OCEANS AND ATMOSPHERE

# Georges River Environment Improvement Program (EIP2)

Prepared for: Illawarra Coal/South32

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# Executive summary

South32/Illawarra Metallurgical Coal (IMC) proposes to continue its underground mining at Appin mine by extracting coal from the Bulli Seam using longwall mining techniques. The Mine has approval under the Commonwealth Environmental Protection and Biodiversity Conservation Act 1999 (EPBC Approval 2010/5350) and Environmental Planning and Assessment Act 1979 (08_0150) An Environmental Protection Licence (2504) is in place for the Bulli Seam Operations (for West Cliff, North Cliff, Appin East and Appin West Mine Sites) which includes licensed points, monitoring and limits for air and water.

The initial monitoring program for IMC's activities were developed in accordance with the Pollution Reduction Program (PRP) 20 Aquatic Health Monitoring Program (AHMP) which was approved by the EPA on 25 September 2013. Given the community's high value for the Georges River catchment, a number of projects have been commissioned to expand upon the original AHMP, with the aim of verifying whether the ecological condition of the system is responding to a reduction in pollutants. This revised program is referred to as the Georges River Environmental Improvement Program (EIP2). Specifically, the EIP2 involves:

- Comparing the Brennans Creek/Georges River sites with reference sites (upstream of the Brennans Creek confluence);
- Examining changes over time in the benthic communities;
- Examining long-term patterns in water quality;
- Assessing the relationship between the downstream gradient and biotic composition; and
- Examining the toxicity of the discharge waters using a range of ecotoxicological assays.

This report examines the biotic and water quality data obtained for the EIP2 in two sections. Firstly, it provides an overview of the long-term trends (2013-2019) in macrobenthos communities, water quality and ecotoxicology data. Secondly, it focuses on the macrobenthic (Autumn and Spring 2018 and 2019) and metabarcoding survey (Spring 2019), as well as the water chemistry data obtained during these sampling events. In addition, the report aims to summarise the long-term trends for the system using a weight of evidence framework drawing from the collective results of the ecological, water quality and ecotoxicological data, and provide recommendations to assist in refining the program. To aid comparisons, in accordance with the EIP2 the macrobenthic and metabarcoding data were examined as two treatments: **Reference** – 3 sites prior to the mine's influence; and **Discharge Monitoring** – 6 sites which capture the gradient from the mine.

The long-term trends in water chemistry showed that conductivity and the concentrations of aluminium, nickel, zinc and ammonia generally declined over time. However, in most Discharge Monitoring sites, metal concentration still remained high, although appreciably lower in the downstream site GRQ18. In contrast, pH appears to have remained unchanged. While highly variable, ammonia concentrations also declined over time, although occasional spikes were observed.

The analysis of the long-term macrobenthic data showed that the Discharge Monitoring treatment had a higher mean abundance of macrobenthic invertebrates than the Reference treatment. Abundances varied greatly in all sites across the sampling period and as such, there were no clear temporal patterns. The long-term trends indicate that Family richness was similar across all treatments. However, as argued in this report, the ecological soundness of both of these endpoints is debatable.

SIGNAL, which is designed to focus the analysis on the sensitivity of macrobenthic taxa to varying ecological conditions, was also examined. There were marked differences in SIGNAL scores between the Reference and Discharge Monitoring treatments, indicating a lower level of ecological integrity in the Discharge Monitoring sites. SIGNAL scores suggested that the ecological integrity of the system improved with downstream distance. However, due to high variability no clear temporal trend was evident.

The ecotoxicological tests on the Point 10 discharge waters showed that historically the waters were toxic. The findings also indicate there has been a decline in toxicity with regards to the *Paratya australiensis* 10-day acute and *Ceriodaphnia dubia* survival assays, however, *Ceriodaphnia dubia* reproduction is still being affected.

Collectively, the long-term ecological, water quality and ecotoxicological data indicates that there is sufficient evidence that the discharge waters continue to pose a significant hazard to the benthic communities and other aquatic biota in the upper-most discharge sites.

The 2018 and 2019 surveys were performed during a protracted drought, with no flow occurring in the Reference sites. In general, pH and number of metals remained elevated in the Discharge Monitoring sites. Some very unusual trends were observed in the water chemistry, most notably, elevated concentrations of nickel in the waters from the Reference sites in both Autumn and Spring 2019. This is the first time this has been observed during this program. Furthermore, nickel concentrations were uncharacteristically low in the Discharge Monitoring sites during these sampling events. Other water quality variables had spikes during 2019, including aluminium and zinc in some sites. Given the overall elevated atmospheric temperature and drought conditions, it is not possible to determine whether the recent water chemistry patterns are a trend or an anomaly.

The recent weather conditions clearly also affected the macrobenthic communities, including one Reference site being completely dry and numerous samples containing no individuals. The Reference sites were particularly affected by the drought, and this was reflected in their lower SIGNAL scores. However, there was a general agreement in the patterns observed by the macrobenthic and metabarcoding surveys for prokaryote and eukaryote communities. All surveys found marked differences between Reference and Discharge Monitoring treatments, with water chemistry explaining a vast majority of the total variation in the ecological data. In particular, pH was shown to be a key correlate of macrobenthic, prokaryote and eukaryote communities. This suggests that the discharge is altering the catchment's aquatic biotic communities, with the effect of the discharge being more pronounced in the upstream Discharge Monitoring sites.

While it is noted that the discharge has been substantially diluted since December 2016, the waters from the upstream Discharge Monitoring sites still consistently exceeded the ANZG values for a range of metrics. Given the relatively brief period since the dilution and the protracted effect of the drought, it is currently not possible to determine whether dilution has had a significant positive effect on the communities. However, it is emphasised that recovery may be slow, and may result in communities which will still be markedly different from those associated with the Reference treatment.

# 1 Introduction

## 1.1 Program requirements

The monitoring program for Illawarra Metallurgical Coal (IMC)'s activities were developed in accordance with the Pollution Reduction Program (PRP) 20 Aquatic Health Monitoring Program (AHMP) which was approved by the EPA on 25 September 2013. Specifically, this report addresses EPL 2504 Condition U3.1 (2) - Conduct Aquatic Health Monitoring Program:

*If and when the EPA approves the monitoring program plan, the licensee must carry out the monitoring program in accordance with the plan. For each monitoring period, the licensee must provide a report detailing the results of the monitoring and assessment in that period to the EPA by 1 December 2013, 1 December 2015, December 2017, December 2019 respectively.*

Note, the reporting deadlines was altered to 31 March each year.
The AHMP included the following:

- Quantitative sampling of macroinvertebrates conducted in line with previous studies undertaken in PRP6, PRP9 and ACARP C15016 (2010);
- Ecological assessment of the sediments using a DNA-based approach, here on referred to as metabarcoding;
- In-stream water quality testing; and
- Laboratory ecotoxicological testing of the discharge water from Point 10.

Given the community's high value for the Georges River catchment, a number of projects have been commissioned to expand the initial monitoring program, with the aim of verifying whether the ecological condition of the system is responding to a reduction in pollutants. The revised program is called the Georges River Environmental Improvement Program (EIP2). Specifically, the EIP2 involves:

- Comparing the Brennan's Creek/Georges River sites with reference sites (upstream of the Brennan's Creek confluence).
- Examining changes over time in the benthic communities;

- Examining long-term trends in water quality;
- Assessing the relationship between the downstream gradient and biotic composition; and
- Examining the toxicity of the discharge waters using a range of ecotoxicological assays.

In March 2020, the EPA imposed Special Conditions to the License including a requirement to install a water treatment plant at Appin North (and amplification of the Appin West water treatment plant). The Conditions also include new water quality, flow limits and additional ecotoxicity monitoring at Point 10.

## 1.2 Objectives of this report

This report examines the abiotic and biotic data obtained for the EIP2 in two sections. Firstly, it provides an overview of the long-term trends (2013-2019) in water chemistry, macrobenthos communities and ecotoxicology data (sections 3.1 to 3.3). Secondly, it focuses on the water chemistry and macrobenthic surveys from Autumn and Spring 2018 and 2019, and the Spring 2019 metabarcoding survey (sections 3.4 to 3.7). In addition, the report aims to summarise the long-term information within a weight of evidence framework, drawing upon the collective results of the water chemistry, community ecology and ecotoxicological data, and provides recommendations to assist in potentially refining the program.

### 1.2.1 Long-term trends (2013-2019) were examined by:

1. Examining long-term patterns in key water quality parameters;
2. Summarising the overall trends in macrobenthic invertebrate abundance and Family richness;
3. Analysing and interpreting long-term patterns in SIGNAL scores. This approach is used to score macrobenthic samples from Australian rivers based on the known sensitivities of specific macrobenthic taxa. SIGNAL predicts that macrobenthic communities with high scores tend to be from sites with low levels of pollution (e.g. nutrients and conductivity) and high dissolved oxygen;

4. Analysing the abundance and occurrences of three Leptophlebiidae genera (*Atelophlebia*, *Ulmerophlebia* and *Koornonga*) (2016-2019); and

5. Interpreting the ecotoxicological tests data performed on waters obtained from the Discharge Monitoring site Point 10.

### 1.2.2 2018-2019 surveys were examined by:

- Summarising the water quality measurements obtained in Autumn and Spring for both 2018 and 2019;

- Exploring trends in macrobenthic invertebrate abundance and richness from samples obtained in Autumn and Spring;

- SIGNAL scores;

- Exploring compositional patterns in macrobenthic invertebrate communities sampled in Autumn and Spring;

- Exploring correlative relationships between water chemistry and macrobenthic communities;

- Exploring compositional patterns in the metabarcoding data for prokaryotes and eukaryotes communities; and

- Exploring correlative relationships between the water chemistry and metabarcoding data.

# 2 Methods

## 2.1 Site locations

The study area is located within the upper Georges River Catchment commencing at Site GR/Q1 and runs down to GRQ18 (Figure 1).

The experimental design consists of two treatments (Table 1)

- **Reference (3 sites)** – GR/Q1, GR/UFS and Point 11; and
- **Discharge Monitoring (6 sites)**, which capture the gradient from the mine - Point 10, Point 12, Jutts Crossing (here on referred to as Jutts); Pool 16, Pool 32 and GRQ18.

Historically, two additional sites have been sampled in Cascade Creek (CC1 and CC2), however, due to logistics, sampling at these sites was discontinued in 2015. The downstream discharge sites GRQ19 and GR/OH have also been discontinued based on the recommendations from the 2018 report (Chariton and Stephenson, 2018).

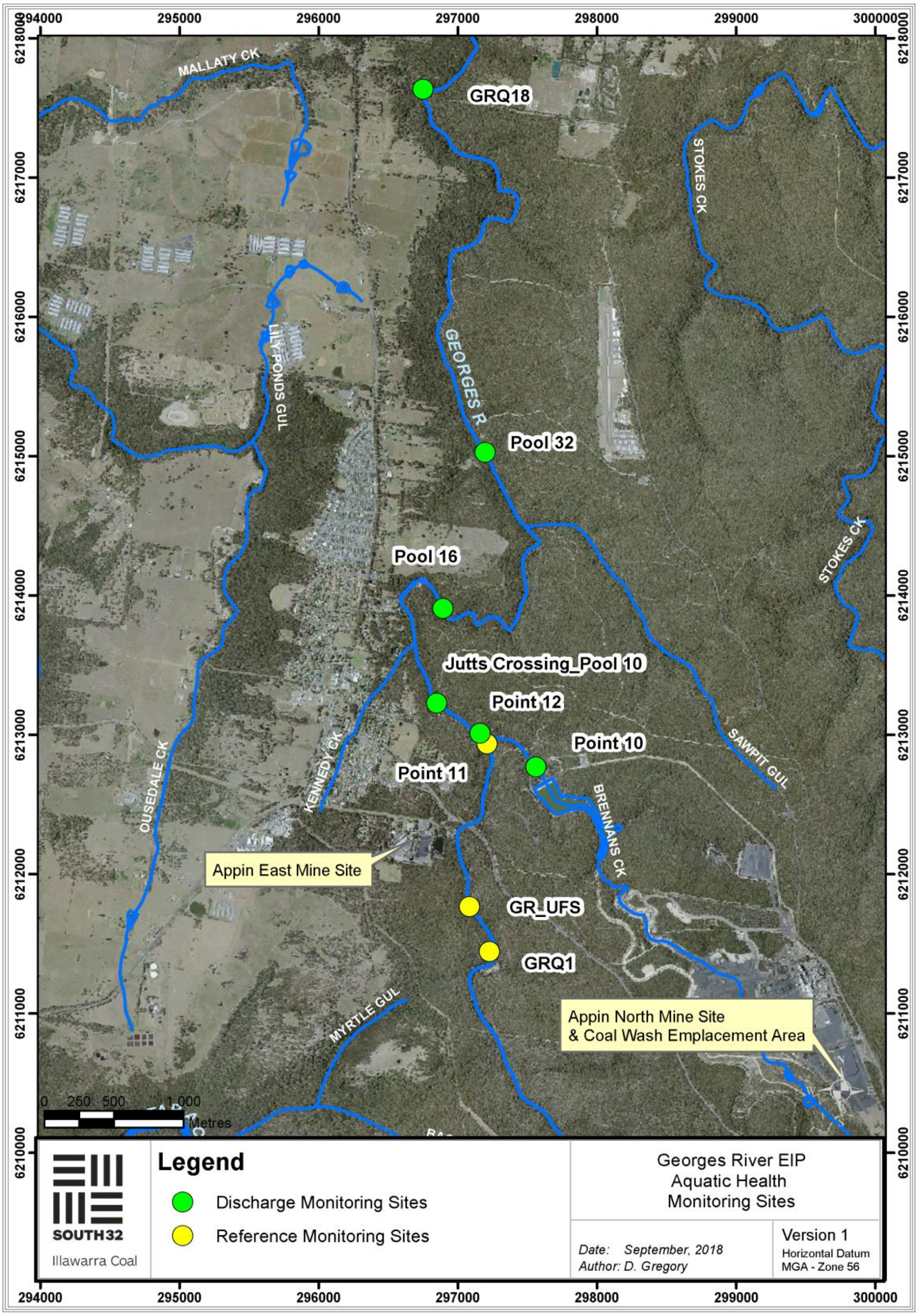


**Figure 1. Location of sampling sites. Reference sites = GR/Q1, GR/UFS and Point 11; Discharge Monitoring sites = Jutts Crossing_Pool10, Point 10, Point 12, Pool 16, Pool 32 and GRQ18**

**Table 1. Location of sampling sites and treatment allocation.**

| Site number | Stream | Location | Easting | Northing | Treatment |
|---|---|---|---|---|---|
| GR/Q1 | Georges R. | U/S of confluence | 297082 | 6211446 | Reference |
| GR/UFS | Georges R. | U/S of confluence | 297082 | 6211771 | Reference |
| Point 11 | Brennans Ck | U/S of Brennans and Georges confluence | 297207 | 6212940 | Reference |
| Point 10 | Brennans Ck | Discharge point (LDP10) | 297558 | 6212772 | Discharge monitoring |
| Point 12 | Georges R. | D/S of Brennans and Georges confluence | 297157 | 6213016 | Discharge monitoring |
| Jutts Crossing | Georges R. | D/S of Jutts Crossings | 296844 | 6213232 | Discharge monitoring |
| Pool 16 | Georges R. | D/S of Kennedy Ck | 296890 | 6213908 | Discharge monitoring |
| Pool 32 | Georges R. | D/S of Sawpit Gully | 297192 | 6215029 | Discharge monitoring |
| GRQ18 | Georges R. | U/S of O'Hares confluence | 296748 | 6217637 | Discharge monitoring |

## 2.2 Macrobenthos sampling

On all occasions (Spring 2013 - Spring 2019), macroinvertebrates were sampled from three random pool edges at each site and combined giving one sample at each site (Downs et al. 2002). The number of replicates was increased to five in 2018. Pool-edge samples were collected from depths of 0.2-0.5 m within 2 m of the bank. A suction sampler described by Brooks (1994) was placed over the substrate and operated for one minute at each sampling location. The sample was washed thoroughly over a 500-µm mesh sieve. All material retained on the 500-µm mesh sieve was preserved in 70% ethanol for laboratory sorting.

Macrobenthic sorting and identification was performed by Niche Environment and the client and provided to CSIRO in a tabulated format. The data was presented at the taxonomic level of Family. In addition, abundances of three potential indicator taxa from Leptophlebiidae (*Atelophlebia*, *Ulmerophlebia* and *Koornonga*) were analysed from the data obtained between 2016 and 2019.

Sampling for the macrobenthic surveys was performed in Autumn 2018 (30 April – 5 May), Spring 2018 (19-21 November), Autumn 2019 (7-9 May) and Spring 2019 (23-24 October). Water chemistry samples were collected at the same time as the macrobenthic samples.

## 2.3 Collection and analysis of DNA samples for metabarcoding

### 2.3.1 DNA sample collection and processing

The collection of samples for the DNA-based eukaryote survey (metabarcoding) was performed concurrent to the Spring 2019 macrobenthic survey. At each site, five sediment samples were collected from the soft-sediment located approximately 1 m from the edge of the water bodies where the water column was approximately 30 to 40 cm deep. Areas of high aquatic vegetation biomass or susceptible to poor sunlight were excluded from sampling. Surficial sediment samples (top 2 cm) were obtained using a clean shallow polycarbonate corer (diameter 10 cm). All samples were transferred into DNA-free sterile 50 mL Greiner tubes and placed on ice immediately, then frozen at -80°C within 8 h of collection. Samples were thawed only just prior to DNA extraction. All materials used for the collection and storage of DNA samples were soaked for at least 24 h in 5% sodium hypochlorite, and rinsed thoroughly five times with Milli-Q water (Millipore, Academic Water Systems, Australia).

Using 10 g of homogenised sediment, DNA was extracted and purified from each using Qiagen DNeasy PowerMax® Soil isolation kits (QIAGEN® Germany) following the manufacturer's protocols. In addition to the sediment samples, three reference samples containing crocodile (*Crocodylus porosus),* a tropical marine Cnidarian (*Carukia barnesi*) and a synthetic bacteria sequence were also processed in three sample replicates as positive controls. Negative water controls were included in all polymerase chain reaction (PCR) experiments to test for biological contamination during amplification.

Conserved regions of two genes were targeted to capture the system's biodiversity: 18S rRNA gene for eukaryotes (Hardy et al., 2010) and the V4 region of the 16SrRNA gene for prokayotes (Caporaso et al 2012).Three identical PCR reactions were undertaken for each gene and the amplicons for the three PCRs were pooled into one library per target gene. Amplification and purification success were interrogated on a MultiNA gel. The three pooled final amplicon library concentrations were measured on the Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). The target gene libraries were prepared with the Illumina Tru-Seq PCR-free library preparation kit and libraries were sequenced over one MiSeq run at 2x 250bp. The Illumina MiSeq sequencing was performed by the Ramaciotti Centre for Genomics, UNSW.

### 2.3.2 Bioinformatics

Sequenced data were processed using a custom pipeline (Greenfield Hybrid Amplicon Pipeline (GHAP) which is based around USEARCH tools (Edgar, 2013). The pipeline is available at https://data.csiro.au/dap/landingpage?pid=csiro:26534. GHAP first demultiplexes the sequence reads to produce a pair of files for each sample. These paired reads were then merged, trimmed, de-replicated, and clustered at 97% similarity to generate a set of representative OTU (Operational Taxonomic Units) sequences which were classified after clustering at 97% similarity in sequences. USearch v8.1.1812 tools (fastq_mergepairs, derep_fulllength and cluster_otus) (Edgar, 2013) were used for the merging, de-replicating and clustering steps. Each OTU sequence was classified in two different ways: first, by using the RDP Classifier (v2.10.2) to determine a taxonomic classification for each sequence, down at best to the level of genus; and second, by using ublast to match a representative sequence from each OTU against a curated set of 18S reference sequences derived from the SILVA v123 SSU reference set (Cole et al. 2014; Quast et al. 2013). This 18S reference set was built by taking all the eukaryote sequences from the SILVA v123 SSU dataset, and removing those sequences found to contain bacterial or chloroplast regions. The pipeline then used usearch_global to map the merged reads from each sample back onto the OTU sequences to obtain accurate read counts for each OTU/sample pairing. The classified OTUs and the counts for each sample were finally used to generate OTU tables in both text and BIOM (v1) file formats, complete with taxonomic classifications, species assignments and counts for each sample. All OTUs with a maximum read abundance of 50 reads, or that were only observed in less than four biological replicates were removed.

**Filtering**

After processing, and prior to statistical analyses, the data sets were filtered to remove potentially erroneous sequences. For all data sets, the proportion of contamination OTU reads in the positive controls (the max read count that is not the positive control divided by the positive control read count) was determined. The proportion of contamination was low in all data sets (between 0.01 – 0.2%) and this value was set as the cut-off for filtering the dataset. The proportion of read counts for each OTU in each sample (the read count for each OTU divided by the total read count for that sample) was determined. If the proportion of read counts for each OTU per sample was less than the proportion of contamination (0.01-0.2%) then those reads were removed from the dataset. After quality control checks were complete, controls were removed from the dataset. Any OTUs that had a match percent of <80 or appeared in less than two samples were all removed.

## 2.4 Ecotoxicological testing

All tests were performed by Ecotox Services, Australasia. Between 2013 and 2017 a range of ecotoxicological assays were performed using discharge waters derived from the Discharge Monitoring site Point 10 (Table 2). However, based on the recommendations of Chariton and Stephenson (2018), ecotoxicological testing was reduced to three assays: *Paratya australiensis* acute; *Ceriodaphnia dubia* survival and *Ceriodaphnia dubia* reproductive impairment. Only the results of these three assays are presented and discussed. As ecotoxicological tests were only performed at the same time as the metabarcoding surveys, the only new results are from Spring 2019.

To enable direct comparisons between the assays, percentage values for the EC/IC10 tests were corrected for dilution values provided by Ecotox Services Australasia, with the final presented data converted to toxic units (TU). This approach of normalizing tests to toxic units (100/EC) is recommended by the ANZECC Water Quality Guidelines Toxicants and Sediments Working Group (Batley et al. 2014; Warne et al., 2015).

**Table 2. Ecotoxicological tests performed on Point 10 waters between 2013-2017.**

| Test organism | Test |
|---|---|
| *Melanotaenia duboulayi* (fish) | 96 hour fish imbalance test |
| *Paratya australiensis* (shrimp) | 10 day acute survival test using the freshwater shrimp |
| *Lemna disperma* (duckweed) | 7-day growth inhibition of the freshwater aquatic duckweed |
| *Ceriodaphnia dubia* (crustacean) | Partial life-cycle 7 day toxicity test using the freshwater cladoceran *Ceriodaphnia dubia* (survival) |
| *Ceriodaphnia dubia* (crustacean) | Partial life-cycle toxicity test using the freshwater cladoceran *Ceriodaphnia dubia* (reproduction) |
| *Ceriodaphnia dubia* (crustacean) | 48hr acute toxicity test using the freshwater cladoceran (*Ceriodaphnia dubia*) |
| *Selenastrum capricornutum* (micro-algae) | 72-hour microalgal growth inhibition test |

## 2.5 Water chemistry

Measurements for water quality were obtained by South32 at the same time as the macrobenthic samples. *In situ* measurements for temperature, conductivity, pH, dissolved oxygen and turbidity were obtained using a Horiba U51 water quality device. Additional laboratory analysis using standard methods for alkalinity, dissolved sulfate, chloride, major cations, dissolved metals, dissolved organic carbon and nutrients were performed by ALS Environmental (Sydney). For all analyses examining the relationships between the benthic biota and water chemistry, measurements from the laboratory analysis were used in preference of the *in situ* measurements, with the exceptions being dissolved oxygen, temperature, turbidity and pH. Given the large number of water quality variables routinely measured, analysis of long-term patterns in water quality (2013-2019) were restricted to a selection of key variables which have historically been shown to be elevated in the discharge waters. These were: conductivity; pH, aluminium, nickel, zinc and ammonia.

## 2.6 Statistical analysis

### 2.6.1 Long-term patterns in macrobenthos

Univariate attributes of the macrobenthos data were obtained using Primer 7's 'Diverse' function. As part of the EIP's requirement to enable a balanced comparison between the Reference and Discharge Monitoring treatments (South32, 2017 see Table 5), differences in total abundance and richness between the three Reference sites and three of the six Discharge Monitoring sites (Point 12, Pool 32 and GRQ18) were examined using a one-way ANOVA. Because of the change in replicates, three prior to 2018 and five subsequent, all univariate metrics are based on site means. Residuals were assessed for skewness, kurtosis, and normality, with homogeneity of variances examined using a modified Levene equal variance test. All univariate analyses were performed using NCSS v12 (Utah, USA).

### 2.6.2 SIGNAL

SIGNAL stands for Stream Invertebrate Grade Number – Average Level, and is simple approach used to score macrobenthic samples from Australian rivers based on the known sensitivities of specific macrobenthic taxa (Chessman, 1995). SIGNAL predicts that macrobenthic communities with high scores tend to be from sites with low levels of pollution (e.g. nutrients and conductivity) and high dissolved oxygen. In this report, scores were calculated using the SIGNAL 2.0 procedure described by Chessman (2003). As the total abundances of the sample varied greatly over time and within sites, here we used unweighted SIGNAL scores, i.e. derived from presence/absence data. SIGNAL scores are then used to putatively classify sites, with a SIGNAL value >6 suggesting clean water; 5-6, doubtful quality, possible mild pollution; 4-5 probable moderate pollution; and less than 4, probable severe pollution.

Comparisons in mean SIGNAL scores between the three Reference sites and three of the six Discharge Monitoring sites (Point 12, Pool 32 and GRQ18) were examined using a one-way ANOVA. Residuals were assessed for skewness, kurtosis, and normality, with homogeneity of variances examined using a modified Levene equal variance test.

Based on the recommendations of Chariton and Stephenson (2018), EPT % has been removed as a metric for the monitoring program. This is because the EPT index was designed for fast moving rivers, and furthermore, plecopterans have never been sampled in this particular system.

### 2.6.3 Macrobenthos data (2018 and 2019)

Because of the low rainfall throughout 2018 and 2019 and the subsequent drying out of some sites, no benthic samples could be obtained from GRUF during Autumn 2018. Furthermore, in Autumn 2019 numerous replicates contained no individuals, specifically: one replicate from GR/Q1, four out of five for GR/UFS, two for Point 10, three for Point 12, one for Pool 16 and one for Pool 32. Consequently, no formal statistics were performed on the univariate attributes (abundance, Family richness and SIGNAL) for the macrobenthic invertebrate samples collected in 2018 and 2019. All univariate comparisons were restricted to graphical interpretations.

Prior to multivariate analysis, the macrobenthos data was log10 transformed. Ordinations of the data were performed by non-metric multidimensional scaling (nMDS) using the Bray-Curtis similarity coefficient. Statistical differences between treatments were tested by permutational multivariate analysis of variance (PERMANOVA), with differences between treatments identified by pairwise a posteriori tests based on 9999 random permutations. The key taxa contributing to significant differences between treatments were identified using Primer's SIMPER function.

The relationships between macrobenthic communities and environmental variables were examined using distance-based linear models (DISTLM) (Legendre and Anderson, 1999). In order to match the number of biological and environmental (physico-chemical) samples, i.e. one sample per site, the similarity matrix for the biological data was recalculated using the distance between centroids for each site derived from the replicate samples. The environmental variables obtained from the monitoring program were both numerous and often strongly correlated, and consequently all highly correlated variables ($r>0.95$) were removed. To reduce over-fitting and to conform to the assumptions of the analysis (number of biological samples > environmental variables), DISTLM was performed using only a limited number of environmental variables, with the variables selected a priori using Primer's BIOENV function. The final variables used in the DISTLM were pH, conductivity, dissolved nickel, dissolved zinc, total nitrogen and total phosphorus. It is emphasised that these variables provide a summary of the discharge water, and it is not possible to robustly quantify the contribution of each measured variable in isolation. All metals and nutrients values were log transformed prior to analysis, with the environmental data normalized prior to computation. The dbRDA option was selected to provide an ordination of the fitted values from the model.

### 2.6.4 Metabarcoding (Spring 2019)

As there is a weak statistical relationship between the number of 18S rDNA sequence reads and organism biomass or abundance (Egge et al., 2013), the 18S rDNA data was converted to presence/absence prior to computation (Chariton et al., 2010). The prokaryote data (16S rDNA) was Hellinger transformed prior to analysis (Sutcliffe et al. 2019). OTUs were assigned to species for the 16S rDNA dataset and Family for the 18S rDNA dataset. Biological replicates were obtained from the sums of the PCR (technical replicates). For the 18S rDNA data, ordination of the OTU data was performed by non-metric multidimensional scaling (nMDS) using the Jaccard similarity coefficient, as was the PERMANOVA analysis. Bray-Curtis distance was used for the 16S rDNA data. The relationships between metabarcoded communities and environmental variables were examined using distance-based linear models (DISTLM) as previously described in section 2.6.3. Putative indicator taxa for the Reference and Discharge Monitoring and Downstream Discharge Monitoring treatments were identified using the R package *Indispecies*.

# 3 Results

## 3.1 Long term water chemistry trends (2013-2019)

In this section we describe the long-term (2013-2019) trends in the key water quality variables: pH, conductivity, aluminium, nickel, zinc and ammonia. Data was unavailable for the Reference site GR/UFS during Spring 2018 as the site was dry.

The Discharge Monitoring sites had a higher pH than the Reference sites (Figure 2. Long-term trends in pH. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value for lowland rivers. ). The pH of these waters frequently exceeded the ANZG (2018) range of pH 6.5 - 8, however, the most downstream Discharge Monitoring site (GRQ18) generally had lower pH values than the other sites in this treatment. Furthermore, the pH of this site was with the guideline range during the most recent sampling event (Spring 2019). In general, there is no clear overall decline in pH over time within the Discharge Monitoring treatment.

In recent years, the conductivity of the Discharge Monitoring sites has been below the ANZG (2018) value for lowland east coast rivers (2,500 uS/cm). However, it was markedly higher in the Discharge Monitoring sites when compared to the Reference sites (Figure 3. Long-term trends in conductivity. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value for lowland rivers.). There was an overall decline in conductivity with distance downstream. In addition, conductivity declined over time in all Discharge Monitoring sites.

Aluminium concentrations were consistently elevated in all Discharge Monitoring sites, with the exception of GRQ18 (Figure 4 Long-term trends in aluminium concentrations. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value.). While measurements varied over time, there was generally a marked decline in aluminium concentrations in the upstream Discharge Monitoring sites. Furthermore, concentrations generally declined with downstream distance.

Nickel concentrations have been historically very high in all Discharge Monitoring sites (Figure 5. Long-term trends in nickel concentrations. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value.). Concentrations were generally similar in the upper Discharge Monitoring sites (Point 10, Point 12 and Jutts), with Pool 16, Pool 32 and GRQ18 also having similar concentrations. While nickel concentrations have generally declined over time, they remained several times above the guideline value in all Discharge Monitoring sites, with the exception of the Autumn and Spring 2019 sampling events. The most salient finding was the very high concentrations of nickel in both Autumn and Spring 2019 in the Reference sites GR/Q1 and GR/UFS. Concentrations were elevated in the Reference site Point 11, although to a lesser extent. Prior to 2019, nickel concentrations in all three Reference sites have been well below the guideline value.

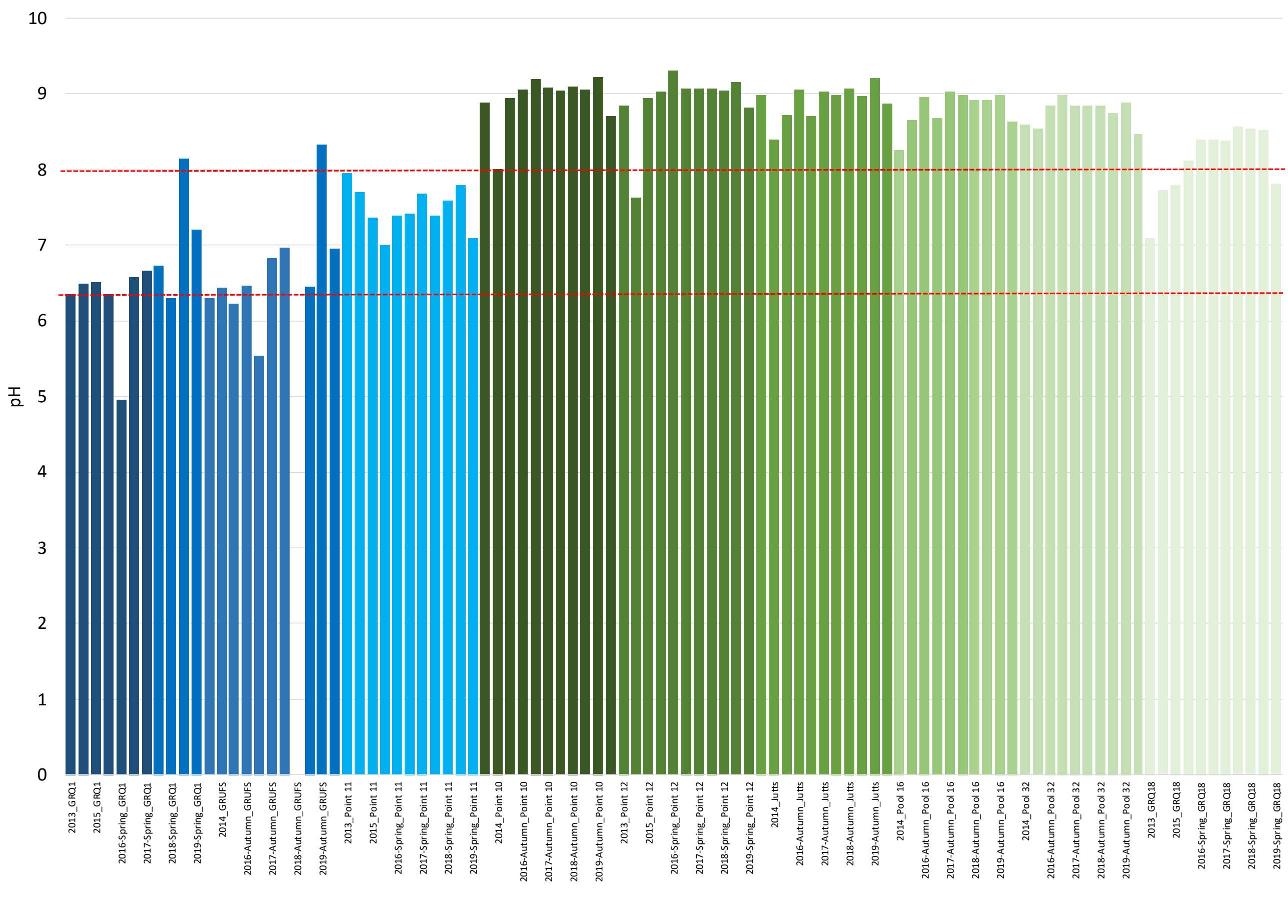


**Figure 2. Long-term trends in pH. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value for lowland rivers.**

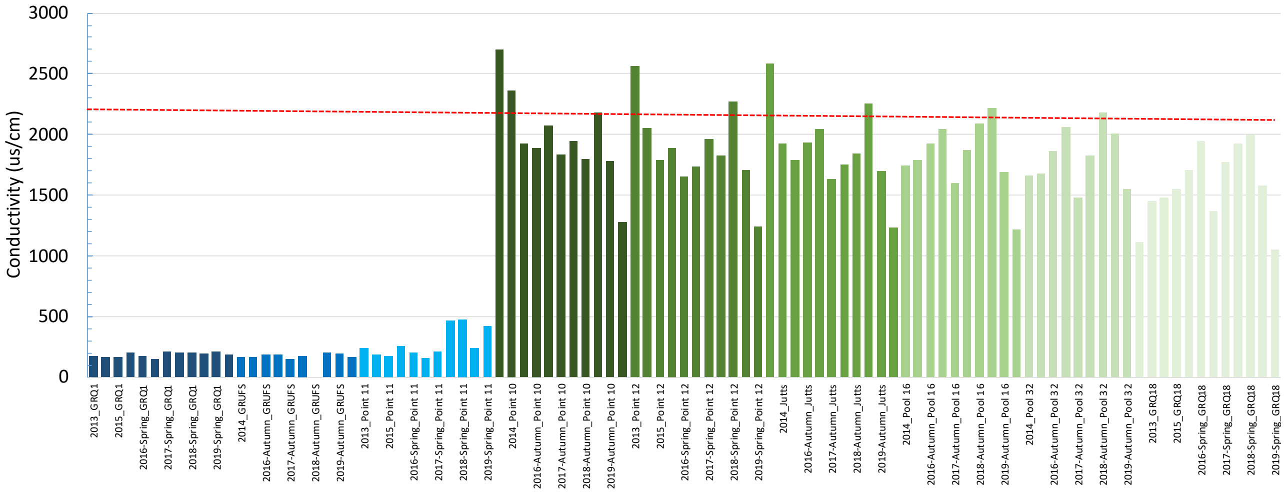


**Figure 3. Long-term trends in conductivity. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value for lowland rivers.**

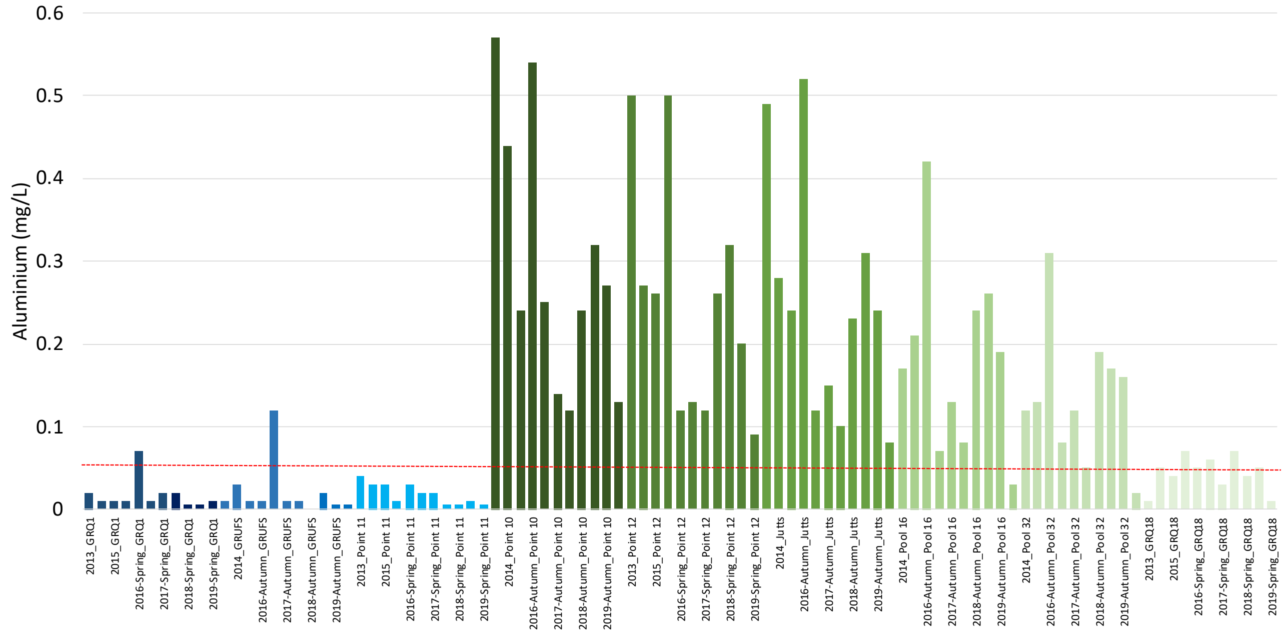


**Figure 4 Long-term trends in aluminium concentrations. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value.**

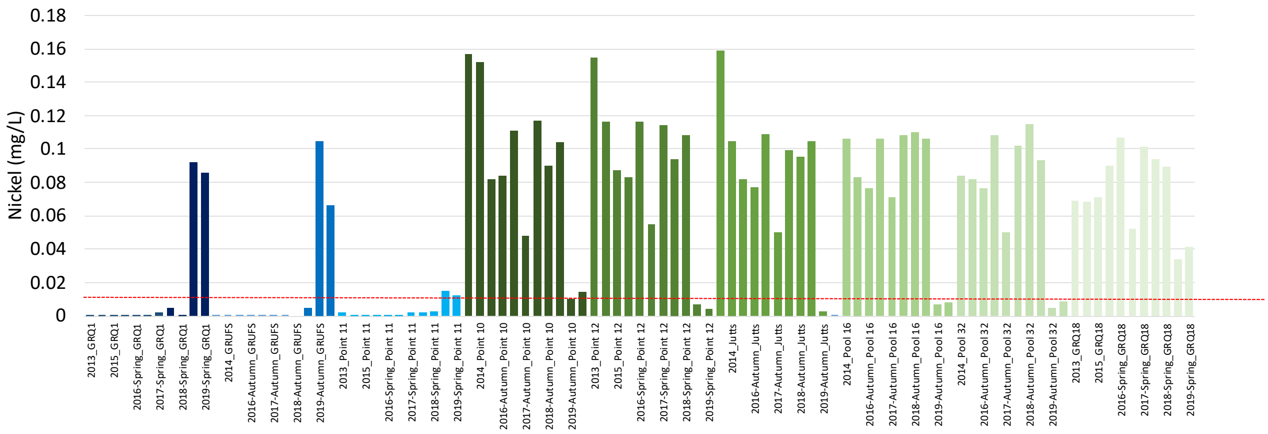


**Figure 5. Long-term trends in nickel concentrations. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value.**

In general, zinc concentrations have declined over time (Figure 6. Long-term trends in zinc concentrations. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value.). However, in Autumn 2019, concentrations were appreciably higher in all Discharge Monitoring sites than previously recorded. Although lower in Spring 2019, zinc concentrations in the waters of all Discharge Monitoring sites remained high and several times above the guideline value.

Ammonia concentrations were generally highest in the upstream Discharge Monitoring site Point 10, declining with downstream distance (Figure 7. Long-term trends in ammonia concentrations. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value for lowland rivers.). There was an overall decline in ammonia concentrations, however, some spikes did occur in all treatments. In recent years (2016-2017), ammonia concentrations were generally below the trigger value. However, there were large spikes in ammonia concentrations in Autumn 2019 in the Discharge Monitoring sites Point 10, Point 12 and Jutts. While lower than Autumn 2019, ammonia concentrations were again elevated in Point 10 in Spring 2019.

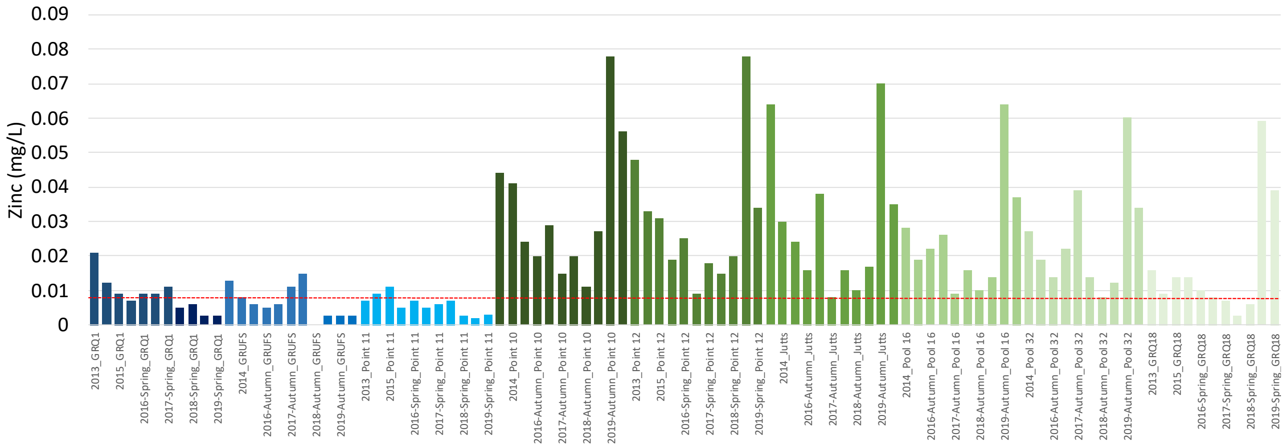


**Figure 6. Long-term trends in zinc concentrations. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value.**

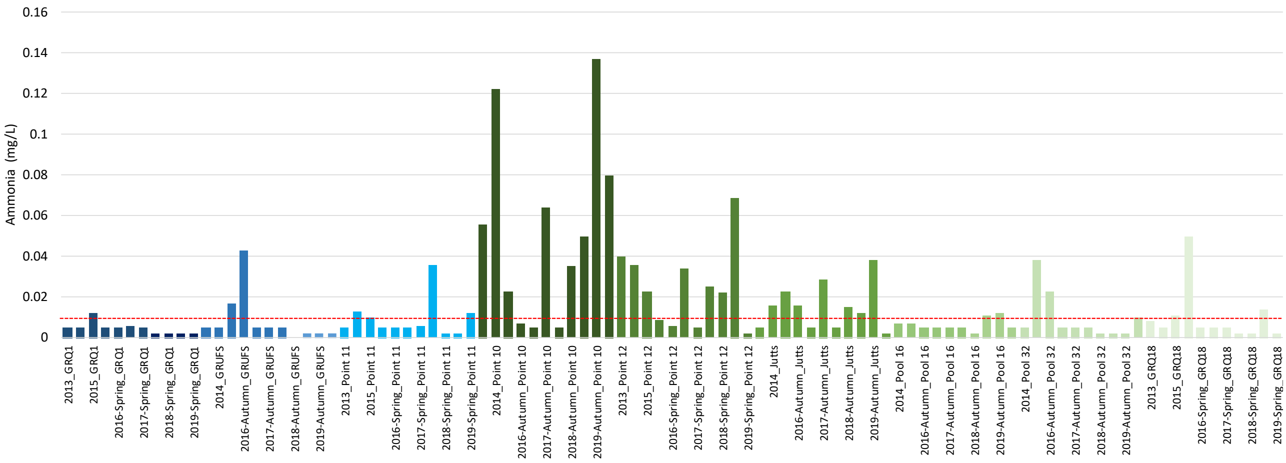


**Figure 7. Long-term trends in ammonia concentrations. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value for lowland rivers.**

## 3.2 Long term patterns in macrobenthic community attributes

### 3.2.1 Abundance and richness (2013-2019)

The abundance of macroinvertebrates varied greatly between sites and across sampling events (Figure 8. Long-term abundance patterns in macrobenthos (2013-2019). Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the mean value for each site. ). The long-term patterns show that the three Discharge Monitoring treatment, based on Point 12, Pool 32 and GRQ18 (91 ± 14 S.E.), had a higher mean abundance than the Reference (57 ± 7 S.E.) treatment (F=4.63, *P*<0.035). It is emphasised that this finding should be taken cautiously given the sample size, even with the reduced number of Discharge Monitoring sites used to balance the analysis.

The mean richness for all sites sampled between 2013 and 2019 is illustrated in Figure 9. Mean Family richness was similar in all treatments, with no significant difference (F= 3.23, $P = 0.0771$) detected between the Reference (11.0 ± 0.91 S.E.) and Discharge Monitoring (13.0 ± 0.61 S.E.) treatments.

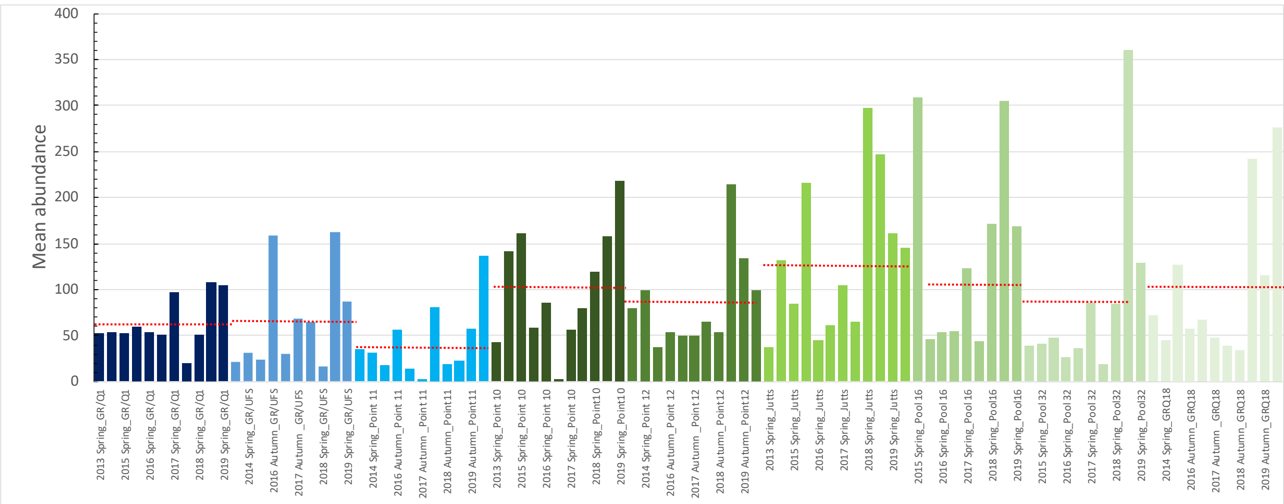


**Figure 8. Long-term abundance patterns in macrobenthos (2013-2019). Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the mean value for each site.**

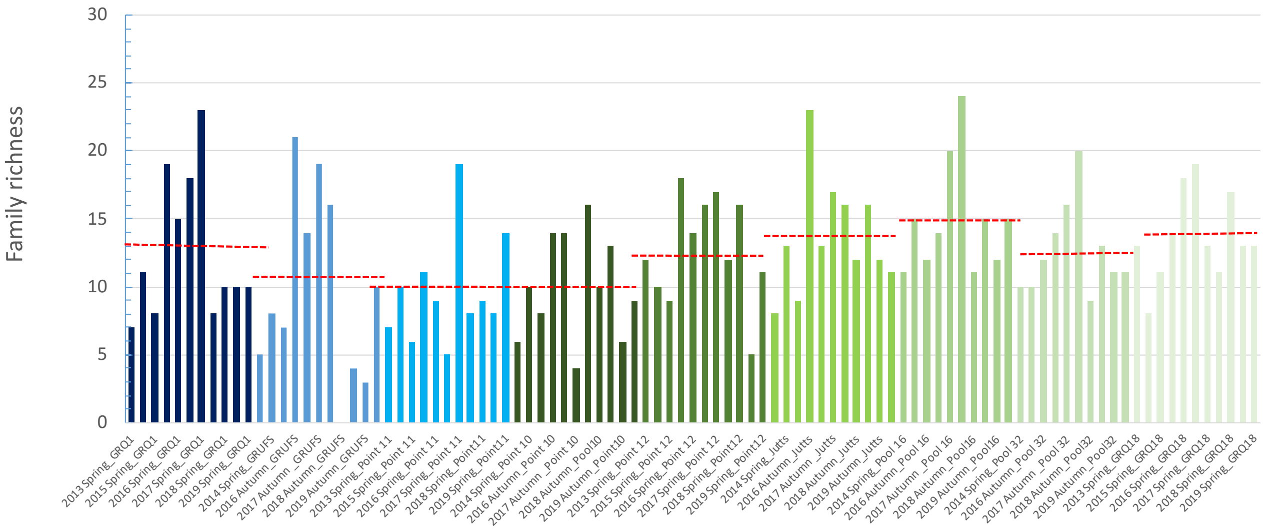


**Figure 9. Long-term Family richness patterns in macrobenthos (2013-2019). Sites were place into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the mean value for each site.**

### 3.2.2 SIGNAL (2013-2019)

Long-term SIGNAL scores for all sites sampled between 2013 and 2019 are illustrated in Figure 10. Long-term SIGNAL scores for sites (2013-2019). Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the mean value for each site. . Based on the arbitrary classifications by Chessman (1995) this suggests, that on average, the Reference sites can be considered to be of 'probable moderate pollution' and the Discharge Monitoring sites of 'probable severe pollution' (Table 3). The exception being the most distant Discharge Monitoring site (GRQ18) which was classified as 'probable moderate pollution'. The reduced analysis comparing three site each from the Reference and Discharge Monitoring treatments found that the long-term mean SIGNAL scores for the Reference treatment (4.65 ± 0.19 S.E.) was significantly greater than the Discharge Monitoring treatment (3.93 ± 0.11 S.E.) (ANOVA: F=10.3, *P*<0.002).

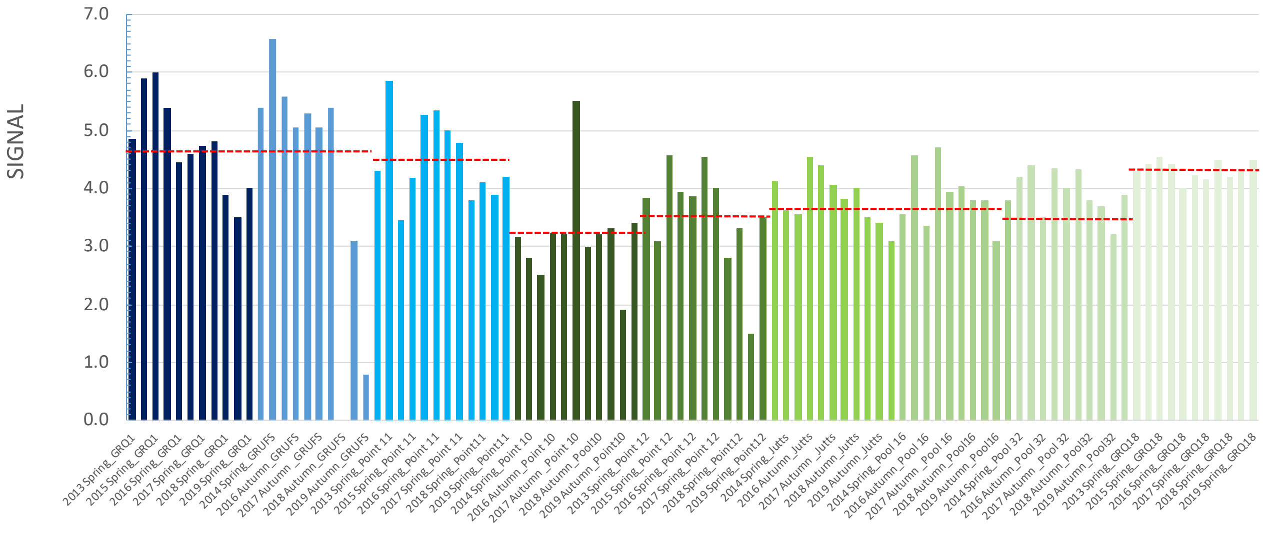


**Figure 10. Long-term SIGNAL scores for sites (2013-2019). Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the mean value for each site.**

**Table 3. Mean SIGNAL scores for each site (2013-2017). *Potential rankings based on Chessman (1995).**

| Treatment | Site | Potential ranking* | Mean SIGNAL | Minimum | Maximum |
|---|---|---|---|---|---|
| Reference | GR/Q1 | Probable moderate pollution | 4.7 | 3.5 | 6.0 |
| Reference | GR/UFS | Probable moderate pollution | 4.7 | 5.1 | 6.6 |
| Reference | Point11 | Probable moderate pollution | 4.5 | 3.4 | 5.9 |
| Discharge Monitoring | Point10 | Probable severe pollution | 3.2 | 1.9 | 5.5 |
| Discharge Monitoring | Point12 | Probable severe pollution | 3.5 | 1.5 | 4.6 |
| Discharge Monitoring | Jutts | Probable severe pollution | 3.6 | 3.1 | 4.5 |
| Discharge Monitoring | Pool 16 | Probable severe pollution | 3.6 | 3.1 | 4.7 |
| Discharge Monitoring | Pool 32 | Probable severe pollution | 3.5 | 3.2 | 4.4 |
| Discharge Monitoring | GRQ18 | Probable moderate pollution | 4.3 | 4.0 | 4.5 |

### 3.2.3 Leptophlebiidae genera of interest (2016-2019)

As indicated in Figure 11, both the abundance and the occurrence of all three genera were higher in the Reference treatment than the Discharge Monitoring treatment. In contrast to 2016-2017 where all three taxa were absent from the Discharge Downstream sites Point 10, Point 12 and Pool 16, a few individuals were sampled in Point 10 (Spring 2019), Point 12 (Autumn and Spring 2019), Jutts (Spring 2019) and Pool32 (Autumn 2019). However, these were always at very low abundances (1-3 individuals per site). Notably, in the most downstream site (GRQ18), *Atelophlebia* was moderately more abundant in 2018/2019 than previous years, however, *Ulmerophlebia* was not sampled during this period despite being present on a previous occasion in Spring 2016. *Kooronga* was only observed in the Reference treatment, with the exception of two individuals being sampled in Point 10 and one in Point 12.

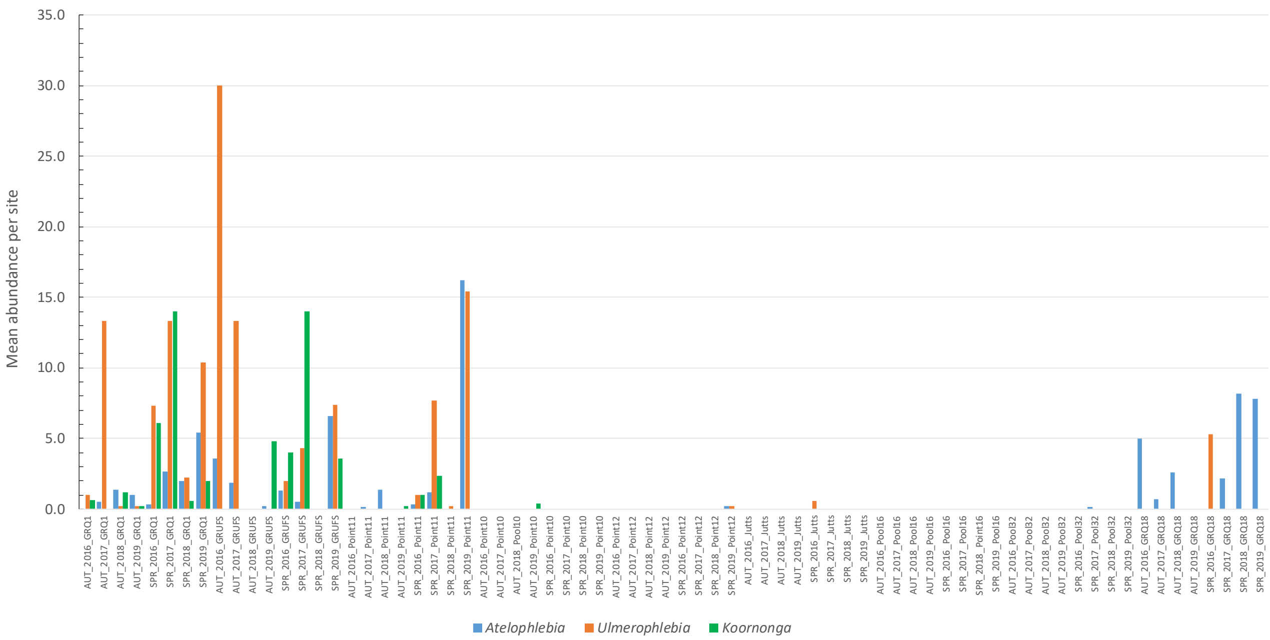


**Figure 11. Abundances of *Atelophlebia* spp, *Ulmerophlebia* spp and *Koornonga* spp (2016-2019).**

## 3.3 Ecotoxicology

**The results from the toxicity tests from Point 10 between 2013 and 2019 are illustrated in**

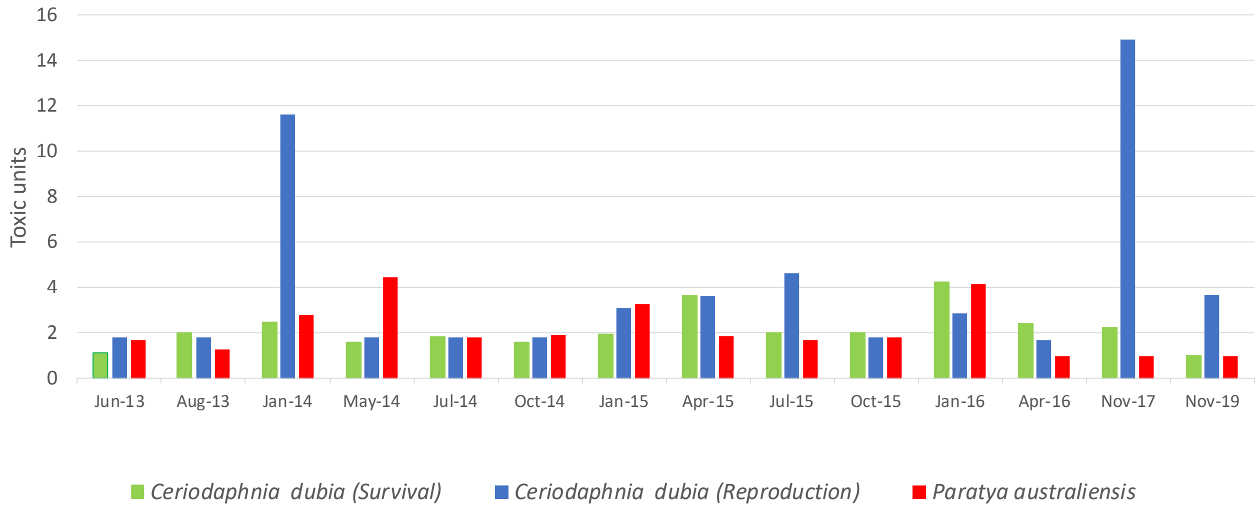


Figure 12. Toxicity of Point 10 waters collected 2013 – 2019. Toxicity is shown as toxic units, higher values are indicative of greater toxicity. . For the *Ceriodaphnia dubia* survival test, toxicity was just observed (toxic units = 1.0) in the 2019 water sample and was below the long-term mean (2013-2017) (mean = 2.23 toxic units ± 0.23 S.E.). Toxicity was also just observed in the *Paratya australiensis* (toxic units = 1.0) and was consistent with the previous two toxicity tests (April 2016 and November 2017) and below the long-term average (mean = 2.11 toxic units ± 0.30 S.E.). The *Ceriodaphnia dubia* reproduction had a marginally lower toxicity value (toxic units = 3.66) than the long-term mean (mean = 4.10 toxic units ± 1.17 S.E.), however, it is not possible to say whether this observation was part of a long-term decline in its toxicity. The Point 10 water is still considered to be toxic for this endpoint.

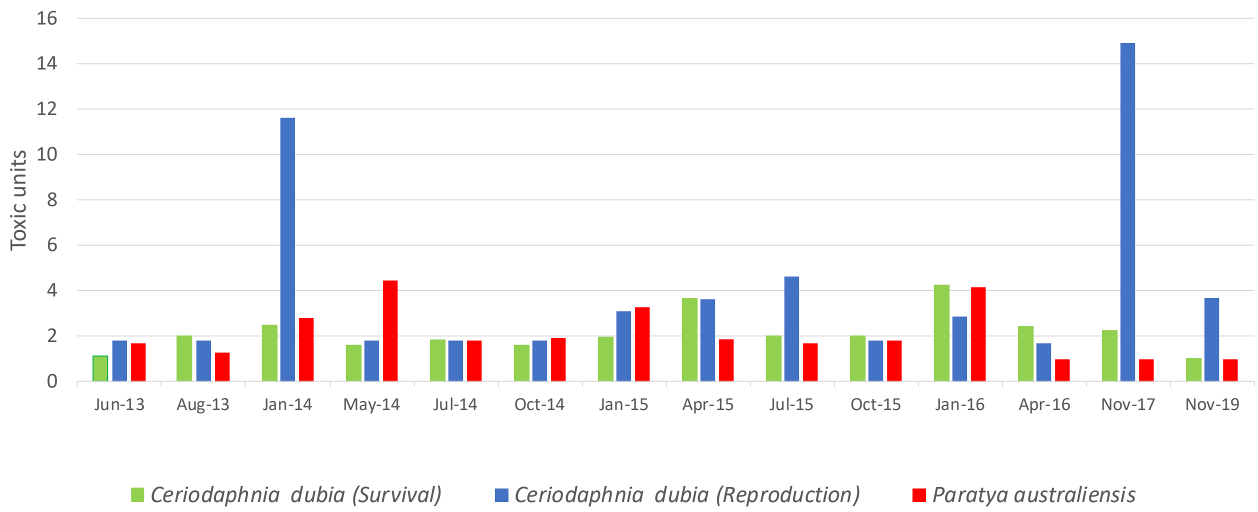


**Figure 12. Toxicity of Point 10 waters collected 2013 – 2019. Toxicity is shown as toxic units, higher values are indicative of greater toxicity.**

## 3.4 Water chemistry (2018-2019)

The water chemistry for 2018 (Autumn and Spring) and 2019 (Autumn and Spring) are summarized in Table 4 and Table 5. No data was available for the reference site GR/UFS in Autumn 2018 as the pool was dry. In both 2018 and 2019, for a number of the water quality variables there were marked differences in mean concentrations between the Reference and Discharge Monitoring sites. In general, concentrations of elevated water quality measurements were lower in the downstream sites of the Discharge Monitoring treatment.

On all sampling occasions, the Reference sites GR/UFS and GR/Q1 had pH values ranging between 6.27 and 6.90. The Point 11 reference site had a higher pH (7.39 – 7.64) than the other Reference sites. The guideline value range for pH (6.5 - 8.0) for lowland rivers (ANZG, 2018) was consistently exceeded in all Discharge Monitoring (range 8.40 – 9.08) sites. In both Autumn and Spring 2018, the pH of the waters was highest in Point 10, 9.1 and 9.05, respectively. In Autumn 2019, the most upstream Discharge monitoring sites (Point 10, Point 12 and Jutts) all had a pH between 9.05 and 9.08. The pH of these sites was lower during Spring (8.81 – 8.84).

Although conductivity only exceeded the upper guideline value in two Discharge Monitoring sites (Point 12 and Jutts) during Spring 2018, it is should be noted that on all occasions, conductivity was generally 5 to 10 times higher in the Discharge Monitoring sites when compared to the Reference sites.

In Autumn 2018, aluminium concentrations exceeded the guideline value in all Discharge Monitoring sites and were below the guideline in the Reference sites. In contrast to the previous variables, the highest concentration of aluminium was not at Point 10 but at Point 12. Although concentrations were lowest at GRQ18, they remained high until Pool 16. This trend was also observed in Spring 2019, however in this case, the aluminium concentration at Point 10 (32 µg/L) were below the guideline value (55 µg/L), with the most distant site (GRQ18) also being below the guideline (40 µg/L). In Autumn 2019, all Discharge Monitoring sites, with the exception of GRQ18, exceeded the guideline value for aluminium. In this case Point 10 did have the highest concentration (270 µg/L). While concentrations were lower in Spring 2019 (130 µg/L), Point 10 (90 µg/L), Point 12 and Jutts (80 µg/L) all exceeded the guideline value.

In both Autumn and Spring 2018, nickel concentrations in all Discharge Monitoring sites exceeded the guideline value (11 µg/L) by almost a magnitude of order (89 -115 µg/L). The results from Autumn 2019 were very unusual, with all three reference sites exceeding the guideline value (GR/UFS = 105 µg/L; GR/Q1 = 92 µg/L; Point 11= 15 µg/L), a trend which was still present in Spring 2019. Furthermore, in Autumn 2019, only the most downstream Discharge Monitoring site, GR18 (nickel = 34 µg/L) had a concentration above the guideline value. In Spring 2019, nickel remained high at this site (41 µg/L), with the nickel being slightly above the guideline value in the most upstream Discharge Monitoring site Point 10 (14 µg/L).

In Autumn 2018, with the exception of GRQ18, all Discharge Monitoring sites marginally exceeded the zinc guideline value of 8 µg/L, with concentrations ranging between 10-15 µg/L. A similar pattern occurred in Spring 2018, although concentrations were generally marginally higher (12 – 27 µg/L). In both Spring and Autumn 2019, all Discharge Monitoring sites exceeded the guideline value for zinc, with concentrations greater than 2018 (Autumn 2019 range 59 - 78 µg/L; Spring 2019 range 34 - 56 µg/L). Reference sites were consistently below the guideline value in both Autumn and Spring in 2018 and 2019.

While there is no formal water quality value for iron as it is currently in draft, in Autumn 2018, the draft concentration of 700 ug/L (pers. comm, Graeme Bately, CSIRO) was exceeded in the Reference site GR/UFS (1030 ug/L). To date, such an exceedance has yet to observed in any of the sites. No further exceedances were observed in 2018 and 2019.

In Autumn 2018, total nitrogen concentrations marginally exceeded the guideline value of 500 µg N/L in the Discharge Monitoring sites in Point 10, Jutts and Pool 16. Both Point 10 and Jutts again marginally exceeded this value in Spring 2018. In Autumn and Spring 2019, total nitrogen concentrations were below the guideline value in all Reference and Discharge Monitoring sites, with concentrations being markedly lower in Autumn 2019. Only on one occasion, Point 10 in Autumn 2019 (131µg P/L), did total phosphorus concentrations exceed the guideline value of 50 µg P/L).

## Table 4. Summary of water quality measurements for 2018 *.

| | | | Autumn 2018 | | | | | | | | | Spring 2018 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Guideline value | Reference | | | Discharge Monitoring | | | | | | Reference | | | Discharge Monitoring | | | | | |
| Analyte | Units | | GR/Q1 | GR/UFS* | Point 11 | Point 10 | Point 12 | Jutts Crossing | Pool 16 | Pool 32 | GRQ18 | GR/UFS | GR/Q1 | Point 11 | Point 10 | Point 12 | Jutts Crossing | Pool 16 | Pool 32 | GRQ18 |
| pH | pH Unit | 6.5-8 | 6.73 | Not availabl | 7.39 | **9.1** | **9.07** | **9.07** | **8.91** | **8.84** | **8.57** | 6.45 | 6.3 | 7.59 | **9.05** | **9.04** | **8.97** | **8.92** | **8.74** | **8.54** |
| Conductivity | µS/cm | 125-2200 | 203 | Not availabl | 462 | 1800 | 1820 | 1840 | 2090 | 2180 | 1920 | 202 | 205 | 478 | 2180 | **2270** | **2250** | 2220 | 2010 | 2000 |
| Hydroxide Alkalinity | mg/L | nv | <1 | Not availabl | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| Carbonate Alkalinity | mg/L | nv | <1 | Not availabl | <1 | 217 | 209 | 222 | 182 | 165 | 57 | <1 | <1 | <1 | 182 | 183 | 198 | 170 | 100 | 43 |
| Bicarbonate Alkalinity | mg/L | nv | 4 | Not availabl | 36 | 217 | 549 | 561 | 679 | 739 | 705 | 8 | 4 | 32 | 544 | 576 | 706 | 693 | 693 | 688 |
| Total Alkalinity | mg/L | nv | 4 | Not availabl | 36 | 741 | 758 | 783 | 862 | 904 | 762 | 8 | 4 | 32 | 726 | 759 | 904 | 864 | 793 | 731 |
| Sulfate | mg/L | nv | 3 | Not availabl | 17 | 23 | 25 | 27 | 24 | 25 | 23 | 6 | 6 | 12 | 23 | 24 | 22 | 21 | 19 | 23 |
| Chloride | mg/L | nv | 52 | Not availabl | 118 | 154 | 164 | 164 | 168 | 178 | 174 | 49 | 51 | 126 | 179 | 178 | 180 | 182 | 170 | 178 |
| Calcium | mg/L | nv | 1 | Not availabl | 22 | 8 | 7 | 7 | 6 | 6 | 7 | 1 | 1 | 28 | 8 | 8 | 7 | 6 | 6 | 8 |
| Magnesium | mg/L | nv | 3 | Not availabl | 9 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 3 | 7 | 3 | 2 | 2 | 3 | 3 | 5 |
| Sodium | mg/L | nv | 27 | Not availabl | 45 | 396 | 391 | 402 | 465 | 484 | 406 | 26 | 27 | 41 | 451 | 465 | 454 | 455 | 406 | 392 |
| Potassium | mg/L | nv | 1 | Not availabl | 3 | 3 | 3 | 3 | 3 | 3 | 3 | <1 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Aluminium | µg/L | 55 | 20 | Not availabl | <10 | **240** | **260** | **230** | **240** | **190** | **70** | 20 | <10 | <10 | 32 | **320** | **310** | **260** | **170** | 40 |
| Arsenic | µg/L | 24 AsIII; 13AsV | <0.1 | Not availabl | <0.1 | 9 | 9 | 9 | 10 | 8 | 4 | <0.1 | <0.1 | <0.1 | 12 | 12 | 11 | 9 | 6 | 2 |
| Cadmium | µg/L | 0.2 | <0.1 | Not availabl | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| Cobalt | µg/L | 4.3 draft | <0.1 | Not availabl | <0.1 | 2 | 2 | 2 | 2 | 2 | 1 | <0.1 | <0.1 | <0.1 | 2 | 2 | 2 | 2 | 1 | 1 |
| Copper | µg/L | 1.4 | <0.1 | Not availabl | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | **4** | **4** | **2** | **2** | <0.1 | 1 |
| Lead | µg/L | 3.4 | <0.1 | Not availabl | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| Manganese | µg/L | 1900 | 79 | Not availabl | 5 | 6 | 4 | 2 | 7 | 5 | 19 | 39 | 159 | <0.001 | 6 | 4 | 2 | 7 | 1 | 2 |
| Nickel | µg/L | 11 | <0.1 | Not availabl | 2 | **90** | **95** | **95** | **110** | **115** | **94** | <0.1 | 1 | 3 | **104** | **108** | **105** | **106** | **93** | **89** |
| Zinc | µg/L | 8 | 5 | Not availabl | 7 | **11** | **15** | **10** | **10** | 8 | <5 | <5 | 6 | <5 | **27** | **20** | **17** | **14** | **12** | 6 |
| Iron | µg/L | 700 draft | **1030** | Not availabl | 50 | 50 | 50 | 50 | 190 | 220 | 320 | 210 | 320 | 50 | 50 | 50 | 50 | 200 | 230 | 190 |
| Barium | µg/L | nv | 8.2 | Not availabl | 126 | 252 | 247 | 237 | 224 | 212 | 162 | 10 | 9.9 | 38 | 317 | 303 | 325 | 274 | 219 | 182 |
| Strontium | µg/L | nv | 13 | Not availabl | 134 | 148 | 148 | 141 | 183 | 189 | 154 | 14 | 13 | 21 | 187 | 186 | 205 | 185 | 159 | 140 |
| Ammonia | µg N/L | 300 draft (pH 8) | <5 | Not availabl | 36 | 35 | 25 | 15 | <5 | <5 | <5 | <5 | <5 | <5 | 50 | 22 | 12 | 11 | <5 | <5 |
| Nitrite + Nitrate NOx | mg/L | nv | <0.002 | Not availabl | 0.062 | 0.067 | 0.12 | 0.137 | 0.162 | 0.126 | 0.017 | 0.012 | 0.04 | 0.069 | 0.111 | 0.117 | 0.109 | 0.087 | 0.021 | 0.04 |
| Total Kjeldahl Nitrogen | mg/L | nv | 0.08 | Not availabl | 0.08 | 0.49 | 0.32 | 0.43 | 0.38 | 0.3 | 0.24 | <0.05 | <0.05 | 0.15 | 0.45 | 0.42 | 0.38 | 0.32 | 0.32 | 0.19 |
| Total Nitrogen | µg N/L | 500 | 80 | Not availabl | 140 | **560** | 440 | **570** | **540** | 430 | 260 | 40 | 40 | 220 | **560** | **540** | 490 | 410 | 340 | 230 |
| Total Phosphorus | µg P/L | 50 | 12 | Not availabl | <5 | 30 | 22 | 22 | 19 | 15 | 6 | <5 | 9 | 5 | 33 | 29 | 23 | 25 | 12 | 6 |
| Total Anions | meq/L | nv | 1.61 | Not availabl | 4.4 | 19.6 | 20.3 | 20.8 | 22.5 | 23.6 | 20.6 | 1.67 | 1.64 | 4.44 | 20 | 20.7 | 23.6 | 22.8 | 21 | 20.1 |
| Dissolved Organic Carbon | mg/L | nv | 6 | Not availabl | 4 | 14 | 4 | 4 | 5 | 5 | 4 | 4 | 5 | 6 | 225 | 312 | 43 | 132 | 12 | 6 |

* Guideline values derived from ANZG (2018). Values in bold text indicate measurements which exceeded the default guideline values for 95% level of protection Bold values indicate measurements which exceeded guideline values. Draft indicates proposed future values (pers comm, Dr Graeme Batley, CSIRO). Values for physico-chemical stressors being the default values for lowland river. **Conductivity for NSW coastal rivers in normally 200–300 µS/cm

**Table 5. Summary of water quality measurements for 2019*.**

| | | | Autumn 2019 | | | | | | | | | Spring 2019 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Guideline value | Reference | | | Discharge Monitoring | | | | | | Reference | | | Dischange Monitoring | | | | | |
| Analyte | **Units** | | GR/Q1 | GR/UFS | Point 11 | Point 10 | Point 12 | Jutts Crossing | Pool 16 | Pool 32 | GRQ18 | GR/UFS | GR/Q1 | Point 11 | Point 10 | Point 12 | Jutts Crossing | Pool 16 | Pool 32 | GRQ18 |
| pH | pH Unit | 6.5-8 | **6.27** | **6.38** | 7.5 | **9.05** | **9.06** | **9.08** | **8.8** | **8.71** | **8.48** | 6.9 | 6.52 | 7.64 | **8.81** | **8.81** | **8.84** | **8.58** | **8.44** | **8.03** |
| Conductivity | µS/cm | 125-2200 | 151 | 155 | 212 | 1730 | 1720 | 1710 | 1740 | 1590 | 1630 | 190 | 185 | 469 | 1360 | 1390 | 1400 | 1350 | 1220 | 1220 |
| Hydroxide Alkalinity | mg/L | nv | 3 | 4 | 29 | 544 | 558 | 540 | 563 | 533 | 540 | 4 | 3 | 32 | 447 | 459 | 464 | 473 | 398 | 395 |
| Carbonate Alkalinity | mg/L | nv | <1 | <1 | <1 | 122 | 120 | 124 | 84 | 64 | 30 | <1 | <1 | <1 | 71 | 74 | 78 | 34 | 19 | <1 |
| Bicarbonate Alkalinity | mg/L | nv | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| Total Alkalinity | mg/L | nv | 3 | 4 | 29 | 666 | 679 | 665 | 647 | 597 | 570 | 4 | 3 | 32 | 518 | 532 | 542 | 507 | 416 | 395 |
| Sulfate | mg/L | nv | 4 | 6 | 7 | 22 | 21 | 22 | 24 | 26 | 17 | 11 | 11 | 12 | 19 | 30 | 20 | 31 | 27 | 24 |
| Chloride | mg/L | nv | 43 | 43 | 42 | 119 | 115 | 115 | 110 | 104 | 119 | 49 | 47 | 101 | 74 | 77 | 76 | 91 | 84 | 123 |
| Calcium | mg/L | nv | 1 | 1 | 11 | 11 | 10 | 8 | 8 | 8 | 7 | 1 | 1 | 25 | 7 | 11 | 11 | 10 | 10 | 8 |
| Magnesium | mg/L | nv | 4 | 4 | 4 | 3 | 5 | 4 | 4 | 4 | 5 | 4 | 4 | 9 | 3 | 4 | 4 | 4 | 4 | 6 |
| Sodium | mg/L | nv | <1 | <1 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 1 | <1 | 3 | 2 | 2 | 3 | 2 | 2 | 2 |
| Potassium | mg/L | nv | 25 | 25 | 27 | 399 | 463 | 420 | 417 | 383 | 360 | 25 | 24 | 42 | 314 | 293 | 300 | 296 | 265 | 235 |
| Aluminium | µg/L | 55 | <10 | <10 | <10 | **270** | **200** | **240** | **190** | **160** | 50 | <10 | <10 | <10 | **130** | **90** | **80** | 30 | 20 | 10 |
| Arsenic | µg/L | 24 AsIII; 13AsV | <0.1 | <0.1 | <0.1 | 9 | 7 | 6 | 4 | 4 | 2 | <0.1 | <0.1 | <0.1 | 2 | 1 | 1 | 2 | 1 | <0.1 |
| Cadmium | µg/L | 0.2 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| Cobalt | µg/L | 4.3 draft | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| Copper | µg/L | 1.4 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | **4** | **2** | **2** | **1** | <0.1 | <0.1 |
| Lead | µg/L | 3.4 | 0.2 | 0.16 | 0.06 | 0.09 | 0.09 | 0.1 | 0.28 | 0.35 | 0.45 | 0.16 | 0.31 | <0.05 | 0.06 | 0.06 | <0.05 | 0.12 | 0.1 | 0.18 |
| Manganese | µg/L | 1900 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| Nickel | µg/L | 11 | **92** | **105** | **15** | 10 | 7 | 3 | 7 | 5 | **34** | **66** | **86** | **12** | **14** | 4 | 1 | 8 | 9 | **41** |
| Zinc | µg/L | 8 | <0.1 | <0.1 | 2 | **78** | **78** | **70** | **64** | **60** | **59** | <0.1 | <0.1 | 3 | **56** | **34** | **35** | **37** | **34** | **39** |
| Iron | µg/L | 700 draft | 6 | <5 | 5 | <5 | <5 | <5 | <5 | <5 | <5 | 6 | 9 | 19 | 6 | 9 | <5 | <5 | <5 | <5 |
| Barium | µg/L | nv | 10.6 | 12.6 | 69.1 | 357 | 340 | 339 | 262 | 225 | 160 | 13.3 | 12.7 | 165 | 384 | 249 | 255 | 190 | 177 | 163 |
| Strontium | µg/L | nv | 14 | 13 | 51 | 225 | 246 | 245 | 176 | 195 | 152 | 10 | 11 | 137 | 363 | 230 | 230 | 180 | 152 | 138 |
| Ammonia | µg N/L | 300 draft (pH 8) | <5 | <5 | <5 | 137 | 69 | 38 | 12 | <5 | 14 | <5 | <5 | 12 | 80 | <5 | <5 | 5 | 10 | <5 |
| Nitrite + Nitrate (NOx) | mg/L | nv | <0.002 | <0.002 | 0.039 | 0.148 | 0.213 | 0.249 | 0.16 | 0.088 | 0.042 | 0.004 | 0.006 | 0.103 | 0.246 | 0.219 | 0.155 | 0.034 | 0.009 | 0.038 |
| Total Kjeldahl Nitrogen | mg/L | nv | <0.05 | <0.05 | 0.12 | 0.55 | 0.37 | 0.36 | 0.24 | 0.25 | 0.18 | 0.06 | 0.06 | <0.05 | 0.21 | 0.24 | 0.24 | 0.34 | 0.41 | 0.17 |
| Total Nitrogen | µg N/L | 500 | <10 | <10 | 160 | 70 | 58 | 61 | 40 | 34 | 22 | 60 | 70 | 120 | 460 | 460 | 390 | 370 | 420 | 210 |
| Total Phosphorus | µg P/L | 50 | 4 | 11 | 5 | **131** | 17 | 16 | 16 | 13 | 9 | <5 | 8 | 7 | 6 | 10 | 6 | 20 | 18 | 7 |
| Total Anions | meq/L | nv | 1.36 | 1.42 | - | 3.13 | 10.1 | 5.78 | 6.8 | 6.27 | 4.42 | - | - | 2.01 | 5.43 | 0.91 | 2.24 | 1.52 | 4.92 | 3.02 |
| Dissolved Organic Carbon | mg/L | nv | 3 | 2 | 3 | 11 | 4 | 4 | 7 | 4 | 4 | 1 | 2 | 9 | 10 | 77 | 8 | 5 | 6 | 5 |

* Guideline values derived from ANZG (2018). Values in bold text indicate measurements which exceeded the default guideline values for 95% level of protection Bold values indicate measurements which exceeded guideline values. Draft indicates proposed future values. Values for physico-chemical stressors being the default values for lowland river. **Conductivity for NSW coastal rivers in normally 200–300 µS/cm

# 3.5 Macrobenthic surveys (2018-2019)

## 3.5.1 Macrobenthos abundance (2018-2019)

A graphical summary of the univariate end-points total abundance and Family richness for all 2018 and 2019 are provided in Figure 13. It should be noted that no macrobenthic data was available for GR/UFS in Autumn 2018 as the waterbody was dry. Furthermore, many of the replicates from Autumn 2019 contained no macrobenthos. These were: GR/UFS (4 replicates); Point 10 (2 replicates); Point 12 (3 replicates), Pool 16 (one replicate) and Pool 32 (one replicate). Consequently, no formal statistics were performed, and results are ambiguous.

In Autumn 2018, there was no clear difference in the total abundances of macrobenthic fauna between the Reference and Discharge Monitoring treatments, nor any clear signal along the gradient from Point 10 to GRQ18. It can be arbitrarily suggested that total abundance was higher in the Discharge Treatment sites in Spring 2018. In Autumn 2019, total abundance is markedly lower in the Discharge Monitoring sites Point 10 and Point 12 than the other sites in this treatment. Abundance appears to be increasing from Jutts to GRQ18. GR/UFS is also lower than the other Reference sites. In Spring 2019, arguably, both the Reference and Discharge Monitoring treatments had similar total abundances. Point 10 had the highest abundance of the Reference sites. While, questionable due to the large number of absences, the lower three Discharge Monitoring sites (Pool 16, Pool 32 and GRQ18) had greater abundances than the other Discharge Monitoring sites.

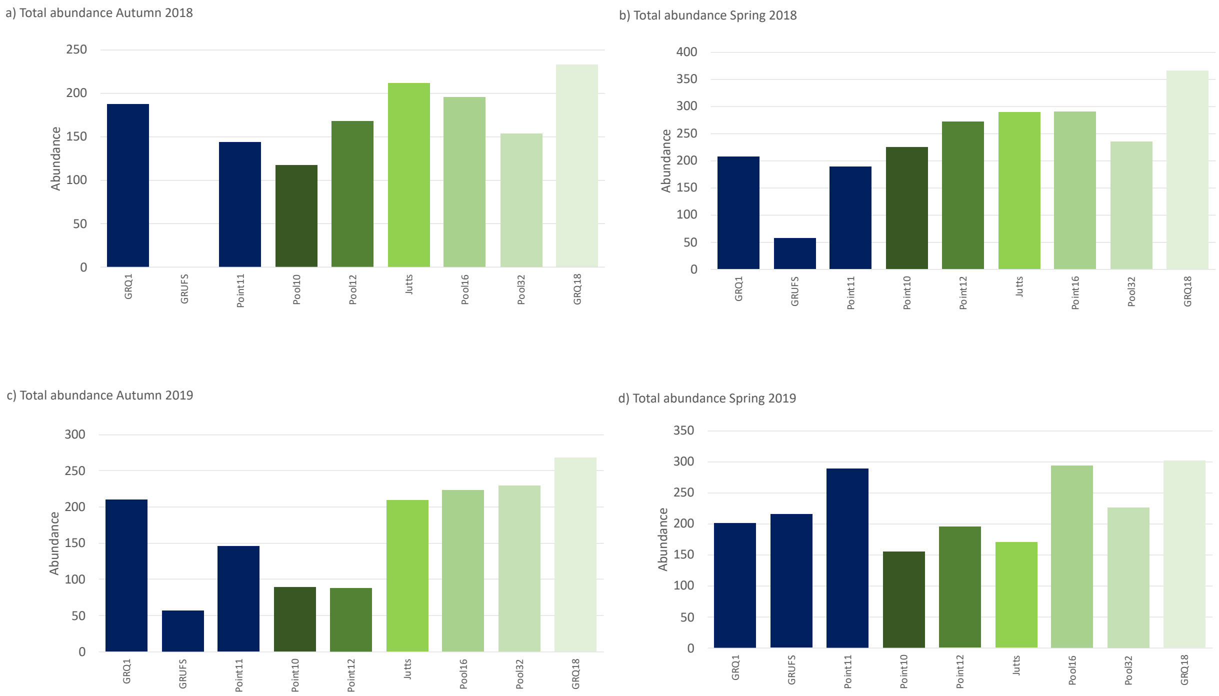


**Figure 13. Abundances of macrobenthic invertebrates (2018-2019). Blue=Reference sites and Green=Discharge Monitoring sites. a) Autumn 2018; b) Spring 2018; c) Autumn 2019; and d) Spring 2019.**

### 3.5.2 Macrobenthos richness (2018-2019)

A summary of Family richness from the macrobenthic data collected in 2018 and 2019 is provided in Figure 14. In Autumn and Spring 2018, richness appears to be lower in the Reference site treatment. This was also the case in Autumn 2019, however, Point 10 and Point 12 had lower richness than the other Discharge Monitoring sites. In Spring 2019, richness appears to be even between the two treatments, and there is no clear indication of gradient within the Discharge Monitoring treatment.

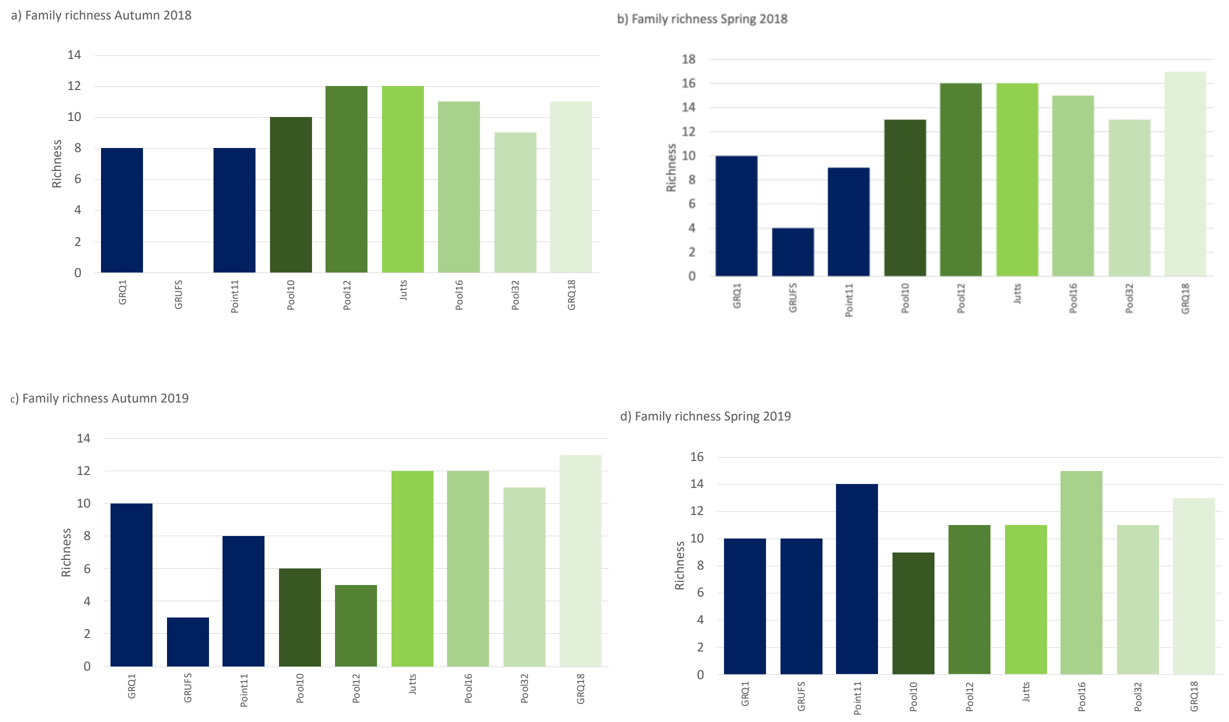


**Figure 14. Family richness of macrobenthic invertebrates (Autumn 2018-2019). Blue=Reference sites and Green=Discharge Monitoring sites. a) Autumn 2018; b) Spring 2018; c) Autumn 2019; and d) Spring 2019.**

### 3.5.3 Macrobenthic composition (2018-2019)

**The ordination plots showing the similarities/differences between macrobenthic assemblages sampled in Autumn and Spring in both 2018 and 2019 are presented in Figure 15. The five top taxa which discriminated between the Reference and Discharge Monitoring treatments on each occasion are shown in**

Table 6. On all four occasions, there were significant differences in the composition in macrofauna communities between the Reference and Discharge Monitoring treatments: Autumn 2019 (Pseudo-F= 6.45, p=0.001); Spring 2018 (Pseudo-F= 12.13, p=0.001); Autumn 2019 (Pseudo-F= 6.16, p=0.001); and Spring 2019 (Pseudo-F= 8.80, p=0.001). In general, the Discharge Monitoring treatment sites were more closely clustered than the Reference sites, indicating that they were more similar to each other.

In Autumn 2018, key taxa which contributed to the observed differences in compositions were: the Ephemeropteran Leptophlebiidae, including Atelophlebia, in the Reference treatment; and

Coenagrionidae (Odonata), Caenidae (Ephemeroptera)and Dytiscidae (Coleoptera) in the Discharge Monitoring treatment. In Spring 2019, differences were primarily due to the higher relative abundances of Caenidae, the chironomids Chironomidae and Tanypodinae, Baetidae (Ephemeroptera) and Dytiscidae in the Discharge Monitoring treatment. In Autumn 2019, Caenidae, Chironominae, Leptoceridae and Tanypodinae were more abundant in the Discharge Monitoring treatment, with Oligochaeta being more abundant in the Reference treatment. In Spring 2019, Leptophlebiidae, including *Ulmerophlebi*a and *Atelophlebia* were characteristic of the Reference treatment, with a relatively higher abundances of Caenidae and Chironominae being indicative of the Discharge Monitoring treatment.

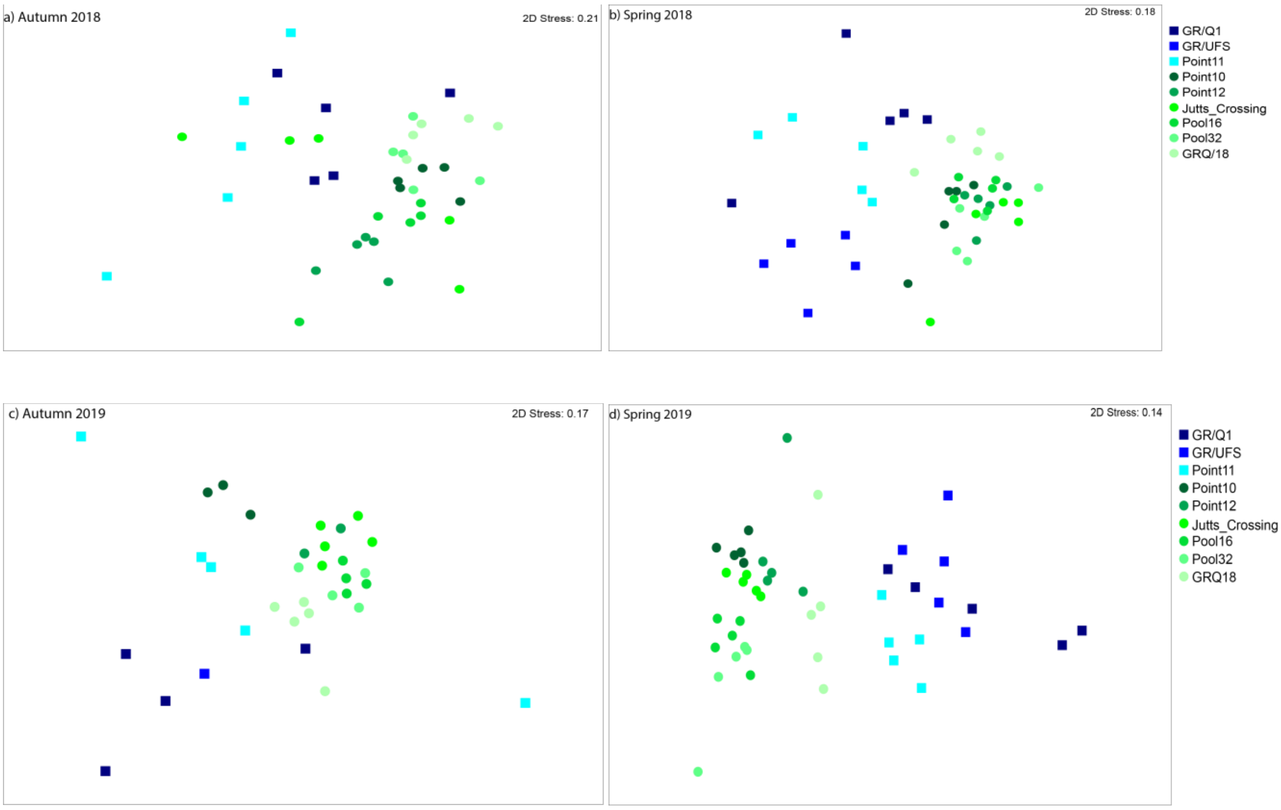


**Figure 15. nMDS of macrobenthic communities (2018-2019). a) Autumn 2018; b) Spring 2018; c) Autumn 2019; and d) Spring 2019.**

**Table 6. SIMPER results illustrating the top 5 taxa which contributed to differences between the Reference and Discharge Monitoring treatments (2018-2019).**

| Year | Season | Family | Reference (Average abundance) | Discharge Monitoring (Average abundance) | Contribution (%) |
|---|---|---|---|---|---|
| 2018 | **Autumn** | Coenagrionidae | 0.48 | 1.32 | 5.91 |
| 2018 | **Autumn** | Caenidae | 0.37 | 1.13 | 5.46 |

| | | | | | |
|---|---|---|---|---|---|
| 2018 | **Autumn** | Leptophlebiidae | 1.04 | 0.12 | 5.14 |
| 2018 | **Autumn** | Dytiscidae | 0.53 | 0.96 | 4.82 |
| 2018 | **Autumn** | *Atelophlebia* AV15 | 0.97 | 0.12 | 4.82 |
| 2018 | **Spring** | Caenidae | 0.41 | 5.90 | 12.9 |
| 2018 | **Spring** | Chironominae | 2.40 | 4.90 | 8.82 |
| 2018 | **Spring** | Tanypodinae | 0.84 | 3.95 | 8.05 |
| 2018 | **Spring** | Baetidae | 0.32 | 3.17 | 7.35 |
| 2018 | **Spring** | Dytiscidae | 0.82 | 2.45 | 4.77 |
| 2019 | **Autumn** | Caenidae | 0.25 | 3.31 | 12.8 |
| 2019 | **Autumn** | Chironominae | 2.40 | 3.39 | 7.88 |
| 2019 | **Autumn** | Leptoceridae | 0.46 | 1.69 | 6.56 |
| 2019 | **Autumn** | Oligochaeta | 1.78 | 0.39 | 6.49 |
| 2019 | **Autumn** | Tanypodinae | 2.35 | 3.50 | 6.34 |
| 2019 | **Spring** | Caenidae | 0.85 | 3.64 | 10.2 |
| 2019 | **Spring** | Leptophlebiidae | 2.89 | 0.34 | 9.24 |
| 2019 | **Spring** | *Ulmerophlebia annulata* | 2.22 | 0.02 | 7.66 |
| 2019 | **Spring** | *Atelophlebia* AV15 | 1.98 | 0.29 | 6.38 |
| 2019 | **Spring** | Chironominae | 1.78 | 2.45 | 5.47 |

### 3.5.4 Correlative patterns between macrobethos and water quality (2018-2019)

Figure 16 illustrates the correlative relationships between the macrobenthic communities and water quality from the Autumn 2018 sampling event. Approximately 94% of the variation in the macrobenthic community data could be explained by the measured environmental variables. The findings suggest that the macrobenthic communities from the Discharge Monitoring sites were being influenced by water quality. The strongest correlations between water quality and macrobenthic communities occurred in the upstream sites from the Discharge Monitoring treatment. When examined individually, pH, conductivity, nickel, and total nitrogen were all shown to correlate significantly with benthic community structure. However, when examined collectively, only pH was shown to significantly contribute to a proportion of the variation in the data, with this variable explaining approximately 37 % in the variation of the macrobenthic community data. This emphasizes then need to consider the discharge as a mixture, rather than focus on the effect of individual variables per se. The key correlates with the communities from Point 11 were difficult to determine.

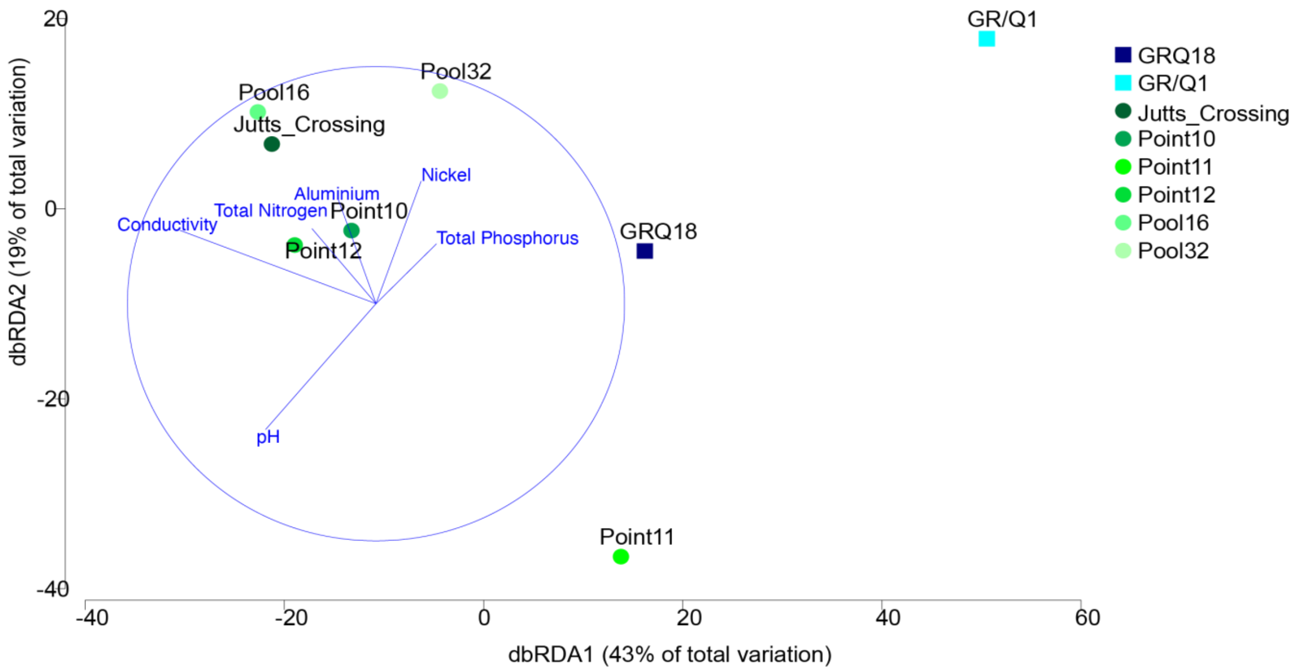


**Figure 16. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and macrobenthic composition sampled in Autumn 2018.**

The ordination plot from distLM analysis of the Spring 2018 data is shown in Figure 17. The measured water quality variables explained 93% of the variation in the macrobenthic data. In general, all the Discharge Monitoring sites were driven by similar water quality parameters, the exception being the most downstream site, GRQ18. When examined collectively only pH was shown to be significant, explaining 37% of the total variation.

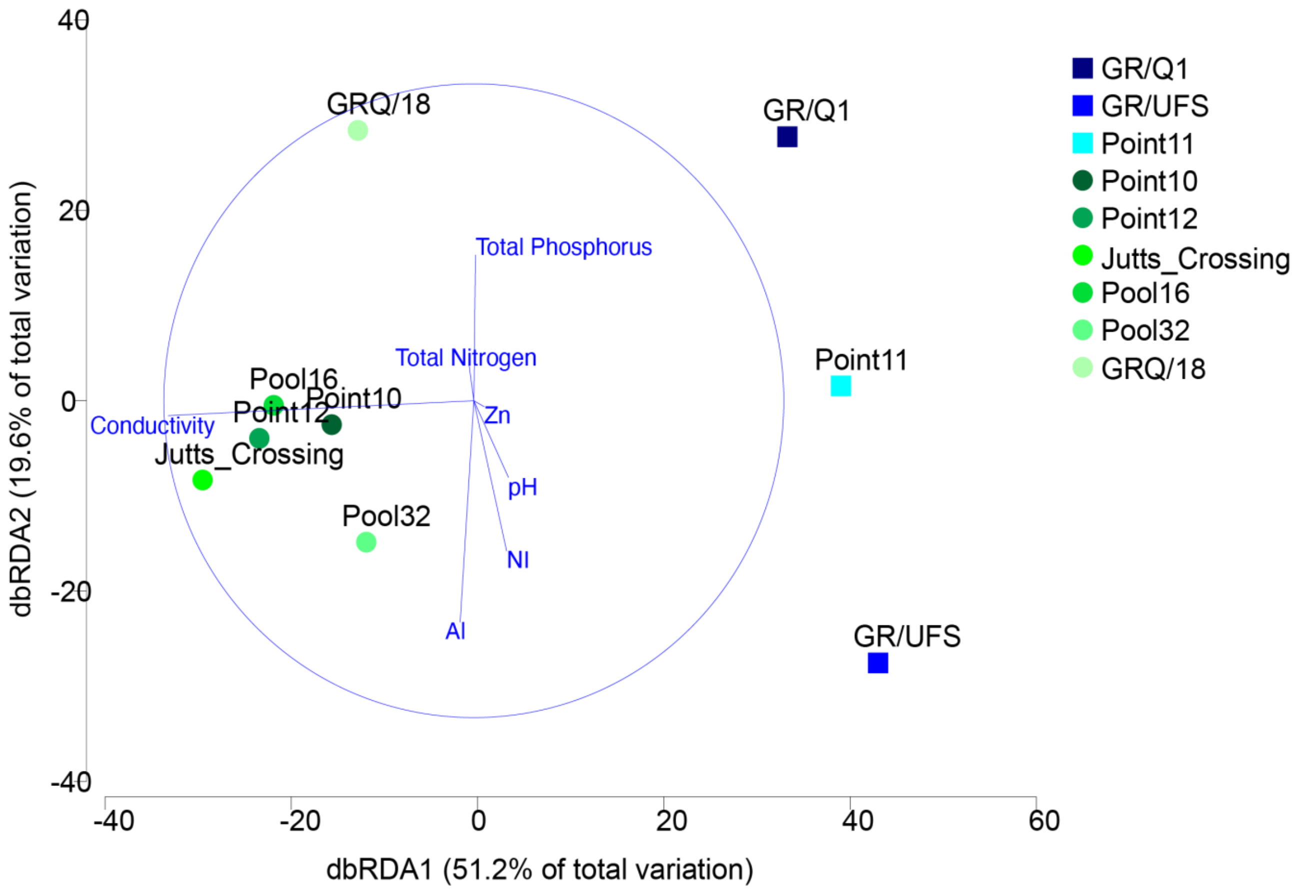


**Figure 17. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and macrobenthic composition from Spring 2018.**

Figure 18 illustrates the correlative relationships between the macrobenthic communities and water quality from the Autumn 2019 sampling event. Approximately 97 % of the variation in the macrobenthic community data could be explained by the environmental variables. The findings suggest that the macrobenthic communities from the upper Discharge Monitoring sites were being influenced primarily by pH and conductivity, with a different combination of water quality parameters influencing the downstream sites Pool 16, Pool 32 and GRQ18. When examined collectively, only pH was shown to significantly contribute to a proportion of the variation in the data, explaining approximately 28 % of the total variation of the macrobenthic community structure.

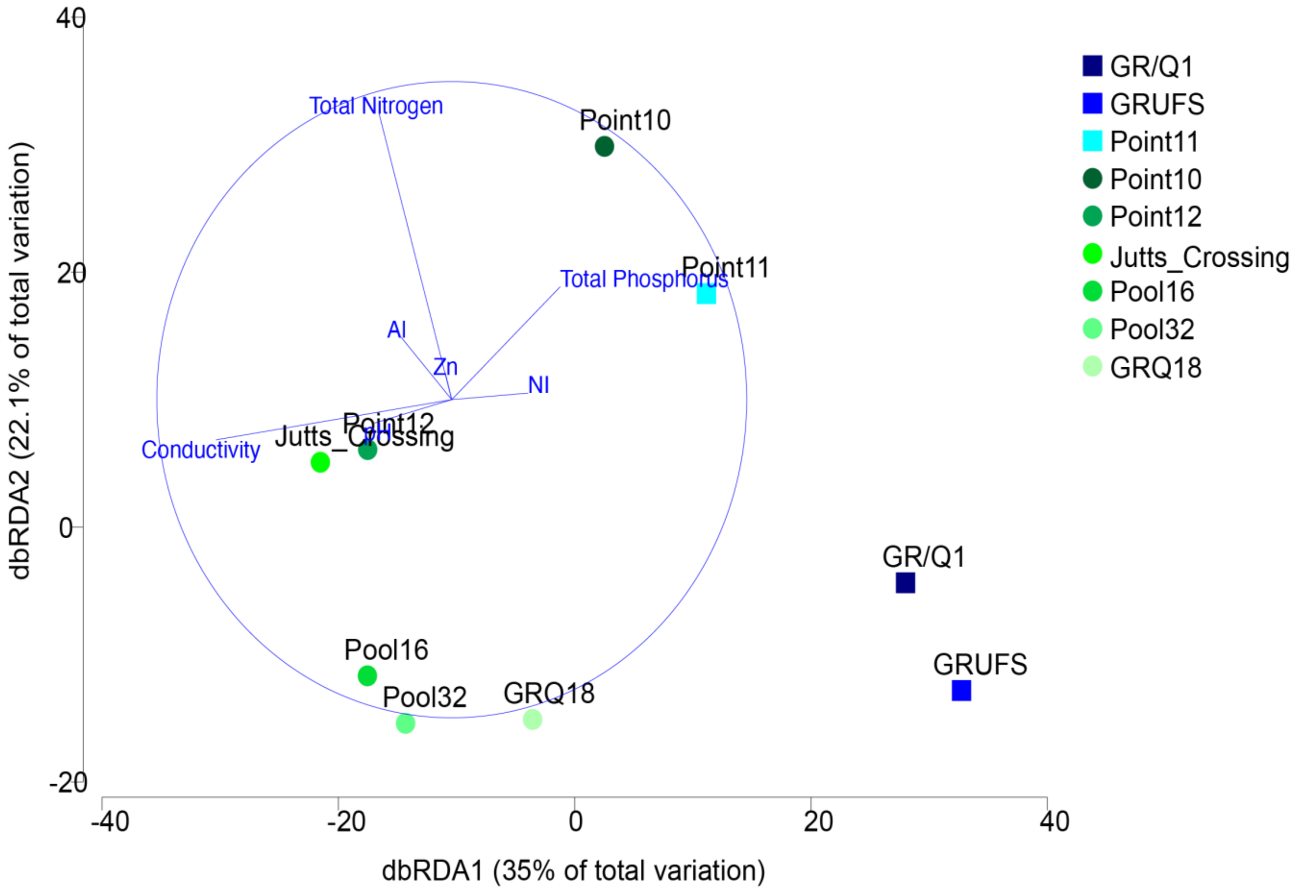


**Figure 18. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and macrobenthic composition from Autumn 2019.**

The distance-based analysis of the Spring 2019 data (Figure 19) found that the measured water quality variables collectively explained 94% of the variation in the macrobenthic data. Three variables were found to be significant, with pH explaining 53%, conductivity 13% and aluminium 11% of the total variation. The composition of the water quality variables driving Point 10, Point 12 and Jutts differed to those for Pool 16, Pool 32 and GRQ18.

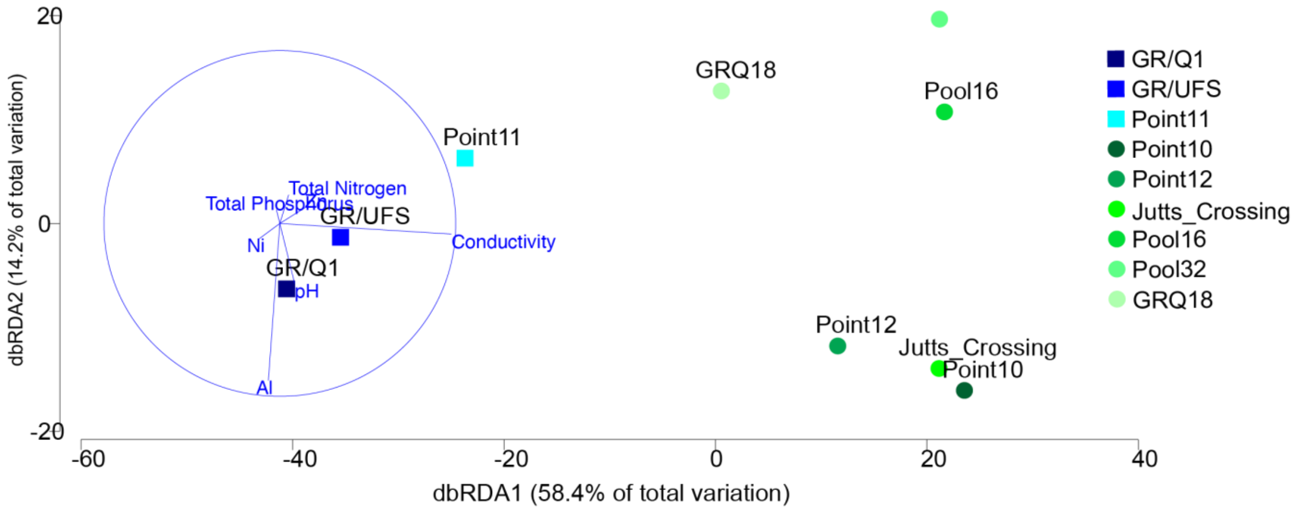


**Figure 19. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and macrobenthic composition from Spring 2019.**

## 3.6 SIGNAL scores (2018-2019)

The SIGNAL scores from the Autumn and Spring macrobenthic surveys performed in 2018 and 2019 are presented in Figure 20. The most salient finding was the comparative lower (based on long-term means) SIGNAL scores in the Reference sites during 2018-2019. The only site which was not below the long-term mean was GR/Q1 in Autumn 2018. In most cases the scores where low enough in this period to reclassify the condition of Reference sites from 'probable moderate pollution' to 'probable severe pollution' (Table 7). SIGNAL scores were similar to the long-term trend for Point 10, the exception being the lower score in Autumn 2019. SIGNAL scores were also lower in Point 12 in August 2018 and 2019. The SIGNAL scores varied across time in Jutts, Point 16 and Point 12, however, were frequently near the long-term mean. In GRQ18, SIGNAL scores were consistently above the long-term average, with the site being potentially ranked as probable moderate pollution', in comparison to 'probable severe pollution' which was generally associated with the Discharge Monitoring sites.

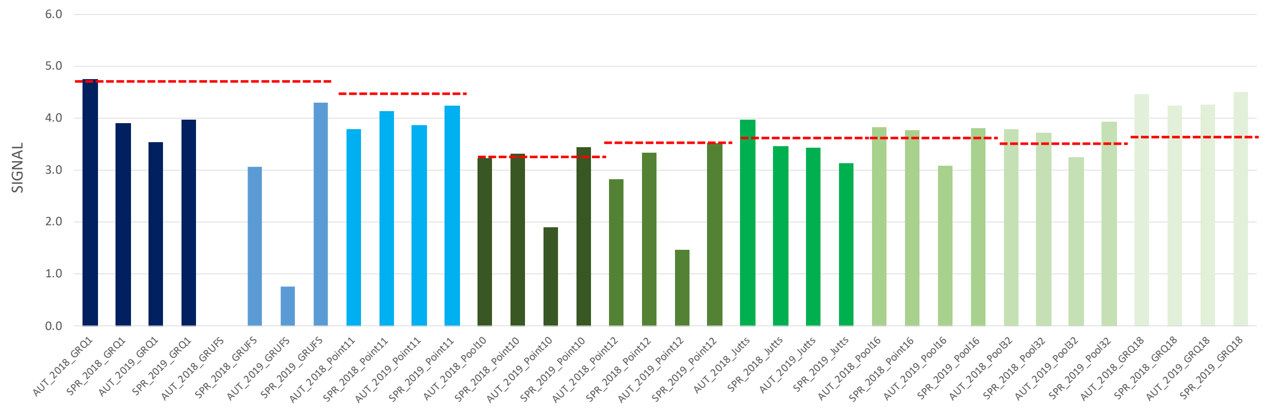


**Figure 20. SIGNAL scores from 2018-2019. Blue=Reference sites and Green=Discharge Monitoring sites. Red dashed line indicates mean SIGNAL scores from (2013-2017).**

**Table 7. SIGNAL scores and rankings for each site (2018-2019). *Potential rankings based on Chessman (1995).**

| Treatment | Year | Season | Site | Potential ranking* | SIGNAL |
|---|---|---|---|---|---|
| Reference | 2018 | Autumn | GR/Q1 | Probable moderate pollution | 4.8 |
| | 2018 | Spring | GR/Q1 | Probable severe pollution | 3.9 |
| | 2019 | Autumn | GR/Q1 | Probable severe pollution | 3.5 |
| | 2019 | Spring | GR/Q1 | Probable moderate pollution | 4.0 |
| Reference | 2018 | Autumn | GR/UFS | | n/a |
| | 2018 | Spring | GR/UFS | Probable severe pollution | 3.1 |
| | 2019 | Autumn | GR/UFS | Probable severe pollution | 0.8 |
| | 2019 | Spring | GR/UFS | Probable moderate pollution | 4.3 |
| Reference | 2018 | Autumn | Point11 | Probable severe pollution | 3.8 |
| | 2018 | Spring | Point11 | Probable moderate pollution | 4.1 |
| | 2019 | Autumn | Point11 | Probable severe pollution | 3.9 |
| | 2019 | Spring | Point11 | Probable moderate pollution | 4.2 |
| Discharge Monitoring | 2018 | Autumn | Point10 | Probable severe pollution | 3.2 |
| | 2018 | Spring | Point10 | Probable severe pollution | 3.3 |
| | 2019 | Autumn | Point10 | Probable severe pollution | 1.9 |
| | 2019 | Spring | Point10 | Probable severe pollution | 3.4 |
| Discharge Monitoring | 2018 | Autumn | Point12 | Probable severe pollution | 2.8 |
| | 2018 | Spring | Point12 | Probable severe pollution | 3.3 |
| | 2019 | Autumn | Point12 | Probable severe pollution | 1.5 |
| | 2019 | Spring | Point12 | Probable severe pollution | 3.5 |
| Discharge Monitoring | 2018 | Autumn | Jutts | Probable moderate pollution | 4.0 |
| | 2018 | Spring | Jutts | Probable severe pollution | 3.5 |
| | 2019 | Autumn | Jutts | Probable severe pollution | 3.4 |
| | 2019 | Spring | Jutts | Probable severe pollution | 3.1 |
| Discharge Monitoring | 2018 | Autumn | Pool 16 | Probable severe pollution | 3.8 |
| | 2018 | Spring | Pool 16 | Probable severe pollution | 3.8 |
| | 2019 | Autumn | Pool 16 | Probable severe pollution | 3.1 |
| | 2019 | Spring | Pool 16 | Probable severe pollution | 3.8 |
| Discharge Monitoring | 2018 | Autumn | Pool 32 | Probable severe pollution | 3.8 |
| | 2018 | Spring | Pool 32 | Probable severe pollution | 3.7 |
| | 2019 | Autumn | Pool 32 | Probable severe pollution | 3.2 |
| | 2019 | Spring | Pool 32 | Probable severe pollution | 3.9 |
| Discharge Monitoring | 2018 | Autumn | GRQ18 | Probable moderate pollution | 4.5 |
| | 2018 | Spring | GRQ18 | Probable moderate pollution | 4.2 |
| | 2019 | Autumn | GRQ18 | Probable moderate pollution | 4.3 |
| | 2019 | Spring | GRQ18 | Probable moderate pollution | 4.5 |

n/a= not available as waterbody was dry.

## 3.7 Metabarcoding survey

### 3.7.1 16S rDNA metabarcoding (prokayotes)

After the removal of potentially erroneous sequences, the prokaryote (16S rDNA) dataset contained >584,000 reads, encompassing 4,660 unique Operational Taxonomic Units (OTUs) from 31 phyla.

The ordination plot for the prokaryote metabarcoded data is provided in Figure 21. As indicated by the figure, the prokaryote communities from the Reference sites were markedly different to those from the Discharge Monitoring sites. This is confirmed by the PERMANOVA which found a significant difference in composition between the two treatments (PERMANOVA: $F= 17.88$, $p<0.001$). In general, each site had its own unique prokaryote community, although there was overlap in the Discharge Monitoring sites Jutts Crossing and Point 12. The communities closer to the discharge (e.g. Point 12 and Jutts) were most dissimilar to the Reference sites. The top ten putative prokaryote indicator taxa for both treatments are provided in Table 8.

Figure 22 illustrates the correlative relationships between the metabarcoded prokaryote communities and water quality from the Spring 2019 sampling event. Approximately 95 % of the variation in the prokaryote community data could be explained by the environmental variables. With the exception of GR/Q1, the ordination plot suggests that the prokaryote communities from the Discharge Monitoring sites are influenced by similar water quality parameters, with a gradient running from Point 10 and Jutts to Pool 32. When examined collectively, three variable explained significant proportions of variation in the prokaryote community data: pH (52%), conductivity (16%) and nickel (10%).

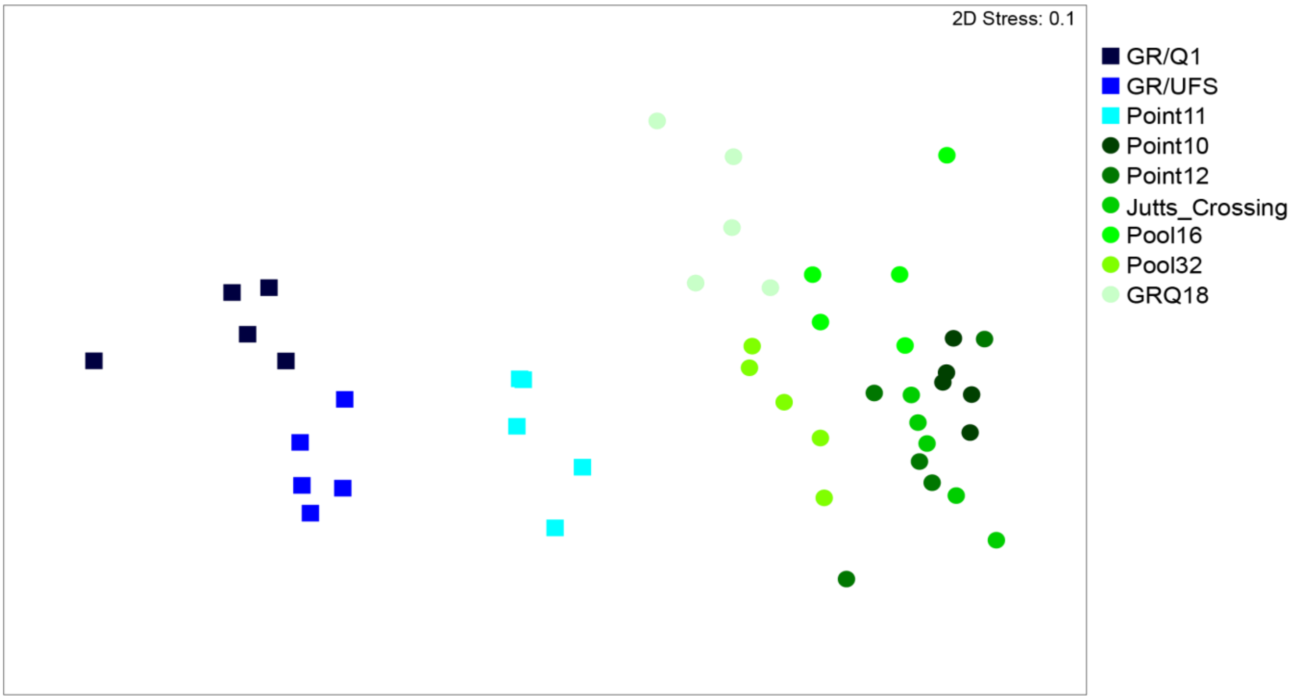


**Figure 21. nMDS of the 16S rDNA (prokaryote) metabarcoding data. Analysis is derived from Hellinger transformed abundance data at the species level.**

**Table 8. Top 10 putative indicator prokaryote taxa for the Reference and Discharge Monitoring treatments.**

| Treatment | Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|---|
| Reference | Actinobacteria | | Actinomycetales | Microbacteriaceae | *Rhodoluna* |
| Reference | Acidobacteria | Acidobacteria_Gp1 | | | *Acidipila* |
| Reference | Proteobacteria | Betaproteobacteria | Burkholderiales | Oxalobacteraceae | |
| Reference | Chloroflexi | Ktedonobacteria | | | |
| Reference | Spirochaetes | Spirochaetia | Spirochaetales | | |
| Reference | Planctomycetes | Planctomycetia | Brocadiales | Brocadiaceae | *Candidatus* |
| Reference | Planctomycetes | Phycisphaerae | Tepidisphaerales | Tepidisphaeraceae | *Tepidisphaera* |
| Reference | Proteobacteria | Betaproteobacteria | Burkholderiales | Oxalobacteraceae | *Massilia* |
| Reference | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Acetobacteraceae | Acidisoma |
| Reference | Thaumarchaeota | | Nitrososphaerales | Nitrososphaeraceae | *Nitrososphaera* |
| Discharge Monitoring | Proteobacteria | Alphaproteobacteria | Caulobacterales | Hyphomonadaceae | *Hyphomonas* |
| Discharge Monitoring | Chloroflexi | Caldilineae | | | |
| Discharge Monitoring | Proteobacteria | Gammaproteobacteri | Xanthomonadales | Xanthomonadaceae | |
| Discharge Monitoring | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Hydrogenophaga* |
| Discharge Monitoring | Bacteroidetes | Cytophagia | Cytophagales | Cyclobacteriaceae | *Aquiflexum* |
| Discharge Monitoring | Proteobacteria | Alphaproteobacteria | Sphingomonadale | Erythrobacteraceae | *Porphyrobacter* |
| Discharge Monitoring | Proteobacteria | Alphaproteobacteria | Sphingomonadale | Sphingomonadacea | *Sphingopyxis* |
| Discharge Monitoring | Bacteroidetes | Flavobacteriia | Flavobacteriales | Flavobacteriaceae | *Arenibacter* |
| Discharge Monitoring | Chloroflexi | Thermomicrobia | Sphaerobacterale | Sphaerobacteracea | *Nitrolancea* |
| Discharge Monitoring | Bacteroidetes | Cytophagia | Cytophagales | Flammeovirgaceae | |

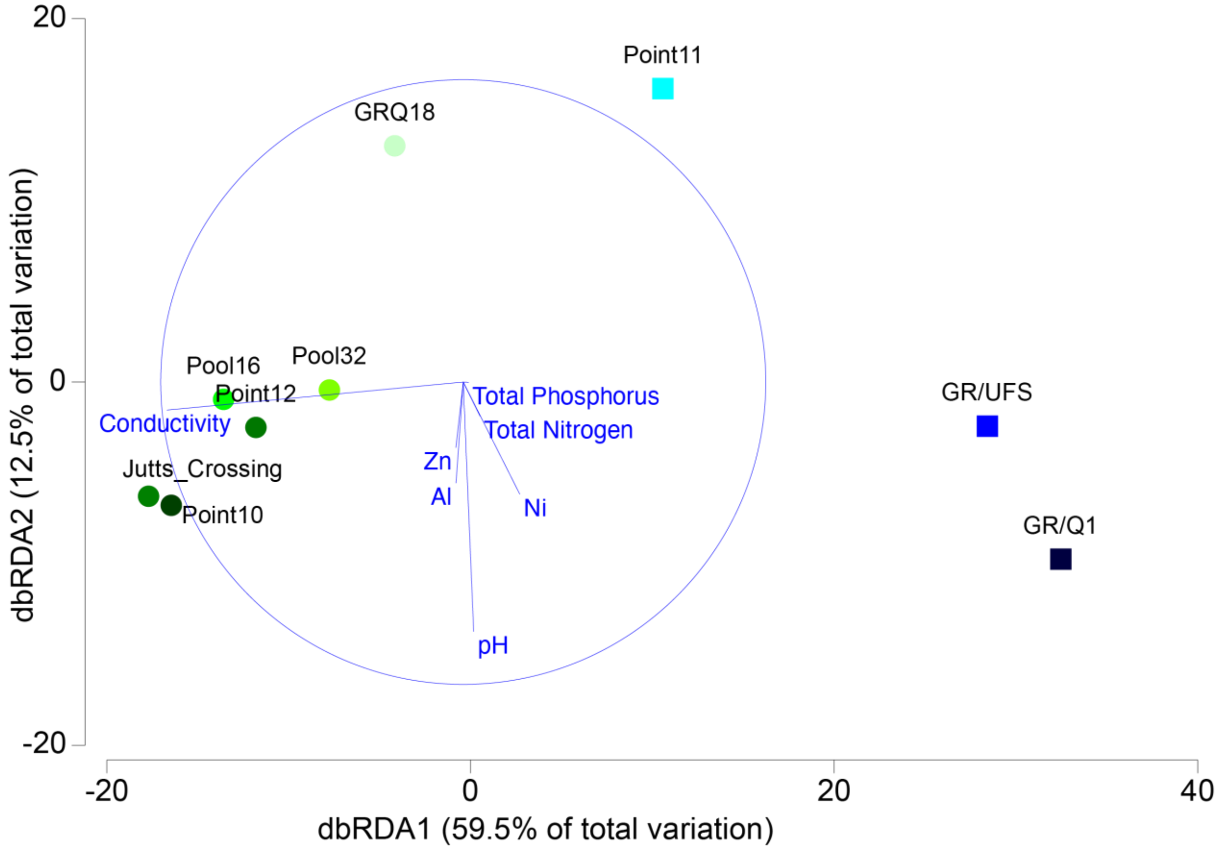


**Figure 22. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and metabarcoded prokaryote composition from Autumn 2018.**

### 3.7.2 18S rDNA metabarcoding (eukaryotes)

After the removal of potentially erroneous sequences, the eukaryote (18S rDNA) dataset contained >1,191,000 reads, encompassing 1,223 unique Operational Taxonomic Units (OTUs) from 50 phyla and other high-level taxonomic groups.

The ordination plot for the eukaryote metabarcoded data is provided in Figure 23. The eukaryote communities from the Reference sites were markedly different to those from the Discharge Monitoring sites, with this confirmed by the PERMANOVA (F= 8.20, p<0.001). Three samples from the Reference site Point 11 were different to all other samples. The top ten putative indicator eukaryote taxa for both treatments are provided in Table 9Table 8.

The ordination plot illustrating the correlative relationships between the metabarcoded eukaryote communities and water quality is provided in Figure 24. Whilst there is correlative evidence that the water chemistry is driving eukaryote composition, there is no clear gradient, with the downstream Discharge Monitoring sites being driven by a different combination of water quality parameters to Point 10, Point 12 and Jutts. Approximately 93 % of the variation in the eukaryote community data could be explained by the environmental variables. Two variables explained significant proportions of variation in the eukaryote community data: pH (28%) and aluminium (17%).

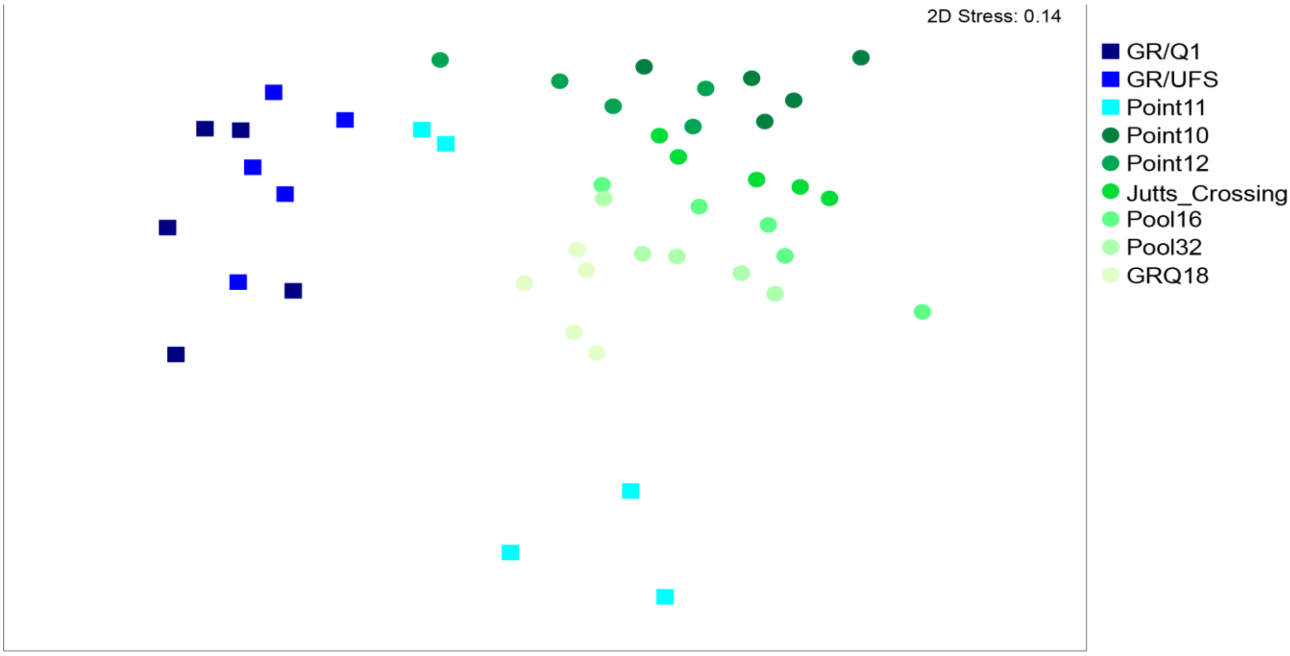


**Figure 23. nMDS of the 18S rDNA (eukaryotes) metabarcoding data. Analysis is derived from presence/absence data at the level of Operational Taxonomic Unit (OTU).**

**Table 9. Top 10 putative indicator eukaryote taxa for the Reference and Discharge Monitoring treatments**

| Treatment | Kingdom | Phylum | Class | Order | Family |
|---|---|---|---|---|---|
| Reference | Metazoa | Nemertea | Enopla | Monostilifera | Tetrastemmatidae |
| Reference | Metazoa | Nemertea | Anopla | Heteronemertea | Gorgonorhynchidae |
| Reference | Stramenopiles | Oomycetes | | | Oomycetes |
| Reference | Metazoa | Platyhelminthes | Turbellaria | | Macrostomidae |
| Reference | Eukaryota | Fungi | Ascomycota | Sordariomycetes | Calosphaeriales |
| Reference | Alveolata | Apicomplexa | Gregarinasina | Neogregarinorida | Syncystidae |
| Reference | Fungi | Basidiomycota | Agaricomycetes | Boletales | Serpulaceae |
| Reference | Stramenopiles | Ochrophyta | Chrysophyceae | | |
| Reference | Metazoa | Arthropoda | Arachnida | Oribatida | Ceratozetidae |
| Reference | Metazoa | Arthropoda | Insecta | Megaloptera | Sialidae |
| Discharge Monitoring | Alveolata | Myzozoa | Dinophyceae | Peridiniales | Glenodiniaceae |
| Discharge Monitoring | Metazoa | Mollusca | Bivalvia | Arcoida | Glycymerididae |
| Discharge Monitoring | Fungi | Ascomycota | Sordariomycetes | Xylariales | Sporocadaceae |
| Discharge Monitoring | Fungi | Basidiomycota | Agaricomycetes | Agaricales | Tricholomataceae |
| Discharge Monitoring | Plantae | Streptophyta | Bryopsida | Grimmiales | Grimmiaceae |
| Discharge Monitoring | Metazoa | Nematoda | Enoplea | Enoplida | Ironidae |
| Discharge Monitoring | Metazoa | Mollusca | Gastropoda | Pulmonata | Planorbidae |
| Discharge Monitoring | Metazoa | Rotifera | Monogononta | Ploima | Microcodonidae |
| Discharge Monitoring | Eukaryota | Fungi | Basidiomycota | Tremellomycetes | Tremellales |
| Discharge Monitoring | Metazoa | Arthropoda | Collembola | Symphypleona | Sminthuridae |

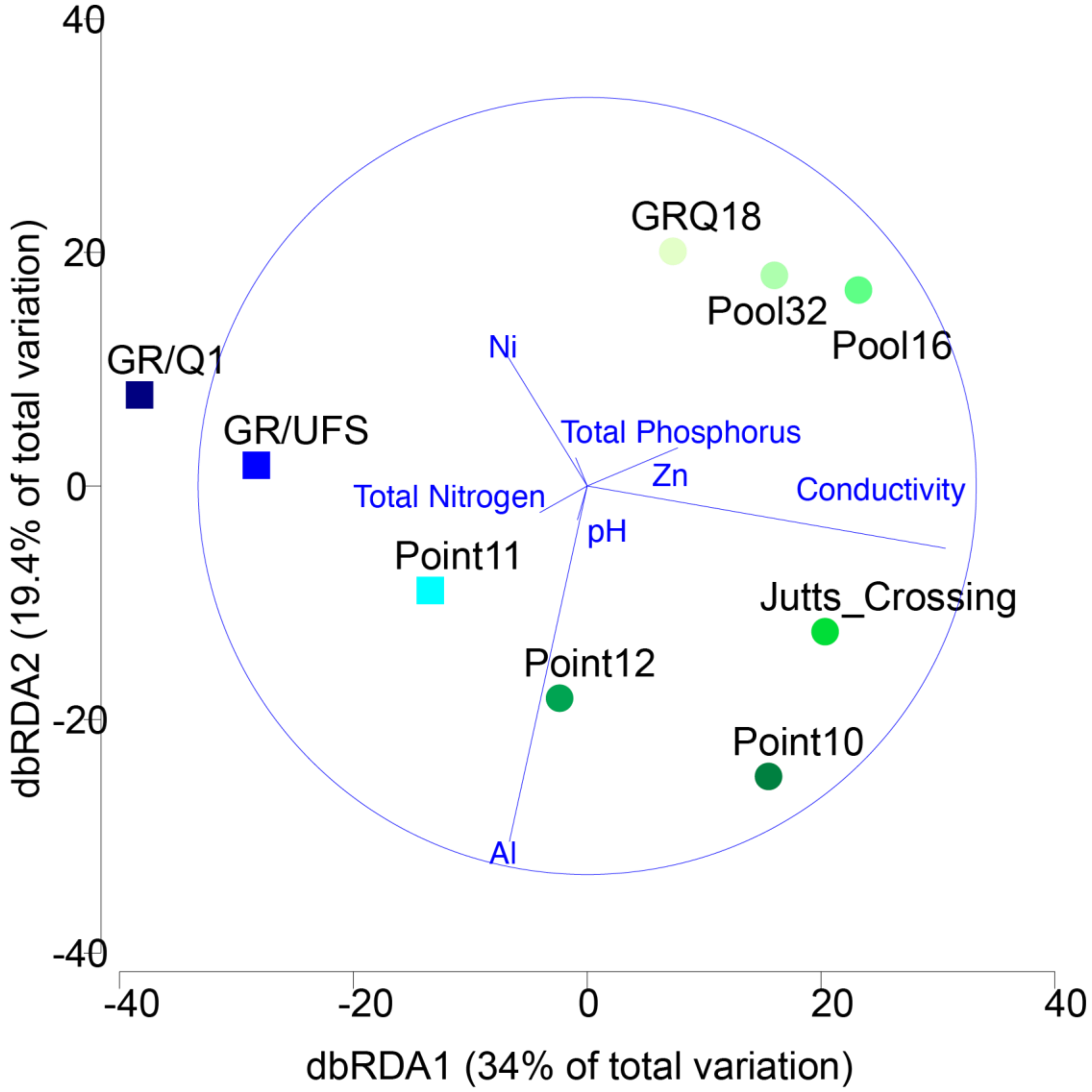


**Figure 24. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and eukaryotic communities (18S rDNA) obtained by metabarcoding.**

# 4 Discussion

## 4.1 Water chemistry

The analysis of the long-term trends in water quality data suggest that there has been a general improvement in water quality over time. The pH of the Discharge Monitoring sites remains comparatively high (often > 8.2 pH), especially given that two of the Reference sites have slightly acidic waters (Figure 2. Long-term trends in pH. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value for lowland rivers. ). There has been a clear reduction in conductivity over time, with Discharge Monitoring sites rarely exceeding the new guideline value of 2250 µS/cm (formally 2500 µS/cm) (Figure 3. Long-term trends in conductivity. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value for lowland rivers.) (ANZG, 2018). However, some caution in interpreting this result is required as the guideline value captures a wide conductivity range (125-2250 µS/cm) and cannot cover all catchments. As the upper Discharge Monitoring sites have around an order of magnitude higher conductivity than the Reference sites, the long-term findings strongly suggest that the catchment has a naturally low conductivity and that mining activities are markedly elevating conductivity. Further evidence of this is the clear gradient from Point 10 to GRQ18.

While aluminium concentrations exceeded the guideline value in the upper Discharge Monitoring sites (Point 10 to Pool 32), there is a clear decline in concentrations over time, with aluminium concentrations returning to around the guideline value in the most downstream Discharge Monitoring site (GRQ18) (Figure 4 Long-term trends in aluminium concentrations. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value.). In the case of nickel, concentrations in all Discharge Monitoring sites were still several times above the guideline value, and pose a risk to aquatic organisms (ANZG, 2018). While exceedances did occur, in general, zinc concentrations have declined over time. However, patterns for nickel and zinc were markedly different in 2019, with this finding discussed in Section 4.4.

Ammonia concentrations have declined over time and decay with distance. However, some patterns in 2018 and 2019 were against this general trend, these are discussed in Section 4.4.

For all analysed variables, there was a general decline in concentrations with downstream distance, however, for most Discharge Monitoring sites, pH, metal concentrations and ammonia were still high enough to be of concern. Collectively, the data suggests that although there has been an overall improvement in water quality, the water quality of the upper Discharge Monitoring sites (e.g. Point 10, Point 12 and Jutts) is likely to impair biological integrity.

## 4.2 Long-term patterns in macrobenthic communities

### 4.2.1 Abundance, richness and composition

The analysis of the long-term macrobenthic dataset indicated that the Discharge Monitoring treatment had a higher mean abundance of macrobenthic invertebrates than the Reference treatment. However, abundances varied greatly over time within all sites across both treatments, and consequently there was no clear temporal trend. Mean Family richness was similar across both treatments.

It is important to note that habitat is also likely contributing to the observed differences between and within treatments. For example, Point 11 is a shallow ephemeral water body which has sporadic periods of no flow (pers. obs. David Gregory, South32). Furthermore, observational evidence (pers. obs. David Gregory, South32) also suggests that the structural complexity of the water bodies varies greatly between the Reference and Discharge Monitoring treatments, with the former containing more complex habitats, including structures such as log jams. Consequently, the observed differences between the two treatments is likely due to a combination of the discharge waters and habitat condition.

### 4.2.2 Long-term trend in SIGNAL

The SIGNAL scores suggested that when examined collectively, the Reference sites were in better ecological condition than the Discharge Monitoring treatments (Table 3). While scores varied, the long-term potential ranking for all Reference sites was ‘probable moderate pollution’. In comparison, all Discharge Monitoring sites, with the exception of GRQ18, we ranked as ‘probable severe pollution’. Although we have provided ecological rankings for each site based on their long-term mean SIGNAL scores (Table 3), as in the case of the univariate metrics, these scores varied widely within sites. Consequently, these rankings should be limited to emphasising that based on the SIGNAL approach, the Reference sites were in better ecological condition than Discharge Monitoring sites Point 10, Point 12, Jutts, Pool 16 and Pool 32, rather than any specific ranking.

There was a marked decline in the SIGNAL scores for the 2018-2019 sampling events, these are discussed in Section 4.6.

### 4.2.3 Leptophlebiidae genera of interest

It has been suggested that specific Leptophlebiidae species are sensitive to conductivity (Cardno, 2010), leading to the recommendation by the Georges River Working Group to examine this group at the species level. The analysis of the 2016-2019 data clearly showed that *Atelophlebia* spp., *Ulmerophlebia* spp. *and Kooronga* spp. were observed far more frequently and in higher abundances in the Reference sites. *Atelophlebia* were more abundant in 2018 and 2019 in GRQ18, and with the exception of a single individual, remained absent from the other Discharge Monitoring sites. Furthermore, only a few individual *Kooronga* were observed in the most recent sampling events in the Discharge Monitoring sites Point 10 and Point 12.

As previously noted by Chariton and Stephenson (2018), specimens were not confirmed by a professional taxonomist, and we strongly recommend that if genus or species level data is required, future identifications should be performed by a professional taxonomist. Given that Leptophlebiidae is captured in the SIGNAL 2.0 analysis, it is unclear if detailed taxonomy of this group is required for future studies. As such, justification for including this component in future surveys is needed.

## 4.3 Ecotoxicology

There appears to be a general reduction in the toxicity of the Point 10 waters in recent years (April 2016-2019) (Figure 12. Toxicity of Point 10 waters collected 2013 – 2019. Toxicity is shown as toxic units, higher values are indicative of greater toxicity. ). The findings from the most recent tests (Spring 2019), suggest that the Point 10 water samples were less toxic than previous years. Toxicity was low for both the *Paratya australiensis* 10-day acute and *Ceriodaphnia dubia* survival tests. Point 10 waters still elicited a toxic response in the *Ceriodaphnia dubia* reproduction test, and while markedly less toxic than the previous test (November 2017), this endpoint was shown to vary greatly over time. Given the complexity of the waters over time, high variability in this assay can be expected. However, without more frequent testing it is not possible to confirm whether the waters from Point 10 are becoming less toxic, especially with regards to the *Ceriodaphnia dubia* reproduction test.

## 4.4 Water chemistry (2018-2019)

It is emphasised that the water samples during this period were collected during a prolonged period of drought. Observational data at the time of collection indicates that there was no flow in the Reference sites, with slow flow occurring in the Discharge Monitoring sites. All water bodies were shallower than previous years. Given the low precipitation and high evaporation rates associated with the warmer climate it is unsurprising that the concentrations of many analytes were elevated in both the Reference and Discharge Monitoring sites.

In all sampling events between 2018-2019, pH was elevated in all Discharge Monitoring sites. Frequently, it exceeded a pH of 9, being up to 2.7 units higher than measurements taken from the Reference sites. Aluminium concentrations remained high in the Discharge Monitoring sites, especially in Autumn 2018, and generally remained at levels to be of significant ecological risk. In some cases, this was 10-fold greater than the threshold for the protection of 95% of aquatic biota (ANZG, 2018). Interestingly, in Spring 2018, Point 10 had a markedly lower concentration than the other Discharge Monitoring sites, however, this only occurred in this single sampling event.

In 2018, nickel concentrations remained high in all Discharge Monitoring sites (Table 4). However, the results from 2019 were extremely unusual, and converse to what was expected (Table 5). Nickel was above the guideline value in all three Reference sites in both Autumn and Spring, with this being most pronounced in GR/UFS and GR/Q1. Furthermore, concentrations were below the guideline in all Discharge Monitoring sites, with the exception of the most downstream site, GRQ18. It should be noted that this is the first time such a finding has been observed since the program commenced (Figure 5. Long-term trends in nickel concentrations. Sites were placed into two treatments: Reference (blue) and Discharge Monitoring (green). Dotted red lines represent the ANZG (2018) guideline value.). While it is possible that concentrations were high in the Reference sites due to evaporation, and possibly the formation of iron oxides, as indicated by the high concentration of iron in GR/Q1 in Autumn 2018, it is unclear why concentrations were so low in the upper Discharge Monitoring sites. Only future monitoring will be able to determine whether this was an anomaly or a true decline in nickel concentrations.

In contrast to previous years, zinc concentrations were markedly elevated in recent years, especially in 2019. Again, it is difficult to determine whether this was due to the drought. Furthermore, it is unclear why the trend was so dissimilar to nickel concentrations, with both metals generally producing similar patterns, albeit at different concentrations.

Given the overriding conditions of the drought, it is hard to draw any definitive conclusions about whether water chemistry has improved or declined. This is especially true when comparing the Reference and Discharge Monitoring treatments, as there was no flow in the Reference sites, and flow in the Discharge Monitoring sites is likely primarily from the mining activity.

## 4.5 Macrobenthic surveys (2018-2019)

To reiterate, macrobenthic surveys were performed during a period of extreme and prolonged drought. In particular, the Reference sites had no flow and in some cases were completely dry (e.g. GR/UFS in Autumn 2018). At the time of sampling minimal flow did occur in the upper Discharge Monitoring sites, however, these sites contained mining discharge, and consequently the study is no longer comparing non-discharge to discharge. While there was evidence that abundance was generally lower in the Reference sites (Figure 13), as with previous years, it was highly variable. The pattern was similar for richness. Given that drought causes a decline in richness and abundance, and dramatically alters composition as well as water chemistry (Boix et al., 2010), no firm conclusions can be drawn from the abundance and richness data for 2018-2019. Furthermore, as suggested by Chariton et al. (2016) and by Chariton and Stephenson (2018), both metrics are flawed indicators of health and should be disregarded in future monitoring events.

Consistent with previous years, there were marked differences in the macrobenthic communities between the Reference and Discharge treatments in Autumn and Spring 2018 and 2019 (Figure 15). In general, a greater abundance of Leptophlebiidae in the Reference treatment, and greater abundances of chironomids and Caenidae in the Discharge Monitoring treatment were key in discriminating between the two treatments. Leptophlebiidae have been identified as a potential indicator of health for this system, with the taxon considered to be pollution intolerant (SIGNAL=8) (Chessman, 2003). In contrast, chironomids are a well-known indicator of systems with a low biotic integrity (Chariton et al., 2016). As reported in previous years (Chariton and Stephenson, 2018), the Ephemeroptera Caenidae were more abundant in the Discharge monitoring treatment. This Family is considered to be moderately insensitive to pollution (SIGNAL=4) (Chessman, 2003).

Although there is disparity in the influence of the abiotic factors between the Reference and Discharge Monitoring treatments, with the former being more likely driven by a lack of water and flow; correlative analysis between the macrobenthic communities and water quality variables consistently demonstrated that pH explained a very large proportion of the total variation in the biological data. Although the findings showed that pH was the key driver, given the complexity of the discharge waters and the tight relationship between pH and metal bioavailability, we recommend viewing the discharge as whole rather than giving weight to any specific variable. Furthermore, the upper Discharge Monitoring sites (Point 10, Point 12 and Jutts) appeared to be shaped by a different composition of the discharge than the downstream sites (Pool 16, Pool 32 and GRQ18).

## 4.6 SIGNAL (2018-2019)

The overall reduction in SIGNAL scores for the Reference sites in 2018 and 2019 suggests that ecological conditions of these sites were degraded compared to previous years (Figure 20). As a result, almost half the sites were downgraded from ‘probable moderate pollution’ to ‘probable severe pollution’ (Table 7). This was most pronounced in GR/UFS in Autumn 2019, although the site’s condition improved greatly by Spring 2019. This is most likely due to the persistent effect of the drought, however, in 2019, it may be attributed to the high nickel concentrations.

While the Discharge Monitoring sites generally had lower SIGNAL scores than previous years, in most cases the decline was not as pronounced as in the Reference sites. This is most likely due to the availability of water associated with the discharge. However, both Point 10 and Point 12 had very low scores in Autumn 2019.

In general, the SIGNAL scores reflected the overall decline in the system. Given the effect was recorded across almost all sites, including the Reference sites, this suggests that it is likely associated with the dry conditions. However, as indicated in the strong correlation between benthos and water chemistry (see Section 4.5), the SIGNAL scores in the upper Discharge Monitoring sites are also likely influenced by the mine discharge.

## 4.7 Metabarcoding survey (Spring 2019)

The metabarcoding (DNA-profiling both eukaryote and prokaryote communities) survey performed in Spring 2019 clearly demonstrated the technique's capacity to capture a diverse range of taxa regardless of the environmental conditions. In contrast to the macrofauna survey, all samples contained several hundred taxa (OTUs), capturing a wide breadth of prokaryotes and eukaryotes.

This was the first time bacterial communities have been sampled for this monitoring program. While providing ecological information on the key bacteria which discriminated between the treatments is beyond the scope of this report, bacteria have been shown to be sensitive to metals and other stressors (Sutcliffe et al., 2019), and consequently, broad changes in their composition may provide an additional line of ecological evidence. The metabarcoded prokaryote data clearly showed that prokaryote composition was markedly different between the Reference and Discharge monitoring treatments (Figure 21). The prokaryote communities from Point 11 were unique, differing from the other two Reference sites. In the Discharge Monitoring treatment, there was a general transition in communities from Point 10 to GRQ18. As in the case of the traditionally obtained macrofauna, a large proportion of the variation in the prokaryote data was explained by pH, and to a lesser degree, conductivity and nickel. Given that bacterial composition is well known to be shaped (filtered) by environmental variables (Stoeck et al., 2018; Sutcliffe et al., 2019), the findings of this survey clearly validate the use of 16S rDNA metabarcoding as an ecological input.

The multivariate analysis of the eukaryote metabarcoding data clearly showed that that eukaryote composition of the Reference sites was markedly different to those sampled from the Discharge Monitoring sites (Figure 23). As in the case of the prokaryote data, Point 11 appeared to contain two unique communities. Furthermore, there was also a general gradient from Point 10/12 to GRQ18. A number of OTUs potentially indicative of each treatment were found (Table 9). However, it is emphasised that these results are only indicative of the time of sampling. In fact, for all treatments the potential indicator OTUs observed in Spring 2019 differed from those previously observed in 2017, 2015 and 2013 (Chariton and Stephenson, 2018; CSIRO, 2014 and 2016). This suggests that there is currently not enough data to firmly establish indicator OTUs associated with each treatment, however, this may be viable once additional surveys are performed.

As in the case of all other ecological lines of evidence (macrofauna and prokaryote), a large proportion of the variation (≈ 93%) in the metabarcoded eukaryote communities could be explained by the selected water quality parameters. The strongest correlates with eukaryote composition were pH and aluminium. These findings support the water chemistry analysis, with both variables being more elevated in the Discharge Monitoring sites.

Collectively, the metabarcoding results indicate that the elevated constituents within the discharge waters were altering both prokaryote and eukaryote composition in the Discharge Monitoring sites. This analysis is in congruence with the water chemistry data, with the Discharge Monitoring sites generally having higher concentrations of metals, nutrients and more alkaline waters than the Reference sites. Some difference in the underlying geology and habitat may have also contributed to these differences (CSIRO, 2016).

As emphasised throughout this report, the water chemistry from the Discharge Monitoring sites were complex, and the focus should be on the composition of the waters rather than any single environmental variable. With this in mind, the metabarcoding data indicated that the discharge waters were influencing the composition of the Discharge Monitoring sites when compared to the Reference sites. Furthermore, the influence of the discharge waters was more pronounced in the upstream Discharge Monitoring sites.

It is important to note that the dry conditions also likely influenced the prokaryote and eukaryote communities. However, data was consistently obtainable. With the exception of Point 11, the replicate samples from each site were all quite similar. This suggests that metabarcoding is a valid approach in dry conditions and is likely to produce more reliable data than macrofaunal surveys where numerous samples contained no individuals.

# 5 Conclusions

## 5.1 Long-term trends

The water quality data indicates that there has been an overall improvement over time. However, a large number of variables were still above, and in many cases, markedly exceeded water quality values. Specifically, conductivity, pH and metal concentrations remained elevated in the upstream Discharge Monitoring sites. This suggests a high likelihood that the discharge waters are impairing the biological integrity of the system, most notably in the upstream Discharge Monitoring sites. It should be noted that the catchment is also influenced by other activities. For example, the Reference site Point 11 is located downstream from the Appin Colliery, which sporadically discharges surfaces waters. While this may explain some of the variation within the Reference sites, the results of the previous campaigns indicate that Point 11 is more similar in composition to the other Reference sites, and for this reason should still be included as a Reference site. For most measurements, there was a clear increase in water quality with downstream distance, suggesting that the likelihood of ecological harm was comparatively lower in the most downstream site (GRQ18).

Examination of the macrobenthic data obtained between 2013 and 2019 showed that macrobenthic abundance was on average higher in the Discharge Monitoring treatment. However, it should be noted that this community attribute varied greatly within treatments and over time. As pollution-tolerant taxa can be frequently found in high abundances, it is our view that this is not a suitable end-point for monitoring the systems (Chariton et al., 2016). Similarly, richness has been shown to be a relatively insensitive metric for monitoring macrobenthic invertebrates and is often correlated with abundance (Chariton et al., 2016). Again, we suggest that further consideration should be given to the suitability of this end-point.

In contrast to total abundance and richness, SIGNAL was designed to focus the analysis on taxa which may be influenced by the ecological condition of the stream. The long-term SIGNAL scores suggest that Reference sites are in better ecological condition than the Discharge Monitoring sites, with the exception of the most downstream site, GRQ18, which had a lower score than the Reference sites, but was still sufficiently high to be classified as 'probable moderate pollution'. Given that SIGNAL is designed specifically for Australian taxa and captures the specific tolerances of taxa rather than aggregating them purely on their taxonomy, we strongly recommend that this approach is continued.

Analysis of the Leptophlebiidae genera *Atelophlebia*, *Ulmerophlebia* and *Koornonga* clearly showed that between 2016 and 2019 these taxa were more abundant and more frequently observed in the Reference sites. However, *Atelophlebia* appears to be occurring more frequently in the most downstream Discharge Monitoring site, GRQ18. While Leptophlebiidae data adds an additional line of evidence, it is arguably redundant, with the overall trend in Leptophlebiidae being detected in the SIGNAL results as well as in the multivariate analyses. While it has been recommended that species-level identification of this group should be used in future monitoring programs (The Georges River Environmental Alliance), it is yet to be ascertained if this is necessary given the sensitivity of the Family as a whole. Furthermore, there are no details regarding the ecotoxicology of the three genera.

Since April 2016, there has been a decline in the toxicity of the waters for both the *Paratya australiensis* 10-day acute and *Ceriodaphnia dubia* survivorship assays; with the toxicity most recently being at 1 toxic unit for both assays. The Point 10 waters still induce a sub-lethal effect using the *Ceriodaphnia dubia* chronic reproduction test, suggesting that Point 10 waters still pose a risk to aquatic biota. Only one battery of ecotoxicological testing (Spring 2019) has been performed since the last report. Given the variability of the endpoints, especially the *Ceriodaphnia dubia* chronic reproduction test, it is not possible to determine whether there has been an overall decline in toxicity, i.e. a reduction in toxicity for all assays. Consequently, we strongly suggest the ecotoxicological testing is performed more frequently, for example, every six months. This is supported by the water chemistry data which also varies greatly over time.

## 5.2 Weight of Evidence (2013-2019)

Table 10 provides a summary of the long-term macrobenthic community, water quality and ecotoxicological data obtained between 2013 and 2019. While we have concerns about the suitability of some of the community end-points, e.g. abundance and richness, there is sufficient correlative evidence from the SIGNAL index to infer that the discharge is altering the communities within the Discharge Monitoring sites. However, the effect of the discharge on these community attributes is more pronounced in the upstream sites Point 10 and Point 12. When combined with the water quality and ecotoxicology data, the evidence strongly suggests that the discharge waters pose a hazard to the benthic communities and other aquatic biota. However, without ecotoxicological testing of downstream sites, the full spatial extent of this impact cannot be elucidated within a weight of evidence framework.

**Table 10. A summary of multiple lines of evidence obtained between 2013 and 2019.**

| Evidence | Attributes | Evidence | Summary |
|---|---|---|---|
| **Macrobenthic communities** | Abundance | Abundance was higher in Discharge Monitoring treatment than other treatments. Varied greatly within and between treatments over time. | Abundance is not a robust measure of environmental stress. |
| | Richness | Richness was similar between all treatments. | Richness is not a robust measure of environmental stress. |
| | SIGNAL | SIGNAL scores were higher in the Reference sites. | Reference sites are in better ecological condition than the Discharge Monitoring sites. However, they have declined in recent years (2018/2019) - most likely drought related. |
| | Leptophlebiidae | This group was far more abundant and frequent in Reference sites. *Kooronga* was only observed in the Reference treatment. | Suggest that this group is sensitive to the discharge waters. However, most Discharge Monitoring sites appear to be also unsuitable for the taxa. |
| **Water chemistry** | Conductivity, pH, metals and nutrients | Overall decline, however, conductivity, pH and metals remain high, and in many cases very high, in the upstream Discharge Monitoring sites. | Water quality in upstream Discharge Monitoring sites is sufficiently poor to cause biological impairment. The effects of the discharge diminish with downstream distance. Unusual results for 2018/2019, most likely drought related. |
| **Ecotoxicology** | 7 tests | For two assays, toxicity has declined in Point 10 waters, however, the waters still affect *Ceriodaphina* reproduction. | Point 10 waters still elicit a sub-lethal toxicological response, (*Ceriodaphina* reproduction). Therefore still poses a risk to aquatic biota. |

## 5.3 2018-2019 surveys

It is emphasised that both 2018 and 2019 were unusual years for the monitoring program, with all four sampling events occurring during a prolonged period of drought. Consequently, the water chemistry and ecological findings from this period may differ from previous years.

There is a general agreement between all approaches that the effect of the discharge was more pronounced in the upstream Discharge Monitoring sites. Furthermore, the environmental variables shaping the downstream Discharge Monitoring sites did not appear to be the same as those driving the communities within the Reference treatment. While it is noted that the discharge has been substantially diluted, most notably in late December 2016, the waters from the upstream Discharge Monitoring sites consistently exceeded the ANZG (2018) values for a range of variables. Given the relatively brief period since the dilution and the sustained effects of the drought, it is not possible to determine whether the dilution has had a significant positive effect on the communities. It is emphasised that recovery will likely be slow and may result in communities which will still be markedly different from those associated with the Reference treatment (Chariton et al., 2016).

Collectively, the macrobenthic and metabarcoding surveys for 2018 and 2019 support the findings of previous reports (CSIRO 2014, 2016; Niche 2014, 2016; Chariton and Stephenson, 2018), providing strong correlative evidence that the discharge is altering the composition of macrobenthic biota within the Discharge Monitoring treatment. This is supported by multiple lines of ecological evidence, including SIGNAL scores, macrobenthic community structure, metabarcoded prokaryote and eukaryote community structure, and correlative patterns between the communities and water quality measurements. Undoubtedly drought has had a major influence on the catchment, although this is likely to be more pronounced in the Reference sites where there was no flow.

## 5.4 Recommendations

- Given the overriding influence of the drought, more details about flow and clearer site description parameters which use semi-quantitative endpoints is required. Currently, field notes are very brief, purely descriptive and lack formal classification.

- Leptophlebiidae should not be analysed at the genus level as there is a lack of scientific literature at the sub-family level. Family level is sufficient as it is captured in both the SIGNAL and multivariate analysis.

- Semi-quantitative measurements of habitat quality should be included in future surveys to assist in identifying the role habitat is playing on the observed differences between the treatments. Furthermore, this may assist in identifying remedial solutions to assist in the ecological recovery of the system.

- Ecotoxicological testing of Point 10 needs to be performed more frequently, e.g. every six months.

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