Saline mine-water alters the structure and function of prokaryote communities in shallow groundwater below a tropical stream

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A B S T R A C T

Bacteria and archaea (prokaryotes) are vital components for maintaining healthy function of groundwater ecosystems. The prokaryotic community composition and associated putative functional processes were examined in a shallow sandy aquifer in a wet-dry tropical environment. The aquifer had a contaminated gradient of saline mine-water, which primarily consisted of elevated magnesium (Mg 2+ ) and sulfate (SO 4 2- ), although other major ions and trace metals were also present. Groundwaters were sampled from piezometers, approximately 2 m in depth, located in the creek channel upstream and downstream of the mine-water influence. Sampling occurred during the dry-season when only subsurface water flow was present. Next generation sequencing was used to analyse the prokaryote assemblages using 16S rDNA and metabolic functions were predicted with FAPROTAX. Significant changes in community composition and functional processes were observed with exposure to mine-waters. Communities in the exposed sites had significantly lower relative abundance of methanotrophs such as Methylococcaceae and methanogens (Methanobacteriaceae), but higher abundance in Nitrososphaeraceae, associated with nitrification, indicating potentially important changes in the biogeochemistry of the exposed sites. The changes were most strongly correlated with concentrations of SO 4 2- , Mg 2+ and Na + . This knowledge allows an assessment of the risk of mine-water contamination to groundwater ecosystem function and aids mine-water management.

1. Introduction

Groundwater is a valuable global resource that is under increasing pressure from disturbances such as extraction and contamination (Griebler et al., 2019). Groundwaters in uncontaminated aquifers are often oligotrophic, with little or no dissolved oxygen, limited carbon, nutrients and energy resources, and the prokaryote communities in these systems are generally dependent on heterotrophy and lithoautotrophy (Goldscheider et al., 2006; Taubert et al., 2019). Prokaryotes are involved in various biogeochemical cycling processes in groundwater ecosystems, particularly playing an important role in carbon and nutrient cycling in groundwaters (Griebler and Leuders, 2009). Groundwater contamination not only affects the quality of groundwater directly but causes changes in prokaryote communities, including loss of biodiversity, thus altering various biogeochemical cycling processes (Hemme et al., 2015). The response of groundwater prokaryote communities to contamination has been the focus of a number of studies (e.g. hydrocarbons, (Grosbacher et al., 2016; Mouser et al., 2005; Stephenson et al., 2013; Wright et al., 2017); uranium and metals, (He et al., 2018; Hemme et al., 2010, 2015); general agricultural/industrial pollutants (Korbel et al., 2013; Smith et al., 2012, 2018); and salinity (Sang et al., 2018)). Changes in physico-chemical conditions result in changes in community composition, e.g. Stephenson et al. (2013) and richness, e.g. Hemme et al. (2015), as well as decreases in functional diversity, e.g. He et al. (2018). Understanding the ecology of groundwater prokaryote communities and the impacts of groundwater contamination on these communities is critical for management and protection of all groundwater values.

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The discharge of saline mine-waters has significantly impacted the quality of many surface- and groundwater ecosystems around the globe (Lottermoser, 2010), resulting in the modification of hydrogeochemistry that can impact on biological communities (Cañedo-Argüelles et al., 2016). Saline mine-waters arise through the dissolution and mobilisation of metals, non-metals and metalloids, and can, depending on the solutes, range from acidic to alkaline (Nordstrom et al., 2015). Knowledge of salinity effects on prokaryote communities in groundwater (often measured as total dissolved solids (TDS) or electrical conductivity (EC)), has mostly been derived from studies of communities along gradients in coastal or estuarine systems, e.g. Héry et al. (2014) and Sang et al. (2018). These studies observed changes in bacterial community composition associated with changes in salinity due to seawater intrusion, for example Héry et al. (2014) noted increases in relative abundance of Gammaproteobacteria associated with increased salinity. However, sodium chloride (NaCl), the key constituent of seawater, is rarely a major component of mine-waters (Nordstrom et al., 2015), and input of higher salinity waters with different ionic composition can have different effects on freshwater biota (Cañedo-Argüelles et al., 2016).

To date, little is known of the groundwater prokaryote community in seasonally-flowing sandy creek channels that are common in the wet-dry tropics. Moreover, there is little understanding of the community response to salinisation and/or other associated contaminants of potential concern (COPCs) from anthropogenic sources such as mine-waters. The impacts of a uranium mine (Ranger) located in the Australian wet-dry tropics, on downstream surface water communities of Magela Creek, have been well documented (Humphrey and Chandler, 2018; McCullough, 2006; Mooney et al., 2020; van Dam et al., 2010), but the impacts on groundwater communities have not been studied. Although the mine has ceased production (in January 2021), and the site is undergoing rehabilitation (to 2026), the site will continue to be a source of salts and other contaminants of potential concern (COPCs) through surface water runoff and exfiltrating groundwater. Although the mine has ceased production (in January 2021), and the site will continue to be a source of salts and other contaminants of potential concern (COPCs) through surface water runoff and exfiltrating groundwater. The major source of salts and other contaminants of potential concern (COPCs) is undergoing rehabilitation (to 2026), and the site will continue to be affected by the mine-waters (Nordstrom et al., 2015), and input of higher salinity waters with different ionic composition can have different effects on freshwater biota (Cañedo-Argüelles et al., 2016).

The sand channel has been filled since the Holocene with medium- to coarse sands (Roberts, 1991). The average thickness of the sands (i.e. depth to original stream bed) has been reported as 5–12 m in sections (Ahmad et al., 1982; Nanson et al., 1993; Roberts, 1991). The sand channel has been described as a strip, unconfined aquifer and has high transmissivities, ranging between 400 and 800 m$^2$ day$^{-1}$ (Ahmad and Green, 1986). To access the groundwater during the dry season, PVC piezometers (Enviroequip 50 mm, 1.5 m PVC bore casings and screens (Thermoﬁsher Scientiﬁc)) were installed to a depth of approximately 2 m within the sand channel (Supplementary Figure 2). A series of piezometers were installed both laterally and longitudinally along the creek from approximately 3 km upstream of Ranger uranium mine to approximately 4 km downstream (Supplementary Figure 1). A 300 m longitudinal section of the shallow groundwaters in Magela Creek is currently contaminated with mine-waters, most likely arising from groundwaters moving through contaminated sediments from the adjacent Coonimbba Billabong. This mine-water source typically contains high concentrations of Mg$^{2+}$, SO$_4^{2-}$ (the dominant contamination signature in the mine-waters) along with elevated levels of Mn, U and ammonia (Supplementary Table 1) (Hart et al., 1992; Noller, 1991; Trenfied et al., 2019).

2.2. Sample collection

Samples of groundwater for water chemistry and molecular analysis were collected monthly from 17 sites, during the dry season (August to November), on 3 occasions in the first sampling year (2017) and 4 occasions in the second year (2018) (Supplementary Table 2). A peristaltic pump (Series IL, Geotech Environmental Equipment, Inc), was used to purge piezometers prior to sample collection by pumping at least three casing volumes (Sundaram et al., 2009). In-situ measurements of physico-chemical variables (Supplementary Table 3) were taken using a multiparameter sonde and flow-through cell (YSI EXO1, Ohio USA). Once purged, and the water quality parameter readings had stabilised, samples for water chemistry and molecular analysis were collected. Samples for water chemistry were collected in 1 L HDPE bottles, ensuring that there was no head space in the bottle. Water samples, for molecular analysis, were collected in sterile 2 L plastic bottles and both were stored on ice until they were processed back in the laboratory.

2.3. Chemical analysis of groundwater samples

A portion of the 1 L water sample was filtered within 4 h of collection using a 150 mL Nalgene™ Rapid-Flow™ Sterile Single Use Vacuum Filter unit (0.45 μm), for analysis of filtered metals and major ions. The remaining sample was decanted into separate bottles for analysis of alkalinity, nutrients and dissolved organic carbon (Supplementary Table 4). Samples were analysed for a suite of metals and major ions (Supplementary Table 3) by inductively coupled plasma mass spectrometry, inductively coupled plasma atomic emission spectrometry and FIA methods, by a NATA-registered laboratory (EnviroLab, Sydney, Australia). Total and dissolved organic carbon (TOC/DOC) samples were analysed in-house using a high-temperature combustion method 5310B (TOC-VCSH, Shimadzu Scientific Instruments, Oceania Pty. Ltd, SD < 0.1 mg L$^{-1}$, maximum CV of 2%).

2.4. DNA extraction and amplification

Water samples were filtered within 4 h of collection, through sterile 0.22 μm mixed cellulose membranes using vacuum filtration, 100 mL increments were added until a total of 1000 mL had been filtered. The filter membranes were placed in sterile petri dishes and stored at –80 °C until DNA extraction. For DNA extraction, membranes were sliced into thin strips, using a maximum of 75% of each membrane. The weight of sediment and filter membrane was recorded, as the recommended 0.25 mg of sediment was generally not collected on the filters. Extractions were performed using the Qiagen DNeasy Powersoil kit, following the
manufacturers protocol with the following modifications; increase in time and intensity for bead beating. 30 min incubation period, 4 min centrifuge period, and only 50 μL of the final C6 solution was used to concentrate the final volume of DNA extracted.

Polymerase chain reaction (PCR) was conducted on all samples using the primer set 515F (50‘-3‘: GTGYCAGCMGCCGCGGTAA) (Parada et al., 2016) and 806R (50‘-3‘: GGACTACNVGGGTWTCTAAT) (Apprill et al., 2015) for the V4 region of the 16S rRNA gene. Amplification used a modified Earth Microbiome Project protocol v4.13 (Caporaso et al., 2012). The sample (2 μL) was added to each reaction, with 15 μL of AmpliTaq Gold 360 Master Mix (Applied Biosystems), 10 μL of ultrapure water (UltraPureTM DNase/RNase-Free Distilled Water, Invitrogen) and 3 μL of a unique combination of forward and reverse tag primers (2 μL) for total PCR reaction volume of 30 μL. A sample specific Geloy barcode was included in the 806R reverse primer. Amplification included both a positive control (Supplementary Figure 3) and negative controls (DNA-free water). Thermal cycling included a simplified hot start at 95 °C and an initial denature cycle at 95 °C for 10 min followed by annealing for 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, followed by elongation at 72 °C for 7 min and a final hold at 4 °C. Amplicon quality and quantity was determined by Quant-it Picogreen assay (Thermofisher Scientific) and agarose gel electrophoresis. PCR products of all samples were pooled in equimolar amounts before being purified, using AMPure XP kit (Beckman Coulter, cat#A63880). The final purified pool was sent to Ramaciotti Centre for Gene Function Analysis (Sydney, Australia) for paired-end sequencing on the Illumina MiSeq using the 250 bp v2.2 process.

2.5. Bioinformatics

A custom pipeline was used to process the amplicon sequence data (Greenfield Hybrid Amplicon Pipeline, GHAP, v2.4) as detailed in Gissi et al. (2019). Usearch v11.0.667 and RDP Classifier (v2.12) were used to classify OTU sequences supplemented with a curated reference set (RefSeq 16S, downloaded September 2019) for the purposes of species-level classification. Complete with taxonomic classifications, species assignments and counts for each sample were generated in OTU tables.

After processing through the GHAP pipeline, and prior to statistical analysis, data were further processed using the R library phyloseq (McMurdie and Holmes, 2013). Rare OTUs that occurred in <2 samples were excluded (10.2% of 37647), as were OTUs that were not classified to a domain (0.19%), and OTUs that matched Eukaryote organelles (i.e. chloroplasts). OTUs that had reads less than 0.01% of total number of sequences were also removed (95.2%) to obtain a total of 1606 different OTUs. Sequences were rarefied to the lowest common number of sequences (7766 sequences/sample) to minimise batch effect due to differences in the numbers of total sequences present in the two sampling years. All subsequent analyses, except the DESeq2 analysis, were based on these rarefied sequences.

2.6. Functional analysis

Functional group prediction was performed using FAPROTAX (Louca et al., 2016). Limitations to this approach include: 1) the FAPROTAX database was constructed primarily to analyse functional profiles of marine and lentic prokaryote communities; and (2) the database is limited with the result that only a small percentage of OTUs in this study could be assigned to at least one functional group. A heatmap illustrating the FAPROTAX was generated using the heatmap() function in R.

2.7. Statistical analyses

Samples were classified a priori according to position in the study reach around a contamination plume (Supplementary Figure 1). Four exposure groups were defined: all sites upstream of contamination plume (Upstream Reference), sites laterally adjacent to contamination plume, in the central channel of the creek (Central Reference), sites downstream of contamination plume (Downstream Reference), and sites within the plume (Exposed).

Patterns amongst the environmental characteristics of the shallow groundwater samples were examined with Principal Components Analysis (PCA), based on Euclidean distances using Primer v7 (Clarke and Gorley, 2015). Data were visualised using draftsman plots, and a log-transform transformation if the data were strongly skewed, before being normalised to account for different measurement scales.

Differences in prokaryote taxonomic and functional communities and environmental variables were compared amongst exposure groups using the Kruskal-Wallis test.

Multivariate statistical analyses were conducted on Hellinger transformed OTU data, and square root transformed functional group data using Primer v7 (Clarke and Gorley, 2015). Non-metric multidimensional scaling (nMDS) was used to visualise the community assemblages of Bray-Curtis resemblance matrices for both the OTU and functional group datasets. PERMANOVA was used to test whether community composition and function differed between year and a priori “exposure groups”. Sample sites were included as a random factor nested in exposure groups. Differences in data dispersion between exposure groups, sites and years were tested with PermutR and the association between environmental variables and prokaryote community composition and function was determined using DISTLM and dbRDA. Highly correlated environmental variables (r > 0.90) were removed from the dataset prior to running DISTLM. DISTLM was used with stepwise selection and Akaike information criterion (AIC). The significance level (α) for all inferential tests was 0.05.

A negative binomial model applied to the non-rarefied OTU data with the DESeq2 implementation in phyloseq (McMurdie and Holmes, 2014) was used to test for differences between exposure groups. P-values were adjusted for multiple tests with the False Discovery Rate Benjamini-Hochberg method.

3. Results

3.1. Overview of chemistry

The data set included exposed sites (n = 5) within a contamination plume on the mine-lease area, as well as reference sites that were upstream (n = 6), downstream (n = 3) and in the central creek channel (n = 3) adjacent to the exposed sites (Supplementary Figure 1). The natural shallow groundwater, observed at the reference sites during the dry season, was generally acidic (pH 4.8–6.4), with low electrical conductivity (EC < 50 μS cm⁻¹), alkalinity as HCO₃⁻ < 10 mg L⁻¹ and concentrations of major ions and metals (Supplementary Figure 4). Mine-water exposed sites had significantly higher concentrations (Kruskal-Wallis, P < 0.001) of known mine-water contaminants, SO₄²⁻, Mg²⁺, and Mn, significantly higher concentrations of Na⁺, Cl⁻ and Fe (Supplementary Figure 4) and lower concentrations of Ca²⁺. The exposed sites also had generally negative redox values (cf. generally positive redox measures for reference sites), and a slightly lower range of pH (4.54–5.93) compared to reference sites. All sites, reference and exposed, had low concentrations of dissolved oxygen (<1 mg L⁻¹), indicating suboxic or anoxic conditions, and were high in temperature (27–32 °C). The sites were typically low in nitrogen with NO₂⁻ and NO₃⁻ concentrations generally below the detection limit (<0.005 mg L⁻¹). Dissolved organic carbon (DOC) was highly variable (0.84–998 mg L⁻¹), with a median value of 15 mg L⁻¹.

Principal Components Analysis (PCA) showed distinct separation of the environmental characteristics of exposed sites and reference sites (Fig. 1). The first PCA axis explained 26.4% of the variation in site environmental variables, and the second axis explained 16%. Separation of samples along the first axis was positively correlated with Mg²⁺ and EC, along with Mn (Pearson correlation coefficient, r > 0.80). The
second axis separated samples, based on Ca$^{2+}$ ($r > 0.7$). Mg concentrations and EC have a strong linear relationship.

A number of chemical variables had lower concentrations in 2018 compared to 2017 (Supplementary Figure 4) presumably due to the slightly higher antecedent rainfall in 2018 and hence dilution available from higher recession flows. However, these differences were not marked and the PCA is notable for the interspersion of 2017 and 2018 data points within each of the exposure groups indicating relatively low interannual variability in water quality.

### 3.2. Taxonomic composition

In the final dataset, 1606 unique OTUs were identified across 17 sites, with Bacteria representing 83% (1332 OTUs) and Archaea 17% (274 OTUs). OTUs were further assigned to lower taxonomic ranks and relative abundance for each rank was estimated across different groups. Across all the sites a total of 31 prokaryote phyla were assigned, consisting of 66 classes, 107 orders, 165 families and 255 genera. Of the 255 genera groups 43% were not classified into a known genus. A large number of unclassified Bacteria (Fig. 2), compared to reference sites. Methylomonas, as well as other methylotrophic families were absent (or had < 1% relative abundance) at the exposed sites, as was the Methanobacteriaceae and Clostridiaceae families. Helicobacteraceae were absent or had <1% relative abundance at the upstream reference sites but were present at both the downstream and central reference sites and the exposed sites.

### 3.3. Community composition

Ordination plots of the prokaryote communities showed a distinct separation of communities between the exposed and the reference site groups (Fig. 3A), with PERMANOVA showing significant differences ($p < 0.01$) amongst the exposure groups, sampling years and sites (Supplementary Table 5). Exposure groups, site and the residual were the largest contributors to variation, and year, although statistically significant ($p = 0.01$) was less important than the other factors (Supplementary Table 5). Pairwise comparisons for exposure groups, indicated that the exposed sites were significantly different from each reference group, and reference groups did not significantly differ from each other. Data dispersion (PermDISP $p > 0.05$) did not significantly differ between exposure groups or years (Supplementary Table 5), suggesting that the results were not influenced by differences in heterogeneity between these groups. However, data dispersion differed significantly amongst sites (PermDISP $p = 0.016$), indicating the PERMANOVA results were also influenced by differences in dispersion (and Supplementary Table 5), indicating spatial heterogeneity, which is also shown in a cluster analysis where samples from each site tended to cluster together (Supplementary Figure 6).

Examination of the association between community structure and environmental variables showed the same strong separation between the exposed and reference groups (Fig. 3B). Samples from the exposed group with higher contaminant concentrations were separated along the first axis (to the right). Na$^+$, SO$_4^{2-}$ and Mg$^{2+}$ concentrations accounted for the largest portion of variation in the community structure, (12.4,
11.8 and 11.2% of the variation, respectively, Supplementary Table 6).

Differential abundance analysis indicated OTUs differed significantly (adjusted \( p < 0.01 \)) in relative abundance between exposed and reference sites (Fig. 4). The reference sites had significantly more OTUs (and in greater relative abundance) assigned to an unclassified Betaproteobacteria family group, Methanobacteriaceae, Methylococccaceae and Methylocystaceae compared to the exposed sites. Whereas the exposed sites had significantly more OTUs, and in greater relative abundance, associated with Nitrospiraceae, an unclassified Thermoproteales family group, Rhodocyclusaceae and an unclassified Campylobacterales family group (Fig. 4).

### 3.4. Prokaryote functional annotation

FAPROTAX assigned 479 out 1606 bacterial OTUs (29.8%) to at least one predicted functional group. A total of sixty-nine functional groups were identified and most groups were represented by more than one OTU (Supplementary Table 7). Chemoheterotrophy was the most dominant function across all samples (17.7% of total OTUs). OTUs assigned to chemoheterotrophy were also associated with other processes, such as methylophyty, hydrocarbon degradation, and aromatic compound degradation. Exposed sites had significantly (Kruskal-Wallis, \( p < 0.001 \)) lower relative abundance of OTUs associated with chemoheterotrophy, hydrocarbon degradation, methylophyty and methanoiphyty, compared to reference sites (Fig. 5), and significantly (Kruskal-Wallis, \( p < 0.001 \)) higher representation of groups involved with nitrification, ureolysis (Fig. 5) and reduction of sulfur compounds along with groups involved in the sulfur cycle (i.e. reduction of sulfur compounds).

Multivariate analysis of the functional data separated the exposed sites from reference sites (Fig. 6 A), although not as strongly as observed for the community composition data. PERMANOVA showed significant difference among the exposure groups, sites and between sampling years (Supplementary Table 8). Exposure groups, site and the residual contributed the largest components of variation in the analysis (Supplementary Table 8). Patterns among exposure groups did not vary between years (Exp x Year, \( p = 0.49 \)) but there was variability in the response of individual sites between years (Site(Exp) x Year \( p = 0.001 \)). Pairwise tests examining the exposure groups showed that each reference group differed significantly from the exposed group. Examination of associations between functional groups and environmental factors (Fig. 6 B) indicated that Na\(^+\) and SO\(_4^2-\) explained most (15 and 13%, respectively) of the variation observed in the functional composition, followed by Mg\(^2+\) (10%) and Cl\(^-\) (9%) (Supplementary Table 9).

### 4. Discussion

We found changes in groundwater prokaryote community assemblages and functional groups strongly correlated with contamination arising from saline expressions of mine waste waters.

Samples from the exposed sites had significantly elevated concentrations of Mg\(^{2+}\), SO\(_4^{2-}\), Na\(^+\), Cl\(^-\), Fe and Mn, compared to concentrations in reference sites, adjacent to and upstream and downstream of the contaminated area. Elevated concentrations of Mg\(^{2+}\) and SO\(_4^{2-}\) are indicative of mine-derived water contaminants. However, while Mn is elevated in some mine-waters, it also occurs naturally in groundwater and samples from one reference site (MCP03) had consistently elevated concentrations of Mn (340–410 µg L\(^{-1}\)). In the reference sites, suboxic redox conditions and low pH may have favoured elevated concentrations of free Mn\(^{2+}\), but there may have also been microbially mediated dissolution from the sands. The exposed sites also had high concentrations of Fe (predominantly as Fe\(^{2+}\), unpublished data), a contaminant not associated with mine-waters, and we observed precipitation of iron oxyhydroxides within the sand channel around these exposed sites, indicating activity of iron oxidising bacteria. Therefore, a combination of input from mine-waters and the influence of local microbial activity and redox conditions may have created the elevated concentrations of Mn and Fe at the exposed sites.

Increases in Mg\(^{2+}\) concentration greater than 3 mg L\(^{-1}\) have been associated with toxic effects on local tropical surface water species (Hogan et al., 2013; Prouse et al., 2015; van Dam et al., 2010). While Mg\(^{2+}\) is strongly associated with SO\(_4^{2-}\) in mine-waters, the SO\(_4^{2-}\) ion has been shown to have low toxicity to surface water organisms (van Dam...
et al., 2010). The sensitivity of Mg$^{2+}$ has been attributed to the extremely soft waters of Magela Creek (van Dam et al., 2010). The high toxicity and high mobility of Mg$^{2+}$ places this major ion as the toxicant of most concern in mine-waters released, or dispersed, to the Magela Creek system. However, elevated concentrations of SO$_4^{2-}$ were more likely to be influencing the groundwater prokaryote communities in this system than Mg$^{2+}$ concentrations, although the two major ions were highly correlated.

Increasing solutes such as Mg$^{2+}$ and SO$_4^{2-}$ can have diverse mechanisms of action on prokaryote organisms, which will be dependent on the concentration. Increased levels of Mg$^{2+}$ have been reported to decrease survival of Staphylococcus aureus cells by disrupting membrane function (Xie and Yang, 2016), as well as decreasing adherence of bacteria to surfaces, impairing biofilm assembly (Demishtein et al., 2019), which can decrease resistance to external stressors. However, the concentrations at which these effects occurred were markedly higher than observed in our samples, indicating direct toxicity from Mg$^{2+}$ isn’t likely to be occurring in these waters. SO$_4^{2-}$ is used by a number of prokaryotes as an energy source, through electron donation, and increasing SO$_4^{2-}$ has been linked to changes in prokaryote community structure and function (discussed further below). While specific mechanisms of influence of increased concentrations of Mg$^{2+}$ and SO$_4^{2-}$ were not examined in this study, we hypothesise that the most likely mechanism for community changes in the shallow groundwater of Magela Creek is through preferential growth of species responding to sulfate, which probably leads to complex biogeochemical processes with other species in synergistic relationships.

Elevated SO$_4^{2-}$ concentrations have been correlated with changes in prokaryote community structure in field (Flynn et al., 2012, 2013) and laboratory (Bethke et al., 2008; Raskin et al., 1996) studies, and our

Fig. 3. nMDS ordination (A) of the prokaryote communities. Dashed line circles represent significant clusters identified by a SIMPROF test for 45% similarity. dbRDA ordination (B) showing relationship between prokaryote communities and environmental variables. Vector overlay of environmental variables identified as significantly correlated using a stepwise DISTLM with AIC.
results concur with these findings. OTUs associated with sulfate-reducing bacteria (SRB) (i.e. Desulfovibrioaceae, Desulfobulbaceae and Desulfobacteraceae) were more prevalent in the exposed sites (Supplementary Figure 7), however they represented only a small percentage (<0.5%) of the overall relative read abundance in the samples. Flynn et al. (2013) observed that SRB were 4x more abundant in assemblages attached to sediments within an aquifer, compared to assemblages suspended in the groundwater fraction. In this study, sulfate reduction was obviously occurring based on observations of strong H2S odour associated with the exposed sites, but organisms associated with the process were not in high abundances and thus were not highlighted as major discriminators in analyses between the exposed and reference sites. However, this study only examined filtered groundwater samples and other attached biota may have been involved in the reduction of sulfur compounds. Future studies aimed at establishing the contribution of attached microbes in these processes would be valuable.

Samples from our exposed sites had reduced relative abundances of OTUs assigned to taxa associated with methanogenesis (e.g. Methanobacteriaceae). Flynn et al. (2013) suggested that the relatively low abundance of methanogens they observed in wells with greater than 0.03 mM of SO4\(^{2-}\) (~3 mg L\(^{-1}\)) could be explained by methanogens being unable to respire quickly enough to maintain a viable population in the presence of active SO4\(^{2-}\) reduction. Raskin et al. (1996) indicated addition of 30 mg L\(^{-1}\) of SO4\(^{2-}\) resulted in decreased concentrations of methanogens (from 25% initial concentration to 8%) and these low concentrations persisted for at least a year. This could explain why, even with measured SO4\(^{2-}\) concentrations decreasing in the second year of our study, these groups were still absent, or in very low abundances at sites within the exposed group.

OTUs assigned to taxa involved with oxidation of methane (i.e. methanotrophs) were also poorly represented in samples from the exposed sites, particularly taxa in the Methylococccaceae family. These taxa were associated with several putative functional groups including chemoheterotrophy and those involved with methane oxidation (i.e. methanotrophy) and oxidation of other carbon compounds (i.e. methylophytroph), and these functions were also lower at the exposed site. Redox potential is one of the most important controls of methane oxidation (Chowdhury and Dick, 2013), therefore the exposed sites having generally negative Eh (ORP) values would not favour growth of methanotrophs.

In addition to Mg\(^{2+}\) and SO4\(^{2-}\), we identified Na\(^+\) as a possible driver, influencing the differences observed between exposed and reference prokaryote communities and functions. Na\(^+\) is highly correlated with Mg\(^{2+}\) and SO4\(^{2-}\) (r = 0.80), and elevated concentrations of Na\(^+\) in the shallow groundwater of the exposed sites in Magela Creek appeared to be mine-water related, as concentrations were higher than reference sites. However, Na\(^+\) is not elevated in surface-water discharges from the mine and has not been identified as a Contaminant of Potential Concern for the Ranger mine surface water monitoring program. Hence, there has been limited site-specific toxicity testing and it is unknown at what concentration Na\(^+\) may result in adverse effects to local surface water organisms. If elevated concentrations of Na\(^+\) were discharged to the surface-waters of the Magela Creek, it is possible the ecosystem may be sensitive to the additions due the extremely soft waters of the stream (i.e. <5 mg L\(^{-1}\) CaCO\(_3\)). There is also limited information on the influence of Na\(^+\) on prokaryote communities, more broadly. Two studies that have focused on the effect of Na\(^+\) (as NaCl) (i.e. Baldwin et al., 2006; Beyer-Robson, 2014) on sediment prokaryote communities in freshwater ecosystems applied NaCl experimentally at up to 100 times higher concentrations than those observed in the Magela Creek shallow groundwater. Baldwin et al. (2006) observed shifts in community structure in wetland sediment bacterial communities only at the highest concentration of NaCl (equivalent to approximately 2352 mg L\(^{-1}\) Na\(^+\)). However, they observed significant changes with increasing NaCl concentrations in the archaeal communities and noted decreased production of methane even at low Na\(^+\) levels (57 mg L\(^{-1}\)).
production of methane was attributed to inhibition of acetoclastic methanogenesis, a process undertaken by archaeal genera *Methanosarcina* and *Methanothrix*. In our study we noted these taxa had generally reduced relative abundance at the exposed sites compared to reference sites but they were in low abundances (\(<1\%) across all samples, indicating that acetoclastic methanogenesis was not a dominant process in the shallow groundwater ecosystem of Magela Creek, and any responses to Na\(^+\) are presumably associated with other taxa and processes. Increased salinity (associated with NaCl) has also been implicated in reduction of nitrification activity (i.e. Nelson et al., 2016) but at concentrations of Na\(^+\) much higher than those measured in our samples. In laboratory studies, using saturated sandy sediments from ephemeral creeks in north Queensland, Australia, Beyer-Robson (2014), noted reduced potential rates of nitrification processes with salinities of 10,000 μS cm\(^{-1}\) (NaCl concentration not reported). In our study, we observed the opposite, with taxa associated with inferred nitrification (i.e. *Nitrososphaera* sp.) having higher relative abundance in the exposed sites (mean of 150 μS cm\(^{-1}\)) compared to the reference sites. It could also be that the high correlations amongst the major ions in our samples are confounding results and reflect a statistical, rather than a true causal effect. Nevertheless, Na\(^+\) and Mg\(^{2+}\) are present in equivalent concentrations in our samples and could be exerting effects independently, working together or could be antagonising any effects observed on the prokaryote communities. The roles of different ions are complex, and it would require further studies to tease out the effects of elevated concentrations of the different ions on the prokaryote communities in these shallow groundwater systems.

Sampling location also had a strong influence on community assemblages, and to a lesser extent functional groups, indicating there was considerable spatial heterogeneity in the prokaryote community structure within the shallow groundwater of the sand channel that was not necessarily associated with water chemistry. The study sites were spread over a 7 km reach of the creek channel, with minimum distance of 10 m between the closest sites (Supplementary Figure 1). Although the sand channel comprises medium-coarse sands (Roberts, 1991) with similarly high hydraulic conductivities along the reach (unpublished data), there is likely to be localised physical differences at each site associated with factors such as differing porosity, and organic content including buried leaf litter and riparian plant roots. Brad et al. (2008) observed location specific grouping of bacteria communities in sediment samples from an aquifer impacted by landfill leachate, even over sampling depth distances of 1 m. Lin et al. (2012) noted spatial heterogeneities, both laterally and vertically in prokaryote communities collected from bores approximately 30 m apart in an unconfined aquifer, and hypothesised the variation was associated with habitat heterogeneities such as voids between gravelly cobble within the aquifer, the study also observed a strong temporal variation governed by fluctuations in groundwater elevation which influenced both geophysical and hydrochemical variables.

While PERMANOVA showed a small but significant contribution of sampling year to variation in the prokaryote community composition, these year-to-year differences were not marked in ordination space (Fig. 3). The ordination depicted a general pattern of interspersion of 2017 and 2018 data points within each of the exposure groups, indicating relatively low interannual variability in community composition. The interannual variability in communities, may be due to changes in water chemistry with differences particularly evident in sites within the contamination plume. These exposed group sites had mean
concentrations of contaminants such as Mg$^{2+}$ and SO$_4^{2-}$ at least 50% lower in the second year of the study. This difference was likely due to the greater rainfall and longer duration of the wet season that preceded the first sampling, which led to larger and longer surface flow, and would have contributed to longitudinal dilution of the contaminant plume. Alternatively, there was a considerable difference in the read depth of samples between the two years (Supplementary Figure 5), which, although rarefaction was done to minimise this batch effect, may have also contributed to the statistical difference between years.

There was considerable taxonomic variability within the putative functional groups, and a number of OTUs were assigned to four or more different functional groups indicating a functional redundancy within

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**Fig. 6.** nMDS (A) and dbRDA (B) ordinations of the predicted functional groups. Dashed line circles represent significant clusters identified by a SIMPROF test for 80% similarity. Vector overlays on the on the nMDS (A) are predicted functional groups with >0.5 correlation, and similar functional processes have been grouped to primary function (indicated by bold font) for clarity. dbRDA has a vector overlay of the environmental variables identified as significantly correlated using a stepwise DISTLM with AIC.
these shallow ground water communities. This might explain why the separation observed between exposure groups was not as strong in the functional data compared to the community composition data. However, much of the diversity that was observed in the prokaryote community ordinations was associated with OTUs that were unclassified beyond phyla level, and FAPROTAX ignored OTUs that were not identified at species or genus level because many functions are only conserved at this level. In studies of prokaryote communities in sediment samples from floodplains (Nelson et al., 2016) and a billabong (Sutcliffe et al., 2017) within Kakadu National Park large proportions of unclassified sequences were also reported, and it was suggested that these sequences may be unique to tropical systems. Sutcliffe et al. (2017) compared shotgun metagenomics with 16S rRNA amplicon data and observed that shotgun metagenomics identified a greater number of genera groupings than the 16S amplicon data. Future studies examining groundwater communities in these understudied regions would benefit from undertaking whole metagenome sequencing.

Given this is the first report, to our knowledge, examining groundwater prokaryote communities in the region, it provides a baseline characterisation of these communities for sand channel streams, as well as empirical evidence that mine-waters with elevated levels of Mg2+ and SO42− changed important biogeochemical processes such as (increased) nitrification and (decreased) methane oxidation, albeit in a localised area. Groundwater prokaryotes are not widely used in assessment of mining impacts, despite having a direct link to changes in biogeochemistry, and this study shows they can provide a useful line of evidence to inform whole of mine-water management.

5. Conclusion

Despite a considerable history of work investigating impacts of mining in the Alligator Rivers Region, NT, this is the first study of responses of groundwater prokaryotes in the region to mine-water contamination. The study demonstrated clear differences in prokaryote community structure and function in response to a contamination plume on the Ranger Project Area. The contamination was associated primarily with Mg2+ and SO42−, although Na+ was identified as the variable most strongly correlated with prokaryote structure and function. This knowledge assists in the assessment of potential mining impacts on groundwater ecosystems and will aid ongoing mine-water management.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117318.

Author contribution

Lisa Chandler: Conceptualization, Methodology, Project administration, Formal analysis, Investigation, Visualization, Writing - Original. Andrew J Harford: Supervision, Conceptualization, Methodology, Project administration, Writing - Reviewing and Editing. Grant C Hose: Supervision, Conceptualization, Methodology, Writing - Reviewing and Editing. Chris L Humphrey: Supervision, Conceptualization, Methodology, Writing - Reviewing and Editing. Anthony Chardon: Resources, Methodology, Writing - Reviewing and Editing. Paul Greenfield: Software, Formal analysis, Writing - Reviewing and Editing. Jenny Davis: Supervision, Conceptualization, Methodology, Writing - Reviewing and Editing.

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