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# Chemical and microbial baseline studies and biodegradation experiments of chemical compounds used in coal seam gas activities in the Narrabri region, NSW

Richard Schinteie, Carla Mariani, Paul Greenfield, Se Gong, Stephen Sestak, Nai Tran-Dinh and David J. Midgley

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# Executive summary

This project aimed to: (1) establish microbial and chemical baselines in aquifer waters, surface waters and soil samples in sites in and around the Narrabri Gas Project area, and (2) assess the capacity of indigenous microbial communities to degrade a range of 'higher hazard' chemicals likely to be used in coal seam gas activities in groundwaters, surface waters and the major soil types (vertosols and sodosols) of the Narrabri Gas Project region.

**Chemical and microbial baselining**

This project generated comprehensive baseline data on groundwater, surface water and soil microbiomes for the Narrabri Gas Project region, though some samples were taken outside its boundaries. Over 50 sites were studied, including 14 groundwater, 16 surface water and 32 soil locations, with most samples collected in both November 2022 and June 2023. From these sites a total of 54 groundwater, 52 surface water and 99 soil samples were collected, across the two sampling campaigns. Flooding and property access issues, however, limited some samples to a single time point. Samples were collected for both physicochemical and microbial analyses.

Comprehensive physicochemical baselining was conducted on groundwaters, surface waters and soils of the region. Groundwaters were generally neutral in pH, relatively fresh (electrical conductivities < 1000 µS $cm^{-1}$), and dominated by sodium and bicarbonate ions. Some variability was noted due to the groundwaters diverse origins. Surface waters were also mostly neutral and low in salinity (electrical conductivities < 700 µS $cm^{-1}$). Surface waters were mostly dominated by sodium cations, though were more variable than groundwaters. Elevated metal concentrations were found in three samples (W3, W5 and W14) collected in November 2022 and June 2023. Repeat testing in 2024 suggested some metals had decreased in concentration, though other analytes, like copper, remained elevated. These findings were reported to the local council and the EPA, though the source of these metals remains unknown.

Soils, primarily sandy sodosols and tenosols with some vertosolic soils included for comparison, were more diverse than the water samples. Soil pH ranged from 4.7 - 9.2, with electrical conductivity between 10 and 294 µS $cm^{-1}$, indicating low soluble salt content.

In terms of biodiversity, across the 14 groundwaters, almost 6000 prokaryotic 'species' were found, with relatively low diversity (averaging about 1000 'species/OTUs' per sample). Common genera included *Gallionella, Arcobacter, Perlucidibaca, Ferrigenum* and *Gemella*. Groundwater communities were dominated by autotrophs, and in shallower aquifers, iron-oxidising taxa.

In contrast, the 16 surface waters had over 24,000 'species/OTUs', averaging ~5100 'species' per sample. Dominant genera included *Acidovorax, Aquirufa, Fluviibacter*, *Polynucleobacter* and *Methylobacillus.* Surface waters were relatively rich in cyanobacteria, though a range of unusual and common heterotrophic species were also detected. Soils had the highest biodiversity, with almost 38,000 'species/OTUs' comprising over 1000 unique genera. Soils were also more variable, reflecting the inclusion of varied soil types (e.g. sodosols and vertosols). Common genera included

*Conexibacter*, *Acidovorax*, *Azospira, Mycobacterium, Bradyrhizobium, Nitrososphaera, Acidobacteria* 'Group 3' and *Rubrobacter*.

Fungal communities in surface waters hosted a diverse array of poorly understood fungi including representatives of most fungal phyla. Freshwater fungal communities are particularly poorly studied, and these data provide the first insights in this region. Common water fungi included various yeasts and chytrids, which are typically more abundant in water than soil habitats.

Soil fungal microbiomes were diverse and dominated by ectomycorrhizal species, many of which appear novel and native, or endemic, to the region. Common ectomycorrhizal genera included *Clavulina, Cortinarius, Inocybe, Laccaria, Lactarius, Pisolithus, Russula, Scleroderma* and *Tometella*, though other mycorrhizal taxa (e.g. *Glomus* spp.) were occasionally detected. Individual soil samples usually were dominated by one or two ectomycorrhizal species, reflecting plant associations. Some fungi, however, were widely distributed across most samples (albeit mostly at lower levels) including various spore-forming ascomycetes *Gibberella, Hypocrea* (or the anamorphic *Fusarium*), *Chaetomium* and *Penicillium* (e.g. *Eupenicillium* and *Talaromyces*). Their presence in many samples likely reflects their aerial dispersal strategies.

**Microbial degradation of chemicals**

For its investigation into chemical degradation, this project focused on chemicals of 'higher hazard potential' regarding their risks to human or environmental health. These included chemicals with established hazards or insufficient hazard data, typically classified as Tier 2 or Tier 3 chemicals in industry risk assessments. The chemicals studied were two alcohol ethoxylates mixtures (AEM), two hydrocarbon mixtures (HCM), monoethanolamine (MEA) and three biocides: dazomet (DAZ), glutaraldehyde (GLU) and 3'3'-methylenebis(5-methyloxazolidine) (MBO). Surfactants and biocides were prioritised due to their known activity against cell membranes and living organisms, respectively.

The project used groundwater, surface water and soil microcosms to assess (1) chemical biodegradation and, where relevant, their breakdown products, and (2) impacts on microbial communities, identifying sensitive taxa for environmental monitoring programmes. Additionally, experiments evaluated whether chemicals could be used as sole carbon sources by microbes to identify degraders.

It is worth noting that hydrocarbon mixture #2 (HCM#2), ordered from an international supplier, was used in all microcosm experiments. However, post-experiment analysis showed the contents did not match the labelled CAS. Results for HCM#2 will be reported in an addendum, expected in early 2025.

After four weeks of incubation in soil microcosms, biocides and HCM#1 were undetectable and no resistant breakdown products of GLU or MBO were found. DAZ, and its active breakdown product methyl isothiocyanate (MITC), were both undetectable. Only trace levels of AEM (<0.01%) were detected after incubation. For MEA, approximately ~75% of added MEA was degraded after incubation. It is noteworthy, however, that MEA was detected in soils to which no MEA was added. MEA concentrations that remained after incubation were not significantly different to background levels found in soil. This is unsurprising as MEA is a key component of bacterial cell

membranes, and prokaryotes found in soil would likely be able to readily degrade and manufacture MEA.

In surface water microcosms, all chemicals were undetectable after three months incubation. In groundwaters, all chemicals except AEM were degraded after three months incubation; AEM#1 degraded by ~20-50%, while AEM#2 showed greater degradation (a loss of 85-90%).

For microbial community impact assessments some chemicals (HCM, MEA and the AEMs) were considered together in mixtures called the 'drilling mixture' and 'workover mixture'. Microbial community changes varied by sample type. For one sample, only DAZ and GLU significantly altered microbial communities. In contrast, the other soil microbial communities were changed by the presence of all chemicals except the drilling mixture. Surface water microbial communities were impacted by most chemicals except MBO. Groundwater microbial communities were not impacted by the drilling or workover mixtures, and biocides had varied impacts.

Microbial sole carbon enrichment experiments revealed many microbes capable of using the tested chemicals as sole carbon sources, though growth on the biocides was generally absent or limited, as expected.

**Conclusions**

Taken together, this study has provided the most comprehensive baseline dataset on soil and surface water microbiology in the region and extends existing groundwater data south and east of Narrabri. It also provides the first insights into the fungal diversity in non-vertosolic/non-cropping soils and surface waters.

The study also demonstrated that most chemicals tested degraded completely in groundwaters, surface waters and soils of the region. As previously observed in other studies, faster degradation occurred in soils than in waters, though significant amounts of AEM persisted in the groundwaters and trace amounts of these same compounds persisted in soils. Finally, this study identified numerous indigenous microorganisms in groundwaters, surface waters and soils that are likely capable of degrading the 'higher hazard potential' chemicals tested; and conversely a number of taxa were identified as sensitive to chemical additions in these environments. Such taxa may be useful targets for monitoring programmes.

In conclusion, the study demonstrates that microbial communities in and around the Narrabri Gas Project area can readily degrade most chemicals tested, offering an additional line of defence against environmental contaminants in the unlikely event of a spill. It also provides a unique, comprehensive baseline dataset and has identified key taxa for environmental monitoring programs.

# 1 Glossary

| Term | Definition |
|---|---|
| **16S rRNA (biology/chemistry)** | A component of prokaryotic ribosomes that is commonly used to study and identify prokaryotes (see also entry for 'Ribosome'). |
| **Alluvium (geology/pedology)** | Sediment that is deposited by rivers or floodwaters. |
| **Anabolism (biology)** | The chemical construction of molecules. |
| **Aquifer (geology)** | A geological formation that can store and transmit groundwater. |
| **Autotrophy (biology)** | A form of nutrition in which organisms produce their own food using inorganic substances. |
| **Back-arc basin (geology)** | A geological depression that forms behind a chain of volcanoes where one tectonic plate is subducted beneath another. |
| **Baselining** | The establishment of a reference point or standard for comparison. |
| **Basement rocks (geology)** | The oldest rocks in the Earth's crust, lying beneath more recently formed sedimentary layers. |
| **Basin (geology)** | An area of the Earth's crust that is typically shaped like a bowl and where sediments accumulate over time. |
| **Biogeochemical cycles (biology/geology/geography)** | Natural pathways through which elements and compounds, such as carbon, nitrogen, sulphur and water circulate through the Earth's ecosystems, atmosphere, hydrosphere, and lithosphere. It involves biological, geological, and chemical processes. |
| **Catabolism (biology)** | The chemical breakdown of molecules. |
| **Catchment (geography)** | An area of land where all rainfall and runoff collect and flow to a common outlet (e.g., into a river, lake, or ocean). |
| **Chemical weathering (geology/geography)** | The break-down of rocks and minerals through chemical reactions. |
| **Ciliate (biology)** | A single-celled organism that is characterized by the presence of hair-like structures called cilia on their surface. Well known examples are *Paramecium* species. |
| **Coal seam (geology)** | A layer of coal embedded within rock layers. |
| **Colluvium (geology/pedology)** | Loose, unconsolidated sediments and soil that accumulates at the base of a slope or hillside. |
| **Dehalogenate/dehalogenation (biological)** | In microbiology, dehalogenation refers to the process by which microorganisms remove halogen atoms (such as chlorine, bromine or fluorine) from organic compounds. This is typically achieved through enzymatic reactions that break carbon-halogen bonds, converting halogenated compounds into less toxic or more biodegradable forms. |
| **DNA (biology/chemistry)** | DNA (deoxyribonucleic acid) is the molecule that carries the genetic instructions for the development, functioning, growth, and reproduction of all known living organisms and many viruses. |

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| **Domain of life (biology)** | The broadest category used to classify living things. There are three domains: Archaea, Bacteria, and Eukarya. |
| **Ectomycorrhiza (biology)** | A type of mutually beneficial relationship between certain fungi and the roots of host plants. The fungi form a protective sheath around the root tips and penetrate the root's outer cells without invading the living tissue. |
| **eDNA** | Environmental DNA (eDNA) is the genetic material left by organisms in their surroundings. It is increasingly used to detect species presence and evaluate biodiversity. |
| **Electrical conductivity (chemistry)** | Also known as EC, it is a measure of a material's ability to conduct electric current. |
| **Electron acceptor (biology/chemistry)** | A molecule that takes in electrons during a chemical reaction to facilitate energy transfer and transformation. |
| **Endosymbiont (biology)** | An organism that lives within the cells or tissues of another organism in a mutually beneficial relationship. |
| **Enzyme (biology)** | A protein that acts as a biological catalyst in living organisms to speed up chemical reactions without being consumed itself. Enzymes are essential for various metabolic processes. |
| **Eukaryote (biology)** | An organism whose cells have a defined nucleus and membrane-bound organelles. Plants, animals and fungi are common and familiar eukaryotes. |
| **Extension (geology)** | The stretching and thinning of the Earth's crust due to tectonic forces. |
| **Fault system (geology)** | A network of interconnected faults. Faults are fractures in the Earth's crust along which movement has occurred. |
| **Flagella (biology)** | Long, whip-like structures that protrude from the surface of some cells and are used for movement. |
| **Fluvial (geography)** | Pertaining to rivers and streams. |
| **Gene (biology)** | Basic unit of inheritance composed of a sequence of DNA. Genes either make proteins or are used to regulate the expression of other genes. |
| **Genome (biology)** | The complete set of genetic material in an organism, encompassing the entirety of its DNA, including all genes. |
| **Geochemistry** | The study of the Earth's chemical composition and processes. |
| **Heterotroph (biology)** | A form of nutrition whereby organisms obtain energy and nutrients by consuming other living organisms or organic matter. Most animals are heterotrophs. Most plants are autotrophs. |
| **Hydraulic conductivity (geology)** | A measure of a material's ability to transmit water or other fluids through its pores or fractures. |
| **Hydrocarbons (chemistry)** | Compounds made up entirely of hydrogen and carbon atoms. |
| **Hydrogeologic (geology)** | Pertaining to the distribution, movement, and quality of groundwater. |
| **Igneous intrusion (geology)** | A body of molten rock (magma) that pushed its way into pre-existing rock formations and solidified beneath the Earth's surface. |
| **Interquartile range (statistics)** | The Interquartile Range (IQR) is a measure of statistical dispersion, representing the range within which the middle 50% of data lies. |

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| **Lithology (geology)** | Pertaining to the physical characteristics of rocks, especially their composition, grain size, texture, and colour. |
| **Loam (pedology)** | A type of soil with a balanced mixture of sand, silt, and clay. |
| **Metabolism (biology)** | The complete set of life-sustaining chemical reactions within organisms. It enables the conversion of food into energy, building and repairing of cells, and regulation of biological functions. |
| **Microaerophilic (biology)** | A physiological preference for a reduced level of oxygen. |
| **Microbiome (ecology)** | The microbiome refers to the entire habitat of microorganisms, their genomes, and the surrounding environmental conditions (physical and chemical). |
| **Microbial community (ecology)** | A group of microbial populations |
| **Microbial population (ecology)** | A group of individuals of the same microbial species. |
| **Mycorrhiza (biology)** | A mutually beneficial relationship between certain fungi and plant roots. The fungi enhances plant nutrient and water uptake, and, in return, the plant provides the fungi with carbohydrates and other organic compounds. |
| **Orogen (geology)** | A region of the Earth's crust where mountains have formed due to tectonic plate movements, such as continental collisions or subduction. |
| **OTU (biology)** | OTU (Operational Taxonomic Unit) is a term used in biology to represent clusters of organisms—typically microorganisms—that share a certain level of genetic similarity. For ease of understanding an OTU can be considered the same as a microbial species. |
| **Outlier (statistics)** | Data point that is significantly different from the other observations in a dataset. Often > 1.5X the interquartile range (IQR). |
| **Overburden thickness (geology)** | The depth of material, such as soil and rock, that lies above a particular deposit or resource. |
| **Paleochannel (geology)** | Ancient and inactive river or stream channels that have been filled with sediments. |
| **PCR (biology)** | PCR (Polymerase Chain Reaction) is a laboratory technique used to rapidly amplify specific segments of DNA for applications such as sequencing. |
| **pH (chemistry)** | Potential of hydrogen; a measure of the acidity or alkalinity of a solution. |
| **Potentiometric head (hydrogeology)** | The height to which aquifer water rises in a well. It describes the potential energy level of water in an aquifer - showing its pressure and elevation. |
| **Primer (biology)** | A short single-stranded sequence of DNA that serves as a starting point for DNA synthesis during processes such as PCR. |
| **Prokaryote (biology)** | Single-celled microorganisms that lack a defined nucleus and membrane-bound organelles. Bacteria and Archaea are the two prokaryotic domains of life. |
| **Rhizobia (biology)** | A group of soil bacteria that form a mutually beneficial relationships with leguminous plants, such as beans and peas. They infect the roots of these plants where they convert atmospheric nitrogen into a form that the plant can use for growth. This process is known as nitrogen fixation. In return, the plants provide the bacteria with habitat, nutrients, and energy. |

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| **Ribosome (biology)** | Cellular structures that serve as the sites of protein synthesis. |
| **Saprolite (geology)** | A chemically weathered rock. |
| **Sedimentary facies (geology)** | Distinct bodies of sediment characterised by particular physical, chemical, and biological attributes. These variations can be indicative of different environments. |
| **SEM**<br>**(multiple meanings)** | Statistics: Standard Error of the Mean is a statistical measure that quantifies the accuracy with which a sample mean estimates the population mean.<br>Physics: Scanning Electron Microscopy |
| **Sodosol (pedosol)** | A soil type enriched in sodium, which affects soil structure and drainage. These soils often have poor physical properties, including low permeability. |
| **Stratigraphic (geology)** | Pertaining to rock layers and layering. |
| **Structural down-warping (geology)** | The bending or sagging of the Earth's crust. |
| **Taxon; Taxa (biology)** | A group of organisms that are classified together based on shared characteristics or evolutionary relationships. Unlike species or genera, or families the terms ‘taxon/taxa’ lack a defined phylogenetic level. |
| **Taxonomy (biology)** | The scientific discipline of classifying and naming living organisms based on shared characteristics and natural relationships |
| **Tenosol (pedology)** | A type of soil with a weakly developed profile and primarily consisting of sandy or loamy textures. It generally has minimal layering or horizon development. |
| **Transgressive (geology)** | A rise in sea level causing the shoreline to move inland. |
| **Tree of life (biology)** | A tree-like model used to describe the evolutionary relationships among all living organisms on Earth. It shows how organisms descended from common ancestors. |
| **Type strain (biology)** | A reference strain of a species used as the definitive example of that species. It is typically the first strain of a species to be studied, formally described and used to represent the species in scientific literature and databases. The type strain holds the species' official name and serves as a standard for comparison in identifying and categorizing other strains within the same species. |
| **Unconformity (geology)** | A rock surface that indicates a period of erosion or non-deposition. |
| **Vertosol (pedology)** | A soil type characterized by a high clay content, leading to significant swelling and cracking during wet and dry conditions, respectively. |

# 2 Aims and introduction

## 2.1 Project aims

In 2020, the Narrabri Gas Project (NGP) was conditionally approved by the Independent Planning Commission (IPC; Santos, 2020)[1]. The NGP, focuses on extracting Permian-age coal seam gas (CSG) from targets in the Gunnedah Basin, NSW (e.g., Salmachi et al., 2021 and references therein). The use of gas from unconventional resources, however, has elicited significant public debate regarding potential environmental impacts, particularly to groundwater resources. One specific concern, is that use of a range of chemicals by CSG companies in drilling, workovers and other activities, including surfactants, biocides, corrosion inhibitors, buffers, friction reducers and viscosity control (e.g., Schinteie et al., 2019 and references therein) may negatively impact the environment.

Unsurprisingly, risks associated with these chemicals have been the focus of numerous reviews into potential environmental and human health impacts (Australian Government Department of the Environment and Energy Reports 2014, 2017)[2]. Regarding the NGP, a chemical risk assessment framework to protect the environment and water resources during CSG operations was assigned to the 2020 environmental approval by the federal government[3].

This project is part of GISERA's efforts to reduce uncertainty around the environmental, social, health, cultural and economic risks associated with CSG activities in the Narrabri local region. In particular, this project aims to:

1. Establish microbial community and chemical baselines in aquifer waters, surface waters and soil samples of sites proximal to future CSG activities.
2. Assess and understand the capacity of these microbial communities to degrade a range of chemicals likely to be used in CSG activities in both the major soil types of the region, in relevant aquifer environments and in surface waters of the region.

[1] https://www.ipcn.nsw.gov.au/cases/2020/03/narrabri-gas-project

[2] https://www.industry.gov.au/publications/environmental-report-2014-15 and https://www.dcceew.gov.au/sites/default/files/documents/annual-report-2017-18-environment-and-energy.pdf

[3] https://www.planningportal.nsw.gov.au/major-projects/projects/narrabri-gas

## 2.2 Land use in the Narrabri Region

The Narrabri region features a mosaic of land use that, in broad terms, mirrors soil types of the region (Figure 1 - Figure 5). Typically, the richer vertosolic soils of the region are used to grow cotton, wheat and other high-value crops, and on these soils the woodlands have mostly been cleared for agriculture, though some remnant patches remain (Figure 1 and Figure 2). In contrast, the other soil types of the region support pastures and grazing (Figure 3) with the mostly sodosolic, and some tenosolic soils, south and southwest of Narrabri having been subject to significant clearing in the areas of Bohena Creek and Yarrie Lake (Figure 1). Further south and southwest of Narrabri the Pilliga woodlands (sometimes called the 'Pilliga scrub') extend towards Coonabarabran (Figure 4 and Figure 5). Much of the NGP lies either in the Pilliga woodlands (itself a mosaic of National Parks and State Forest with a combined area of over 500,000 ha (Cleland, 2015); or in the grazing and cropping areas in Bohena Creek or Yarrie Lake (Figure 1).

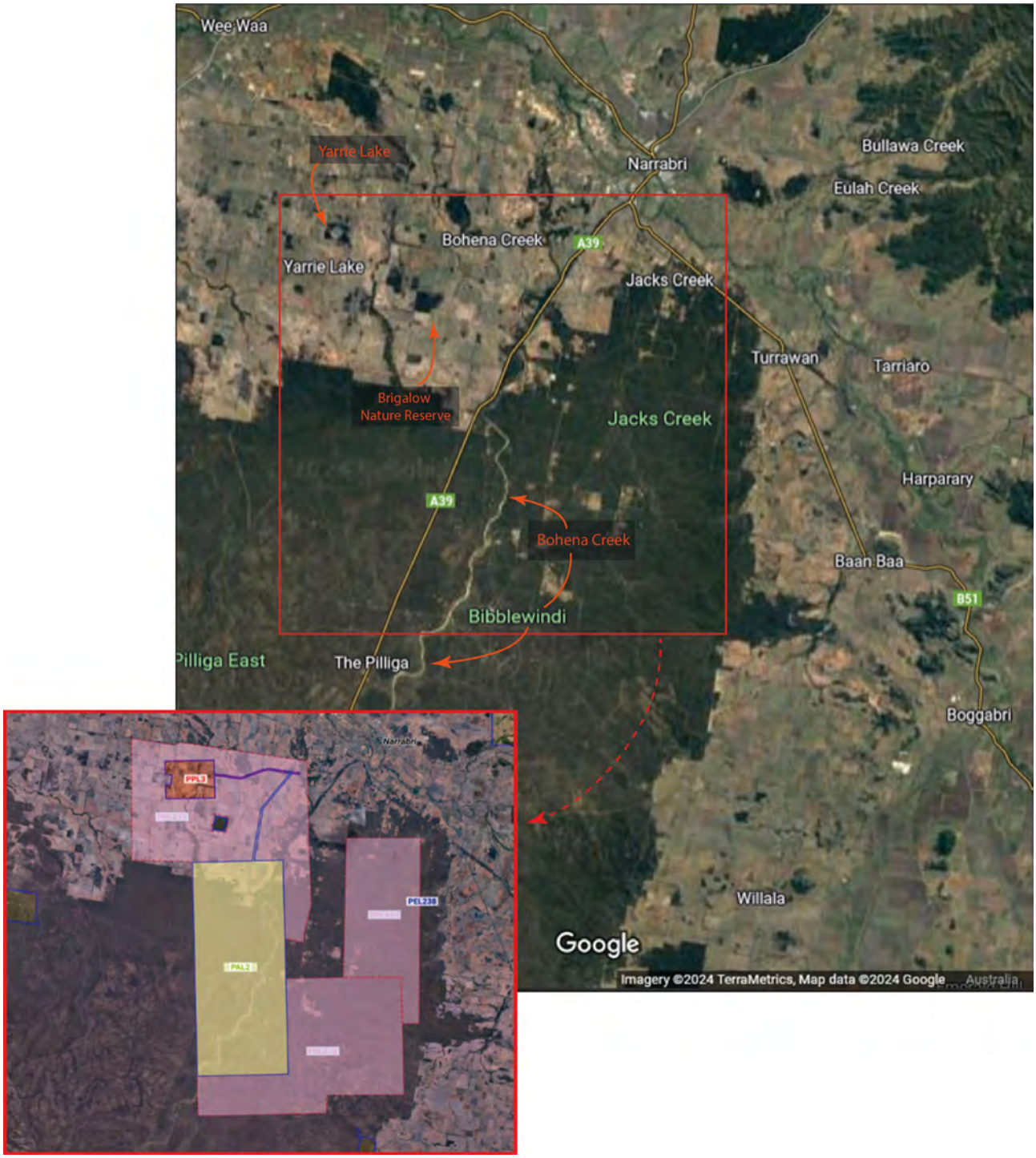



**Figure 1 Satellite photos of the Narrabri region**

Note: Clearing in areas southwest of Narrabri (regional areas of Bohena Creek and Yarrie Lake) and large stands of woodlands southwest towards Coonabarabran. Map courtesy of Google Maps, inset map (red) of the Narrabri Gas Project area reproduced from the NSW Government. (https://meg.resourcesregulator.nsw.gov.au/mining-and-exploration/significant-projects/narrabri-gas-project)

Google (n.d.). [Narrabri]. Retrieved September 2024.

In the Pilliga woodlands themselves, disturbance is generally high with the existence of hunting, sporadic logging and a significant dirt road network off the Newell Highway (OEH, 2017)[4]. The soils in the Pilliga woodlands are primarily sodosols, characterised by a high sodium content with generally poor soil structure and low nutrient status. Despite these challenging soil conditions, the Pilliga woodland supports a distinctive semi-arid woodland ecosystem. This woodland has changed significantly over time, and there are debates about the scope and nature of this change (for a detailed analysis see: Whipp et al., 2012) over the last ~250 years.

Dominant vegetation includes various *Eucalyptus*, *Angophora*, *Casuarina* species and *Callitris glaucophylla* (White Cypress Pine), with the latter sometimes forming dense stands (Figure 4). The understory features a mosaic of sclerophyllous shrubs, grasses and herbs including several endangered plant species. A floral study by Idyll Spaces Environmental Consultants (2008)[5] encompassing parts of Bibblewindi and Pilliga East State Forests, road and travelling stock reserves and nearby private properties, provided more detailed vegetation descriptions into the area proximal to gas production activities. Varied vegetation communities were noted in that study, depending on a range of ecological factors that encompasses both the physical environment (e.g., drainage, soil types) and any other local biological characteristics (e.g. associated plant species). In cleared areas, paddock trees including *Casuarina cristata* and occasional clumps of *Acacia harpophylla* young coppice growth was observed. In low open forest, the brigalow *Acacia harpophylla* was dominant although *Casuarina cristata* may be dominant in localised areas of poor drainage. The dry open forest was dominated by the narrow leaved ironbark *Eucalyptus crebra*, the Pilliga box *Eucalyptus pilligaensis*, and a rough-barked apple hybrid *Angophora floribunda x leiocarpa*. In woodland, the red gum *Eucalyptus dwyeri* was observed to be dominant. For a more detailed description of these communities, including midstratum and ground layer vegetation, consult the report by Idyll Spaces Environmental Consultants (2008).

Overall, it is important to note that the Pilliga-area vegetation cover is highly non-homogenous and plant communities can differ markedly depending on the ecological characteristics in the region (Figure 4 and Figure 5).

[4] https://www.environment.nsw.gov.au/-/media/OEH/Corporate-Site/Documents/Parks-reserves-and-protected-areas/Parks-statement-of-management-intent/pilliga-outwash-parks-statement-management-intent-170686.pdf

[5] https://narrabrigasproject.com.au/wp-content/uploads/2015/06/NSW_CSG_Narrabri_19_Environmental_Assessment_Specialist_Report_2_Flora.pdf

**Figure 2 Farmland in the Narrabri region on vertosolic soils that support cotton and other high-value crops**

This photo shows cotton growing on the side of the Kamilaroi Highway. Photo by Kate Ausburn.

**Figure 3 Grazing is also a significant industry in the Narrabri region**

**Figure 4 Photograph showing White Cypress Pine (*Callitris glaucophylla*) forming dense stands**

**Figure 5 Parts of the Pilliga woodland with dense stands of eucalypts**

This photo shows Dandry Gorge Creek in Pilliga Nature Reserve. Compared to Figure 4, parts of the Pilliga woodland feature few *Callitris*. Photo by Margaret Donald.

## 2.3 Basic geology of the Narrabri region

The regional geology of the Narrabri area is dominated by three geological systems, which are the 1) Permian to Triassic Gunnedah Basin, the 2) Jurassic to Cretaceous Surat Basin, and 3) overlying Cenozoic alluvium (Figure 6). All these systems are underlain by Palaeozoic basement rocks of the Lachlan Orogen. Coal resources in that area are located in the Gunnedah Basin with economic seams in the Black Jack Group and the Maules Creek Formation (Northey et al., 2014). As already mentioned, in 2020, the Narrabri Gas Project was conditionally approved by the Independent Planning Commission (IPC; Santos, 2020). This project, focusing on extracting gas from Permian coal seams, is considered as highly prospective (e.g., Salmachi et al., 2021 and references therein). By contrast, no coal or CSG resource development from seams in the Surat Basin are currently under development. The Late Jurassic Pilliga Sandstone of the Surat Basin and the Cenozoic alluvium form the main groundwater conduits in the Narrabri region and do not contain coal. In the following sections, the basin systems and alluvium are briefly reviewed. The geological sections focus on key stratigraphic aspects to provide a broad understanding of the hydrogeology of the Narrabri region.

### Geology of the Gunnedah Basin

The early Permian saw the initiation of the Gunnedah Basin in a back-arc setting whereby an extensional event resulted in the deposition of interlayered volcanic and lacustrine sedimentary rocks (Totterdell et al., 2009). In the western regions, this basin rests unconformably (i.e. deposited on an erosional surface or a pause in sediment accumulation) on basement rocks of the Palaeozoic Lachlan Orogen, while in the east it sits next to the New England Orogen along the Hunter-Mooki thrust fault system (Welsh et al., 2014). This fault system terminates the basin at each end and separates from the adjacent Werrie Basin (Hill, 2002). The Gunnedah Basin architecture consists of three north-north-westerly oriented sub-basins lying between basement ridges (Tadros, 1988, 1993). Coal resources are located in the Maules Creek and Mullaley sub-basins (Herr et al., 2018b).

Early sedimentation in the Gunnedah Basin, marked by the Leard Formation, consists of localised deposition of both alluvium and colluvium in palaeo-valleys on the weathered surface of the Palaeozoic basement rocks (Gurba et al., 2009). A subsequent influx of sediments of predominantly volcanic origin from adjacent highlands resulted in the lower Permian Maules Creek Formation and contains numerous coal seams. Most structural activity such as faulting occurred in the Narrabri region during the early to mid-Permian (Tadros, 1993). Most significant is the Boggabri thrust fault system located within the Boggabri Volcanics of the Boggabri Ridge, approximately underlying the Namoi River between Gunnedah and Narrabri (Herr et al., 2018b). Fault activation raised the Maules Creek sub-basin strata by up to several hundred metres higher than equivalent strata in the Mullaley sub-basin (Herr et al., 2018b; Hill, 2002).

The sedimentary facies of the Maules Creek Formation consist of a diverse range of fluvial settings that range from alluvial fans to peat swamps (Gurba et al., 2009; Totterdell et al., 2009). Transgressive marine conditions in the Lower Permian resulted in the overlying Porcupine and Watermark formations. A subsequent marine regression gave rise to a fluvial depositional setting, which included peat swamp deposits that formed the precursor of the Hoskissons Coal in the Black

Jack Group (Totterdell et al., 2009). Sediments of the Black Jack Group, especially the upper portions, originated from the New England Orogen in the eastern part of the Narrabri region and deposited as part of an alluvial system (Totterdell et al., 2009).

The two principal coal-bearing sequences of interest to CSG production are the Early Permian Leard and Maules Creek Formations and the Late Permian Black Jack Group (Gurba and Weber, 2001; Tadros, 1995). With approximately 500 Gt of coal in these Permian strata, these formations are of significant economic value (e.g., Gurba and Weber , 2001 and references therein; Salmachi et al., 2021).

The Late Permian saw the basin undergo structural contraction and deformation followed by erosion (Hill, 2002). Consequently, the Early Triassic Digby and Napperby formations rest unconformably on an erosional surface (Totterdell et al., 2009). Late Triassic to Early Jurassic structural down-warping of the area resulted in the Surat Basin, which is situated in the western part of the Gunnedah Basin. The geologic aspect of the Surat Basin is described below.

### 2.3.1 Geology of the Surat Basin

As described above, structural down-warping in the Late Triassic to Early Jurassic resulted in the formation of the Surat Basin in the western part of the Gunnedah Basin. Structurally, the younger Surat Basin is less deformed than the underlying Gunnedah Basin (Herr et al., 2018b). The basal lithology of the Surat Basin generally consist of the Garrawilla Volcanics (Totterdell et al., 2009). This volcanic unit is unconformably overlain by the Purlawaugh Formation, which has been interpreted as deeply eroded prior to the deposition of the younger Pilliga Sandstone (Totterdell et al., 2009). This overlying sandstone unit itself underwent significant erosion, resulting in a saprolite (i.e., chemically weathered rock) layer of variable thickness and extent (Herr et al., 2018b). The Pilliga Sandstone forms one of the two major aquifers of the Narrabri region. A water resource assessment by Smerdon et al., (2012) noted that the overlying Late Jurassic to Cretaceous Rolling Downs Group, composed of mudstone, siltstone and fine sandstone, acts as the aquitard of the Pilliga Sandstone aquifer, while the underlying Purlawaugh Formation is the aquiclude. This is in contrast to other studies (Schlumberger Water Services, 2012; Sanderson, 2013), which describe the Purlawaugh Formation as a negligibly transmissive unit or an aquifer, respectively. However, this discrepancy may be due to uncertainty regarding the boundaries between different strata (Hawke and Cramsie, 1984; Herr et al., 2018b). Regardless, recent studies by Raiber et al. (2022) have shown that the hydrogeological connectivity between the Pilliga Sandstone and underlying Gunnedah Basin formations in the eastern and southern part of the Narrabri Gas Project (NGP) area. However, localised connectivity likely exists near the Plumb Road NSW DPIE groundwater monitoring site, due to a spatially extensive igneous intrusion emplaced into the shallow Gunnedah Basin may facilitate some connection (Raiber et al., 2022). This connectivity is also supported geochemically, with an increase in chloride anion concentrations and the presence of methane gas (Raiber et al., 2022).

### 2.3.2 Geology of Cenozoic alluvium

The Cenozoic era saw the deposition of more alluvium being deposited in the Narrabri region. This alluvial deposition resulted in the formation of the Upper and Lower Namoi alluvial aquifers. Volcanism during that era subsequently affected the topography of the area with volcanic features seen today as hills around the margins of the Narrabri region (Wellman and McDougall, 1974). Furthermore, the weathering of these volcanic remnants over time resulted in the formation of rich and fertile soils of the Liverpool Plains (Welsh et al., 2014). The alluvium consists of the uppermost Narrabri Formation and the underlying Gunnedah Formation with the strata composed of gravel- and sand-rich layers that are usually near the base and overlain by fine-grained floodplain silts and clays deposited in a lower energy environment (Berrett, 2012; Herr et al., 2018b; Thoms et al., 1999). Based on the relatively large size of the Cenozoic paleochannels compared to those of today, Young et al. (2002) deduced that larger river flows of about twice the magnitude of modern flows in the Namoi river basin.

## 2.4 Hydrogeology of the region

The two major aquifers in the Narrabri region are the Pilliga Sandstone aquifer of the Surat Basin and the Naomi Alluvial aquifer of the Cenozoic alluvium (Herr et al., 2018b). Numerous other aquifers, such as the Mooga Sandstone, can be geographically extensive but not as high yielding (NSW DPE, 2022; Figure 6) Several studies exist on groundwater quality and chemistry of the Narrabri area, including in the deeper underlying Gunnedah Basin. These studies were conducted previously by government departments, consultants, and research organisations. Many of these studies are reviewed below.

Currently, limited information exists about the aquifers and groundwater flows of the Gunnedah Basin and are rarely utilised for stock and domestic purposes (Herr et al., 2018b). Within this basin, porous and permeable rock sequences within the Digby Formation and Clare Sandstone (part of the Black Jack Group), and the Permian coal beds serve as aquifers (Herr et al., 2018b). These rock sequences do not comprise a single aquifer, but numerous overlapping and stacked together units that are commonly separated by lower-permeability layers. Importantly, these rock aquifers and coal beds are believed to have significantly lower hydraulic conductivity than the overlying alluvial aquifers (Herr et al., 2018b). As noted in the geological section above, the boundary of the Gunnedah Basin is defined by the Hunter-Mooki thrust fault system, which is considered to be an impermeable boundary that hydrogeologically isolates the basin from the adjacent Werrie Basin (Herr et al., 2018b). Little is known about any aquifers in the Lachlan Orogen basement, which underlies the Gunnedah Basin (Herr et al., 2018b). Since the sub-basins architecture of the Gunnedah Basin were compartmentalised, it has been speculated that the sub-basin strata may also be divided into discrete sections with respect to regional groundwater flow systems (Herr et al., 2018b). Such a compartmentalisation of deep groundwater flow systems may therefore limit potential groundwater impacts from coal resource developments. Nevertheless, there may be areas where deep groundwater flow may be connected such as between the Maules Creek and Mullaley sub-basins where the Boggabri Ridge that separates these two areas is not continuous (Tadros, 1993), or where flow proceeds around the ridge (Schlumberger Water Services , 2012), or

through overlying alluvium (Herr et al., 2018b). Fault activity, mostly during the Permian, may have also had a significant effect on groundwater flow characteristics at a local scale (Schlumberger Water Services, 2011).

As noted above, the Surat Basin hosts one of the major aquifers in the Narrabri region. Groundwater in this aquifer, the Pilliga Sandstone, flows from south-east to west and north-west (Herr et al., 2018b; Smerdon et al., 2012). Potentiometric heads of the Pilliga Sandstone do not generally show any obvious surface water to groundwater interaction along the Namoi River or the Pian Creek with the only exception being downstream of Narrabri at the confluence with Bohena Creek (Herr et al., 2018b). Due to the intense chemical weathering resulting in saprolite formation of the Pilliga Sandstone, a reduced connectivity is assumed with the alluvium (Herr et al., 2018b).

The aquifer system of the Cenozoic alluvium occurs along river valleys and floodplains and consequently play a central role for agriculture and the local ecosystems (Herr et al., 2018b). Simplistically, the alluvium could be seen as a stacked aquifer system consisting of the uppermost Narrabri Formation (≤ 30 to 40 m deep) and the underlying Gunnedah Formation (≤ 170 m deep) (Berrett, 2012; Herr et al., 2018b). In reality, the stacked aquifer system model is likely more complex with an interplay between the clay, sand and gravel beds occurring (Kelly et al., 2007). Indeed, in some areas there may not be any hydraulic separation between the Narrabri and Gunnedah formations, with both units acting as a single aquifer (Herr et al., 2018b). The groundwater gradients in the alluvial aquifers indicate flow from the east in a north-westerly to westerly direction (Welsh et al., 2014). While the hydraulic conductivity of the Cenozoic alluvium generally increases with depth and in the paleochannels, it can vary depending on the presence of sand or clay lenses (Herr et al., 2018b). Generally, groundwater levels in the Cenozoic alluvium correspond to rainfall variability and groundwater use with the water table level ranging approximately from 4 to 12 m below ground near Narrabri but progressively deepens (≤ 34 m) to the west (Herr et al., 2018b).

A hydrogeochemical project by Parsons Brinckerhoff (2011) indicated that the Narrabri Formation is dominated by sodium and chloride with major ion chemistry in the Gunnedah Formation being spatially variable, which indicates that the aquifer is laterally discontinuous and exhibits zones of differing ion compositions and salinity. The report also observed deteriorating groundwater quality in these formations that make them unsuitable for stock and/or grazing. In addition, a long-term trend of increasing salinity was recorded in some Gunnedah Formation bores and attributed to vertical leakage of saline water from the upper aquifer and saline intrusion of pore waters. A similar trend was also recorded in one Narrabri Formation and one Cubbaroo Formation bore. Finally, the report pointed to processes influencing the major ion composition of groundwater in this region and include mixing, ion exchange, reverse ion exchange, and dissolution and precipitation of minerals such as carbonates and gypsum (Parsons Brinckerhoff, 2011). Such reports provide useful context, but they generally are not focused around CSG development.

The degree of connectivity between the groundwater and coal measures is dependent, among other factors, on the overburden thickness (Herr et al., 2018b). Therefore, the overburden

depends on the combined thickness of the overlying Watermark, Porcupine, Digby and Napperby formations, Surat Basin stratigraphic sequences (wherever present), and the Cenozoic sediments (Herr et al., 2018b). The overburden thickens rapidly to the west of the Boggabri Ridge (Herr et al., 2018b). More recently, Raiber et al. (2022) pointed out that this overburden thickness acts as an aquitard that appears to be continuous with limit hydrogeological connectivity occurring between the Pilliga Sandstone of the Surat Basin and underlying Gunnedah Basin formations in the eastern and southern part of the Narrabri gas project area. Some localised hydrogeological connectivity was noted between the Gunnedah and Surat basins near the Plumb Road groundwater monitoring site where a spatially extensive igneous intrusion occurs. Potential hydrogeological connectivity between the Pilliga Sandstone and deeper formations was also inferred chemically through an increase in chloride anion concentrations and the presence of low concentrations of methane within the Pilliga Sandstone in the north-west of the Narrabri gas project area (Raiber et al., 2022).

## 2.5 Surface waters of the region

The area around Narrabri and beyond, also known as the Northwest Slopes region, is characterised by a broad floodplain in the west and highlands in the east and south (Welsh et al., 2014). This landform pattern is overlain by an east-to-west climatic and hydrologic asymmetry. With an annual rainfall band of 500–750 mm, the area contains dry inland and the wetter coastal zone further east (Cleland, 2015). Mean annual rainfall is recorded at approximately 900 mm on the Nandewar Range to the north, 700 mm at Narrabri further south, and 480 mm at Walgett to the west. This disparity in rainfall pattern is due to the boundary conditions between semiarid and humid climates, which runs through the central part of the region (Wray, 2009; Young et al., 2002). Consequently, this region experiences variable drought risks (Hoque et al., 2021) while the township of Narrabri is highly exposed to flooding (WRM, 2016; Yeo, 2002).

The major fluvial system in this area is the Namoi River, which is part of the Barwon catchment of the Murray-Darling basin (Figure 7). This river originates on the western slopes of the Moonbi Range in the New England Plateau among crystalline and metamorphic rocks of the New England Fold Belt at an elevation of approximately 1300 m (Young et al., 2002). From these headwaters, the river flows initially south westward to Gunnedah and then north westward to Narrabri among sedimentary and volcanic rocks such as the Jurassic Pilliga sandstone and Miocene-age trachytes and basalts. This flow occurs largely in a relatively narrow, albeit deeply alluviated (i.e. sedimented) valley. From Narrabri, the river flows westwards across a broad alluvial plain to join the Barwon River at Walgett (Young et al., 2002). In this westward course, the Naomi River is joined by twenty-seven tributaries that include the Peel (Calga), Manilla and Mooki rivers as well as the Bohena, Coghill, Baradine, Etoo and Tullaba creeks that deliver significant volumes of water into the Naomi River (Figure 7) (WRM, 2016; NSW Natural Resources Commission, 2023).

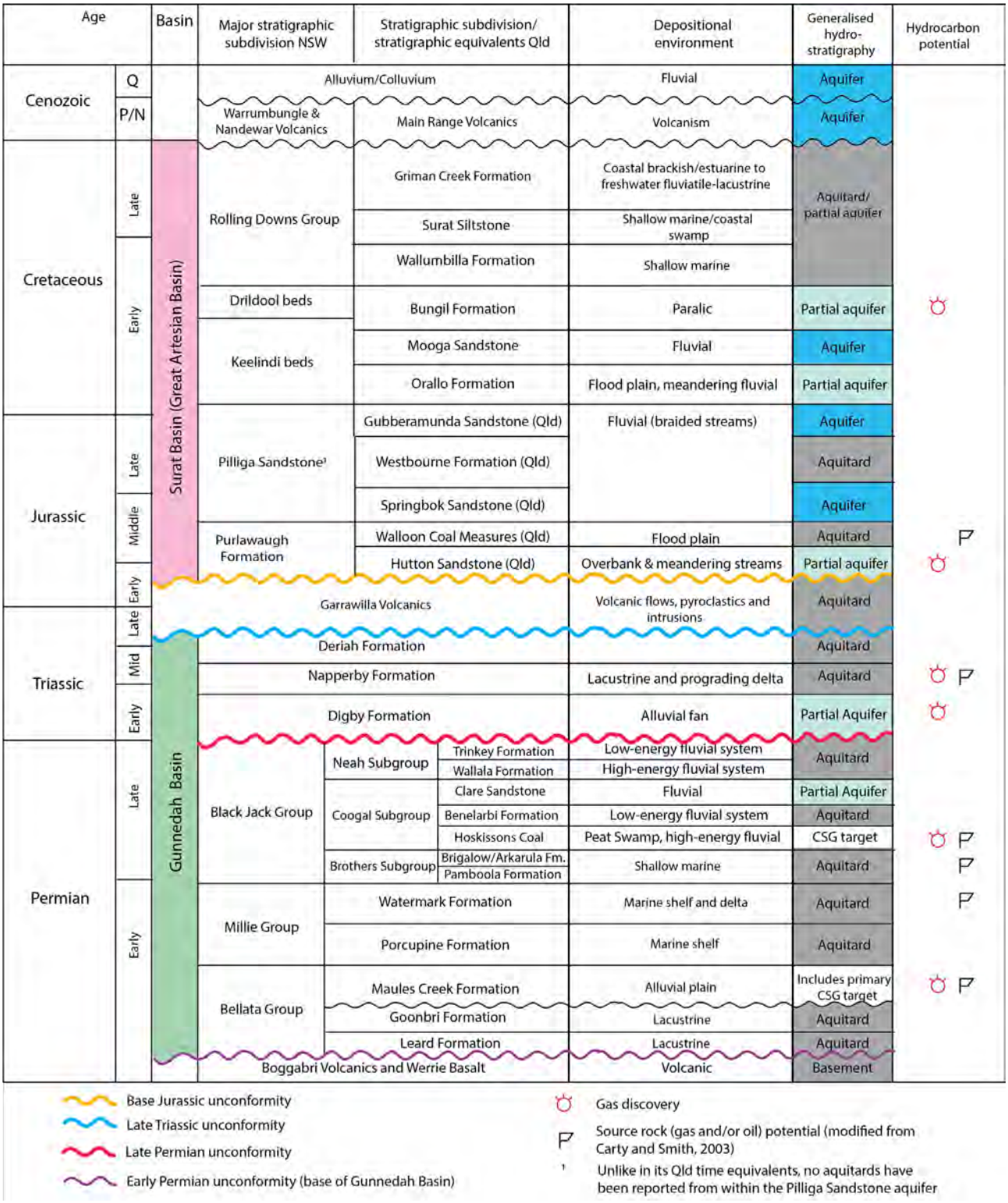



**Figure 6 Stratigraphy, including hydrostratigraphy, of the Surat and Gunnedah basins, NSW**

Adapted with permission from Raiber et al. (2022) and compiled from Tadros (1993, 1995), Carty and Smith (2003), Korsch and Totterdell (2009), Totterdell et al. (2009), Geoscience Australia (2013) and Ruming (2015) and modified from Raiber and Suckow (2018)). Q and P/N in the Cenozoic era denote Quaternary and Palaeogene/Neogene, respectively.

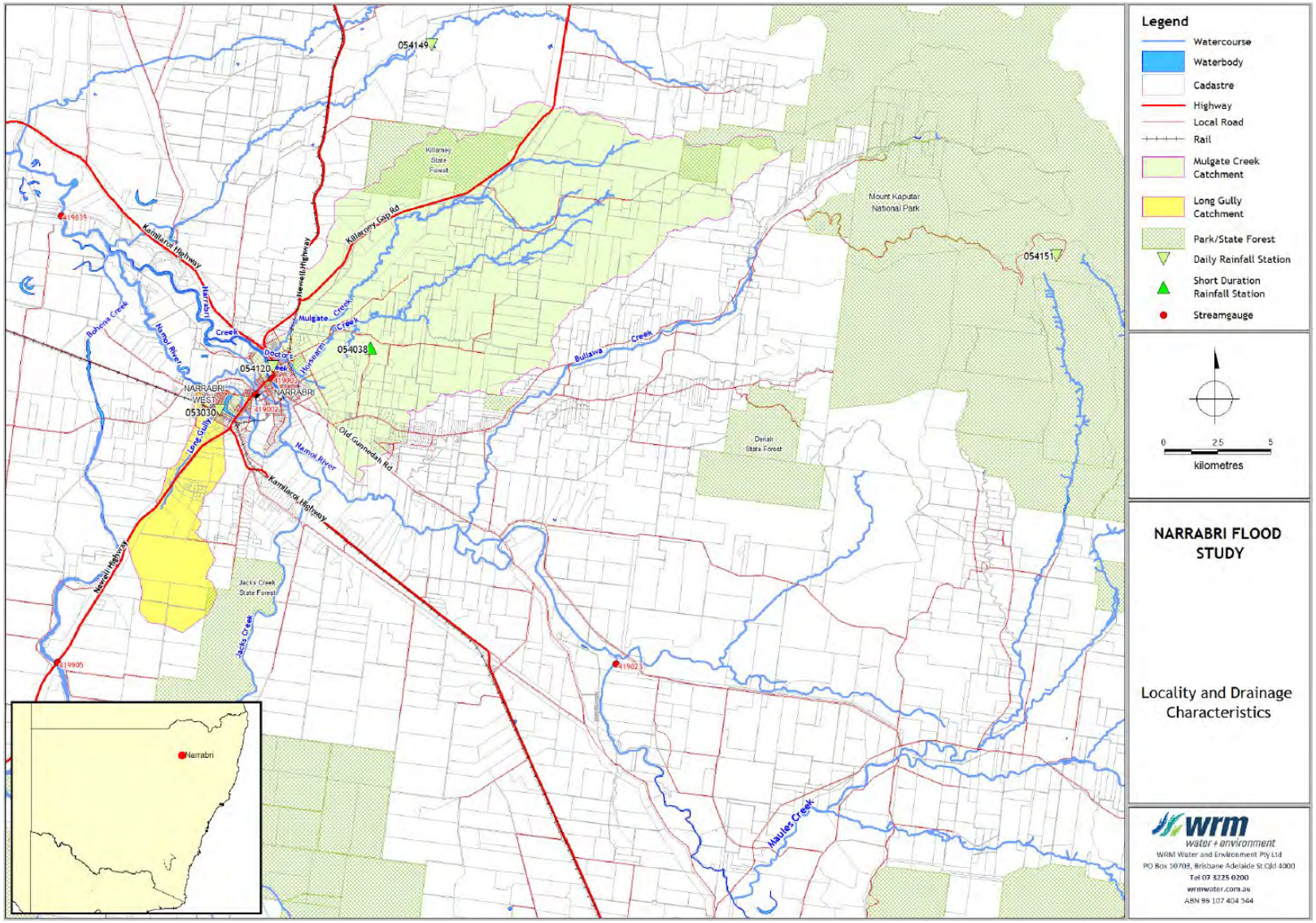



**Figure 7 Narrabri water catchment map**

Reproduced with permission from Narrabri Shire Council (WRM, 2016).

Upstream of the township of Narrabri, the Naomi River channel splits in two with the northern channel (Narrabri Creek) carrying most of the flow. Narrabri Creek eventually rejoins the Namoi River at Mollee Weir (Green et al., 2011). That the flow is principally through Narrabri Creek and not the Namoi River is surprising given the names of these waterways. Local knowledge suggests human activity may have altered the flow of water through these waterways in the 1950s, though this remains speculative and evidence of its occurrence is not readily available. Other prominent freshwater bodies in the Narrabri area are Yarrie Lake (a purported crater lake), 17 km southeast of the township of Wee Waa and currently used for recreational purposes, and 'Dripping Rock', a waterfall and waterhole situated on crown land 10 km southeast of Maules Creek (OzArk Environment and Heritage, 2020). Downstream of Narrabri, many small wetlands and lagoons, anabranches (diversions from the main channel), as well as floodplain woodlands occur (Green et al., 2011). Previous studies have shown that the Naomi River floodplain contains numerous river channels that used to be active in the past but are now extinct. On the western edge of the township of Narrabri, for example, an artificial lake, named 'Narrabri Lake', was created on swampy wetlands in 1982 on one of these now extinct channels (Wray, 2009). This lake is currently being used for a range of recreational activities, including water sports.

The Namoi River is highly regulated as it is dammed in its upper reaches and, for much of its length, affected by extensive storage and agricultural use of both surface and groundwater (Wray, 2009). Therefore, typical river discharge is below what would be expected under unaffected, natural flow conditions.

In the NGP, southwest of the township of Narrabri, Bohena Creek is an important water course and tributary of the Namoi River. Originating in the dry, semi-arid Pilliga woodlands, Bohena Creek has a stream catchment of around 830 $km^2$ that is situated at low elevation, of which less than 160 $km^2$ is cleared and used for sheep and cattle grazing with the remainder designated for biodiversity conservation (Green et al., 2011; Welsh et al., 2014). Bohena Creek flows northeast towards Narrabri (Herr et al., 2018a) and generally contributes little inflow under normal conditions although during wet times, significant flood inflows occur (Green et al., 2011). A recent hydrochemical assessment showed that very fresh groundwaters occur within Bohena Creek catchment including within the area of the proposed NGP (Raiber and Suckow, 2018).

## 2.6 Introduction to soils

Soil is a complex mixture of mineral material, organic matter, gas, water, and living organisms (Needelman, 2013). These constituents can vary significantly along with their relative abundances and this diversity in soil composition results in different soil 'types' that vary in physical, chemical and biological qualities.

In terms of the inorganic components, different sizes of particles e.g. clay, silt or sand can affect soil texture (Figure 8). The mineralogy of this fraction can affect the chemical and physical properties of the rock such as shrinking and swelling phenomena during wetting and drying depending on the content of individual clay minerals (Needelman, 2013).

In addition to inorganic components, the amount of soil organic carbon that is at varying stages of decomposition, by contrast, is a key determinant for soil quality. Soil carbon plays important roles

in soil aggregation, water-holding properties, fertility and can (depending on the soil) also be an important sink for metals which can be immobilised in metal-polyphenolic complexes (Kumar et al., 2022).


**Figure 8 USDA Soil texture triangle**

(Ditzler et al., 2017)

The proportion of organic matter can vary markedly by soil type. Some organic rich peats, for example, are largely organic matter, while many other soils are principally mineral matter and contain low percentages of organic matter. Soils in general tend to become more depleted in organic matter content by depth. The carbon in soils at the surface is typically more labile than the carbon at depth.

### 2.6.1 Soils in the present study

Soils in the Narrabri region are typically described as clays, clay loams or sandy loams (Welsh et al., 2014). The quality of the soil can be variable and depending on land use can be compacted by stock and grazing activities and impacted by erosion (Tongway and Ludwig, 1990). According to the Australian Soil Resource Information System (ASRIS, 2011), 55 soil descriptions across the Namoi river basin have been described (Welsh et al., 2014). According to the State of the Catchments (2010) statement on soil condition in the Narrabri region, the soils in the area are generally regarded as 'Fair' - 'Noticeable loss of soil function. Noticeable deterioration against reference condition'- to 'Good' - 'Slight loss of soil function. Noticeable but not significant deterioration against reference condition'. The 'Fair' soil classification is typically on grazing land close to Gunnedah and Narrabri and also on forestry land (State of the Catchments, 2010; Welsh et al., 2014).

The most abundant soil type in the Narrabri region are clay-rich vertosols in the northern agricultural-dominant area and sodosols in the south where the Pilliga Forest is located. A high

clay content in soils indicates high water holding capacities when saturated, which impeded drainage and hard setting surfaces and restrictive infiltration during heavy rainfall (State of the Catchments, 2010; Welsh et al., 2014). Among the sodosols of the Pilliga Plateau, by contrast, most of the land is characterised as having considerable limitations to agricultural land use. Nevertheless, these soils do support a variety of low intensity land uses, such as grazing, forestry and nature conservation (Welsh et al., 2014).

Based on an NSW Land and soil capability assessment (OEH, 2012), most of the vertosols of Narrabri region belong to Classes 3 and 4 with a narrow belt of Class 2 along major river valleys (Welsh et al., 2014). Class 2 denotes very good cropping land with fertile soils, while Class 3 supports most land uses with intensive management practices. Class 4 lands, by contrast, are incapable to sustain a high impact land use without the need of specialised management practices (OEH, 2012; Welsh et al., 2014). On the sodosols of the Pilliga Plateau, the land is generally characterised as having severe limitations (Class 5), with localised areas being classified as very severely limited land (Class 6). While these lands can support a range of low intensity land uses, including grazing, forestry and nature conservation, highly specialised management practices can overcome some limitations (OEH, 2012; Welsh et al., 2014).

### Vertosol

Vertosolic soils of the region are typically used for cotton and other high-value cropping. These soils occur in a band running approximately NNW towards Wee Waa, and SSE towards Tarriaro. These soils are characterised by their high clay content, with pronounced shrink-swell behaviour, leading to the formation of deep cracks (gilgai) during dry periods (Figure 9). Unlike many Australian soils, the black vertosols of the Narrabri region are relatively nutrient-rich, particularly for phosphorus content.


**Figure 9 An example of the black, vertosolic soil (S4) collected in the present study**

This soil has high clay content, and relatively low content of coarser (sandy) material (see Table 9 for details of composition).

### Sodosols and other soils

The area to the south of Narrabri is more varied with respect to soil type, though the region is typically lower in clay content (Figure 10). Indeed, through most of the Pilliga scrub sandy, sodosolic soils are relatively common. These are soils that have high sodium or magnesium content and are mostly nutrient poor.


**Figure 10 An example of the lighter coloured coarser textured, sodosolic soil (S24) collected in the present study**

This soil has considerably more coarse, sandy material with less clay content (see Table 9 for details of composition).

In addition, the region also contains chromosols, dermosol, kurosols and rudosols, and some plays that include significantly higher clay content, particularly around creeks (Figure 11).

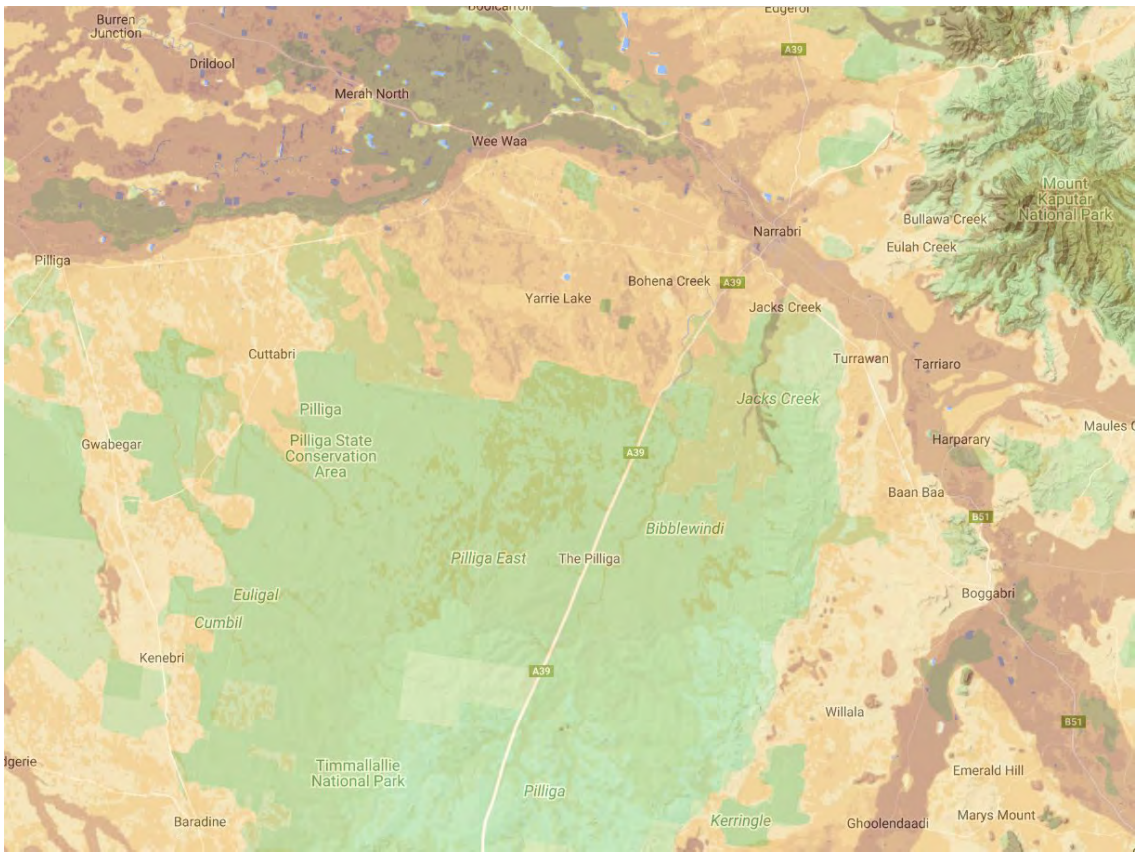



**Figure 11 The clay content of soils shown as a yellow (min) to brown (max) overlay over a map of the region**

Note the dark band of clay-rich vertosols that occur between Wee Waa, through Narrabri and Tarriaro, and the low clay content of soils of the Pilliga to the south. Data from eSPADE © State of NSW and Department of Planning, Industry and Environment 2024.

## 2.7 Introduction to microbial diversity and ecosystem function

Over the last 50 years, it has become clear that all ecosystems are underpinned by microbial communities which provide vital ecosystem services (Ali, 2023; Delgado-Baquerizo et al., 2016; Philippot et al., 2021). Microorganisms in these communities include bacteria, archaea and fungi and a range of other microscopic organisms[6]. The compositions of microbial communities are highly variable and their individual richness (a count of its species, and evenness (how equally abundant (or not) species are) determine the microbial biodiversity of a given environment.

The results of these two properties are that different communities can have different biodiversity but contain the same number of species. Below, for example, is a hypothetical community with 10 different coloured species (Figure 12). Community 1 is, however, more biodiverse than community 3 (as community 3 is mostly comprised of species 1).

[6] Specifically a large diversity of non-fungal, disparate microbial eukaryotes. This group includes various well known singled celled organisms such as paramecia or amoebas.

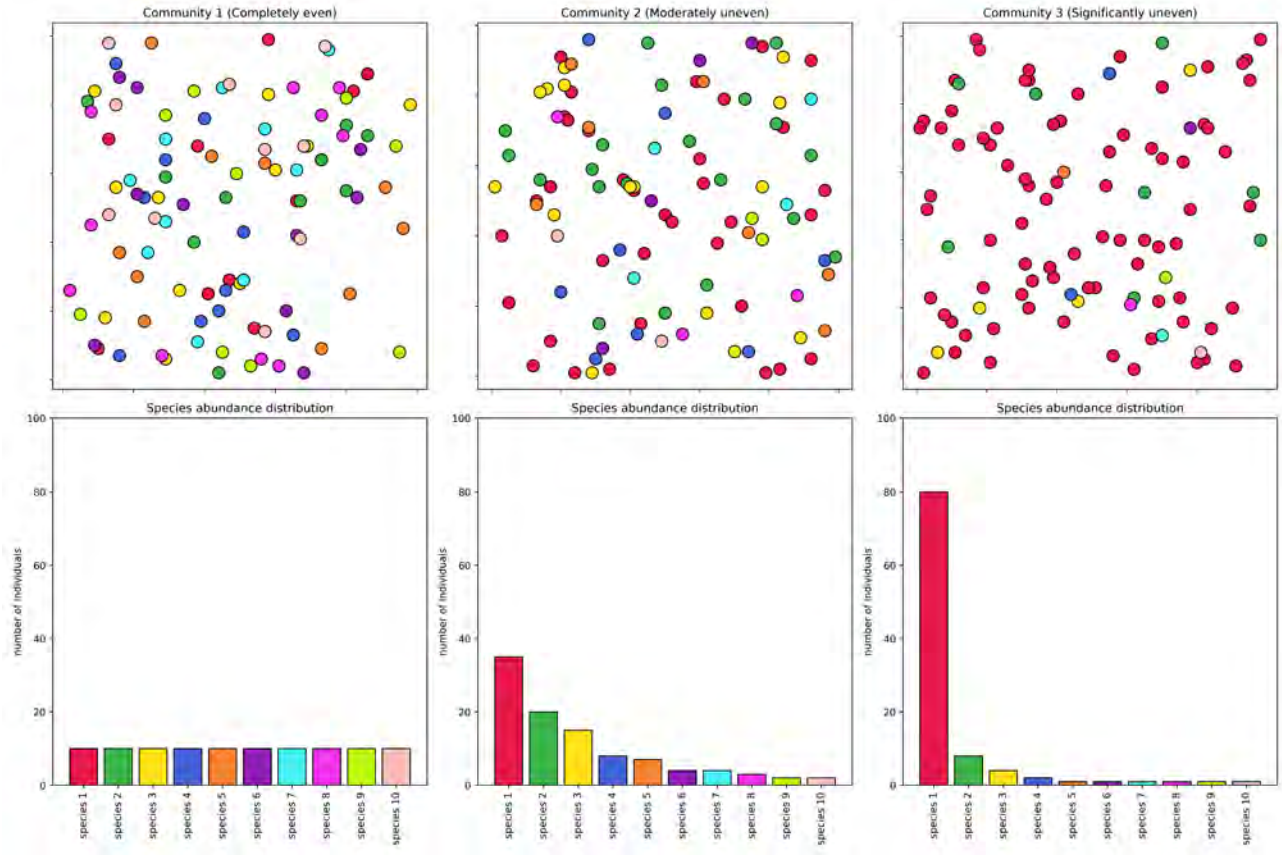



**Figure 12 Explainer for diversity (richness vs evenness)**

Recreated using Matplotlib in Python 3, adapted from a figure by David Zelený (davidzeleny.net/anadat-r)

Unsurprisingly, different environments host different numbers of microbes. Of the three sample types examined in the present study, soils host the highest microbial (and other) biodiversity. Indeed, soils have among the highest biodiversity of all environments (Anthony et al., 2023; Bardgett, 2023). For example, a teaspoon of soil (5g) may host ~5000 species and over $10^8$ cells $g^{-1}$. In contrast, aquatic environments are both less biodiverse and typically less cell dense. A study of bacteria in the Namoi River, for example, found post-flood cell counts of $3x10^5$ cells $mL^{-1}$, and outside of flood a cell density of ~$7.5x10^5$ cells $mL^{-1}$ (Balzer et al., 2021). Groundwaters typically have lower cell density still, though there are few reports of cell densities in waters of the Narrabri region. In the subsurface, these densities have been estimated at $10^2$-$10^5$ cells $mL^{-1}$ of groundwater, though densities in this environment ignore microbes attached to surfaces (Alfreider et al., 1997), and total subsurface cell densities may be more comparable with surface waters when these are considered.

Microbes are critical to life on Earth in that they underpin biogeochemical cycles on Earth (Prescott, 1999). These include the global carbon, nitrogen, sulphur and phosphorus cycles. They do this through their metabolic abilities which are distinct from those in multicellular organisms. Such processes are collectively referred to as metabolisms and are designed to provide the cells with the essentials, such as energy and nutrients. Metabolism is generally divided into catabolic and anabolic processes, which 'break down' or 'build-up' components, respectively. For example, microbial degradation of a compound like starch into simpler sugars (e.g. maltose or glucose)

through the use of amylase enzymes is a catabolic process and these type of processes are referred to as catabolism. In contrast, when bacteria take in sugars excess to their requirements, they use enzymes to make various energy storage compounds such as glycogen or poly-β-hydroxybutyrate. This construction of storage compounds like glycogen is an anabolic process, and the part of metabolism this refers to is known as anabolism.

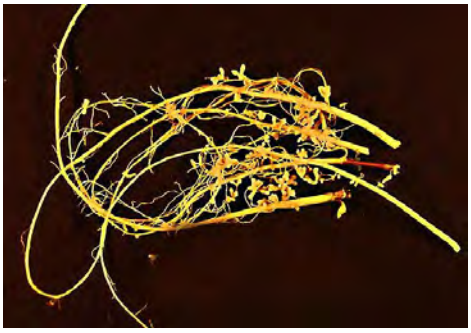
While all animals, including humans, solely use oxygen as the key compound (electron acceptor) in their energy metabolism, microorganisms, depending on their species, can use a more diverse range of components (Gibbons and Gilbert, 2015). This can include various other inorganic compounds such as nitrate, sulphate or a range of oxidised metals (like iron III or manganese IV).

Unlike animals or plants, microbes can degrade resistant compounds including even quite recalcitrant compounds like aromatic carbon compounds (e.g. benzene) or halogenated compounds. Many can also do this in environments without oxygen (called anoxic environments), meaning that microorganisms have the capacity to markedly exploit the local geology of an area, essentially remobilising ancient compounds.

To add to this complexity, various microbes engage in complex relationships with other organisms including animals or plants (Prescott, 1999). Notable examples of these processes are mycorrhizal formation and root nodulation in plants, which enable the recycling or fixing of nutrients from otherwise plant-inaccessible nutrients (Kennedy, 1999; Martin and van der Heijden, 2024; van der Heijden et al., 2015).

It is important to explain that the term ‘microorganism’ or ‘microbe’ is a generic term that includes a range of very different types of organisms united by their small size. Microbes come from all the ‘Domains’ (the highest/broadest level grouping) of life. These are the Bacteria, Archaea and the Eukarya. The last contains organisms most readers will be familiar with, e.g. fungi, plants, animals, (Table 1) while the two former domains, Bacteria and Archaea, contain organisms that look superficially similar (single celled organisms) but are genetically unrelated.

**Table 1 Information about the domains of life**

| Domain | Description | Study relevant examples |
|---|---|---|
| **Bacteria** | Bacteria lack a membrane-bound nucleus and other organelles. They are the most diverse group of life on Earth. Typically they are single-celled, though they can form filaments and complex multispecies aggregations. | Among the most common microorganisms detected in this study is *Bradyrhizobium liaoningense*. This species was originally described from soybean roots in Liaoning Province in China but has since been found globally. Like many 'rhizobia', *B. liaoningense* is known from root nodules of leguminous plants, but (given its ease of culture) presumably readily occurs in non-root associated forms in the environment (called free living). It is worth noting that Australia hosts many native nodulating, non-agricultural 'pea' species with which the species may associate.<br>  <br>The photo above shows root nodules formed by *Bradyrhizobium* species on soybean. |
| **Archaea** | Like Bacteria, Archaea lack a membrane-bound nucleus and other organelles. They were (until recently) considered to be different bacteria, but advances in our understanding of their biology and genetics indicate, while they look like bacteria, Archaea are completely unrelated. | The most common Archaea detected in this study was a novel (new) species of *Nitrososphaera*, related somewhat to *N. viennensis*. *Nitrososphaera* species are mostly ammonia-oxidising species that use energy from oxidation of ammonia ($NH_3$) to nitrite ($NO_2^-$) to power carbon ($CO_2$) fixation. |
| **Eukarya** | All other organisms, other than Bacteria and Archaea, are eukaryotes. These organisms have membrane-bound nuclei and other organelles. Most of the living organisms familiar to most people are eukaryotes.<br><br>Microbial eukaryotes include fungi and a host of other organisms that were part of a group known as 'protists'. For the purpose of this report, only fungi are considered, though soils and waters of the Narrabri region undoubtedly host a vast diversity of 'protist' taxa. | An example of a fungus relevant to this study is *Pisolithus marmoratus*. This fungus forms associations (ectomycorrhiza) with Eucalyptus, Casuarina and other Australian native tree species. It was observed in many baseline samples from the Narrabri region.<br>  <br>*Pisolithus marmoratus*. Photo by Martin Lagerwey |

Both the Archaea and Bacteria are single celled (Table 1), and it is thought that the Eukarya may have arisen (in the distant geologic past) through endosymbiosis between the two groups (Figure 13). It is important for readers to note that while Archaea were originally regarded as occurring in

extreme environments (like hot springs), many non-extreme Archaea have since been identified in more mundane locations including soils and groundwater.

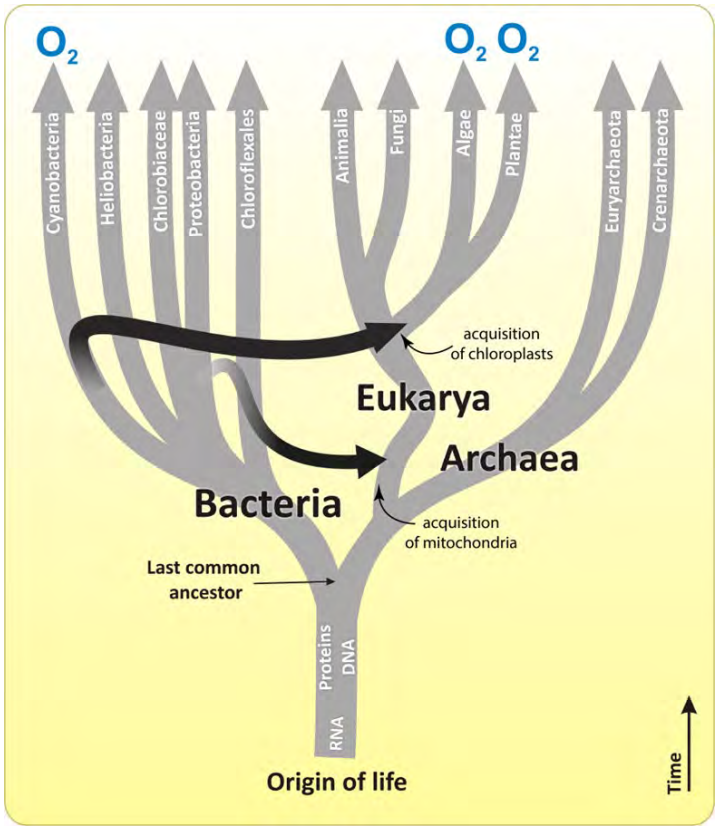



**Figure 13 The metaphorical 'Tree of Life' of common descent**

Grey arrows highlighting the three domains: Bacteria, Archaea, and Eukarya. This diagram emphasizes the significance of horizontal gene transfer in evolutionary processes (black arrows) and the role of oxygen ($O_2$) production in algae, plants, and cyanobacteria. Additionally, it explores the foundational interplay between RNA, DNA, and proteins in the origins of life.

Modified from Govindjee and Shevela (2011)

## 2.8 Molecular approaches to microbiology

Modern microbiological studies are underpinned by the application of molecular, DNA-based approaches in identifying what microbial species occur in a certain environment and their relationship to other microorganisms (also known as phylogenetics). Unlike studies using animals and plants with often well-defined morphologies and more easily discernible physiologies, microbial cells, especially prokaryotic ones, exhibit very simple cell morphologies. Consequently, it is difficult to ascertain even basic taxonomic status (e.g., domain, kingdom, phylum, and class) based on morphology alone. Furthermore, only a very small percentage (< 1%) of prokaryotic microorganisms have been cultured under laboratory conditions (Schloss and Handelsman, 2004), prohibiting any classification using their physiology. However, it is important to state that while more than 99% of these microbes have remained elusive so far, they are not "unculturable"; rather their range of growth conditions have either not yet been determined or been replicated for them to survive and grow under laboratory conditions (Wang et al., 2021a).

The difficulty in discerning microbial diversity can largely be resolved through the application of culture-independent DNA-based techniques provided through the polymerase chain reaction

(PCR) amplification technique (Olsen et al., 1986; Pace, 1997). A relatively cheap and rapid option involves the extraction of DNA directly from the environment (e.g., soil, water) and the sequencing of a region of the microbial genome that encodes the structure of 16S rRNA (ribosomal RNA). All bacteria and archaea share this gene, yet each variant involving different species or strains is unique in barcoding individual taxonomic groups (Pace, 1997). Ideally, these sequences can be compared to those that were able to be cultured and their metabolism elucidated or at least predicted (Newman et al., 2012). However, microorganisms can be either metabolically diverse (i.e., able to use multiple metabolisms depending on substrate availability) or are poorly characterised with no close taxa for comparison. In addition, since PCR amplification employs DNA sequences called primers that are designed to amplify conserved segments of 16S rRNA genes, it may miss taxa that do not possess these sequences in their genes (Newman et al., 2012).

In this study the 16S rRNA gene, and an analogous nuclear ribosomal region called Internal Transcribed Spacer (ITS) in fungi, are used to determine what microorganisms occur in each natural environment or in one of the many subsequent experimental microcosm studies. As mentioned previously, these genes contain highly conserved regions that are similar across all microorganisms, as well as variable regions that can provide more specific taxonomic differentiation. These variable regions are used to determine the taxonomic grouping to which microorganisms belong (e.g., what species, genus, family etc).

Microbial baselining is the process of establishing the normal composition and variability of microbial communities in a specific environment. This typically involves collecting comprehensive data on the types and abundances of microorganisms present in a particular setting, such as soil (Cluzeau et al., 2012), water (Pinar-Méndez et al., 2022), the subsurface (Amann et al., 1997), the bodies of humans and other animals (Ross et al., 2019) or built environments like hospitals or food production facilities (Vargas et al., 2023).

The last few decades have seen biological assessment techniques reach a state where it has been rated more heavily than physicochemical measures in determining the environmental health (Pawlowski et al., 2018). This outcome has largely been achieved through advances in genomic techniques and their application to cost-effectively and comprehensively assess biological "health" indicators in a timely manner (Pawlowski et al., 2018). DNA-based baseline assessments rely on developments in next-generation sequencing which enable the comprehensive "counting" of all organisms in a given environment using DNA extracted directly from the environment (also known as ‘environmental DNA’ or ‘eDNA’). Consequently, this allows the identification of all members of various microbial groups present in a particular environment using informative marker genes such as 16S (see Figure 14). In addition to using fluctuations in microbial communities to measure change, these methods allow the identification of organisms that may be sensitive to particular environmental contaminants. Indeed, these more sensitive organisms may then be a specific focus of monitoring efforts. This field is generally known as either biomonitoring or metabarcoding and increasingly comprises a body of research which demonstrates its efficacy for monitoring and managing environmental change (Bohmann et al., 2014; Cristescu, 2014; Darling et al., 2017; Deiner et al., 2017; Keck et al., 2017; Leese et al., 2018; Valentini et al., 2016).

With regards to CSG activity, defining baseline microbial compositions allow for a better understanding of the impacts of an accidental chemical spillage. Therefore, before any spillage occurs, detailed samples are collected from the unaffected natural environment to identify and quantify the types of microorganisms present under normal conditions. This may include identifying prevalent microbial species and understanding their roles in the native ecosystem.

If a chemical spillage occurs, the same sampling techniques as used in the baselining work are applied to detect any changes in the microbial community. In such studies, shifts in microbial diversity, decreases or increases in specific populations, and changes in microbial activities are investigated (Overholt et al., 2019). By comparing post-spill conditions to the baseline, it is possible to identify which microorganisms that are most affected by the spillage, how community composition has changed, and potential ecological impacts.

Indeed, microbial baselining serves as a crucial tool for quantifying the ecological impacts of chemical spills and guiding effective environmental management and restoration efforts. Understanding baseline conditions aids in designing intervention strategies, helping to determine which aspects of the microbial community are critical for ecosystem functions that need to be restored.

## 2.9 State of knowledge: microbial communities in the NGP

The Narrabri region has been host to many studies on microbiology. The vast majority of these studies, however, are focussed on vertosolic soils used for cotton (and other) cropping. These include numerous studies on plant pathogens (Knox et al., 2006; Le et al., 2022; Mondal et al., 2004; Nehl et al., 2004; Ogle et al., 1993), mycorrhizas (Knox et al., 2008; Loke, 2007; McGee et al., 1997; Pattinson and McGee, 1997; Rosendahl et al., 2009) nitrogen-fixing bacteria (Diatloff and Brockwell, 1976; Oparah et al., 2024) and general soil microbiology (Gupta et al., 2018; Knox et al., 2016; Knox et al., 2014; Midgley et al., 2007a; Polain et al., 2021).

Outside of agricultural soils, however, there is a dearth of information on microbiology in the region, especially in the non-vertosolic soils. There are a handful of studies that warrant discussion, including two studies of groundwater microbes or microbial capabilities in groundwaters by Iverach et al. (2020) and Korbel et al. (2013). Of the two studies, Iverach et al. (2020) identified microbes in 28 groundwaters (and also included a sample from the Namoi River for comparison). In contrast, Korbel et al. (2013) examined 15 groundwaters in a somewhat overlapping region. It is noteworthy that Korbel et al. (2013) characterised microbial activity and capacity (on biology plates and against cellulose strips) rather than microbial identity.

There is a single study of archaeal diversity in soils of the region, though it contains no data from non-vertosolic soils in the NGP (Midgley et al., 2007a). It does, however, include two remnant forest soils (also vertosols) from within the project area both of which are from vertosolic soils in Brigalow Nature Reserve and Brigalow Park State Conservation Area (formerly Claremont Nature Reserve) southeast of Yarrie Lake.

There does not appear to be any studies of the bacterial diversity in soils of the region outside of studies of agricultural soils. In addition, prokaryotic microbiology on the surface waters of the area

is also extremely limited, noting the single sample of the Namoi River examined by Iverach and co-workers.

While no other studies appear to have described bacterial communities in surface waters, there are several other microbial investigations of the Namoi River. For example, Mitrovic et al. (2014) undertook microbial cell counts (but not identification) in the Namoi River near Boggabri. The Namoi River was also included in a study of microbial gene cassettes (Stokes et al., 2001), though it is unclear where on the Namoi River this sample was taken. There are also numerous bacterial isolates which have been obtained from undisclosed locations in the Namoi River. For example, *Vibrio cholerae* 'N60' was isolated from the Namoi River in 1981 (Desmarchelier and Reichelt, 1982). No studies appear to have been conducted into the fungal ecology or fungal diversity in surface waters of the region.

In terms of soil fungi, there are two studies by Midgley and co-workers, in 2007b and 2011, which explore basidiomycete (Midgley et al., 2007b) and ascomycete communities (Midgley et al., 2011), at a single site in Jack's Creek State Forest. Like Midgley et al. (2007a), these studies also sampled Brigalow Nature Reserve and Brigalow Park State Conservation Area, however, while these sites are within the boundaries of the NGP area both sites host vertosolic soils. It is important to note that Brigalow Nature Reserve is specifically excluded from the project area, though it is surrounded by the project area (Figure 1). Regardless, both studies demonstrate that significant fungal diversity occurs in the sodosolic soils of the region, including numerous novel species.

There has also been one sporocarp survey in the broader region, though these were in Mount Kaputar National Park (Danks et al., 2013). This study recorded a number of novel, truffle-like taxa, though the soil type (mainly rudosols) (NSW DPE, 2022) and vegetation likely differ substantially from the area in the NGP.

In addition to this sporocarp survey, a novel species of morel (*Morchella australiana*) was collected (and described) in the southwestern area (near Bugaldie) of the Pilliga (Elliott et al., 2014), though this was outside of the NGP area. No other fungal collections were apparent at the time of writing.

There are also other incidental reports of fungi in the region, these include brief mentions in papers with other purposes, or citizen science initiatives. One example of the former is a report of fungus beetles in association with '*Polyporus*' (now *Laccocephalum*) *mylittae* in an undescribed location in Pilliga East State Forest (Webb and Simpson, 1991). Examples of the latter can be found via FungiMap[7] and iNaturalist[8], however, these data are mostly photos of sporocarps and common (and easily identified) fungi such as *Pycnoporus cinnabarinus* predominate these datasets in this region. It should be noted that along with these more common fungi, FungiMap and iNaturalist also host a number of unidentified fungal photos from the region with location identifiers. Once more significant data is available on the fungi of the region, these may prove valuable in understanding the range of poorly understood species in the region.

[7] https://fungimap.org.au/

[8] https://www.inaturalist.org/

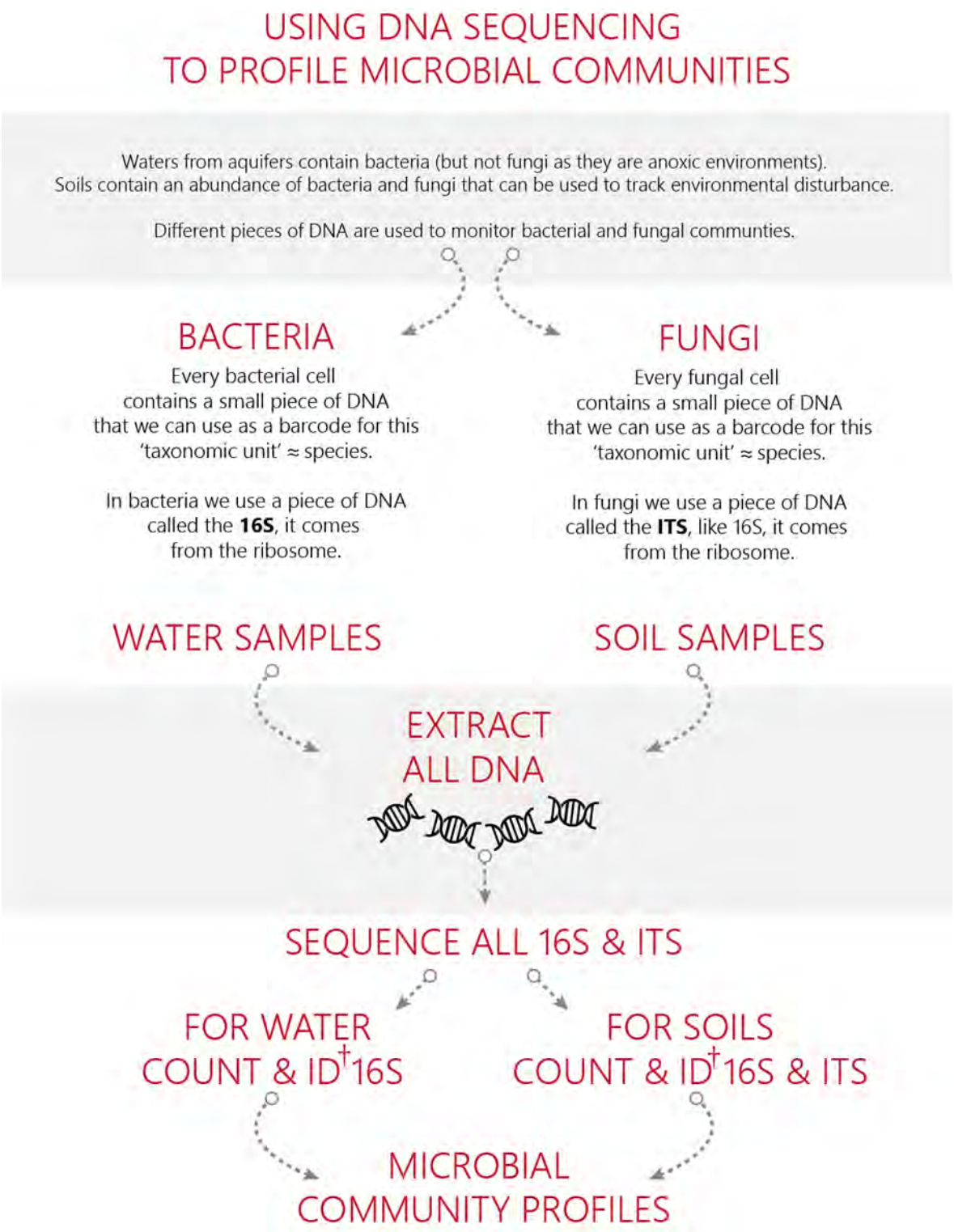



**Figure 14 Microbial community profiling using environmental DNA and next-generation DNA sequencing approaches**

† ID- Identification using Bayesian classifiers and a bacterial 16S database (Cole et al., 2014) and a fungal ITS database (Deshpande et al., 2016)

## 2.10 Chemicals of potential concern used in gas production

The production of natural gas in onshore settings requires the use of various chemicals that perform a range of functions. These chemicals are required at various stages of the gas production process, for example, some chemicals are only used during drilling, while others are used during the production phase. Furthermore, other chemicals are situationally used as required for individual projects (Schinteie et al., 2019). For example, some biocides are used only when odours are detected in surface water facilities. Most of these chemicals have relatively limited risk profiles and are known to degrade in the environment, however, some chemicals have elevated risks.

Drilling fluids, used to assist in the drilling of boreholes, are essentially a mud composed of a mix of clay minerals and either water, oil or synthetic organic matter (Hyne, 2019). To these muds, various chemicals additives are added to produce a desired effect such as thinners, foaming agents, flocculants, pH controls, and emulsifiers to disperse mixtures (Hyne, 2019; Schinteie et al., 2019). Workover fluids are employed in an already completed well for maintenance and to increase productivity (Fink, 2015). These fluids can be water, oil, emulsions or foam-based and the final makeup depends on the structure and composition of the well (Speight, 2016). In addition, various biocides are used to kill or control harmful organisms. Such biocides are chemical substances that have the capacity to destroy, deter, or exert a controlling effect on any harmful organisms. The current study focussed on a range of chemicals used in the above-mentioned processes. For introductory purposes, each of the chemicals examined in this study are described below and in Table 2.

Table 2 Chemicals examined in this study

| Chemical | Abbreviation | CAS # | Additive role | Notes |
|---|---|---|---|---|
| **Alcohol ethoxylate mixture #1** | AEM#1 | 78330-21-9 | Surfactant | |
| **Alcohol ethoxylate mixture #2** | AEM#2 | 69011-36-5 | Surfactant | |
| **Hydrocarbon mixture #1** | HCM#1 | 64742-47-8 | Workover | |
| **Hydrocarbon mixture #2** | HCM#2 | 90622-58-5 | Workover | |
| **Monoethanolamine** | MEA | 141-43-5 | Viscosity management/ drilling additive | |
| **3,5-dimethyl-1,3,5-thiadiazinane-2-thione** | DAZ | 533-74-4 | Biocide | The breakdown product of DAZ, methyl isothiocynate, was tested in addition to DAZ itself |
| **Glutaraldehyde** | GLU | 111-30-8 | Biocide | |
| **3,3'-methylenebis(5-methyloxazolidine)** | MBO | 66204-44-2 | Biocide | The breakdown product of MBO, 5-methyloxazolidine, was tested in addition to MBO itself |

## 2.10.1 Alcohol ethoxylates

In this study two different types of alcohol ethoxylates were used as non-ionic surfactants (see below). These molecules include *n* ethyoxlate units with 'R' representing a long hydrocarbon chain (Figure 15). Alcohol ethoxylates are non-ionic surfactants, the long hydrocarbon chain is hydrophobic while the repeated ethoxylate units are hydrophilic.

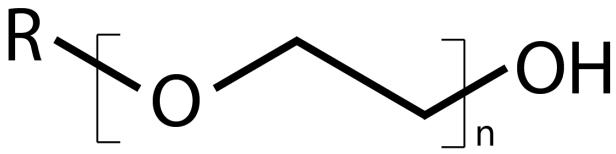



Figure 15 General chemical structure of alcohol ethoxylates

See text for description.

## Alcohol ethoxylate mixture #1

An alcohol ethoxylate mixture #1 (AEM#1) with CAS number 78330-21-9 was used in this study. It consists of $C_{11-14}$ iso-alkyl ethers with the $C_{13}$ iso-alkyl ether (polyoxyethylene (10) tridecyl ether) predominating (Figure 16). It is worth noting that the position of the methyl groups is unknown. The hydrophobic tail is derived from tridecyl alcohol, which is a 13-carbon long alkyl chain (13 = tridecyl). This long chain is responsible for the hydrophobic affinity of the surfactant with oils and

nonpolar substances. The hydrophilic head portion is composed of a polyoxyethylene chain. The '10' in the compound name indicates that there are 10 ethylene oxide units in this chain (Figure 16). Each ethylene oxide unit consists of two carbon atoms and one oxygen atom forming an -O-$CH_2$-$CH_2$- linkage. This mixture of hydrophobic and hydrophilic structures allows the compound to act effectively as a surfactant by reducing surface tension and enhancing the mixing and dissolution of different phases, such as oil and water.

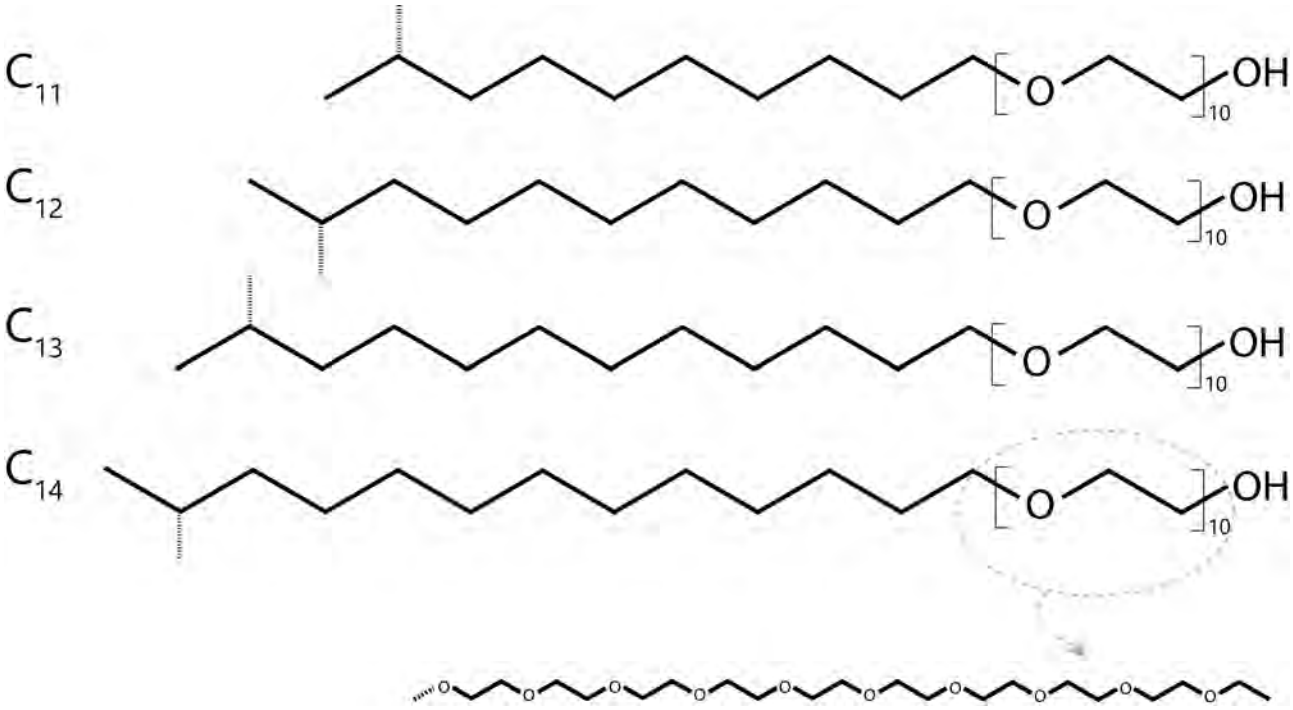



**Figure 16 Chemical structure of Alcohol Ethyoxylate mixture #1**

Alcohol Ethyoxylate mixture #1 (AEM#1) contains polyoxyethylene (10) undecyl ether, polyoxyethylene (10) dodecyl ether, polyoxyethylene (10) tridecyl ether and polyoxyethylene (10) myristyl ether.

## Alcohol ethoxylate mixture #2

Another alcohol ethoxylate mixture (AEM#2) with CAS number 69011-36-5, used in workover processes (EHS, 2024a), was examined in this study. This mixture includes isotridecanol monoethoxylate, diethoxylate and triethoxylate (Figure 17). Similar to the other alcohol ethoxylate mixture, AEM#2 is a surfactant with a hydrophobic tail consisting of a branched alkyl chain attached to a hydrophilic polyoxyethylene chain. The relative proportions of these three compounds within the mixture are unknown.

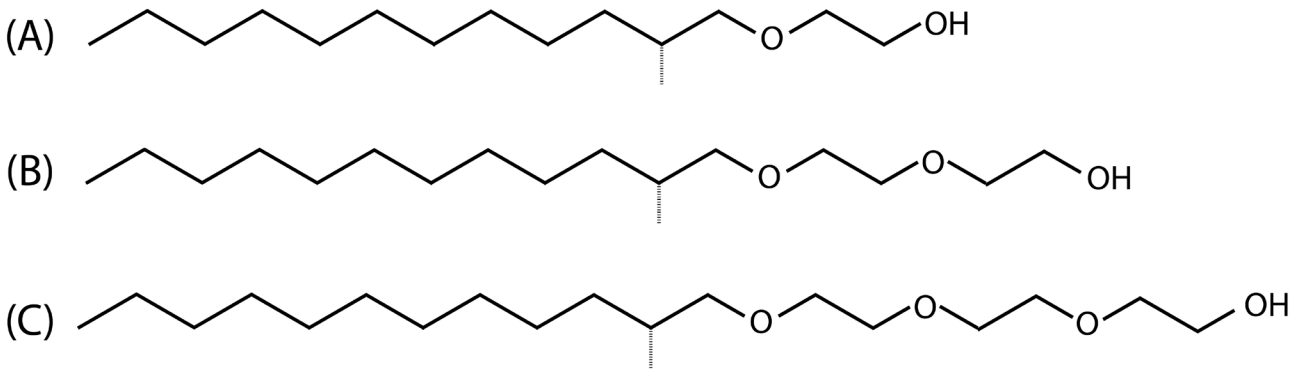



**Figure 17 Chemical structure of Alcohol Ethoxylate mixture #2**

Contains a mixture of isotridecanol monoethyoxylate (A), isotridecanol dioethyoxylate (B) and isotridecanol triethyoxylate (C) (referred to in this study as AEM#2)

### 2.10.2 Hydrocarbons

Two different hydrocarbon mixtures were used in this study, which are both used in the workover process. One mixture, with CAS number 64742-47-8, is referred to as hydrocarbon mixture #1 (HCM#1), including $C_{12\text{-}15}$ *n*-alkanes, iso-alkanes, cyclics, and less than 2% aromatics. The other mixtures, with CAS number 90622-58-5, is referred to as hydrocarbon mixture #2 (HCM#2) including $C_{11\text{-}15}$ iso-alkanes.

Hydrocarbons are a group of compounds made up entirely of hydrogen and carbon atoms (McMurry, 2023) (Figure 18). These compounds can be classified into different types, such as aliphatic and aromatic compounds (Figure 18). Alkanes, major aliphatic compounds, are hydrocarbons of carbon and hydrogen atoms where all the carbon-carbon bonds are single bonds and have the general chemical formula $C_nH_{2n+2}$ (Figure 18). They can have a straight-chain appearance without any branches called *n*-alkanes, side branches from a straight-chain appearance called *iso*-alkanes and cyclic appearance where the carbon atoms are arranged in a ring structure called cyclic alkanes (Figure 18). Aromatic compounds consist primarily of carbon and hydrogen atoms with a stable, planar ring structure with alternating double carbon bonds (McMurry, 2023) (Figure 18).

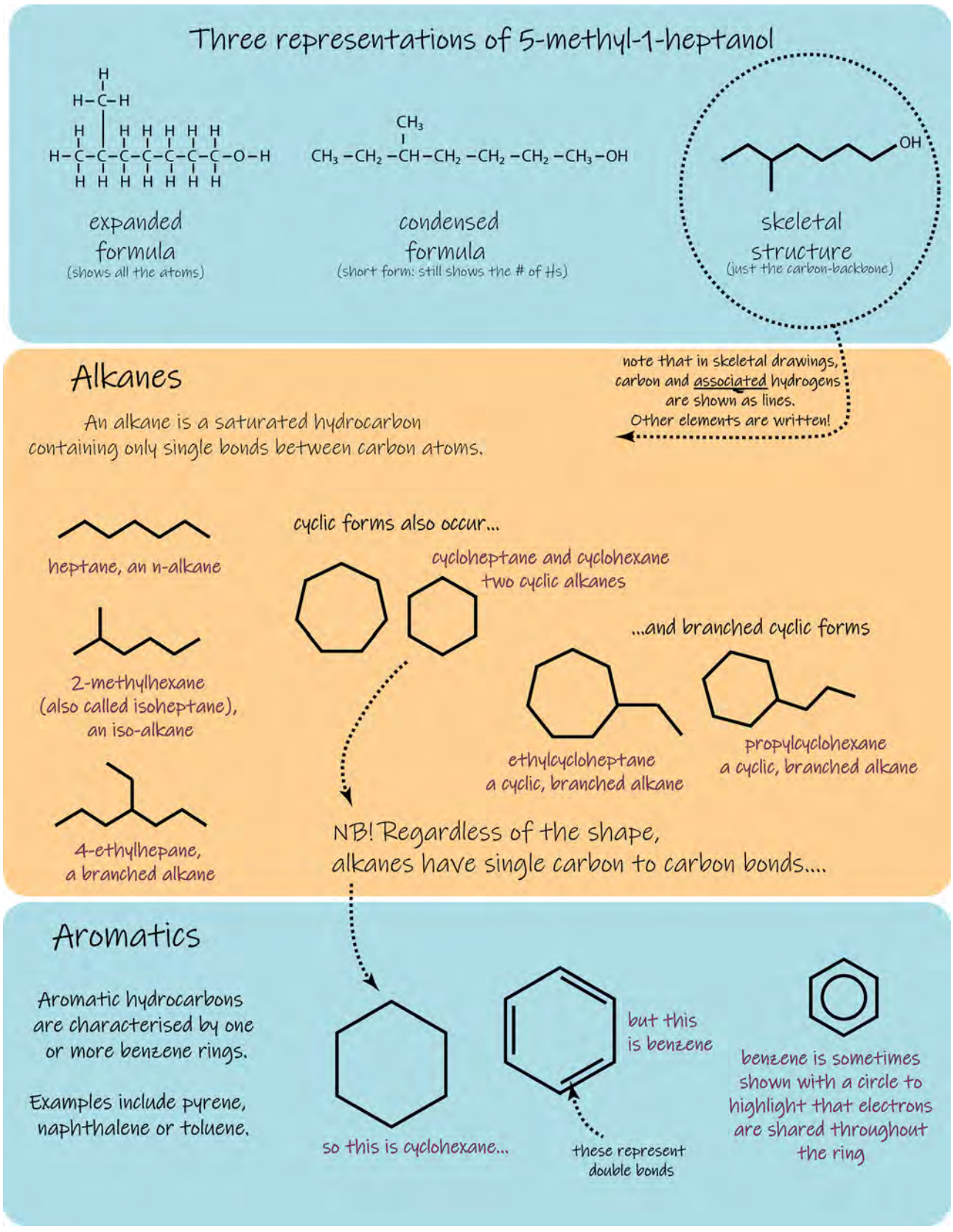



**Figure 18 Primer to hydrocarbons, drawing chemical structures and alkanes and aromatic compounds**

### 2.10.3 Monoethanolamine

Monoethanolamine, also known as ethanolamine, the mono prefix is used to distinguish it from di- and tri- ethanolamines. The sourced product has CAS number 141-43-5. Structurally it is a two-carbon chain with an amino and a hydroxy substituent at either end making it both a primary amine and a primary alcohol (Figure 19). This compound is used as a component in drilling fluids (EHS, 2024b).

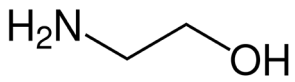
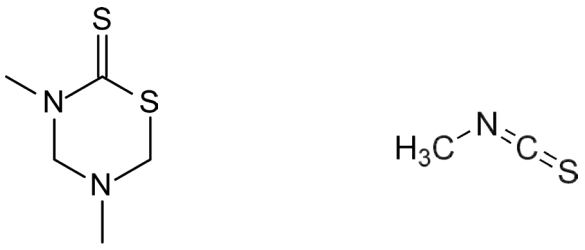



**Figure 19 Chemical structure of monoethanolamine**

### 2.10.4 Dazomet and methyl isothiocyanate

Dazomet (DAZ) is the trade name for a biocide and fumigant (CAS # 533-74-4). Structurally, DAZ is a thiadiazine (NCBI, 2024b). Structurally, DAZ is a heavily modified thiadiazine ring that includes two methyl groups and a doubled-bonded sulphur attached to the ring (NCBI, 2024b)(Figure 20). DAZ is unstable on contact with water and breaks down in a series of hydrolysis reactions to methyl isothiocyanate (MITC) (Gadagbui et al., 2014), the principal active compound that is acutely toxic to a range of organisms. It also produces other breakdown products including formaldehyde, monomethylamine and hydrogen sulphide.




Dazomet Methyl isothiocyanate

**Figure 20 Chemical structures of dazomet and methyl isothiocyanate**

### 2.10.5 Glutaraldehyde

Glutaraldehyde (GLU) is a commonly used biocide (CAS #111-30-8). Structurally, GLU is simple compound that is essentially a pentane with aldehyde functional groups on either end (Figure 21). GLU is a highly effective biocide in that is readily reacts with organic compounds, such as proteins, often forming cross-links which disrupt cellular function (NCBI, 2024a) (Matei et al., 2020).


**Figure 21 Chemical structure of glutaraldehyde**

### 2.10.6 3,3'-Methylenebis(5-methyloxazolidine)

3,3'-Methylenebis (5-methyloxazolidine) described here as MBO, is a molecule that is comprised of two 5-methyl oxazolidine units joined by a methylene ($CH_2$) 'bridge' between the two nitrogen atoms. MBO is unstable in water, which attacks the N-C bonds in the methylene bridge, breaking the compound into two methylated oxazolidines. These methylated oxazolidines are then subject to further attack by water, resulting in the release of formaldehyde and the production of 1-aminopropan-2-ol (Figure 22) (EHS, 2024c) (EHS, 2022; Madan and Beck, 2006).

Due to this instability, MBO was not used for analysis. For this study, 5-methyloxazolidine was used as the analytical target compound in the degradation experiments of this study.

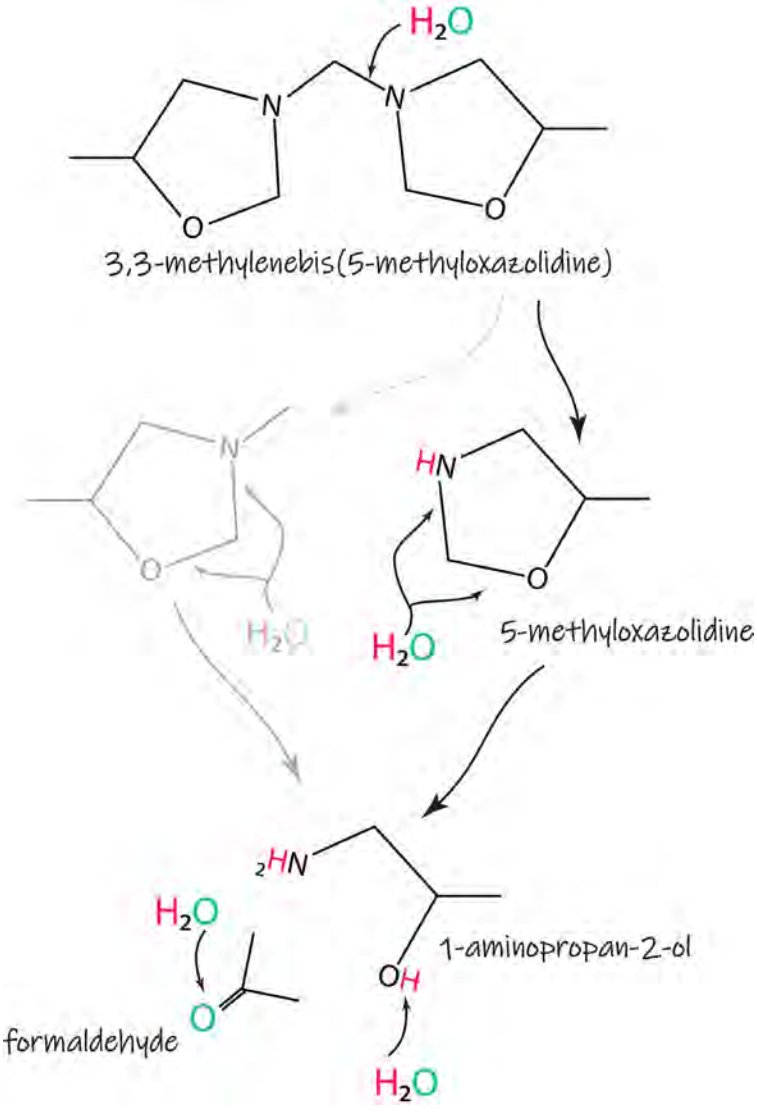



**Figure 22 Chemical structure of the oxazolidine compound used in this study**

## 2.11 Microbial degradation of chemicals

The diversity of microbes within soil, surface water, or groundwater is directly related to the capacity of a given sample or environment to degrade a given xenobiotic (a non-indigenous) chemical. This is because an individual microbial genome contains 1,500 to 8,000 genes for bacteria and 10,000 to 60,000 genes in most fungi. These genes have specific roles and capabilities and having more genes available at a community level enables the community to catabolise (degrade) more substrates. It is also important to note that chemicals that are harmful to humans and other organisms, are not necessarily harmful to bacteria, archaea or fungi. For example, the compound benzene while a known carcinogen, numerous microbes (especially in the Desulfobacteraceae for example) can use benzene as a sole source of carbon for growth. Such factors are important to keep in mind when natural soils or waters become contaminated by xenobiotic chemicals used in various human-led processes such as agricultural usage or onshore gas exploration and/or production.

### 2.11.1 Microbial degradation of alcohol ethoxylates

To degrade alcohol ethoxylates under oxic conditions, microbes typically use monooxygenases, that specifically target the ether bonds within the ethoxylate chains of the surfactants. This enzymatic action cleaves ethers in the centre of the molecule, producing by-products such as polyethylene glycols (PEGs) and alcohols (Budnik et al., 2016; Cain, 1994; Ji et al., 2019a; Tidswell et al., 1996; Witkowska et al., 2018; Zembrzuska et al., 2016) (Figure 23).

The chain length of the alkyl group and the number of ethoxylate units in the molecule play significant roles in governing how easily individual compounds are degraded. In general, pure cultures cannot mineralise the entire molecule, and as such multiple species working in concert may be required for complete degradation (Cain, 1994). Generally, shorter and simpler ethoxylate chains are more susceptible to microbial attack and degrade more quickly. Branched chains can hinder enzymatic access to these ether bonds, limiting degradation. This may be more of an issue under anoxic conditions (Mösche, 2004).

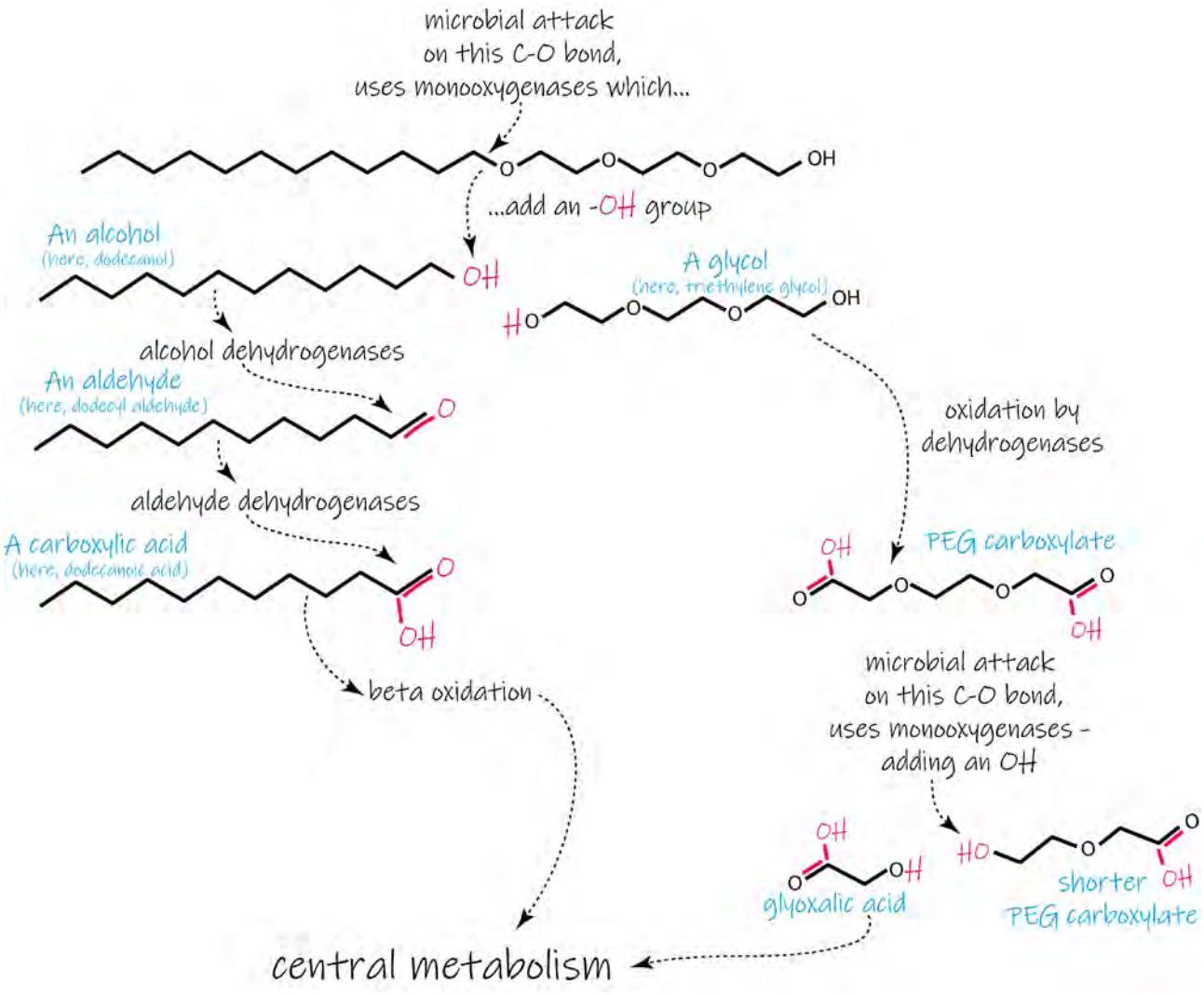



**Figure 23 Schematic for the central fission pathway for degradation of detergents**

Adapted from Cain, 1994.

## 2.11.2 Microbial degradation of hydrocarbons

Microbes have evolved numerous metabolic pathways to degrade hydrocarbons under oxic and anoxic conditions, using different aliphatic and aromatic hydrocarbon strategies (Ghosal et al., 2016; Ławniczak et al., 2020; Leahy and Colwell, 1990).

### Aerobic hydrocarbon degradation

In the presence of oxygen, bacteria can efficiently degrade aliphatic and aromatic hydrocarbons. Aliphatic hydrocarbons, which consist of straight or branched chains, are typically broken down through a series of oxidation reactions. The process often begins with an enzyme called monooxygenases, which incorporates an oxygen molecule into the hydrocarbon. In terminal oxidation, the oxygen is added to the terminal (end) carbon of the chain, converting it into an alcohol (Figure 24). This alcohol is subsequently oxidised into an aldehyde and then into a fatty acid (Markovetz and Kallio, 1971; Rojo, 2009; Singh et al., 2011; Wang and Shao, 2013). The fatty acid enters the β-oxidation pathway, where it is broken down step by step to produce acetyl-CoA, a

key molecule that feeds into the tricarboxylic acid (TCA) cycle for energy production and biomass synthesis.

In subterminal oxidation, the initial oxygenation occurs at a carbon near the end of the chain, forming secondary alcohols (Figure 24). These alcohols are further converted into aldehydes and are then cleaved at an ether bond, resulting in acetate (which is funnelled to central metabolism) and a terminal alcohol which can then be processed via terminal oxidation.

Aromatic hydrocarbons, with their stable ring structures, are more complex to break down. Under aerobic conditions, bacteria often employ monooxygenases to transform these compounds into simpler, more reactive intermediates like catechol (Díaz et al., 2013; Fuchs et al., 2011). Catechol serves as a key gateway molecule that undergoes enzymatic ring cleavage. These cleavage reactions produce smaller molecules that can be processed through the TCA cycle, enabling the complete mineralization of the aromatic structure.

**Anaerobic hydrocarbon degradation**

When oxygen is not available, bacteria use alternative strategies to degrade hydrocarbons. The anaerobic breakdown of aliphatic hydrocarbons often begins with a unique process called fumarate addition. In this pathway, bacteria add fumarate to the hydrocarbon, forming an alkylsuccinate (Ji et al., 2019b; Tan et al., 2014; Tan et al., 2015) (Figure 24). This compound undergoes subsequent reactions that convert it into fatty acids, which can then be metabolized through other pathways to yield energy and cellular building blocks.

Aromatic hydrocarbons present an even greater challenge under anaerobic conditions due to their stable ring structures. One of the primary strategies bacteria use is to activate these compounds by forming benzoyl-CoA, a central intermediate. Benzoyl-CoA is reduced by benzoyl-CoA reductase, which catalyses the dearomatisation of the ring, making it more amenable to breakdown (Boll et al., 1997; Breese et al., 1998; Porter and Young, 2014). The products of these reactions are linear molecules that can be further processed into acetyl-CoA and incorporated into the cell's energy and biosynthesis pathways.

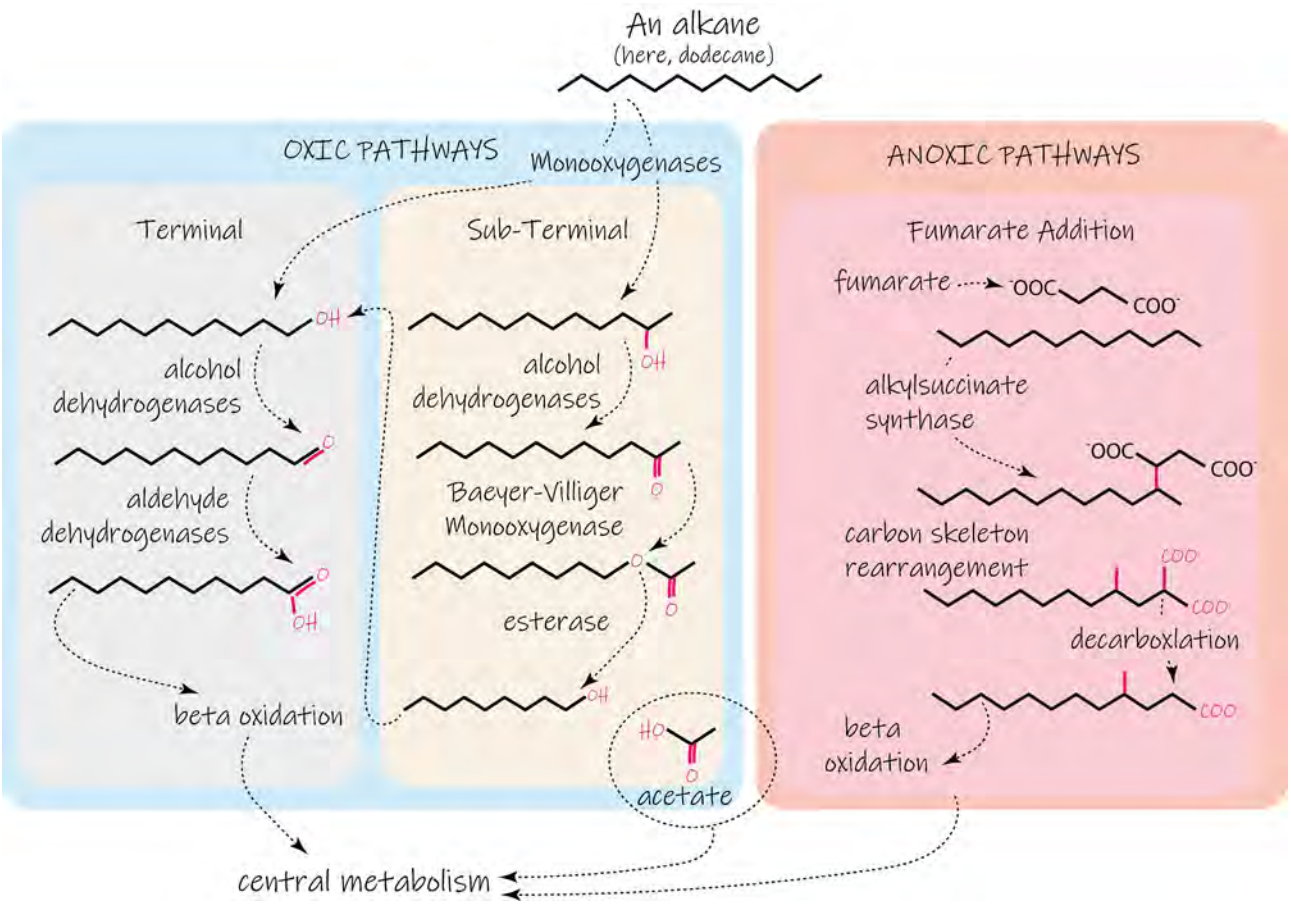



**Figure 24 Schematic showing microbial degradation of n-alkanes via terminal and subterminal oxidation processes, along with the anaerobic fumarate addition pathway**

### 2.11.3 Microbial degradation of monoethanolamine

Monoethanolamine is two carbon compound with a hydroxyl group on one end and an amine group on the other end. Growth, at least for some bacteria, appears to be facilitated by an ethanolamine-ammonia lyase. This pathway is best studied in a range of environmentally-less-relevant clinical species such as *Salmonella*. Regardless, this enzyme activity cleaves ethanolamine into ethanol and ammonia, the latter is then used as a source of nitrogen. The former is converted to acetaldehyde and acetic acid, by alcohol and aldehyde dehydrogenases (Figure 25). The best studied organisms for its degradation are *Escherichia coli* or *Salmonella enterica* (Blackwell et al., 1976; Blackwell and Turner, 1978; Chang and Chang, 1975), though other bacteria have been studied (Bradbeer, 1965a, b) and for bacteria at least, there have been a small number of reviews (Garsin, 2010; Kaval and Garsin, 2018). The fungal literature demonstrates that some fungi appear to be able to use this compound including *Aspergillus nidulans*, *Papiliotrema* (syn. *Cryptococcus*) *laurentii* (Flipphi et al., 2002; Middelhoven et al., 1985). In the case of *A. nidulans*, this fungus is also able to use ethanolamine as a sole source of nitrogen (Flipphi et al., 2002). At least some fungi, for example, *Blastobotrys* (syn. *Arxula*) *adeninivorans* can use the compound as a sole source of nitrogen, but not a sole source of carbon (Middelhoven et al., 1991). Its use by fungi as a sole source of carbon, nitrogen, or both is almost certainly more widespread than the limited

reports suggest. This lack of reporting is likely because ethanolamine is not a commonly tested compound.

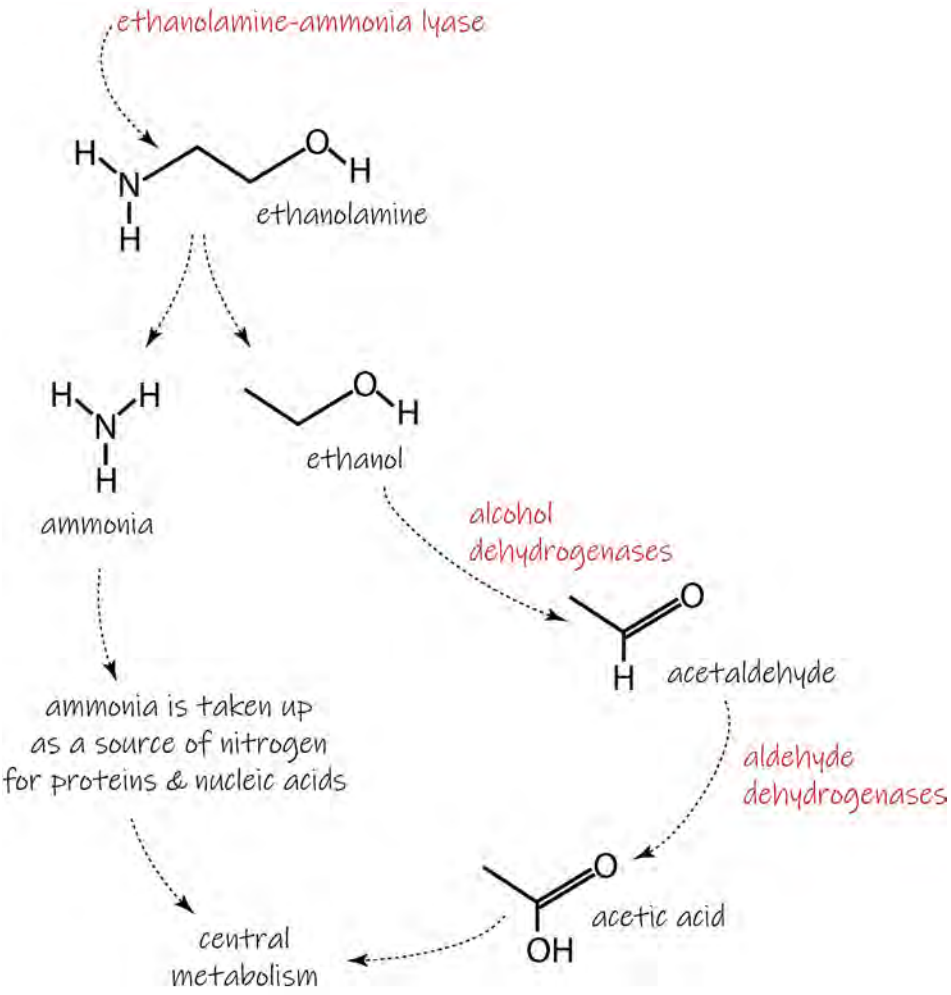



**Figure 25 (Mono)Ethanolamine utilisation as it occurs in *Salmonella* species**

Enzymes are shown in red.

## 2.11.4 Microbial degradation of dazomet or methyl isothiocyanate (MITC)

Microbial growth on or with DAZ or MITC is poorly studied. There are only a small number of studies that discuss catabolism of these compounds (Babu et al., 1996; Cobb, 1973). These two studies focussed on microbial access to sulphur and nitrogen in MITC. Cobb (1973), for example, showed that MITC toxicity in *Fusarium oxysporum* was related to the general sulphur status of the fungus. When cellular levels of sulphur were low, growth was stimulated by the presence of MITC in axenic culture, and the converse was true when, other, non-MITC sulphur levels were high. In the absence of other sulphur sources, *F. oxysporum* only degraded MITC to meet its sulphur needs (Cobb, 1973). Similarly, but from the perspective of nitrogen, *Pseudomonas putida* was demonstrated to use nitrogen from MITC as a sole source of nitrogen for growth (Babu et al.,

1996). Furthermore, cell-free extracts of culture fluids were able to extract ~20 µmol $NH_3$ released protein$^{-1}$ $h^{-1}$, illustrating that *P. putida* does this using extracellular enzymes.

### 2.11.5 Microbial degradation of glutaraldehyde

In oxic conditions, microbial catabolism of glutaraldehyde begins with its oxidation to glutaric acid, typically catalysed (Figure 26). Glutaric acid can be further converted in compounds like succinate which enter central metabolism, completely mineralising glutaraldehyde. Under anoxic conditions, however, glutaraldehyde is reduced to 1,5-pentanediol via 5-hydroxypentanal (Figure 26). In general, the pathway terminates at 1,5-pentanediol (Leung, 2001a), though some organisms can metabolise the compound further to 5-hydroxypentanoic acid (syn 5-hydroxyvaleric acid; Tanaka, 1992). The half-life of glutaraldehyde in the environment is short, in the order of hours, due to its ready metabolism and reactivity (Leung, 2001a). Anoxically, glutaraldehyde can abiotically degrade to form 3-formyl-6-hydroxy-2-cyclohexene-1-propanal, a complex, cyclic dimer of glutaraldehyde.

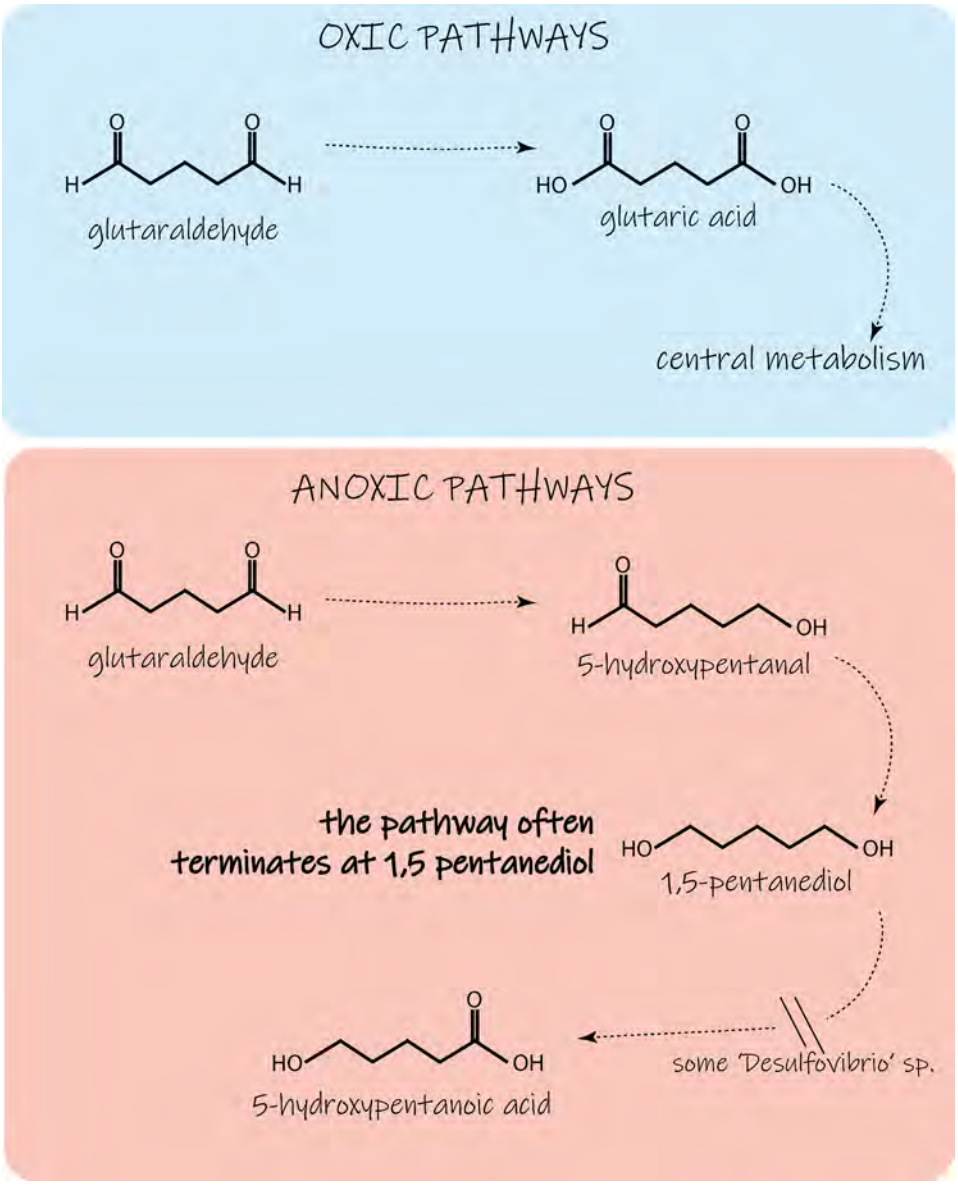



**Figure 26 Microbial degradation of glutaraldehyde under oxic and anoxic conditions**

### 2.11.6 Microbial degradation of 3,3'-methylene-bis(5-methyloxazolidine)

There is very limited information about degradation of 3,3'-methylene-bis(5-methyloxazolidine), and the literature is similarly sparse for its principal breakdown product: 5-methyloxazolidine. The breakdown product, 5-methyloxazolidine, then undergoes further abiotic degradation to produce formaldehyde and 1-aminopropan-2-ol (described in some studies as 2-hydroxypropylamine). Both these initial reactions occur in water spontaneously and are abiotic. It is unknown whether microbes can accelerate this process.

Microbial use of formaldehyde is well-established and it can be altered or used as a carbon source by numerous microbes (Husárová et al., 2011; Klein et al., 2022; Yurimoto et al., 2005). Like other aldehydes, it tends to be converted into a carboxylic acid prior to further modification. The 1-aminopropan-2-ol breakdown product is chiral with two different forms (D and L). Growth on 1-aminopropan-2-ol is well established, with studies from the 1970s demonstrating growth by Pseudomonas isolates (culture collection numbers: NCIB 10431 & NCIB 8858; Jones and Turner, 1973; Faulkner and Turner, 1974) on both chiral forms.

## 2.12 Other microbial or ecological responses to chemicals

Along with direct catabolism (degradation) of chemicals described, some other microbial or ecological responses are introduced below.

### 2.12.1 Resistance to chemicals

Some microbes can exhibit inherent or acquired resistance to certain chemicals. *Inherent resistance* to chemicals implies a natural ability of a microorganism to withstand chemical compounds without needing to acquire new genetic material or mutations (Salam et al., 2023). This natural ability is expressed in biological features such as certain cell wall structures acting as potent barriers to chemicals and effective efflux pumps proteins that actively export a range of substances out of the cell (Madigan et al., 2022; Salam et al., 2023). *Acquired resistance* occurs when microbes gain new genetic traits that confer the ability to survive  exposure of toxic chemicals (Arzanlou et al., 2017). Such resistance can occur as a result of mutations leading to altered target sites for chemicals, transformation or horizontal gene transfer through the uptake of free DNA from the environment containing genes conferring some resistance to the chemical, transfer of plasmids carrying resistance genes between bacterial cells through direct contact via conjugation, transduction or the transfer of genes between bacteria via bacteriophages (i.e. viruses that infect bacteria), and gene amplification, which increases the number of copies of resistance genes or the upregulation of their expression (Arzanlou et al., 2017; Dordet-Frisoni et al., 2019; Madigan et al., 2022).

### 2.12.2 Ecological considerations

Adding a new chemical to a soil can suppress or kill particularly susceptible microbes in that soil. This suppression can lead to changes in competition and open-up new ecological niches. Including catabolism of organisms that have died as a result of the chemical addition. Organisms that are either resistant to the chemical or can catabolise the chemical can subsequently capitalise on the reduced competition and increased availability of nutrients or niches (Bauer et al., 2018). Other ecological factors include the loss of individual partners in various symbiotic or commensal relationships (Haque and Haque, 2017), a loss of one partner, even when the other is resistant to the chemical may disadvantage some organisms.

### 2.12.3 Alteration of groundwater, surface water or soil chemistry

The breakdown of some chemicals can lead to a change in chemical conditions of the surrounding environment (Barman and Preston, 1992), potentially creating conditions more favourable for certain microbial taxa. Such microorganisms may prefer or tolerate shifts in soil/water pH, nutrient availability, or other chemical parameters caused by applying such chemicals. Certain microbial taxa may be able to utilise specific breakdown byproducts of contaminating chemicals as energy or nutrient sources, which can lead to an increase in their relative abundance over other less adaptable species.

# 3 Brief methods

## 3.1 Sampling information

Groundwater, surface water and soil samples were collected at two time points representing seasonal variations (November 2022 and June 2023) across the Narrabri region (Table 3 - Table 5). The November 2022 sampling campaign was impacted by flooding in the area. This flood meant that many of the areas more distantly located in the Pilliga woodlands were inaccessible. As such, more samples were obtained from areas adjacent to larger roadways. The second sampling campaign, which aimed to assess temporal effects, was thus bound (to some extent) by the sampling campaign of 2022. Regardless, the methods used were determined by downstream applications (chemical or biological baselining or a requirement for "live microbiological" collection from groundwater, surface water or all). Regardless of the type of sample, all sampling was undertaken aseptically, and sampling equipment was cleaned and decontaminated between the collection of samples.

### 3.1.1 Chemical baseline sampling

All samples for chemical baselining were collected in bottles/containers provided by Australian Laboratory Services (ALS), following guidelines provided.

### 3.1.2 Preservation of microbial communities

All samples for microbial baselining 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 (both groundwater and surface) samples were collected using prepared Schott bottles containing 200 mL of DESS. For groundwater sampling, bores were allowed to purge (minimum time of 5 minutes) to ensure samples were collected directly from the aquifers and not stagnant water in the pipelines, unless the bore/well was under continuous production. Samples were sealed in Schott bottles with silicone rubber seals.

Approximately 5 g of soil were sampled using sanitised utensils into 15 mL centrifuge tubes and overlayed with DESS (~10 mL) and mixed thoroughly. The surface litter was removed prior to collection.

### 3.1.3 Collection of 'live' microbes

For "live" microbial sampling of groundwater, bores were allowed to purge for five minutes to ensure samples were collected from the aquifers. "Live" anoxic microbial samples of ~1 L volumes were collected (under a $CO_2$ atmosphere). Water was bubbled vigorously with $CO_2$ and the headspace filled with $CO_2$ prior to being sealed in Schott bottles with silicone rubber seals. "Live" surface water samples were collected in Schott bottles. Sealed bottles were transported inside hard shell insulated containers back to the CSIRO laboratory at Lindfield, NSW. On receipt at Lindfield, the groundwater sample bottles were vented inside an anaerobic chamber where the atmosphere comprised ~95% argon, 1-2% hydrogen and the balance nitrogen (~3-4%). These "live" samples were used as inoculum in microcosm experiments. "Live" surface water samples were stoppered with cotton wool to allow air to enter and leave the sampling containers once in the laboratory.

"Live" soils were sampled using sanitised spades into a sanitised polypropylene bucket (approximately 12kg). The surface litter was removed prior to collection. These "live" samples were used as inoculum in microcosm experiments.

### 3.1.4 Sample locations

Samples for the present study were collected from a range of groundwater, surface water and soil locations (Tables 3-5). Only locations of samples on public or state controlled land is described. For all groundwater samples bore depth is provided. For private bores, this was rounded to the nearest 10 m.

**Table 3 Groundwater samples from the present study, their proximal land use, location, bore depths, and interpreted aquifers. Cenozoic undifferentiated = unable to be attributed to a specific aquifer of Cenozoic age.**

| Identifier | Location | Description | Depth (m) | Aquifer* | Notes |
|---|---|---|---|---|---|
| **G1** | CSIRO site (Australia Telescope Compact Array) | Site bore | 41.7 | Cenozoic undifferentiated | |
| **G2** | Australian Cotton Research Institute | Groundwater bore #1 for site use | 49.6 | Cenozoic undifferentiated | |
| **G3** | Farm | Private landowner bore | ~100 | Cenozoic undifferentiated | Collected only during November 2022, due to change of ownership. |
| **G4** | Farm | Private landowner bore | ~60 | Cenozoic undifferentiated | |
| **G5** | Farm | Private landowner bore | ~40 | Cenozoic undifferentiated | |
| **G6** | Farm | Private landowner bore | ~40 | Cenozoic undifferentiated | |
| **G7** | Farm | Private landowner bore | ~50 | Cenozoic undifferentiated | |
| **G8** | Farm | Private landowner bore | ~140 | Cenozoic undifferentiated | |
| **G9** | Gwabegar | Domestic water supply (pretreatment) bore | 296.2 | Orallo Formation | |
| **G10** | Pilliga | Domestic water supply (pretreatment) bore | 518.2 | Pilliga Sandstone | |
| **G11** | Pilliga Artesian Bore Bath | Outlet flowing into bath | 564.5 | Pilliga Sandstone | |
| **G13** | Australian Cotton Research Institute | Groundwater bore #2 for site use | 72.5 | Cenozoic undifferentiated | Collected only during June 2023, due to flooding in November 2022. |
| **G14** | Narrabri Lawn Cemetery | Bore for site use | 84 | Cenozoic undifferentiated | Collected only during June 2023. |

*** likely aquifers were identified from depth, however, exact prediction is challenging due to the complexity of the system and topography features in the landscape.**

**Table 4 Surface water samples from the present study, their location and description**

| Identifier | Location | Description | Notes |
|---|---|---|---|
| **W1** | Yarrie Lake | Lake water, ~1 m off the shore | Sample from the northern shore, off Lake Cct, Yarrie Lake. |
| **W2** | Bohena Creek | Creek water sample | South of Westport Road, Narrabri |
| **W3** | Long Gully | Creek water sample | Near Gould St, Narrabri |
| **W4** | Pine Creek | Creek water sample | Off Pine Creek Road, Turrawan |
| **W5** | Tulla Mullen Creek | Creek water sample | Off Yarranabee Road, Baan Baa |
| **W6** | Andy's Creek | Creek water sample | Andy's Creek in the Pilliga woodland, southwest of Old No 1 Break Road, off the Newell Hwy. Collected only during November 2022, due to an absence of water in June 2023. |
| **W7** | Wiggans Creek | Creek water sample | Off Delwood Road, The Pilliga. Collected only during November 2022, due to an absence of water in June 2023. |
| **W8** | Duck Creek | Creek water sample | South of Oil Well Road, The Pilliga. Collected only during November 2022, due to an absence of water in June 2023. |
| **W9** | Farm Dam | Private farmland, dam water sample | |
| **W10** | Etoo Creek Dam | Dam water sample | ENE of Etoo Creek Road, Gwabegar |
| **W11** | Tinegie Creek | Creek water sample | NE of Tinegie Creek Road, Gwabegar. Collected only during November 2022, due to an absence of water in June 2023. |
| **W12** | Roadside dam/ large pond | Pond water sample | SW of Crow Road on the eastern side of the Newell Highway |
| **W13** | Spring Creek | Creek water sample | NE of Cains Crossing Road, Narrabri. Only collected in June 2023. |
| **W14** | O'Briens Creek | Creek water sample | Near Gould Street, Narrabri West. Only collected in June 2023. |
| **W15** | Namoi River | Creek water sample | Near Bridge Street, Narrabri. Only collected in June 2023. |
| **W16** | Narrabri Creek | River water sample | Near Cameron Street, Narrabri. Only collected in June 2023. |

**Table 5 Soil samples from the present study, their location and description**

| Identifier | Location | Description |
|---|---|---|
| **S1** | Smiths Lane, Bohena Creek | In remnant woodland with *Acacia harpophylla* on roadside. Dark cracking clay soil, likely vertosol. |
| **S2** | CSIRO Australian Telescope Compact Array site | In *Callitris* woodland on sandy textured, likely sodosolic soil. |
| **S3** | Gun Club Road, Narrabri | Jack's Creek State Forest sample in woodland. |
| **S4** | Australian Cotton Research Institute | Black vertosol, tilled soil used for agricultural/crop research. Used as one of the main soils for numerous experiments in the present study. |
| **S5** | Pilliga Forest Way, near Staffords Well Road | Woodland soil, sandy textured, sodosolic soil. In Pilliga East State Forest. |
| **S6** | Corner of Kurrajong Creek Rd and Mayfield Rd. | Probably chromosol soil. |
| **S7** | North of Rocky Glen, Roadside | Borah Creek Rd. Possible ferrosol soil. Collected only in November 2022. Limited access to the Pilliga forest due to flooding. |
| **S8** | Off No 1 Break Rd, Roadside | Likely tenosol soil. |
| **S9** | Off Newell Highway | Probably tenosol soil. |
| **S10** | East into the Pilliga Woodland near, Yamminba Rest area. | Probably sodosolic soil. |
| **S11** | Off Newell Highway, south west of Wiggans Creek crossing. | Probably tenosol soil. Not collected in June 2023 due to roadworks limiting access to the forest. |
| **S12** | Near Station Creek. | Probably tenosol soil. Not collected in June 2023 due to roadworks limiting access to the forest. |
| **S13** | Pilliga woodland, near Pilliga Rest Area | Soil type unclear, tenosol or sodosol soil. Not collected in June 2023 due to roadworks limiting access to the forest. |
| **S14** | Pilliga woodland, west of the Newell Highway, south of Garlands Road. | Soil type unclear, likely tenosol. Not collected in June 2023 due to roadworks limiting access to the forest. |
| **S15** | Off X-Line Rd, The Pilliga. | Soil type unclear, likely tenosol. Not collected in June 2023 due to roadworks limiting access to the forest. |
| **S16** | Roadside, into the Pilliga woodland off the Newell Highway | Soil type unclear, likely tenosol or sodosol. |
| **S17** | Pilliga woodland near William Bridges Rest Area | Sodosolic soil. |
| **S18** | Farm in the Tarrario area | Vertosol soil under crop production. Not collected in June 2023 due to change of ownership. |
| **S19** | Farm soil | Sandy loam, possible tenosol or sodosol soil. |

| | | |
|---|---|---|
| **S20** | Etoo Creek Dam | Sodosol soil. |
| **S21** | Tiengie Creek, Roadside | Unknown soil type, possible tenosol soil, contains some alluvium from the waterway. |
| **S22** | South of Yarrie Lake | Vertosol soil. |
| **S23** | Southeast of Yarrie Lake, Rosevale Road | Vertosol soil. |
| **S24** | SW of Crow Road on the eastern side of the Newell Highway | Sodosolic soil. Used as one of the main soils for numerous experiments in the present study. |
| **S25** | Delwood Road, The Pillaga. | Sodosolic soil. Not collected in June 2023 due to roadworks limiting access to the forest. |
| **S26** | Borah Creek Road | Sodosolic soil. Not collected in November 2022 due to access issues. |
| **S27** | Weeping Myall Woodland soil | Soil from under Weeping Myall (*Acacia pendula*), northwest of Narrabri. Not collected in November 2022 due to access issues. |
| **S28** | Tuppiari Road | Mixed land use, likely sodosolic soil. Not collected in November 2022 due to access issues. |
| **S29** | West Port Road | Sodosolic soil. Not collected in November 2022 due to access issues. |
| **S30** | Dog Proof Fence Road | Sodosolic soil. Not collected in November 2022 due to access issues. |
| **S31** | North-eastern most corner of Dog Proof fence road | Possible rudosol soil. Not collected in November 2022 due to access issues. |
| **S32** | Nuable Road, roadside adjacent to Brigalow Nature Reserve | Vertosol soil. Not collected in November 2022 due to access issues. |

## 3.2 Chemical baselining

Water chemistry, including pH, electrical conductivity, alkalinity, various carbon pools, nitrogen, phosphorus, sulphate, chloride, mercury, fluoride, ammonia, bromide, dissolved major cations and total metals were quantitatively analysed in a NATA credited laboratory (ALS).

The analytical procedures used by ALS have been developed in-house from established internationally recognized procedures such as those published by the USEPA (U.S. Environmental Protection Agency), APHA (American Public Health Association) and NEPM (National Environmental Protection Measure) with details in Table 6.

Table 6 Method details (ALS) for water chemistry analytes

| Analytes | ALS methods |
|---|---|
| **pH** | EA005P |
| **Electrical Conductivity** | EA010P |
| **Bromide** | ED009 |
| **Alkalinity** | ED037P |
| **Sulphate as $SO_4^{2-}$** | ED041G |
| **Chloride** | ED045G |
| **Dissolved major cations** | ED093F |
| **Total metals** | EG020T |
| **Total recoverable mercury** | EG035T |
| **Fluoride** | EK050P |
| **Ammonia** | EK055G |
| **Nitrite as N** | EK057G |
| **Nitrate as N** | EK058G |
| **Nitrite plus Nitrate as N (NOx)** | EK059G |
| **Total Kjeldahl Nitrogen as N** | EK061G |
| **Total nitrogen as N** | EK062G |
| **Total phosphorus as P** | EK067G |
| **Reactive total phosphorus** | EK071G |
| **Ionic balance** | EN055 |
| **Dissolved organic carbon** | EP002 |
| **Total organic carbon** | EP005 |
| **Total inorganic carbon** | EP006 |
| **Total carbon** | EP007 |

## 3.3 Baselining prokaryotic and fungal communities

Microbial baselining in the current study was all conducted using eDNA approaches regardless of whether the samples were surface water, groundwaters or soils. All baseline microbial community profiling was analysed by Molecular Research LP DNA, Texas, USA (MR DNA) using Illumina Tru-Seq PCR-free library preparation kits and sequencing was conducted on an Illumina miSEQ DNA sequencer using 250 paired end read length.

### 3.3.1 DNA extractions

All DNA in this project was extracted using the DNeasy PowerSoil® kit (Qiagen Cat No./ID: 12855), using the manufacturers method[9], with minor modifications. The input material differed depending on the sample type.

For surface and groundwater samples (300 mL), all samples were filtered through Millipore 0.1 µm polyvinylidene difluoride (PVDF) filters under gentle vacuum. These filters were aseptically moved into sterile, 9 cm petri dishes which were stored at -20°C prior to DNA extraction. Immediately prior to DNA extraction, the filter was sliced into 5 mm strips using a new sterile scalpel blade. Approximately ½ of the PVDF filter was added to the tubes used in the DNA extraction kit for bead beating using sterilised forceps. The bead beating tubes were then processed as per the manufacturer's instructions.

For soils, no pre-extraction concentration was undertaken. Instead, 0.25 g of soil was added directly to the bead beating tubes using a sterilised spatula. The bead beating tubes were then processed as per the manufacturer's instructions.

### 3.3.2 16S or ITS PCRs

Two different primer sets were used in this study, both assays followed the Earth Microbiome Project.

#### 16S

The primers used in this study were: 515F: GTGYCAGCMGCCGCGGTAA; 806R: GGACTACNVGGGTWTCTAAT. These primers amplify both bacterial and archaeal phyla from samples. The primers have been extensively tested, and while all primers have some bias (towards particular microbial groups), these biases are somewhat limited using these primers. The Earth Microbiome Project 16S Illumina amplicon protocol was followed in this study[10].

#### ITS

The ITS primers are designed to amplify fungal microbial eukaryotic lineages from samples. The primers target the ITS region, which has been shown to be variable between fungal species and relatively unchanged within individuals from the same species. The Earth Microbiome Project ITS Illumina amplicon protocol was followed in this study[11].

[9] Qiagen DNeasy PowerSoil® kit protocol https://www.qiagen.com/au/Resources/ResourceDetail?id=329362e4-03e6-4ae1-9e4e-bbce41abe4b7&lang=en

[10] https://earthmicrobiome.org/protocols-and-standards/16s/

[11] https://earthmicrobiome.org/protocols-and-standards/its/

### 3.3.3 DNA sequencing analysis and bioinformatics

16S and ITS amplicon datasets were processed using the Greenfield Hybrid Amplicon Pipeline (GHAP)[12]. Analyses for 16S and ITS 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.

## 3.4 Microcosm experiments

This study aimed to simulate chemical contamination events by collecting samples of soils and waters from their natural environments, placing them in sterile containers and spiking them with chemicals used in the CSG industry. These 'microcosms', represented smaller, encapsulated versions of natural water and soil systems from the Narrabri area and allowed laboratory analyses of indigenous microbial communities (Mandelbaum et al., 1997). Microcosm experiments also mimicked the natural environment using a night-day cycle for soils with an air atmosphere, and an oxygen-free atmosphere in darkness for groundwaters.

Establishment of microbial degradation experiments involved spiking of selected groundwater (G2 and G8), surface water (W12) and soil (S4 and S24) samples with the chemicals of interest (Figure 27 and Table 7).

All groundwater microcosms were established in 50 mL glass serum vials under an anoxic atmosphere that comprised ~95% argon, 1-2% hydrogen and the balance nitrogen, at atmospheric pressure. Each vial used for the experiment contained 21 mL of filter-sterile groundwater. This was subsequently inoculated with 4 mL of "live" groundwater collected under an $CO_2$ atmosphere (see Section 3.1) and chemicals of interest were added to achieve concentrations as shown in Table 7. All vessels were established in replicates of ten. Vessels were incubated under static conditions for approximately 90 days, at ~22°C in the dark.

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

Table 7 Concentration (mg $L^{-1}$) of chemicals for the community impact assessment experiment

| Chemical | Concentration (mg $L^{-1}$) |
|---|---|
| **AEM#1** | 4.02 |
| **AEM#2** | 50 |
| **HCM#1** | 50 |
| **MEA** | 0.46 |
| **DAZ** | 0.11 |
| **GLU** | 30 |
| **MBO** | 0.7 |

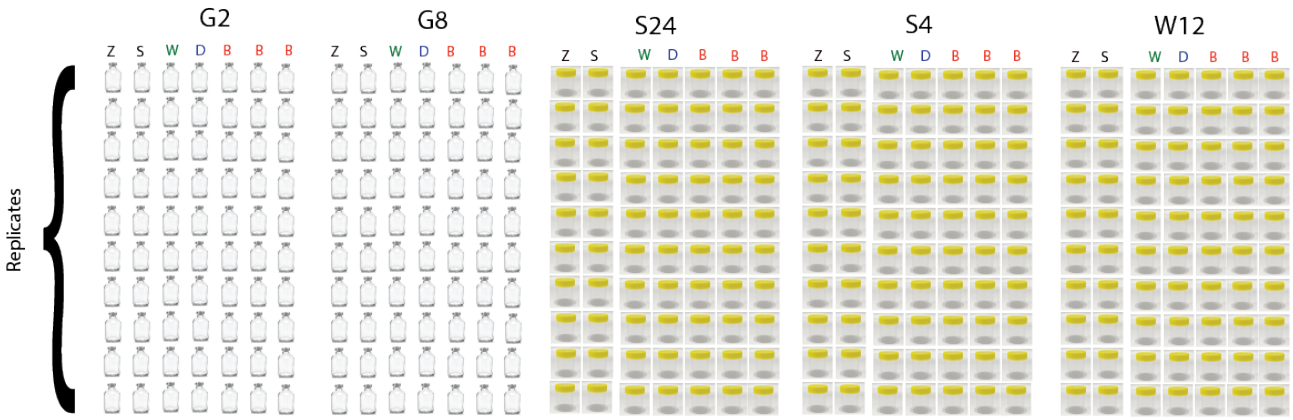
All surface water microcosms were established in 70 mL gamma-sterile specimen jars (Figure 27). Each jar used for the experiment contained 25 mL of surface water. Chemicals of interest were added to achieve concentrations as shown in (Table 7). All experiments were established in replicates of ten. Jars were incubated under static conditions with lids slightly loose to allow oxygen transfer for approximately 90 days, at ~22°C with a day/night cycle (12hr/12hr).

All soil microcosms were also established in 70 mL gamma-sterile specimen jars (Figure 27). Each jar used for the experiment contained 25 mL of soil. Chemicals of interest were added to achieve concentrations as shown in Table 7. All experiments were established in replicates of ten. Jars were incubated under static conditions with lids slightly loose to allow oxygen transfer for approximately 30 days, at ~22°C with a day/night cycle (12hr/12hr).

Zero-time controls for all microcosm experiments 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 (see Figure 27). Zero-time controls were set up in replicates of ten. The zero-time controls represent the original microbial community present in the samples prior to the chemical degradation experiments.

Storage controls for all microcosm experiments were set up without the addition of any chemicals (see Figure 27). These controls investigated the changes in the microbial community composition resulting from the experimental procedure. Storage controls were set up in replicates of ten and were incubated with experimental microcosms. In this way, the storage control provides an indication of how microbial communities change during the incubation period, without the addition of any chemicals.

At the end of incubations, all material was harvested for DNA extraction by filtration through a 0.1 µm PVDF filter for surface water and groundwater samples and subjected to DNA extraction using the DNeasy PowerSoil® kit (Qiagen Cat No./ID: 12855), following the manufacturer's method (see Section 3.3.1 for further details). DNA extractions for soil experiments were carried out using 0.25 g of soil directly (see Section 3.3.1 for further details). Microbial community profiling of the resultant DNA was done by Molecular Research LP DNA, Texas, USA (MR DNA) (see Section 3.3 for further details).




W27 Chemicals

W = Workover cocktail

HCM #1
+
HCM #2 *
+
AEM #2

D = Drilling cocktail

MEA
+
AEM #1

B = Biocide (separate)

DAZ
GLU
MBO

Zero time (Z): 21 mL of water sample + 4 mL of inoculum and filter straight away; or subsample from 25 mL of soil

Storage control (S): 24 mL of sample + 1 mL of inoculum and incubate

**Figure 27 Microbial degradation experiment schematic**

## 3.5 Chemical assessment of microbial degradation of specific compounds

Microbial degradation of target chemicals was assessed by chemical analyses after microcosm experiments were completed. Chemical analyses for AEM#1 (69011-36-5), monoethanolamine (141-43-5), AEM#2 (78330-21-9), dazomet (533-74-4) and methyl isothiocynate (556-61-6), MBO (66204-44-2) and 5-methyl-oxazolidine (58328-22-6) were undertaken by ACS Laboratories Australia. Chemical analyses for total petroleum hydrocarbons (HCM#1 and #2) and glutaraldehyde (111-30-8) were undertaken by Australian Laboratory Services (ALS).

## 3.6 Microbial use of chemicals as a sole source of carbon

A laboratory trial known as a 'sole carbon growth experiment' was conducted to examine the ability of individual microbial species or strains to utilise a specific chemical as their sole source of carbon and energy. The objective was to identify microbes capable of degrading specific chemicals, such as pollutants that could pose a health or environmental risk.

These experiments used defined mineral medium that lacks a carbon source. A single, known carbon source was added to this medium. Growth on this medium thus indicated the use of this carbon source to create biomass. In this experiment, a modified minimal salts medium (MSM)

(Coleman et al., 2002; Hartmans et al., 1992) was used. To carbon-free MSM, various carbon sources (dazomet, oxazolidine, glutaraldehyde, ethoxylated alcohols, isotridecanol) were individually added (see Figure 28). To reduce the effect of carry over carbon from the initial inoculum (a dilution of soil or water), all inoculated cultures in MSM were sub-cultured weekly into fresh MSM with each of the specific carbon sources for a total of eight weeks and only the final weeks growth was considered (see Figure 28).

Since soil harbours a large microbial biomass, soils were diluted prior to starting the sole carbon experiments. To achieve this, 10 g of soil was placed in 90 mL of growth medium to which approximately 50 µL of Tween 80 (a surfactant) was added. This was subsequently mixed by hand to disperse the soil and 1 mL used in 24 mL of growth medium/sole carbon chemical mix. This final mix represents a sole carbon microcosm for the period of one week.

Waters, by contrast, were not diluted by this method as they harbour a lower microbial biomass. For the waters, 1 mL from each source was directly placed in 24 mL growth medium/sole carbon chemical mix to act as microcosms.

After one week, 1 mL of liquid was taken from each microcosm and placed into a fresh mixture of growth medium/sole carbon chemical. This was continued over a period of seven weeks by which the surviving microbial biomass would be sequentially diluted (see Figure 28). This dilution removed heterotrophs feeding on any dead biomass and selecting for the growth of microorganisms solely capable of degrading the chemical of interest.

At the end of eight weeks, all material was harvested for DNA extraction by filtration through a 0.1 µm PVDF filter for water samples and subject to DNA extraction using the DNeasy PowerSoil® kit (Qiagen Cat No./ID: 12855), according to the manufacturer's method (see Section 3.3.1 for further details). DNA extractions for soil experiments were carried out using 0.25g of soil directly (see Section 3.3.1 for further details). Microbial community profiling of the resultant DNA was done by Molecular Research LP DNA, Texas, USA (MR DNA) (see Section 3.3 for further details).

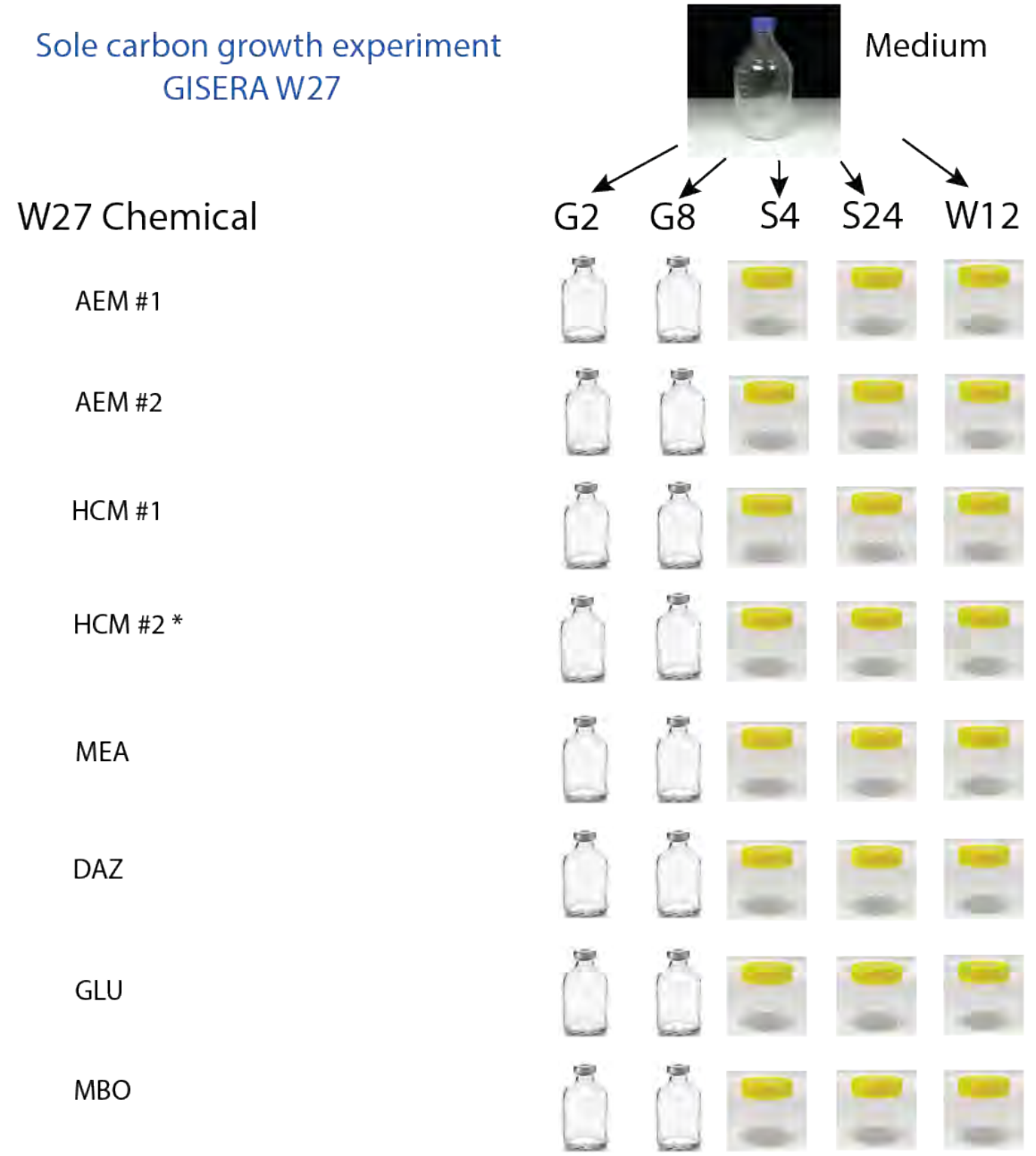



**Figure 28 Sole carbon growth experiment schematic**

Groundwater samples G2 and G8 used in experiments; soil samples S4 and S24 used in experiments; surface water sample W12 used in experiments.

## 3.7 Statistics

Various statistical approaches were used in the current study including independent t-tests, correlations, PERMANOVA and SIMPER. Various statistical ordinations were used including Principal Component Analysis (PCAs) and Non-metric Multidimensional Scaling (nMDS). These are briefly introduced below.

### 3.7.1 Independent t-tests

An independent t-test (also called the student t-test) is a statistical test used to compare the averages (means) of two separate groups to see if they are significantly different from each other (Student, 1908). It is called 'independent' because the two groups have had separate processes (often a treatment and control) applied. This is different compared to paired t-tests which are used to test the same sample, before and after some experimental change.

### 3.7.2 Correlations

Pearson's correlation coefficient (often represented as $r$ or r) is a statistical measure that quantifies the strength and direction of the linear relationship between two continuous variables (Pearson, 1904). An r-value of 1 indicates the two variables are completely positively correlated, while a value of -1 indicates the variables are completely negatively correlated (Figure 29).

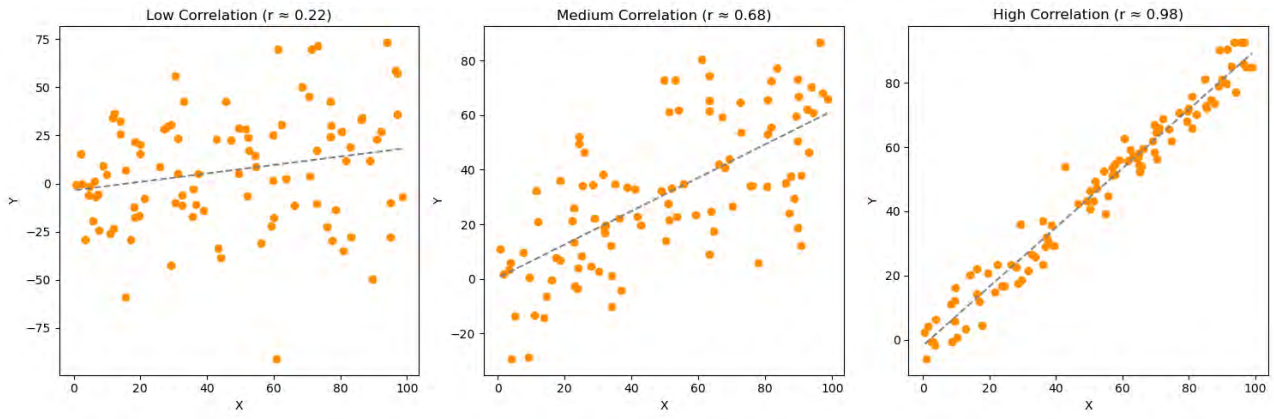



**Figure 29 Example scatter plots (with regression lines) showing correlation of two variables at different strengths**

### 3.7.3 PERMANOVA

PERMANOVA (Permutational Multivariate Analysis of Variance) is a statistical test used to assess whether there are significant differences between groups based on multivariate data (Anderson, 2017). It is a non-parametric method that compares the dissimilarity between samples within and between groups by permuting the data to generate a distribution of test statistics under the null hypothesis.

The technique is particularly useful when the data involve multiple variables or measurements (such as community composition, species abundance etc), and the assumptions of traditional

ANOVA (such as normality) are not met. PERMANOVA is undertaken on a distance matrix (a dataset that records how similar different samples are). This distance matrix used commonly for ecology data is the Bray-Curtis measure, which is a mathematical approach for comparing the number of shared and different species between communities (Beals, 1984; Bray and Curtis, 1957).

### 3.7.4 SIMPER

When a significant difference between communities is detected using PERMANOVA, the SIMPER (SIMilarity PERcentage) method is used to determine which species are most responsible for this difference (Clarke, 1993). SIMPER is applied to identify which species are representative of the different treatments.

### 3.7.5 Ordination methods (PCA and nMDS)

nMDS (Non-metric Multidimensional Scaling) is a non-parametric technique used to reduce the dimensionality of complex data while preserving the rank order of dissimilarities (i.e., how different the samples are from one another). Like other PERMANOVA, nMDS uses a distance matrix as a starting point. One similar technique is principal components analysis (PCA). PCA is a linear dimensionality reduction technique used to transform a set of possibly correlated variables into a smaller number of uncorrelated variables called principal components. The goal of PCA is to simplify the dataset by reducing its dimensionality while retaining as much of the variability (or information) as possible. Both techniques, essentially reduce multidimensional data to simpler (fewer dimensions) representations that approximate the major trends within the data. Of the two techniques, nMDS is used more frequently in ecological settings. For a discussion of both see Jackson, (1993), Smith and Mather, (2012), and Roberts, (2020).

### 3.7.6 Software used in the present study

Both 16S and ITS amplicon datasets were processed using the Greenfield Hybrid Amplicon Pipeline (GHAP)[13]. DNA sequence analyses followed the typical workflow. In brief, data was demultiplexed, subjected to quality control prior to merging, OTU clustering and mapping reads back to the resultant OTU table.

Analyses in the present study were conducted in a range of programs including Microsoft Excel and Past 3 (Hammer et al., 2001), along with a range of customised analysis and visualisations in Python 3.7.3 and R languages (R Core Team, 2017). Most visualisations were conducted in Python using Matplotlib (Hunter, 2007), with Pandas and, in some cases, Seaborn (McKinney, 2010; Waskom, 2021). Statistics in Python were undertaken using SciPy and Scikit-learn (Pedregosa et

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

al., 2011; Virtanen et al., 2020). For R, the vegan module (Oksanen et al., 2017) was also used for some ecological statistics.

# 4 Results and discussion

## 4.1 Physico-chemistry of water and soil samples

### 4.1.1 Groundwater samples of the region

Of the groundwater samples examined in the present study, the majority are relatively soft, neutral pH waters. The mean pH of these groundwater samples is 7.6 (±0.16 Standard Error of the Mean; SEM), however, the distribution of pH values has a wide range from 6.03 (G5-Jun 23) to 8.48 (G10- Jun 23), as a number of moderately acidic and mildly alkaline groundwaters were observed (Figure 30). For example, G5 and G6, collected at shallow depths (~40 m) from a private landholder in the Jack's Creek area have pH values ranging from 6.03 to 6.58 regardless of time of sampling. In contrast, G1 and G7 (the bore at the CSIRO telescope site and a sample from a different landholder bore) are both consistently alkaline, with pH values ranging from 8.06 to 8.4.

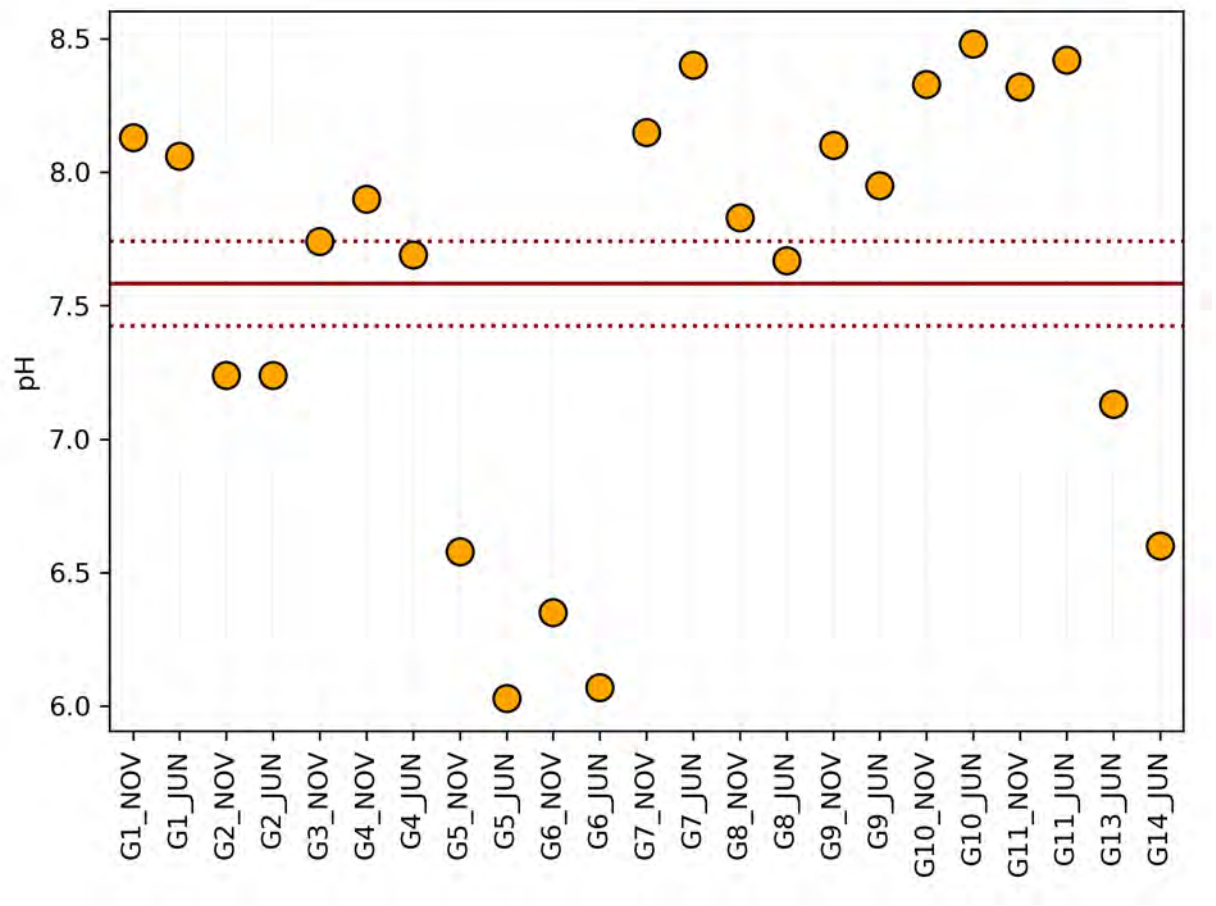



**Figure 30 Dot plot of groundwater samples from the present study and their pH**

The mean is shown as an unbroken red line, while horizontal dotted lines show the standard error of the mean.

Unsurprisingly, there was very little variation between the samples collected in November 2022 and June 2023, except G5 with 0.55 pH unit difference between the two time points. The G5

sample was collected from a relatively shallow (~40 m) bore and its difference in pH between the time points may be related to meteoric water ingress during the heavy rainfall that proceeded the November 2022 sampling or may simply represent natural variation in this aquifer.

The groundwater samples were also relatively fresh as evidenced by the electrical conductivity (EC) measurements, a measure of the saltiness of the water. The EC of the groundwater samples are all under 1150 µS $cm^{-1}$ with a mean EC of 699 µS $cm^{-1}$ (Figure 31). For reference, pure water has an EC close to zero, typical seawater has an EC of ~50,000 µS $cm^{-1}$, while the EC of most municipal tap water is between 100-1000 µS $cm^{-1}$, thus the groundwater samples here are similar in their dissolved salt content to tap water. EC of the groundwater samples did not change over time.


**Figure 31 Dot plot of groundwater samples from the present study and their electrical conductivity (in µS $cm^{-1}$)**

The mean is shown as an unbroken red line, while horizontal dotted lines show the standard error of the mean.

There is a strong relationship between groundwater sample pH and EC (Figure 32). This is considered to be driven by bicarbonate and sodium contents as demonstrated by the strong correlation between EC and bicarbonate as well as sodium ($r > 0.9$) (Figure 32) since it is obvious that bicarbonate and sodium are the main drivers of EC in the groundwater samples.

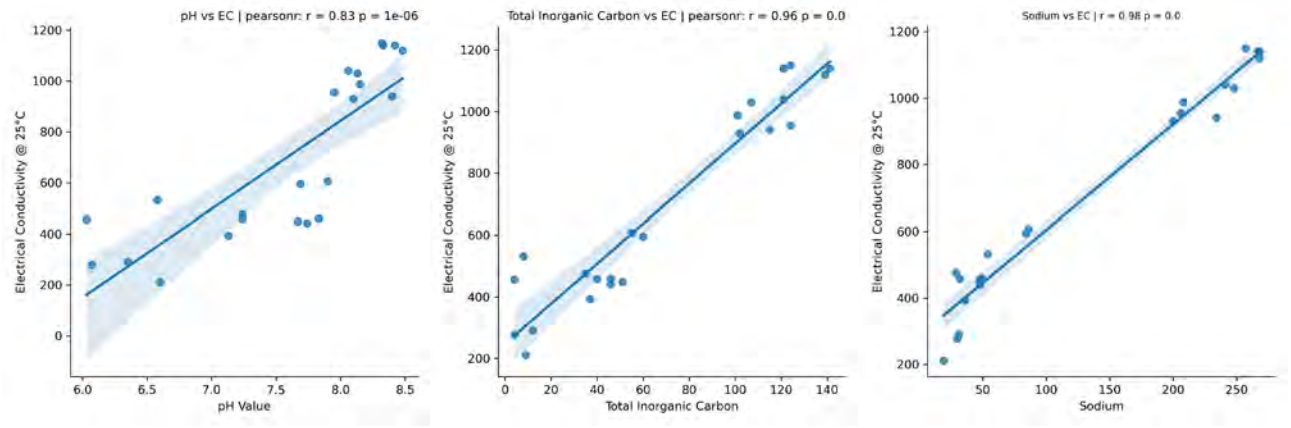



**Figure 32 Linear regression plots for pH, total inorganic carbon (TIC; mg $L^{-1}$) and sodium (mg $L^{-1}$), all vs electrical conductivity on the y-axis (in µS $cm^{-1}$)**

Pearsons R value and a p-value for the correlation are shown for each plot in their respective titles.

As one may expect, most of the carbon associated with these groundwaters is total inorganic carbon (TIC) and only small amounts of organic carbon were detected, but not in all samples. Indeed, most samples contained no detectable organic carbon and those that did ranged from 2-12 mg $L^{-1}$. To put that concentration in context, the sample G9 (the bore that supplies domestic water supply for Gwabegar) had 12 mg $L^{-1}$ of organic carbon but 124 mg $L^{-1}$ of TIC. TIC values were consistently higher in samples collected in winter compared to samples collected in summer except G5 and G6 samples. TIC values in most of the samples collected in winter were half or one-third of TIC in samples collected in summer, apart from the fact that TIC values in both G5 and G6 samples (4-18 mg $L^{-1}$) regardless of time were much lower than other groundwater samples (35-141 mg $L^{-1}$) except G14 (9 mg $L^{-1}$).

In terms of the dominant cations, most groundwaters were proportionally dominated by sodium, or to a lesser extent, calcium ions, with all waters having relatively lower magnesium concentrations (Figure 33). Typically, sodium arises in groundwater by the weathering of evaporites or silicates, while calcium in groundwater is likely from the weathering of various carbonates and silicates. Mostly the weathering zone would be deeper in the subsurface, but some samples may pick up minerals from meteoric water recharge through soils. Groundwater sample G5, for example, is only at 40 m depth and contains very little calcium, a small proportion < 20% magnesium and the remainder as sodium. Given the widespread nature of sodosolic soils in the region this is unsurprising.

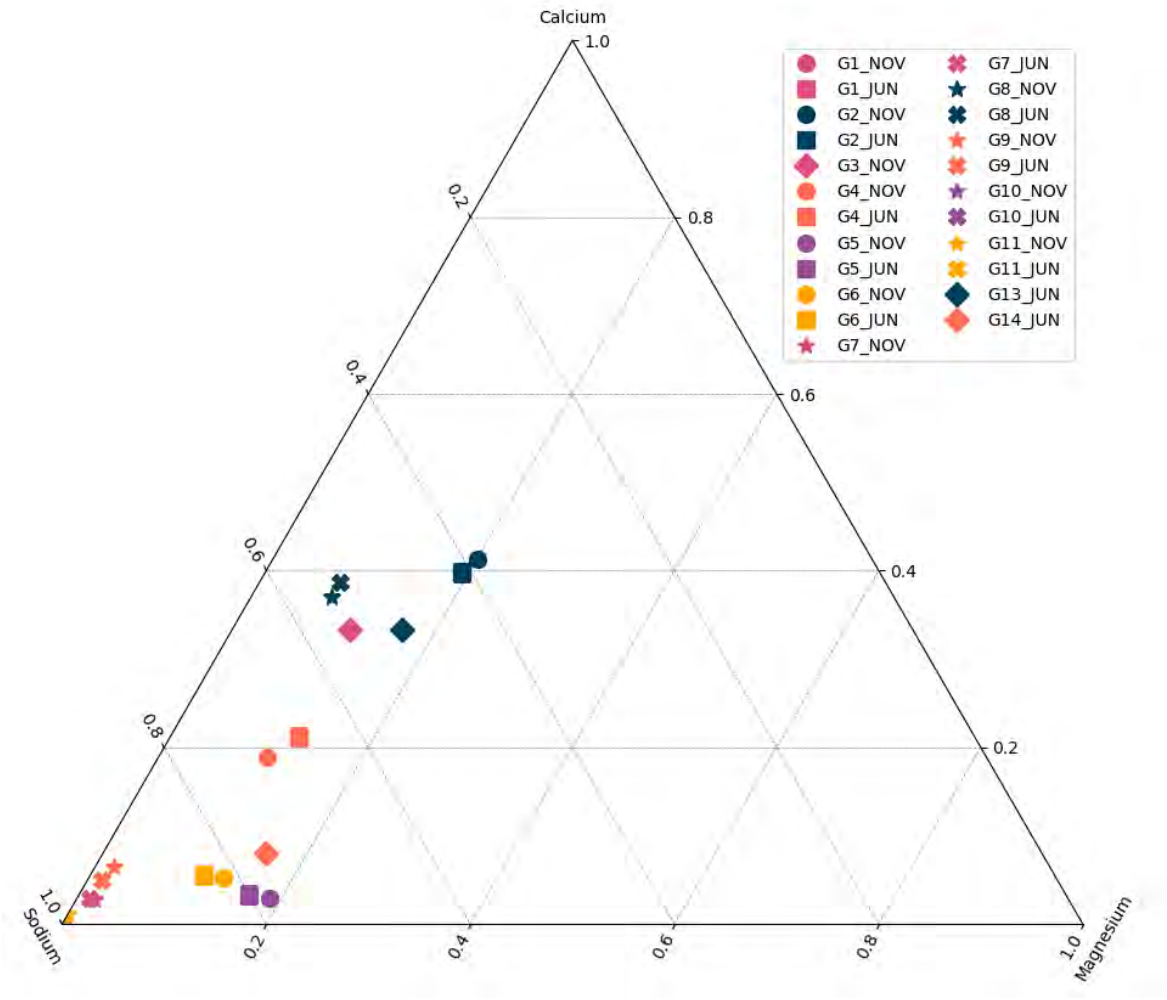



**Figure 33 Ternary plot of the proportions of major cations ($Ca^{2+}$, $Mg^{2+}$ and $Na^{+}$) for groundwater samples**

With regard to anions, most samples were dominated by bicarbonate (Figure 34). Indeed, proportionally bicarbonate makes up more than 85% of the samples. Some notable exceptions to this were G4, G5 and G14. G5 was the most distant to the other samples, being proportionately dominated by chlorides (Figure 34).

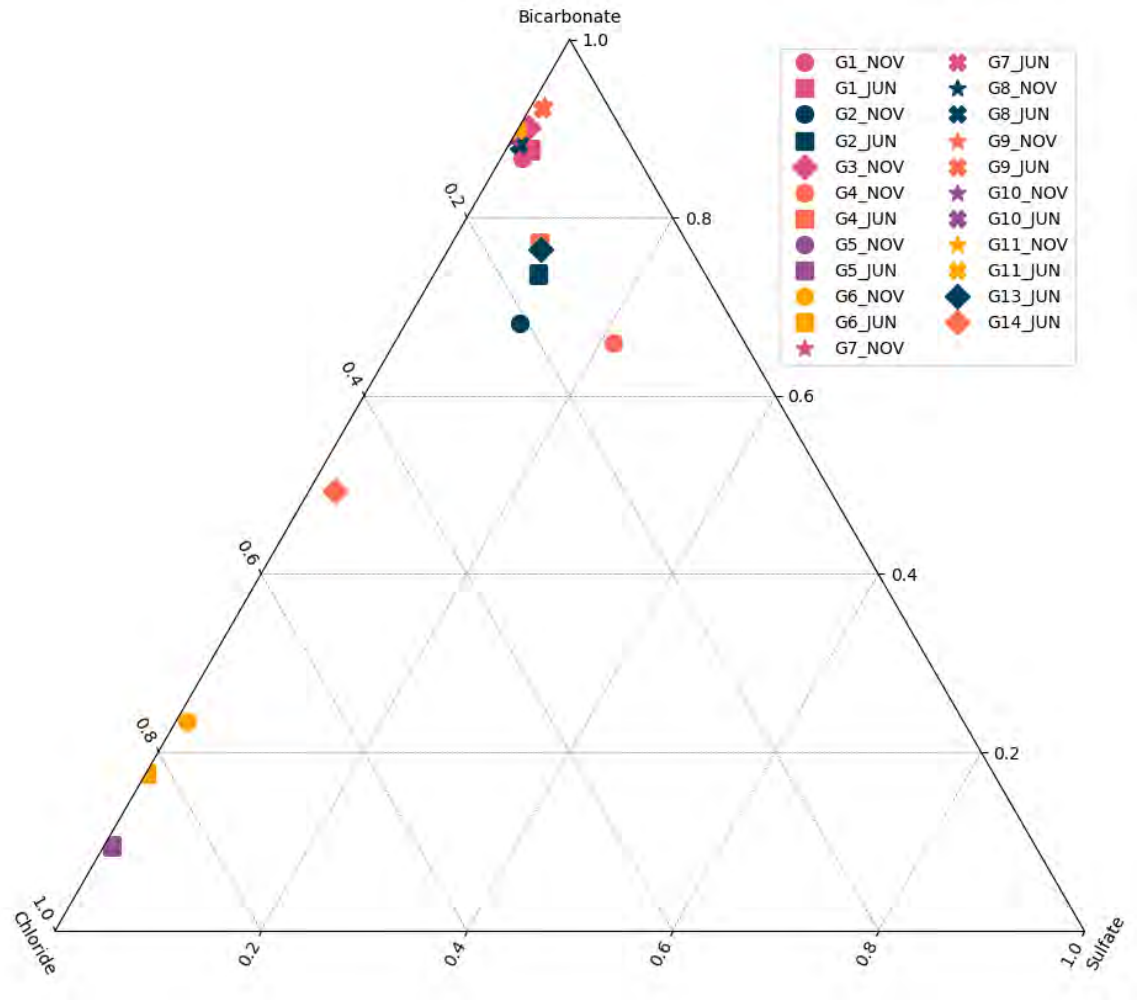



**Figure 34 Ternary plot of the proportions of major anions ($HCO_3^-$, $Cl^-$ and $SO_4^{2-}$) for groundwater samples**

In terms of other metals, apart from calcium, magnesium and sodium mentioned in sections above, potassium is also detected in the groundwater samples with concentrations mostly from 1 to 9 mg $L^{-1}$ (Figure 35). The G5 and the related G6 samples, again as the exceptions, have considerably higher potassium with concentrations from 15 to 22 mg $L^{-1}$ (Figure 35), further illustrating the different geological situation of these samples compared to other samples in the study. Only G14, a sample from the Lawn Cemetery bore (Kamilaroi Hwy) in Narrabri, contains similar potassium concentration (16 mg $L^{-1}$). Iron concentrations ranged between 0 to 1.42 mg $L^{-1}$ and iron was not detected in G1, G5, G10, G11 and G14's seasonal collections, present in G2, G6, G7 for one of the seasonal collections and present in G4, G8 and G9 for both seasonal collections (Figure 36). The biggest seasonal variation for iron is in G6, with iron absent in the November 2022 sampling and 1.42 mg $L^{-1}$ in the June 2023 sampling. Aluminium is not detected in most of the samples except G2, G4, G5 and G13 with trace levels from 0.01 to 0.03 mg $L^{-1}$ (data not shown). Although samples were analysed for antimony, beryllium, cadmium, mercury, selenium, silver, uranium and vanadium, none of these analytes were detected in any of the groundwaters examined (data not shown).

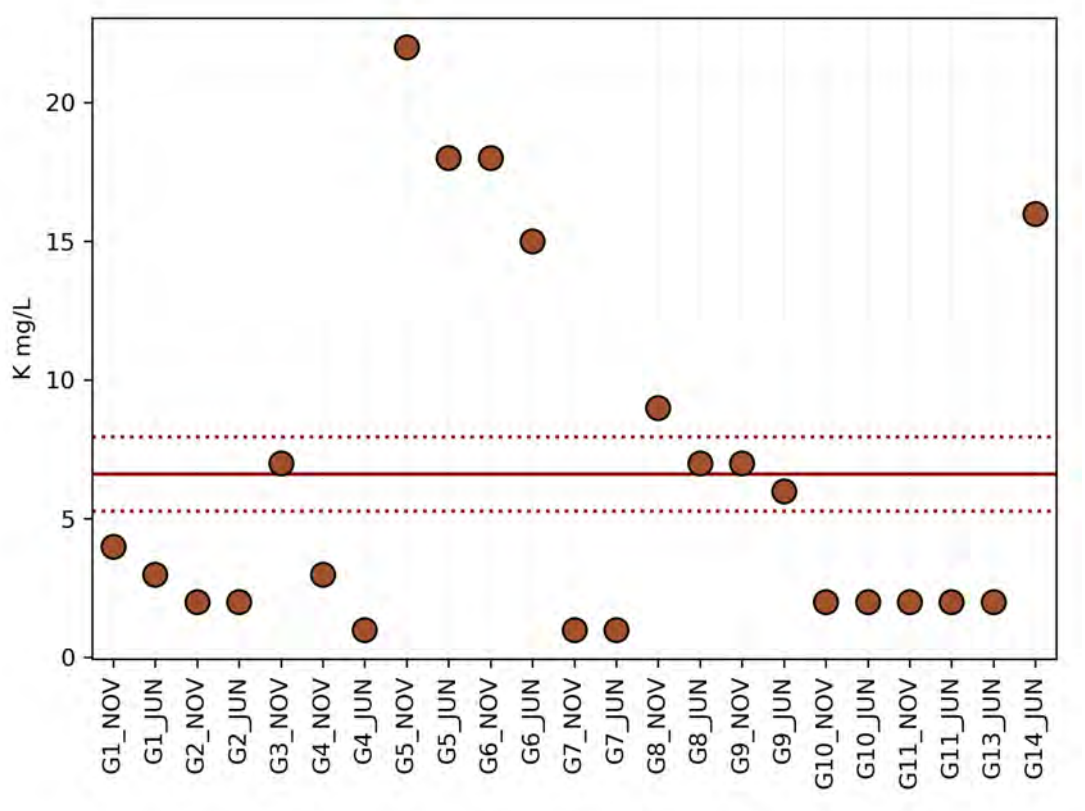



**Figure 35 Dot plot showing the concentration of potassium in mg $L^{-1}$ in the groundwater samples for this study**

The mean is shown as an unbroken red line, while horizontal dotted lines show the standard error of the mean.

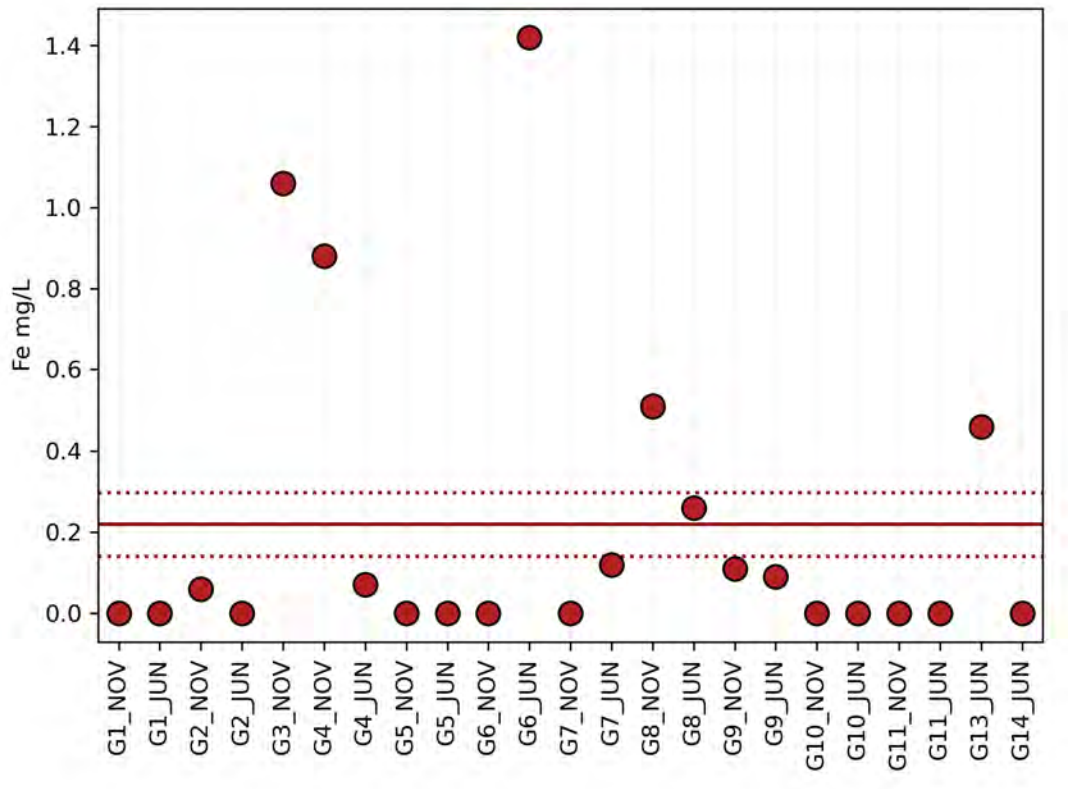



**Figure 36 Dot plot showing the concentration of iron in mg $L^{-1}$ in the groundwater sample for this study**

The mean is shown as an unbroken red line, while horizontal dotted lines show the standard error of the mean.

Nitrogen in groundwater samples of the region was generally low, even in areas with significant agricultural activity on the surface. For example, samples from G2, a bore at ACRI had an average of 0.045 mg $L^{-1}$ ammonia across the two sampling times, while nitrate for this sample was 2.24 mg $L^{-1}$ on average (data not shown). For comparison, a survey of nitrate concentrations in Australia showed that a substantial number (~11%) of the groundwaters in Queensland had medium (20-50 mg $L^{-1}$) or elevated (>50 mg $L^{-1}$) concentrations of nitrate (Thorburn et al., 2003).

### 4.1.2 Surface water samples of the region

Regardless of time of collection, most of the surface waters examined in the present study had moderately neutral pH (Figure 37). The mean pH was 6.84 (±0.11 SEM) with the lowest pH (5.29) being detected in W1 (Yarrie Lake) collected in November 2022 and the highest pH (7.76) being detected in W15 collected from the Namoi River via access from Bridge St in Narrabri. The low pH of Yarrie Lake sample collected in November 2022 represents something of an outlier, and a collection from the same location in 2023 had a pH above 7. The reasons for this low pH are unclear, but maybe related to rainwater ingress into the lake during the flood event of 2022 or the presence of significant tannic or humic acids from plant material. No published data could be found on water chemistry of Yarrie Lake for comparison. Apart from the outlier W1, for the samples with seasonal collections, most of these samples have higher pH in November 2022 compared to June 2023 except W3 and W12.

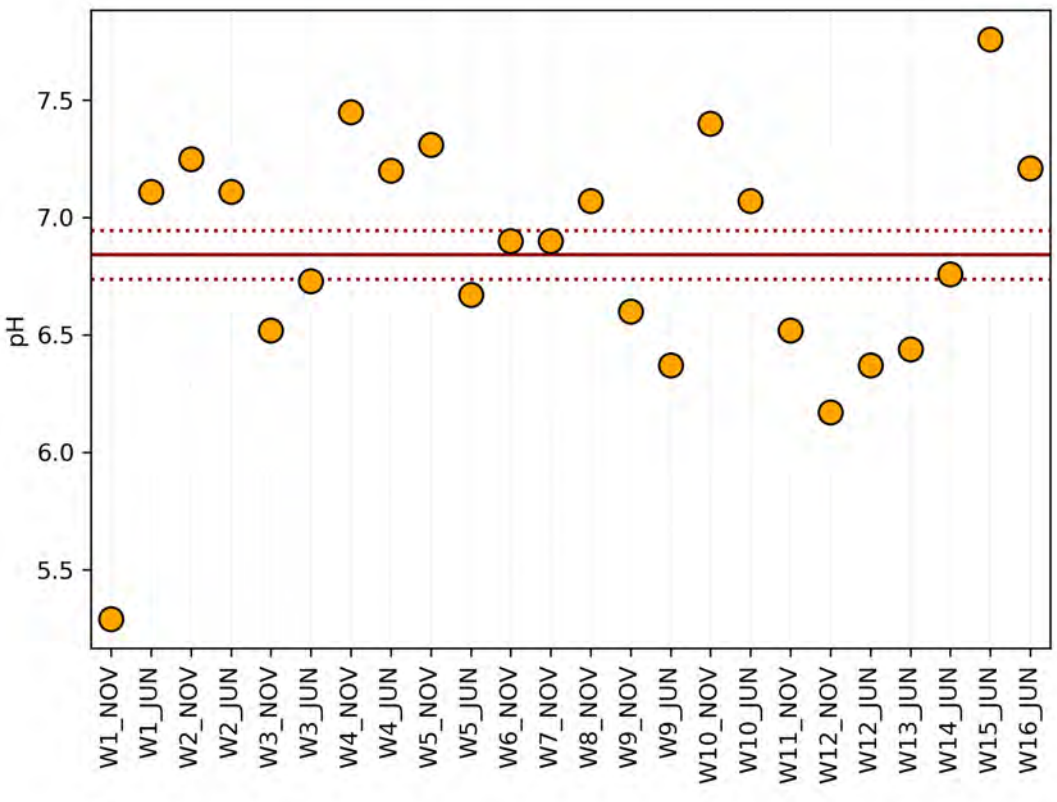



**Figure 37 Dot plot of pH of surface water samples collected in this present study**

The mean is shown as an unbroken red line, while horizontal dotted lines show the standard error of the mean.

The surface water EC values in the present study had a mean of 212 µS $cm^{-1}$ (±32 SEM) (Figure 39). Due to the dilution effect from flooding and ingress of freshwater, samples with seasonal

samplings demonstrate significant changes in EC with samples collected from November 2022 consistently lower than samples collected in June 2023 except W5 (Table 8). This is also reflected from the mean EC values of all the samples from the November 2022 and June 2023 collections which were 153 µS $cm^{-1}$ in November 2022, and 270 µS $cm^{-1}$ in June 2023.

**Table 8 Changes in EC between samples collected from November 2022 and June 2023**

| Sample | Change in EC %* |
|---|---|
| **W1** | 55 |
| **W2** | 45 |
| **W3** | 83 |
| **W4** | 80 |
| **W5** | -1.7 |
| **W9** | 77 |
| **W10** | 50 |
| **W12** | 92 |

*Change is the percentage change between June 2023 and November 2022. For example, 55% is a 55% increase in EC in June 2023 compared to November 2022. While -1.7% is a 1.7% decrease in EC from June 2023 compared to the value in November 2022.

There were some samples, notably W9 and W12, that had quite low EC values (Figure 38). Both W9 and W12 were collected from dams, with W9 being on private farmland and W12 being located off the Newell Highway southwest of Crow Road. Conversely, the higher EC samples from W15 and W16 were from Namoi River and Narrabri Creek sites near town. Same as groundwater samples, EC values of the surface water samples are positively correlated with both TIC and sodium concentrations, however, do not seem to be correlated with pH values as groundwater samples do (Figure 39).

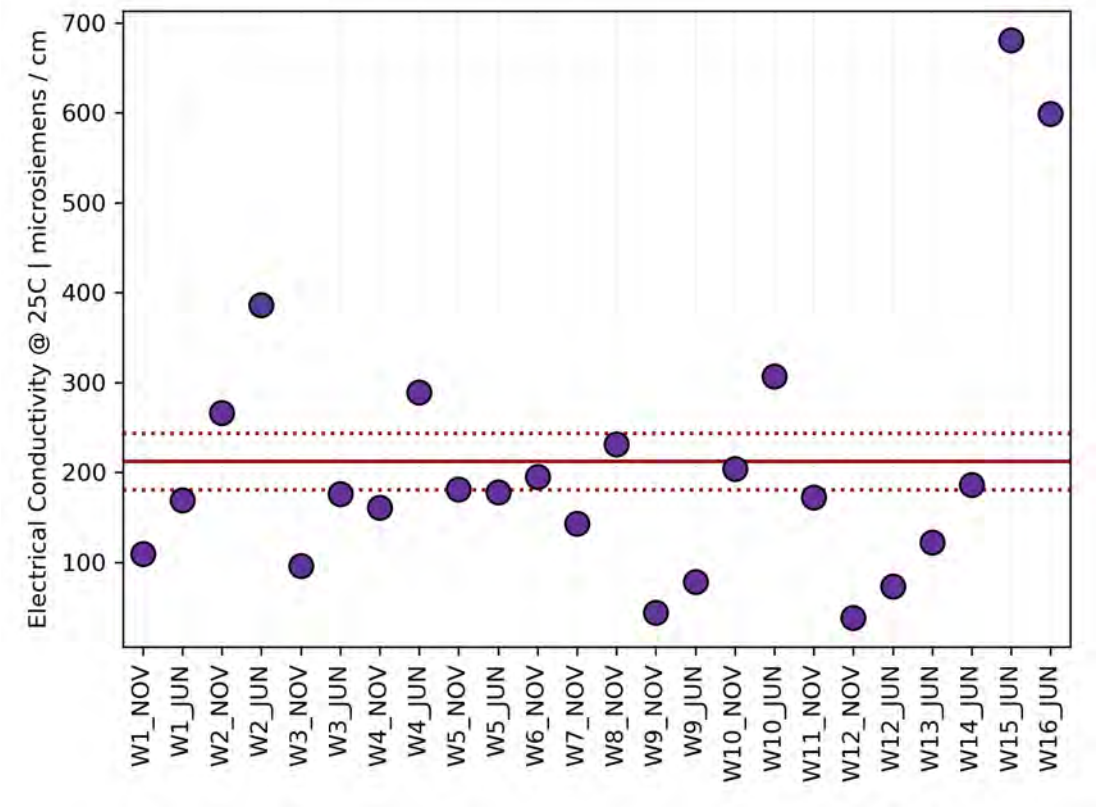



**Figure 38 Dot plot of EC of surface water samples collected in this present study**

The mean is shown as an unbroken red line, while horizontal dotted lines show the standard error of the mean.

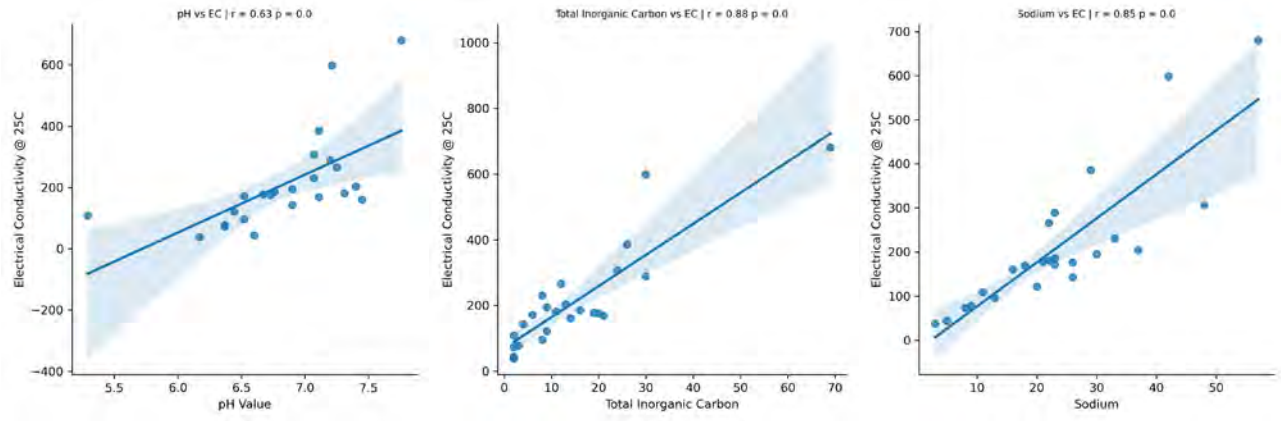



**Figure 39 Linear regression plots for pH, total inorganic carbon (TIC mg $L^{-1}$) and sodium (mg $L^{-1}$) vs electrical conductivity on the y-axis (in µS cm-1)**

The proportions of the major cations (sodium, calcium and magnesium) varied among the surface water samples with sodium being the predominant cation except W15 and W16 (Figure 40). all three major cations in W15 and W16 are in much higher concentrations (sodium: 42-57 mg $L^{-1}$; magnesium: 22-23 mg $L^{-1}$; calcium: 40-43 mg $L^{-1}$) compared to the rest of the surface water samples with sodium, magnesium and calcium ranging from 3 to 33 mg $L^{-1}$, 0 to 16 mg $L^{-1}$ and from 0 to 15 mg $L^{-1}$, respectively. Sodium, as the predominant cation in the surface water samples is in

much lower concentrations (24 mg $L^{-1}$ in average) compared with sodium the groundwater samples (130 mg $L^{-1}$ in average). W9, a sample taken from a dam near the site of collection of G5 and G6 on the same farmland, contained no calcium or magnesium and only small amounts (7 mg $L^{-1}$ ± 1 SEM) of sodium. Consistently, G5 and G6 also had very little calcium (2 mg $L^{-1}$), a small amount of magnesium (4-13 mg $L^{-1}$) and a lower amount of sodium (30-54 mg $L^{-1}$) (presumably from dissolution of minerals in rocks), suggesting likely connectivity between the surface and ground waters, however, further work would be required to confirm this supposition. Seasonal variations of major cation proportions are not obvious for most of the samples except W4 with elevated calcium in sample collected in June 2023.

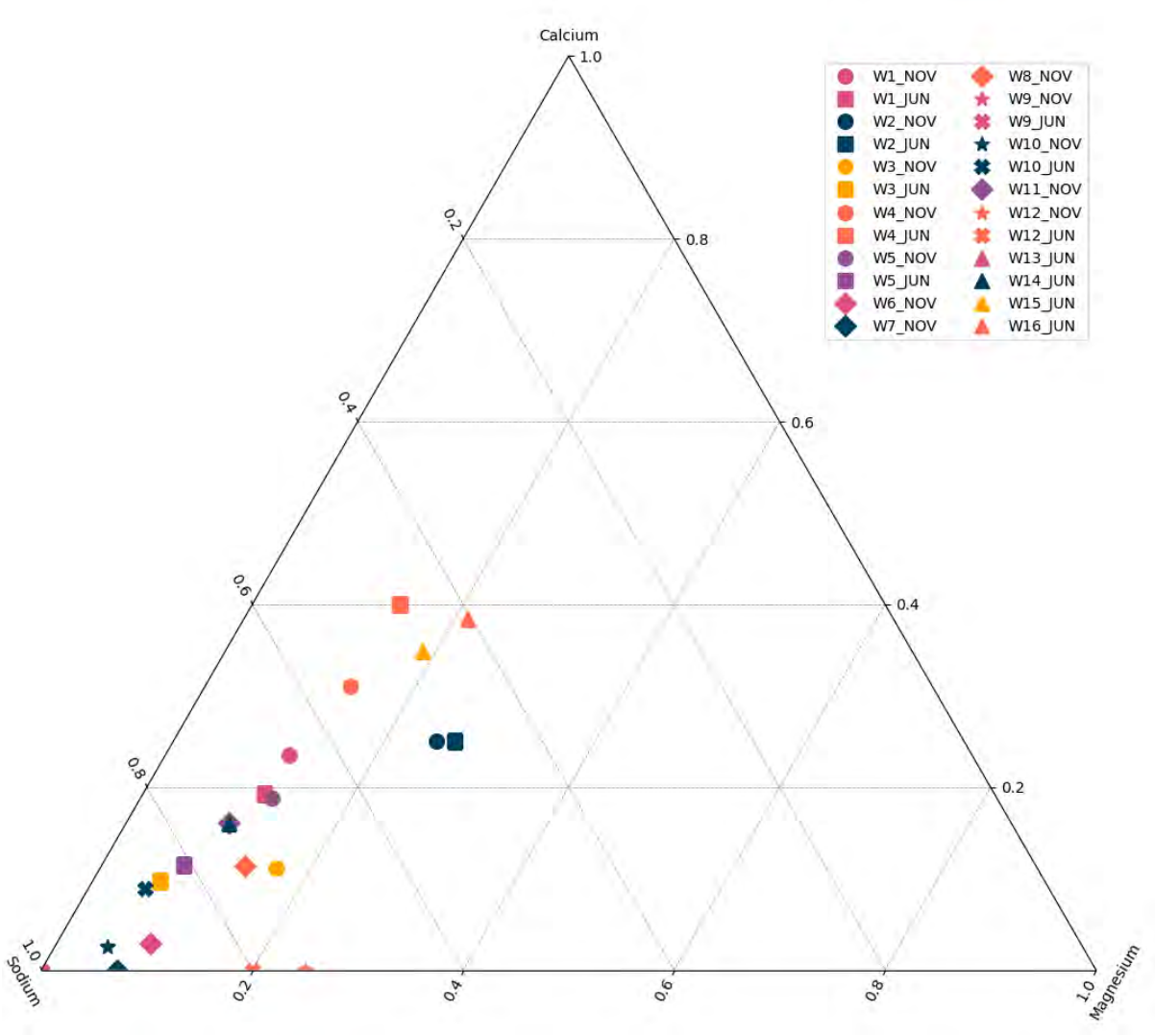



**Figure 40 Ternary plot of the proportions of major cations ($Ca^{2+}$, $Mg^{2+}$ and $Na^{+}$) for surface water samples**

Iron and aluminium are the other two abundant metals in the surface water samples apart from the above major cations. W1, W3, W5 and W14 contains much higher iron (16.9 to 45.4 mg $L^{-1}$) and aluminium (19.6 to 40.1 mg $L^{-1}$) than the rest of the surface water samples with iron and aluminium ranging from 0.12 to 13.2 mg $L^{-1}$ and 0.3 to 8.2 mg $L^{-1}$, respectively (Figure 41 and Figure 42). The surface water samples contain much higher iron and aluminium compared to the ground water samples in which Iron and aluminium are either absent or only in trace amounts.

Concentrations of these two metals varied differently between seasons in different sampling locations. Concentrations of iron and aluminium from summer to winter decrease in W1, W2, W4 and W12, while increase in W3, W5 and W9 (Figure 41 and Figure 42). This is probably related to the amount of rainfalls and inputs of these metals from surrounding environment.

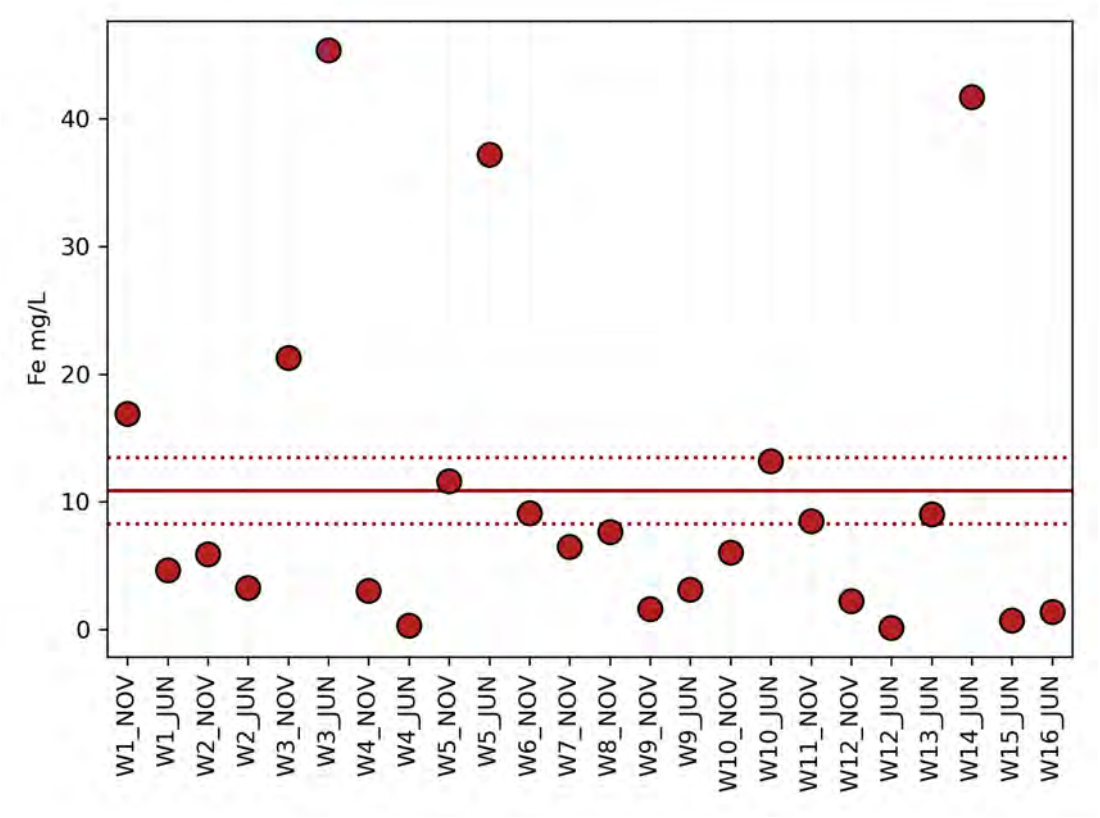



**Figure 41 Dot plot showing iron concentration (mg $L^{-1}$) of surface water samples from this study**

The mean is shown as an unbroken red line, while horizontal dotted lines show the standard error of the mean.

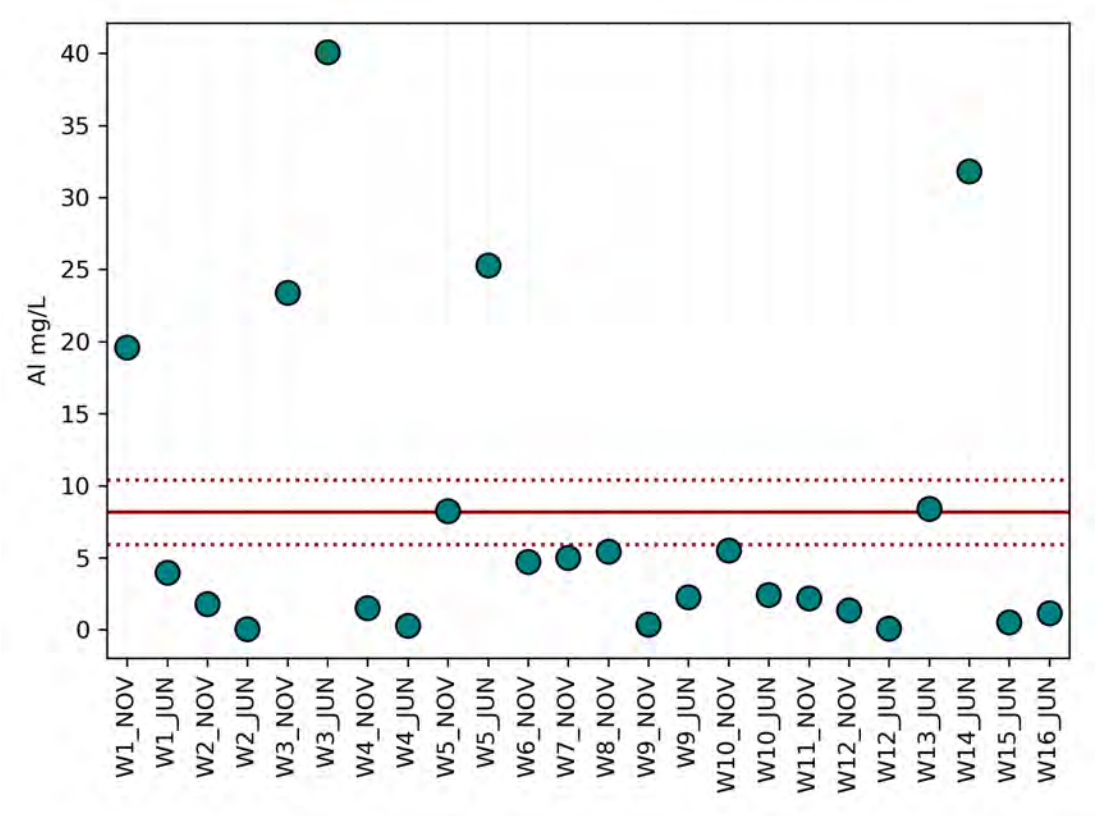



**Figure 42 Dot plot showing aluminium concentration (mg $L^{-1}$) of surface water samples from this study**

The mean is shown as an unbroken red line, while horizontal dotted lines show the standard error of the mean.

Some metals, such as antimony, cadmium, mercury, selenium or silver, were not detected in any surface water sample collected in 2022 and 2023 (Figure 43). Other metals such as beryllium, boron, molybdenum, tin and uranium, were only detected in trace amounts. Beryllium was detected in four samples (W1, W3, W5 and W14) with a maximum concentration of 0.002 mg $L^{-1}$. Boron was only detected in W1 and W10 with concentrations being 0.05-0.06 mg $L^{-1}$ and molybdenum was detected in W4 and W15 with concentration being 0.001 mg $L^{-1}$. Tin and uranium were only detected in single waters (W1 and W7, respectively) with both concentrations being 0.001 mg $L^{-1}$.

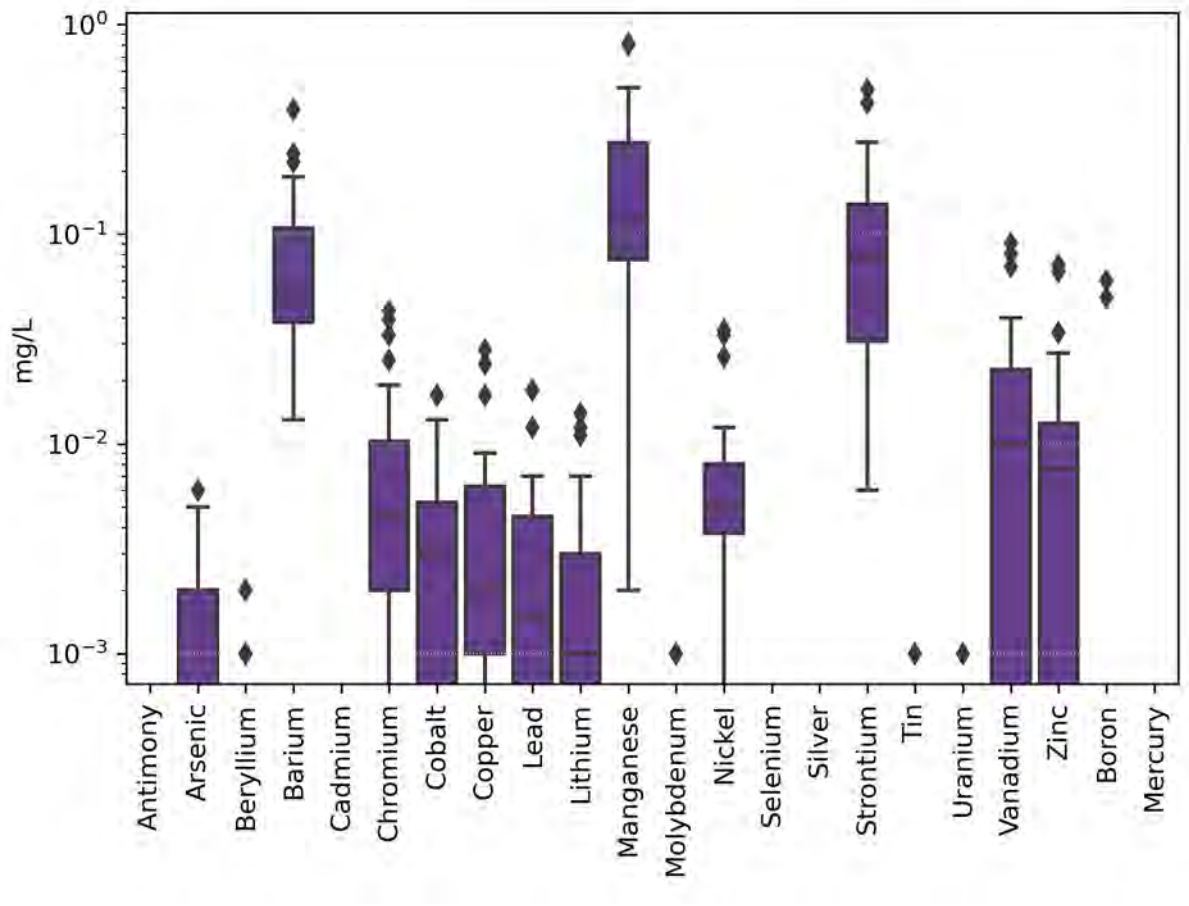



**Figure 43 Box plot of metal content of surface water samples collected in this present study**

Various other metals were also examined. Prior to their discussion, it should be noted that samples were not filtered and thus, some suspended soil/sediment particles may be included in the measurement of total metals, potentially increasing their concentrations. It does, however, seem reasonable to assume that this phenomenon, if it occurred, would be broadly consistent across the samples. The majority of samples using this approach had metal concentrations at or near ANZECC[14] default guidance values for freshwater environments that were slightly to moderately disturbed. Three notable exceptions to this observation were samples from W3 (Long Gully, near Gould St, Narrabri West), W5 (Tulla Mullen Creek near Baan Baa) and W14 (O'Briens Creek near Mooloobar Rd in Narrabri West) which showed significantly higher concentrations of chromium, cobalt, copper, nickel, lead, vanadium and zinc (Figure 43 and Figure 44).

Indeed, these three samples (W3, W5 and W14) were consistently higher in these metals compared to other surface waters examined. To illustrate this observation, a dot plot which highlights W3, W5 and W14 compared to all other samples (in light blue) has been constructed (Figure 44). These elevated concentrations were more pronounced in the June 2023 sampling than the November 2022 sampling presumably due to the flooding that was occurring at this earlier

[14] Australian and New Zealand Environment and Conservation Council.

https://www.waterquality.gov.au/anz-guidelines/resources/previous-guidelines/anzecc-armcanz-2000

timepoint. Even with the flooding, the November 2022 samples had more elevated metal concentrations than most other samples examined (Figure 44).

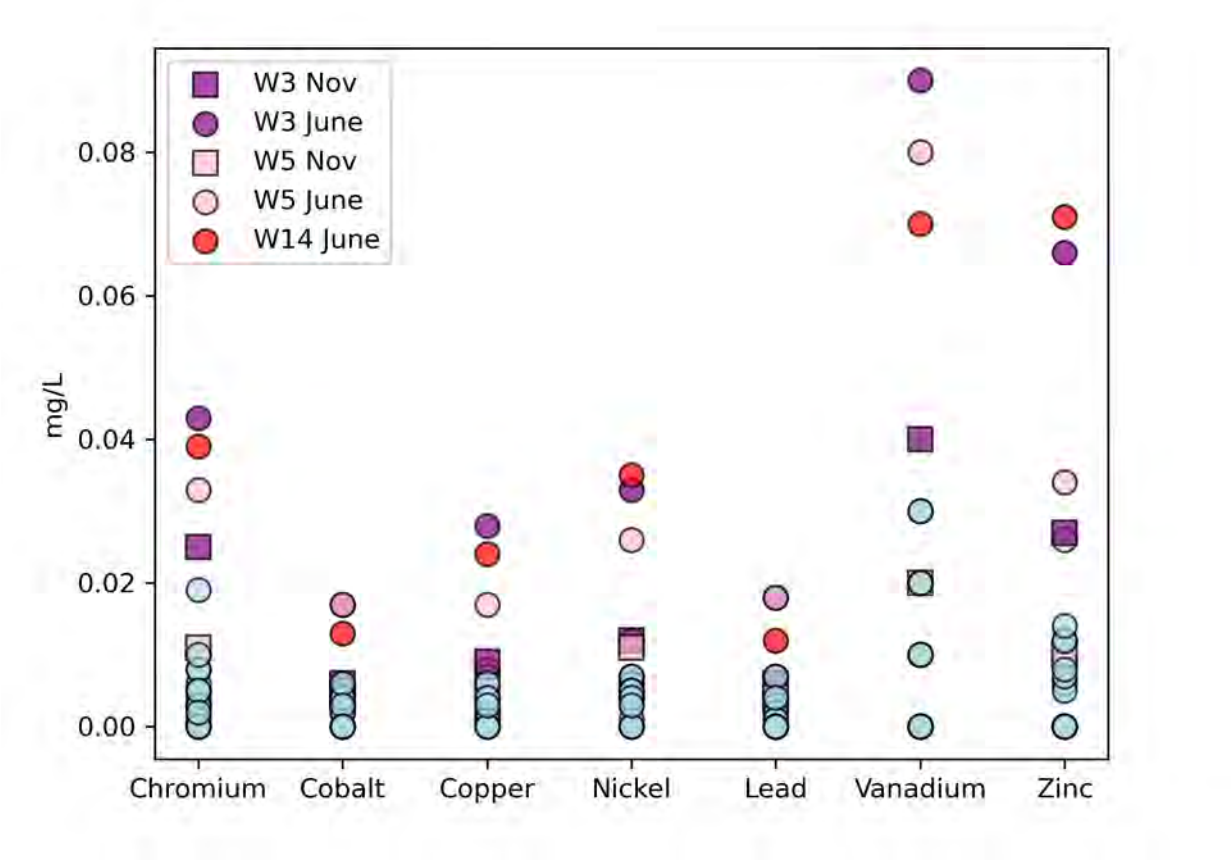



**Figure 44 Dot plot of chromium, cobalt, copper, nickel, lead, vanadium and zinc concentrations for surface water samples**

All collected surface water samples (light blue circles), except for W3, W5 and W14.

Given the nature of the testing undertaken, it seems likely that either these water samples or sediment particles associated with these samples are enriched in a fairly diverse range of metals. This may be naturally occurring or may be the result of some other environmental contamination. At the time of writing, the cause of the elevated levels is unknown. The local council and NSW EPA were advised of these observations and an additional resampling was undertaken (see Box 1).

## Box 1. 2024 Resampling to test for elevated total metals

On becoming aware of these results, GISERA commissioned a small follow up study to resample W3, W5 and W14 and to collect some additional samples from around Narrabri Lake adjacent to W14.

### Samples

Duplicate samples were collected at W3, W5 and W14. In addition, multiple samples were collected from Narrabri Lake in September 2024 (Figure B1).


**Figure B1 Map showing the resampling locations in Narrabri West (left) and near Baan Baa (right)**

Note that for W3 (Long Gully) and W5 (Tulla Mullen Creek) duplicate samples were taken, while for W14 (O'Briens Creek) five extra locations were sampled along with the original W14 location.

Note that the original site of collection of W14 is represented by W14.S2 on the map. Maps reproduced using Google Maps (2024).

### Results

These samples were retested for dissolved metals at a NATA credited laboratory (ALS). In broad terms W3 and W14 had lower total metal concentrations in 2024 than in 2022/2023, though some metals for W5 were largely unchanged (Figure B2).

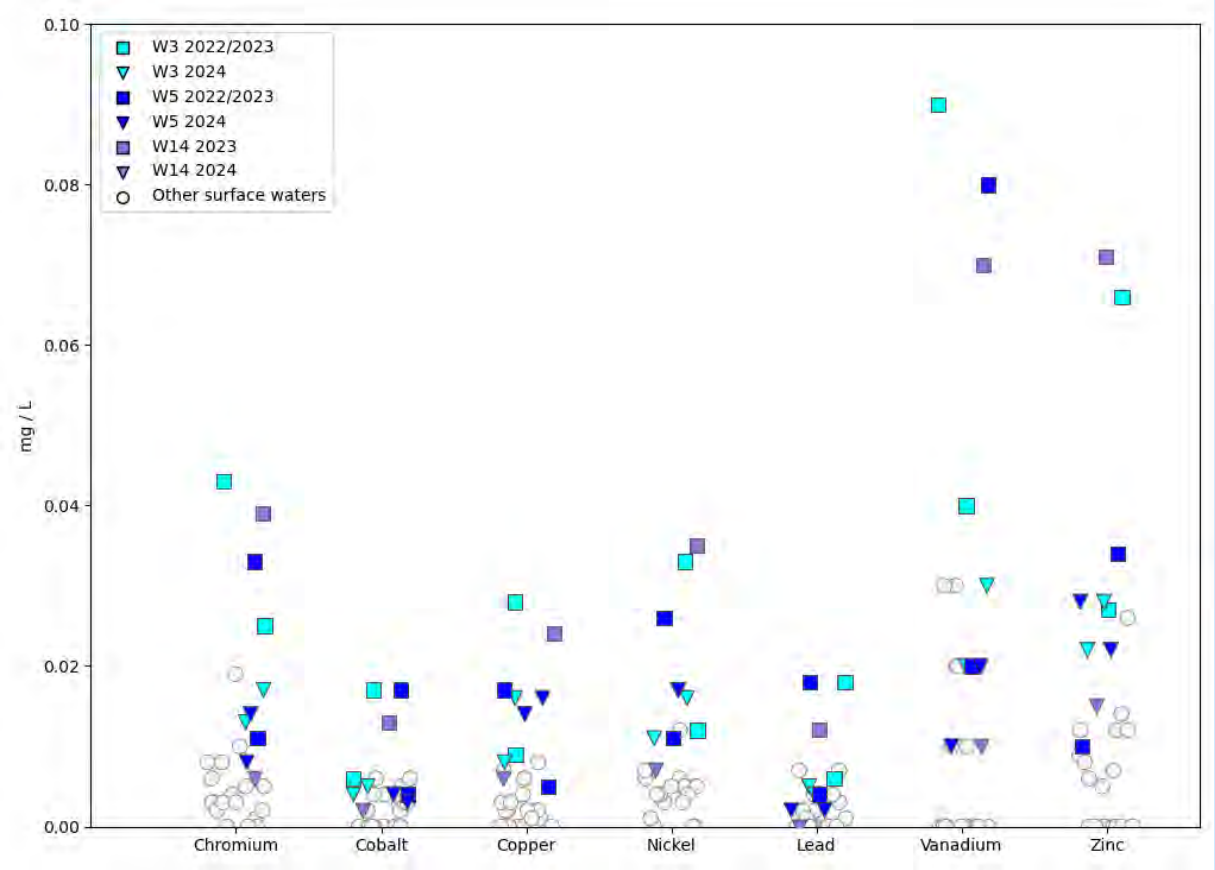



**Figure B2 Strip plot of total metals analyses of surface water samples**

W3, W5 and W14 from 2022/2023 (shown as squares) and retesting of these same three samples in 2024 (shown as triangles). Other samples from surface waters in the study are shown as light grey circles for comparison. Note that W14.S2 (2024) is the same location as W14_JUN from 2023.

Data from around Narrabri Lake (W14.S3, W14.S4, W14.S5 and W14.S6) along with a sample from O'Briens Creek under the Newell Hwy (W14.S1) reveal that all these locations have considerably lower dissolved metals compared to their measures in 2023 (Figure B3). Note that W14.S2 (2024) is the same location as W14_JUN from 2023.

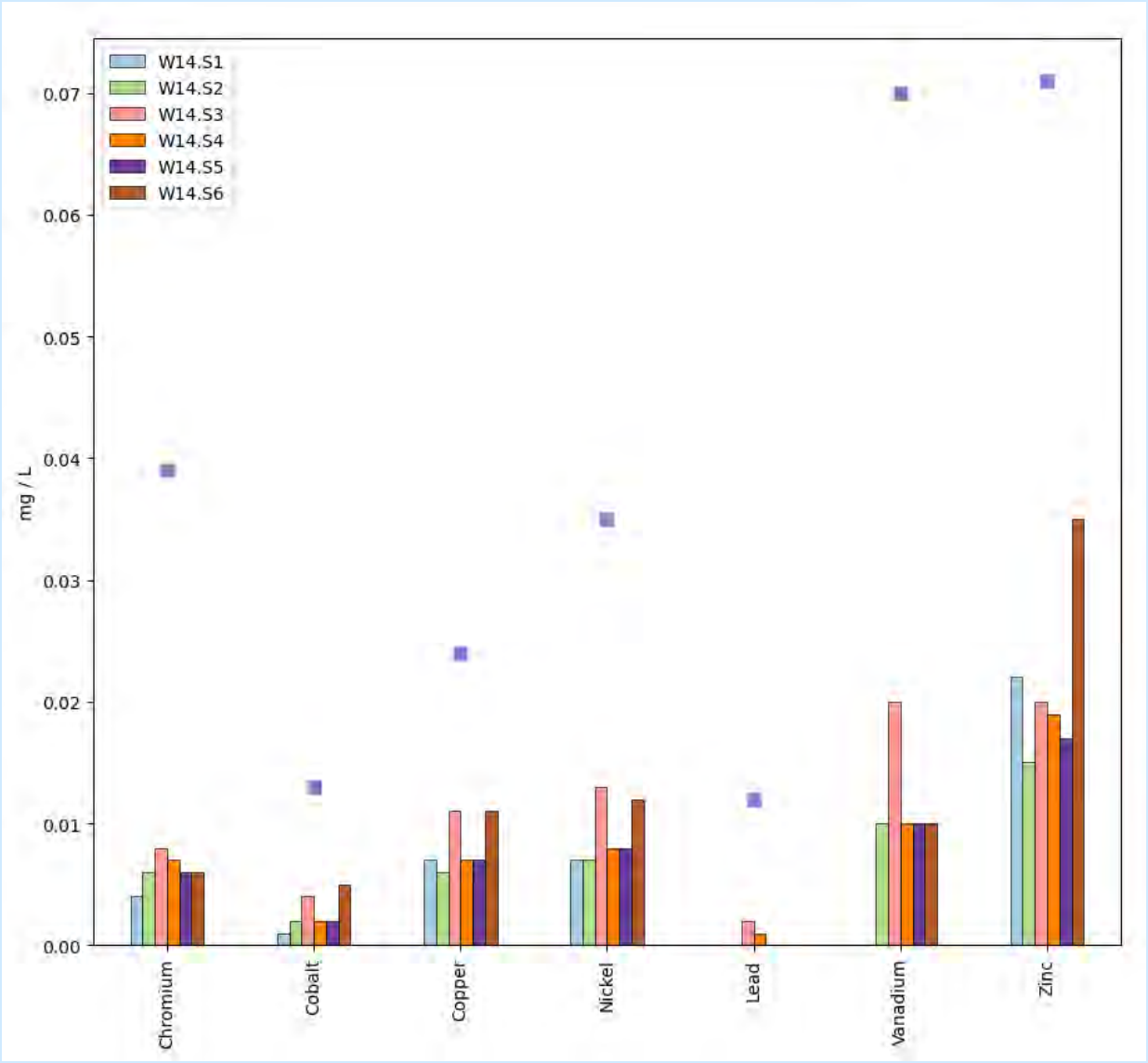



**Figure B3 Bar plot showing the concentrations of total metals in individual surface water samples around Narrabri Lake or O'Briens Creek**

The purple square points show the observed mean concentration in 2023 in O'Briens Creek.

In terms of major anions (bicarbonate, chloride and sulphate), most samples of the region were dominated by bicarbonate (e.g. W1, W4 or W15) or an approximately even spread of bicarbonate to chloride (e.g. W2, W8 or W11) and most samples either completely lacked sulphate or had less than a 20% contribution by this anion (Figure 45). The most sulphate rich sample was W16 (Narrabri Creek near Cameron St in Narrabri) which had ~17% sulphate as a major anion (Figure 45).

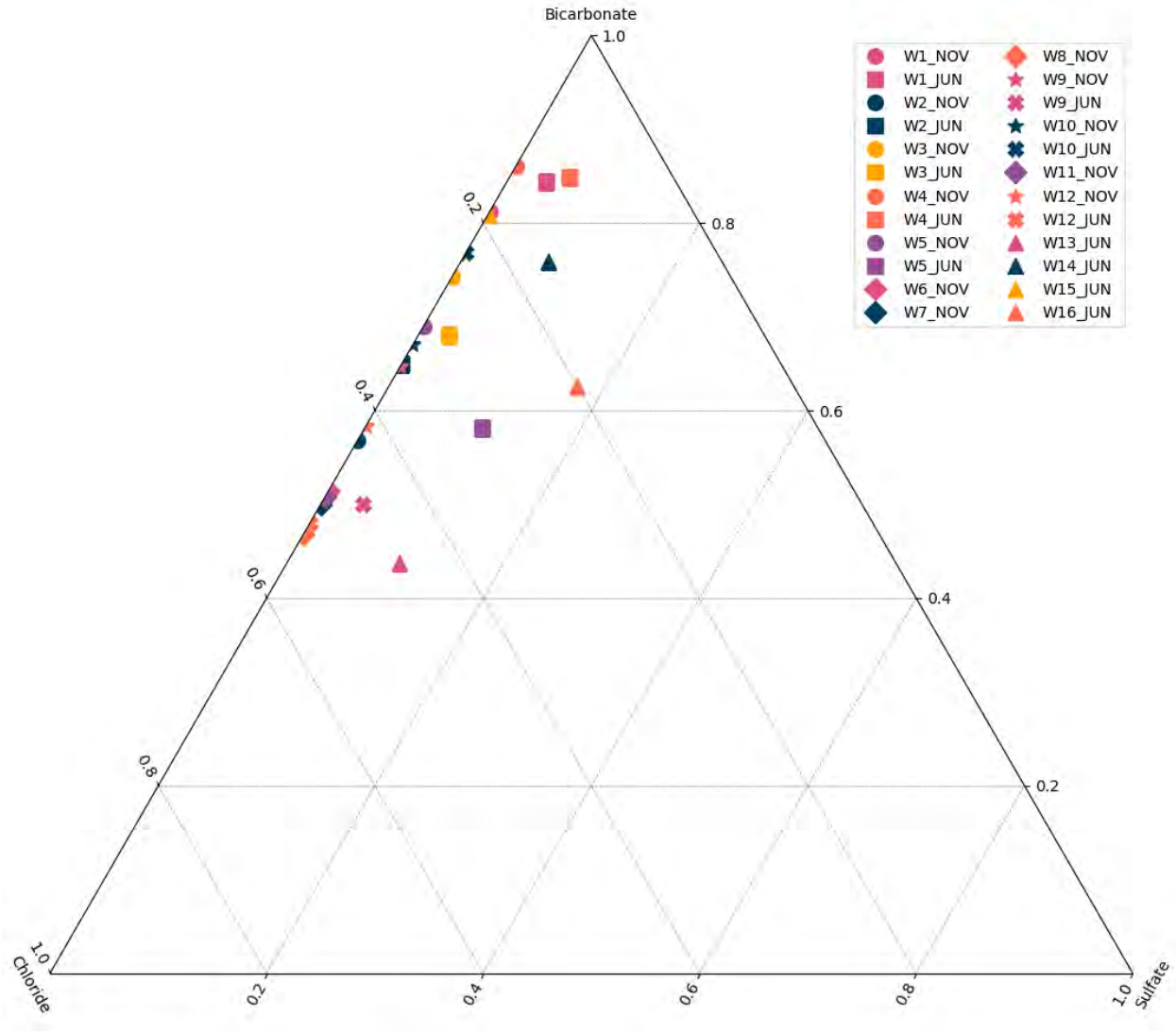



**Figure 45 Ternary plot of the proportions of major anions ($HCO_3^-$, $Cl^-$ and $SO_4^{2-}$) for surface water samples**

### 4.1.3 Soil samples of the region

In terms of structure, the most common soil types collected contained significant amounts of coarse sand (<0.2 mm-2 mm) (Table 9 and Figure 46). In some cases, sand comprised a large portion of the inorganic matter in the soils. For example, S10, taken near Yamminba Rest area in the south of the Narrabri Gas Project area contained over 80% coarse sand, and the median proportion for coarse sand in soils collected for this study was over 50% (Table 9).

Table 9 Particle sizes (%) in soils collected during the June 2023 sampling

| Sample | Clay (<2 µm) | Silt (2-20 µm) | Fine Sand (0.02-0.2 mm) | Coarse Sand (0.2-2.0 mm) | Gravel (>2 mm) |
|---|---|---|---|---|---|
| **S1** | 38 | 19 | 19 | 23 | 1 |
| **S2** | 16 | 10 | 8 | 65 | 1 |
| **S3** | 26 | 22 | 12 | 38 | 2 |
| **S4** | 49 | 30 | 19 | 2 | 0 |
| **S5** | 20 | 13 | 12 | 54 | 1 |
| **S6** | 36 | 28 | 15 | 16 | 5 |
| **S8** | 9 | 8 | 8 | 74 | 1 |
| **S9** | 4 | 11 | 7 | 77 | 1 |
| **S10** | 9 | 7 | 3 | 81 | 0 |
| **S16** | 20 | 10 | 13 | 57 | 0 |
| **S17** | 15 | 5 | 8 | 65 | 7 |
| **S19** | 20 | 7 | 17 | 54 | 2 |
| **S20** | 19 | 8 | 13 | 59 | 1 |
| **S22** | 21 | 8 | 9 | 61 | 1 |
| **S23** | 25 | 13 | 15 | 47 | 0 |
| **S24** | 15 | 8 | 9 | 67 | 1 |
| **S26** | 19 | 10 | 8 | 58 | 5 |
| **S27** | 53 | 28 | 8 | 8 | 2 |
| **S28** | 19 | 7 | 27 | 46 | 1 |
| **S29** | 30 | 22 | 20 | 25 | 3 |
| **S30** | 26 | 12 | 11 | 50 | 1 |
| **S31** | 24 | 9 | 14 | 52 | 1 |
| **S32** | 41 | 21 | 23 | 15 | 0 |
| | | | | | |
| **Median** | **20** | **10** | **12** | **54** | **1** |

Some samples were, however, collected outside of the NGP area to act as controls and comparisons to the sandier soils of the region. For example, S4, a vertosol from the Australian Cotton Research Institute comprised almost 50% clay (Table 9). Other clay rich soils were also included in the study for example, S27 and S32 were both roadside samples taken outside of a stand of Weeping Myall Woodland and near the Brigalow Nature Reserve. Both these soils were similar to S4 in that they represent remnant woodland on the main agriculturally important soil in the region.

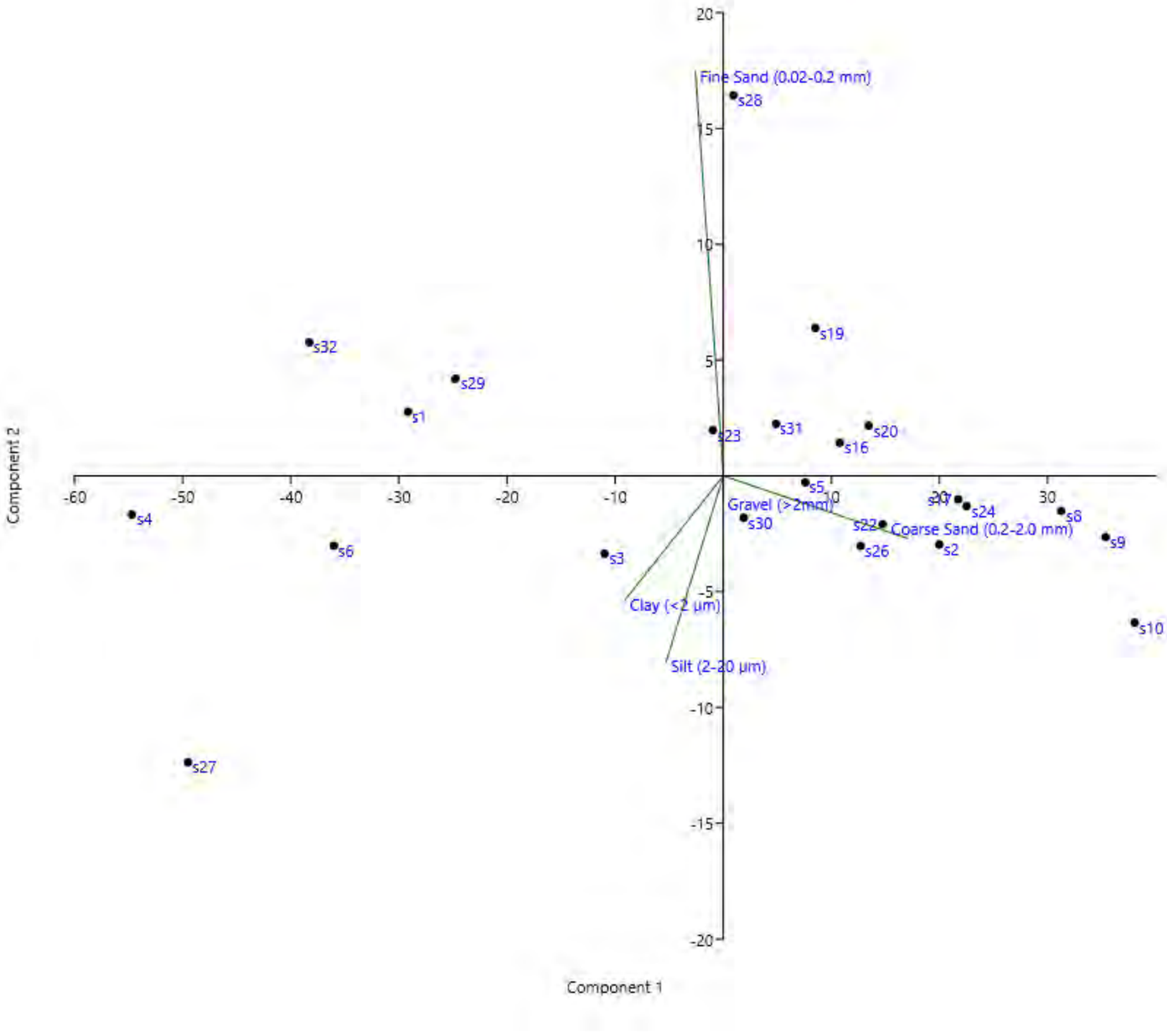



**Figure 46 Principal Component Analysis (PCA) showing an ordination of the particle size distribution from the June 2023 soil samples**

PC1 and PC2 are shown and represent 94% and 3% of the variance in the dataset. Samples close together in the ordination are similar for these characteristics. It should be noted that as almost 95% of the variance in the data is captured on the x axis (PC1). Vertical differences (PC2) are, in general, much less important in terms of sample difference.

This difference between the vertosols and sodosol of the region was carried through to other experiments in the current study with microbial degradation of chemicals tested in S4 and S24. The difference between these two important soils in this study is marked with S4 being mostly clay and silt, while S24 is mostly coarse and fine sand (Table 9 and Figure 46).

Another important determinant of soil structure (other than particle size distribution) is the amount of organic matter (or total organic carbon (TOC)). The majority of samples examined in the present study contained 1-4% TOC (Figure 47). Some samples, particularly associated with woodland soils (e.g. S1, S8 and S10) contained 10-12% TOC, mostly associated with litterfall and recalcitrant carbon. TOC is highly variable between replicates, within some sample locations, such as S10 collected in November 2022 and S26 collected in June 2023 with TOC differences of 8.9 and 7.4, respectively (Figure 47).

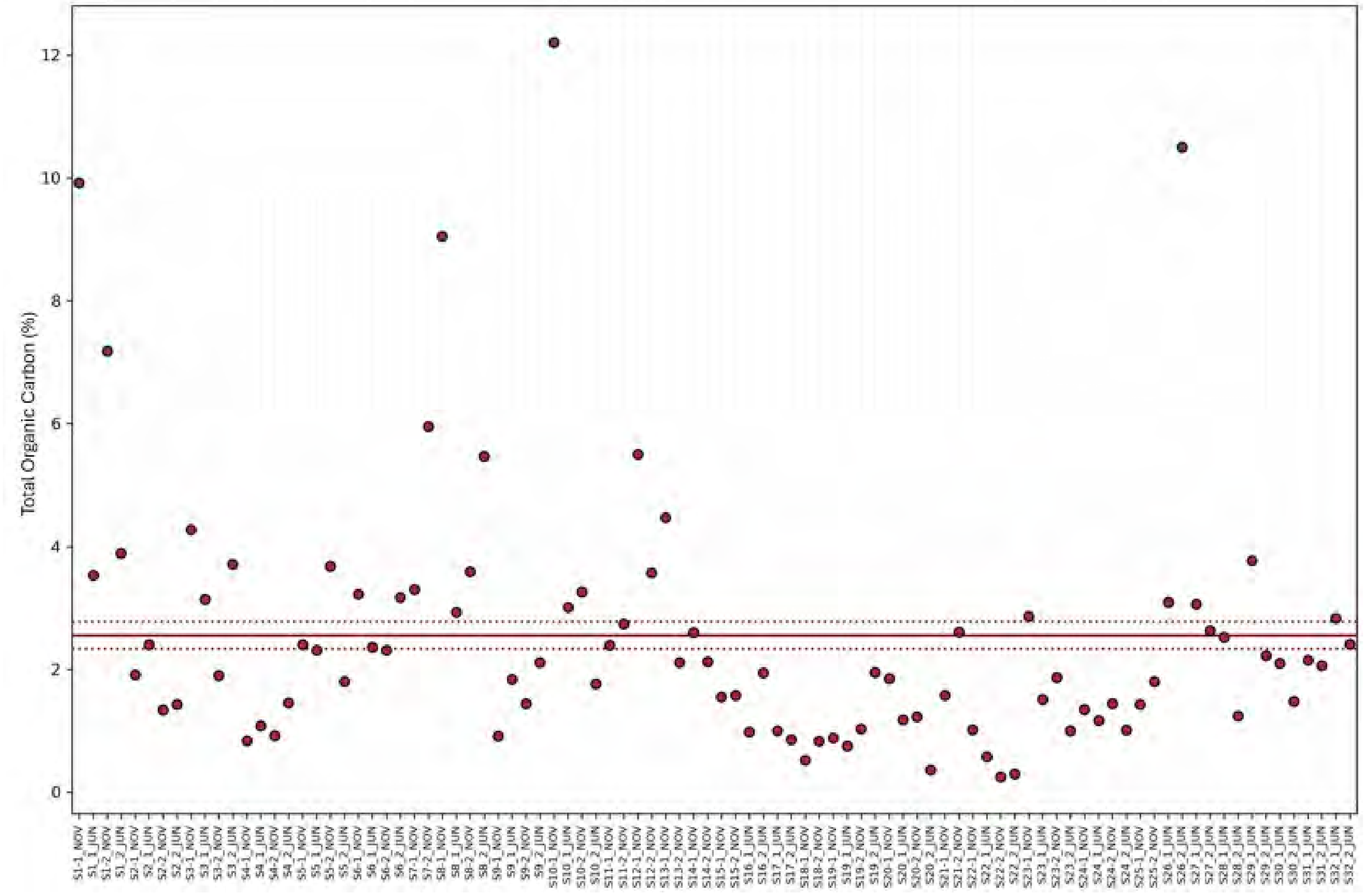



**Figure 47 Dot plot showing concentration of organic matter (%) across the 92 soil samples collected in the present study**

The mean is shown as an unbroken red line, while horizontal dotted lines show the standard error of the mean.

In addition to organic carbon, a range of other chemical analytes were tested for in the 92 soil samples in the present study. In broad terms, the chemistry of the samples was largely unaffected by the season of collection and most analytes were not significantly different between time points. There were, however, some exceptions. For example, moisture content of soils differed significantly ($p<0.00001$) between the two seasonal collections with soils (Figure 48).

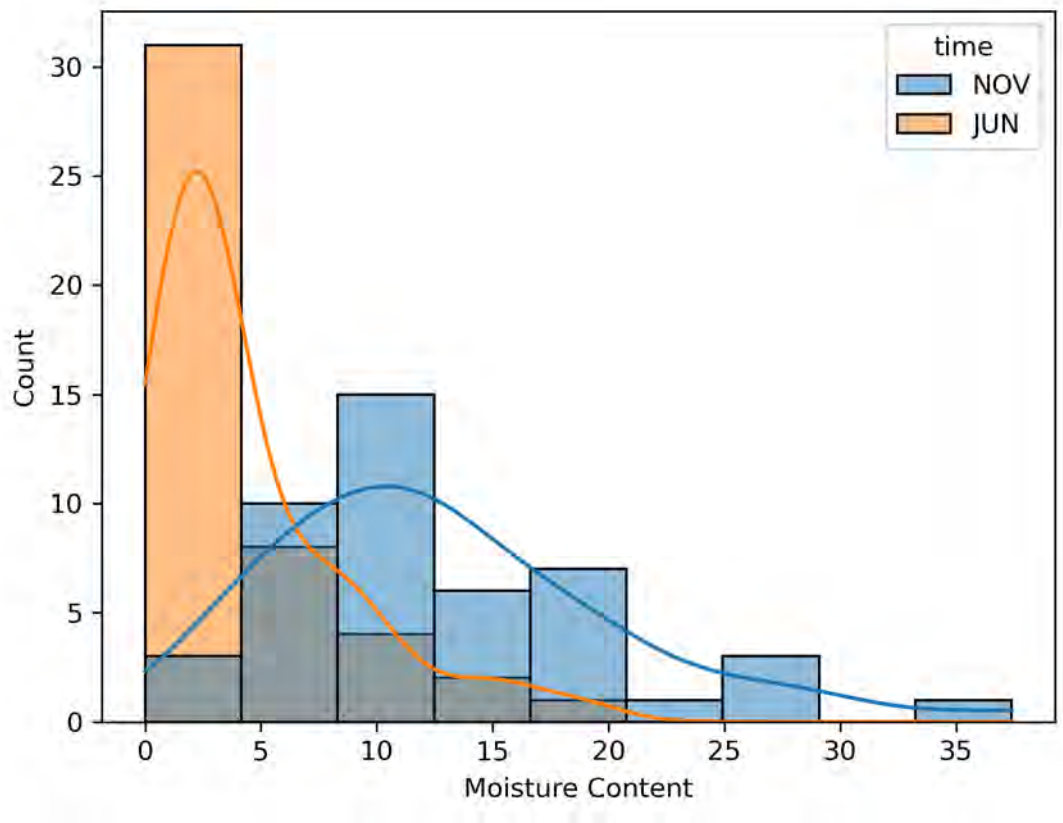



**Figure 48 Histogram with KDE (an estimate of the distribution as a line) for the moisture content (%) across the 92 soil samples collected in the present study**

Overlapping bars are shown in grey.

Unsurprisingly, given the high rainfall and flooding, moisture was significantly elevated and most samples collected during the November 2022 time point had moisture contents at or above 10% (Figure 48). In contrast, the majority of samples from June 2022 had moisture content less than 5% (Figure 48). The only other significantly altered chemistry between the two time points were chloride and soluble nitrite/nitrate. Likely correlated with this event were the lower chloride content of some soils at the November sampling. This, presumably, was a result of leaching of this chloride by rainwater (Figure 49, left). Conversely, some samples from the November collection showed elevated nitrite and nitrate (Figure 49, right), which may have been the result of nitrification of ammonia.

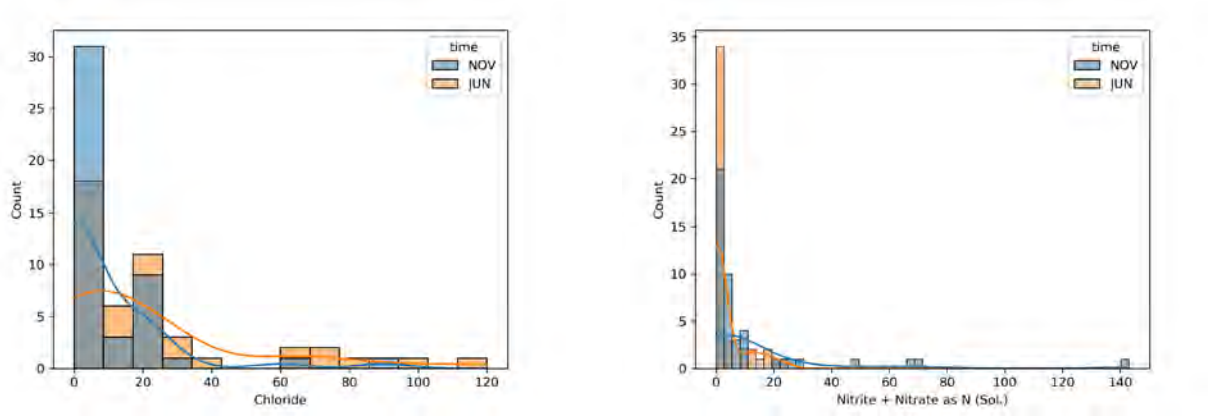



**Figure 49 Histograms with a KDE (an estimate of the distribution as a line) for chloride (left) and nitrite + nitrate (right)**

Overlapping bars are shown in grey.

Chloride analyses (left); Nitrite + Nitrate analyses (right). Soil samples collected in November 2022 (blue) or June 2023 (orange).

Units for these plots are both ppm (mg $L^{-1}$).

All other analytes examined (see Section 3.2) were not significantly different between the seasonal collections. Most soils were mildly acidic in pH, though some alkaline soils were also identified (Figure 50). This correlated somewhat with soil type, with the vertosolic soils tending to be less acidic than the sandy soils. For example, S4 from the Australian Cotton Research Institute were around ~pH 8, this is similar to previous work at this site which suggests pH above 7.5 (Hulugalle et al., 1999).

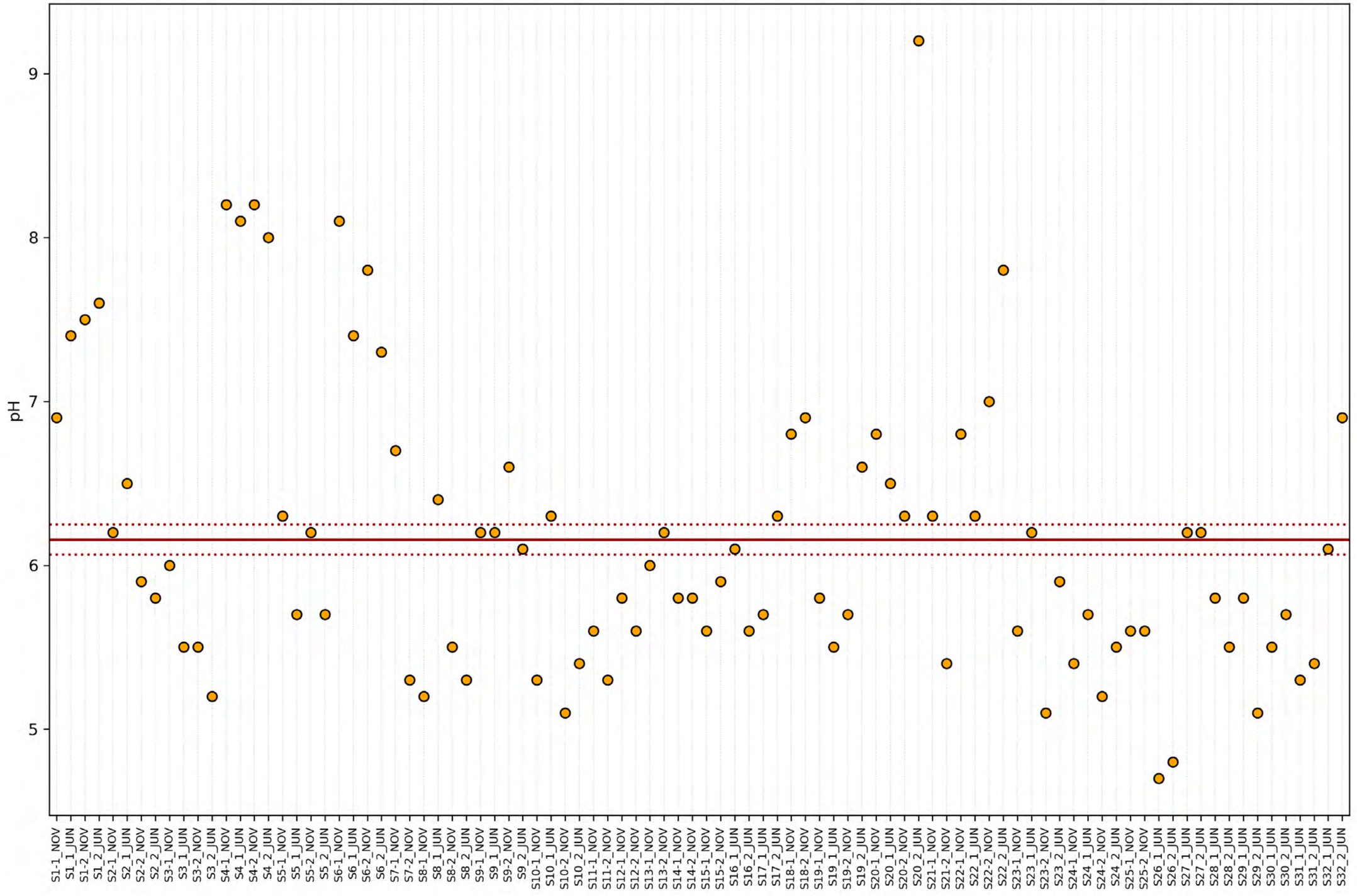



**Figure 50 Dot plot showing the pH across the 92 soil samples collected in the present study**

The mean of the entire dataset (n=92) is shown as an unbroken red line, while horizontal dotted lines show the standard error of the mean.

A range of metals were examined in the soil samples including barium, calcium, magnesium, manganese, potassium and sodium (Figure 51); as well as arsenic, chromium, cobalt, copper, lead, nickel, vanadium and zinc (Figure 52). In addition, boron, cadmium, mercury and selenium were analysed, though, these metals were below their limits of reporting (50, 1, 0.1 and 5ppm, respectively) in all soil samples. Beryllium was only detected in two soils S1 and S4, at 2 and 1 ppm, respectively (data not shown).

The levels observed are broadly consistent with other soils. For example, the mean chromium concentration of soil samples from this study was ~14 mg $kg^{-1}$ (± 1.6 mg SEM). In a broad study of soils from the US the mean concentration was ~37 mg $kg^{-1}$ (Shacklette and Boerngen, 1984). Some of these other metals are low by international standards, for example, average vanadium content of soils in China are ~82 mg $kg^{-1}$, and while three soils had concentrations of vanadium higher than this, most the median vanadium concentration was around 20 mg $kg^{-1}$ (Li et al., 2020). Lead and copper were also observed in all soils at fairly low levels, for example, the mean for lead and copper in this study were 5 (± 0.52 SEM) and 5 (± 0.97 SEM), respectively. This is less than some international norms; for example, lead and copper in UK soils average ~42mg $kg^{-1}$ (Davies, 1983) and 10 mg $kg^{-1}$ (Reaves and Berrow, 1984), respectively.

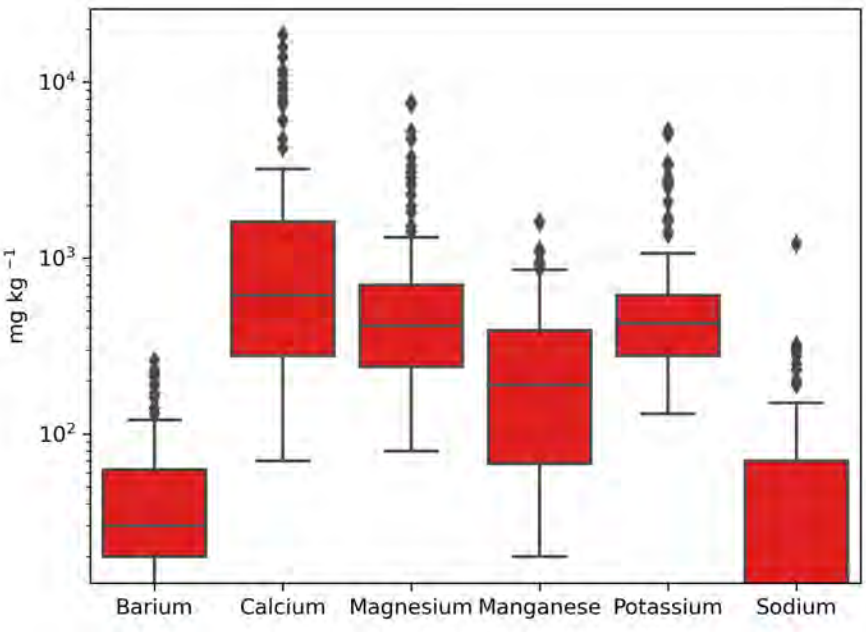



**Figure 51 Boxplot of barium, calcium, magnesium, manganese, potassium and sodium content of soil samples collected in this present study**

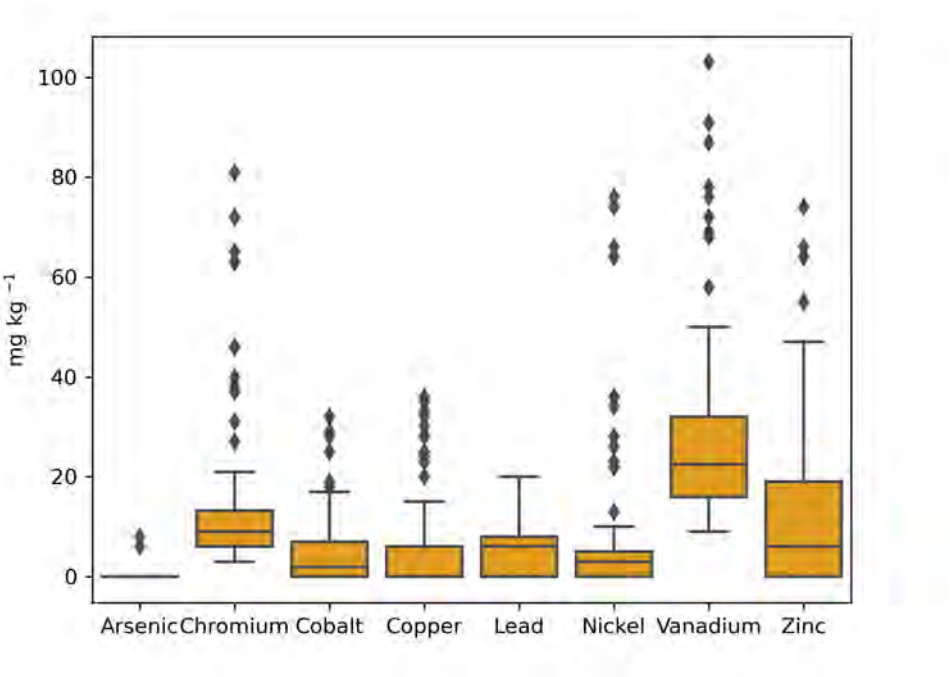



**Figure 52 Boxplot of arsenic, chromium, cobalt, copper, lead, nickel, vanadium and zinc content of soil samples collected in this present study**

## 4.2 Microbial baseline data for the Narrabri region

### 4.2.1 Groundwater bacterial baseline data

The cell numbers in groundwaters are generally low (Pedersen, 1996). Typically this is due to the low nutrient loading and organic carbon content of the water that would be required to support higher cell numbers. Additionally, given that a significant portion of the subsurface microbiome is adhered to surfaces of minerals, sampling the water alone likely represents something of an underestimate (Escudero et al., 2018; McMahon and Parnell, 2014; Patel et al., 2024). This low cell number meant that some samples did not yield DNA that could support analyses, particularly in the November 2022 collection.

Regardless, significant baseline data were collected for groundwater bacterial communities in the region. Almost 6,600 prokaryotic species were detected from the groundwaters, with individual ~300 mL samples hosting an average of just under 1000 OTUs (≈ species). There was a significant difference ($p<0.01$) between the samples collected in November 2022 and June 2023, though the reason for these differences is unclear.

The most abundant phyla across the samples were the Campylobacterota (formerly a class, Epsilonproteobacteria, within the Proteobacteria) (see Supplementary data). Interestingly, despite its high mean phylum abundance, it was represented by only a small number of OTUs (n=9). These included sequences from the *Campylobacter, Helicobacter, Malaciobacter, Sulfuricurvum, Sulfurimonas* and *Sulfurispirillum* genera. The next most abundant group was the Nitrososphaerota, a group that includes various ammonia-oxidising, autotrophic species (Tourna et al., 2011). Such species have been observed in other aquifers, for example, in the Northern Territory GISERA has previously seen numerous ammonia-oxidising *Nitrososphaeria* species were observed in aquifers in the Beetaloo Basin region in central Australia (Tran-Dinh et al., 2022). Other phyla observed included the Pseudomonadota, Nitrospira, Chlorobiota, Bacillota, and Fusobacteriota. The distribution of these phyla differed across the samples. For example, the Pseudomonadota were observed at similar levels across most samples in the present study (see Supplementary data). In contrast, other phyla (for example the Fusobacteriota) were much more common in some samples (for example, G10), while largely absent from G2, G5 and G8 samples (see Supplementary data).

Examination of the data at genus level reveals that the most abundant genera were *Gallionella, Arcobacter, Perlucidibaca, Ferrigenum* and *Gemella*. There are some trends in the kinds of microbes that are being observed. For example, there appears to be an iron-oxidising community that includes some taxa known to produce stalks (Chan et al., 2011). This community includes: a strain of *Ferrigenium kumadai*, *Gailonella capsiferriformans*, and a *Ferriphaselus* species (see Supplementary data; Table 10). All three species are reported to oxidise iron under hypoxic conditions (Fabisch et al., 2016; Garry et al., 2024; Khalifa et al., 2018) and most of these taxa are absent from the deeper bore samples where oxygen is likely excluded (G9, 10 and 11). Indeed, these species are particularly common in shallow (~40 m) depths. Such depths are likely unconfined, alluvial aquifers and the presence of these organisms in these samples makes more sense in the light of oxygen entering into this environment through recharge.

Table 10 Taxonomic information of top 10 most abundant prokaryotic OTUs in groundwater samples

| OTU | Closest match* | Identity (%)† |
|---|---|---|
| **OTU49** | *Ferrigenium kumadai* An22 (NR 179187) | 100 |
| **OTU26** | *Gallionella capsiferriformans* ES-2 (NR 074658) | 99.2 |
| **OTU2** | *Curvibacter delicatus* NBRC 14919 (NR 113696) | 100 |
| **OTU7** | *Ralstonia pickettii* ATCC 27511 (NR 043152) | 100 |
| **OTU729** | *Sideroxydans lithotrophicus* ES-1 (NR 074731) | 98.8 |
| **OTU77** | *Amnimonas aquatica* HR-E (NR 165751) | 99.2 |
| **OTU23** | *Sulfurisoma sediminicola* BSN1 (NR 125471) | 98.4 |
| **OTU141** | *Sideroxydans lithotrophicus* ES-1 (NR 074731) | 97.2 |
| **OTU3479** | *Serpentinimonas maccroryi* B1 (NR 181589) | 98.4 |
| **OTU7331** | *Ferriphaselus amnicola* OYT1 (NR 114334) | 98.8 |

* Closest matches on GenBank database. † Percentage identity match to GenBank sequence.

Another key physiological character of the species detected in groundwaters was autotrophy; the ability to fix inorganic carbon (carbon dioxide or bicarbonate) to organic carbon. This makes sense in an environment like the subsurface where outside of organic rock formations, organic carbon is relatively scarce. Of the most common species for example, *Ferrigenium*, *Sideroxydans*, *Sulfuristona* and *Ferriphaselus* are likely capable of at least facultative autotrophy. Similarly, the common phylum Nitrosospheria also includes a range of ammonia-oxidising autotrophs, highlighting the importance of this process in aquifer environments. Indeed, it may be that autotrophic microbes represent primary producers in this environment and subsequently support other heterotrophic species. Thus the presence of taxa like *Amnimonas aquatica* in some samples, a taxon not known for autotrophy (Lee et al., 2019), may be supported through the growth of key autotrophic species and their production of biomass and extracellular materials.

### 4.2.2 Surface water bacterial data

Given the significant flood event in late 2022, it is unsurprising that the microbiomes detected in the surface waters between the two sampling times were significantly different ($p<0.0006$). One feature of surface waters is the presence of a variety of eukaryotes including various algal species along with bacteria. It is noteworthy that the photosynthetic components of algal cells (chloroplasts) are bacterial in origin and have their own DNA. Some of this DNA matches the primers (16S) used in this study to profile bacteria (Apprill et al., 2015; Parada et al., 2016), as such, algal OTUs can be co-reported with bacterial OTUs. Thus, the total OTUs reported for this environment include a number of non-bacterial OTUs. These include some relatively abundant sequences recovered from various surface water samples (Table 11).

Table 11 A selection of algal OTUs recovered from surface water samples from 16S DNA of chloroplasts

| OTU | Closest match* | Identity (%)† |
|---|---|---|
| **OTU40** | *Cyclotella cryptica* chloroplast | 100 |
| **OTU1085** | *Pseudo-nitzchia* sp. chloroplast | 98.8 |
| **OTU65** | *Synura* sp. chloroplast | 100 |
| **OTU89** | *Micractinium* sp. chloroplast | 100 |
| **OTU59** | Unknown algal chloroplast | 98.8 |
| **OTU95** | *Synura* sp. chloroplast | 96.0 |

* Closest matches on GenBank database. † Percentage identity match to GenBank sequence.

In general, microbial ecology of freshwater habitats (especially the water column itself) are more poorly studied than soils, sediments or even marine waters. Consequently, microbes that occur in these environments tend to be less well characterised and less well understood in terms of their functional roles in this habitat.

The identification of algae is mirrored by the bacterial communities which include many photosynthetic cyanobacterial species. Indeed, Cyanobacteriota were the most commonly identified phylum (see Supplementary data). While cyanobacteria were the most commonly identified phylum across the study, in broad terms they were more common in June than November. This is likely also due to the flood event which in part increased the abundance of non-cyanobacterial taxa through the incorporation of soil and sediment into fast-flowing waters during the flood, this disturbance and the lack of light penetration into the water also likely disadvantaged the cyanobacteria (and algae more generally) and may have also contributed to their generally lower abundance in November.

In addition to Cyanobacteriota, numerous other phyla were detected commonly including the Actinomyceota, Fusobacteriota, Campylobacteriota, Pseudomonadota and Verrucomicrobiota (see Supplementary data). Some of these phyla were relatively uniformly observed across the samples e.g. the Actinomyceota or the Pseudomonadota, while others like the Fusobacteriota had a more uneven distribution, being relatively common in some samples but absent (or nearly so) from other samples.

Among the more commonly detected species in the current study was a strain of *Polynucleobacter acidiphobus* (OTU17)(Table 12). This genus is known principally as endosymbionts of freshwater ciliates from the genus *Euplotes* (*Hypotrichia*) (Hahn et al., 2011a), though the type strain of *P. acidiphobus*, at least, appears to be a free-living organism. Given the genetically close relationship between OTU17 and *P. acidiphobus* the strain here likely engages in the same behaviour as the described species. This species appears to be widespread occurring in all water samples examined in the present study regardless of time point. It is also widespread in other freshwater environments globally, occurring in Africa (Hahn et al., 2011a), the Americas (Hahn et al., 2011b; Horner-Devine et al., 2003; Shaw et al., 2008; Simpson et al., 2004), Asia (Wu et al., 2006; Zhou et al., 2017) in Europe (Hahn et al., 2011a), on Pacific islands (Donachie et al., 2004), and here in the

present study in Australia. It is noteworthy that here one water sample (W1; Yarrie Lake; November 2022) was pH ~5, and numerous samples were in the pH 6 range, calling into question observations (from the organism's description) that this taxon does not occur in mildly acidic waters.

Like *P. acidophobus*, OTU41 (a member of the *Fonsibacter* LD12 subclade, and probably a strain of *F. ubiquis* that is closely related to the type strain LSUCC0530. Like OTU17, this strain appears to be a common member of the bacterial community in freshwater environments in a range of habitats globally (Newton et al., 2011), including environments in North America (Henson et al., 2018) and Europe (Salcher et al., 2011), in the present study, in Australia. Interestingly, W1 (Yarrie Lake) aside, this taxon was absent from other surface water samples in November 2022 (see Supplementary data). It is, however, present in most samples from June 2023 (see Supplementary data). It is notable that most surface water samples, except W1, W10 and W12 came from rivers, streams or creeks in the region and it may be that microbial communities in creeks, streams and rivers were disproportionately impacted by the flood event in 2022. Similarly, OTU1864, which is species of *Verrucomicrobiota* and perhaps part of the class Spartobacteria, only occurred in the June 2023 collection and was completely absent from all November collections, suggesting it too is sensitive to flooding.

In addition to microbes that appear to be affected negatively by the flooding of 2022, numerous microbes appear to benefit from this event. For example, OTUs 63 and 110 appear to be more common (at least in some samples; W9 and W11) during the flooding (see Supplementary data). OTU110 is a Rhodocyclaceae species that is probably in a novel genus. Its closest relatives were sequences from the Karasawa River in Japan (Genbank: LC094645), from floating rice-beds in China (Genbank: JQ994356) and Étang de Thau (a string of lagoons in coastal France; Genbank: JN679957). This taxon appears to be most commonly associated with water and presumably its presence here is enhanced by the increased water availability during the flood. Like OTU110, OTU_63 also appears to be a water-associated taxon, this species has no close described relatives, however, its 16S sequence is 100% identical to sequences (Genbank: KY516348, KP687139, JF488106 and MF438632) recovered from lakes and rivers (Balmonte et al., 2016; Martinez-Garcia et al., 2012; Schiff et al., 2017).

Regardless, surface water microbial community baseline data reveal a host of typical, but poorly studied, bacteria that form part of the bacterioplankton in the Narrabri region. In general, waterways of this region exceed the trigger values set for eutrophication set by ANZECC [15]and used in NSW. This is, in part, unsurprisingly, given the moderate disturbance that has occurred in the region for some time in association with cropping, grazing, forestry and other practices.

[15] Australian and New Zealand Environment and Conservation Council. https://www.waterquality.gov.au/anz-guidelines/resources/previous-guidelines/anzecc-armcanz-2000

Table 12 Taxonomic information of top 10 most abundant prokaryotic OTUs in surface water samples

| OTU | Closest match* | Identity (%)† |
|---|---|---|
| **OTU17** | *Polynucleobacter acidiphobus* MWH-PoolGreenA3 (NR 125545) | 100 |
| **OTU41** | *Fonsibacter* sp. (SAR11) | 100 |
| **OTU83** | *Planktophila sulfonica* | 100 |
| **OTU69** | *Aquirufa antheringensis* 30S-ANTBAC (NR 165029) | 100 |
| **OTU522** | *Rhodoluna planktonica* (NR 125488) | 98.4 |
| **OTU63** | *Fontisphaera persica* B-154 (NR 189261) | 90.1 |
| **OTU110** | *Burkholderia pyrrocinia* ATCC 15958 (NR 112060) | 95.3 |
| **OTU323** | *Rhodoluna lacicola* MWH-Ta8 (NR 125493) | 98.0 |
| **OTU58** | *Sediminibacterium salmoneum* NBRC 103935 NJ-44 (NR 044197) | 100 |
| **OTU145** | *Ramlibacter monticola* G-3-2 (NR 159166) | 100 |

* Closest matches on GenBank database. † Percentage identity match to GenBank sequence.

### 4.2.3 Soil bacterial data

Across the soils examined in this study a total of 37,754 distinct OTUs (≈ species) were found. The most abundant phyla were the Actinobacteria, followed by the Proteobacteria, which, represented ~37% and 24% of all OTUs. There were significant differences between the different collections, however, these were relatively minor in terms of the quantum of difference, i.e. no abundant phyla differed, for instance, by more than an order of magnitude. For example, Actinobacteria represented ~32% of all OTUs in June 2023, and 42% of all OTUs in November 2022. Conversely, the Acidobacteria represented ~16% of all OTUs in June 2023, but only 9% of OTUs in November 2022. These high-level differences could be related to temperature differences between the two timepoints (the average min/max in November is 15-30°C, compared to the June average min/max of 5-19°C) or maybe related to soil moisture, with the flood event of November 2022 increasing soil moisture markedly around the time of collection. Both abundant phyla were bacterial, though the *Thaumarchaeota*, an archaeal phylum was also common representing between four and two percent of OTUs at the November 2022, and June 2023, timepoints, respectively.

Across the OTUs, ~1191 bacterial genera were observed. This is a relatively high number for a single study, though it should be noted that this study takes in a range of soils from different habitats and land-use types. From these phyla, the most common genera detected was the actinobacterial taxon *Conexibacter* (Table 13). Indeed, there were 156 OTUs that were related to *Conexibacter*. *Conexibacter* is a relatively newly described taxon, having been first identified in forest soils near Milan, Italy (Monciardini et al., 2003). *Conexibacter* species have also been found in numerous other environments, including soils from Senegal were they comprise quite significant amounts of the bacterial microbiome (Diatta et al., 2020). The type species of this genus, *C. woesei* is characterised by its long flagella which form spiral networks through which individual cells of this species can aggregate. The species observed in the soils of the Narrabri region were not *C. woesei* but were likely in the genus. Abundance across the study area varied, with some samples having quite significant numbers of *Conexibacter*. For example, S3 (a sandy soil

from farmland in the Jack's Creek area) had high abundance of *Conexibacter* (>10% of all bacteria detected) regardless of the sampling time, suggesting this is a common taxon in these soils (see Supplementary data). Other soils had relatively low abundances for this genus, S4 for instance (which was a vertosol soil collected at the ACRI research site) had less than 0.5% *Conexibacter* species, across both time points) (see Supplementary data).The function of this genus is unclear, however, some members are capable of degrading complex carbon sources. Lei et al., (2023) for example, detail a strain of *Conexibacter* (LD01) that appears to be able to grow on lincomycin (a pyrrolidine linked pyranose moiety). Such complex carbon may occur in moribund organic matter or may be included in plant root exudates.

Other commonly detected genera include the members of subdivision 3 of the Acidobacteria another actinobacterial genus, *Rubrobacter* and numerous species from the archaeal genus *Nitrososphaera* (Table 13). Knowledge of the ecology and function of Acidobacteria genera is limited (Fierer et al., 2007; Kuramae and de Assis Costa, 2019). The genus *Rubrobacter* is known for its radiation tolerance, halotolerance and moderate thermotolerance (Hatzenpichler et al., 2008). It may be that these species occur in the top layers of soil, where they are exposed to heat and ultraviolet radiation from the sun, or it may be that the occurrence of these species is related to the sodic soils of the region having high sodium concentrations, relative to other cations. Further work would be required to confirm this speculation. For the acidobacterial species, subdivision 3 likely represents a family (the Bryobacteraceae), which has been proposed by Dedysh et al., 2017, after the type species *Bryobacter* which was described as moss-associated (and comes from a sphagnum bog). Given the paucity of information about these species its role in the Narrabri area is unclear. Lastly, the *Nitrososphaera* species are mostly ammonia oxidising autotrophs that use the energy from oxidising ammonia to nitrite in order to fix $CO_2$ (Tourna et al., 2011), they are ubiquitous in oxic environments and their range includes the project area in this study.

**Table 13 Taxonomic information of top 10 most abundant prokaryotic OTUs in soil samples**

| OTU | Closest match* | Identity (%)† |
|---|---|---|
| **OTU44** | *Conexibacter woesei* ID131577; DSM 14684; JCM 11494 (NR 028979) | 96.8 |
| **OTU25** | *Baekduia soli* BR7-21 (NR 178337) | 96.4 |
| **OTU37** | uncultured *Holophaga* sp. (AJ519386) | 95.7 |
| **OTU5** | *Bradyrhizobium liaoningense* 2281; USDA 3622 (NR 041785) | 100 |
| **OTU43** | *Crossiella cryophila* NRRL B-16238 (NR 024964) | 97.2 |
| **OTU3** | *Arthrobacter nitrophenolicus* SJCon (NR 178397) | 100 |
| **OTU15** | *Rubrobacter spartanus* HPK2-2 (NR 158052) | 97.2 |
| **OTU312** | *Conexibacter woesei* DSM 14684 (NR 074830) | 94.1 |
| **OTU31** | *Nitrososphaera viennensis* EN76 (NR 134097) | 94.8 |
| **OTU57** | *Angustibacter speluncae* YC2-20 (NR 158006) | 98.8 |

* Closest matches on GenBank database. † Percentage identity match to GenBank sequence.

### 4.2.4 Surface waters fungal data

In total 1586 species (fOTUs) were detected from the surface water samples examined in the present study. The samples included representatives from the Ascomycota and Basidiomycota as well as the Chytridiomycota and Zygomycota. Unlike soils, the Glomeromycota were not detected in the surface waters, despite the 2022 flood event that likely mobilised at least some soil materials into the surface waters of the region. This may be in part due to the relatively large size of the arbuscular mycorrhiza spores which are typically >100 µm in diameter (Davison et al., 2018; Paz et al., 2021).

Regardless, this flood event strongly impacted the diversity of fungi detected in the surface waters of the region ($p<0.0004$). Aggregating the fungi to genera for example, demonstrated that surface waters collected during the flooding in November 2022 had a significantly increased fungal diversity compared to June 2023 (Figure 53).

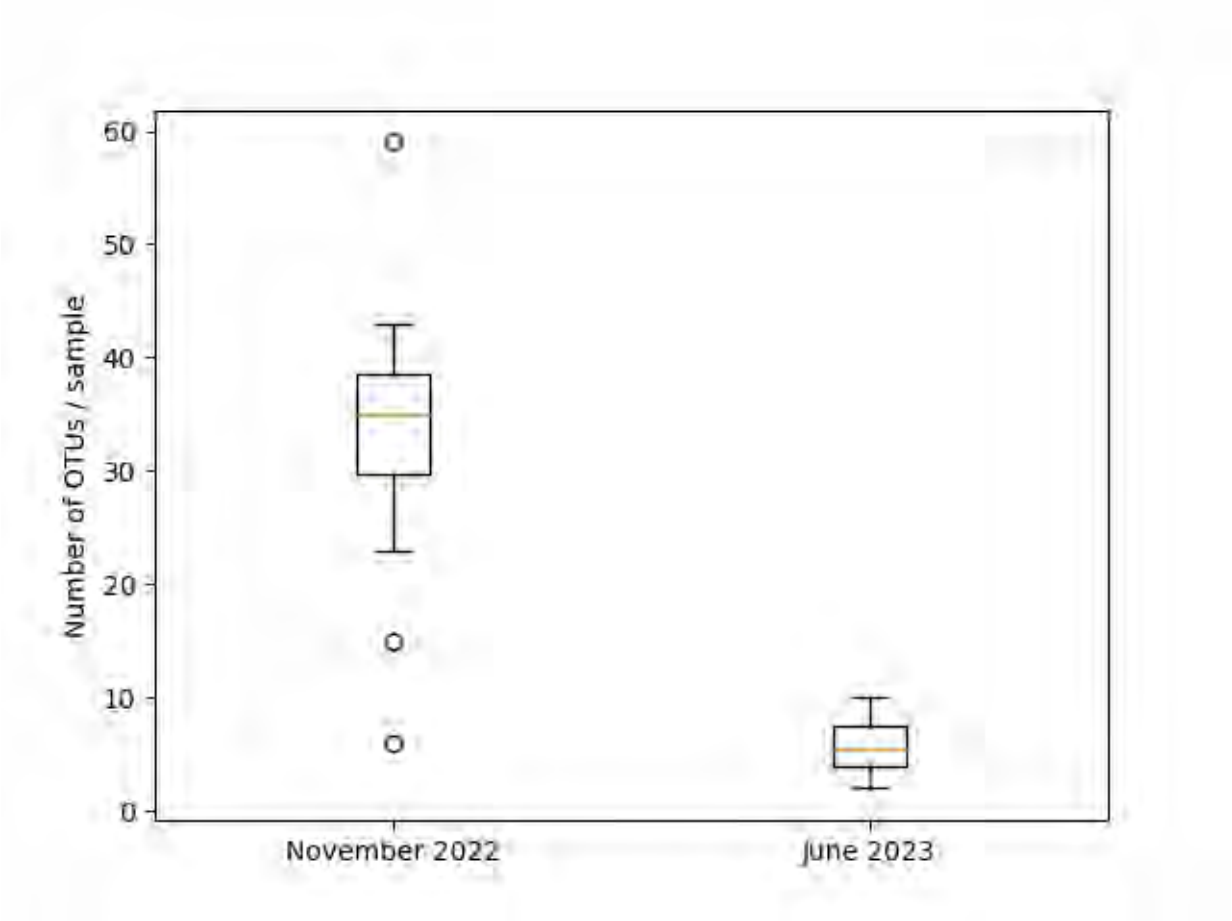



**Figure 53 Boxplot showing fungal diversity in surface waters per sample in the November 22 vs June 23 field campaigns**

The number of species detected at these two timepoints differed significantly ($p<0.0004$).

Along with each sample in June 2023 being less diverse, the water samples collected in November 2022 were also more distinct from each other (Figure 54). In other words, the flooding event acted to increase fungal diversity and distinctiveness between different creeks and other surface waters in the Narrabri region (Figure 53 and Figure 54). These events are relatively common in the region

due to the relatively flat geography of the region[16]. This is likely due to the recruitment of soil material into flood waters which had localised effects on which microbes were present in individual samples. For this reason, flooding likely masks any apparent seasonality of samples and it is not possible to describe seasonal changes in surface water fungal communities. Furthermore, for the purposes of microbial baselining of the region, the June 2023 samples should be considered a useful starting point for profiling the relatively low fungal diversity in surface waters of the region.

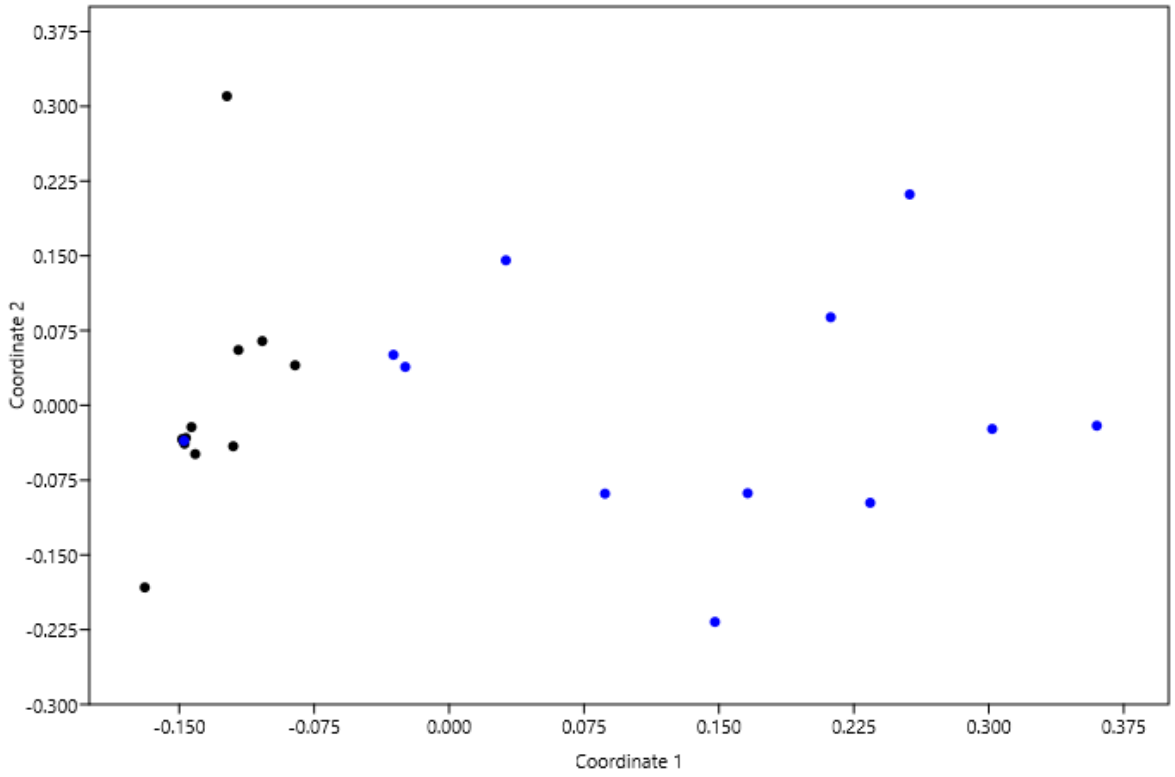



**Figure 54 Non-metric MultiDimensional Scaling (nMDS) ordination of the fungal communities in surface waters in the November 22 vs June 23 field campaigns**

The blue dots show samples collected during the flood event in November 2022, the black dots show samples collected in June 2023. In this plot, dots that are close to each other show similar microbial communities.

Examining the microbial diversity in the June 2023 surface waters reveals that most of the common fungi detected in water samples were poorly classified. Generally, the fungal microbiomes for surface waters in the Narrabri region broadly mirror some other studies. The key property of freshwater fungal communities are (1) that they are poorly studied (Bärlocher and

[16] NSW State Emergency Service brochure. https://www.ses.nsw.gov.au/media/1954/brochure-narrabri_res.pdf

Boddy, 2016), and (2) they contain a large number of poorly defined fungal taxa with more yeast and chytrid taxa than are detected in soils (Bärlocher and Boddy, 2016; Sutcliffe et al., 2018).

Starting first with taxa that were detected across many samples, the only species that was detected across all the samples was fOTU12292. This species was a yeast from the *Saccharomyces* genus most closely related to *S. cariocanus* (Naumov et al., 2000). Similarly, a chytrid, fOTU7167 was detected in ~60% of samples and is most closely related to, but distinct from *Rhizophydium carpophilum* (Letcher et al., 2006).

These chytrid fOTUs can also be quite abundant in individual samples, but not common to all samples. For example, a species of chytrid (fOTU72) was found to represent almost a quarter of all fungal sequences from two of the samples (W4 and W15). These samples were from the Namoi River in Narrabri township and Pine Creek southeast of Turrawan, a distance of ~20 km. This suggests this taxon is widespread in the region.

Finally, it should be noted that some of the recovered amplicons may not be fungal at all. For example, fOTU5439 was found at 75% of all sites, however, it is unclear which organism this DNA sequence was recovered from, it may be an algal sequence. Similarly, other non-fungal species recovered include species that maybe amoebal or rhizarian. This is not uncommon as other eukaryotic groups have the ITS region and subsequently can be amplified in the place of the target organisms (fungi) where fungal diversity is low.

### 4.2.5 Soil fungal data

Across the soils of the region, 2846 distinct fungal OTUs (fOTUs) were detected comprising ~270 described fungal genera. These included representatives from the Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and the Zygomycota. For the majority of samples, the Ascomycota or Basidiomycota were the most abundant phyla, and have been discussed below. The most abundant Chytridiomycota, Glomeromycota and Zygomycota species were members of the *Spizellomyces,* (fOTU416), *Glomus* (fOTU748), and *Mortierella* (fOTU66) genera, respectively.

Overall, the most abundant genus detected in the samples was a group of ascomycetous Peziza-like genera. Peziza-like fungi include a range of saprotrophic and ectomycorrhizal species (Hansen and Pfister, 2006; Tedersoo et al., 2006). Most species detected in the Narrabri region were only distantly related to known Peziza species and may fall outside this genus. DNA comparisons of some of the Peziza-like fungi detected in the current study indicated that they are related to other fungi previously detected in association with roots. For example, fOTU9 (the most abundant of these fungi) was detected in a range of soils during both the November and June sampling trips. It is most closely related to root associated fungi previously detected in northern Australia (Raghavendra et al., 2017).

Along with these fungi, a range of known ectomycorrhizal taxa were among the most commonly identified fungi in the present study. These included species of *Clavulina, Cortinarius, Inocybe, Laccaria, Lactarius, Pisolithus, Russula, Scleroderma* and *Tometella*. These species were very abundant in individual samples but were often not abundant across every sample. This is likely due

to different ectomycorrhizal relationships between individual host plants and their associated fungi.

It should also be noted that many of these ectomycorrhizal fungi appear to be novel. For example, the two most common *Russula, Lactarius* and *Laccaria* species are only distantly related to known species. For example, fOTU1, a *Lactarius* species is only distantly shares ~90% ITS identity with *Lactarius clarkae* (*Lactifluus clarkae*), its closest relative. Comparisons of the ITS DNA region with known species reveal that this species is ~96% identical to *L. clarkae* and *L. subclarkae*. This level of identity likely suggests this is a novel *Lactarius* species. One notable exception to this were some of the *Pisolithus* OTUs which mapped closely to known species. This species has been subject to more work in Australia (Cairney, 2002; Hitchcock et al., 2003; Hitchcock et al., 2011; Leonard and McMullan-Fisher, 2013) and numerous Australian species such as *P. albus* (fOTU1635), *P. marmoratus* (fOTU27) or *P. microcarpus* (fOTU536) were detected in the current study. Overall, however, this study demonstrates that the Narrabri region hosts a range of diverse and poorly characterised ectomycorrhizal fungal species.

In contrast to this pattern of sporadic high abundance of ectomycorrhizal fungi, a number of other fungi were abundant at in a relatively large number (15+) samples. These fungi included various species of fusaria in the genera *Gibberella, Hypocrea* (or the anamorphic *Fusarium*). Similarly, various *Chaetomium* species and *Penicillium* species (including some *Eupenicillium* and *Talaromyces* spp.) were also abundant in many samples. This is in keeping with the life-strategy of these species which involves the production of vast numbers of air or dust dispersed spores (conidia) which mean these species commonly occur in, but may not be metabolically active in, many different soils (Cooke and Rayner, 1984; Warcup, 1967). It should be noted that the microbial community profiling carried out using DNA sequencing, is unable to distinguish between DNA from spores and DNA from metabolically active fungal cells.

### Seasonality

Between the November 2022 and June 23, along with expected differences in the climate at these times, i.e. cooler weather in June, the November 2022 sampling occurred after a very significant rainfall event that caused widespread flooding in the Narrabri region. In terms of soil fungi, there was a significant difference ($p<0.003$) between the types of fungi detected at these time points, though some species appear to be present, regardless of season.

For example, across the soil samples collected in November (2022) ectomycorrhizal *Russula* species comprised ~8.6% of fungal OTUs detected, this was not markedly different to the June (2023) sampling campaign when *Russula* species comprised 9.5% of all fungal OTUs detected in soils. This suggests that the extramatrical mycelium of ectomycorrhizal *Russula* species is present regardless of soil temperature or rainfall events. It is important to note that here, data presented are aggregated to the genus level, and there may be patterns of abundance below this level that are not described. Regardless, there are also clear examples of the opposite phenomenon. For instance, *Lactarius* (*Lactifluus*) constituted ~3.7% of detected fungal OTUs in the June (2023) sampling but are largely absent from the November (2022) sampling, representing just ~0.01% of fungal OTUs at this time point. Similar seasonal changes in abundance have been observed in other *Lactarius* species. The work of Castaño et al., 2017, for example, previously demonstrated

significant changes in biomass of *L. vinosus* in Spanish forests across a 12 month period related to seasonality.

## 4.3 Chemical degradation and community impact assessment

Following the method described in section 3.4, community impact assessment experiments were set up (Figure 27), aiming to investigate the chemical degradation and microbial community impact by adding these chemicals into the soil, surface water, and groundwater samples collected from the Narrabri region. Briefly, an aliquot of an aqueous solution of either a specific chemical (biocides – DAZ, GLU, or MBO) or a mixture of chemicals (the drilling or the workover cocktails) was added to microcosms of each of two groundwaters (G2 and G8), a surface water (W12) and soils (S4 and S24). Residue chemicals and microbial community in microcosm experiments were analysed after incubation (30 days for soil experiments and 90 days for groundwater and surface water experiments) and compared with zero time and storage controls.

**Ground water samples**

Biocides (DAZ, GLU and MBO), HCM#1 and MEA were not detected in groundwater samples, while AEMs were detected in both groundwater samples, with AEM#1 ranging from 0.84 to 1.83 mg $L^{-1}$ and AEM#2 ranging from 5.2 to 7.4 mg $L^{-1}$ (Table 14). Absence of tested biocides and MEA indicated that these chemicals were completely biodegraded (Table 14, Figure 55).

The absence of DAZ in both groundwater samples after the experiment might not be due to microbial degradation since DAZ tends to be hydrolysed to methyl isothiocynate (MITC), formaldehyde and other compounds (Consolazio et al., 2019) upon reaction with water. MITC was also analysed, and it is also absent in both groundwater samples.

AEMs seemed more resistant to microbial degradation in these groundwater samples compared to other chemicals with 54 to 79% of AEM#1 and 85 to 90% of AEM#2 being degraded during the experiment period (Figure 55). It is worth noting that biodegradation of HCM#1 and HCM#2 in the groundwater samples will be included in the addendum to the report.

**Surface water samples**

All tested chemicals or chemical mixtures were not detected in the surface water sample after the community impact assessment experiment (Table 14), indicating complete degradation of these chemicals in the surface water sample during the experiment (Table 14, Figure 55). MITC, the breakdown product of DAZ, was also not detected in the surface water after the experiment. It is noted that biodegradation of HCM#1 and HCM#2 in the surface water sample will be included in the addendum to the report.

Table 14 Mean (± std. error of the mean) of chemicals in groundwater and surface water microcosm experiments after incubation

| Sample | AEM#1 | AEM#2 | MEA | MBO | DAZ | GLU |
|---|---|---|---|---|---|---|
| **G2** | 0.84 (±0.03) | 5.2 (±0.45) | <0.01 | <0.01 | <0.005 | <1 |
| **G8** | 1.83 (±0.42) | 7.4 (±4.6) | <0.01 | <0.01 | <0.005 | <1 |
| **W12** | <0.01 | <0.01 | <0.01 | <0.01 | <0.005 | <1 |

All concentrations in mg $L^{-1}$

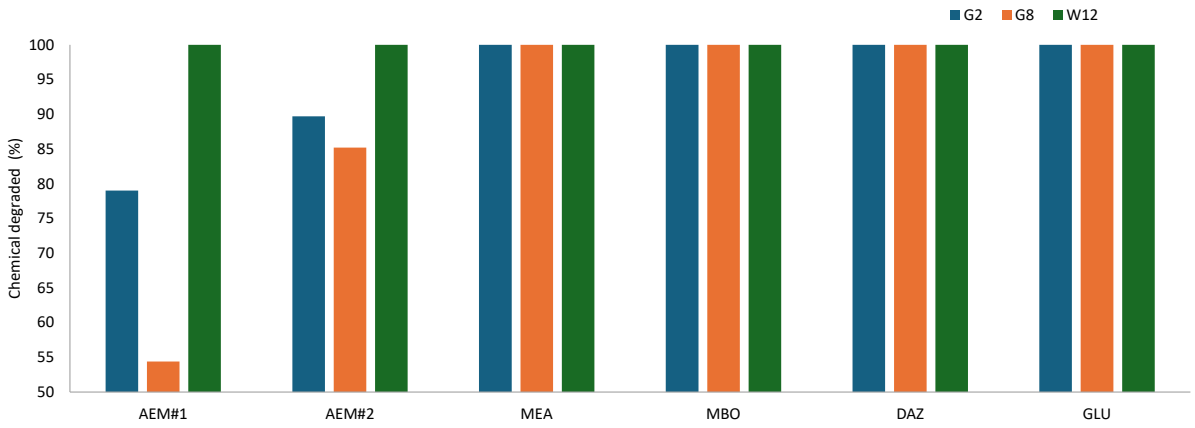



Figure 55 Percentages of chemicals biodegraded during the community impact assessment experiment in water samples

## Soil samples

All tested biocides (DAZ, MBO and GLU) and HCM#1 were not detected in both soil samples after the community impact assessment experiment (Table 15), indicating the added MBO, GLU and HCM#1 were completely biodegraded during the experiment (Table 16 and Figure 56). For DAZ, because of the rapid hydrolysis as mentioned above, the absence of DAZ in both soil samples after the experiment was unlikely due to biodegradation. This is also consistent with the lack of microbial growth detected in the experiment with DAZ as the sole carbon source. MITC, the breakdown product of DAZ, was not detected in both soils either after the experiment.

AEM#1 was not detected in S4 soil but detected in S24 with trace amounts (0.15-0.2 µg $kg^{-1}$), while AEM#2 were detected in trace amounts (0.09-4.12 µg $kg^{-1}$) in both soil samples (Table 15). Trace amounts of detected AEMs indicate >99.99 % of the added AEMs were biodegraded during the experiment (Table 16 and Figure 56).

MEA was detected in both soil samples with an average of 0.03 mg $kg^{-1}$ in S24 and 0.12 mg $kg^{-1}$ in S4 (Table 15), however, MEA was also detected in both soils not used in the experiment which have similar MEA concentrations with an average of 0.05 mg $kg^{-1}$ in S24 and 0.12 mg $kg^{-1}$ in S4 . Since phosphatidylethanolamine is part of cell membrane of microbes (Sohlenkamp and Geiger, 2016), it is considered that the detected MEA in both soils used in the experiment was mainly

attributed to cell membrane of the microbes in soils. Hence, it is likely that the added MEA was completely biodegraded in S4 and 96% biodegraded in S24 during the experiment (Table 16 and Figure 59) after removing the contribution from cell membrane.

It is noted that biodegradation of HCM#2 in soil samples will be included in the addendum to the report.

**Table 15 Mean (± std. error of the mean) of chemicals in soil microcosm experiments after incubation**

| Sample | AEM#1 (µg $kg^{-1}$) | AEM#2 (µg $kg^{-1}$) | MEA (mg $kg^{-1}$) | MBO (mg $kg^{-1}$) | DAZ (mg $kg^{-1}$) | GLU (mg $kg^{-1}$) | HCM#1 (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|
| **S4** | <0.05 | 0.23 (±0.13) | 0.12 (±0.01) | <0.01 | <0.02 | <50 | <1 |
| **S24** | 0.17 (±0.02) | 2.66 (±1.46) | 0.03 (±0.01) | <0.01 | <0.02 | <50 | <1 |

**Table 16 Percentages of chemicals being biodegraded during the community impact assessment experiment**

| Sample | AEM#1 | AEM#2 | MEA | MBO | DAZ | GLU | HCM#1 |
|---|---|---|---|---|---|---|---|
| **G2** | 79 | 90 | 100 | 100 | 100 | 100 | n/a |
| **G8** | 54 | 85 | 100 | 100 | 100 | 100 | n/a |
| **W12** | 100 | 100 | 100 | 100 | 100 | 100 | n/a |
| **S4** | 100 | 99.9996 | 100 | 100 | 100 | 100 | 100 |
| **S24** | 99.996 | 99.9953 | 96 | 100 | 100 | 100 | 100 |

n/a: not available

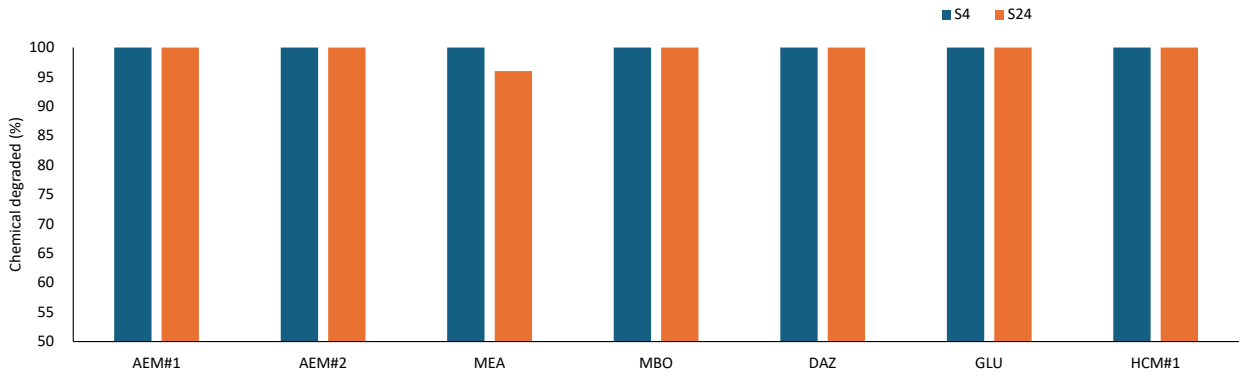



**Figure 56 Percentages of chemicals biodegraded during the community impact assessment experiment in the soil samples**

### 4.3.1 Community impact assessment

The microbiological effects of adding chemicals to groundwaters, surface waters, and soils of the Narrabri region were investigated to determine how the indigenous microbial communities present in each sample type would be impacted by chemicals entering these environments. Statistical analyses were used to determine the impact on microbial communities relative to the zero time and storage controls.

An important difference between the sole carbon experiments and these community impact assessments were that for the community impact assessment, some chemicals were considered in mixtures. These two mixtures were the 'drilling' mixture: which included MEA and AEM#2, along with the 'workover' mixture which included hydrocarbons and AEM#1. All three biocides: DAZ, GLU and MBO were considered separately.

The impacts of chemicals or chemical mixtures varied with sample type (Table 17). For the soil sample S4, only the biocides DAZ and GLU significantly altered the microbial community (Table 17). In contrast S24 was impacted by the presence of all chemicals except the mixture used for drilling (Table 17). The surface water sample W12 was impacted by the addition of all chemicals other than MBO. While neither groundwater sample, G2 and G8, was impacted by the drilling or workover mixture, the biocides had varied effects in these samples (Table 17).

It should be noted that PERMANOVA, like many statistical tests, uses an alpha level of 0.05. The difference between 0.049 and 0.051 is negligible, and as such values close to 0.05 are worth consideration. In the present study, there were some results above 0.05, i.e. 0.062 and 0.0691, while these results were not statistically significantly different, there may still have been effects here, but the impact was more subtle and so a decisively statistically, different community was not indicated. Further work may be required here to exclude community change as a result of addition of particular chemicals.

**Table 17 PERMANOVA post hoc comparisons between the addition of a chemical treatment with the storage control for bacterial communities**

| Chemical Treatment | G2 | G8 | W12 | S4 | S24 |
|---|---|---|---|---|---|
| DAZ | **0.001** | 0.2345 | **0.0009** | **0.0054** | **0.0003** |
| Drilling mix | 0.2679 | 0.1476 | **0.031** | 0.5022 | 0.062 |
| GLU | **0.0008** | **0.0116** | **0.0006** | **0.0007** | **0.0007** |
| MBO | **0.0048** | 0.1343 | 0.2516 | 0.2469 | **0.0262** |
| Workover mix | 0.2236 | 0.0691 | **0.0006** | 0.0837 | **0.0031** |

Significant p-values (<0.05) are in bold

### Dazomet

The presence of DAZ altered the microbial communities in all samples except for G8 (Table 17). In G2, large changes were observed in response to DAZ. For example, OTU2 had a median abundance in the G2 storage control of ~50% (Table 18). In contrast, when DAZ was present this taxon was reduced to median abundances under 3% (Table 18). This suggests that OTU2, which was a strain of *Curvibacter delicatus*, was strongly negatively impacted by DAZ or its breakdown products, a similarly strongly negative response was observed to DAZ by OTU8, which was a strain of *Methylocystis echinoides*, which declined from almost 10% abundance to close to zero when DAZ was added (Table 18). Some other species did increase in response to DAZ, the most significant of which was OTU6, a novel species of *Dechloromonas* most closely related to but distinct from *D. agitate*. *Dechloromonas* species are best known for their abilities to dehalogenate a range of halogenated organic compounds (Fetzner, 1998; Mohn and Tiedje, 1992; Smidt and De Vos, 2004). It should be noted though, that neither DAZ or its breakdown products contain halogenated components, and as such growth of *Dechloromonas* on DAZ and its breakdown products likely indicates use of these components by OTU8.

**Table 18 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the DAZ treatment relative to the storage control in G2 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | DAZ median ± IQR | DAZ Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU2** | 20.89 | 25.15 | 25.15 | 2.89 ± 4.47 | 0.0 - 17.32 | 51.01 ± 16.43 | 5.46 - 72.69 |
| **OTU6** | 9.57 | 11.53 | 36.68 | 13.0 ± 25.04 | 0.0 - 63.92 | 3.75 ± 3.16 | 0.33 - 10.87 |
| **OTU8** | 8.19 | 9.87 | 46.55 | 0.0 ± 0.12 | 0.0 - 11.93 | 9.61 ± 16.74 | 0.73 - 53.48 |
| **OTU10** | 3.77 | 4.53 | 51.08 | 3.21 ± 7.14 | 0.0 - 33.5 | 0.0 ± 0.66 | 0.0 - 9.61 |
| **OTU12** | 3.74 | 4.50 | 55.58 | 0.16 ± 2.9 | 0.0 - 42.03 | 0.0 ± 0.49 | 0.0 - 8.14 |
| **OTU5** | 2.87 | 3.46 | 59.04 | 0.82 ± 3.87 | 0.0 - 6.18 | 1.93 ± 1.78 | 0.0 - 29.14 |
| **OTU42** | 1.67 | 2.01 | 61.05 | 4.1 ± 2.62 | 0.0 - 13.81 | 2.67 ± 2.21 | 0.57 - 5.02 |
| **OTU111** | 1.63 | 1.96 | 63.01 | 0.9 ± 5.05 | 0.0 - 11.07 | 0.37 ± 1.01 | 0.0 - 5.08 |
| **OTU64** | 1.48 | 1.79 | 64.8 | 0.0 ± 2.4 | 0.0 - 5.51 | 0.0 ± 0.4 | 0.0 - 13.03 |
| **OTU19** | 1.44 | 1.73 | 66.53 | 0.0 ± 0.0 | 0.0 - 13.87 | 0.0 ± 0.0 | 0.0 - 8.8 |
| **OTU124** | 1.43 | 1.73 | 68.26 | 0.2 ± 2.27 | 0.0 - 15.04 | 0.25 ± 0.88 | 0.0 - 1.7 |
| **OTU54** | 1.31 | 1.57 | 69.83 | 0.0 ± 3.47 | 0.0 - 11.29 | 0.0 ± 0.0 | 0.0 - 0.06 |
| **OTU117** | 1.27 | 1.53 | 71.36 | 1.22 ± 1.36 | 0.0 - 12.75 | 0.0 ± 0.02 | 0.0 - 0.08 |
| **OTU185** | 1.25 | 1.51 | 72.87 | 0.0 ± 0.23 | 0.0 - 12.67 | 0.0 ± 0.0 | 0.0 - 6.39 |
| **OTU78** | 1.19 | 1.43 | 74.30 | 0.0 ± 2.71 | 0.0 - 11.16 | 0.0 ± 0.0 | 0.0 - 0.0 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; DAZ median ± IQR – DAZ median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

In W12, similar patterns were observed with OTUs 136, 5 and 61 being sensitive to DAZ presence (Table 19). OTUs 136, 5 and 61 were a possible *Nitrobacter* species, a strain of *Bradyrhizobium liaoningense* and a poorly described microbe that is known from bacterioplankton in other sites,

respectively. Interestingly both the OTU136 and 5 were probably nitrogen fixing species and it may be that DAZ poses greater risks to these organisms in some way, though a broader analysis would be required to confirm this observation. Such observations of DAZ impact on microbes important in the nitrogen cycle has been recorded previously. For example, Fang and co-workers demonstrated that the presence of DAZ and its breakdown products adversely impacted the abundance of 16S and nitrogen cycling genes, though the same study demonstrates that DAZ in parallel temporarily increased bacterial diversity – presumably through growth of other taxa able to capitalise either on the moribund cells of affected bacteria or on the breakdown products of DAZ (Fang et al., 2018). Further work is required to ascertain the holistic impacts of DAZ. In addition to OTUs 136, 5 and 61, OTUs 52 and 88 were also negatively impacted and, like OTUs 136 and 5, were related to each other, with both being cyanobacteria and either strains of *Leptodemis alaskaensis* (OTU88) or a new species of *Leptodemis*. Regardless, the presence of two species of the Leptolyngbyaceae family in the most affected species raises the possibility that like nitrogen-fixing Pseudomonadota, DAZ has phylogenetically-targeted effects on groups of microbes.

In contrast, several taxa increased in the presence of DAZ. These included OTUs 4, 45 and 39, which were a strain of *Mycolicibacterium smegmatis*, a novel species of *Reyranella*, and a novel species of *Pseudorhodoplanes* (Table 19). No literature is available on these genera with respect to their DAZ tolerance or catabolism, however, their presence here clearly indicates they benefit from the presence of DAZ and its breakdown products in some way.

**Table 19 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the DAZ treatment relative to the storage control in W12 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | DAZ median ± IQR | DAZ Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU4** | 4.04 | 5.33 | 5.33 | 3.4 ± 2.41 | 1.13 - 23.07 | 1.61 ± 5.08 | 0.67 - 33.14 |
| **OTU45** | 3.49 | 4.60 | 9.93 | 4.14 ± 9.98 | 0.0 - 25.61 | 1.44 ± 1.83 | 0.09 - 4.84 |
| **OTU39** | 3.39 | 4.48 | 14.41 | 6.56 ± 8.34 | 0.0 - 15.81 | 1.1 ± 0.71 | 0.12 - 2.36 |
| **OTU136** | 2.50 | 3.30 | 17.71 | 0.45 ± 2.58 | 0.0 - 5.27 | 5.16 ± 7.08 | 0.26 - 13.49 |
| **OTU61** | 1.75 | 2.31 | 20.02 | 0.41 ± 0.63 | 0.0 - 3.12 | 1.71 ± 3.7 | 0.35 - 14.74 |
| **OTU5** | 1.56 | 2.06 | 22.08 | 0.98 ± 0.75 | 0.0 - 3.17 | 2.28 ± 1.86 | 0.25 - 14.88 |
| **OTU126** | 1.50 | 1.99 | 24.06 | 0.0 ± 0.0 | 0.0 - 0.02 | 0.03 ± 1.62 | 0.0 - 17.77 |
| **OTU52** | 1.50 | 1.98 | 26.04 | 0.0 ± 0.57 | 0.0 - 1.1 | 2.25 ± 4.22 | 0.27 - 8.35 |
| **OTU7813** | 1.48 | 1.96 | 28.00 | 0.04 ± 1.14 | 0.0 - 18.42 | 0.02 ± 0.06 | 0.0 - 0.09 |
| **OTU88** | 1.37 | 1.81 | 29.82 | 0.0 ± 0.16 | 0.0 - 0.18 | 2.89 ± 1.04 | 0.68 - 4.28 |
| **OTU109** | 1.09 | 1.44 | 31.26 | 0.0 ± 0.01 | 0.0 - 0.02 | 0.95 ± 2.78 | 0.09 - 7.08 |
| **OTU82** | 0.94 | 1.24 | 32.49 | 0.68 ± 3.03 | 0.0 - 5.83 | 0.2 ± 0.22 | 0.0 - 0.4 |
| **OTU92** | 0.93 | 1.22 | 33.71 | 1.16 ± 3.31 | 0.0 - 5.37 | 0.18 ± 0.21 | 0.0 - 0.51 |
| **OTU17** | 0.92 | 1.21 | 34.92 | 0.32 ± 2.04 | 0.0 - 5.67 | 1.31 ± 1.13 | 0.29 - 2.87 |
| **OTU174** | 0.88 | 1.16 | 36.08 | 0.01 ± 0.43 | 0.0 - 11.15 | 0.09 ± 0.12 | 0.0 - 0.19 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; DAZ median ± IQR – DAZ median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

For S4, presence of DAZ caused the common taxon, OTU3, to almost halve in abundance (Table 20). This taxon was present at ~16% in the storage control soils, but the addition of DAZ caused its abundance to decrease to a median of ~9%. This taxon, a strain of *Arthrobacter nitrophenolicus*, is capable of degradation of quite complex material. For example, the species is known to degrade chlorinated nitrophenols (Arora and Jain, 2013). Data presented here, however, suggests the taxon was sensitive to the presence of DAZ. Other sensitive taxa included OTU3350 (a *Falsirhodobacter* species), which declined ~10X in the presence of DAZ. Unlike some of the other chemicals examined, few obviously advantaged species were detected in this soil.

**Table 20 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the DAZ treatment relative to the storage control in S4 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | DAZ median ± IQR | DAZ Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU3** | 3.67 | 14.36 | 14.36 | 8.76 ± 4.92 | 3.15 - 16.73 | 14.76 ± 3.07 | 11.37 - 24.14 |
| **OTU14** | 1.09 | 4.25 | 18.61 | 7.22 ± 2.25 | 3.75 - 11.41 | 7.02 ± 2.01 | 6.37 - 10.75 |
| **OTU15** | 0.99 | 3.87 | 22.48 | 6.33 ± 1.5 | 4.26 - 10.99 | 5.03 ± 1.68 | 4.06 - 6.53 |
| **OTU24** | 0.67 | 2.61 | 25.09 | 3.33 ± 1.76 | 0.92 - 5.8 | 3.57 ± 0.68 | 2.88 - 4.92 |
| **OTU66** | 0.34 | 1.32 | 26.41 | 1.84 ± 0.73 | 1.06 - 3.65 | 2.22 ± 0.29 | 1.43 - 2.63 |
| **OTU3350** | 0.32 | 1.26 | 27.67 | 0.05 ± 0.06 | 0.01 - 0.16 | 0.08 ± 0.05 | 0.04 - 4.27 |
| **OTU74** | 0.30 | 1.19 | 28.85 | 1.81 ± 0.67 | 0.25 - 2.77 | 1.85 ± 0.29 | 1.47 - 2.23 |
| **OTU269** | 0.27 | 1.05 | 29.91 | 0.52 ± 0.29 | 0.12 - 3.22 | 0.51 ± 0.08 | 0.41 - 0.65 |
| **OTU326** | 0.25 | 0.96 | 30.87 | 1.39 ± 0.45 | 0.81 - 2.01 | 0.86 ± 0.17 | 0.74 - 1.16 |
| **OTU121** | 0.23 | 0.91 | 31.78 | 0.62 ± 0.36 | 0.13 - 2.53 | 0.6 ± 0.12 | 0.36 - 0.69 |
| **OTU145** | 0.23 | 0.89 | 32.67 | 1.18 ± 0.29 | 0.69 - 2.29 | 1.48 ± 0.23 | 1.07 - 1.73 |
| **OTU36** | 0.20 | 0.79 | 33.45 | 0.68 ± 0.35 | 0.26 - 1.26 | 0.93 ± 0.17 | 0.55 - 1.89 |
| **OTU776** | 0.17 | 0.66 | 34.11 | 0.97 ± 0.35 | 0.68 - 1.85 | 0.82 ± 0.26 | 0.65 - 1.09 |
| **OTU287** | 0.16 | 0.63 | 34.75 | 0.49 ± 0.35 | 0.09 - 1.41 | 0.39 ± 0.13 | 0.22 - 0.51 |
| **OTU129** | 0.16 | 0.62 | 35.36 | 0.88 ± 0.37 | 0.4 - 1.7 | 0.91 ± 0.28 | 0.81 - 1.18 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; DAZ median ± IQR – DAZ median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

These observations contrasted with the microbial community from S24 which showed both the increase and decrease in abundance of certain taxa when DAZ was present. For example, in the presence of DAZ, OTU193 a strain of *Geomesophilobacter sediminis*, decreased from almost 3% in the storage control to 0.007%, a decrease of over 400X (Table 21). The converse was true for OTU234, a strain of *Tumebacillus ginsengisoli*, which was only present at ~0.07% in the storage control microbial community, but increased to ~1.5% in those microcosms that were dosed with DAZ. Because DAZ produces a number of breakdown products: methyl isothiocyanate, methylamine, formaldehyde and others, it is difficult to know which specific breakdown products, or the conditions they create, are advantageous for species like *T. ginsengisoli.*

**Table 21 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the DAZ treatment relative to the storage control in S24 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | DAZ median ± IQR | DAZ Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU37** | 1.64 | 4.43 | 4.43 | 7.32 ± 2.71 | 4.64 - 8.72 | 3.76 ± 1.28 | 2.06 - 6.37 |
| **OTU193** | 1.48 | 3.97 | 8.40 | 0.01 ± 0.0 | 0.0 - 0.01 | 2.36 ± 3.96 | 0.01 - 6.92 |
| **OTU20** | 0.82 | 2.20 | 10.59 | 1.2 ± 1.19 | 0.98 - 3.01 | 1.17 ± 0.6 | 0.4 - 7.81 |
| **OTU56** | 0.80 | 2.15 | 12.74 | 1.8 ± 1.13 | 1.01 - 3.07 | 0.61 ± 0.44 | 0.29 - 5.63 |
| **OTU234** | 0.74 | 1.99 | 14.73 | 1.03 ± 1.97 | 0.38 - 3.31 | 0.05 ± 0.03 | 0.03 - 0.24 |
| **OTU75** | 0.65 | 1.76 | 16.49 | 1.77 ± 1.93 | 0.57 - 3.97 | 0.66 ± 0.73 | 0.35 - 2.98 |
| **OTU123** | 0.62 | 1.67 | 18.16 | 1.89 ± 0.13 | 1.68 - 2.15 | 0.69 ± 0.11 | 0.43 - 0.84 |
| **OTU91** | 0.57 | 1.54 | 19.70 | 0.85 ± 0.29 | 0.37 - 1.44 | 2.05 ± 0.76 | 0.77 - 3.08 |
| **OTU36** | 0.56 | 1.52 | 21.21 | 1.53 ± 2.03 | 0.47 - 3.71 | 1.19 ± 0.64 | 0.73 - 2.04 |
| **OTU451** | 0.50 | 1.35 | 22.56 | 0.23 ± 0.1 | 0.15 - 0.44 | 0.22 ± 0.18 | 0.09 - 6.59 |
| **OTU558** | 0.48 | 1.28 | 23.84 | 0.0 ± 0.0 | 0.0 - 0.0 | 0.0 ± 0.03 | 0.0 - 6.6 |
| **OTU5** | 0.47 | 1.27 | 25.12 | 2.99 ± 0.5 | 2.61 - 4.15 | 3.52 ± 1.22 | 2.15 - 4.76 |
| **OTU44** | 0.37 | 0.98 | 26.10 | 1.72 ± 0.46 | 1.07 - 2.01 | 0.89 ± 0.19 | 0.48 - 1.14 |
| **OTU86** | 0.33 | 0.88 | 26.98 | 0.65 ± 0.11 | 0.56 - 1.13 | 0.07 ± 0.04 | 0.03 - 0.09 |
| **OTU128** | 0.31 | 0.83 | 27.81 | 0.73 ± 0.18 | 0.68 - 1.09 | 0.44 ± 0.42 | 0.15 - 2.48 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; DAZ median ± IQR – DAZ median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

### Drilling mixture: MEA and AEM#2

For all groundwaters and soils no significant effect was observed on the microbial community after adding these chemicals (Table 17). Only W12 showed microbial community alteration from the presence of these chemicals. This change was characterised by a small number of species that changed significantly. For example, OTU4 decreased in abundance in the presence of MEA and AEM#2 (Table 22). Other notable taxa that decreased in the presence of these chemicals were OTUs 52, 126 and 88 (Table 22). Conversely, OTUs 10 and 81 increased in the presence of MEA and AEM#2 (Table 22).

When examining taxa sensitive to these chemicals, OTU4 (*Mycolicibacterium smegmatis*) and a taxon related to the genus *Sandarakinorhabdus* were identified. The reason for their sensitivity is not known and while the effect was relatively modest for OTU4, the effect on OTU126 was substantial, and this taxon dropped in abundance by 100x in W12 that was stored in the laboratory (0.03%) compared with MEA and AEM#2 added (0.00023%)(Table 22). The reasons for these changes are not well understood. While *M. smegmatis* is relatively well studied, it is a non-pathogenic model for other Mycobacterium species, data on the taxon in non-clinical environments and its tolerance of stressors are lacking. *Sandarakinorhabdus* is much more poorly studied, and the reasons for its intolerance of these chemicals is unknown. A further complication is that it is not clear what contribution the components in this mixture had towards this negative impact. It may be for example, that strains were sensitive to MEA or AEM#2 alone, or that their presence together had additional negative effects.

In contrast, OTUs 10 and 81 increased significantly in the presence of these compounds. OTU81 is a particularly poorly understood actinomycetous member of the bacterioplankton, and as such the reasons for its enrichment in the presence of the drilling mixture compounds was unknown. OTU10 by contrast, is from the comparatively well-studied *Corynebacterium* genus and was conspecific with *C. suicordis*. This is somewhat problematic, as *Corynebacterium* can come from human skin as well as from the environment. Its presence here, in the W12 samples requires at least some exploration. This species has been detected in a range of aquatic samples in association with humans and animals (Vela et al., 2003). Its presence here in W12 may be the result of its natural occurrence in this water body, or it may be the result of animals living in this water body. Regardless, in this experiment this taxon increased in abundance in the presence of MEA or AEM#2 suggesting the taxon benefits in some manner from these additions. Either through direct catabolism of the components of the drilling mixture or as a result of reduced competition or some other advantage under these settings. For MEA at least, it would appear that some *Corynebacterium* have the genes (ethanolamine-ammonia lyases) to degrade these products (Swaney and Kalan, 2020).

**Table 22 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the drilling mixture treatment relative to the storage control in W12 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | D median ± IQR | D Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU4** | 3.38 | 4.76 | 4.76 | 0.92 ± 0.95 | 0.02 - 8.15 | 1.61 ± 5.08 | 0.67 - 33.14 |
| **OTU136** | 2.74 | 3.86 | 8.62 | 8.13 ± 7.63 | 0.06 - 13.13 | 5.16 ± 7.08 | 0.26 - 13.49 |
| **OTU10** | 2.01 | 2.84 | 11.45 | 0.0 ± 5.03 | 0.0 - 17.98 | 0.0 ± 0.08 | 0.0 - 0.16 |
| **OTU52** | 1.81 | 2.55 | 14.00 | 0.54 ± 2.91 | 0.12 - 11.3 | 2.25 ± 4.22 | 0.27 - 8.35 |
| **OTU81** | 1.80 | 2.54 | 16.54 | 2.26 ± 6.24 | 0.0 - 10.56 | 0.1 ± 0.35 | 0.0 - 0.72 |
| **OTU61** | 1.69 | 2.38 | 18.92 | 1.71 ± 1.55 | 0.0 - 3.08 | 1.71 ± 3.7 | 0.35 - 14.74 |
| **OTU109** | 1.69 | 2.37 | 21.30 | 0.61 ± 3.89 | 0.0 - 11.74 | 0.95 ± 2.78 | 0.09 - 7.08 |
| **OTU5** | 1.67 | 2.36 | 23.65 | 0.42 ± 0.88 | 0.0 - 4.2 | 2.28 ± 1.86 | 0.25 - 14.88 |
| **OTU19** | 1.51 | 2.13 | 25.78 | 0.0 ± 4.02 | 0.0 - 13.08 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU126** | 1.51 | 2.12 | 27.90 | 0.0 ± 0.0 | 0.0 - 0.0 | 0.03 ± 1.62 | 0.0 - 17.77 |
| **OTU122** | 1.29 | 1.81 | 29.71 | 2.29 ± 1.76 | 0.01 - 9.1 | 0.35 ± 0.33 | 0.05 - 1.65 |
| **OTU88** | 1.09 | 1.53 | 31.24 | 0.37 ± 0.97 | 0.0 - 3.59 | 2.89 ± 1.04 | 0.68 - 4.28 |
| **OTU99** | 1.05 | 1.47 | 32.72 | 0.24 ± 1.99 | 0.0 - 9.78 | 0.25 ± 0.26 | 0.09 - 1.75 |
| **OTU157** | 1.02 | 1.43 | 34.15 | 0.8 ± 2.04 | 0.0 - 8.64 | 0.12 ± 0.22 | 0.01 - 0.41 |
| **OTU45** | 0.94 | 1.32 | 47.00 | 0.05 ± 2.39 | 0.0 - 4.53 | 1.44 ± 1.83 | 0.09 - 4.84 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; D median ± IQR – Drilling median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

### Workover mixture: Hydrocarbons and AEM#1

The workover mixture in the current study included HCM#1, HCM#2 (see discussion in Section 4.4.4) and AEM#1. Only W12 and S24 had microbial communities that were significantly altered by addition of these compounds.

For W12, OTUs 17 and 98 increased significantly in abundance; these were a strain of *Polynucleobacter acidiphobus* and a novel freshwater alphaproteobacterial species (Table 23). Unfortunately, OTU98 is so poorly defined (i.e. it's not related to any known taxa at family level) that it is difficult to speculate on its potential to use these chemicals for growth. In contrast, *Polynucleobacter* species have been experimentally demonstrated to be enriched when hydrocarbons were present (Xin et al., 2023). This suggests that *Polynucleobacter* benefits in some fashion from the presence of these compounds, though it is unclear which chemical and whether direct catabolism is the cause of its increase. This is compounded by the lack of physiological/genetic information for *P. acidiphobus*. Related *Polynucleobacter* species do not appear to host genes for aromatic or aliphatic (alkane) degradation at least in those strains currently available in the KEGG database (Kanehisa et al., 2016a; Kanehisa et al., 2016b; Ogata et al., 1999). In contrast to OTUs 17 and 98, several species were adversely impacted by the presence of these chemicals, these included OTUs 52, 88, 61 and 136, which were also all sensitive to DAZ (Table 19) and are variously discussed above.

**Table 23 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the workover mixture treatment relative to the storage control in W12 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | W median ± IQR | W Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU17** | 6.29 | 7.42 | 7.42 | 9.61 ± 11.87 | 1.05 - 31.0 | 1.31 ± 1.13 | 0.29 - 2.87 |
| **OTU4** | 3.37 | 3.98 | 11.39 | 0.35 ± 1.56 | 0.0 - 5.11 | 1.61 ± 5.08 | 0.67 - 33.14 |
| **OTU98** | 3.15 | 3.72 | 15.11 | 7.29 ± 7.37 | 0.0 - 13.97 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU5** | 3.14 | 3.70 | 18.81 | 1.85 ± 4.36 | 0.04 - 26.59 | 2.28 ± 1.86 | 0.25 - 14.88 |
| **OTU136** | 2.71 | 3.21 | 22.02 | 0.03 ± 0.42 | 0.0 - 1.08 | 5.16 ± 7.08 | 0.26 - 13.49 |
| **OTU39** | 2.15 | 2.54 | 24.56 | 5.56 ± 3.82 | 1.28 - 9.89 | 1.1 ± 0.71 | 0.12 - 2.36 |
| **OTU61** | 1.90 | 2.25 | 26.81 | 0.0 ± 0.0 | 0.0 - 0.0 | 1.71 ± 3.7 | 0.35 - 14.74 |
| **OTU52** | 1.62 | 1.91 | 28.72 | 0.0 ± 0.0 | 0.0 - 0.0 | 2.25 ± 4.22 | 0.27 - 8.35 |
| **OTU126** | 1.51 | 1.78 | 30.50 | 0.0 ± 0.0 | 0.0 - 0.0 | 0.03 ± 1.62 | 0.0 - 17.77 |
| **OTU10** | 1.38 | 1.63 | 32.13 | 0.75 ± 1.67 | 0.0 - 15.31 | 0.0 ± 0.08 | 0.0 - 0.16 |
| **OTU88** | 1.35 | 1.60 | 33.73 | 0.0 ± 0.21 | 0.0 - 0.41 | 2.89 ± 1.04 | 0.68 - 4.28 |
| **OTU111** | 1.33 | 1.58 | 35.30 | 1.34 ± 2.04 | 0.0 - 8.21 | 0.0 ± 0.0 | 0.0 - 0.01 |
| **OTU109** | 1.05 | 1.24 | 36.54 | 0.01 ± 0.16 | 0.0 - 0.86 | 0.95 ± 2.78 | 0.09 - 7.08 |
| **OTU100** | 0.98 | 1.16 | 37.69 | 0.05 ± 0.34 | 0.0 - 12.97 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU96** | 0.93 | 1.10 | 38.79 | 0.0 ± 0.04 | 0.0 - 0.09 | 1.24 ± 1.96 | 0.66 - 4.3 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; W median ± IQR – Workover median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

Results from S24 for the workover mixture showed a high level of intra-treatment variation (Table 24). That is, within the seven replicates for the workover mixture for S24 there was high degrees of variation in the range of relative abundances for some OTUs. For instance, OTUs 193 and 188, 0.01 – 6.21 and 0.0 – 14.3, respectively (Table 24). This may be due to the relatively hydrophobic nature of S24 and its inability to mix well with the workover mixture. Given this high degree of variation, inferring which taxa responded in particular ways was problematic. One example of relative clarity was for OTU75, which was notably higher when the workover mixture was present than in its absence. OTU75 was a strain of *Massilia horti*, a soil bacterium recently described from a sandy loam in North Carolina, USA (Peta et al., 2021). At least some *Massilia* species appear to host activity against hydrocarbons. For example, *Massilia varians* has a complete pathway for benzoate degradation (Kanehisa et al., 2016a; Kanehisa et al., 2016b; Ogata et al., 1999) , while *Massilia* sp. W5 hosts these genes along with additional pathways for benzene and polyaromatic degradation (Kanehisa et al., 2016a; Kanehisa et al., 2016b; Lou et al., 2016; Ogata et al., 1999). This observation demonstrates that *M. horti* occurs in Australia and that it too appears to host significant capacity for degrading hydrocarbons.

**Table 24 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the workover mixture treatment relative to the storage control in S24 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | W median ± IQR | W Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU193** | 1.40 | 4.25 | 4.25 | 0.02 ± 2.58 | 0.01 - 6.21 | 2.36 ± 3.96 | 0.01 - 6.92 |
| **OTU188** | 1.05 | 3.19 | 7.44 | 0.0 ± 0.04 | 0.0 - 14.32 | 0.0 ± 0.0 | 0.0 - 0.47 |
| **OTU75** | 0.94 | 2.85 | 10.29 | 3.32 ± 1.11 | 1.0 - 3.64 | 0.66 ± 0.73 | 0.35 - 2.98 |
| **OTU20** | 0.84 | 2.56 | 12.84 | 1.45 ± 1.46 | 0.31 - 2.63 | 1.17 ± 0.6 | 0.4 - 7.81 |
| **OTU56** | 0.74 | 2.25 | 15.09 | 1.33 ± 0.99 | 0.39 - 3.12 | 0.61 ± 0.44 | 0.29 - 5.63 |
| **OTU37** | 0.70 | 2.11 | 17.20 | 3.69 ± 1.56 | 2.64 - 5.36 | 3.76 ± 1.28 | 2.06 - 6.37 |
| **OTU36** | 0.59 | 1.80 | 19.00 | 0.11 ± 0.04 | 0.08 - 0.21 | 1.19 ± 0.64 | 0.73 - 2.04 |
| **OTU5** | 0.54 | 1.64 | 20.65 | 2.65 ± 0.35 | 2.03 - 3.61 | 3.52 ± 1.22 | 2.15 - 4.76 |
| **OTU451** | 0.54 | 1.63 | 22.28 | 0.2 ± 0.37 | 0.07 - 0.68 | 0.22 ± 0.18 | 0.09 - 6.59 |
| **OTU558** | 0.48 | 1.44 | 23.72 | 0.0 ± 0.0 | 0.0 - 0.0 | 0.0 ± 0.03 | 0.0 - 6.6 |
| **OTU94** | 0.35 | 1.07 | 24.79 | 2.24 ± 0.21 | 1.91 - 2.5 | 1.6 ± 0.32 | 1.08 - 1.87 |
| **OTU128** | 0.32 | 0.98 | 25.77 | 0.61 ± 0.58 | 0.07 - 1.34 | 0.44 ± 0.42 | 0.15 - 2.48 |
| **OTU91** | 0.32 | 0.97 | 26.74 | 2.25 ± 0.25 | 1.52 - 2.52 | 2.05 ± 0.76 | 0.77 - 3.08 |
| **OTU123** | 0.32 | 0.97 | 27.72 | 1.32 ± 0.13 | 1.12 - 1.46 | 0.69 ± 0.11 | 0.43 - 0.84 |
| **OTU48** | 0.27 | 0.80 | 28.53 | 1.14 ± 0.23 | 0.87 - 1.41 | 0.68 ± 0.14 | 0.34 - 0.9 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; W median ± IQR – Workover median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

### Glutaraldehyde

Glutaraldehyde impacted microbial communities whenever it was present (Table 17; Table 25 - Table 29). This was not surprising as GLU is acutely toxic and highly reactive (Leung, 2001b). It is highly likely that its half-life in soils was in the order of low numbers of hours, while it may have persisted more in waters, particularly groundwater due to their inherently low microbial cell densities and organic matter content. It is somewhat difficult to study tolerance of, or degradation of GLU due to the widespread use of GLU as a fixative in preparation for Scanning Electron Microscopy (SEM). Such papers which include the names of various bacterial species and their fixation for imaging using SEM make searching for literature on GLU more problematic than for other chemical additives.

For G2, taxa that were particularly sensitive to GLU were OTUs 2 and 8, with the former declining from relative abundances of ~50% to under 6%, and the latter declining from over 9% to close to zero (Table 25). OTUs 2 and 8 have been discussed previously and declined significantly when DAZ was present, highlighting that these taxa appear to be sensitive to a range of biocides. OTU4 in contrast increased from almost zero to just under 50% of the relative abundance (Table 25). This OTU (a strain of *Mycolicibacterium smegmatis*) has been discussed previously as it was sensitive to the addition of the drilling mixture. Its significant increase here demonstrates that this taxon can dynamically respond to different stressors with different responses, and its growth here indicates that the taxon is advantaged by the presence of GLU.

**Table 25 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the GLU treatment relative to the storage control in G2 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | GLU median ± IQR | GLU Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU2** | 18.35 | 22.74 | 22.74 | 5.85 ± 12.86 | 1.58 - 28.83 | 51.01 ± 16.43 | 5.46 - 72.69 |
| **OTU4** | 17.26 | 21.40 | 44.13 | 48.94 ± 36.94 | 0.0 - 60.77 | 0.0 ± 0.01 | 0.0 - 2.92 |
| **OTU8** | 8.52 | 10.56 | 54.70 | 0.0 ± 0.0 | 0.0 - 0.01 | 9.61 ± 16.74 | 0.73 - 53.48 |
| **OTU5** | 6.28 | 7.788 | 62.49 | 13.04 ± 11.39 | 0.0 - 27.74 | 1.93 ± 1.78 | 0.0 - 29.14 |
| **OTU80** | 3.89 | 4.83 | 67.31 | 7.06 ± 7.75 | 0.0 - 26.19 | 0.0 ± 0.0 | 0.0 - 0.03 |
| **OTU6** | 3.21 | 3.97 | 71.29 | 5.3 ± 2.56 | 0.0 - 30.36 | 3.75 ± 3.16 | 0.33 - 10.87 |
| **OTU12** | 2.16 | 2.68 | 73.97 | 0.03 ± 0.75 | 0.0 - 22.99 | 0.0 ± 0.49 | 0.0 - 8.14 |
| **OTU189** | 1.51 | 1.88 | 75.84 | 0.0 ± 0.0 | 0.0 - 21.19 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU34** | 1.49 | 1.85 | 77.69 | 1.38 ± 2.57 | 0.0 - 13.83 | 0.0 ± 0.0 | 0.0 - 1.76 |
| **OTU197** | 1.47 | 1.82 | 79.51 | 0.0 ± 0.01 | 0.0 - 20.58 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU2521** | 0.99 | 1.23 | 80.75 | 1.71 ± 1.05 | 0.0 - 6.05 | 0.0 ± 0.0 | 0.0 - 0.06 |
| **OTU64** | 0.99 | 1.23 | 81.97 | 0.0 ± 0.0 | 0.0 - 0.0 | 0.0 ± 0.4 | 0.0 - 13.03 |
| **OTU42** | 0.97 | 1.20 | 83.17 | 0.98 ± 1.87 | 0.0 - 4.4 | 2.67 ± 2.21 | 0.57 - 5.02 |
| **OTU19** | 0.87 | 1.08 | 84.25 | 0.0 ± 0.0 | 0.0 - 4.74 | 0.0 ± 0.0 | 0.0 - 8.8 |
| **OTU106** | 0.85 | 1.05 | 85.31 | 0.0 ± 0.0 | 0.0 - 11.89 | 0.0 ± 0.0 | 0.0 - 0.0 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; GLU median ± IQR – Glutaraldehyde median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

For G8, as for G2, there were a number of negatively impacted taxa including OTUs 1, 11 and 23 (Table 26). These were a strain of *Azospira oryzae*, and two novel species of *Oryzomonas* and *Sulfurisoma*, respectively. In contrast, OTU2750 increased markedly when GLU was present. This strain of *Methyloversatilis* – and the genera more broadly, are known for their use of single carbon compounds, though they also have genes for the manipulation of glutarate (Aranda et al., 2024; Kalyuzhnaya et al., 2006). The conversion of GLU to glutarate typically occurs abiotically under oxic conditions, the absence of such oxygen from the groundwater microcosms likely means that degradation proceeds differently. In anoxic sediment, for example, anaerobic metabolism of GLU results in the production of 1,5 pentanediol (Leung, 2001b). The present study did not test for 1,5 pentanediol, so it is unclear whether this degradation product accumulated in groundwater microcosms.

**Table 26 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the GLU treatment relative to the storage control in G8 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | GLU median ± IQR | GLU Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU1** | 12.21 | 15.96 | 15.96 | 9.3 ± 40.38 | 0.0 - 51.3 | 32.29 ± 13.08 | 22.03 - 57.66 |
| **OTU11** | 8.99 | 11.75 | 27.71 | 0.75 ± 3.28 | 0.0 - 5.06 | 25.72 ± 14.29 | 0.82 - 28.34 |
| **OTU4** | 4.50 | 5.88 | 33.59 | 0.06 ± 1.11 | 0.0 - 54.02 | 0.09 ± 1.1 | 0.0 - 9.13 |
| **OTU23** | 4.27 | 5.58 | 39.18 | 0.21 ± 0.51 | 0.0 - 1.52 | 9.06 ± 6.75 | 0.02 - 18.41 |
| **OTU2750** | 3.76 | 4.91 | 44.09 | 3.15 ± 13.59 | 0.0 - 21.7 | 0.08 ± 0.04 | 0.02 - 0.72 |
| **OTU16** | 3.11 | 4.06 | 48.15 | 0.0 ± 0.0 | 0.0 - 43.49 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU10** | 2.80 | 3.66 | 51.81 | 0.04 ± 0.09 | 0.0 - 27.08 | 0.0 ± 3.71 | 0.0 - 9.51 |
| **OTU5** | 2.66 | 3.47 | 55.28 | 0.38 ± 0.78 | 0.0 - 33.31 | 0.0 ± 0.39 | 0.0 - 2.98 |
| **OTU19** | 1.80 | 2.35 | 57.63 | 0.0 ± 0.0 | 0.0 - 17.7 | 0.0 ± 1.51 | 0.0 - 7.41 |
| **OTU244** | 1.78 | 2.33 | 59.96 | 0.01 ± 0.07 | 0.0 - 0.12 | 0.04 ± 3.79 | 0.0 - 17.31 |
| **OTU603** | 1.76 | 2.30 | 62.25 | 0.96 ± 7.47 | 0.0 - 8.96 | 0.28 ± 0.17 | 0.12 - 0.46 |
| **OTU78** | 1.68 | 2.19 | 64.44 | 0.01 ± 2.96 | 0.0 - 14.65 | 0.0 ± 0.88 | 0.0 - 5.19 |
| **OTU6** | 1.50 | 1.96 | 66.41 | 0.93 ± 2.4 | 0.0 - 5.48 | 2.29 ± 5.0 | 0.25 - 7.82 |
| **OTU173** | 1.46 | 1.91 | 68.31 | 0.0 ± 0.0 | 0.0 - 20.44 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU55** | 1.39 | 1.82 | 70.13 | 0.06 ± 0.18 | 0.0 - 0.35 | 1.14 ± 4.71 | 0.0 - 8.93 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; GLU median ± IQR – Glutaraldehyde median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

The microbial community in W12, like G2, responded to GLU addition by an increase in relative abundance of OTU4 (Table 27). The reasons for its growth are unclear, but its presence here and in sole-carbon assays with GLU (see Section 4.4) suggest that *Mycolicibacterium smegmatis* is at least tolerant of GLU and may be able to degrade this compound as a sole carbon source. The presence of OTU5 which increased a similar quantum to *M. smegmatis*, is somewhat puzzling as the taxon

*Bradyrhizobium liaoningense*, is not known to be tolerant of GLU, however, related species of *Bradyrhizobium* can tolerant quite high concentrations of other biocidal components (Shahid and Khan, 2019). Shahid and Khan (2019) for instance, demonstrated that *B. japonicum* was tolerant of 2.4 g $L^{-1}$ of hexaconazole. It may be that, like *B. japonicum, B. liaoningense* are tolerant of GLU. W12 also had taxa that were sensitive to the presence of GLU. For example, the cyanobacteria OTU52 (a *Leptodesmis* spp.,) was shown to decline significantly in the presence of GLU. This taxon was also sensitive to DAZ and may be represent a useful indicator species for a range of chemicals.

**Table 27 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the GLU treatment relative to the storage control in W12 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | GLU median ± IQR | GLU Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU4** | 7.90 | 9.14 | 9.14 | 19.97 ± 10.01 | 5.79 - 31.96 | 1.61 ± 5.08 | 0.67 - 33.14 |
| **OTU5** | 7.25 | 8.39 | 17.54 | 15.91 ± 8.32 | 1.66 - 30.59 | 2.28 ± 1.86 | 0.25 - 14.88 |
| **OTU20** | 4.03 | 4.66 | 22.20 | 0.03 ± 1.18 | 0.0 - 53.67 | 0.05 ± 0.73 | 0.0 - 1.0 |
| **OTU136** | 2.94 | 3.41 | 25.61 | 0.19 ± 2.34 | 0.0 - 15.35 | 5.16 ± 7.08 | 0.26 - 13.49 |
| **OTU113** | 2.88 | 3.33 | 28.94 | 1.39 ± 7.74 | 0.0 - 23.44 | 0.0 ± 0.01 | 0.0 - 0.02 |
| **OTU1054** | 2.84 | 3.29 | 32.23 | 4.61 ± 2.15 | 1.23 - 19.41 | 0.23 ± 0.25 | 0.1 - 1.21 |
| **OTU72** | 1.91 | 2.21 | 34.44 | 3.29 ± 2.85 | 0.0 - 11.67 | 0.01 ± 0.02 | 0.0 - 0.37 |
| **OTU61** | 1.90 | 2.21 | 36.65 | 0.0 ± 0.0 | 0.0 - 0.0 | 1.71 ± 3.7 | 0.35 - 14.74 |
| **OTU34** | 1.66 | 1.92 | 38.56 | 1.84 ± 4.79 | 0.01 - 8.22 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU52** | 1.62 | 1.87 | 40.43 | 0.0 ± 0.0 | 0.0 - 0.0 | 2.25 ± 4.22 | 0.27 - 8.35 |
| **OTU126** | 1.51 | 1.74 | 42.18 | 0.0 ± 0.0 | 0.0 - 0.0 | 0.03 ± 1.62 | 0.0 - 17.77 |
| **OTU88** | 1.41 | 1.63 | 43.81 | 0.0 ± 0.0 | 0.0 - 0.0 | 2.89 ± 1.04 | 0.68 - 4.28 |
| **OTU4646** | 1.16 | 1.34 | 45.15 | 0.09 ± 0.8 | 0.0 - 14.38 | 0.01 ± 0.0 | 0.0 - 0.04 |
| **OTU109** | 1.09 | 1.27 | 46.41 | 0.0 ± 0.0 | 0.0 - 0.0 | 0.95 ± 2.78 | 0.09 - 7.08 |
| **OTU144** | 1.06 | 1.23 | 47.64 | 0.0 ± 2.9 | 0.0 - 9.04 | 0.0 ± 0.0 | 0.0 - 0.0 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; GLU median ± IQR – Glutaraldehyde median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

In comparison to other samples, the effect of GLU on S4 was negative for many species examined and no obvious GLU-degrading taxa were identified. Among the more negatively impacted taxa were OTUs 3, 14, 15 and 24 (Table 28). These were a strain of *Arthrobacter nitrophenolicus* (OTU3), two related archaeal taxa that were the Nitrososphaeraecae family (OTUs 14 and 24) and a species of *Rubrobacter* (OTU15).

**Table 28 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the GLU treatment relative to the storage control in S4 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | GLU median ± IQR | GLU Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU3** | 5.06 | 10.97 | 10.97 | 5.79 ± 4.22 | 1.16 - 9.26 | 14.76 ± 3.07 | 11.37 - 24.14 |
| **OTU10** | 1.92 | 4.17 | 15.14 | 0.0 ± 3.54 | 0.0 - 19.82 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU14** | 1.82 | 3.94 | 19.08 | 6.68 ± 5.23 | 0.01 - 10.56 | 7.02 ± 2.01 | 6.37 - 10.75 |
| **OTU19** | 1.40 | 3.03 | 22.11 | 0.0 ± 0.88 | 0.0 - 17.8 | 0.01 ± 0.0 | 0.0 - 0.01 |
| **OTU15** | 0.97 | 2.11 | 24.21 | 5.84 ± 1.19 | 0.41 - 8.82 | 5.03 ± 1.68 | 4.06 - 6.53 |
| **OTU24** | 0.74 | 1.60 | 25.82 | 3.23 ± 2.18 | 0.0 - 4.55 | 3.57 ± 0.68 | 2.88 - 4.92 |
| **OTU47** | 0.59 | 1.29 | 27.10 | 0.0 ± 0.05 | 0.0 - 8.21 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU345** | 0.58 | 1.26 | 28.36 | 0.67 ± 1.74 | 0.0 - 3.53 | 0.27 ± 0.11 | 0.1 - 0.29 |
| **OTU36** | 0.47 | 1.01 | 29.37 | 1.21 ± 0.69 | 0.0 - 4.15 | 0.93 ± 0.17 | 0.55 - 1.89 |
| **OTU53** | 0.46 | 1.00 | 30.37 | 0.0 ± 0.78 | 0.0 - 4.89 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU145** | 0.46 | 0.99 | 31.36 | 0.55 ± 0.57 | 0.0 - 1.28 | 1.48 ± 0.23 | 1.07 - 1.73 |
| **OTU54** | 0.38 | 0.82 | 32.18 | 0.0 ± 0.0 | 0.0 - 5.31 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU66** | 0.36 | 0.78 | 32.96 | 2.13 ± 0.73 | 0.0 - 2.93 | 2.22 ± 0.29 | 1.43 - 2.63 |
| **OTU73** | 0.36 | 0.77 | 33.73 | 0.02 ± 0.01 | 0.0 - 4.94 | 0.03 ± 0.01 | 0.02 - 0.05 |
| **OTU74** | 0.37 | 0.73 | 34.46 | 1.71 ± 0.74 | 0.0 - 2.51 | 1.85 ± 0.29 | 1.47 - 2.23 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; GLU median ± IQR – Glutaraldehyde median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

Unlike S4, S24 microcosms showed both taxa that increased and decreased in abundance when GLU was present (Table 29). In brief, OTU193 decreased significantly, while OTUs 234 and 46 both increased in the presence of GLU. OTU193 was a strain of *Geomesophilobacter sediminis* which is presumably sensitive to the presence of GLU. OTU234 has been discussed previously as it increases in S24 in response to DAZ addition. This strain of *Tumebacillus ginsengisoli* appears to response positively to a range of chemical stressors and may be an important cataboliser of unusual compounds in this environment. OTU46 is a strain of *Sphingomonas limnosediminicola*, which like *T. ginsengisoli*, benefits in some way from the presence of GLU.

**Table 29 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the GLU treatment relative to the storage control in S24 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | GLU median ± IQR | GLU Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU193** | 1.477 | 3.876 | 3.876 | 0.0 ± 0.0 | 0.0 - 0.01 | 2.36 ± 3.96 | 0.01 - 6.92 |
| **OTU234** | 1.28 | 3.358 | 7.234 | 1.67 ± 1.78 | 1.24 - 5.8 | 0.05 ± 0.03 | 0.03 - 0.24 |
| **OTU46** | 0.8551 | 2.243 | 9.477 | 1.78 ± 1.35 | 1.17 - 3.57 | 0.59 ± 0.13 | 0.35 - 0.99 |
| **OTU20** | 0.7469 | 1.959 | 11.44 | 0.34 ± 0.25 | 0.26 - 0.71 | 1.17 ± 0.6 | 0.4 - 7.81 |
| **OTU36** | 0.7192 | 1.887 | 13.32 | 2.74 ± 1.06 | 1.67 - 3.59 | 1.19 ± 0.64 | 0.73 - 2.04 |
| **OTU123** | 0.6113 | 1.604 | 14.93 | 2.01 ± 0.49 | 0.82 - 2.76 | 0.69 ± 0.11 | 0.43 - 0.84 |
| **OTU37** | 0.6065 | 1.591 | 16.52 | 3.93 ± 0.53 | 2.29 - 4.98 | 3.76 ± 1.28 | 2.06 - 6.37 |
| **OTU56** | 0.5818 | 1.526 | 18.04 | 0.24 ± 0.22 | 0.11 - 1.63 | 0.61 ± 0.44 | 0.29 - 5.63 |
| **OTU451** | 0.5444 | 1.428 | 19.47 | 0.02 ± 0.02 | 0.0 - 0.07 | 0.22 ± 0.18 | 0.09 - 6.59 |
| **OTU558** | 0.4755 | 1.248 | 20.72 | 0.0 ± 0.0 | 0.0 - 0.0 | 0.0 ± 0.03 | 0.0 - 6.6 |
| **OTU91** | 0.4202 | 1.102 | 21.82 | 1.76 ± 1.15 | 0.92 - 2.75 | 2.05 ± 0.76 | 0.77 - 3.08 |
| **OTU75** | 0.4058 | 1.065 | 22.89 | 1.24 ± 0.73 | 0.41 - 1.9 | 0.66 ± 0.73 | 0.35 - 2.98 |
| **OTU5** | 0.3993 | 1.048 | 23.93 | 3.81 ± 0.42 | 3.31 - 4.22 | 3.52 ± 1.22 | 2.15 - 4.76 |
| **OTU44** | 0.3962 | 1.04 | 24.97 | 1.67 ± 0.53 | 1.1 - 2.38 | 0.89 ± 0.19 | 0.48 - 1.14 |
| **OTU201** | 0.3532 | 0.9266 | 25.9 | 1.2 ± 0.26 | 0.69 - 1.53 | 0.32 ± 0.69 | 0.09 - 1.12 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; GLU median ± IQR – Glutaraldehyde median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

### MBO

Only microbial communities from G2 and S24 were significantly perturbed with the addition of the biocide MBO (Table 17).

The presence of MBO affected G2 communities, pushing the abundance of OTUs 6 and 58 higher and negatively impacting a number of species including OTU2 (Table 30). OTU2 is a strain of *Curvibacter delicatus*, and as discussed previously this taxon declined significantly when either GLU or DAZ was present. Its sensitivity here to MBO further highlights the intolerance of this taxon to all tested biocides. This taxon may thus be a useful indicator taxon to monitor environmental disturbance. Interestingly, the presence of MBO, like DAZ, appears to benefit OTU6 (a species of *Dechloromonas*). OTU58, which is a strain of *Sediminibacterium salmoneum*, the type species of which was isolated from sediment of the Guanting Reservoir in China (Qu and Yuan, 2008), presumably benefits in some fashion from the presence of MBO. As for other positively responding taxa, its increase may be due to direct catabolism of the compound, tolerance of the compound creating new niches or other physicochemical effects of the chemical on the groundwater in the microcosms.

**Table 30 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the MBO treatment relative to the storage control in G2 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | MBO median ± IQR | MBO Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU_2** | 19.12 | 26.05 | 26.05 | 8.26 ± 11.57 | 0.0 - 26.27 | 51.01 ± 16.43 | 5.46 - 72.69 |
| **OTU_8** | 9.89 | 13.47 | 39.52 | 12.21 ± 25.21 | 0.0 - 54.11 | 9.61 ± 16.74 | 0.73 - 53.48 |
| **OTU_6** | 6.50 | 8.85 | 48.37 | 11.38 ± 17.17 | 0.0 - 31.98 | 3.75 ± 3.16 | 0.33 - 10.87 |
| **OTU_12** | 3.83 | 5.21 | 53.58 | 6.05 ± 12.97 | 0.01 - 20.71 | 0.0 ± 0.49 | 0.0 - 8.14 |
| **OTU_5** | 2.74 | 3.74 | 57.32 | 0.47 ± 1.38 | 0.0 - 4.13 | 1.93 ± 1.78 | 0.0 - 29.14 |
| **OTU_10** | 1.50 | 2.05 | 59.37 | 0.05 ± 4.03 | 0.0 - 7.65 | 0.0 ± 0.66 | 0.0 - 9.61 |
| **OTU_42** | 1.45 | 1.98 | 61.35 | 4.03 ± 2.58 | 0.0 - 9.84 | 2.67 ± 2.21 | 0.57 - 5.02 |
| **OTU_19** | 1.43 | 1.95 | 63.3 | 0.0 ± 0.09 | 0.0 - 13.63 | 0.0 ± 0.0 | 0.0 - 8.8 |
| **OTU_90** | 1.41 | 1.93 | 65.22 | 0.0 ± 0.0 | 0.0 - 19.79 | 0.0 ± 0.0 | 0.0 - 0.0 |
| **OTU_149** | 1.28 | 1.74 | 66.96 | 0.0 ± 1.06 | 0.0 - 14.33 | 0.63 ± 0.75 | 0.0 - 2.38 |
| **OTU_35** | 1.25 | 1.70 | 68.66 | 0.51 ± 5.15 | 0.0 - 6.61 | 0.01 ± 0.4 | 0.0 - 1.6 |
| **OTU_30** | 1.05 | 1.43 | 70.09 | 0.01 ± 0.34 | 0.0 - 12.41 | 0.07 ± 0.7 | 0.02 - 1.39 |
| **OTU_58** | 1.05 | 1.42 | 71.51 | 2.29 ± 2.64 | 0.0 - 7.3 | 0.82 ± 1.44 | 0.0 - 3.44 |
| **OTU_64** | 0.99 | 1.35 | 72.86 | 0.0 ± 0.0 | 0.0 - 0.0 | 0.0 ± 0.4 | 0.0 - 13.03 |
| **OTU_159** | 0.88 | 1.21 | 74.06 | 0.0 ± 0.0 | 0.0 - 12.38 | 0.0 ± 0.0 | 0.0 - 0.0 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; MBO median ± IQR – MBO median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

The most negatively impacted taxon from S24 on addition of MBO was OTU193 (Table 31). This strain of *Geomesophilobacter sediminis* would appear to be sensitive to additions of all the biocides and declined in DAZ, GLU and here when MBO was present. Like OTU2, OTU193 may be a useful indicator taxon for biocide presence in soils of the region. Though it should be noted that the taxon is largely absent from the vertosolic soils of the region and may only be useful in sodsolic soil regions.

**Table 31 SIMPER analysis of the top 15 taxa that account for most of the dissimilarities between the MBO treatment relative to the storage control in S24 microbial communities**

| Taxon | Av. dissim | Contrib. % | Cumulative % | MBO median ± IQR | MBO Minimum - Maximum | S median ± IQR | S Minimum - Maximum |
|---|---|---|---|---|---|---|---|
| **OTU193** | 1.45 | 4.64 | 4.64 | 0.03 ± 0.12 | 0.0 - 0.34 | 2.36 ± 3.96 | 0.01 - 6.92 |
| **OTU36** | 1.11 | 3.54 | 8.18 | 2.51 ± 2.65 | 0.34 - 6.89 | 1.19 ± 0.64 | 0.73 - 2.04 |
| **OTU20** | 0.76 | 2.43 | 10.61 | 1.59 ± 0.61 | 0.52 - 2.24 | 1.17 ± 0.6 | 0.4 - 7.81 |
| **OTU37** | 0.63 | 2.02 | 12.63 | 4.42 ± 0.76 | 3.1 - 4.9 | 3.76 ± 1.28 | 2.06 - 6.37 |
| **OTU56** | 0.57 | 1.82 | 14.45 | 0.96 ± 0.37 | 0.24 - 2.26 | 0.61 ± 0.44 | 0.29 - 5.63 |
| **OTU451** | 0.50 | 1.60 | 16.05 | 0.24 ± 0.1 | 0.13 - 0.41 | 0.22 ± 0.18 | 0.09 - 6.59 |
| **OTU558** | 0.48 | 1.52 | 17.57 | 0.0 ± 0.0 | 0.0 - 0.0 | 0.0 ± 0.03 | 0.0 - 6.6 |
| **OTU5** | 0.43 | 1.37 | 18.94 | 3.07 ± 0.6 | 2.89 - 3.71 | 3.52 ± 1.22 | 2.15 - 4.76 |
| **OTU75** | 0.39 | 1.24 | 20.18 | 1.21 ± 0.8 | 0.27 - 1.62 | 0.66 ± 0.73 | 0.35 - 2.98 |
| **OTU91** | 0.37 | 1.19 | 21.37 | 2.36 ± 0.74 | 0.78 - 2.52 | 2.05 ± 0.76 | 0.77 - 3.08 |
| **OTU46** | 0.32 | 1.01 | 22.38 | 0.8 ± 0.61 | 0.49 - 2.68 | 0.59 ± 0.13 | 0.35 - 0.99 |
| **OTU128** | 0.27 | 0.87 | 23.25 | 0.62 ± 0.12 | 0.46 - 1.12 | 0.44 ± 0.42 | 0.15 - 2.48 |
| **OTU334** | 0.26 | 0.83 | 24.08 | 0.02 ± 0.13 | 0.0 - 0.24 | 0.82 ± 0.76 | 0.01 - 1.1 |
| **OTU1320** | 0.23 | 0.73 | 24.81 | 0.01 ± 0.01 | 0.0 - 0.03 | 0.07 ± 0.3 | 0.01 - 2.45 |
| **OTU145** | 0.22 | 0.72 | 25.53 | 0.44 ± 0.24 | 0.23 - 1.27 | 0.39 ± 0.72 | 0.29 - 1.57 |

Av. dissim – Average dissimilarity; Contrib. % - Contribution percentage; Cumulative % -Cumulative percentage; MBO median ± IQR – MBO median ± Interquartile Range; S median ± IQR – Storage median ± Interquartile Range

### Summary of microbial community impact of chemical additions

Overall, data from the present study demonstrated that the effect of chemicals on microbial communities in groundwater, surface water or soil depend on the individual chemical and the microbial community with which it is interacting. Numerous microbial communities showed no perturbation with chemical addition (summarised in Table 17). For those microbiomes that were altered by chemical addition, most microbial communities had taxa that responded positively or negatively to these additions.

Three species were identified that were sensitive to more than one chemical addition. For example, OTUs 2, 52 and OTU193 (from G2, W12 and S24, respectively) were sensitive to the addition of three of the five chemicals. OTU2, for example, was sensitive to the addition of all the biocides (DAZ, GLU and MBO) as was OTU193. In contrast, OTU52 was sensitive to both dazomet and glutaraldehyde, but insensitive to MBO. It was, however, negatively impacted by the presence of the drilling mixture of chemicals.

In contrast, a small number of taxa (OTU6 and OTU234) were detected that appeared to respond positively to the presence of more than one chemical. OTU6, for example, increased in abundance when DAZ or MBO were present, while OTU234 increased when DAZ or GLU was present.

There were also some taxa that responded positively in the presence of some chemicals and negatively in the presence of others. For example, OTU4 increased in abundance when DAZ or GLU were present but decreased in the presence of the drilling mixture. Conversely, OTU5 decreased in the present of DAZ but increased in the presence of GLU.

### 4.3.2 Fungal community changes

In comparison to bacterial communities, fungal communities were less affected by addition of chemicals (Table 32). Regardless of whether soils or surface waters were tested, the drilling chemicals (MEA and AEMs) had no discernible impact on fungal communities in soils or surface water. The same pattern was observed for MBO which did not alter fungal communities in either soils or surface waters. Generally, there is no evidence of microbial effects from the additions of MEA, AEMs or MBO beyond the effect of adding water to the sample (in the case of soils) and incubating these samples in the laboratory. One possibility is that addition of these chemicals did cause a change in the community, but this was transient, and this change was reversed during the month-long (e.g. three months in the case of W12) incubation, however, other experiments would be required to confirm this speculation.

**Table 32 PERMANOVA post hoc comparisons between the addition of a chemical treatment with the storage control for fungal**

| Chemical Treatment | S4 | S24 | W12 |
|---|---|---|---|
| DAZ | **0.0015** | **0.0007** | 0.1581 |
| Drilling mix | 0.5565 | 0.7396 | 0.1607 |
| GLU | 0.0871 | **0.0007** | **0.0007** |
| MBO | 0.4524 | 0.8616 | 0.1659 |
| Workover mix | 0.1268 | **0.0014** | **0.0017** |

Significant p-values (<0.05) are in bold

Other chemicals had mixed effects. For example, DAZ addition affected fungal communities in soils, but not the surface water tested, while addition of GLU inconsistently affected fungal communities in soil, but also affected the community in the surface water (Table 32). That DAZ had no impact on water communities likely reflects the rapid hydrolysis of DAZ in water. This hydrolysis is pH dependant, with faster rates at more moderate pH levels. For example, a study by Consolazio et al. (2019), demonstrated that dazomet had a half-life of 3-8 hours in water. Further, Consolazio et al. (2019) report that ferrous ($Fe^{2+}$) iron concentrations of 0.08 mM (~4.5 ppm) resulted in catalysis of the process, increasing the rate of reaction almost 200%. In contrast, DAZ significantly impacted fungal community structure in both S4 and S24 (Table 32). These impacts were bidirectional, with DAZ additions increasing the relative abundance of some species and decreasing the abundance of others.

For example, fOTU6 and fOTU41 increased by ~10% each when DAZ was added to S24. Interestingly, both fOTU6 and fOTU41 were *Talaromyces* (anamorph[17] *Penicillium*) species, which must benefit in some manner from the addition of DAZ (see Section 2.9). There are, however, reports of *Penicillium* species increasing in abundance after DAZ application (Gao et al., 2022; Zhu et al., 2020). For example, Zhu et al., (2020) report this increase in soils treated to supress watermelon wilt (Zhu et al., 2020). Similar results have also been reported in studies of soil fumigation in other crops. For example, Wang et al., (2021b) reported increases in *Penicillium* species abundance when dazomet is applied to soils (Wang et al., 2021b). fOTU6 is genetically identical with three sequences available in Genbank (MK808942, OZ012304 & MK808941, two of which were recovered from roots of *Schizachyrium scoparium* (Rudgers et al., 2022), while OZ012304 comes from water associated with industrial activity in Spain (unpublished). fOTU41 appears to be a novel species of *Talaromyces*, most closely related to, but distinct from, *Talaromyces purpureus*. In contrast, in the same soil, fOTUs 24, 39 and 62 all decreased by ~7-9% when DAZ was present. Two of these fOTUs (fOTU24 and 62) though are sequences that do not appear to be fungal, so it is difficult to determine their origin and importance. fOTU39 appears to be a poorly identified species of Lasiosphaeriaceae. Most members of this large family are saprobes, though this taxon is likely conspecific (>99% identity) with a root-associated fungus (EP1-7; Genbank EU677764) recovered from *Epacris pulchella* (Ericaceae) at Lovers Jump Creek in Sydney (Curlevski et al., 2009).

It should be noted that while a similar level of community alteration was observed in S4 compared to S24, the taxa that changed were mostly different species. This is unsurprising as the two soils are structurally and chemically quite distinct, with S4 being a vertosol, while S24 was a sodosol. In S4, fOTUs 43, 118 and 139 all increased their abundance significantly in the presence of DAZ. fOTU43 appears to be a *Gyrothrix* species previously (and repeatedly) recovered from cotton growing regions in the south of the USA (Davinic et al., 2012). This presumably indicates this taxon is associated with leaves of cotton, as S4 is from the Australian Cotton Research Institute. Both the other fOTUs detected were relatively well-known saprobes, for example fOTU118 was a species of *Rhizopus* (*Rhizopus sexualis*) while fOTU139 is a *Chaetomium* species, probably *C. subaffine* (JN209929). Increases in abundance of *Rhizopus* and *Chaetomium* species, like *Penicillium* species, have been recorded previously. Eo and co-workers (2014) for example, note that *Rhizopus* is resistant to DAZ treatment and has been isolated from treated Ginseng crop soils, similar observations have been made for Chaetomium species (Gao et al., 2022). Like S24, there were numerous fOTUs which were sensitive to DAZ addition, including fOTUs 17, 154 and fOTU5198. Some of these are fairly novel species, for example, fOTU17, appears to be a novel taxon from the Xylariales, likely in its own family, while fOTU154 is also a Xylariales species, probably a novel genus in the *Microdochiaceae*, and notably most of its closest related sequences were also recovered from cotton growing areas in the southern USA (Davinic et al., 2012). fOTU5198 is a possibly chytrid, though it would appear to be poorly described as its closest relatives come from a

[17] In mycology, an anamorph refers to the asexual, form of a fungus. Note that these anamorphic fungi still produce 'spores', though they are asexually derived. Some fungi are only known from the anamorphic form. In contrast the sexual form is called the teleomorph. In pre-molecular taxonomy, anamorphic and telemorphic forms of the same fungus had different names.

range of different chytrid orders. Like some of the other fungi here, the sequences most related to this taxon were recovered from the southern USA under cotton by Davinic and co-workers (2012).

The responses observed for DAZ were the same for GLU, which despite its 'generalised' mode of action, did appear to positively and negatively impact select taxa. For example, for S24, fOTUs 5, 6 and 142 benefited from the addition of GLU. For fOTU5, for example, after storage this taxon comprised just 0.3% of the fungal amplicon pool. The addition of GLU, however, resulted in this taxon increasing to over 29% of the fungal amplicon pool. This taxon is an *Umbelopsis* species from the Mucoromycota (formerly the Zygomycetes). fOTU6 has been discussed previously for S4 (and DAZ), its presence here in a different (but geographically only 40 km distant) soil is not that unusual. This is because *Penicillium* species (and their close relatives the *Aspergilli*) produce vast quantities of air borne spores which are spread by wind (Ramírez-Camejo et al., 2012). It is also challenging to search the literature for GLU degradation as the compound is used widely as a biological cross-linker and fixative and used in preparation of samples for microscopy, though significant literature exists of resistance to GLU by microbes in clinical or veterinary settings (Collins, 1986; Huang et al., 2019; Simões et al., 2011; Tennen et al., 2000). One further complication is that GLU is a highly reactive molecule where the mode of biological action is to chemically bind irreversibly to organic matter. This binding not only destroys the GLU molecule, but also results in the formation of a vast array of different compounds.

## 4.4 Sole carbon microbial chemical use study

In these experiments a carbon-free medium was used, to which a known concentration of a particular carbon source was included (see Section 3.6). It is important to note that some of these carbon sources were not single compounds, but instead represent mixtures. For example, in the treatments with MEA, it represents the sole source of carbon in the microcosm. In contrast, for AEMs, this carbon source was a mixture of other ethoxylated alcohols.

Provision of a sole carbon source, regardless of whether the addition is a compound or a mixture, means that in these experiments microbial growth is contingent on microbes able to degrade that compound (or those compounds) and use the carbon for metabolism and biomass production. So for example, growth on MEA (Figure 19) requires ethanolamine ammonia-lyase activity or analogous enzyme activity (Kaval and Garsin, 2018). This specific enzyme splits ethanolamine into ammonia/ammonium and acetaldehyde. This provides not only a source of carbon (in acetaldehyde) but also a source of nitrogen. Acetaldehyde is generally then converted to acetate prior to its use in central metabolism.

These experiments by their nature are reductionist. Growth here indicates use of the compound or mixture as a sole source of carbon. There are some important limitations to this approach:

1) Frequently microorganisms that degrade breakdown products of the target compound are often recruited with the 'main' degrader(s).
2) Growth does not necessarily indicate that the organisms that grow in these experiments are the main degraders/users of these compounds in groundwaters, surface waters or soils, but it does indicate that microbes in these environments include this capacity.

### 4.4.1 General overview

Microbes were enriched in culture using various chemicals. Growth was more pronounced in oxic conditions (using soil or surface water as inoculum) compared to anoxic conditions (using groundwaters). Under anoxic conditions, growth was difficult to observe within the experimental timeframe, and at the conclusion of the experiment it was determined that no enrichment had occurred and that no taxa capable of utilising any of the tested substrates as sole carbon sources were identified in the anoxic cultures. This result is likely due to the combination of low microbial cell numbers in groundwaters from the region and the limited nutrient availability in the waters.

### 4.4.2 Contaminant taxa

On some substrates, visible microbial growth was difficult to detect. Despite this, DNA sequencing of these samples consistently revealed the presence of numerous, sometimes similar, microorganisms. While these taxa undoubtedly have some catabolic abilities their presence should be viewed somewhat sceptically given their routine occurrence in air samples (see for example, Chan et al., 2009) or filtered water (Da Silva et al., 2022; Hahn, 2004; Phelps et al., 1980). This distinction between genuine microbial growth and the 'contaminant signature' is particularly critical when drawing conclusions from experiments involving low biomass systems. These taxa are noted here for the readers convenience, and while results that include these species should not be excluded completely, one should be cautious in suggesting that these taxa are important, naturally occurring organisms that could degrade xenobiotics. These taxa include:

- *Corynebacterium* species (*C. afermentans*, *C. suicordis* or *C. tuberculostearicum*),
- *Micrococcus luteus*
- *Kocuria tytonis*
- *Moraxella* (often *M. osloensis*)

### 4.4.3 Alcohol ethoxylates

Two different alcohol ethoxylate mixtures (AEMs) were examined in the present study (see Section 2.10.1); the first (AEM#1) was a mixture of isoalkyl ethers, where the alkyl component (the hydrocarbon chain) varied in length between 11 and 14 carbons, though the most common alkyl length was 13 carbons. For this mixture the number of 'ethylene oxide' repeats was set to 10 and did not vary regardless of alkyl chain length.

The second mixture (AEM#2) contained three compounds where the length of the alkyl chain was set (in all compounds) at 13 carbons, but the number of 'ethylene oxide' repeated units varied from 1-3; yielding a mixture of an isotriadecanol mono-, di-, and triethoxylate.

Under anoxic conditions it is possible to degrade alcohol ethoxylates, however, the level of branching in the alkyl chain and inclusion of propylene oxide units inhibit degradation in a more substantial way than in oxic conditions (Mosche, 2004), which may be due to steric interference or oxygen availability for monooxygenase enzymes.

Using AEM#1 as the sole source of carbon resulted in the enrichment of a mixture of taxa that included: *Stenotrophomonas cyclobalanopsidis, S. maltophilia, Cupravidus oxalaticus* and two species of *Pseudomonas*: *P. monteilii*, and *P. viridiflava* (Table 33). Using AEM#2, similar taxa were enriched (Table 34). In addition, *Ralstonia* species, specifically *R. picketti* and *R. syzygii*, were also enriched in the presence of alcohol ethoxylates.

*S. cyclobalanopsidis* and *S. maltophilia* are both motile, facultatively anaerobic bacterial species (Bian et al., 2020; Palleroni and Bradbury, 1993) with members of this genus occupying a wide range of habitats including soil (Kumar et al., 2023). Numerous *Stenotrophomonas* have been demonstrated to use alcohol ethoxylates. For example, *S*. sp. Z5 (a relative of *S. maltophilia*) isolated from the River Warta in Poland, was demonstrated to degrade a $C_{12}$ alkyl ethoxylate with 10 ethoxylate units to PEG like compounds (Budnik et al., 2016). While no direct evidence of catabolism of alcohol ethoxylates has been demonstrated for OTU5482, its enrichment here, and its activity against complex xenobiotics (Chen et al., 2021) suggests it too likely degrades these compounds. Multiple *Pseudomonas* species have been implicated in the degradation of alcohol ethyoxylates. For example, along with *Stenotrophomonas* species, Budnik and co-workers isolated *Pseudomonas* species from river water that engage in central fission of alcohol ethoxylates (Budnik et al., 2016).

**Table 33 Taxa able to grow on AEM#1 as a sole source of carbon**

| Sample | OTU | Abd (%) | Taxonomic match \| Identity (%) |
|---|---|---|---|
| **S4** | OTU105 | 20.1 | *Stenotrophomonas cyclobalanopsidis* TPQG1-4 (NR 180613) \| 100% |
| **S4** | OTU120 | 19.29 | *Pseudomonas viridiflava* (NR 117825) \| |
| **S4** | OTU5482 | 13.05 | *Cupriavidus oxalaticus* DSM 1105 (NR 025018) |
| **S4** | OTU1961 | 10.76 | *Stenotrophomonas hibiscicola* ATCC 19867 (NR 024709) |
| **S4** | OTU22 | 9.19 | *Pseudomonas monteilii* CIP 104883 (NR 024910) |
| **S24** | OTU7 | 86.14 | *Ralstonia pickettii* ATCC 27511 (NR 043152) |
| **S24** | OTU5 | 7.33 | *Bradyrhizobium liaoningense* 2281; USDA 3622 (NR 041785) |
| **S24** | OTU154 | 1.58 | *Pseudolabrys taiwanensis* CC-BB4 (NR 043515) |
| **S24** | OTU18038 | 1.06 | *Pseudolabrys taiwanensis* CC-BB4 (NR 043515) |
| **S24** | OTU578 | 0.89 | *Azospirillum brasilense* ATCC 29145; NCIMB 11860 (NR 042845) |
| **W12** | OTU5 | 58.86 | *Bradyrhizobium liaoningense* 2281; USDA 3622 (NR 041785) |
| **W12** | OTU120 | 26.8 | *Pseudomonas viridiflava* (NR 117825) |
| **W12** | OTU5123 | 5.43 | *Mesorhizobium huakuii* NBRC 15243 (NR 113738) |
| **W12** | OTU5455 | 2.58 | *Pseudomonas meliae* MAFF 301463 (NR 178225) |
| **W12** | OTU2216 | 2.51 | *Bradyrhizobium japonicum* USDA 6 USDA 6 (NR 112552) |

Abd (%) – relative abundance

Using AEM#1 as the sole source of carbon resulted in the enrichment of a mixture of taxa that included: Stenotrophomonas cyclobalanopsidis, S. maltophilia, Cupravidus oxalaticus and two species of Pseudomonas: P. monteilii, and P. viridiflava (Table 33). UUsing AEM#1 as the sole source of carbon resulted in the enrichment of a mixture of taxa that included: Stenotrophomonas cyclobalanopsidis, S. maltophilia, Cupravidus oxalaticus and two species of Pseudomonas: P. monteilii, and P. viridiflava (Table 33). UChemical and microbial baseline studies and microbial degradation experiments of CSG chemicals in Narrabri NSW | 

Table 34 Taxa able to grow on AEM#2 as a sole source of carbon

| Sample | OTU | Abd (%) | Taxonomic match \| Identity (%) |
|---|---|---|---|
| **S4** | OTU5482 | 30.69 | *Cupriavidus oxalaticus* DSM 1105 (NR 025018) |
| **S4** | OTU22 | 30.58 | *Pseudomonas monteilii* CIP 104883 (NR 024910) |
| **S4** | OTU120 | 10.97 | *Pseudomonas viridiflava* (NR 117825) |
| **S4** | OTU105 | 6.21 | *Stenotrophomonas cyclobalanopsidis* TPQG1-4 (NR 180613) |
| **S4** | OTU25465 | 4.93 | *Stenotrophomonas maltophilia* LMG 958 (NR 119220) |
| **S24** | OTU7 | 62.80 | *Ralstonia pickettii* ATCC 27511 (NR 043152) |
| **S24** | OTU118 | 15.68 | *Rhizobium tropici* CIAT 899 CIAT 899 (NR 102511) |
| **S24** | OTU5 | 14.96 | *Bradyrhizobium liaoningense* 2281; USDA 3622 (NR 041785) |
| **S24** | OTU578 | 2.03 | *Azospirillum brasilense* ATCC 29145; NCIMB 11860 (NR 042845) |
| **S24** | OTU1131 | 1.89 | *Ralstonia syzygii* ATCC 49543 (NR 040803) |
| **W12** | OTU5 | 57.10 | *Ralstonia pickettii* ATCC 27511 (NR 043152) |
| **W12** | OTU120 | 35.25 | *Rhizobium tropici* CIAT 899 CIAT 899 (NR 102511) |
| **W12** | OTU5455 | 2.12 | *Bradyrhizobium liaoningense* 2281; USDA 3622 (NR 041785) |
| **W12** | OTU22 | 1.62 | *Azospirillum brasilense* ATCC 29145; NCIMB 11860 (NR 042845) |
| **W12** | OTU12 | 1.40 | *Ralstonia syzygii* ATCC 49543 (NR 040803) |

Abd (%) – relative abundance

It is also noteworthy that multiple Ralstonia species were enriched when AEM were present. This is perhaps unsurprising as *Ralstonia* species, including *R. picketti*, have previously been implicated in the catabolism of a range of complex organic compounds (Kasuya et al., 1998; Kotik et al., 2009; Matsumoto et al., 2008). Their enrichment here, on both AEM#1 and AEM#2, likely indicates these taxa are also capable of degradation of various type of alcohol ethyoxylates.

### 4.4.4 Hydrocarbons

Two mixtures of hydrocarbons were obtained for this study. The first was a mixture of aliphatic components (from $C_{12\text{-}15}$) including n-alkanes, branched (and iso-) alkanes and cyclic alkanes (HCM#1). The second was a mixture of isoalkanes ($C_{11\text{-}15}$; HCM#2). After completion of the experimental program, it was noticed that some of the species present in enrichments with HCM#2 were quite different from microbes in HCM#1. This was surprising as the chemical composition of HCM#2 should, in large part, be present in HCM#1 and one might expect the degrading/enriched microbes to be similar. This prompted a check of material purchased for the present study from an international, chemical supplier. Despite being labelled correctly, including the CAS RN number, GCMS analyses of contents of this material, did not contain any hydrocarbons and instead appear to be a mixture of siloxanes.

Three outcomes and/or caveats of this discovery are discussed below:

1. The results for HCM#2 (which was a mislabelled mixture of siloxanes) are not included in this report as siloxanes are not used by the coal seam gas industry in Narrabri.

2. Regardless of their omission, the results from HCM#1 should be broadly indicative of HCM#2 as both include isoalkanes. This was confirmed by GCMS.
3. The experiments that include HCM#2 will be repeated using a newly obtained and confirmed source of these chemicals and the results supplied as an addendum to the final report for this project.

For S4, growth on HCM#1 enriched a species of *Rhodococcus*, likely a strain of *R. qingshengii*. The type strain of this *Rhodococcus* species was originally described from carbendazim-contaminated soils, and it possesses suitable genes for the conversion of n-alkanes and cyclic alkanes to alcohols (alkane 1-monooxygenases) (Xu et al., 2007). This species also appears to have the capacity to degrade monoaromatic compounds under oxic conditions, for example, it has the complete pathway of genes for the breakdown of benzoate via catechol (Kanehisa et al., 2016a; Kanehisa et al., 2016b; Ogata et al., 1999).

Use of HCM#1 also enriched another *Rhodococcus* species, likely a strain of *R. marinonascens* (Table 35). Like *R. qingshengii*, *R. marinonascens* likely can degrade alkanes, supposition that is supported by genetic evidence for this taxon (Táncsics et al., 2015). Along with these rhodococci, a strain of *Pedobacter chitinilyticus* was also detected on HCM#1 in S4. *Pedobacter* have been repeatedly demonstrated to occur in oil-contaminated soils and degrade aromatic hydrocarbons (Chang et al., 2017; Kwon et al., 2019; Sun et al., 2015; Vázquez et al., 2013; Yang et al., 2015; Zhao et al., 2013), though their ability to degrade alkanes does not appear to be universal, as many species appear to lack alkane 1-monooxygenases. It may be that these *Pedobacter* species are engaged in niche partitioning and instead of utilising the alkanes as *Rhodococcus* does are instead focussed on aromatic components, though further work would be required to confirm this speculation. '*Curvibacter' delicatus* was also identified in this hydrocarbon enrichment. This taxon is likely not a *Curvibacter* species (Van Le et al., 2023), and its ability to use hydrocarbons at this time is unknown.

Growth of microbes from S24 with HCM#1 also enriched essentially a dual culture that included a strain of *R. qingshengii* and a strain of *Cupriavidus necator* (Syn. *Alcaligenes eutrophus*, *Ralstonia eutropha*). The redetection of *R. qingshengii* further emphasises this taxon's ubiquity and its role in hydrocarbon degradation. This taxon is reasonably widespread across soils in the present study occurring in most soils of the region at relatively low abundance (see Supplementary data). The presence of *C. necator* is somewhat intriguing as the species has not been reported to degrade alkanes. It has, however, been shown to degrade a range of hydrocarbon including ethylene and the aromatic compounds benzene and xylene (Berezina and Paternostre, 2010; Pérez-Pantoja et al., 2008), benzoic acid, benzaldehyde and toluene (Berezina et al., 2015), polyethylene (Montazer et al., 2019), polychlorinated biphenyls (Van Dyke et al., 1996), and dichlorodiphenyltrichloroethane (DDT) (Pan et al., 2016). Inspection of the KEGG annotation of the *C. necator* N-1 genome reveals a host of aromatic hydrocarbon degrading genes, though the taxon appears to lack specific alkane degrading genes (Kanehisa et al., 2016a; Kanehisa et al., 2016b; Ogata et al., 1999). It may be that like *Pedobacter* species, this taxon is growing on the aromatic fraction present in HCM#1 added to these enrichment cultures. Other strains were detected in the soil 24 enrichment on HCM#1, including a *Bosea* species, and strains of *Bradyrhizobium*

*liaoningense* and *Phenylobacterium haematophilum*. These species represent minor components of the S24 enrichment on HCM#1 and, given the paucity of information about these taxa, their roles are unclear, though they may be degrading components within the mixture or breakdown products. Unsurprisingly, the bacterial species enriched on HCM#1 from W12 are quite different from those taxa identified in the soils, though *Bradyrhizobium liaoningense* occurred in both enrichments. The dominant taxon for W12 growing on HCM#1 was a strain of *Mycolicibacterium smegmatis*. This species is known to have a significant array of genes that have activity against both aliphatic and aromatic hydrocarbons (Kanehisa et al., 2016a; Kanehisa et al., 2016b; Ogata et al., 1999), and its growth here presumably indicates these genes are being used to access carbon from HCM#1.

**Table 35 Taxa able to grow on HCM#1 as a sole source of carbon**

| Sample | OTU | Abd (%) | Taxonomic match \| Identity (%) |
|---|---|---|---|
| **S4** | OTU16 | 51.35 | *Rhodococcus qingshengii* JCM 15477 djl-6 \| 100% |
| **S4** | OTU2 | 17.88 | *Curvibacter delicatus* NBRC 14919 (NR 113696) \|100% |
| **S4** | OTU216 | 5.8 | *Pedobacter chitinilyticus* CM134L-2 (NR 180020) \| 99.2% |
| **S4** | OTU434 | 4.29 | *Pseudomonas nitroreducens* DSM 14399 (NR 114975) \| 100% |
| **S4** | OTU3349 | 3.99 | *Rhodococcus marinonascens* DSM 43752 (NR 026183) \| 99.6% |
| **S24** | OTU29 | 43.47 | *Cupriavidus necator N-1; ATCC 43291 (NR 028766)* \|100% |
| **S24** | OTU16 | 43.34 | *Rhodococcus qingshengii JCM 15477 djl-6 (NR 043535)* \| 100% |
| **S24** | OTU291 | 2.85 | *Phenylobacterium haematophilum (NR 041991)* \| 99.6% |
| **S24** | OTU5 | 2.58 | *Bradyrhizobium liaoningense 2281; USDA 3622 (NR 041785)* \| 100% |
| **S24** | OTU17957 | 1.13 | *Bosea minatitlanensis AMX51; DSM 13099 (NR 028787)* \| 98.4% |
| **W12** | OTU4 | 44.91 | *Mycolicibacterium smegmatis* ATCC 19420 (NR 025311) \| 100% |
| **W12** | OTU5 | 37.37 | *Bradyrhizobium liaoningense 2281; USDA 3622 (NR 041785)* \| 100% |
| **W12** | OTU33 | 14.35 | *Lysinibacillus sphaericus DSM 28 (NR 042073)* |
| **W12** | OTU3747 | 0.93 | *Mycolicibacterium mucogenicum ATCC 49650 (NR 042919)* |
| **W12** | OTU687 | 0.85 | *Nitrobacter vulgaris Z (NR 042449)* |

Abd (%) – relative abundance

### 4.4.5 Monoethanolamine

MEA is both an alcohol and an amine, essentially the compound is a short, two-carbon chain with an alcohol group (-OH) on one end and an amine group (-NH2) on the other end (Figure 19). From a metabolic perspective, the compound is used by cells to make phosphatidylethanolamine, a major cell membrane lipid in most living organisms. For this reason, growth on MEA is reasonably widespread. In model (and pathogenic) species like *Salmonella*, MEA is degraded to ammonia and ethanol by ethanolamine-ammonia lyases. Ethanol is then routinely converted to acetaldehyde and acetate which then enters central metabolism. Use of ammonia/ammonium by bacteria is almost universal. Using MEA as a sole carbon source a strain of *Ochrobactrum (Brucella) pseudogrignonensis* was enriched and was the dominant taxon enriched from soil 4. Rarer taxa

including S. cyclobalanopsidis, a species of *Shinella* along with strains of *Bosea vestrisii*, *Neobacillus bataviensis* were also enriched, though they collectively made up only 10% of amplicons from the recovered enrichment. There are little data on *Ochrobactrum*/*Brucella* species in terms of their ability to use ethanolamine and other species in this group seem to lack ethanolamine-ammonia lyases. It may be that their growth on this substrate leverages other enzymes, or that their ethanolamine-ammonia lyases are divergent and not readily recognised by KEGG and the annotation pipelines it uses. The same group of taxa were enriched on monoethanolamine for soil 24 and water 12. This is somewhat surprising, but given that these samples are collocated (both come from a site near X-line Road) it may be that water 12 (which was turbid) includes significant soil/sediment microbes. Like soil 4, however, those microbes that were enriched are not known to degrade ethanolamine, at least via the typical pathway. Regardless, growth on ethanolamine was marked and obvious, suggesting the compound is readily utilised, though further work is required to ascertain the mechanistic aspects that underpin this ability.

**Table 36 Growth on monoethanolamine (MEA) as a sole source of carbon**

| Sample | OTU | Abd (%) | Taxonomic match \| Identity (%) |
|---|---|---|---|
| **S4** | OTU_13 | 90.61 | *Brucella pseudogrignonensis* CCUG 30717 (NR 042589) \|100% |
| **S4** | OTU_105 | 5.31 | *Stenotrophomonas cyclobalanopsidis* TPQG1-4 (NR 180613) \| 100% |
| **S4** | OTU_1652 | 1.36 | *Shinella zoogloeoides* NBRC 102405 (NR 114067) \| 98.4% |
| **S4** | OTU_423 | 0.77 | *Bosea vestrisii* 34635 34635 (NR 028799) \| 100% |
| **S4** | OTU_36 | 0.7 | *Neobacillus bataviensis* NBRC 102449 (NR 114093) \| 100% |
| **S24** | OTU_35 | 46.79 | *Sphingomonas aquatilis JSS-7; KCTC 2881 (NR 024997)* \| 100% |
| **S24** | OTU_30 | 32.8 | *Mesorhizobium terrae* NIBRBAC000500504 (NR 180479) \| 100% |
| **S24** | OTU_219 | 11.39 | *Aliterella atlantica CENA595 (NR 177002)* \| 93.7% |
| **W12** | OTU_35 | 42.85 | *Sphingomonas aquatilis JSS-7; KCTC 2881 (NR 024997)* \| 100% |
| **W12** | OTU_30 | 13.06 | *Mesorhizobium terrae* NIBRBAC000500504 (NR 180479) \| 100% |
| **W12** | OTU_219 | 8.03 | *Aliterella atlantica CENA595 (NR 177002)* \| 93.7% |

Abd (%) – relative abundance

### 4.4.6 Dazomet

No obvious growth was observed on DAZ and taxa identified were those described in Section 4.4.2. This occurred regardless of whether the sample was from S4, S24 or W12. If growth does occur on dazomet it is minimal and slow. It should be noted that in water, DAZ is rapidly hydrolysed to MITC, the active compound, and any growth, were it to occur, would likely be on MITC or other dazomet breakdown products. Further work on dazomet would be useful to determine if lower concentrations of the compound can be catabolised by the taxa detected.

### 4.4.7 Glutaraldehyde

Taxa detected when glutaraldehyde was the sole carbon source and W12 was the inoculum were those suspect taxa described in 4.4.2. Growth for W12 was minimal, and like dazomet it seems unlikely that these taxa were growing in the presence of this biocide. For S4 and S24, however, a range of other taxa were detected including most prominently the closely related *Cupriavidus necator* (S4) and *Ralstonia picketti* (S24). *Cupriavidus* and *Ralstonia* are sister genera within the Burkholderiaceae, and there has been some fairly reclassification (and movement between these two genera) within these taxa (Moriuchi et al., 2019). Both these taxa contain fairly extensive catabolic capabilities (Sohn et al., 2021), so their growth on glutaraldehyde or its hydration product glutaric acid is not surprising. Both taxa encode complete pathways for the degradation of glutarate to glutaryl-CoA, then to crotonoyl-CoA before conversion to acetyl-CoA and degradation in the citric acid cycle (Kanehisa et al., 2016a; Kanehisa et al., 2016b; Ogata et al., 1999).

### 4.4.8 3,3’-methylenebis(5-methyloxazolidine)

For S4 it is unclear whether growth occurred when 3,3’-methylenebis(5-methyloxazolidine) was the sole source of carbon. As for dazomet, growth was limited on this substrate and for S4 at least, those taxa that were detected using this inoculum were the suspect taxa described in Section 4.4.2. For S24, however, a mixture of other taxa including *Mycolicibacterium smegmatis*, *Bradyrhizobium liaoningense* and *Lysinibacillus sphaericus* were detected. The breakdown products of this biocide are 5-methyloxazolidine and, in turn, formaldehyde. None of these taxa are particularly well known for their degradation of these kinds of compounds, though most (like other prokaryotes) have many genes for recycling and reorganising heterocyclic compounds. These are principally used for creating and manipulating compounds into nucleic acid purines/pyrimadines. The presence of these taxa here is somewhat surprising, however, it suggests that they are capable of growth on 3,3’-methylenebis(5-methyloxazolidine) and its breakdown products as a sole source of carbon.

# 5 Conclusions

This study has extensively sampled soils, surface waters and groundwaters from in and around the Narrabri Gas Project area. The work has provided extensive baselining of both chemistry and microbial community data; indeed, it has provided the most comprehensive baseline dataset on soil and surface water microbiology in the region and extends existing groundwater data south and east of Narrabri. The fungal community dataset is the first, large scale study reported for non-vertosolic/non-cropping soils and surface waters from the region.

The microcosm experiments which aimed to test the biodegradability of 'chemical of potential concern' used by the gas industry demonstrated that most of these chemicals tested completely degraded in the region's groundwater, surface waters and soils. This chemical degradation was more rapid in soils than in waters, and was consistent with results from previous GISERA studies. It is noteworthy, however, that significant amounts of AEM persisted in groundwater microcosms, and trace amounts of this mixture persisted in soils after three months (one month for soils) incubation. It may that degradation in groundwater is simply slow, and would be completed with sufficient time. Conversely, it may be that degradation would not proceed further, and may remain incomplete for significant periods of time. Experimental data would be required to resolve this uncertainty.

Further the study identified numerous indigenous microorganisms in groundwaters, surface waters and soils that are likely capable of degrading the 'higher hazard potential' chemicals tested; and conversely a number of taxa were identified as sensitive to chemical additions in these environments. Such taxa may be useful targets for biomonitoring for potential chemical contamination events.

It is worth noting that while this study provides important baseline data, and identifies useful taxa for biomonitoring such datasets and species represent a snapshot in time (in this case 2022 and 2023). Additional programs developed by other stakeholders may be valuable in extending these data especially in the event of further development in the region.

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**For further information**
**CSIRO Energy**
Richard Schinteie
+61 2 9413 7954
richard.schinteie@csiro.au
csiro.au/energy