc was sliced into small pieces using a sterile scalpel blade and half of each filter disc was used for DNA extraction. All DNA extractions from aquifer samples used a 96-well plate format and the ZymoBIOMICS 96 DNA Kit. DNA extractions were carried out following the manufacturer's instructions[10] using the recommended lysis protocol for bead-beating with the Mini-BeadBeater-96 (Biospec).

2.2.2 DNA quantification and cell number estimations

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

For all DNA samples, the PicoGreen reagent was diluted 200-fold. Also included in the Quaint-it™ PicoGreen Kit was a λ DNA standard (100µg mL^{-1}) which was diluted to make 5mL of a 4µg mL^{-1} solution. The 4µg mL^{-1} λ solution was then used to make appropriate standards.

All wells measured had 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 measured using the means of each standard. Unknown concentrations were derived from the standard curve associated with that plate.

[10] ZymoBIOMICS 96 DNA kit protocol https://files.zymoresearch.com/protocols/_d4303_d4307_d4309_zymobiomics_96_dna_kit.pdf

Cell number estimations were calculated from DNA concentration assuming an average bacterial genome size of 5 x 10^6 base pairs (Land et al., 2015).

2.2.3 DNA processing and sequencing

Microbial community profiling was performed for bacteria and archaea using the V4 region of the 16S rDNA gene for prokaryotes with the PCR 16S primers 515F (Parada; GTG**Y**CAGCMGCCGCGGTAA) and 806R (Apprill; GGACTAC**N**VGGGTWTCTAAT) (Apprill et al., 2015; Caporaso et al., 2011). The 16S gene libraries of DNA samples were prepared with Illumina Tru-Seq PCR-free library preparation kits and sequencing was conducted on an Illumina miSEQ DNA sequencer at 250 paired end. All baseline microbial community profiling was done by Molecular Research LP DNA, Texas, USA (MR DNA).

An internal control was used to correct for sample contamination on the Illumina sequencing platform. In brief, each sequencing run included an artificial chimeric bacterial amplicon control (containing fungal mycorrhizal species, *Cairneyella variabilis*) with its own unique barcode. The presence of this chimeric amplicon with alternative barcodes would identify the amount of 'bleed' between samples (i.e., cross-contamination of samples).

2.3 Aquifer water chemistry

Characterisation of the chemical properties of the aquifer water samples was undertaken by ALS Environmental. The schedule of tests and their method references are shown in Appendix A. All chemical analyses were done in triplicate by ALS. Initial information on water chemistry of bores, at their time of drilling, were obtained from Water Connect, Department of Water, Government of South Australia[11].

[11] Know Your Bore: https://www.waterconnect.sa.gov.au/Systems/GD/Pages/Default.aspx

2.4 Degradation and microbial growth trials

2.4.1 Chemicals used in this project

Chemicals used in this project were sourced from chemical suppliers as shown in Table 3. C12 alcohol ethoxylate was used to represent C6-12 ethoxylated alcohols. Pristane (branched C15) and eicosane (linear C20) were used to represent alkanes, C12-26 branched and linear compound, respectively.

Table 3: Information about 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%
benzisothiazolinone	BZ	2634-33-5	Sigma-Aldrich, 97%
bronopol	BR	52-51-7	Sigma-Aldrich, 98%
c12 alcohol ethoxylate	AL	68439-45-2	Oleum, 100%
diesel fuel	DS	68334-30-5	Commercial fuel supplier, premium diesel
diethylene glycol ethyl ether	DG	111-90-0	Sigma-Aldrich 99%
d-limonene	DL	138-86-3	Sigma-Aldrich, 90%
eicosane	EC	112-95-8	Fluka, 97%
ethylene glycol	EG	107-21-1	Sigma-Aldrich, 99%
glutaraldehyde	GA	111-30-8	Sigma-Aldrich, 50%
glyoxal	GO	107-22-2	Sigma-Aldrich, 40%
hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine	HT	4719-04-4	Combi-Blocks, 75%
hydrotreated light petroleum distillate	HP	64742-47-8	Sigma-Aldrich, 99.9%
isopropanol	IP	67-63-0	BDH, 100%
methanol	MT	67-56-1	Ajax, 99.8%
methylchloroisothiazolinone	MC	26172-55-4	Combi-Blocks, 68%
methylisothiazolinone	MI	2682-20-4	Sigma-Aldrich, 95%
naphthalene	NP	91-20-3	BDH, 100%
o-cresol	OC	95-48-7	Sigma-Aldrich, 99%
polyacrylamide	PC	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%

CAS #- Chemical abstract society identifying numbers

2.4.2 Establishment of aquifer microcosm experiments

Establishment of microbial degradation experiments involved spiking of samples with the chemicals of interest (Table 1). All aquifer microcosms were established in 50mL glass serum vials under an anoxic atmosphere that comprised ~95% argon, 1-2% hydrogen and the balance nitrogen, at atmospheric pressure. Six aquifer water samples were used for the establishment of aquifer microcosms: PA19.1A, OR09.2A, PA04.3A, SS07.4A, SS11.6A and VE15.6A. Each vial used for the experiment contained 10.0mL of filter-sterile aquifer water. This was subsequently inoculated with ~2.4 mL of aquifer water collected under an CO_2 atmosphere (see section 2.1.2) and chemicals of interest were mostly added as solutions (100µl) to achieve concentrations as shown in Table 4. Five chemicals varied from this procedure as follows: polyacrylamide (200µL of 5% polyacrylamide solution); pristane (11µL neat pristane); eicosane (11µL of neat eicosane that had been heated to 70°C to liquify); d-limonene (0.2µL of neat d-limonene); naphthalene (added as a solid at ~2.75mg ± 0.25; n=10). All vessels were established in replicates of ten. Vessels were incubated under static conditions for approximately 90 days, at 20°C in the dark.

Table 4: Initial concentrations of chemicals used in microcosm experiments.

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

2.4.3 Establishment of storage control/zero-time control

Zero-time controls for aquifer microcosms were set up without the addition of any chemicals, and immediately harvested to determine the composition of the microbial communities at the start of the experiments (Figure 3A). Zero-time controls were set up in replicates of ten. These zero-time controls represent the original microbial community present in the aquifer samples prior to the chemical degradation experiments.

Storage controls for aquifer microcosms were set up without the addition of any chemicals. These controls allowed us to investigate and compare the changes in the microbial community composition resulting from the experimental procedure (Figure 3B). Storage controls were set up in replicates of ten and were incubated with aquifer experimental microcosms. In this way, the storage control provides an indication of how aquifer microbial communities change during the 90-day incubation period, without the addition of any chemicals. An infographic explaining the purpose of these various controls and treatments is shown in Figure 3.

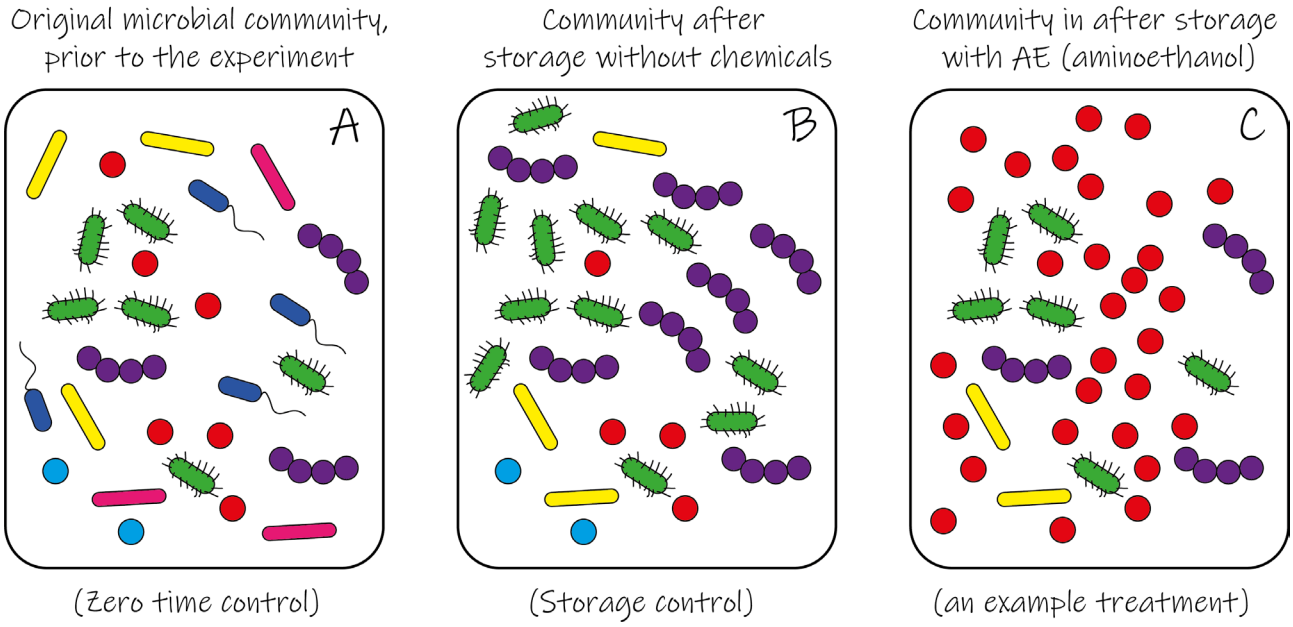

Figure 3: Aquifer microcosm establishment infographic.

A) Zero-time control; B) Storage control; C) Treatment. This infographic depicts a simplified microbial community. In the zero control , there are seven "species" shown in various colours and shapes. During the experiment, the microbial community will change even if chemicals are not added. For example, in the storage control, the several species of microbes have been lost (pink and dark blue rods) and other species (e.g., green and purple shapes) have increased in number, while other species remain unchanged by storage (blue circles). To determine the effect of chemicals on aquifer microbiomes, the storage microbial community is compared with an otherwise identical treatment where a chemical is added. In the example treatment (C), the red circular species have increased in number and the blue circular species have been lost in response to the chemical treatment.

2.4.4 Harvesting aquifer microcosms for DNA samples and microbial community profiling

Initial and final compound measurements

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

DNA recovery, extraction, and processing

After the incubation period, the aquifer microcosms were vacuum filtered to capture all microorganisms onto a sterile 25mm PVDF disc (0.1µm pore size) using a sampling manifold (Millipore). All discs were then frozen at -20°C prior to DNA extraction. For DNA extraction, the discs were sliced into small pieces using a sterile scalpel blade and added directly into 96-well plates for extraction using the ZymoBIOMICS 96 DNA Kit following the manufacturer's protocols (see section 2.2.1; Zymo Research).

Microbial community profiling was performed for bacteria and archaea using the V4 region of the 16S rDNA gene for prokaryotes (see section 2.2.3). Amplification and purification success was verified by DNA quantification (see section 2.2.2). The 16S gene libraries of DNA samples were prepared with Illumina Tru-Seq PCR-free library preparation kits and sequencing was conducted on an Illumina miSEQ DNA sequencer at 250bp paired end. All DNA from microcosm experiments in this project was sequenced by Ramaciotti Centre for Genomics (https://www.ramaciotti.unsw.edu.au/).

Target gene libraries of DNA samples were then prepared with the Illumina Tru-Seq PCR-free library preparation kit and libraries were sequenced over one MiSeq run at 2x 250bp. The Illumina MiSeq sequencing was performed by the Ramaciotti Centre for Genomics, UNSW, Australia.

2.5 Metagenomics

DNA from aquifers that were spiked with diesel, ethylene glycol, hydrotreated light petroleum distillate, isopropanol, methylisothiazolinone, and polyacrylamide were subject to NovaSeq 6000 SP (2x 150bp) metagenomic sequencing at Ramaciotti Centre for Genomics, UNSW.

2.6 Data analysis

2.6.1 Bioinformatics

16S amplicon datasets were processed using the Greenfield Hybrid Amplicon Pipeline (GHAP)[12]. Analyses for 16S followed the typical workflow: in brief, data was demultiplexed, subjected to quality control prior to merging, clustered into Operational Taxonomic Units (OTUs) and mapped back to the resultant OTU table. For ease of understanding an OTU can be considered the same as a microbial species.

Sample contamination was addressed by an in-house method which determines the level at which a given OTU signal is untrustworthy.

2.6.2 Metagenomics

Detailed metagenomic analyses methods, with references and software are in Appendix A. In brief, however, the workflow is described here. The reads (150bp) from the DNA sequencer were assembled into contigs (longer sequences of DNA). These longer sequences (called contigs) were analysed for genes and the genes identified using Prokka (Seemann, 2014). The resultant gene calls were summarised into gene tables (Appendix A).

To bin the genomes to contigs, a method that uses trinucleotide frequency was used (senso Dick et al., 2009). This was implemented using a custom Python script. Bins were then determined visually, using contig coverage and 3-mer signature to assign bins. Bins were manually inspected to determine if they represented single genomes or multiple genomes, and to determine the level of completeness.

To examine gene function, a combinatory approach that used KO (Kegg Ontology mapping) and the gene calls from Prokka was used (Seemann, 2014). For the KO mapping, translated amino acid sequences were submitted to GhostKoala for mapping (Kanehisa et al., 2016).

[12] https://researchdata.edu.au/greenfield-hybrid-analysis-pipeline-ghap/981523

2.6.3 Statistical analyses and plotting

Ordinations of aquifer communities subject to chemical treatments

Two types of ordinations are included in this report, PCAs and nMDS ordinations. The former, Principal Component Analysis is used for chemical data or for comparison for 3mer signatures in the metagenomic analysis. Non-metric multidimensional scaling (nMDS) was exclusively used for visualising differences in OTU abundance (relative abundance) between treatments, sites, or land use. Ordinations were completed in either Python or R. Detailed methods for these ordinations are in Appendix A.

Simpson's Index

Simpson's Index (1-D) was calculated for the microbial baselines of aquifer samples (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.

2.6.4 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.

3 Results and Discussion

3.1 Sampling

In total, 21 samples were collected in replicates (Table 5). These samples included representatives from hydrogeological zones 1A through 6A, with one sample collected from within zone 6B.

Table 5: Details of samples, describing the hydrogeological zone of their origin.

Hydrogeological Zone	Sites
1A	PA19.1A, PA20.1A, PA21.1A
2A	OR09.2A, SS01.2A, SS02.2A, SS03.2A, VE16.2A, VE17.2A, VE18.2A
3A	PA04.3A, SS05.3A, SS06.3A
4A	SS07.4A
5A	PA10.5A, SS08.5A
6A	SS11.6A, SS13.6A, VE14.6A, VE15.6A
6B	SS12.6B

These hydrogeological zones (Figure 4) are all from Hydrogeological Provinces 1 and 2 and are parts of the unconfined TLA. As such, many of these samples are, at least in part, vertically recharged. This recharge may mean that local conditions, as opposed to hydrogeological zones, may in part describe any microbial or geochemical conditions at that site.

The samples are associated with different land use types; this is denoted by the prefixes of the site names and refer to land used for orchards, pasture, small seed farming, and vegetable cropping (Table 6).

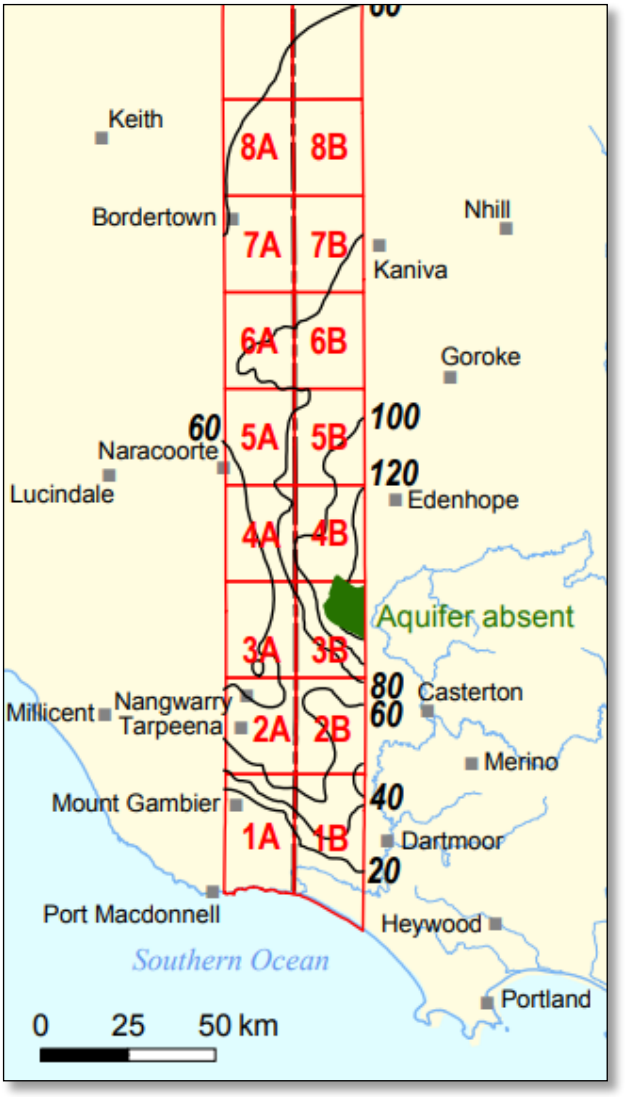

Figure 4: Hydrogeological zones of the Tertiary Limestone Aquifer on the Limestone Coast[13].

Table 6: Details of samples, describing the land use at the site of collection.

Site code	Land use	Number of collections
OR	Orchards	1
PA	Pasture	5
SS	Small seeds	10
VE	Vegetable cropping	5

[13] Department for Environment and Water, Government of South Australia. https://cdn.environment.sa.gov.au/environment/docs/groundwaters-agreement-tertiary-limestone-aquifer-fact.pdf

3.2 Aquifer chemistry

Water bore and groundwater information for each site sampled was accessed through the South Australian Government Water Connect and the Visualising Victoria's Groundwater websites. Table 7 provides publicly available characteristics of each bore sampled (maximum depth, standing water level, total dissolved solids, EC, and pH). Table 7 also lists the formation that the bore intersects.

In the present study, detailed water chemistry was determined for 21 collected samples (Table 8). In broad terms, the samples collected from the Limestone Coast region were similar to each other, exhibiting near neutral to moderately alkaline pH. The measured EC indicated a low salinity and varied from 669 to just over 3000 µSiemens cm^{-1}. Both the pH and EC ranges in the samples collected are within suitable parameters for microbial growth. Microbial activity in aquifers is also dependent on other geochemical factors, particularly the presence of important macronutrients such as nitrogen or phosphorus and a source of accessible organic carbon. Total nitrogen varied from less than the limit of reporting (0.1 mg L^{-1}; SS12.6B) to 14.2 mg L^{-1} (SS05.3A). Total phosphorus was generally low across all samples with the majority of samples below the limit of reporting (0.01 mg L^{-1}) and a maximum of 0.05 mg L^{-1} (SS01.2A). Most samples were below the limit of reporting (1 mg L^{-1}) for dissolved organic carbon, with the highest concentration being 3 mg L^{-1} (SS07.4A and VE16.2A). The low availability of these macronutrients and a carbon source is likely to limit microbial activity in these groundwaters.

Table 7: Water bore information and aquifer details.

Sample	Aquifer	Maximum depth (m)*	Standing Water Level (m)*	Total Dissolved Solids (mg/L)*	EC (µS/cm)*	pH
SS01.2A	Gambier Limestone	60	5	380	690	ND
SS02.2A	Bridgewater Formation	10	ND	1070	1940	7.6
SS03.2A	Gambier Limestone	30	ND	660	1200	ND
PA04.3A	Gambier Limestone	10	5	770	1400	7
SS05.3A	Pleistocene marine sediments and Gambier Limestone	20	1	1050	1900	ND
SS06.3A	Pleistocene marine sediments	10	1	1130	2050	7.4
SS07.4A	Gambier Limestone	40	10	1060	1920	ND
SS08.5A	Gambier Limestone	70	15	930	1680	ND
OR09.2A	Gambier Limestone	40	10	620	1120	ND
PA10.5A	Gambier Limestone	20	15	1480	2670	ND
SS11.6A	Gambier Limestone	10	ND	ND	ND	ND
SS12.6B	Gambier Limestone	ND	ND	ND	ND	ND
SS13.6A	Gambier Limestone	30	20	1660	3000	7
VE14.6A	Pleistocene marine sediments	20	ND	980	1770	7.2
VE15.6A	Gambier Limestone	80	10	880	1600	
VE16.2A	Bridgewater Formation	10	ND	970	1750	6.8
VE17.2A	Bridgewater Formation and Gambier Limestone	20	1	460	840	ND
VE18.2A	Bridgewater Formation	10	1	660	1200	7.8
PA19.1A	Gambier Limestone	220	55	ND	ND	ND
PA20.1A	Gambier Limestone	60	ND	400	730	7
PA21.1A	Gambier Limestone	70	30	420	760	ND

* All values have been rounded to the nearest 10m (Depth), 5m (Standing Water Level), 10 mg L^{-1} (Total dissolved solids), 10 µS cm^{-1} (EC).

ND- Not disclosed.

Table 8: Water chemistry of the aquifers sampled in this study.

Analyte (mg L^{-1}, exceptions marked with *)	LOR	SS01.2A	SS02.2A	SS03.2A	PA04.3A	SS05.3A	SS06.3A	SS07.4A	SS08.5A	OR09.2A	PA10.5A	SS11.6A	SS12.6B	SS13.6A	VE14.6A	VE15.6A	VE16.2A	VE17.2A	VE18.2A	PA19.1A	PA20.1A	PA21.1A
pH*	0.01	7.92	7.95	7.64	7.92	8.06	7.93	7.94	8.06	7.82	7.66	7.82	7.95	7.92	7.93	7.76	7.87	7.96	7.93	8.07	8.09	7.87
Electrical Conductivity **	1	844	1210	1770	1660	2190	1920	2160	1820	1180	3090	2410	1980	3000	2320	1650	1590	1430	1210	669	810	832
Total Alkalinity	1	269	268	408	409	347	329	433	365	347	468	384	376	373	390	418	334	382	355	230	250	282
Sulfate as SO_4^-	1	30	139	179	63	71	40	67	63	42	98	86	67	140	64	40	177	123	21	11	11	10
Chloride	1	116	175	273	289	454	396	423	363	146	683	532	411	680	508	288	239	188	183	66	79	78
Calcium	1	86	139	210	105	92	95	106	76	134	150	112	99	97	136	133	139	130	126	69	110	105
Magnesium	1	14	15	21	31	50	40	52	52	19	69	71	61	86	60	41	24	18	19	19	11	13
Sodium	1	65	98	165	218	303	241	288	223	94	432	299	225	411	259	177	181	163	119	42	46	51
Potassium	1	1	<1	2	5	6	4	7	8	2	7	7	6	10	7	6	2	2	2	<1	<1	1
Aluminium	0.01	0.06	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Antimony	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	<0.001
Arsenic	0.001	0.001	<0.001	<0.001	0.001	0.005	0.002	0.001	<0.001	<0.001	<0.001	0.001	0.002	0.001	0.001	0.002	0.002	<0.001	<0.001	<0.001	<0.001	<0.001
Beryllium	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Barium	0.001	0.01	0.015	0.028	0.019	0.08	0.031	0.022	0.011	0.017	0.025	0.026	0.023	0.029	0.042	0.02	0.017	0.019	0.01	0.008	0.007	0.009
Cadmium	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Chromium	0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	0.003	0.001	0.002	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cobalt	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Copper	0.001	<0.001	0.003	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Lead	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Lithium	0.001	<0.001	<0.001	0.002	0.004	0.016	0.017	0.008	0.021	<0.001	0.007	0.012	0.01	0.017	0.009	0.007	<0.001	<0.001	0.001	<0.001	<0.001	<0.001
Manganese	0.001	0.022	0.026	0.063	0.002	<0.001	<0.001	<0.001	0.001	0.001	0.004	<0.001	0.012	0.002	<0.001	0.015	0.069	0.038	0.01	<0.001	<0.001	<0.001
Molybdenum	0.001	<0.001	<0.001	<0.001	0.006	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002
Nickel	0.001	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.003	0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Selenium	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Silver	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Strontium	0.001	0.582	0.31	1.09	0.787	1.65	1.31	1.45	1.54	0.33	1.24	2.25	2.05	2.51	1.64	1.26	0.513	0.302	0.883	0.411	0.312	0.258
Tin	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.001	0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Uranium	0.001	0.001	<0.001	0.001	0.027	0.002	0.002	0.002	0.002	<0.001	0.002	0.003	0.002	0.003	0.002	<0.001	<0.001	0.005	<0.001	<0.001	<0.001	<0.001
Vanadium	0.01	0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Zinc	0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	0.006	<0.005	0.008	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	0.03	0.008	0.006	<0.005
Boron	0.05	<0.05	<0.05	<0.05	0.07	0.12	0.08	0.31	0.25	<0.05	0.2	0.2	0.16	0.28	0.16	0.1	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Fluoride	0.1	0.2	<0.1	0.2	0.4	1	0.7	0.6	0.9	0.2	0.4	0.7	0.7	0.8	0.5	0.6	0.1	0.2	0.3	0.3	0.2	0.1
Ammonia as N	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.1	<0.01	0.05	<0.01	<0.01	<0.01
Nitrite + Nitrate as N	0.01	2.56	2.07	0.02	1.52	13.4	11.1	3.43	0.28	7.42	1.53	0.12	0.07	0.1	1.32	0.36	0.02	3.09	0.03	5.02	13.5	9
Total Kjeldahl Nitrogen as N	0.1	0.4	0.4	0.1	0.3	0.8	0.9	0.4	<0.1	0.6	0.2	<0.1	<0.1	<0.1	0.2	<0.1	0.4	0.4	0.1	0.4	0.6	0.7
Total Nitrogen as N	0.1	3	2.5	0.1	1.8	14.2	12	3.8	0.3	8	1.7	0.1	<0.1	0.1	1.5	0.4	0.4	3.5	0.1	5.4	14.1	9.7
Total Phosphorus as P	0.01	0.05	<0.01	0.03	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Reactive Phosphorus as P	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	0.02	0.02	0.03	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Total Anions	0.01	9.27	13.2	19.6	17.6	21.2	18.6	22	18.8	11.9	30.6	24.5	20.5	29.5	23.4	17.3	17.1	15.5	12.7	6.69	8.41	8.04
Total Cations	0.01	8.3	12.4	19.4	17.4	22	18.6	22.3	18	12.4	32.1	24.6	19.9	30	23.2	17.9	16.8	15.1	13.1	6.83	8.4	8.55
Dissolved Organic Carbon	1	<1	2	<1	1	<1	<1	3	<1	<1	<1	<1	<1	1	<1	<1	3	<1	<1	<1	<1	<1

*pH units, ** µSiemens / cm

To determine if land use altered microbial community structure, land use was examined as a factor for statistical differences between the samples. Unfortunately, the study was only able to obtain a single sample from orchards in the region, as such, this sample was excluded from the analyses. When compared by land use, most water chemistry components were not significantly different, however, a small number of components (ammonia, molybdenum, and strontium) differ significantly between pasture, small seed farming and vegetable farming (p-values: 0.042, 0.042, 0.045, respectively). Ammonia was considerably higher in groundwater associated with vegetable farming than either small seed or pasture land use. In contrast, strontium was significantly higher in groundwaters associated with pasture than small seeds ($p < 0.028$), but not significantly different to groundwater from vegetable farms. Conversely, molybdenum was significantly higher in groundwater from small seeds than from groundwater at either vegetable farms or pasture. It should be noted that while the number of replicate samples is sufficient to establish a significant difference, further sampling may be required to better establish if land use type impacts groundwater chemistry in these ways. Assuming these differences are genuine, it may be that land use practices such as irrigation, fertilisation or mineral supply for livestock may affect local groundwater chemistry.

Analysis of the same data by hydrogeological zone suggested that water chemistry does differ significantly by zone. For example, electrical conductivity (an indirect measure of dissolved ions in the water) is significantly lower in zone 1 than in other zones and, for the most part, increases as the zone number increases (though zone 6 is similar to zone 5) (Figure 5).

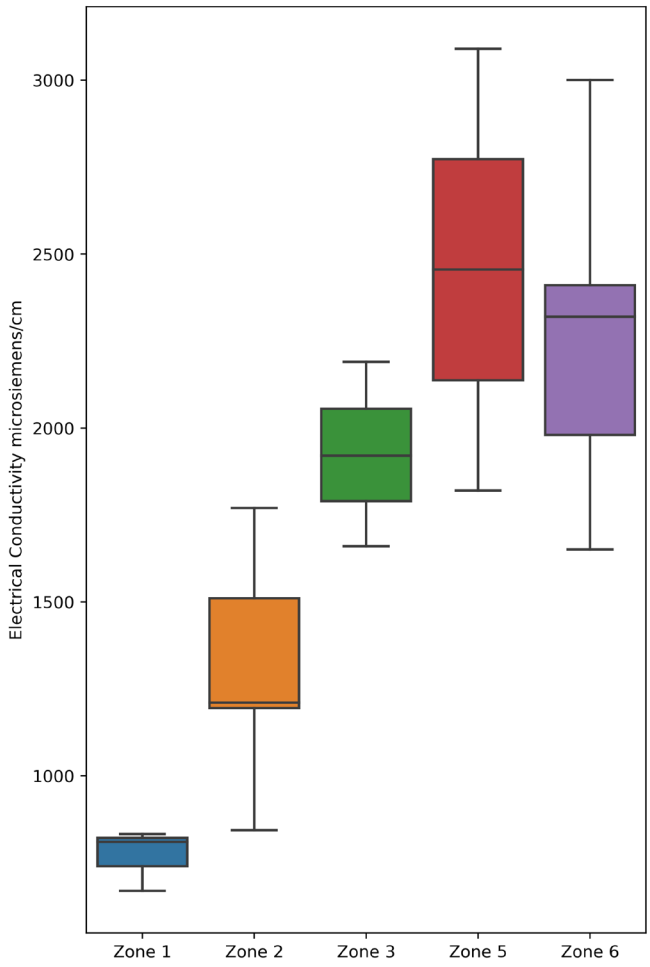

Figure 5: Boxplot showing electrical conductivity for samples from five of the six zones examined in the present study.

Note: Zone 4 was omitted due to a lack of replicate samples from this zone.

This trend observed in EC is underpinned by significant differences between zones in terms of potassium, sodium, magnesium, and chloride concentrations (Figure 6).

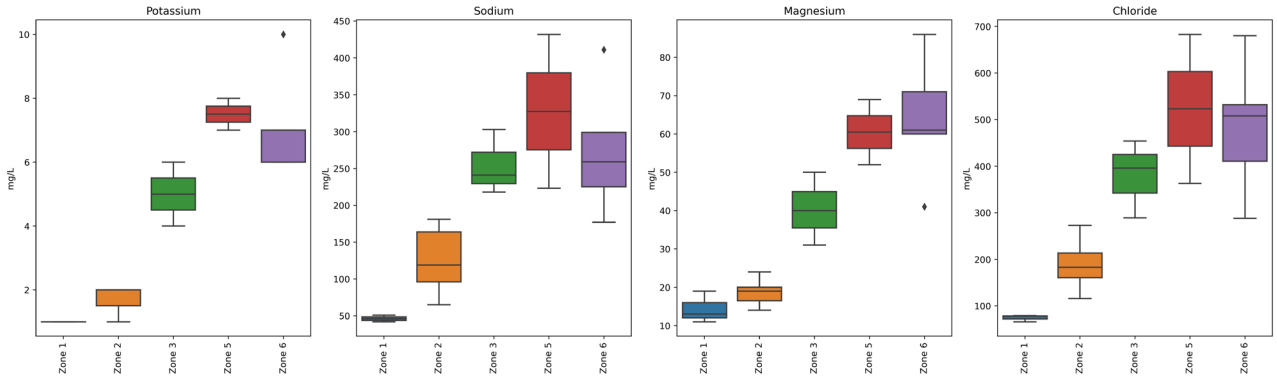

Figure 6: Boxplots showing potassium, sodium, magnesium and chloride for samples from five of the six zones examined in the present study.

Note: Zone 4 was omitted due to a lack of replicate samples from this zone.

In broad terms, these analytes demonstrate a similar profile as electrical conductivity (Figure 5 and Figure 6). There are, however, some chemical components for which this trend was not consistent. For example, barium and manganese were both notably higher at a single site and did not follow the trend for EC of broadly increasing concentration with zone (Figure 7). In zone 3 there is an elevated concentration of barium, and manganese is elevated in zone 2. Outside of these individual zones, the broad trend is of increasing concentration with increasing zone number (Figure 7).

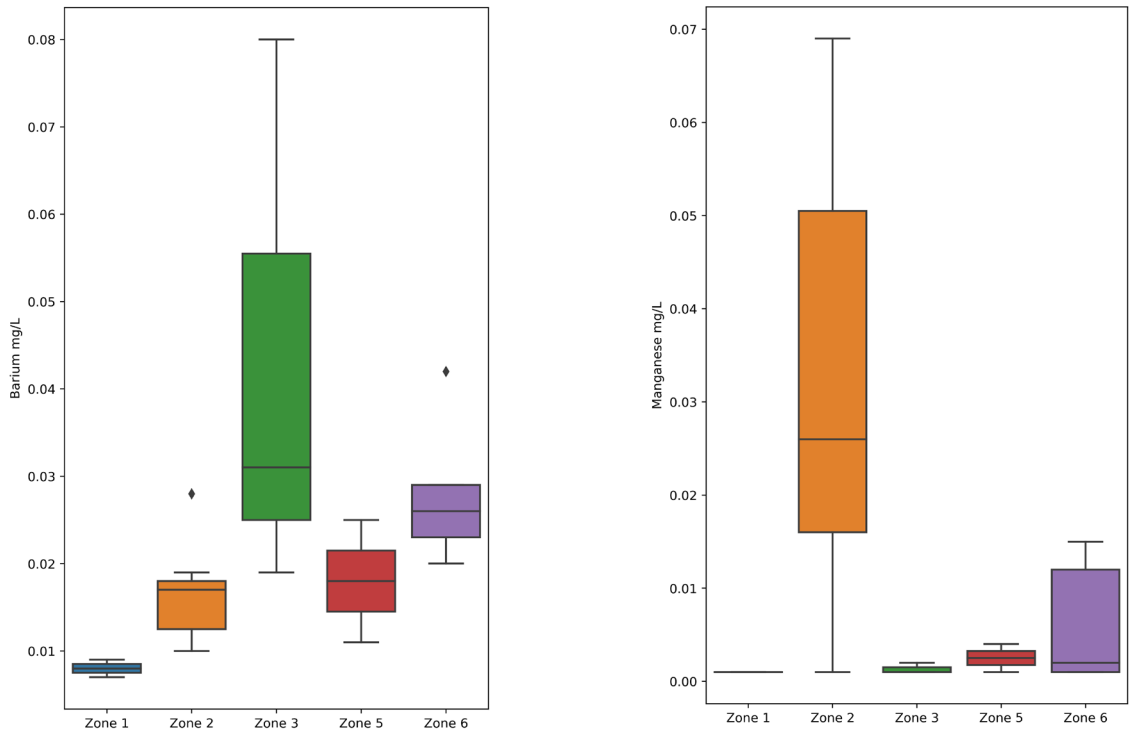

Figure 7: Boxplots for barium (left) and manganese (right) concentration (mg/L) by zone.

The concentration of nitrogen did not mirror the pattern observed for other chemical analytes. Indeed, nitrogen appears to show a relationship that is inversely correlated with zone number, i.e., nitrogen decreases in concentration in groundwater, generally, as zone number increases (Figure 8).

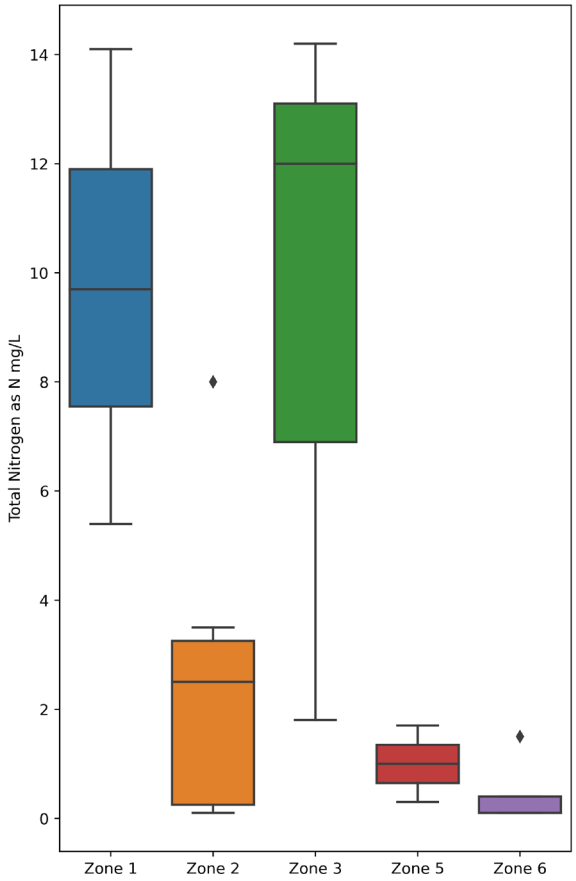

Figure 8: Boxplot showing total nitrogen (mg/L) for samples from Zones 1, 2, 3, 5 and 6.

Principal components analysis (PCA) was conducted to compare the water chemistry data from the 21 aquifer samples as a whole (Figure 9). The PCA separated the aquifer samples from hydrogeological provinces 1 and 2, and broadly separated the zones, however, the samples from zones 4A, 5A and 6A clustered together (Figure 9). The trends seen in the PCA broadly reflect those seen in the individual analytes discussed above.

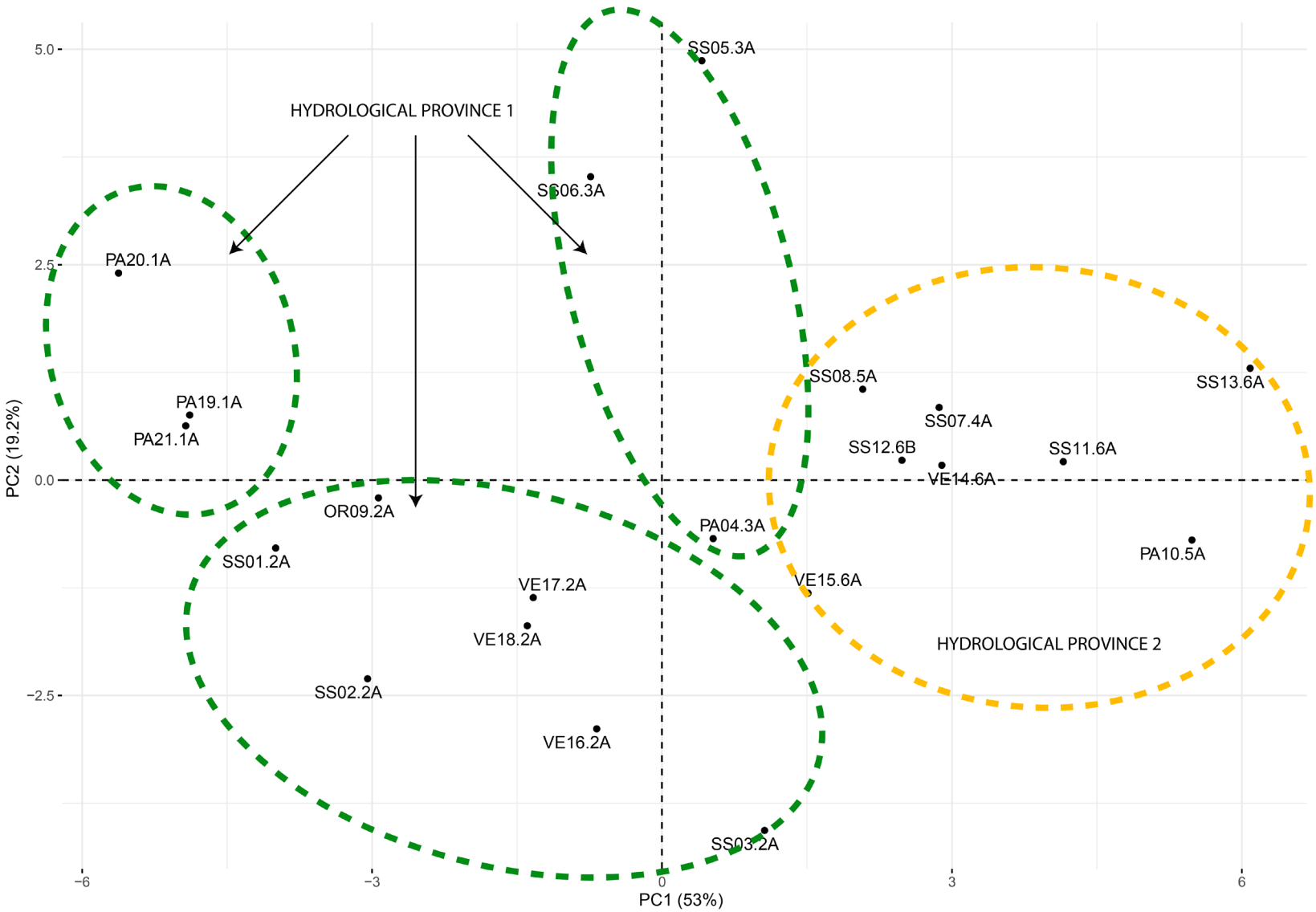

Figure 9: Principal components analysis of baseline water chemistry data by hydrological province.

3.3 Microbial community baselines

3.3.1 Cell numbers and physical niches

Microbial communities found in aquifers and groundwater are typically low in cell densities, ranging from 10^2 and 10^6 cells per ml of water (Alfreider et al., 1997; Griebler and Lueders, 2009; Whitman et al., 1998). In South Australian aquifers, approximately 300 km north-west of this study's sampling sites, microbial community cell density was recorded in the range 5.2×10^3 to 6.1×10^5 cells ml^{-1} (Smith et al., 2018). In this study, estimates of cell numbers in the sampled Limestone Coast aquifers ranged from 5.04×10^5 to 8.66×10^5 cells mL^{-1}, which are in agreement with this observation (Table 9). Importantly, cell density is really a measure of abundance (how many of an organism there are per unit volume) and differs from diversity (which is a measure of how many species occur in a given area).

Importantly, the low cell number observed in this study is likely a reflection only of those microbes that occur in the water, however, it should be noted that biofilms (basically a consortium or community of microorganisms) represent the most common form of microbial occurrence (Costerton et al., 1995; Davey and O'toole, 2000; Makin and Beveridge, 1996).

Table 9: Cell number estimates based on DNA concentration.

Sample	Cell density (cells per mL)
OR09.2A	8.66×10^5
PA04.3A	7.54×10^5
PA19.1A	5.73×10^5
SS07.4A	5.72×10^5
SS11.6A	6.05×10^5
VE15.6A	5.04×10^5

Biofilms are structures that are formed by bacteria on surfaces or interfaces; such films are typically comprised of multiple microbial species that live in close proximity to each other, embedded in biologically produced, but non-living material e.g., sticky polysaccharides or proteins

(Stoodley et al., 2002). There are significant advantages in living in biofilms, as organisms in close proximity to one another can engage in syntrophic (cross-feeding) relationships, cell-to-cell signalling (Davey and O'toole, 2000) and engage in environmental modification between the biofilm and attached interface (Dopffel et al., 2021; Ghosh et al., 2019; Gupta and Anand, 2018). Such environmental modifications can facilitate access to biologically important materials (macro or micronutrients) held in otherwise insoluble materials (Dang and Lovell, 2016; Yin et al., 2019).

In the subsurface, microbial communities are demonstrably separated into physical niches with significant evidence that planktonic communities (in groundwater) differ markedly from attached (biofilm forming) counterparts (McLeish et al., 2021; Vick et al., 2019, 2016). Therefore, the low cell number in Limestone Coast aquifers may be related to a low cell number in the planktonic community and data presented here may underestimate microbial abundance in these settings.

Other potential contributors to the low cell number may include the limited dissolved organic carbon content of these groundwaters (ranging from below the limit of report to 3 mg L^{-1}).

3.3.2 Prokaryotic diversity in aquifers of the Limestone Coast region

Across the 21 aquifer samples that were collected, a total of almost 26,000 OTUs were detected. Of these OTUs, almost 2700 were Archaea while the remainder were Bacteria (Figure 10).

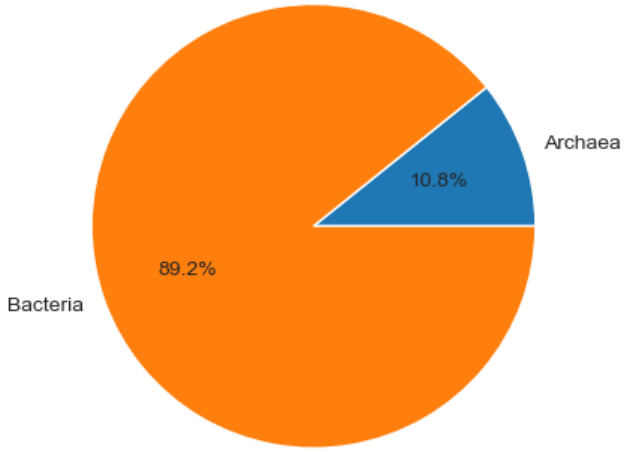

Figure 10: Pie chart showing the proportion of Archaea to Bacteria in the aquifers of the Limestone coast.

Somewhat strikingly, regardless of the aquifer studied, the samples were characterised by very significant numbers of novel species even at high phylogenetic levels. For instance, almost 45% of OTUs were novel at the phylum level. In a previous South Australian survey of 54 groundwater microbial communities from eight sites within a ~60 km radius of Adelaide CBD, Smith et al., (2018) also reported a high proportion of unknown/unassigned/novel OTUs, particularly from more rural sites.

In general, all aquifer samples exhibited a relatively high biodiversity with all sites having Simpson's indices greater than 0.7 (Figure 11). Biodiversity varied by individual site rather than by hydrogeological zones or land use practices. For this reason, local recharge, and variables associated with this recharge, are likely to be the main drivers of microbial diversity.

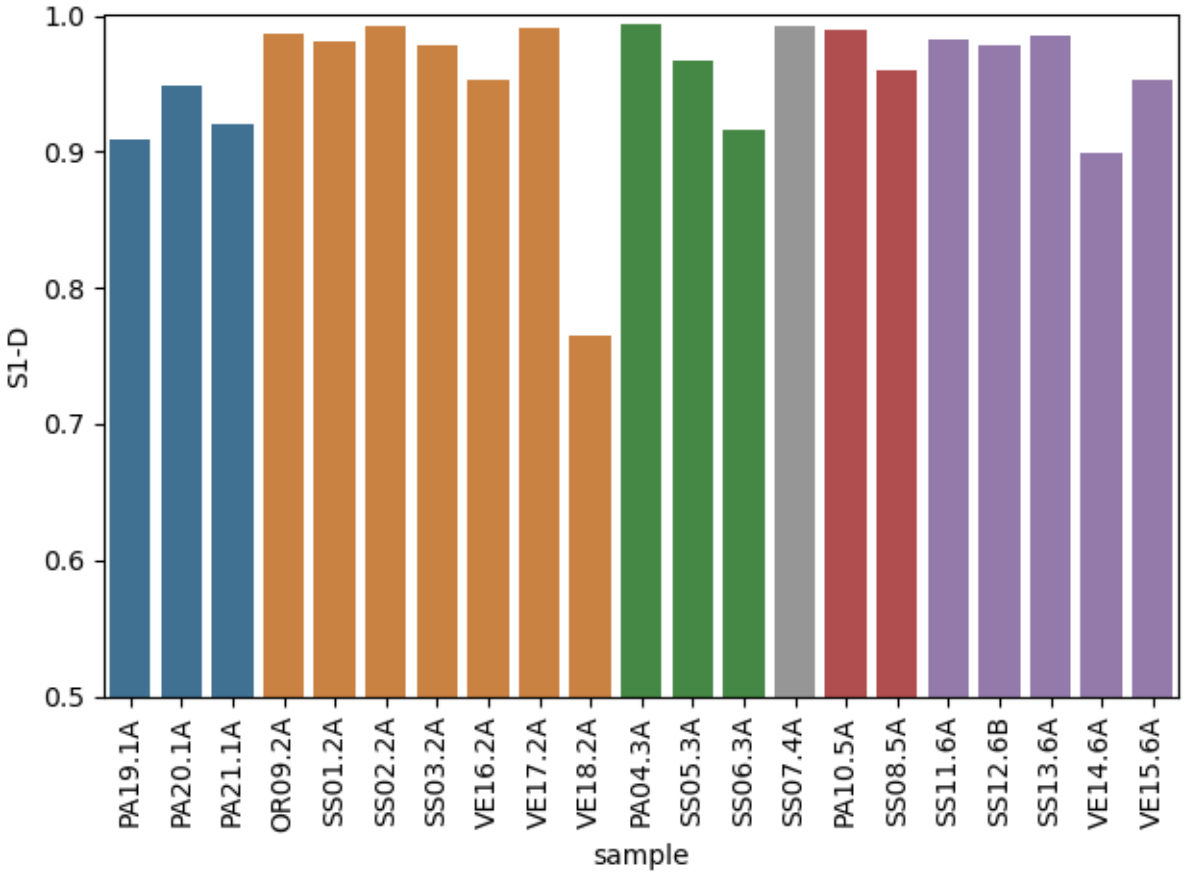

Figure 11: 16S rDNA bacteria and archaea OTU richness.

The NMDS (non-metric multidimensional scaling) ordination shows the interrelatedness of the microbiomes from the aquifer samples collected in this study (Figure 12). Factors that may influence the microbiome found in an aquifer include recharge rate, connectivity with other aquifers, or hydrogeological discontinuities.

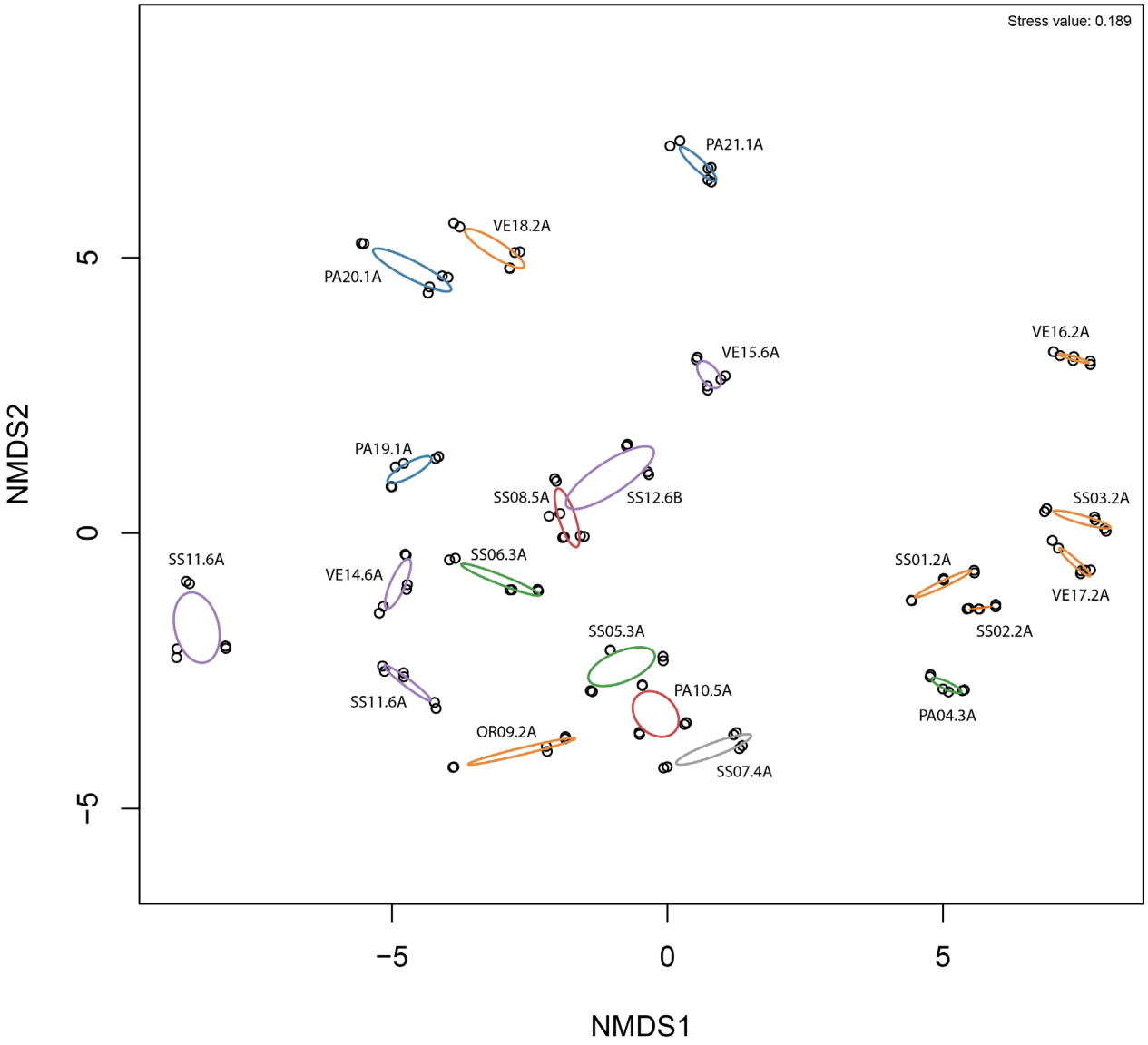

Figure 12: NMDS of microbiome data from the 21 aquifers examined in the present study.

Colouring represents the different hydrological zones of aquifer samples.

The majority of OTUs observed belonged to the Proteobacteria and Firmicutes phyla (Figure 13). These two phyla commonly dominate subsurface environments (Soares et al., 2023) and their detection here is unsurprising given their ubiquity. Interestingly, the third and fifth most populous phyla were unusual archaeal phyla: the Pacearchaeota and Woesearchaeota (Figure 13). Both phyla are poorly understood; what little information available seems to suggest limited genome size, and as a result limited metabolic capability, especially regarding catabolism. It is unclear what the lineages detected here do in this environment, however, the Woesearchaeota at least may be engaged in symbiotic or parasitic lifestyles in association with bacteria (Castelle et al., 2015; Rinke et al., 2013). It is intriguing that both lineages were previously shown to be abundant in low-nutrient, high altitude lakes, and it may indicate a specialisation in low nutrient environments including the aquifers of the Limestone Coast, South Australia (Ortiz-Alvarez and Casamayor, 2016). One complication with this idea, is the relative lack of diversity of these groups in other Australian aquifers which are also low in nutrient loads. It may be that there are specific environmental conditions in the Limestone Coast area which encourage diversity of these groups.

Further evidence that a low-nutrient groundwater environment is driving diversity of taxa with small genomes can be seen in the prevalence of the Parcubacteria and Microgenomates in the aquifers of the region (Figure 13). Like their archaeal counterparts, the Pacearchaeota and Woesearchaeota, the known Parcubacteria and Microgenomates (a bacterial, not archaeal lineage) also have small genomes (~1Mbp) (Tian et al., 2020).

The classes observed in aquifer samples simply reflect the phyla detected, with the most abundant being the Delta-, Alpha- and Gamma- proteobacterial classes (Figure 14). Further, the Firmicutes are mostly represented by members of the Clostridia, as opposed, to the Bacilli class, presumably due to the anoxic nature of the environment. One important note, however, is that some of the more novel taxa discussed previously do not yet have classes, and so can be obscured when examining this taxonomic lineage.

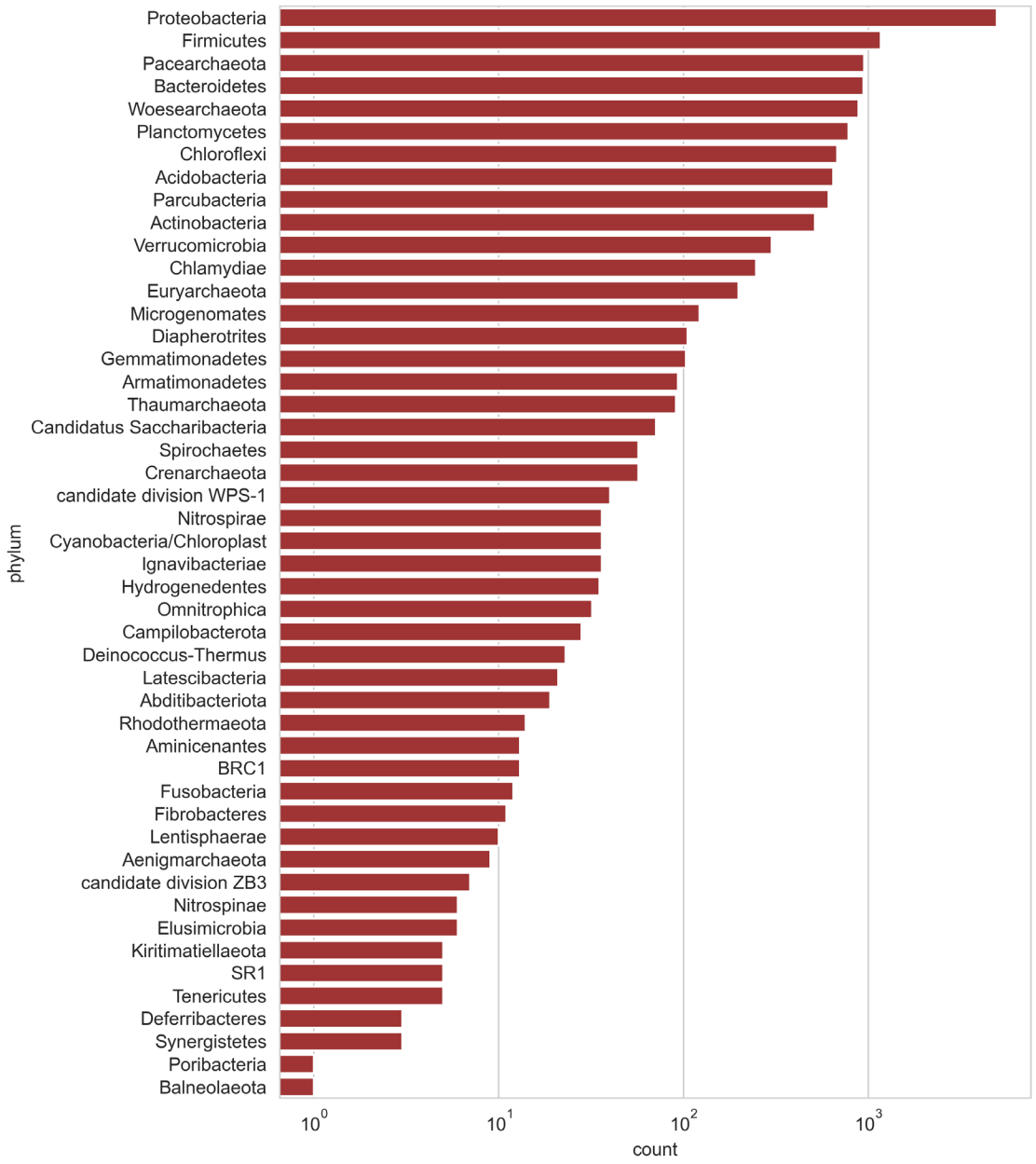

Figure 13: All phyla detected in the aquifers of the Limestone Coast and the number of 'species' (OTUs) that were detected from each phylum.

Note that the x axis is in log scale.

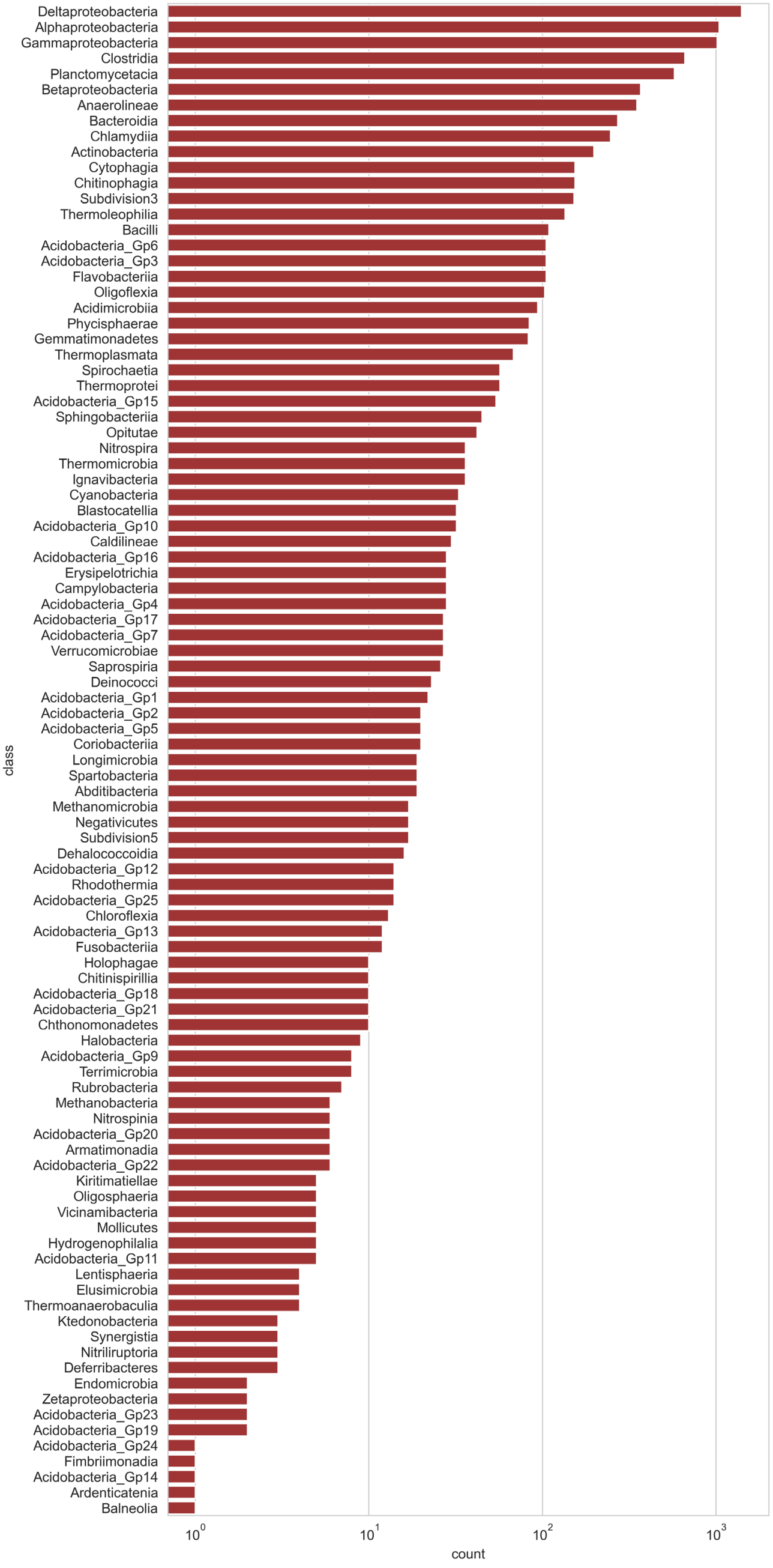

Figure 14: All classes detected in the aquifers of the Limestone Coast and the number of 'species' (OTUs) that were detected from each class.

Note that the x axis is in log scale.

The most abundant organisms in each aquifer sample (Table 10 - Table 16) largely reflect the observations previously made in this section i.e., many novel taxa and a surprising abundance of less common groups. Sometimes the novelty is at a lower taxonomic level. For example, in PA19.1A, the most abundant taxon (OTU_23) is clearly a xanthomonad (in the Gammaproteobacteria) whose closest matches are to *Coralloluteibacterium* species (see Table 10). From the identity though, it is clear at a 93.3% identity that this is not a *Coralloluteibacterium* species. Historically, 16S sequence identities >95% represent the same genus, and sequences >97% identity represent the same species (Schloss and Handelsman, 2005). More recently a higher threshold range of 98.2-99.0% was suggested for species classification based on comparisons of pairs of strains (Meier-Kolthoff et al., 2013). Its closest blast matches (99.6-100% identity) are to a range of environmental sequences (GenBank accessions: KT911490, KP143940 and KX972423) from river biofilms (for KT911490 & KP143940) or a tropical water catchment (KX972423). Similarly, lower taxonomic level novelty can be seen in other more abundant taxa. For example, OTU_59 is the most abundant taxon in PA04.3A (Table 12), and its closest described relatives are *Sulfuritalea* species, but its low identity almost certainly means that while this taxon is a nitrosomonad (in the Betaproteobacteria), it is not a *Sulfuritalea*, and like OTU_23, it is much more closely matched with environmental sequences recovered from a range of environments (data not shown).

The converse (i.e., sequences that very closely matched known species) were also observed, though their presence is not always straightforward to explain. For example, in PA20.1A, the dominant taxon observed is OTU_37 (Table 10). This is a strain of *Tsuneonella*, previously belonging to the genus *Altererythrobacter* (Fan et al., 2011) that is conspecific (the same species) with *T. dongtanensis*, which was isolated from tidal flats in Dongtan Wetland (China). The second most common taxon in this sample was conspecific with *Blastocatella fastidiosa*, an organism previously isolated from semiarid soils (Foesel et al., 2013). While it may seem counter intuitive, the presence of these organisms in aquifers is not completely surprising, as aquifers represent comparatively poorly studied environments, particular in Australia, so it is unclear whether these two species are common elsewhere. Further, while organisms can sometimes be described from specific environments, they are often later found elsewhere in a way that makes their original naming somewhat incongruous.

While local conditions largely appeared to dictate which bacteria came to dominate individual sites, there were some exceptions. Of note were the presence of OTUs 5 and 26 among the most

dominant organisms at each site (Table 10 - Table 16), occurring at four and three of the sites, respectively. OTU_5 is a strain of *Sideroxydans lithotrophicus*, an organism isolated from previous groundwater environments and shown to grow autotrophically (fix CO_2) by using energy from oxidation of iron (Emerson et al., 2013). This study did not specifically measure iron content of the aquifers of the region, but it may be that OTU_5 also grows autotrophically in the aquifers of the Limestone Coast and oxidises iron under low-oxygen conditions. Smith et al., (2018) analysed aquifer microbiomes at a site ~350 km northwest of Penola and revealed the presence of some Gallionellaceae species at that location, though the sequence identities in that work cannot be directly compared with those reported in this study, as Smith et al., (2018) used a different set of primers to the Earth Microbiome primers used in this study. Similarly, OTU_26 was found at several locations, this taxon is probably a *Paraburkholderia* species, related to, but distinct from *P. ribeironis* based on the match percentage.

Hydrogeological Zone 1A

Table 10: Top 5 most abundant Hydrogeological Zone 1A aquifer taxa and their closest matches on Genbank with their percentage identity (over ~290bp).

PA19.1A	Best species match	Match%	Abundance (%)
OTU_23	*Coralloluteibacterium thermophilus* B51-30 (NR 165760)	93.3	28.06
OTU_120	Unknown (*)	0	5.11
OTU_279	Unknown (*)	0	3.79
OTU_131	uncultured Acidobacteriaceae bacterium (AY225644)	88.9	3.30
OTU_228	*Caldimonas brevitalea* DSM 7029 (NR 175563)	98	2.33

PA20.1A	Best species match	Match%	Abundance (%)
OTU_37	*Tsuneonella dongtanensis* JM27 (NR 108695)	100	12.49
OTU_73	*Blastocatella fastidiosa* A2-16 (NR 118350)	99.2	10.53
OTU_245	*Sphingomonas echinoides* DSM 1805 (NR 114697)	100	7.77
OTU_211	*Spirosoma flavum* Y4AR-5 (NR 159210)	92.9	5.97
OTU_157	*Sphingomonas fonticola* TNR-2 (NR 145865)	100	4.96

PA21.1A	Best species match	Match%	Abundance (%)
OTU_43	*Rugosibacter aromaticivorans* Ca6 (NR 156019)	97.6	14.45
OTU_9	*Nitrosarchaeum koreense* MY1 (NR 177299)	99.6	11.61
OTU_40	*Azospira restricta* SUA2 (NR 044023)	98	8.59
OTU_61	*Nitrospira japonica* J1 (NR 114396)	94.1	7.85
OTU_63	*Nitrosomonas aestuarii* Nm36 (NR 114769)	97.6	6.08

Hydrogeological Zone 2A

Table 11:Top 5 most abundant Hydrogeological Zone 2A aquifer taxa and their closest matches on Genbank with their percentage identity (over ~290bp).

OR09.2A	BestSpecies	Match%	Abundance (%)
OTU_51	*Arcobacter cloacae* SW28-13 (NR 117570)	99.6	9.74
OTU_6	*Curvibacter fontanus* AQ9 (NR 112221)	100	1.55
OTU_83	*Staphylococcus aureus* MVF-7; ATCC 35844 (NR 036828)	100	0.91
OTU_24	*Ileibacterium valens* NYU-BL-A3 (NR 156909)	100	0.91
OTU_68	*Allobaculum stercoricanis* DSM 13633 (NR 042110)	92.1	0.85

SS01.2A	BestSpecies	Match%	Abundance (%)
OTU_26	*Paraburkholderia ribeironis* STM 7296 (NR 156098)	96	9.93
OTU_34	*Nitrospira lenta* BS10 (NR 148573)	97.2	5.76
OTU_67	(*)	0	2.86
OTU_25	uncultured Acidobacteria bacterium (EF457492)	88.8	2.40
OTU_81	*Moorella humiferrea* 64-FGQ (NR 108634)	85.4	2.26

SS02.2A	BestSpecies	Match%	Abundance (%)
OTU_46	(*)	0	4.34
OTU_50	(*)	0	3.90
OTU_41	(*)	0	3.35
OTU_6556	(*)	0	2.30
OTU_78	*Candidatus Kuenenia* (CT573071)	95.7	1.81

SS03.2A	BestSpecies	Match%	Abundance (%)
OTU_4	*Desulfatiglans parachlorophenolica* DS (NR 126176)	87.4	12.67
OTU_31	*Thermodesulfovibrio yellowstonii* YP87 (NR 041318)	88.1	3.99
OTU_26	*Paraburkholderia ribeironis* STM 7296 (NR 156098)	96	2.58
OTU_17	*Sideroxyarcus emersonii* MIZ01 (NR 179387)	98.4	2.07
OTU_25	uncultured Acidobacteria bacterium (EF457492)	88.8	1.80

VE16.2A	BestSpecies	Match%	Abundance (%)
OTU_5	*Sideroxydans lithotrophicus* ES-1 (NR 115756)	98.8	19.89
OTU_33	*Sulfuricurvum kujiense* DSM 16994 (NR 074398)	100	7.08
OTU_41	(*)	0	1.85
OTU_11	*Gallionella capsiferriformans* ES-2 (NR 074658)	98.4	1.62
OTU_320	(*)	0	1.40

VE17.2A	BestSpecies	Match%	Abundance (%)
OTU_55	(*)	0	4.18
OTU_76	(*)	0	3.63
OTU_26	*Paraburkholderia ribeironis* STM 7296 (NR 156098)	96	3.40
OTU_110	(*)	0	2.43
OTU_56	(*)	0	2.13

VE18.2A	BestSpecies	Match%	Abundance (%)
OTU_17	*Sideroxyarcus emersonii* MIZ01 (NR 179387)	98.4	44.85
OTU_11	*Gallionella capsiferriformans* ES-2 (NR 074658)	98.4	13.15
OTU_5	*Sideroxydans lithotrophicus* ES-1 (NR 115756)	98.8	10.02
OTU_33	*Sulfuricurvum kujiense* DSM 16994 (NR 074398)	100	4.26
OTU_247	*Desulfoprunum benzoelyticum* KoBa311 (NR 178703)	96.4	2.31

Hydrogeological Zone 3A

Table 12: Top 5 most abundant Hydrogeological Zone 3A aquifer taxa and their closest matches on Genbank with their percentage identity (over ~290bp).

PA04.3A	BestSpecies	Match%	Abundance (%)
OTU_59	*Sulfuritalea hydrogenivorans* sk43H (NR 113147)	93.7	3.66
OTU_89	*Methanomassiliicoccus luminyensis* B10 (NR 118098)	85.4	2.31
OTU_107	*Porphyromonas canoris* JCM 16132 (NR 104686)	99.6	2.25
OTU_96	*Thiobacter subterraneus* C55; JCM 12421 (NR 024834)	92.9	2.09
OTU_88	*Fusobacterium russii* ATCC 25533 (NR 176801)	99.6	2.08

SS05.3A	BestSpecies	Match%	Abundance (%)
OTU_38	*Actimicrobium antarcticum* KOPRI 25157 (NR 118029)	98.8	12.96
OTU_44	*Agitococcus lubricus* DSM 5822 (NR 104868)	97.6	8.84
OTU_4424	*Polaromonas jejuensis* JS12-13; NBRC 106434 (NR 044379)	98.8	7.40
OTU_249	*Lentimicrobium saccharophilum* TBC1 (NR 149795)	87.7	1.81
OTU_125	*Undibacterium parvum* CCUG 49012 (NR 115015)	99.6	1.57

SS06.3A	BestSpecies	Match%	Abundance (%)
OTU_9	*Nitrosarchaeum koreense* MY1 (NR 177299)	99.6	19.48
OTU_15	*Nitrosarchaeum koreense* MY1 (NR 177299)	92.1	18.79
OTU_4	*Desulfatiglans parachlorophenolica* DS (NR 126176)	87.4	3.21
OTU_109	*uncultured Acidobacteria* bacterium (EF457308)	97.6	2.85
OTU_90	*Immundisolibacter cernigliae* TR3.2 (NR 156801)	91.3	2.77

Hydrogeological Zone 4A

Table 13: Top 5 most abundant Hydrogeological Zone 4A aquifer taxa and their closest matches on Genbank with their percentage identity (over ~290bp).

SS07.4A	BestSpecies	Match%	Abundance (%)
OTU_106	*Sulfurisoma sediminicola* BSN1 (NR 125471)	100	6.50
OTU_108	*Dawidia soli* PWU37 (NR 181493)	94.1	2.96
OTU_129	*Chryseolinea soli* KIS68-18 (NR 165708)	90.1	2.20
OTU_39	*Methyloversatilis discipulorum* FAM1 (NR 136517)	100	1.24
OTU_13425	*Chryseolinea soli* KIS68-18 (NR 165708)	92.1	1.03

Hydrogeological Zone 5A

Table 14: Top 5 most abundant Hydrogeological Zone 5A aquifer taxa and their closest matches on Genbank with their percentage identity (over ~290bp).

PA10.5A	BestSpecies	Match%	Abundance (%)
OTU_128	*Cellvibrio mixtus* ACM 2601 (NR 041884)	100	3.01
OTU_95	*Dissulfurirhabdus thermomarina* SH388 (NR 149782)	91.7	2.69
OTU_3	*Pseudomonas aeruginosa* NBRC 12689 (NR 113599)	100	2.66
OTU_144	*Geobacter sulfurreducens* PCA (NR 075009)	90.9	1.86
OTU_162	*Ignavibacterium album* JCM 16511 (NR 074698)	96	1.64

SS08.5A	BestSpecies	Match%	Abundance (%)
OTU_28	*Dechloromonas hortensis* MA-1 (NR 042819)	100	11.92
OTU_2	*Leptonema illini* DSM 21528 3055 (NR 043139)	100	8.81
OTU_52	*Anaeromyxobacter dehalogenans* 2CP-1 (NR 074927)	90.1	8.27
OTU_75	*Capillibacterium thermochitinicola* UUS1-1 (NR 180889)	88.5	5.65
OTU_99	*Zoogloea caeni* EMB43 (NR 043795)	100	4.20

Hydrogeological Zone 6A

Table 15: Top 5 most abundant Hydrogeological Zone 6A aquifer taxa and their closest matches on Genbank with their percentage identity (over ~290bp).

SS11.6A	BestSpecies	Match%	Abundance (%)
OTU_24	*Ileibacterium valens* NYU-BL-A3 (NR 156909)	100	7.38
OTU_24467	*Aquabacterium parvum* B6; DSM 11968 (NR 024874)	96.8	4.19
OTU_80	(*)	0	2.87
OTU_94	*Turicibacter bilis* MMM721 (NR 181198)	98.4	2.05
OTU_102	*Lactococcus lactis* NCDO 604 (NR 040955)	100	1.95

SS13.6A	BestSpecies	Match%	Abundance (%)
OTU_111	(*)	0	5.18
OTU_148	(*)	0	4.82
OTU_54	*Magnetovibrio blakemorei* MV-1 (NR 118660)	94.5	3.42
OTU_423	*Noviherbaspirillum agri* K-1-15 (NR 156921)	96.8	3.22
OTU_7	*Hydrogenophaga taeniospiralis* CCUG 15921 NBRC 102512 (NR 114131)	98.4	3.20

VE14.6A	BestSpecies	Match%	Abundance (%)
OTU_13	*Sphingobium naphthae* K-3-6 (NR 157779)	100	28.83
OTU_77	*Gordonia polyisoprenivorans* Kd2; DSM 44302 (NR 026500)	100	6.64
OTU_70	*Phenylobacterium immobile* E; DSM 1986 (NR 026498)	99.2	5.03
OTU_322	*Larkinella ripae* 15J11-11 (NR 158087)	98.4	1.84
OTU_187	*Caulobacter segnis* MBIC 2835 (NR 040819)	100	1.67

VE15.6A	BestSpecies	Match%	Abundance (%)
OTU_5	*Sideroxydans lithotrophicus* ES-1 (NR 115756)	98.8	15.08
OTU_11	*Gallionella capsiferriformans* ES-2 (NR 074658)	98.4	13.42
OTU_154	(*)	0	2.86
OTU_176	(*)	0	2.10
OTU_199	*Pacearchaeota Incertae* (KP308750)	85.8	2.05

Hydrogeological Zone 6B

Table 16: Top 5 most abundant Hydrogeological Zone 6B aquifer taxa and their closest matches on Genbank with their percentage identity (over ~290bp).

SS12.6B	BestSpecies	Match%	Abundance (%)
OTU_6	*Curvibacter fontanus* AQ9 (NR 112221)	100	7.49
OTU_5	*Sideroxydans lithotrophicus* ES-1 (NR 115756)	98.8	4.90
OTU_47	*Acetobacteroides hydrogenigenes* RL-C (NR 133950)	94.9	4.87
OTU_84	*Thiobaca trueperi* BCH; DSM 13587 (NR 028959)	100	4.61
OTU_54	*Magnetovibrio blakemorei* MV-1 (NR 118660)	94.5	3.74

3.4 Degradation of chemicals by microbes from aquifers of the Limestone Coast

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

Glycols

Microbiomes from Limestone Coast aquifers varied in their ability to biodegrade the glycols tested, ethylene glycol and propylene glycol. No biodegradation of ethylene glycol was observed in the 90-day experiment in any of the aquifer samples tested (Figure 15). The biodegradation of ethylene glycol under anaerobic conditions has been shown via methanogenesis or fermentation (Carnegie and Ramsay, 2009; Dwyer and Tiedje, 1983; Schink and Stieb, 1983; Straß and Schink, 1986). It is likely that microbes present in the aquifer are able to access ethylene glycol as a carbon source. Their ability to do so, however, may be limited due to the low concentration of other essential nutrients such as nitrogen and phosphorus in the aquifer waters. Additionally, low cell numbers in aquifers may also result in slower catabolism of chemicals. Ethylene glycol has been shown to be readily biodegraded under anoxic conditions using groundwater samples in laboratory experiments (Klotzbücher et al., 2007) and in batch studies (McGahey and Bouwer, 1992). Extensive anaerobic biodegradation of ethylene glycol has also been reported in various test systems (Kameya et al., 1995) including anaerobic treatment lagoons (Hovious et al., 1973) and using active anaerobic microbial consortia (Dwyer and Tiedje, 1983).

Despite the chemical similarity between ethylene and propylene glycols, the aquifer experiments demonstrated biodegradation of propylene glycol by some Limestone Coast aquifer microbes. Of the initial propylene glycol dose of ~600 mg L^{-1}, less than 300 mg L^{-1} was detected in the OR09.2A aquifer and ~ 100 mg L^{-1} was detected in the SS07.4A aquifer, indicating that in these two treatments more than half of the propylene glycol was biodegraded (Figure 15). In contrast, the aquifer experiments with PA19.1A, PA04.3A, SS11.6A and VE15.6A showed little or no biodegradation of propylene glycol over the 90-day time course. Anaerobic degradation of propylene glycol follows similar pathways to the decomposition of ethylene glycol. For propylene glycol, degradation produces propionate (propionic acid at lower pH) and n-propanol (Veltman et

al., 1998). Both components are readily used by numerous microbial lineages (Blevins and Perry, 1972; Bustard et al., 2000; Vermorel et al., 2017; Vestal and Perry, 1969).

Alcohols

Generally, all the alcohols were degraded by aquifer microbiomes of the Limestone Coast, though the degree of degradation varied for individual alcohols and aquifer samples (Figure 15). While alcohols are readily used by environmental microbes for cell growth and metabolism (Christensson et al., 1994; Wiedemeier, 1999), at high concentrations, alcohols have been used in numerous applications as stabilisers, solvents, and primarily as disinfectants. The bactericidal (bacterial killing) activity of alcohols is due to several factors, but the main modes of action are through disruption of membrane structure or function (Barker and Park, 2001; Fried and Novick, 1973; Silveira et al., 2004), interference of cell division (Fried and Novick, 1973), and inhibition of nutrient transport via protein transporters bound in the membrane (Bowles and Ellefson, 1985). The microbial inhibitory effects of alcohols have been reported to increase with increasing chain length (Jia et al., 2010).

Degradation of 2-butoxyethanol varied between aquifer samples, with the greatest degradation from the initial dose of 150 mg L^{-1} occurring in VE15.6A to approximately 80 mg L^{-1} (Figure 15). Significant degradation also occurred in aquifers OR09.2A (90 mg L^{-1}) and PA04.3A (100 mg L^{-1}) (Figure 15). Degradation of 2-butoxyethanol has not been extensively investigated, however some researchers have demonstrated an anaerobic pathway in seawater (Rhiner, 2014).

For isopropanol, ~50% of the isopropanol had been degraded across all the aquifer samples tested after the 90-day incubation period (Figure 15). There is scarce reporting of anaerobic degradation of isopropanol, although there are several studies successfully showing the biodegradation of isopropanol in anaerobic batch reactors (Vermorel et al., 2017), under denitrifying conditions (Fida et al., 2017) and in wastewater treatments (Fox and Ketha, 1996).

Similar to the isopropanol result, the methanol aquifer experiments showed that approximately 50% of the methanol was degraded in all samples tested (Figure 15). In anoxic settings, methanol degradation can be complex and syntrophic, whereby some species live off the metabolic products of other species(Goorissen et al., 2004; Keller et al., 2019; Paulo et al., 2004). Anaerobic degradation can occur via several pathways including methanogenesis to CO_2 and CH_4 (Goorissen et al., 2004; Liamleam and Annachhatre, 2007; Paulo et al., 2004), acetogenesis with acetate as an

intermediate (Balk et al., 2002; Keller et al., 2019), oxidation utilising electron acceptors such as sulfate, nitrate and iron (Claus and Kutzner, 1985; Goorissen et al., 2004; Liamleam and Annachhatre, 2007; Lovley and Phillips, 1986; Paulo et al., 2004), and degradation to propionate and butyrate intermediates (Dijkhuizen et al., 1985; Schmidt and Ahring, 1993).

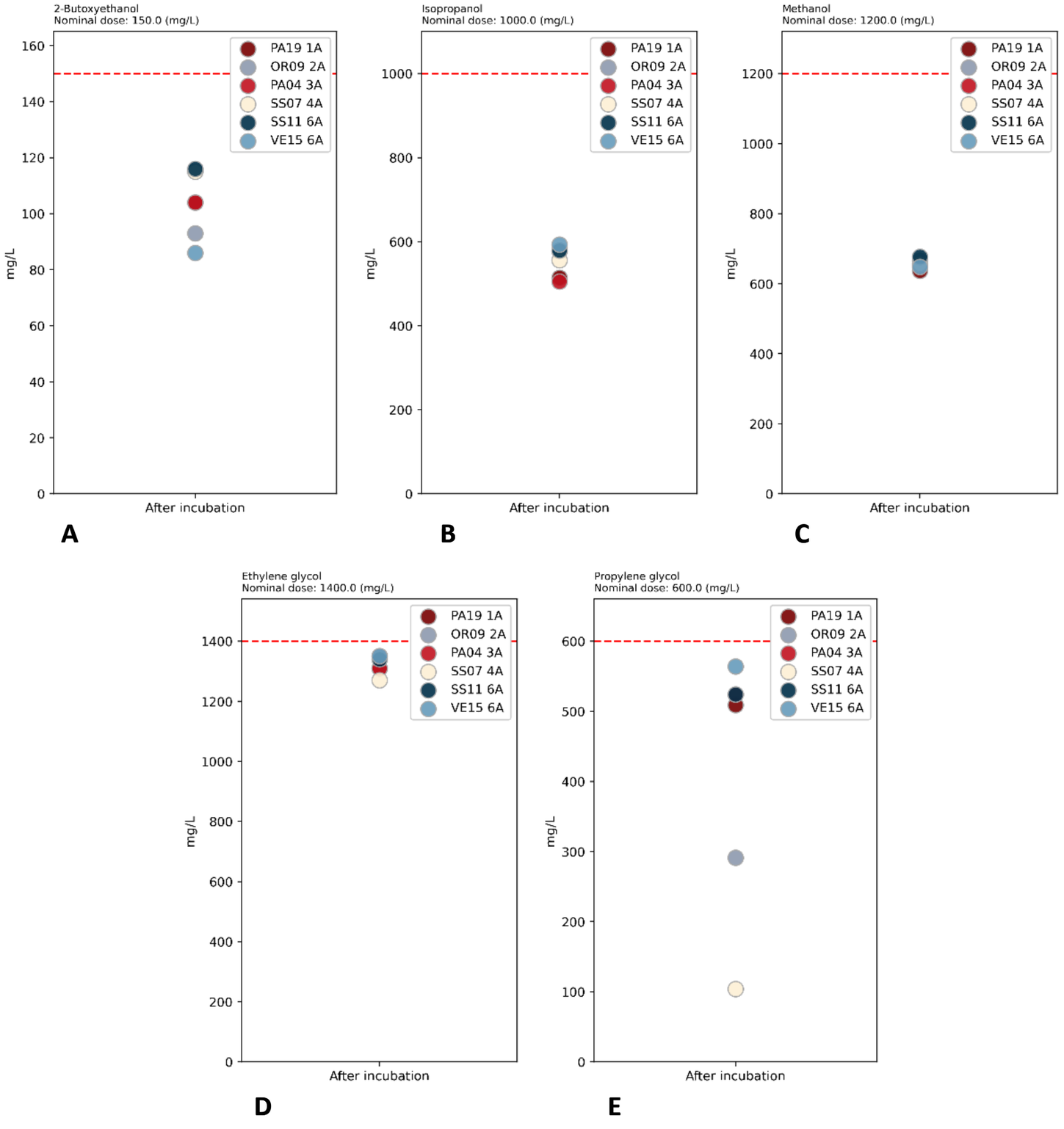

Figure 15: Chemical biodegradation in Limestone Coast aquifers.

A) 2- Butoxyethanol; B) Isopropanol; C) Methanol; D) Ethylene glycol; E) Propylene glycol. Nominal initial dose indicted by red dotted line.

3.5 Response of aquifer microbiomes to chemicals

In this study, the response of Limestone Coast aquifer microbiomes to chemical additions in microcosms were compared to two controls: a storage control and a zero-time control (Figure 3). The storage control was established for each aquifer sample and was a microcosm experiment but without the addition of any chemicals. This control was incubated under the same conditions alongside other samples in the experiment and harvested at the same time. In this way, the storage control provides an indication of how aquifer microbial communities change after the 90-day incubation period without the addition of any chemicals. The zero-time control for each aquifer sample was a microcosm experiment without the addition of any chemicals but was immediately harvested after establishment (Figure 3). Therefore, the zero-time control provides an indication of the microbial community at the start of the experiment.

The addition of chemicals to most microcosms in this study caused a statistically significant shift ($p < 0.05$) in the microbial community profile and all chemicals caused a significant change in a minimum of two of the aquifer samples tested (Table 17). Some chemicals significantly affected the microbiomes of all aquifer samples tested:c12 alcohol ethoxylate, bronopol, benzisothiazolinone, d-limonene, glutaraldehyde, glyoxal, hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine, methylchloroisothiazolinone, naphthalene, polyacrylamide, polyoxypropylene diamine. The chemicals that caused the least changes to the tested aquifer microbiomes were 2-butoxyethanol and ethylene glycol (Table 17). All chemicals added to the PA19.1A aquifer sample had a significant effect on its microbiome, while the other aquifer microbiomes were significantly changed by different numbers of chemicals SS07.4A (22 chemicals), OR09.2A (21 chemicals), VE15.6A (20 chemicals), SS11.6A (19 chemicals), and PA04.3A (16 chemicals) (Table 17).

For the analysis of results, the chemicals added to microbial microcosm experiments were divided into groups based on chemical structure (Table 17). These groups were alcohols (2-aminoethanol, c12 alcohol ethoxylate, 2-butoxyethanol, 2-ethylhexanol, isopropanol, and methanol), amines (polyoxypropylene diamine and triethanolamine), biocides (bronopol, benzisothiazolinone, glutaraldehyde, hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine, and methylchloroisothiazolinone, methylisothiazolinone), glycols (diethylene glycol ethyl ether, ethylene glycol, and propylene glycol), hydrocarbons (d-limonene, diesel fuel, eicosane, hydrotreated light petroleum distillate, naphthalene, and o-cresol, pristane) and miscellaneous chemicals (glyoxal and polyacrylamide).

Generally, the biocides and the miscellaneous group of chemicals had the largest effects on aquifer microbiomes with statistically significant effects observed across all treatments except for methylisothiazolinone in aquifer PA04.3A. Mixed effects were observed for chemical treatments with both the alcohols, amines, and hydrocarbons across the aquifer samples. Indeed, some specific alcohols or hydrocarbons having marked effects on the microbiomes, while others had limited impacts. The glycols, in general, had a limited effect on aquifer microbiome structure with most treatments not significantly different from the storage control.

For the alcohols, ethoxylated alcohols significantly changed the aquifer microbiomes relative to the storage control across all tested aquifers. Lesser impacts were observed for amino ethanol and ethyl hexanol.

Within the amine group of chemicals, the most consistent changes were seen with the addition of polyoxypropylene diamine. By contrast, triethanolamine only significantly changed the microbiomes in four out of the six aquifer samples (PA19.1A, SS07.4A, SS11.6A and VE15.6A) tested.

For the hydrocarbons, regardless of the aquifer sample, d-limonene and naphthalene both had significant impacts on the microbial communities relative to the storage control.

Table 17: PERMANOVA results for microbial community profile changes relative to the storage control.

Chemical	Group	PA19.1A	OR09.2A	PA04.3A	SS07.4A	SS11.6A	VE15.6A
2-aminoethanol	Alcohols	**	**	ND	**	**	**
c12 alcohol ethoxylate	Alcohols	**	**	*	**	**	*
2-butoxyethanol	Alcohols	**	ND	ND	ND	ND	*
2-ethylhexanol	Alcohols	**	*	ND	**	*	**
isopropanol	Alcohols	**	*	ND	ND	ND	**
methanol	Alcohols	**	*	ND	**	*	ND
polyoxypropylene diamine	Amines	**	**	**	**	**	**
triethanolamine	Amines	**	ND	ND	**	*	**
bronopol	Biocides	***	**	*	**	**	**
benzisothiazolinone	Biocides	**	*	**	**	**	**
glutaraldehyde	Biocides	**	**	*	**	**	**
hexahydro-1,3,5-tris(2-hydroxyethyl)-sym-triazine	Biocides	**	**	**	**	**	**
methylchloroisothiazolinone	Biocides	**	**	*	**	**	**
methylisothiazolinone	Biocides	**	**	ND	**	**	**
diethylene glycol ethyl ether	Glycols	**	*	ND	**	ND	ND
ethylene glycol	Glycols	**	ND	*	ND	ND	ND
propylene glycol	Glycols	**	ND	ND	**	ND	**
d-limonene	Hydrocarbons	**	**	**	**	**	**
diesel fuel	Hydrocarbons	**	*	ND	*	*	*
eicosane	Hydrocarbons	**	*	*	**	**	ND
hydrotreated light petroleum distillate	Hydrocarbons	**	*	*	ND	ND	**
naphthalene	Hydrocarbons	**	**	**	**	*	*
o-cresol	Hydrocarbons	**	ND	**	**	**	ND
pristane	Hydrocarbons	**	**	*	**	ND	ND
glyoxal	Misc.	**	*	*	**	**	**
polyacrylamide	Misc.	**	**	**	**	**	**

ND = no significant difference, * 0.05 – 0.01, ** 0.009-0.001, *** < 0.001.

The taxa or species driving the changes in microbial communities in the aquifer sample treatments can be seen from the SIMPER analyses which shows the dissimilarity between treatments relative to the storage control (see Figure 16 for an explanation of SIMPER heatmaps and Figure 17). The SIMPER heat maps indicate that significant changes are generally associated with the growth of a number of taxa that respond positively to the addition of chemicals and a small number of taxa showing sensitivity to chemical additions (Figure 17). For instance, with the OR09.2A sample (Figure 17A), OTU_1 responded negatively (i.e., decreased in relative abundance) to a range of chemical additions and remained relatively unchanged when compared to the storage control (note its negligible change in the zero time control). In fact, OTU_1 decreased significantly in relative abundance in 20 of the 26 chemical additions and is an example of a taxon that appears to be impacted widely. By contrast, other taxa appear to decrease in relative abundance in response to the addition of only a select few chemicals, e.g., OTU_74 (for example bronopol, glutaraldehyde, glyoxal, methylisothiazolinone and polyacrylamide).

Conversely, some taxa were observed to respond very positively to the addition of chemicals, e.g., OTU_8 markedly increased in abundance with the addition of alcohols (isopropanol and methanol) and hydrocarbons (d-limonene, diesel fuel, eicosane and naphthalene). Interestingly, OTU_8 also responded positively to several biocides (benzisothiazolinone, methylchloroisothiazolinone, and methylisothiazolinone). This may suggest that OTU_8 is a putative generalist cataboliser of these chemicals.

Looking at microbial community responses across all the samples examined in this study reveals some broad trends that may be applicable to the TLA more generally. In this study, taxa that were catabolisers of chemicals of similar structure were identified. For example, OTU_6 and OTU_3 appear to show a broad preference for hydrocarbons and other aliphatic compounds (Figure 17). OTU_3 is most probably a strain of *P. knackmussii* and this taxon has been shown to degrade chlorinated hydrocarbons (Stolz et al., 2007). OTU_6 was identified as an *Acidovorax* species and has been shown to degrade hydrocarbons in various microaerophilic conditions (Révész et al., 2020) and to be the dominant members of microbial communities able to degrade petroleum hydrocarbons (Daghio et al., 2015; Popp et al., 2006).

Other taxa appear to degrade different chemicals in different aquifer samples and may represent generalist taxa with broad catabolic activities towards a range of chemicals. For example, OTU_8 appears to grow on a range of different chemicals in various aquifer samples. OTU_8 was identified as a *Caulobacter* species and from the SIMPER analyses it appears to grow well on a

range of chemicals, particularly polyacrylamide (Figure 17). In fact, related *Caulobacter* species have been shown to have amidase activity against a range of substrates including acrylamides (Kanehisa et al., 2016).

In contrast, some taxa were identified as highly specialised catabolists of specific chemicals or classes of chemicals. For example, OTU_16 appears mostly to respond positively to the addition of methylisothiazolinone in several aquifer samples (OR09.2A, PA19.1A, SS11.6A, and VE15.6A). OTU_16 was identified as a *Vogesella* species and probably a strain of *V. indigofera*. This is the first report to our knowledge of a relationship between isothiazolinone biocides and this taxon.

Taxa that are sensitive, i.e., they decrease in relative abundance, in response to chemical additions in the present study were generally rare and behaved inconsistently across the aquifer samples. For example, OTU_1 appeared to be sensitive to a range of chemicals in OR09 but was unaffected in other aquifer samples (PA04.3A, PA19.1A, SS11.6A). OTU_1 was identified as *Leptonema illini,* a spirochetous bacterium, which is only distantly related to other spirochetes (Huntemann et al., 2013).

This plot is a heatmap of a SIMPER results.

It simply shows change in a species (OTUs)
abundance for a given chemical, compared to the storage control.
SIMPER shows which species contribute most to the observed changes.

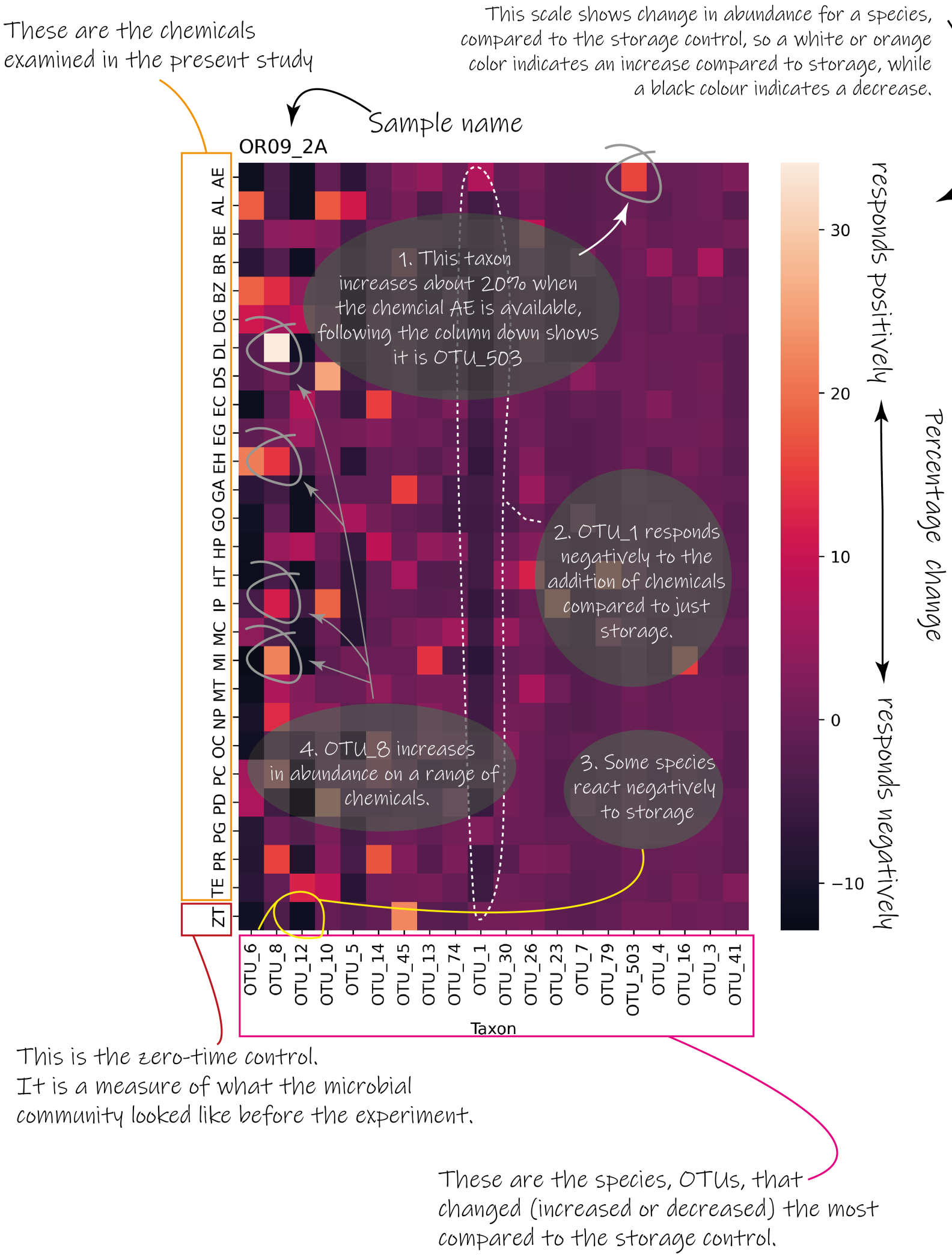

Figure 16: SIMPER analysis of the top 20 taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in the OR09_2A aquifer microbial degradation experiments.

This infographic shows how to interpret SIMPER heatmaps from this project. The scale on the right shows changes in abundance for a species in a chemical treatment, shown on the right. The values in this heatmap are relative to the storage control (see Figure 3 for an explanation of the controls used in this project), therefore a value of 20 (shown as a tan colour) indicates a 20% increase in the abundance of that taxon in a chemical treatment relative to the storage control. For example, see speech bubble 1. Some taxa decrease in abundance in response to numerous chemical additions, i.e., the taxa is sensitive to the chemical; this is seen as dark vertical columns in the heatmap. For example, see speech bubble 2. It is important to note that some taxa responded negatively to the experimental conditions. For example, see speech bubble 3 that shows OTU_12 decreases in abundance relative to the storage control. Some taxa increase in abundance in multiple chemical additions. For example, see speech bubble 4 that shows OTU_8 that increases in abundance on multiple chemical additions (DL, EH, IP and MI). These taxa can be thought of as catabolisers of these chemicals.

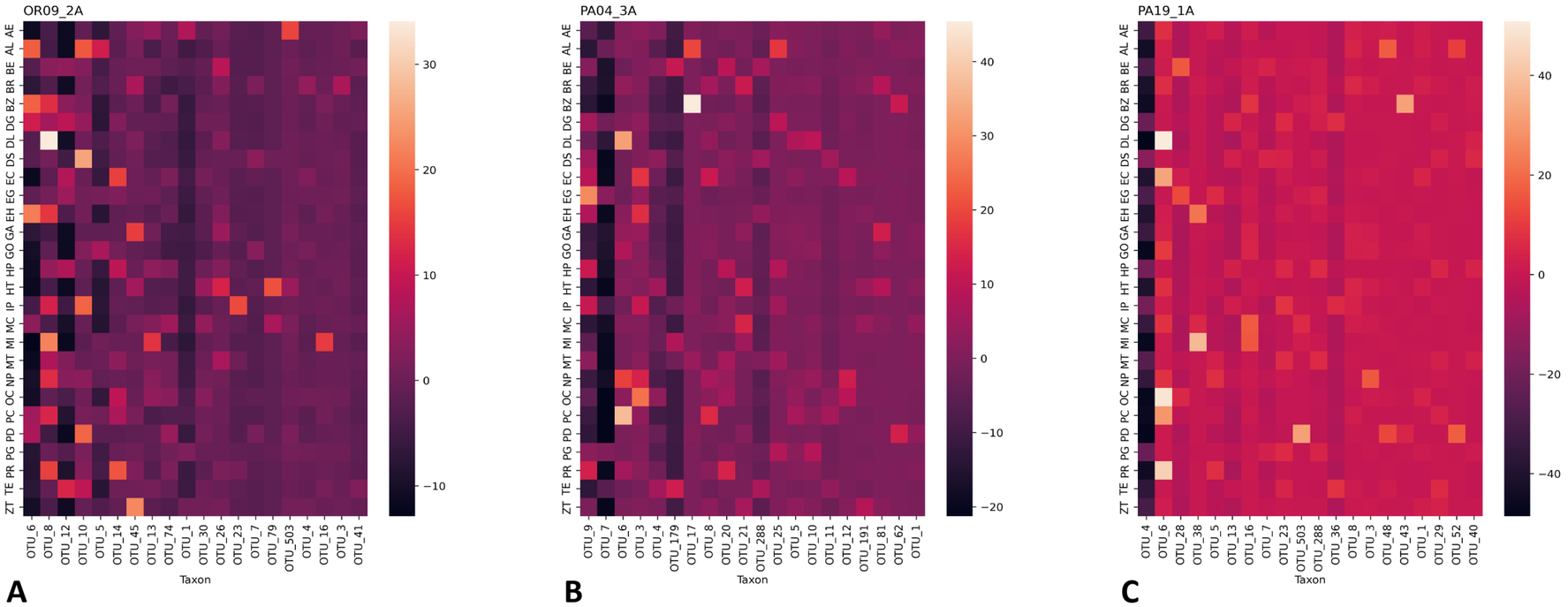

Figure 17: SIMPER analysis of the top 20 taxa that account for most of the dissimilarities between chemical treatments relative to the storage control in aquifer microbial degradation experiments.

A) OR01.2A; B) PA04.3A; C) PA19.1A; D) SS07.4A; E) SS11.6A; F) VE15.6A. AE: 2-aminoethanol; AL: C12 alcohol ethoxylate; BE: 2-butoxyethanol; BR: bronopol; BZ: benzisothiazolinone; DG: diethylene glycol ethyl ether; DL: d-limonene; DS: diesel fuel; EC: eicosane; EG: ethylene glycol; EH: 2-ethylhexanol; GA: glutaraldehyde; GO: glyoxal; HP: hydrotreated light petroleum distillate; HT: hexahydro-1,3,5-tris(hydroxyethyl)-S-triazine; IP: isopropanol; MC: methylchloroisothiazolinone; MI: methylisothiazolinone; MT: methanol; NP: naphthalene; OC: o-cresol; PC: polyacrylamide; PD: polyoxypropylene diamine; PG: propylene glycol; PR: pristane; TE: triethanolamine; ZT: zero time control

Scale bar indicates percentage change relative to the storage control.

3.5.1 Metagenomic analysis of microbial communities from chemical degradation trials

In total, 10 samples were subject to metagenomic analysis. Samples selected were those that had DNA concentrations that were significantly increased compared to the storage controls. The logic behind this selection was that in these samples, cells had increased significantly in number, and these represented the best opportunities to explore the genetic underpinning of catabolism (break down) of chemicals examined in the present study. The samples that underwent metagenomic analyses included four locations and six chemicals (Table 18). The total size of the metagenomic assemblies ranged from 72 Mbp (PA04.EG and PA04.PC) to 474 Mbp (PA19.IP) (Table 19).

The individual assembly statistics depend on a range of factors but are shown in Table 19. These include the amount of DNA sequence data obtained (which affects the depth of coverage for a given sequence) and the phylogenetic diversity of organisms within the metagenome. In general, organisms with higher abundance have higher sequence coverage and assemble better as a rule. There are, however, some exceptions to this rule. For instance, if the numerically dominant organism in a sample is really a population of closely related strains of a single species, then this tends to make assembly problematic. In contrast, if an organism is relatively rare in the consortium but is both phylogenetically distinct from all other taxa in the sequencing pool, then these taxa can sometimes assemble well.

All samples were binned to genomes (data not shown); an example of the binning analysis is shown in Table 20 for the hydrotreated light petroleum distillate addition experiments for aquifer sample SS07.4A. Typically binning recovers a mixture of complete, incomplete, and chimeric (mixed) genomes. For example, from the hydrotreated light petroleum distillate metagenome data for SS07.4A, 11 bins were recovered; these included at least five near complete genomes (bins 6, 7, 8, 9, and 11), three partially complete genomes (bins 1, 2 and 10), and the remainder being represented by mixed bins of unknown completeness and contamination (Table 20).

Table 18: Samples examined by metagenomics.

Site	Chemical
PA04	Ethylene glycol
	Isopropanol
	Polyacrylamide
PA19	Diesel
	Isopropanol
SS07	Diesel
	Hydrotreated light petroleum (kerosene)
	Polyacrylamide
VE15	Diesel
	Methylisothiazolinone

Table 19: Statistics of the metagenomic assemblies.

	PA04 EG	PA04 IP	PA04 PC	PA19 DS	PA19 IP	SS07 DS	SS07 HP	SS07 PC	VE15 DS	VE15 MI
Total size Mbp	72	96	72	86	474	352	175	151	180	143
# of contigs	50,654	58,662	86,527	102,441	625,298	446,203	243,358	203,357	180,323	144,552
Mean contig length	1429	1640	842	842	759	791	719	746	999	989
Median contig length	311	304	312	313	300	305	285	303	306	327
N50	23118	21881	2443	2130	1753	1811	1708	1485	4335	3557
Maximum contig length	1,451,439	2,677,838	705,967	801,129	1,109,993	1,658,425	1,243,167	506,081	950,720	898,559
Mean GC	62.7	60.5	61.9	63.2	62.1	61.8	62.0	62.5	63.3	62.3
Number of genes	63277	87046	56716	73755	396448	325323	148191	116887	148384	4727401

Table 20: SS07.4A_HP metagenome bins, closet species matches.

Sample	Bin	Size (bp)	OUT	Closet species match	Notes
SS07_4A_HP_5	Bin_1	2395995	mOTU_16	*Nocardioides sp*	Likely incomplete genome.
SS07_4A_HP_5	Bin_2	4915392	mOTU_47	*Bradyrhizobium* taxon	Likely incomplete genome. (All *Bradyrhizobium* WGS are > 7Mbp)
SS07_4A_HP_5	Bin_3	2585630	mOTU_23	*Caulobacter* taxon	Mixed bin, but the majority seem to be *Caulobacter* sequences. Likely incomplete genome.
SS07_4A_HP_5	Bin_4	3266286	mOTU_32	*Rhizobiales* taxon	Novel taxon with closest matches to order Rhizobiales. Possibly a combined bin with Bin_5.
SS07_4A_HP_5	Bin_5	3760436	mOTU_32	*Rhizobiales* taxon	Novel taxon with closest matches to order Rhizobiales. Possibly a combined bin with Bin_4.
SS07_4A_HP_5	Bin_6	3521883	mOTU_29	*Actinobacteria*	Novel taxon with closest matches to order *Candidatus Nanopelagicales* or *Candidatus Nanopelagicaceae* other relatives are*: Candidatus Planktophila versatilis*
SS07_4A_HP_5	Bin_7	5288022	mOTU_14	*Anaerolineales*	A novel taxon within the Anaerolineales order. Possibly a complete genome.
SS07_4A_HP_5	Bin_8	4251299	mOTU_7	Ignavibacteriaceae taxon	Probable near complete genome.
SS07_4A_HP_5	Bin_9	3174953	mOTU_22	*Sediminibacterium* species	Probable near complete genome.
SS07_4A_HP_5	Bin_10	3291930	mOTU_38	*Hydrogenophaga* species	Partially complete genome.
SS07_4A_HP_5	Bin_11	3213496	mOTU_26	Novel bacterium	Novel taxon *Patescibacteria*

3.5.2 Metagenomic analysis of microbial communities based on chemical treatment

Ethylene glycol

For the degradation of ethylene glycol, several pathways are known. The pathways in aerobes are best studied and use a range of genes (FucO, AldA, various Ped genes E, F, H, and I) to convert ethylene glycol to glyoxylate (Mückschel et al., 2012; Panda et al., 2021). Glyoxylate is in turn converted to malate and enters the tricarboxylic acid (TCA) cycle where it can be used for energy metabolism or put to other cellular uses. Under anaerobic conditions, however, alternative strategies are required. The most common of which is dehydration of ethylene glycol to acetaldehyde by propanediol dehydratase (EC. 4.2.1.28) enzymes (Pdu C, D and E), and its subsequent conversion to acetate (Forage and Foster, 1982; LaMattina et al., 2016). In the present study, limited evidence exists to support that ethylene glycol is degraded using aerobic pathways, and in general, these genes were rare or absent from the PA04 metagenome. Instead, propanediol dehydratase appears to be relatively common in the PA04 metagenome, and maps to gene LLBNCJHH_08290, which is from the *Propionicimonas* binned genome (Appendix A). This *Propionicimonas* is one of the less common organisms present in the metagenome. It may be that this organism is solely responsible for degradation of ethylene glycol in the sample, and the other taxa are feeding on the subsequent breakdown products. Alternatively, given the novelty of the species that occur in PA04, it may be that the organisms in the consortium can use alternative, novel pathways for ethylene glycol catabolism under anaerobic conditions.

Isopropanol

For a relatively common alcohol, there is surprisingly little data on microbes that can use isopropanol as a sole source of carbon. In one pathway, isopropanol is converted to acetone via an alcohol dehydrogenase, which then is degraded to hydroxyacetone (acetol), methylglyoxal and finally pyruvate using some form of acetone monooxygenase, an acetol dehydrogenase and methylglyoxal dehydrogenase, respectively. Other possible pathways exist, for example, acetone can be converted to methyl acetate using an alternate acetone monooxygenase, which is then further converted to methanol and then formate (which can be used for many cellular processes). There are other possibilities as well for isopropanol catabolism, for example, a group of methanotrophs (organisms capable of using compounds with single carbon atoms, including

methane) from the Verrucomicrobia were shown to use particulate methane-monooxygenase to mediate the conversion of acetone to acetol (Awala et al., 2021).

In the PA04 metagenome, an appropriate alcohol dehydrogenase was detected (COMKNFCE_28562; adh_6, an NAD-dependent alcohol dehydrogenase) that was likely part of a relatively rare taxon, mOTU_33, whose closest relative is *Hyphomicrobium facile* subsp. *tolerans.* This same taxon likely also has a methyl acetate-forming acetone monooxygenase (COMKNFCE_10875), and methyl acetate hydrolase (COMKNFCE_09292) which make methyl acetate from acetone, and methanol from methyl acetate, respectively.

While no verrucomicrobial methanotrophs were detected in this sample, there were several thaumarchaeal species that do possess particulate methane/ammonia-monooxygenases (see for example: COMKNFCE_39722). It may be that these organisms can also use compounds like acetone, however, further work would be required to confirm this speculation.

Polyacrylamide

The degradation of polyacrylamide is not completely understood, but under oxic conditions, the compound is rapidly deaminated by a range of amidase enzymes leaving polyacrylates (Xiong et al., 2018). This process removes the amide side groups leaving polyacrylate, a polymer that is somewhat resistant to microbial degradation. There are conflicting reports of acrylamide release when polyacrylamide is degraded anaerobically (Nyyssölä and Ahlgren, 2019). At the level of fundamental chemistry, this seems highly unlikely due to the release of a higher energy compound after microbial degradation on polyacrylamide (a lower energy compound). Instead, it is more likely that the measured acrylamide, previously reported, represents mobilisation of acrylamide left occluded within the polyacrylamide matrix, rather than cleavage from the polymer. It is also likely that deamination occurs prior to, or at least in tandem with attacks on the carbon backbone, rendering the production of acrylamide monomers even less likely.

In the metagenomic analysis for PA04, 20 amidases were identified, while in the SS07 sample, 83 amidases were detected. These are high counts, but not necessarily higher than in other, non-polyacrylamide containing treatments. This result may be due to the widespread possession of amidase enzymes in prokaryotic organisms to obtain nitrogen for use in proteins or DNA in the environment. As there are so many amidases, the current study has looked at the more abundant organisms (by examining coverage) and hereafter discussion relates to these taxa in the metagenome.

For the PA04 metagenome with polyacrylamide, a taxon related to *Propionicimonas paludicola* (the most abundant taxon in the metagenome), was shown to possess amidase (NKMMEHBP_17849). While there are no reports of *Propionicimonas* degrading acrylamide in the literature, its abundance in this sample and the presence of amidases suggests at the very least the taxon is likely capable of deaminating polyacrylamide. Further work would be required to determine if this taxon can also use the carbon backbone. For the SS07 metagenome with polyacrylamide, for example, the dominant organism appears to be a *Variovorax* species, and two genes from this organism were shown to encode amidases (IIFLDFGI_04554 and IIFLDFGI_04555). *Variovorax* are known to degrade acrylamides, and some species, for example, *Variovorax boronicumulans* CGMCC 4969 can use acrylamide as a sole source of nitrogen and carbon (Liu et al., 2013). This indicates not just deamination of the acrylamide to acrylic acid by this taxon, but additionally use of acrylic acid itself as a carbon source.

Methylisothiazolinone

Methylisothiazolinone is a biocide used to supress microbial growth, particular in surface facilities but also to prevent reagents from fouling (Silva et al., 2020). While there is much work published on the action of this chemical as a skin sensitisation agent (Lundov et al., 2011), little work exists on its fate in the natural environment. This biocide works via oxidation of thiol-containing proteins within microbial cells (e.g., amino acid cysteine) (Denyer and Stewart, 1998; Park and Seong, 2020; Williams, 2007). The chemical itself is destroyed during this reaction, leaving a range of potential degradation products such as mercaptoacrylamide. Like methylisothiazolinone, there is little information on the degradation of these compounds. The dominant organism in the VE15 aquifer sample was a *Pseudomonas* species that was conspecific with *P. linyingensis*. This species is part of the '*P. aeruginosa* group', members of which have been previously demonstrated to be tolerant to a range of biocides. Future work to understand whether these taxa can use isothiazolinone biocides as a sole source of carbon would be valuable.

Diesel

Diesel is a complex mixture of aliphatic (oily) and aromatic (compounds with a carbon ring with alternating double bonds) compounds. In general, diesel is approximately 75:25 aliphatic: aromatic, and the carbon compounds contained therein are normally longer/larger than those present in petrol, however, the biodegradation of petrol and diesel are comparable (Das and Chandran, 2011; Leahy and Colwell, 1990).

While the aliphatic component of fuels such as diesel or petrol are complex, alkane degradation represents a good model for understanding their degradation by bacteria as they are likely shuffled by microbes towards common intermediates (Rojo, 2009). In alkane degradation under oxic conditions, molecular oxygen is used for oxidation of the alkanes via enzymes called monooxygenases (Moreno and Rojo, 2017; Wang and Shao, 2013). This reaction generally results in the formation of some kind of alcohol (primary in terminal oxidation, secondary in subterminal), which are in turn further oxidised to an aldehyde before being converted to a fatty acid. Fatty acids can then be further manipulated though β oxidation and used for a range of cellular processes.

By contrast, under anoxic conditions, molecular oxygen is not available. While a variety of pathways may occur, the best studied is arguably fumarate addition, likely through the generation of a radical by the enzyme alkylsuccinate synthase (Callaghan, 2013; Ji et al., 2019). Subsequently, the 'fumarated' alkane gets linked to coenzyme A and is then subject to β oxidation.

As with the aliphatic compounds, there are thousands of potential aromatic compounds in diesel. To deal with this great diversity of aromatic compounds, bacteria and archaea use a system where they simplify these compounds to a central metabolite, and then use a common pathway for its degradation.

The catechol and benzoyl-CoA pathways represent two distinct strategies employed by microorganisms to break down aromatic compounds, each specialised to specific environmental conditions (Figure 18). The catechol pathway predominantly operates in oxic settings characterised by ample oxygen availability (Gibson, 1968; Seo et al., 2009). Here, aerobic bacteria funnel aromatic compounds towards catechol, the central metabolite in aerobic aromatic degradation.

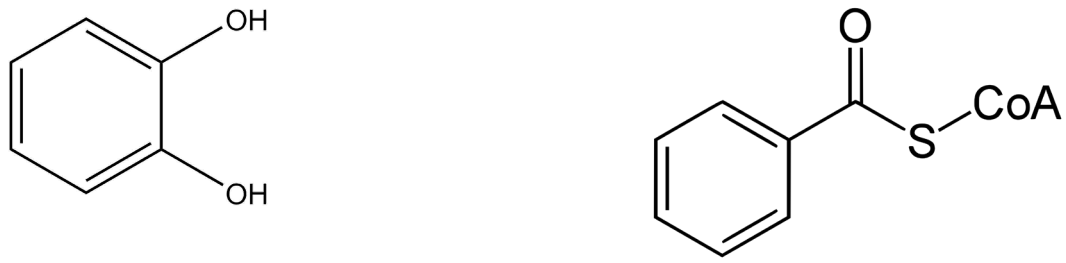

Figure 18: Catechol (left) vs benzoyl-CoA (right) are the central intermediates for oxic and anoxic biodegradation of aromatic compounds. Note that CoA abbreviates a reasonably complex molecular structure.

Subsequently, the aromatic ring structure undergoes cleavage, generating smaller compounds that can enter central metabolic pathways for energy production or to use the carbon in the compound for alternative anabolic uses within the cell.

Conversely, in anoxic environments, benzoyl-CoA replaces catechol as the central metabolite (Harwood et al., 1999). Benzoyl-coA is a benzoate molecule that has been joined to coenzyme A via a ligase enzyme. Despite the 'enzyme' component in its name, coenzyme-A is a non-protein cofactor, essentially a biological helper molecule (Porter and Young, 2014).

In the PAA19, SS07 and VE15 metagenomes, complete pathways were observed for aromatic degradation both under oxic conditions (via catechol) and under anoxic conditions (via benzoyl-CoA). In terms of aliphatic degradative abilities, all three metagenomes also encode alkane 1-monooxygenases, and downstream genes for conversion of resultant alcohols to aldehydes and fatty acids. While genes for anoxic aliphatic degradation where not found, this may be due to the relative novelty of the species detected in the aquifer microbiomes.

Hydro-treated light petroleum distillate

The degradation of this compound largely mirrors diesel, though the product contains fewer aromatic compounds and more cyclic aliphatic compounds (Gad and Pham, 2014). The degradation of cyclic aliphatics (using cyclohexane as a model) proceeds in a similar fashion to alkanes, with oxic conversion to an alcohol, aldehyde and then to fatty acids. Similarly, in anoxic conditions, fumarate-addition is likely involved in its degradation. The complete oxic pathway was detected in the SS07 HP metagenome.

For a summary of the conclusions, caveats and options for future work please see the executive summary.

Glossary

Term	Meaning
16S rRNA	16S ribosomal RNA genes used in taxonomy and molecular phylogeny of bacteria.
aquifer	An underground geological formation containing water.
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.
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
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.
PCA	Principal components analysis
PCR	Polymerase Chain Reaction is a laboratory technique used to amplify or copy a segment of DNA.
prokaryotic	Refers to organisms without membrane bound organelles. Bacteria and archaea.
RO water	Reverse osmosis water.
TLA	Tertiary Limestone Aquifer.

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C**SIRO ENERGY**
Nai TRAN-DINH
+61 2 9**413** 7**873**
n**ai.tran-dinh**@csiro.au
csiro.au/energy