Georges River Environment Improvement Program (EIP2)

Prepared for: Illawarra Coal/South32

Commercial-in-confidence

14 April, 2018

Dr Anthony Chariton[1,2] and Sarah Stephenson[2]

[1]Environmental Genomics, Ecology and Ecotoxicology Lab (EGEEL)

Department of Biological Sciences

Macquarie University

[2] CSIRO Oceans and Atmosphere, Lucas Heights, New South Wales

ENVIRONMENTAL GENOMICS, ECOLOGY & ECO TOXICOLOGY LAB

MACQUARIE University SYDNEY · AUSTRALIA

Citation

CSIRO (2018). Georges River Environment Improvement Program (EIP2). Prepared for Illawarra Coal/South32. CSIRO Oceans and Atmosphere.

All enquiries should be made to Dr Anthony Chariton, Macquarie University: anthony.chariton@mq.edu.au

Contents

Figures

Tables

Executive summary

South32/Illawarra Coal proposes to continue its underground mining at West Cliff mine by extracting coal from the Bulli Seam using longwall mining techniques. Under the Commonwealth Environmental Protection and Biodiversity Conservation Act 1999 (EPBC Approval 2010/5350) a Project Approval for the Bulli Seam Operations was granted by the Department of Environment, Climate Change and Water (DECCW), now known as the NSW Office of Environment and Heritage (OEH). 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 Illawarra Coal'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 George's 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 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 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 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 (2013-2017) in macrobenthos communities, water quality and ecotoxicology data. And secondly, focusses on the macrobenthic (autumn and spring) and metabarcoding surveys (spring) performed in 2017 (summarized in section 1.2.2.). In addition, the report aims to summarize this information within a weight of evidence framework drawing upon the collective results on the community, water quality and ecotoxicological data, and provides recommendations to assist in potentially refining the program. To aid comparisons, in accordance with the EIP2 the macrobenthic and metabarcoding data were examined as three treatments: **Reference**, 3 sites prior to the mine's influence; **Discharge Monitoring**, 6 sites which capture the gradient from the mine; and **Downstream Discharge Monitoring**, 2 sites not directly associated with the Discharge Monitoring gradient.

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

The two indices, EPT % and SIGNAL, which are designed to focus the analysis on the sensitivity of taxa to varying ecological conditions were also examined. For both indices, there were marked differences between the Reference and Discharge Monitoring treatments, indicating a lower level of ecological integrity in the Discharge Monitoring sites. In particular, the SIGNAL scores suggested that the ecological integrity of the system improved with downstream distance. However, due to high variability no clear temporal trend were evident.

Collectively, the long-term macrobenthic data indicates that the discharge waters are impairing macrobenthic communities, with the effect being more pronounced in the upstream sites Point 10 and Point 12. However, given the variability of the data, it remains unclear if there have been any significant changes in the composition of macrobenthic communities since the conductivity of the discharge waters was reduced from 2500 to 2000 µS/cm.

The long-term trends in water chemistry showed that conductivity and the concentrations of aluminium, nickel, zinc and ammonia generally declined overtime. 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 ecotoxicological tests on the Point 10 discharge waters shows that historically the waters were toxic. The findings also indicate that the reduction in conductivity has had no significant influence on the *Paratya australiensis* 10-day acute and *Ceriodaphnia dubia* reproduction tests, with the latter being particularly sensitive. However, survivorship of *Ceriodaphnia dubia* appears to have improved.

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. However, without ecotoxicological testing of downstream sites the full spatial extent of this impact cannot be determined. Therefore we recommend that Discharge Monitoring site GRQ18 be included in future ecotoxicological tests.

The macrobenthic and metabarcoding surveys for 2017 support the findings of previous surveys, providing strong correlative evidence that the discharge was altering the composition of macrobenthic biota within the Discharge Monitoring treatment. This is supported by multiple lines of ecological evidence, including EPT%, SIGNAL scores, macrobenthic community structure, metabarcoding community structure, and correlative patterns between the communities and water quality measurements.

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 do not appear to be the same as those driving the communities within either the Reference or Discharge Monitoring treatments. 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 still consistently exceeded the ANZECC/ARMCANZ (2000) trigger values for a range of metrics. Given the relatively brief period since the dilution, and the high inter-and intra-variability in the data, it is not currently 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

South32/Illawarra Coal proposes to continue its underground mining at Appin mine by extracting coal from the Bulli Seam using longwall mining techniques. Under the Commonwealth Environmental Protection and Biodiversity Conservation Act 1999 (EPBC Approval 2010/5350) a Project Approval for the Bulli Seam Operations was granted by the Department of Environment, Climate Change and Water (DECCW), now known as the NSW Office of Environment and Heritage (OEH). 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 monitoring program for Illawarra Coal'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. However, the reporting deadlines was altered to the 31st 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 (details on this approach can be found in Appendix B);
- In-stream water quality testing; and
- Laboratory ecotoxicological testing of the discharge water from Point 10.

The complete requirements of the AHMP are documented in EPL 2504.

Given the community's high value for the George's 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 ecotoxicological assays.

1.2 Objectives of this report

This report examines the biotic data obtained for the EIP2 in two sections. Firstly, it provides an overview of the long-term trends (2013-2017) in macrobenthos communities and ecotoxicology data (summarized in section 1.2.1). And secondly, focusses on the macrobenthic (autumn and spring) and metabarcoding surveys (spring) performed in 2017 (summarized in section 1.2.2.). In addition, the report aims to summarize this information within a weight of evidence framework drawing upon the collective results on the community and ecotoxicological data, and provides recommendations to assist in potentially refining the program.

1.2.1 Long-term trends (2013-2017) were examined by:

1. Summarizing the overall trends in macrobenthic invertebrate abundance and Family richness;

2. Analysing and interpreting long-term patterns in EPT % scores. This approach compares the condition of sites based on their relative abundances of aquatic insects from the Orders Ephemeroptera (mayflies); Plecoptera (stoneflies); and Trichoptera (caddisflies). The underpinning assumption is that a greater proportion of EPT taxa will be in sites of higher quality;

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*) (2016-2017 only)

5. Analysing long-term compositional patterns in macrobenthic invertebrates;

6. Examining long-term patterns in key water quality parameters; and

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

1.2.2 2017 surveys were examined by:

- Summarizing the water quality measurements obtained in autumn and spring;

- Exploring trends in macrobenthic invertebrate abundance and richness from samples obtained in autumn and spring;

- Assigning EPT % and SIGNAL scores to 2017 macrobenthic invertebrate data;

- Exploring compositional patterns is macrobenthic invertebrate communities sampled in autumn and spring;
- Exploring correlative relationships between water chemistry and macrobenthic communities;
- Exploring compositional patterns in the metabarcoding data; 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 GRQ/1 and runs for 21 km to Site GR/OH, just downstream of the confluence with O'Hares Creek (Figure 1). Sites GR/OH and GRQ19 are downstream of the West Cliff licensed discharge Point 10 (Table 1).

The experimental design consists of three treatments (Table 1):

- **Reference (3 sites)** – GRQ/1, GR/UFS and Point 11;
- **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; and
- **Downstream Discharge Monitoring (2 sites)**, these sites are not directly associated with the Discharge Monitoring gradient– GRQ19 and GR/OH. GRQ19 is upstream of Spring Creek and the confluence with O'Hares Creek, receiving storm water inflows from Campbelltown. GR/OH is slightly downstream of the O'Hares Creek confluence, and is therefore more influenced by the natural surrounding catchment.

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. Consequently, these sites are not included in the analysis.

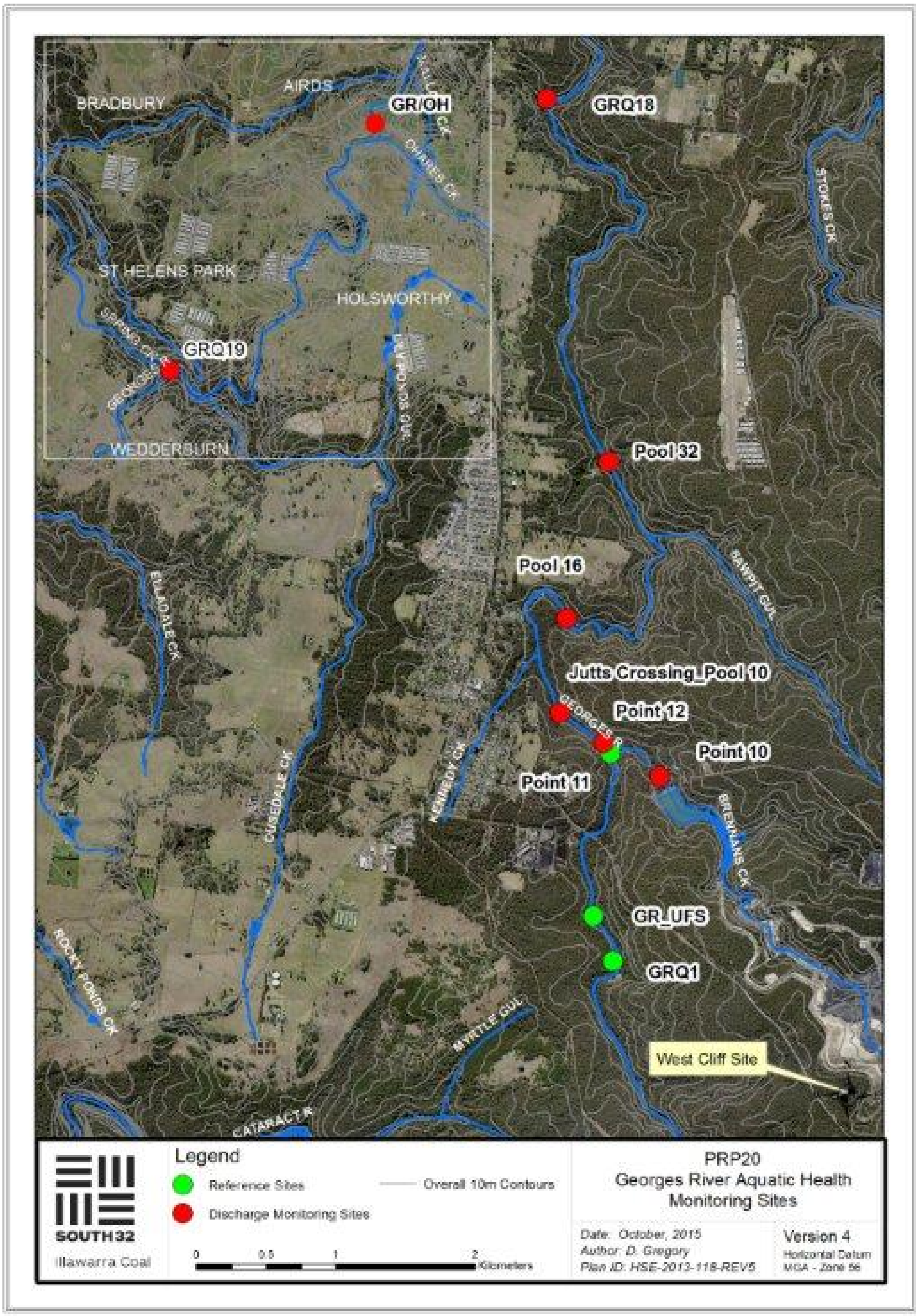

Figure 1. Location of sampling sites. Reference sites = GR/Q1, GR/UFS and Point 11; Discharge Monitoring sites = Jutts Crossing_Pool10, Point 10, Point 12, Pool 16, Pool 32, GRQ18; Downstream Discharge Monitoring sites = GRQ19 and GR/OH.

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
GRQ19	Georges R.	U/S of Spring Ck	298747	6223615	Downstream Discharge Monitoring
GR/OH	Georges R.	D/S of O'Hares confluence	300156	6225390	Downstream Discharge Monitoring

2.2 Macrobenthos sampling

On all occasions (Spring 2013 - Spring 2017), macroinvertebrates were sampled from three random pool edges at each site and combined giving one sample at each site (Downs et al. 2002). 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 2017.

Sampling for the 2017 macrobenthic surveys was performed in Autumn (8-11th May) and Spring (17-19th October) using the protocol described above.

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 2017 macrobenthic survey. At each site, five sediment samples were collected from the soft-sediments 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, two reference samples containing crocodile (*Crocodylus porosus)* and the marine mussel (*Mytillus edulis*) 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.

For each sediment sample, three identical replicate polymerase chain reaction (PCR) amplifications of a 200-350-bp fragment of the 18S rRNA gene were carried out with the 'universal' primers All18SF-TGGTGCATGGCCGTTCTTAGT and All18SR-CATCTAAGGGCATCACAGACC (Hardy et al., 2010), using the AmpliTaq (Thermo Fisher Scientific, Waltham, MA USA) modified PCR protocols and conditions described by Baldwin et al. (2013). Subsequent to amplification, pooled PCR products were purified using the QIAGEN QIAquick® PCR purification kit (QIAGEN®, Germany). Amplification and purification success was interrogated on a MultiNA gel. The three final amplicon library concentrations were measured on the Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). The three pooled libraries of 62 samples 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 MOTU (Molecular 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 MOTU 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 MOTU 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 MOTU sequences to obtain accurate read counts for each MOTU/sample pairing. The classified MOTUs and the counts for each sample were finally used to generate MOTU tables in both text and BIOM (v1) file formats, complete with taxonomic classifications, species assignments and counts for each sample. All MOTUs with a maximum read abundance of 50 reads, or that were only observed in less than four biological replicate were removed.

2.4 Ecotoxicological testing

Between 2013 and 2017 a range of ecotoxicological assays were performed using discharge waters derived from the Downstream Discharge Monitoring site Point 10. All tests were performed by Ecotox Services Australasia. A summary of the tests is provided in Table 2. The provided results were summarized in terms of: Effective concentrations (EC), concentrations that has a sub-lethal effect on 10,25 and 50 % of the test organisms; Inhibiting concentrations (IC), concentrations that inhibits or impairs a biological function of 10 and 25 % the test organisms; LOEC, lowest observed effect concentration where there was an observable impact that was significantly different from control; and NOEC, no observed effect concentration - concentration where there is no observable impact that is significantly different to the control.

Based on discussions with CSIRO Land and Water's Ecotoxicology Team, all unreliable tests were identified and removed from the analysis. To enable direct comparisons between the tests, 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).

From April 2016, the ecotoxicological testing was reduced to three assays (*Paratya australiensis* acute, and *Ceriodaphnia dubia* survival and reproductive impairment). Additional analysis was performed on these three assays to identify potential correlations between their toxicity units and the conductivity of the test waters.

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 s
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. *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 (macrobenthic Autumn and Spring 2017 and metabarcoding Spring 2017), 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 (2013-2017) 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. Differences in long-term (2014-2017) mean abundances and Family richness between treatments 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. All univariate analysis were performed using NCSS v8 (Utah, USA).

Multivariate analysis of the macrobenthos data was performed using the Primer 7+ statistical package (Plymouth Marine Laboratory, UK). Ordination was 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) using the same design as the ANOVAs. Differences between treatments were identified by pairwise a posteriori tests based on 9,999 random permutations.

2.6.2 EPT and SIGNAL

Using the data provided by the client, EPT % scores were calculated for each site at each time point. EPT is named after the three orders of aquatic insects which are used in the index: Ephemeroptera (mayflies); Plecoptera (stoneflies); and Trichoptera (caddisflies). The underpinning assumption is that the proportion of EPT taxa will be higher in sites of higher quality (Barbour and Stribling, 1991). The following formula was used to calculate EPT % scores

EPT % = (the abundance of EPT taxa / total abundance) x 100.

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, 2003). 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.

The statistical analysis of the complete dataset for both EPT % and SIGNAL scores are as described for the long-term abundance and richness data. As part of the EIP's requirement to enable a balanced comparison between the Reference and Discharge Monitoring treatments (South32, 2017 see Table 5) additional statistical analysis (ANOVAs) were performed between the three Reference sites and three of the six Discharge Monitoring sites (Point 12, Pool 32 and GRQ18). These additional analyses were performed on the long-term data set as well as the data obtained in Autumn and Spring 2017.

2.6.3 Macrobenthos data (Autumn and Spring 2017)

Because of the low number of replicates, no formal statistics were performed on the univariate attributes (abundance and Family richness) for the macrobenthic invertebrate samples obtained in Autumn and Spring 2017. Consequently, all univariate comparisons between treatments are purely derived from 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, total phosphorus and dissolved organic carbon. 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 2017)

As there is a weak statistical relationship between the number of sequence reads and organism biomass or abundance (Egge et al., 2013), all OTU data were converted to presence/absence prior to computation (Chariton et al., 2010). Biological replicates were obtained from the sums of the PCR (technical replicates). The ordination of the OTU data was performed by non-metric multidimensional scaling (nMDS) using the Jaccard similarity coefficient, as was the PERMANOVA analysis. The relationships between eukaryote communities and environmental variables were examined using distance-based linear models (DISTLM) as previously described in section 2.6.3. Potential indicator OTUs for each treatment (Reference, Discharge Monitoring and Downstream Discharge Monitoring) were identified using the R package *Indispecies*.

3 Results

3.1 Long term patterns in macrobenthic community attributes

3.1.1 Abundance and richness (2013-2017)

Long-term abundance patterns for all sites sampled between 2013 and 2017 are illustrated in Figure 2. The abundance of macroinvertebrates varied greatly between sites and across sampling events. In general, the Discharge Monitoring sites (133 ± 12 S.E.) had a higher mean abundance than both the Reference (79 ± 16 S.E.) and Downstream Discharge Monitoring sites (66 ± 21 S.E.) (F=5.85, P<0.004). It is emphasised that this finding should be taken cautiously given the sample size, unbalanced design and high variability.

The mean Family richness for all sites sampled between 2013 and 2017 are illustrated in Figure 3. Mean Family richness was similar in all treatments, with no significant difference (F= 0.47, P = 0.626) detected between the Reference (12.31 ± 1.05 S.E.), Discharge Monitoring (13.53 ± 0.76 S.E.) and Downstream Discharge Monitoring (12.83 ± 1.39 S.E.) treatments.

The ordination plot in Figure 4 summarizes the macrobenthic communities from all samples obtained between 2013 and 2017. The over-arching trend throughout the sampling program is that the composition of macrobenthic invertebrates from the Reference treatment differ to those from both the Discharge Monitoring and Downstream Discharge Monitoring treatments (PERMANOVA: F_{psuedo}=12.1, P=0.01). While different to each other, macrobenthic communities from the Discharge Monitoring treatment were more similar to the Downstream Discharge Monitoring treatment than they were to the Reference treatment. Two samples (Point 10 and Point 11) obtained in August 2017 appear to stand out from all the other samples, with both of these samples also having relatively low abundances and richness (Figure 2 and Figure 3).

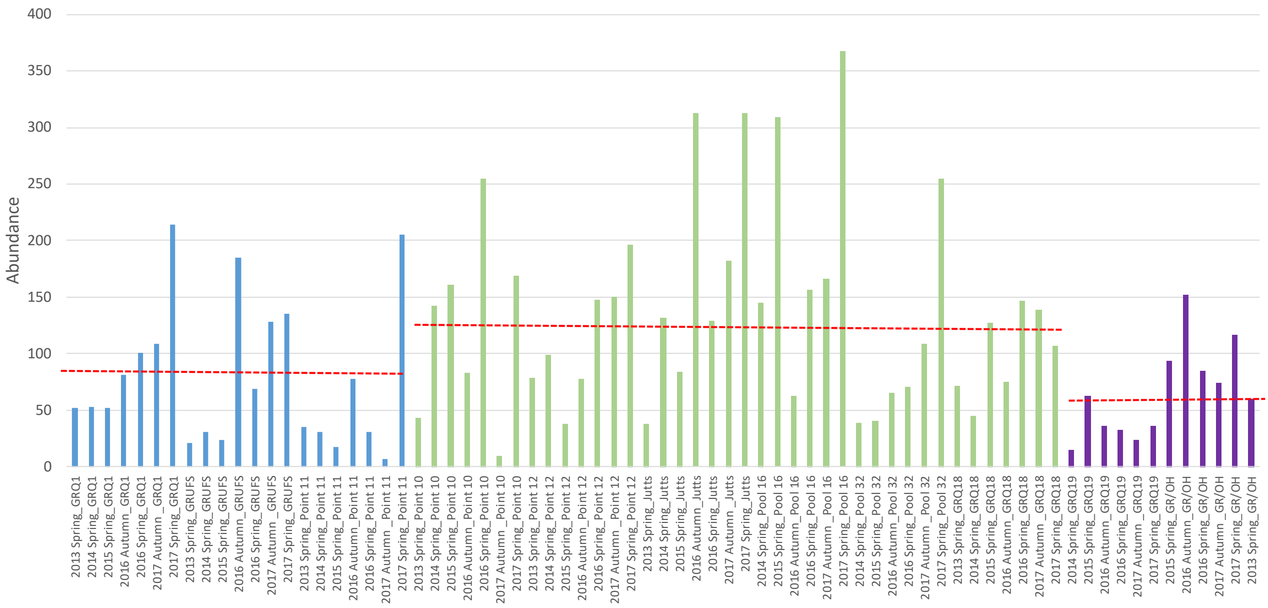

Figure 2. Long-term abundance patterns in macrobenthos (2013-2017). Sites were place into three treatments: Reference (blue); Discharge Monitoring (Green) and Downstream Discharge Monitoring (Purple). Dotted red lines represent the mean value for each treatment.

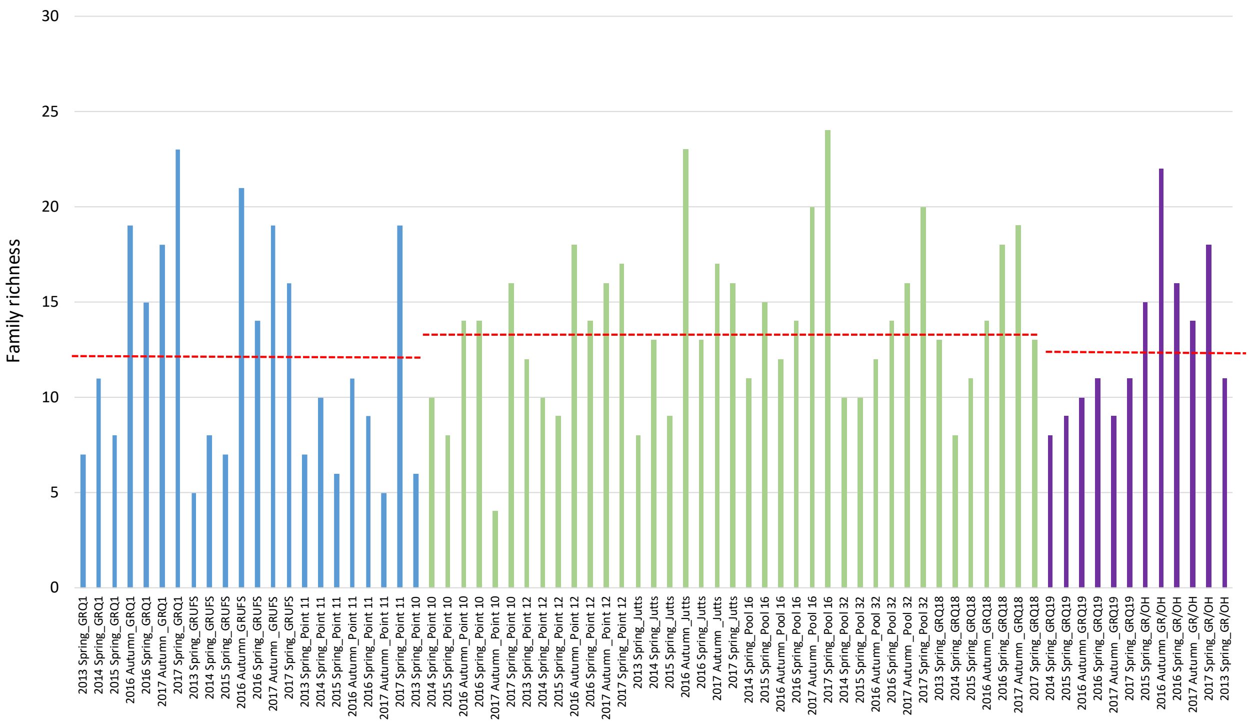

Figure 3. Long-term Family richness patterns in macrobenthos (2013-2017). Sites were place into three treatments: Reference (blue); Discharge Monitoring (Green) and Downstream Discharge Monitoring (Purple). Dotted red lines represent the mean value for each treatment.

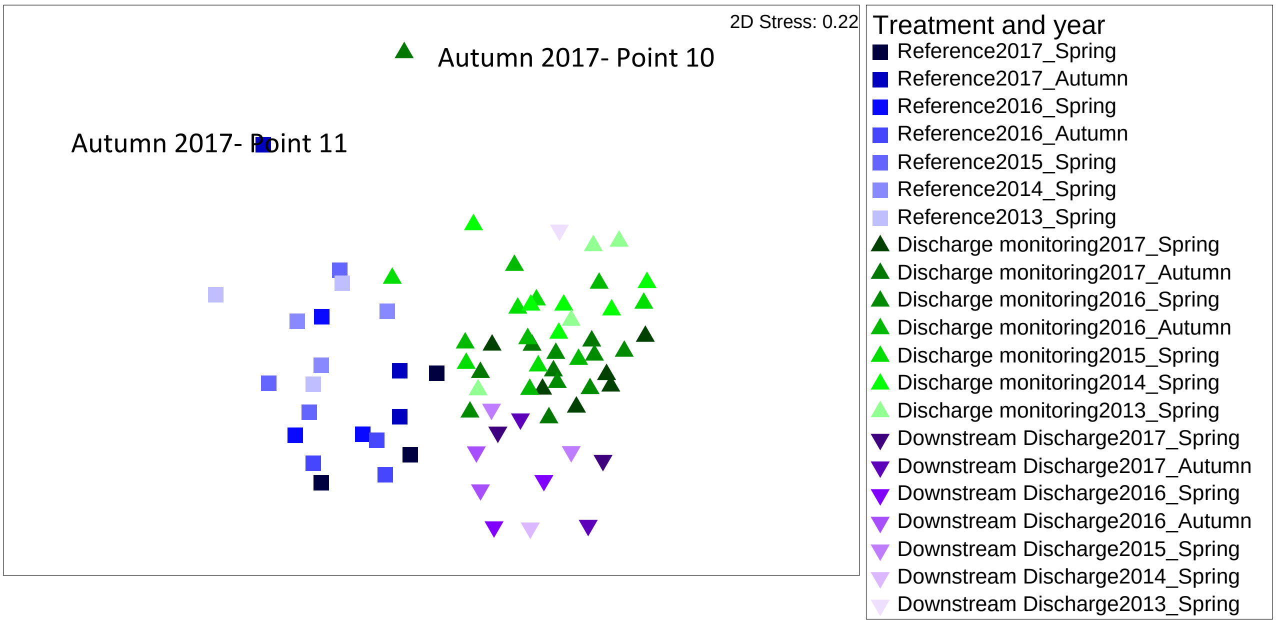

Figure 4. Long-term compositional patterns in macrobenthos. Blue= Reference sites, Green= Discharge Monitoring Sites and Purple = Downstream Discharge Monitoring sites. Later years are darker than earlier years.

3.1.2 EPT (2013-2017)

A summary of the mean EPT % scores for each site sampled between 2013 and 2017 are summarized in Figure 5. When examined collectively (2013-2017), the mean EPT % for the Reference treatment was significantly greater (56.8 % ± 3.1 S.E) than the Discharge Monitoring treatment (30.5 % ± 2.5 S.E), with both treatments having a greater mean EPT % than the Downstream Discharge Monitoring treatment (18.7 % ± 4.6 S.E) (ANOVA: F=26.69, P<0.001). The figure also suggest that the EPT % is increasing with downstream distance from the discharge point, with this being most notable in GRQ18.

A summary of the EPT % for each site averaged across all years is provided in Table 3. It is worth noting that EPT % varied greatly within sites across time. For example, the Reference site Point 11, which had a mean EPT % of 50, also had EPT scores ranging from 24 to 83 %. In the Discharge Monitoring treatment, EPT % scores generally increased with downstream distance.

The reduced analysis comparing three site each from the Reference and Discharge Monitoring treatments found that the long-term mean EPT % for the Reference treatment (56.8 % ± 2.94 S.E.) was significantly greater than the Discharge Monitoring treatment (36.6 ± 3.01 S.E.) (ANOVA: F=23.09, *P*<0.001).

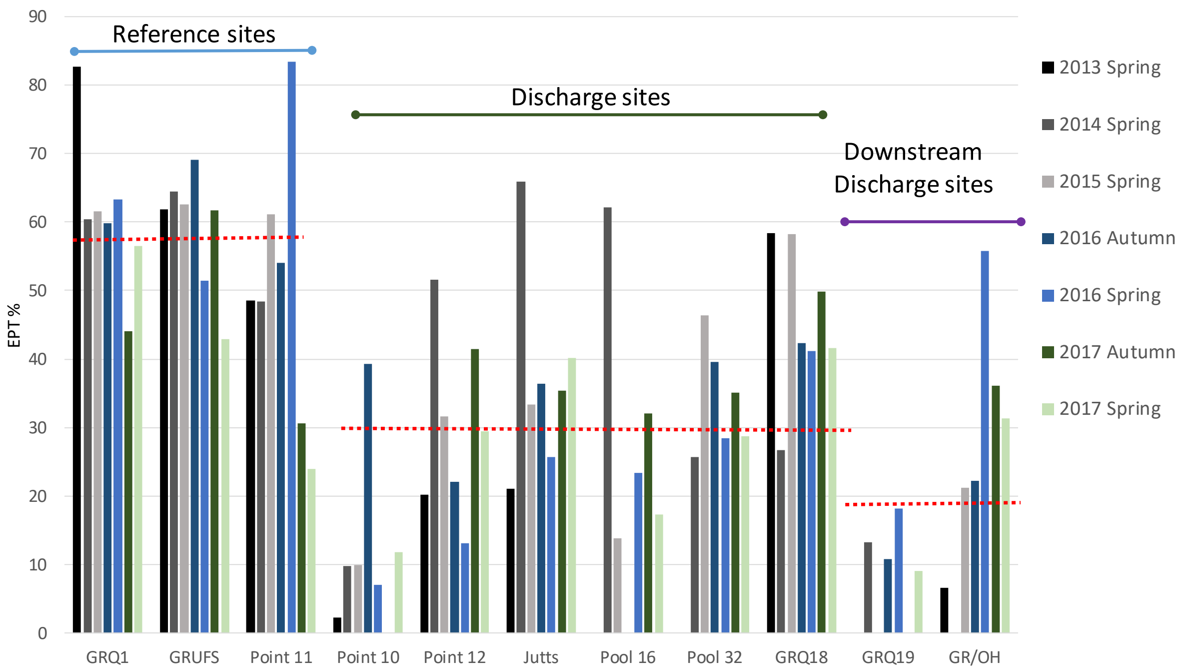

Figure 5. Mean EPT % for each site on each sampling occasion. The red dash lines represent the mean EPT % for each treatment over the entire sampling period

Table 3. Mean EPT % scores of sites from Spring 2013-Spring 2017

Treatment	Site	Mean (S.E)	Min	Max
Reference	GRQ1	61.2 (4.3)	44.1	82.7
Reference	GRUFS	59.2 (3.4)	42.9	69.1
Reference	Point 11	50.0 (7.4)	24.0	83.3
Discharge Monitoring	Point 10	11.5 (4.9)	0.0	39.3
Discharge Monitoring	Point 12	29.9 (5.0)	13.1	51.5
Discharge Monitoring	Jutts	36.8 (5.4)	21.1	65.9
Discharge Monitoring	Pool 16	24.8 (8.6)	0.0	62.1
Discharge Monitoring	Pool 32	34.0 (3.2)	25.6	46.3
Discharge Monitoring	GRQ18	45.5 (4.2)	26.7	58.3
Downstream Discharge Monitoring	GRQ19	8.6 (3.0)	0.0	18.2
Downstream Discharge Monitoring	GR/OH	28.9 (6.8)	6.7	55.8

3.1.3 SIGNAL (2013-2017)

Long-term SIGNAL scores for all sites sampled between 2013 and 2017 are illustrated in Figure 6. When examined collectively at the treatment level, the Reference treatment (mean = 5.16 ± 0.14 S.E.) had a significantly greater mean SIGNAL score than both the Discharge Monitoring (mean = 3.96 ± 0.10 S.E.) and Downstream Discharge Monitoring treatments (mean = 4.25 ± 0.19 S.E.) (ANOVA: F=24.3, P<0.001). No difference in mean SIGNAL scores were found between the Discharge Monitoring and Downstream Discharge Monitoring treatments. Based on the classifications by Chessman (1995), this arbitrarily suggests, that on average, the Reference sites can be considered to be of “doubtful quality, possible mild pollution”; the Discharge Monitoring sites generally ranged from “probable severe pollution” to “probable moderate pollution”; and the Downstream Discharge sites are “probable moderate pollution”.

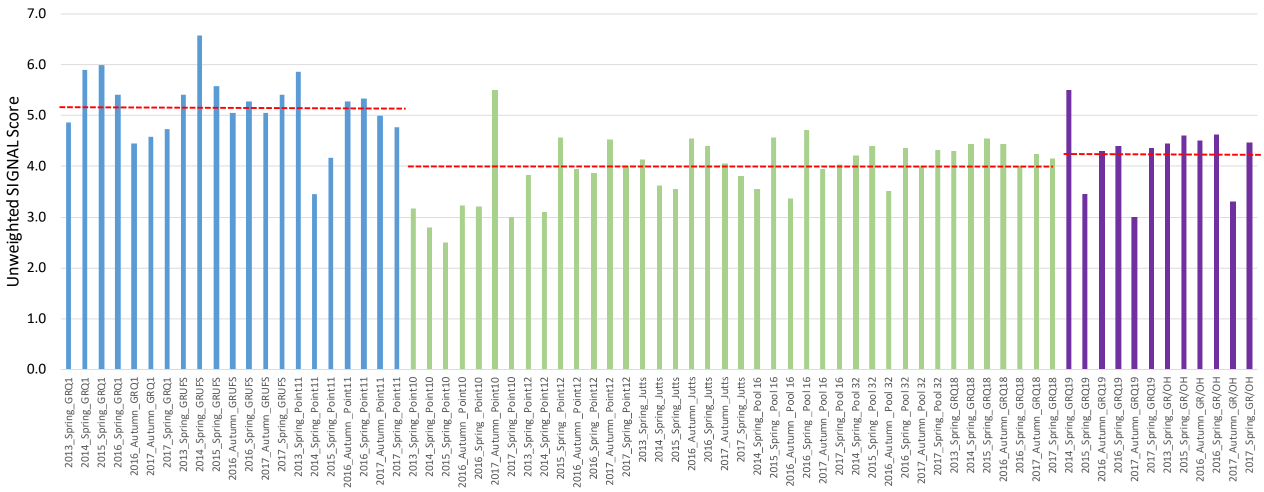

Figure 6. Long-term SIGNAL patterns (2013-2017). Sites were place into three treatments: Reference (blue); Discharge Monitoring (Green) and Downstream Discharge Monitoring (Purple). Dotted red lines represent the mean value for each treatment.

All Reference sites had greater mean SIGNAL scores than the Discharge Monitoring and Downstream Discharge Monitoring sites (Table 4). However, scores within sites varied greatly over time. For example, the SIGNAL score for the Reference Site Point 11 ranged from 3.44 to 5.86, similarly the Discharge Monitoring site Point 10 ranged from 2.5 to 5.5. The mean SIGNAL scores for the Downstream Discharge Monitoring sites were towards the higher end of the Discharge Monitoring sites. As with EPT %, SIGNAL scores from the Discharge Monitoring treatment appeared to increase with distance from the discharge source, with the "probably severe pollution" ranking restricted to Point 10 and Point 12.

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 (5.16 ± 0.12 S.E.) was significantly greater than the Discharge Monitoring treatment (4.13 ± 0.13 S.E.) (ANOVA: F=34.45, P<0.001).

Table 4. Mean SIGNAL scores for each site (2013-2017). *Potential rankings based on Chessman (1995).

Treatment	Site	Potential ranking*	Mean SIGNAL	Standard Error	Minimum	Maximum
Reference	GRQ1	Doubtful quality, possible mild pollution	5.13	0.24	4.44	6.00
Reference	GRUFS	Doubtful quality, possible mild pollution	5.48	0.20	5.05	6.57
Reference	Point11	Probable moderate pollution	4.84	0.30	3.44	5.86
Discharge Monitoring	Point10	Probable severe pollution	3.34	0.37	2.50	5.50
Discharge Monitoring	Point12	Probable severe pollution	3.97	0.19	3.10	4.56
Discharge Monitoring	Jutts	Probable moderate pollution	4.01	0.14	3.56	4.55
Discharge Monitoring	Pool 16	Probable moderate pollution	4.03	0.22	3.36	4.71
Discharge Monitoring	Pool 32	Probable moderate pollution	4.13	0.14	3.50	4.40
Discharge Monitoring	GRQ18	Probable moderate pollution	4.30	0.07	4.00	4.55
Downstream Discharge Monitoring	GRQ19	Probable moderate pollution	4.17	0.35	3.00	5.50
Downstream Discharge Monitoring	GR/OH	Probable moderate pollution	4.33	0.21	3.31	4.63

3.1.4 Leptophlebiidae genera of interest (2016-2017)

As indicated in Figure 7, both the abundance and the occurrence of all three genera were higher in the Reference treatment than either the Discharge Monitoring and Downstream Discharge Monitoring treatments. *Kooronga* was only observed in the Reference treatment. All three taxa were absent from the Discharge Downstream sites Point 10, Point 12 and Pool 16, with *Atelophlebia* and *Ulmerophlebia* being rarely observed in the other sites, most notably in the most downstream site (GRQ18). *Atelophlebia* and *Ulmerophlebia* were not sampled in the Downstream Discharge Monitoring site GRQ19.

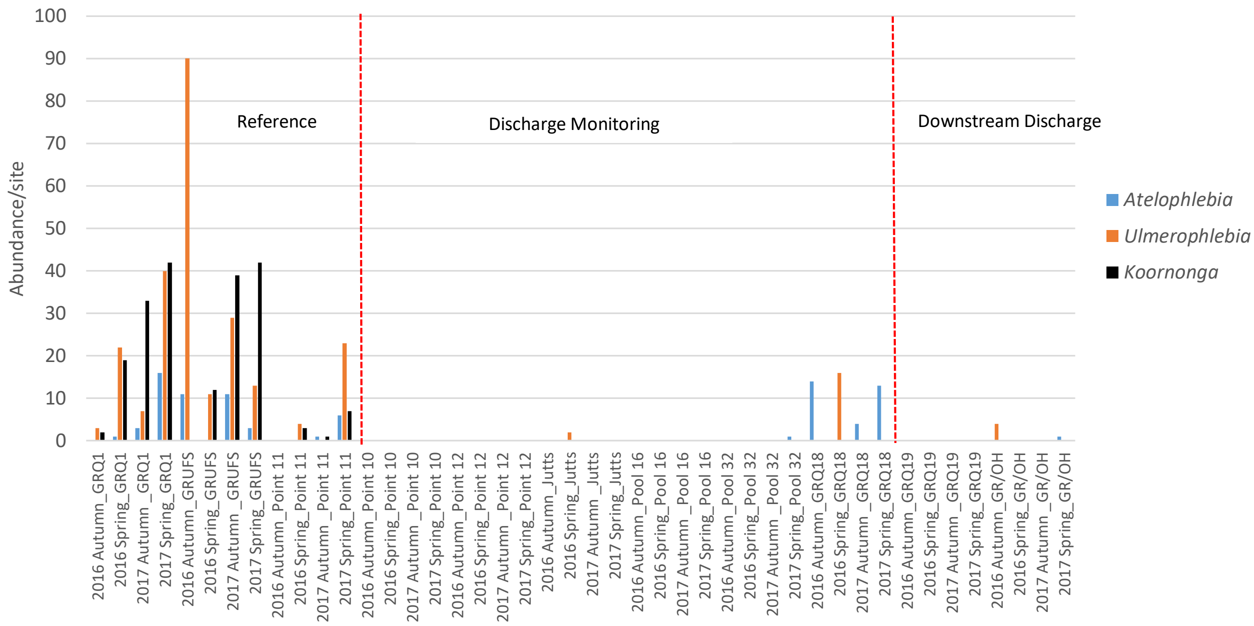

Figure 7. Abundances of *Atelophlebia* spp, *Ulmerophlebia* spp and *Koornonga* spp (2016-2017). The dotted vertical line separates sites from the Reference, Discharge Monitoring and Downstream Discharge Monitoring treatments.

3.2 Water chemistry

In this section we describe the long-term (2013-2017) trends in the key water quality variables: pH, conductivity, aluminium, nickel, zinc and ammonia. Our analysis showed that both the Discharge Monitoring and Downstream Discharge Monitoring sites had a higher pH than the Reference sites (Figure 8). The pH of these waters frequently exceeded the ANZECC/ARMCANZ (2000) trigger value range of between 6.5 and 8, however, the most downstream Discharge Monitoring site (GRQ18) generally had lower pH values than other sites in this treatment. There was no clear overall decline over time in pH within either the Discharge Monitoring or the Downstream Discharge Monitoring treatments.

Conductivity was markedly elevated in all Discharge Monitoring and Downstream Discharge Monitoring sites (Figure 9). There was an overall decline in conductivity with distance downstream. In addition, there appears to be a general decline in conductivity over time, with this being most evident in the upstream Discharge Monitoring sites (e.g. Point 10, Point 12 and Jutts).

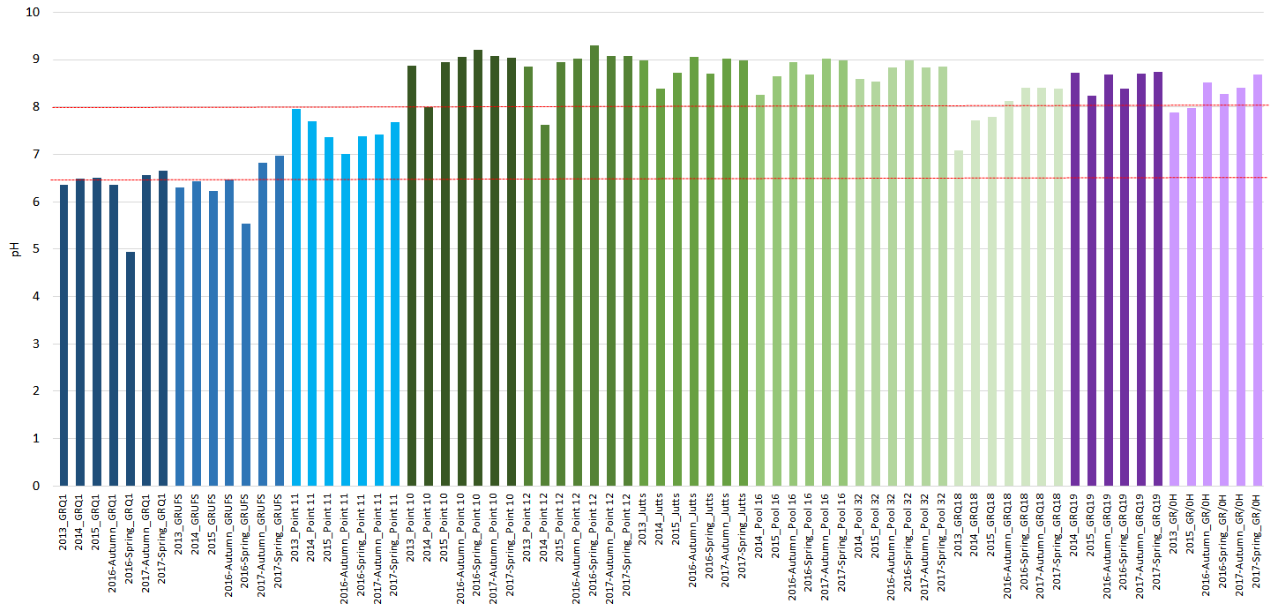

Figure 8. Long-term trends in pH. Sites were place into three treatments: Reference (blue); Discharge Monitoring (Green) and Downstream Discharge Monitoring (Purple). Dotted red lines represent the ANZECC/ARMCANZ (2000) trigger value for lowland rivers.

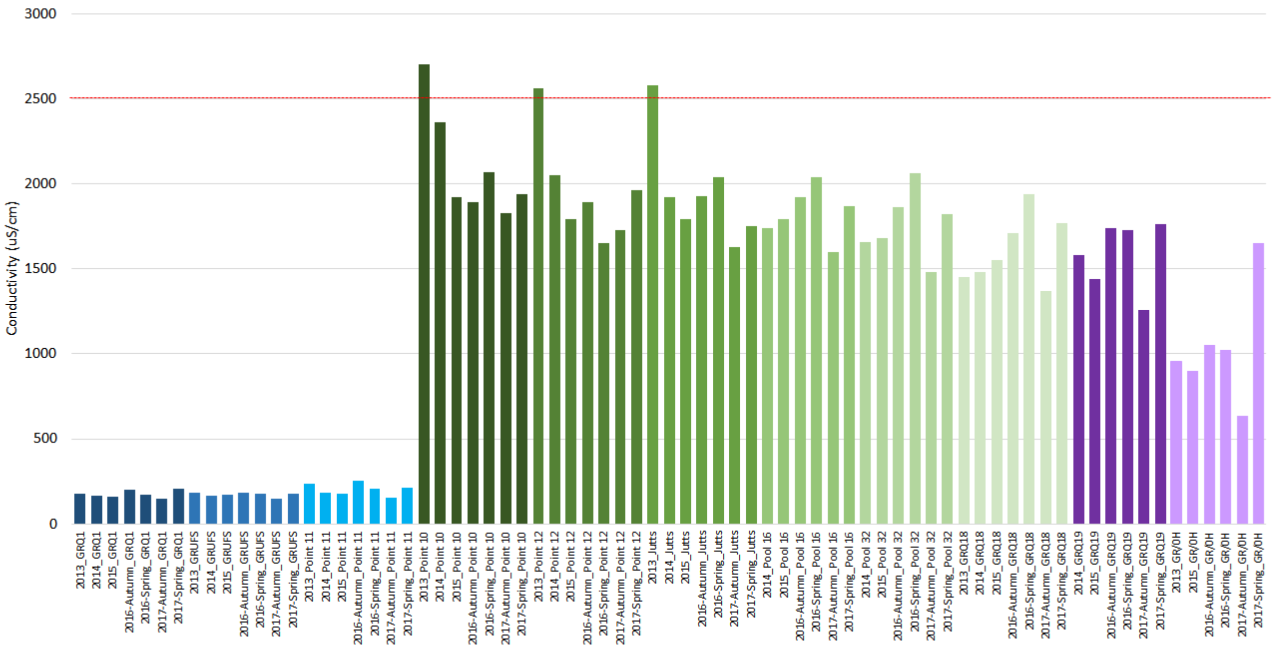

Figure 9. Long-term trends in conductivity. Sites were place into three treatments: Reference (blue); Discharge Monitoring (Green) and Downstream Discharge Monitoring (Purple). Dotted red lines represent the ANZECC/ARMCANZ (2000) trigger value for lowland rivers.

Aluminium concentrations were consistently elevated in all Discharge Monitoring sites, with the exception of GRQ18 (Figure 10). 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 were consistently very high in all Discharge Monitoring sites, and the Downstream Discharge Monitoring site GRQ19 (Figure 11). 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 generally declined over time, they remained several times above the guideline value in all Discharge Monitoring sites.

With the exception of GRQ18, zinc concentrations in all Discharge Monitoring sites generally exceeded the guideline value (Figure 12). However, in all Discharge Monitoring sites there was a marked overall decline in zinc concentrations over time, with concentrations in the more recent years frequently being close, or in some cases, below the trigger value.

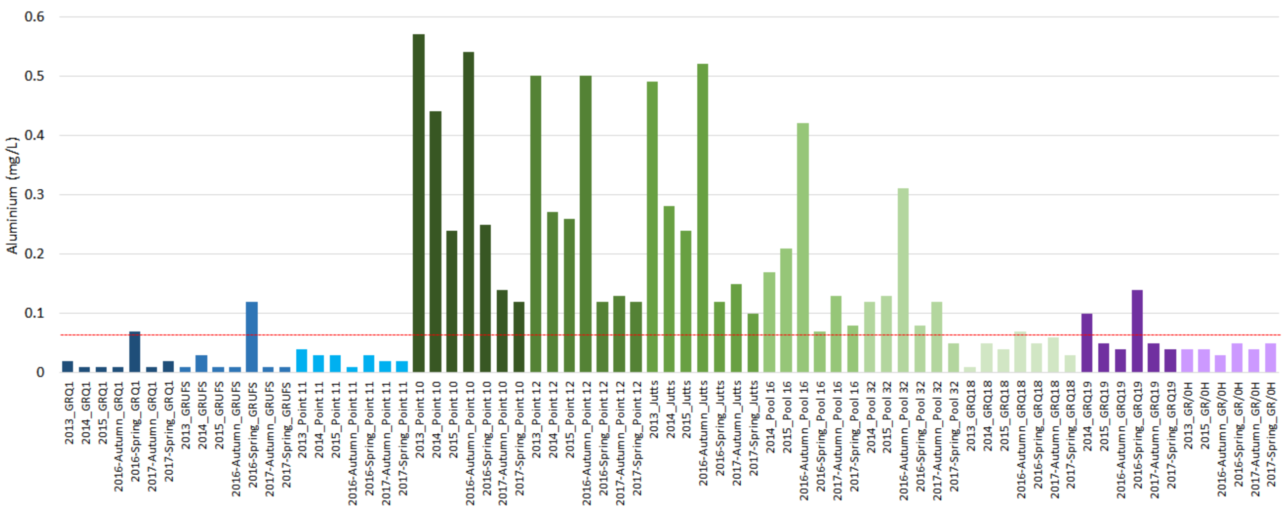

Figure 10. Long-term trends in aluminium concentrations. Sites were place into three treatments: Reference (blue); Discharge Monitoring (Green) and Downstream Discharge Monitoring (Purple). Dotted red lines represent the ANZECC/ARMCANZ (2000) trigger value for lowland rivers.

Ammonia concentrations were generally highest in the upstream Discharge Monitoring site Point 10, and declined with distance downstream (Figure 13). In general, 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.

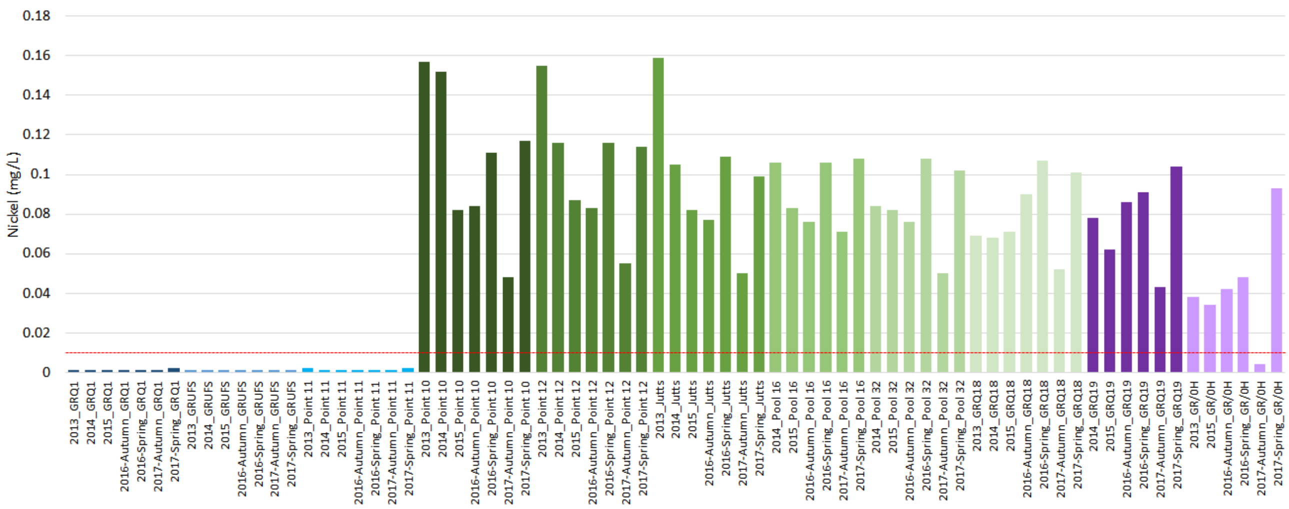

Figure 11. Long-term trends in nickel concentrations. Sites were place into three treatments: Reference (blue); Discharge Monitoring (Green) and Downstream Discharge Monitoring (Purple). Dotted red lines represent the ANZECC/ARMCANZ (2000) trigger value for lowland rivers.

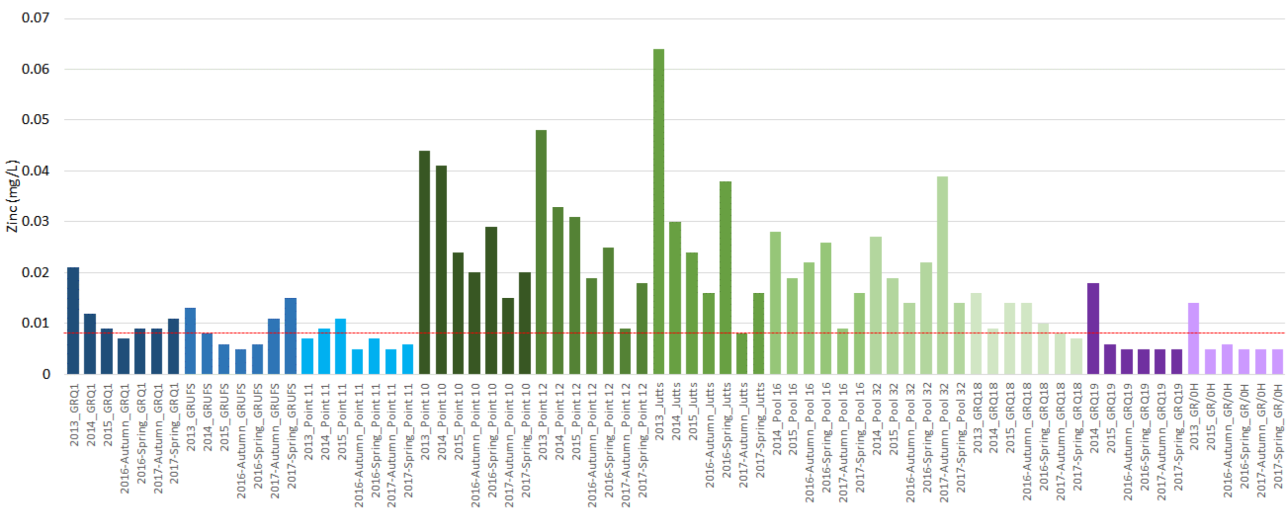

Figure 12. Long-term trends in zinc concentrations. Sites were place into three treatments: Reference (blue); Discharge Monitoring (Green) and Downstream Discharge Monitoring (Purple). Dotted red lines represent the ANZECC/ARMCANZ (2000) trigger value for lowland rivers

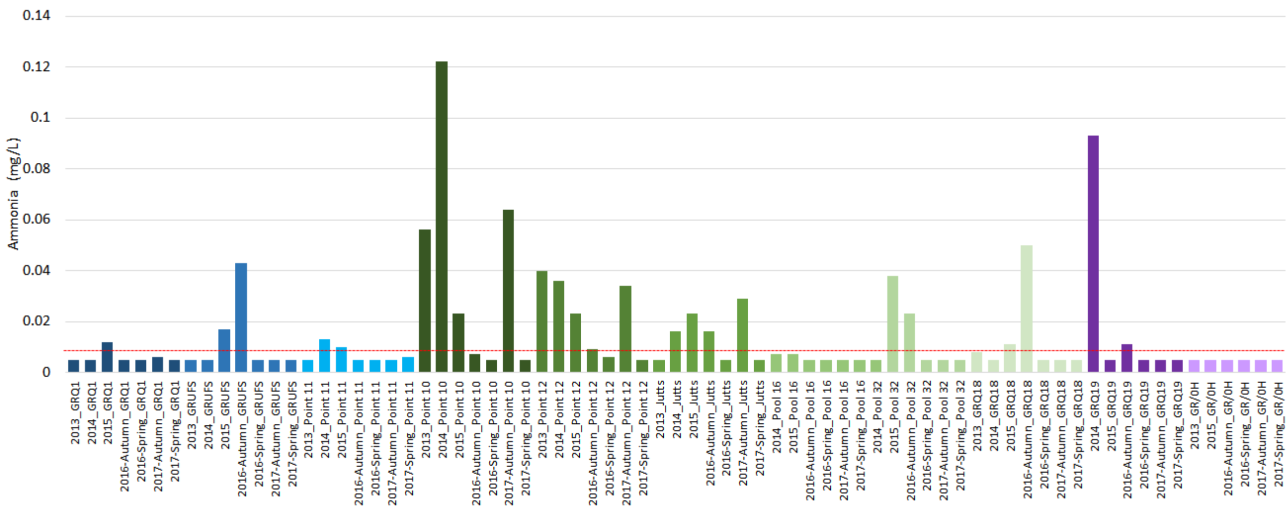

Figure 13. Long-term trends in ammonia concentrations. Sites were place into three treatments: Reference (blue); Discharge Monitoring (Green) and Downstream Discharge Monitoring (Purple). Dotted red lines represent the ANZECC/ARMCANZ (2000) trigger value for lowland rivers.

3.3 Ecotoxicology

The ecotoxicology data for all tests, including those no longer used in the EIP2 is provided in Appendix A. Figure 14 summarizes the findings for all included ecotoxicological tests performed on the discharge waters from Point 10.

The most sensitive tests were the *Selenastrum capricornatum*, *Lemna disperma* and the *Ceriodaphnia dubia* chronic reproduction tests. From July 2014, the discharge samples from Point 10 become more toxic to both *Selenastrum capricornatum,* however, this test was not performed from mid-2016. In contrast, the samples appear to be less toxic to *Lemna disperma* from January 2016, however, this test was no longer performed from mid-2016. For the remaining tests, due to the high variability between sampling events it is not possible to state any clear trends, however, the toxic units for the tests were generally low. The exception being the 96-hr fish imbalance, which peaked in January 2015, but has steadily declined since. Again, this test is no longer performed.

The more detailed analysis of the relationships between the three currently being used assays and conductivity are presented in Figure 15. There was no significant correlation between conductivity with either the *Paratya australiensis* 10-day acute test (r^2=0.002, *P*=0.902) or *Ceriodaphnia dubia* reproduction test (r^2=0.090, *P*=0.318). However, a weak but significant correlation was found between conductivity and toxicity units from the *Ceriodaphnia dubia* survival tests (r^2=0.361, *P*=0.029), indicating that survivorship of this species has increased with the reduction in conductivity and its correlates.

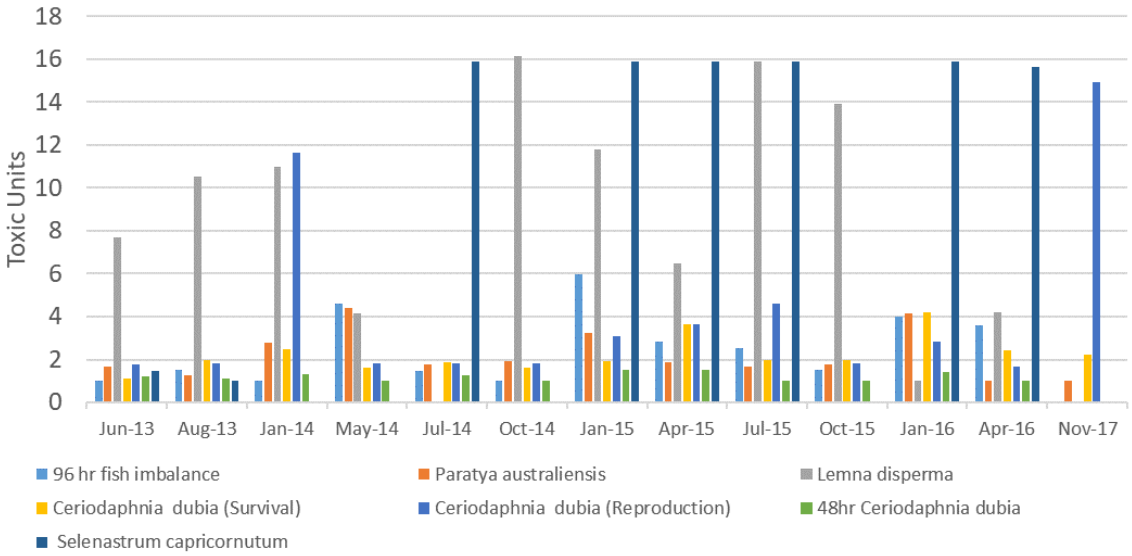

Figure 14. Toxicity of Point 10 waters collected 2013 – 2017. Toxicity is shown as toxic units, higher values are indicative of greater toxicity.

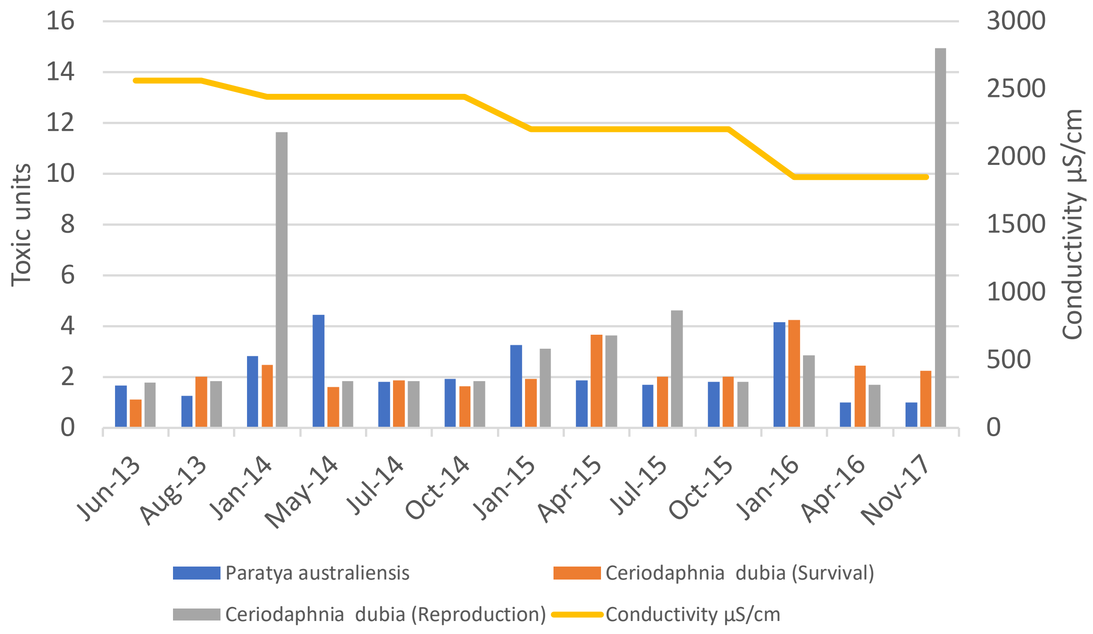

Figure 15. Trends in three ecotoxicological end-points as response to long-term patterns in conductivity in waters obtained from Point 10.

3.4 2017 Water chemistry

In both Autumn and Spring 2017, for a large number of water quality variables, there were marked differences in mean concentrations between the Reference, Discharge Monitoring and the Downstream Discharge Monitoring sites. A summary of the water quality for each season is provided in Table 5 and Table 6. In general, concentrations of elevated water quality measurements were lower in the downstream sites of the Discharge Monitoring treatment.

In Autumn 2017, the default trigger value for pH for lowland rivers (ANZECC/ARMCANZ, 2000), was exceeded in all Discharge Monitoring (range 8.40 – 9.08) and Downstream Discharge Monitoring sites (range 8.4 – 8.7), with no exceedances observed in the reference sites (range 6.57 – 7.41). Dissolved nickel concentrations exceeded the trigger value in all Discharge Monitoring sites ((range 0.048-0.071 mg/L), as was the case for the Downstream Discharge Monitoring site GRQ19. All Reference sites had dissolved nickel concentrations below are at the detection limit of 0.001 mg/L. Zinc concentrations were marginally above the guideline value in a number of Discharge Monitoring sites, as was the case for two of the Reference sites. Aluminium concentrations exceeded the trigger value in all Discharge Monitoring sites (range 0.06-0.15 mg/L), with no other treatments having exceedances. Three Discharge Monitoring sites also showed relatively higher concentrations of ammonia, with all Discharge Monitoring sites having nitrate + nitrite concentrations which exceeded the trigger value. No elevated concentrations of nutrients were detected in the Downstream Discharge Monitoring sites.

Similarly, in Spring 2017, the default trigger value for pH for lowland rivers (ANZECC/ARMCANZ, 2000), was exceed in all Discharge Monitoring (range 8.38 – 9.07) and Downstream Discharge Monitoring sites (range 8.68 – 8.74), with no exceedances observed in the Reference sites (range 6.66 – 7.68). Similarly, dissolved nickel concentrations exceed the trigger value in all Discharge Monitoring (range 0.099 –0.117 mg/L) and Downstream Discharge Monitoring sites (range 0.093 – 0.104 mg/L), with no exceedances observed in the Reference sites. Zinc concentrations were also above the trigger value in all Discharge Monitoring sites (0.007 – 0.02 mg/L), the exception being the most downstream site (GRQ18), with some exceedances also occurring in two of the Reference sites (0.011 – 0.015 mg/L), but not in the Downstream Discharge Monitoring sites. The Discharge Monitoring sites also had exceedances in aluminium and copper concentrations. Nitrate + nitrite concentrations were also elevated in the Discharge Monitoring sites, however, one site in both the Reference and Downstream Discharge Monitoring sites also showed elevated concentrations of nitrate + nitrite. Total nitrogen was above the trigger value in four of the Discharge Monitoring sites, with one of these sites also exceeding the trigger value for total phosphorus.

Table 5. Summary of water quality measurements for Autumn 2017 [a].

Variable	Trigger value		Reference			Discharge Monitoring						Downstream Discharge Monitoring	
	(Lowland rivers)	Units	GR/UFS	GR/Q1	Point 11	Point 10	Point 12	Jutts	Pool 16	Pool 32	GRQ18	GRQ19	GR/0H
pH*	6.5-8.0		6.83	6.57	7.41	**9.08**	**9.07**	**9.02**	**9.02**	**8.84**	**8.4**	**8.7**	**8.4**
Conductivity*	125-2,500	µs/cm	150	150	153	1830	1730	1630	1600	1480	1370	1260	636
Carbonate Alkalinity		mg/L	<1	<1	<1	139	134	125	116	86	31	42	<1
Bicarbonate Alkalinity		mg/L	4	3	8	640	613	586	582	538	536	463	51
Total Alkalinity		mg/L	4	3	8	779	747	711	698	624	567	505	51
Turbidity*		NTU	0	0.4	4.6	1.6	2.9	0	0	2.4	2.8	25.4	0.1
Dissolved Oxygen*		%	45.2	34.4	43.1	47	83.6	n/a	68	62	39	65.6	56.6
Temperature*		C°	11.69	11.41	13.82	n/A	n/a	14.06	13.32	14	13.15	12.58	14.34
Sulfate		mg/L	5	5	6	26	25	24	24	21	21	19	5
Chloride		mg/L	36	35	39	129	124	126	123	119	123	113	36
Calcium		mg/L	<1	<1	2	5	4	4	4	4	7	7	2
Magnesium		mg/L	3	3	3	2	2	2	2	2	4	4	3
Sodium		mg/L	16	16	20	420	397	344	338	310	335	294	34
Potassium		mg/L	<1	<1	<1	3	3	3	3	2	2	2	<1
Aluminium	0.055 (pH > 6.5)	mg/L	0.01	0.01	0.02	**0.14**	**0.13**	**0.15**	**0.13**	**0.12**	**0.06**	0.05	0.04
Arsenic	0.024	mg/L	<0.001	<0.001	<0.001	0.003	0.003	0.003	0.004	0.003	0.002	<0.001	<0.001
Cobalt		mg/L	<0.001	<0.001	<0.001	0.002	0.001	0.002	0.002	0.002	0.001	0.001	<0.001
Copper	0.0014	mg/L	<0.001	<0.001	<0.001	0.001	0.001	0.001	**0.002**	**0.002**	<0.001	<0.001	<0.001
Lead		mg/L	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Manganese	1.9	mg/L	0.086	0.071	0.025	0.008	0.008	0.006	0.007	0.004	0.016	0.003	0.008
Nickel	0.011	mg/L	<0.001	0.001	<0.001	**0.048**	**0.055**	**0.05**	**0.071**	**0.05**	**0.052**	**0.043**	0.004
Zinc	0.008	mg/L	**0.011**	**0.009**	<0.005	**0.015**	**0.009**	0.008	**0.009**	**0.039**	0.008	<0.005	0.005
Iron		mg/L	0.59	0.48	0.17	<0.05	0.08	0.06	0.17	0.21	0.34	0.29	0.3
Ammonia	0.013	mg/L	<0.005	0.006	<0.005	**0.064**	**0.034**	**0.029**	<0.005	<0.005	<0.005	<0.005	<0.005
Nitrite + Nitrate	0.015	mg/L	<0.002	<0.002	0.003	**0.054**	**0.076**	**0.086**	**0.032**	**0.036**	**0.018**	0.005	0.003
Total Kjeldahl Nitrogen		mg/L	<0.05	<0.05	<0.05	0.29	0.22	0.3	0.15	0.12	0.15	0.16	0.12
Total Nitrogen	0.5	mg/L	0.03	<0.01	<0.01	0.34	0.3	0.39	0.18	0.16	0.17	0.17	0.12
Total Phosphorus	0.05	mg/L	0.006	<0.005	0.01	0.024	0.015	0.016	0.013	0.006	0.007	0.02	0.007
Total Anions		meq/L	1.2	1.15	1.38	19.7	18.9	18.2	17.9	16.3	15.2	13.7	2.14
Total Cations		meq/L	0.94	0.94	1.22	18.8	17.7	15.4	15.1	13.9	15.3	13.5	1.82
Organic Carbon		mg/L	2	3	8	<1	31	5	<1	<1	2	<1	4

[a] Trigger values for metals were obtained from ANZECC/ARMCANZ (2000), with the values for physico-chemical stressors being the default values for lowland rivers. Values in bold text indicate measurements which exceeded the default guideline values for 95% level of protection. * values obtained from *in situ* measurements.

Table 6. Summary of water quality measurements for Spring 2017[a].

Variable	Trigger value	Units		Reference				Discharge Monitoring				Downstream Discharge Monitoring	
	(Lowland rivers)		GR/UFS	GR/Q1	Point 11	Point 10	Point 12	Jutts	Pool 16	Pool 32	GRQ18	GRQ19	GR/0H
pH*	6.5-8.0		6.97	6.66	7.68	**9.04**	**9.07**	**8.99**	**8.98**	**8.85**	**8.38**	**8.74**	**8.68**
Conductivity*	125-2,500	µs/cm	178	1720	210	1940	1960	1750	1870	1820	1770	1760	1650
Carbonate Alkalinity		mg/L	<1	<1	<1	192	204	133	132	106	51	94	69
Bicarbonate Alkalinity		mg/L	4	3	16	609	598	659	700	735	726	661	642
Total Alkalinity		mg/L	4	3	16	801	801	792	832	842	777	755	711
Turbidity*		NTU	0	0	0	0.1	0	0	0	0	0	0	0
Dissolved Oxygen*		%	69.6	46.6	62.1	97.2	131.0	91.4	104.0	103.1	62.2	81.3	95.8
Temperature*		C°	16.96	16.44	15.41	17.95	18.96	17.29	18.49	19.23	17.19	18.55	19.3
Sulfate		mg/L	8	8	11	14	12	21	23	22	20	21	20
Chloride		mg/L	46	45	40	162	163	130	142	140	178	176	169
Calcium		mg/L	1	1	5	11	8	8	6	7	8	8	8
Magnesium		mg/L	4	3	4	4	3	3	3	3	5	5	5
Sodium		mg/L	26	25	27	453	456	410	428	423	387	418	383
Potassium		mg/L	<1	<1	1	3	3	2	3	3	2	2	2
Aluminium	0.055 (pH > 6.5)	mg/L	<0.01	0.02	0.02	**0.12**	**0.12**	**0.1**	**0.08**	0.05	0.03	0.04	0.05
Arsenic	0.024	mg/L	<0.001	<0.001	<0.001	0.01	0.01	0.008	0.008	0.008	0.003	0.001	0.001
Cadmium		mg/L	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Cobalt		mg/L	<0.001	<0.001	<0.001	0.002	0.002	0.002	0.002	0.001	0.001	0.001	<0.001
Copper	0.0014	mg/L	<0.001	<0.001	<0.001	**0.002**	**0.002**	**0.002**	**0.002**	**0.002**	0.001	0.001	0.001
Lead		mg/L	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Manganese	1.9	mg/L	0.108	0.098	0.059	0.003	0.005	0.001	0.006	0.011	0.021	0.005	0.009
Nickel	0.011	mg/L	<0.001	0.002	0.002	**0.117**	**0.114**	**0.099**	**0.108**	**0.102**	**0.101**	**0.104**	**0.093**
Zinc	0.008	mg/L	**0.015**	**0.011**	0.006	**0.02**	**0.018**	**0.016**	**0.016**	**0.014**	0.007	0.005	<0.005
Iron		mg/L	0.12	0.28	0.15	<0.05	<0.05	<0.05	0.16	0.15	0.28	0.17	0.18
Ammonia	0.013	mg/L	<0.005	<0.005	0.006	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Nitrite + Nitrate	0.015	mg/L	0.003	<0.002	**0.029**	**0.402**	**0.388**	**0.352**	**0.272**	**0.165**	**0.109**	**0.021**	0.004
Total Kjeldahl Nitrogen		mg/L	<0.05	0.07	<0.05	0.22	0.2	0.22	0.68	0.34	0.2	0.26	0.34
Total Nitrogen	0.5	mg/L	0.04	0.07	0.05	**0.62**	**0.59**	**0.57**	**0.95**	0.5	0.31	0.28	0.34
Total Phosphorus	0.05	mg/L	0.007	<0.005	<0.005	<0.005	<0.005	0.006	**0.21**	<0.005	<0.005	<0.005	<0.005
Total Anions		meq/L	1.54	1.5	1.68	20.9	20.8	19.9	21.1	21.2	21	20.5	19.4
Total Cations		meq/L	1.51	1.38	1.78	20.6	20.6	18.5	19.2	19.1	17.7	19	17.5
Organic Carbon		mg/L	4	4	4	5	4	5	4	6	21	8	5

[a] Trigger values for metals were obtained from ANZECC/ARMCANZ (2000), with the values for physico-chemical stressors being the default values for lowland rivers. Values in bold text indicate measurements which exceeded the default guideline values for 95% level of protection. * values obtained from *in situ* measurements.

3.5 2017 Macrobenthic surveys

3.5.1 Macrobenthos Autumn 2017

In Autumn 2017, the abundances of macroinvertebrates varied greatly among sites and within treatments (Figure 16). The mean abundance across all sites was 100 individuals, however, abundances were very low in the Reference site Point 11 (7 individuals) and the Discharge Monitoring site Point 10 (10 individuals). Anecdotally, the Downstream Discharge Monitoring sites appeared to have lower abundances at the time of sampling than the Discharge Monitoring sites.

A similar pattern was observed with richness (Family level) (Figure 17), with both Point 11 (5 families) and Point 10 (4 families) having substantially lower richness that the other sites.

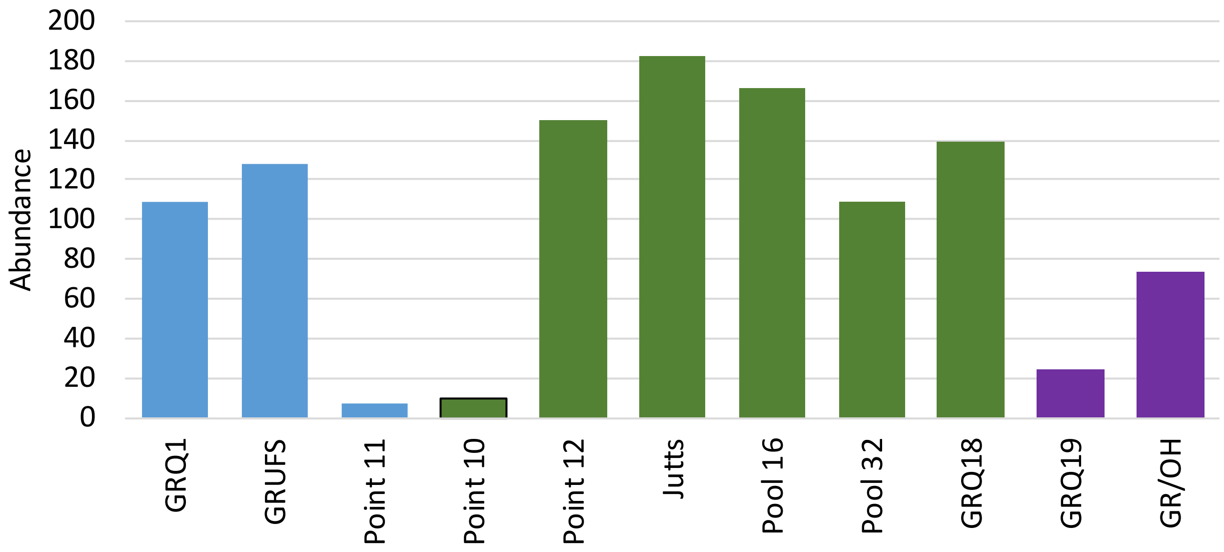

Figure 16. Abundances of macrobenthic invertebrates (Autumn 2017). Blue=Reference sites, Green=Discharge Monitoring sites and Purple=Downstream Discharge Monitoring sites.

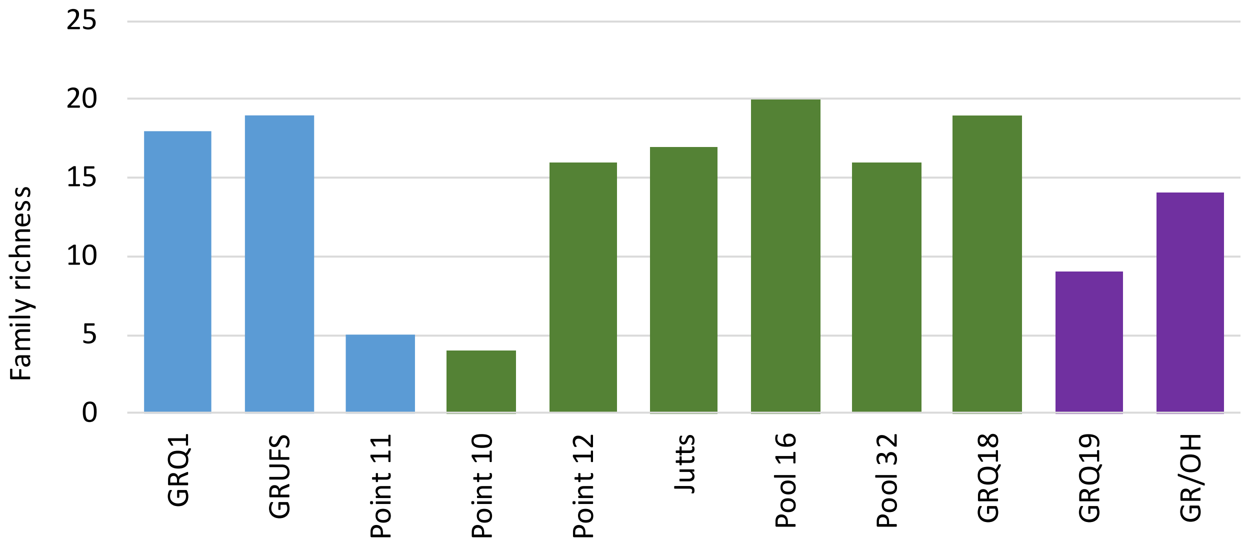

Figure 17. Family richness of macrobenthic invertebrates (Autumn 2017). Blue=Reference sites, Green=Discharge Monitoring sites and Purple=Downstream Discharge Monitoring sites.

Examination at the community level (Figure 18) again highlights differences in the Point 11 and Point 10 communities from the other sampled communities. All other Discharge Monitoring sites appeared to contain similar assemblages, however the two Downstream Discharge Monitoring sites were quite dissimilar. The two remaining Reference sites (GRUFS and GRQ1) had similar compositions at the time of sampling. PERMANOVA results added credence to these findings, with a significant difference detected among the treatments (PERMANOVA: F=2.14, *P*=0.02). Subsequent post hoc analysis confirmed differences were between the Reference and Discharge Monitoring treatments, with no other differences in composition detected between the three treatments. Communities sampled from the Downstream Discharge Monitoring treatment were more similar to the Discharge Monitoring treatment (34.5%) than they were with the Reference treatment (25.1%).

A summary of the key taxa contributing to the differences between the Reference and Discharge Monitoring treatments is provided in Table 7. Notably, a higher average abundance of Leptophlebiidae (Ephemeroptera) was observed in the Reference treatment. The Discharge Monitoring treatment had higher average abundances of Caenidae (Ephemeroptera), Libellulidae (Odonata) and Hydrophilidae (Coleoptera), with all three of these families being absent or rarely sampled in the Reference treatment.

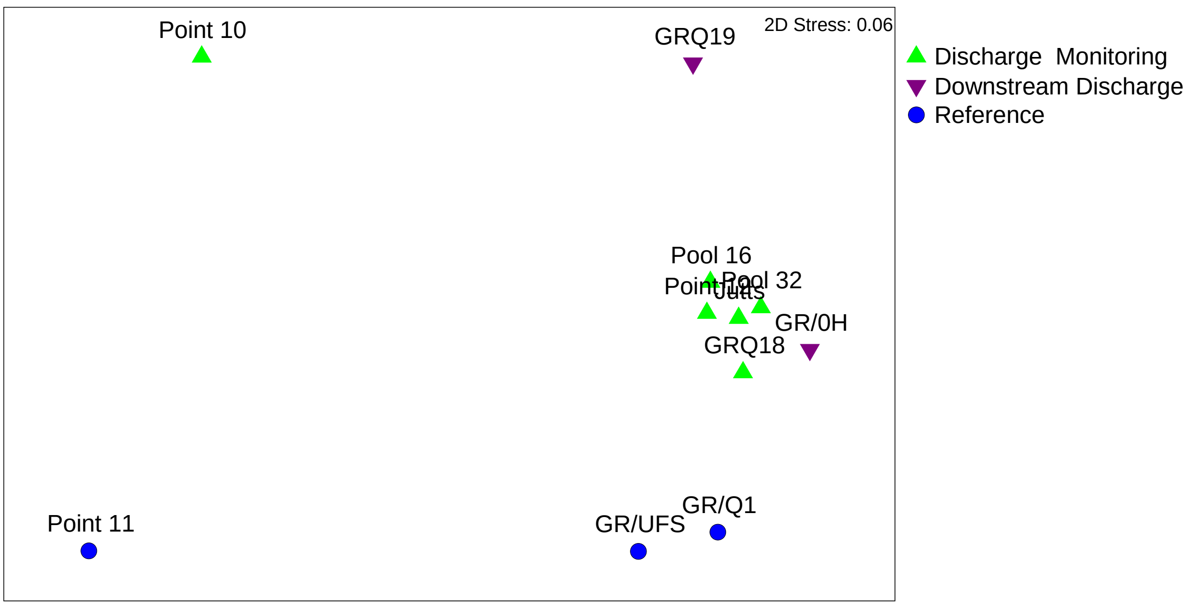

Figure 18. nMDS of macrobenthic communities (Autumn 2017).

Table 7. SIMPER results illustrating the families which contributed to differences between the Reference and Discharge Monitoring treatments (Autumn 2017).

Family	Reference (Average abundance)	Discharge Monitoring (Average abundance)	Contribution (%)
Leptophlebiidae	3.06	0.27	8.92
Caenidae	0	2.91	8.46
Libellulidae	0	1.51	5.7
Hydrophilidae	0	1.92	5.69
Baetidae	0.83	2.18	5.3
Dytiscidae	0.37	1.81	5.15
Chironomidae	1.64	1.85	4.8
Coenagrionidae	0.73	1.45	3.9
Leptoceridae	0.88	1.48	3.75
Megapodagrionidae	1.34	0.91	3.4
Diphlebiidae	0.23	1.19	3.28
Copepod (subclass)	1.09	0.66	3.24
Austrocorduliidae	1.11	0	3.04
Gyrinidae	0.6	0.32	3.01
Atyidae	1.06	0	2.9

The ordination plot (Figure 19) illustrates the correlative relationships between the macrobenthic communities and water quality from the Autumn 2017 sampling event. Approximately 73 % of the variation in the macrobenthic community data could be explained by the environmental variables. The findings suggest that the macrobenthic communities from the Discharge Monitoring sites, and to a less degree, the Downstream Discharge Monitoring sites are 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 20 % in the variation of the macrobenthic community data. This emphasizes then need to consider the effect of the discharge as a mixture, rather than the effect on individual variables per se.

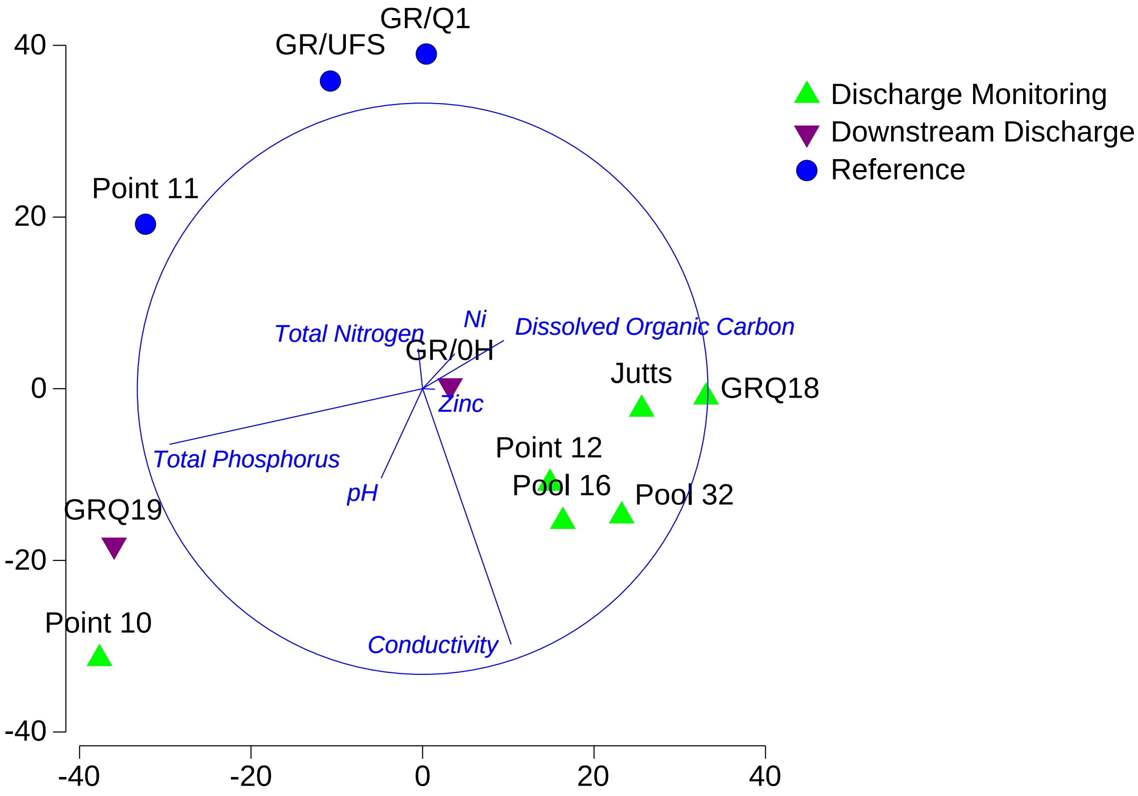

Figure 19. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and macrobenthic composition from Autumn 2017. The horizontal and vertical axes explain 28 % and 21 % of the total variation, respectively.

3.5.2 Macrobenthos Spring 2017

In Spring, the abundances of macroinvertebrates varied greatly among sites and within treatments (Figure 20), but to a lesser extent than the Autumn 2017 sampling event (Figure 16). The mean abundance across all sites was 192 individuals, however, abundance was very low in the Downstream Discharge Monitoring site GRQ19 (36 individuals).

Richness (Figure 21) was greater across all sites than during Autumn 2017 (Figure 17). As in the case of abundance, GRQ19 had a lower richness in Spring 2017 than the other sites.

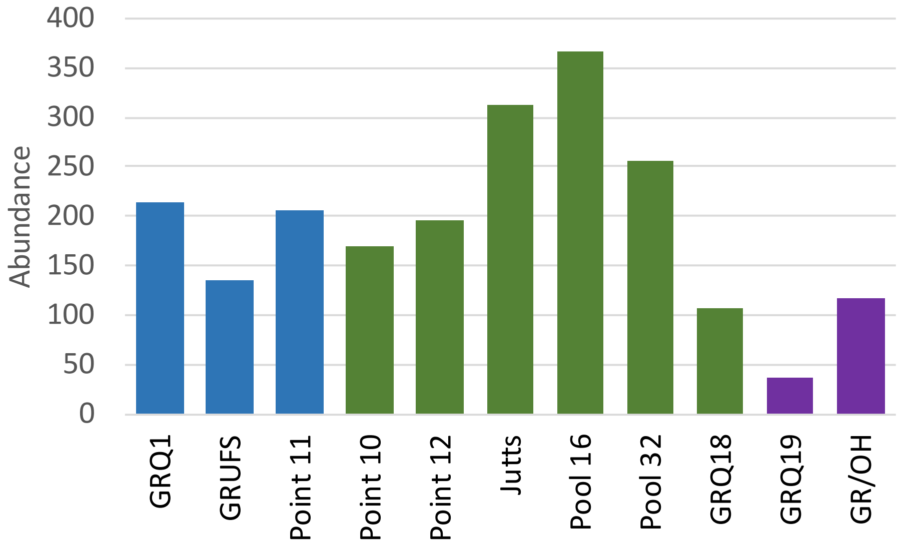

Figure 20. Abundances of macrobenthic invertebrates (Spring 2017). Blue=Reference sites, Green=Discharge Monitoring sites and Purple=Downstream Discharge Monitoring sites.

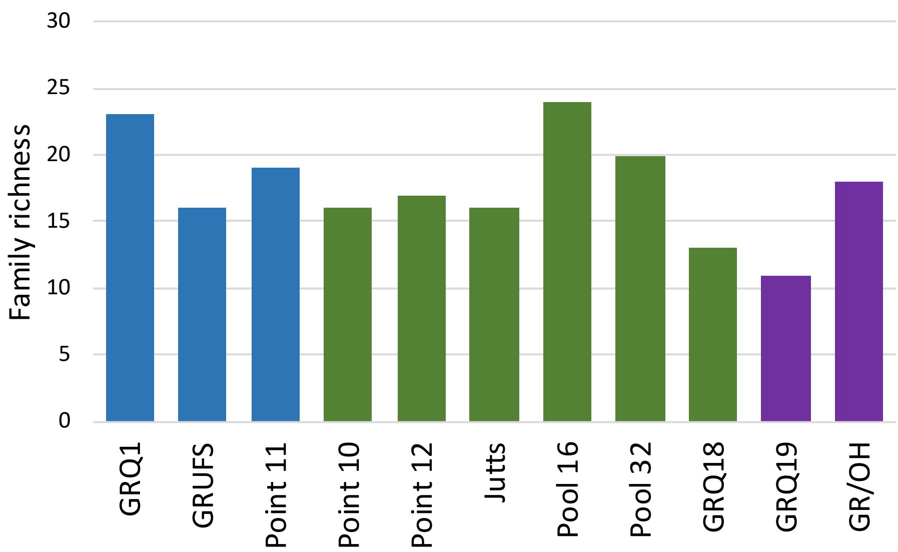

Figure 21. Family richness of macrobenthic invertebrates (Spring 2017). Blue=Reference sites, Green=Discharge Monitoring sites and Purple=Downstream Discharge Monitoring sites.

The ordination plot of the macrobenthic communities sampled in Spring 2017 indicates differences in the communities sampled between the Reference and Discharge Monitoring treatments (Figure 22). The two Downstream Discharge Monitoring sites appeared to contain markedly different assemblages. PERMANOVA and subsequent post hoc analysis indicated that the Reference and Discharge Monitoring treatments contained significantly different macrobenthic communities (T=2.58, P=0.015), with no differences detected between the other treatments. The Downstream Discharge Monitoring treatment was more similar to Discharge Monitoring treatment (58.9%) than it was to the Reference treatment (45.7 %).

A summary of the key taxa contributing to the differences between the Reference and Discharge Monitoring treatments is provided in Table 8. Notably, a higher average abundance of Leptophlebiidae (Ephemeroptera) was observed in the Reference treatment. While the Discharge Monitoring treatment had higher average abundances of Ceinidae (Amphipoda), Caenidae (Ephemeroptera) and Dytiscidae (Coleoptera).

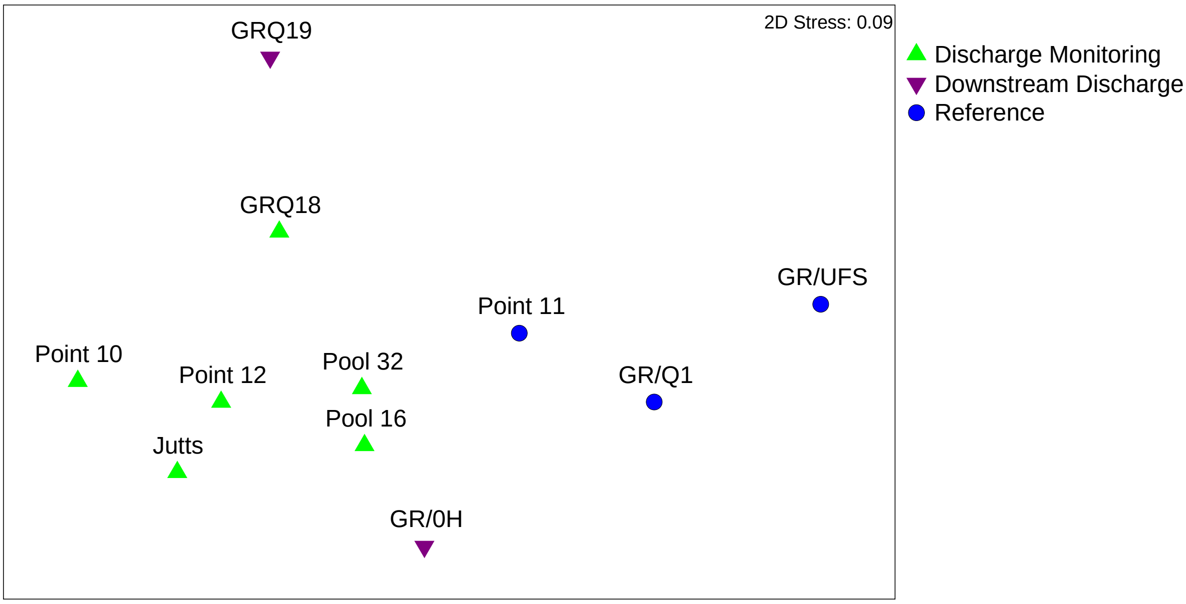

Figure 22. nMDS of macrobenthic communities (Spring 2017).

Table 8. SIMPER results illustrating the families which contributed to differences between the Reference and Discharge Monitoring treatments (Spring 2017).

Family	Reference (Average abundance)	Discharge Monitoring (Average abundance)	Contribution (%)
Ceinidae	0.46	4.04	8.27
Caenidae	0.46	4.04	8.27
Leptophlebiidae	4.09	0.56	8.18
Dytiscidae	1.11	3.36	5.84
Megapodagrionidae	2.55	0.23	5.45
Copepod(subclass)	3.19	1.51	4.82
Gripopterygidae	1.85	0	4.33
Chironomidae	1.19	2.84	4.05
Corixidae	0	1.6	3.87
Culicidae	1.76	0.27	3.59
Coenagrionidae	0	1.56	3.47
Hemicorduliidae	2.1	1.77	3.36
Arrenuridae	1.94	1.06	2.97
Oligochaeta	0.6	1.41	2.8
Austrocorduliidae	1.06	0	2.57

The dbRDA ordination plot (Figure 23) illustrates the correlative relationships between macrobenthic communities and water quality from the Spring 2017 sampling event. Approximately 86 % of the variation in the macrobenthic community data could be explained by the environmental variables. The findings suggest that the macrobenthic communities from the Discharge Monitoring sites, and to a less degree, the Downstream Discharge sites are being influenced by water quality. In contrast to Autumn 2017, in the Discharge Monitoring treatment there was no clear pattern between downstream distance and the influence of the discharge waters on macrobenthic communities, however, the results suggested that the communities from GRQ18 and Pool16 were being less influenced by the discharge waters. When examined individually, pH, conductivity, nickel, and total phosphorus were all shown to correlate significantly with benthic community structure. However, as in the case of the Autumn 2017 sampling event, when examined collectively, only pH was shown to significantly contribute to a proportion of the variation in the data, explaining approximately 40 % of the variation in the macrobenthic data.

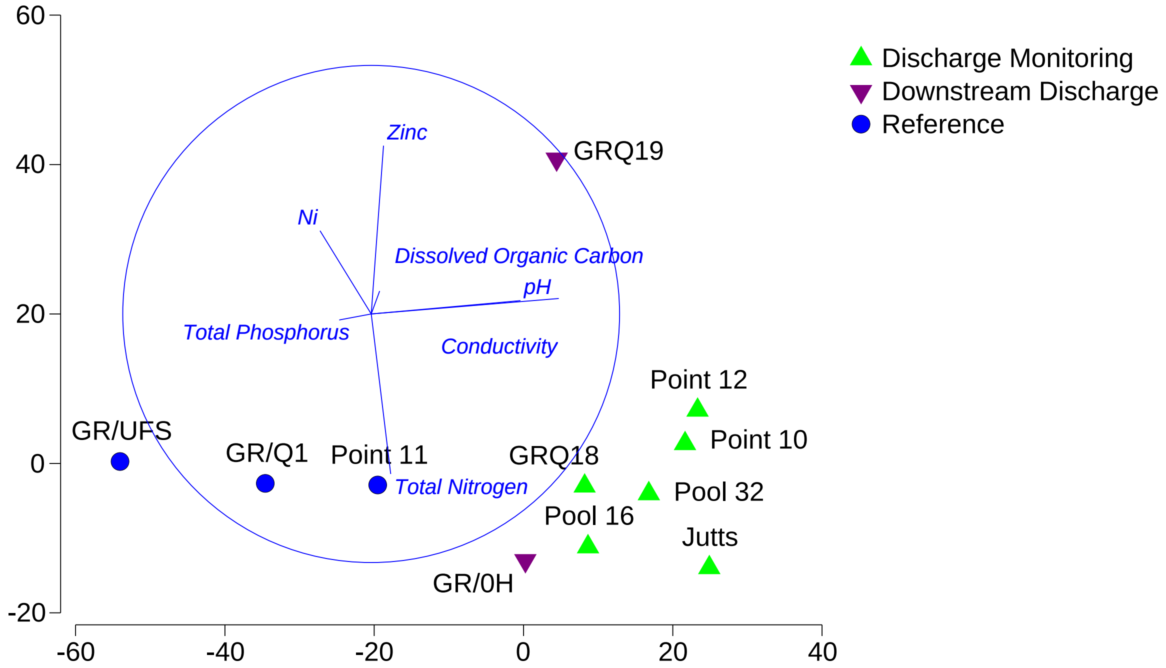

Figure 23. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and macrobenthic composition from Spring 2017. The horizontal and vertical axes explain 45 % and 15 % of the total variation, respectively.

3.6 EPT % and SIGNAL scores (Autumn and Spring 2017)

The EPT % scores the Autumn and Spring 2017 macrobenthic invertebrate surveys are provided in Figure 24. On both sampling occasions, the EPT % scores for the Reference site Point 11 were well below their long-term mean. In Autumn, both the Point 10 (Discharge Monitoring) and GRQ19 (Downstream Discharge Monitoring) sites contained no EPT taxa. There was considerable variation within sites, however, overall, the Reference sites had higher EPT % values than the other treatments. The findings suggested an overall increase in EPT % with distance downstream within the Discharge Monitoring treatment.

Analysis between the three a prior selected Reference (GRQ1, GRUFS and Point 11) and Discharge Monitoring sites (Point 12, Pool 32 and GRQ18) found that on both sampling occasions found no significant difference (ANOVAs: Autumn F=0.11, P=0.757; Spring F=0.59, P=0.484) between the

EPT % of the Reference (Autumn, mean = 45.5%; Spring, mean = 41.1 %) and Discharge Monitoring (Autumn, mean = 45.5%; Spring, mean = 41.1 %) treatments. However, it is emphasised that the number of replicates (i.e. 3) is insufficient to produce robust results, and little weight should be given to the findings.

For both survey events, SIGNAL scores for all sites were generally similar to the long-term means (Figure 25). Notable exceptions were the relatively low SIGNAL scores in both Downstream Discharge Monitoring sites (GRQ19 and GR/OH) in autumn. In contrasts to EPT % scores, SIGNAL scores were produced for each site on each occasion. SIGNAL scores were generally higher for the Reference sites, and there was a small increase in SIGNAL scores with downstream distance within the Discharge Monitoring treatment. Of note was the relatively high SIGNAL score for the Autumn Point 10 sample, however, it is emphasised that this discrepancy is likely an artefact of the sample's particularly low abundance and richness, and therefore should be discarded.

The comparisons between the three Reference and Discharge Monitoring sites found that on both occasions SIGNAL scores were significantly greater (ANOVAs: Autumn $F=10.0$, $P=0.034$; Spring $F=11.52$, $P=0.027$) in the Reference treatment (Autumn, mean = 4.57; Spring, mean = 4.97) than the Discharge Monitoring treatment (Autumn, mean = 4.23; Spring, mean = 4.17). Again, because of the small size we emphasise caution in interpreting the ecological significance of these findings.

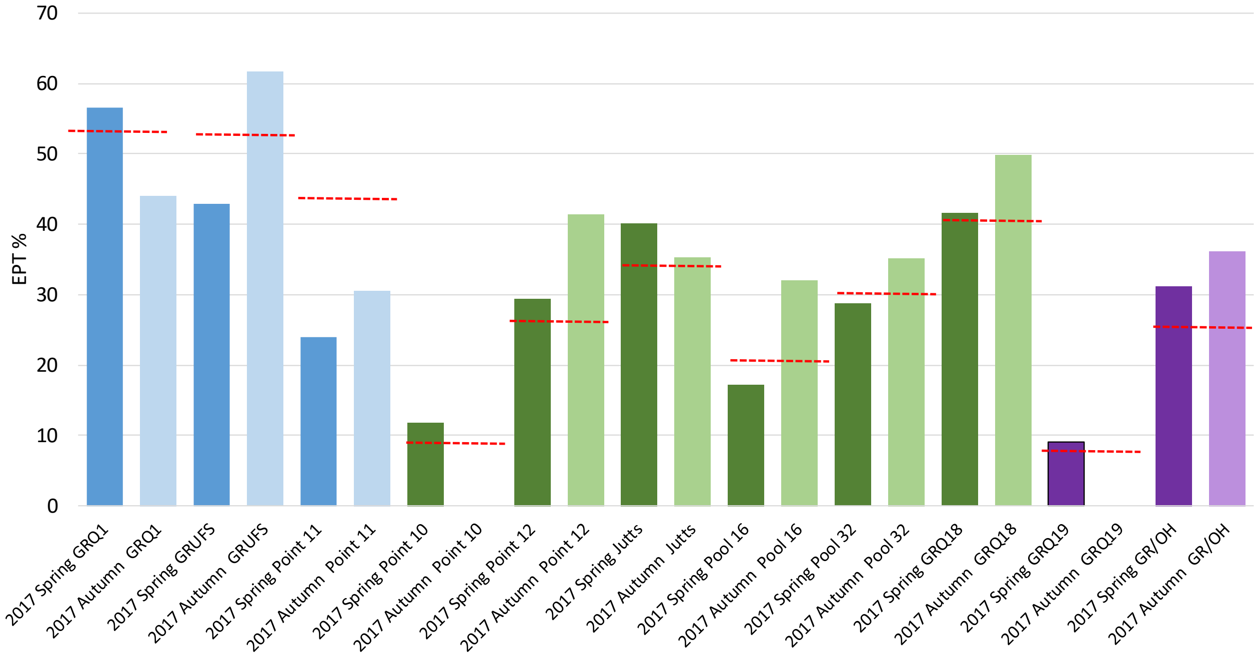

Figure 24. EPT % for Autumn and Spring 2017. Dotted red lines indicate the long-term (2013-2017) mean value for each site (2013-2017). Blue=Reference sites, Green=Discharge Monitoring sites and Purple=Downstream Discharge Monitoring sites.

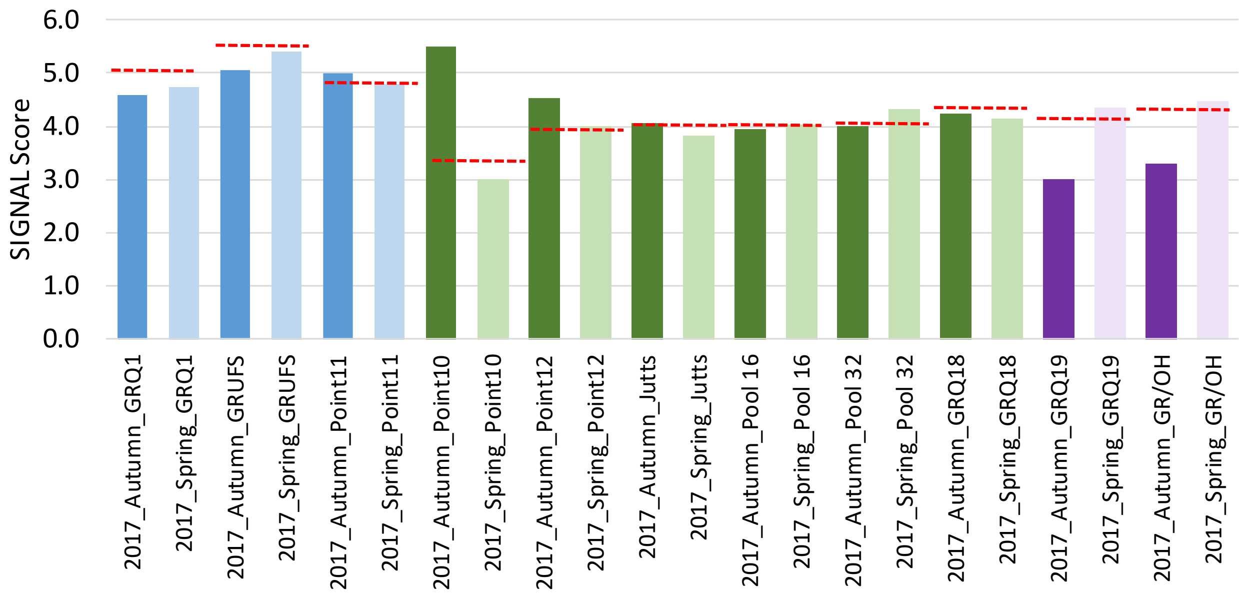

Figure 25. SIGNAL scores for Autumn and Spring 2017. Dotted red lines indicate the long-term (2013-2017) mean value for each site (2013-2017). Blue=Reference sites, Green=Discharge Monitoring sites and Purple=Downstream Discharge Monitoring sites.

3.7 Metabarcoding survey (18S rDNA)

After the removal of potentially erroneous sequences, the sequenced data set contained >11 million reads, encompassing 763 unique Operational Taxonomic Units (OTUs) from 36 eukaryote phyla. Of the 95% of OTUs that could be confidently assigned to a Kingdom, the largest proportion belonged to the phylum Arthropoda (21 %) and Bacillariophyta (17 %) (Figure 26).

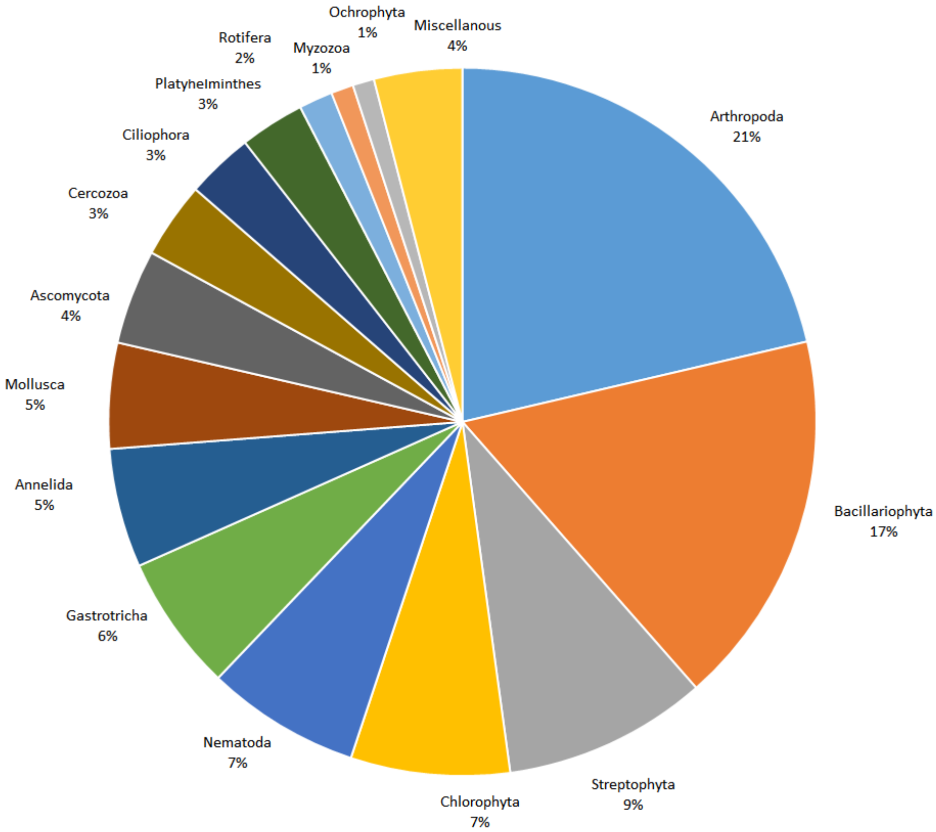

Figure 26. Summary of the OTU data illustrating the proportion of unique OTUs associated with each major taxonomic group. To aid interpretation data is aggregated at phylum and above. Miscellaneous encompasses all taxonomic groups represented by a small number of OTUs.

At the phylum level, there were very few notable differences between the treatments (Figure 27). Exceptions were, Bacillariophyta, which was richer in the Discharge Monitoring treatment (70 OTUs) than the Reference treatment (57 OTUs); and Ochrophyta, which was richer in the Reference treatment (41 OTUs), than both the Discharge Monitoring (21 OTUs) and Downstream Discharge Monitoring (28 OTUs) treatments.

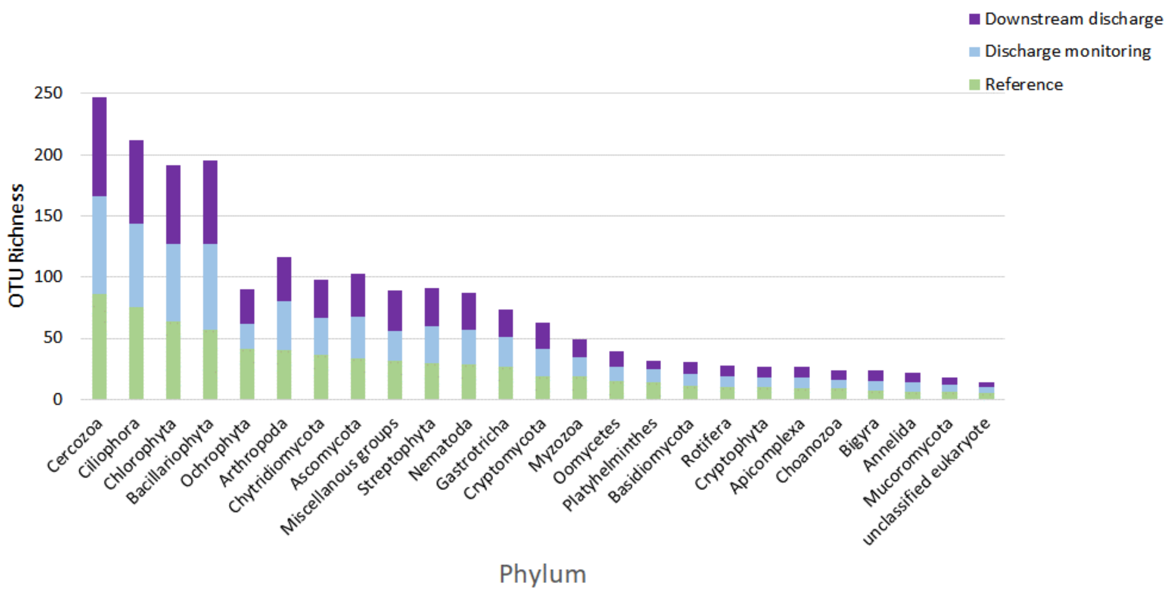

Figure 27. OTU richness of each phyla observed in each treatment.

The ordination plot of the metabarcoding data clearly shows that eukaryote communities from the Reference treatment were markedly different to those from both the Discharge Monitoring and Downstream Discharge Monitoring treatments (Figure 28). With the exception of one sample from Point 12, all sites and replicates from all three treatments were relatively clustered, indicating a high level of similarity between replicates and sites within treatments. PERMANOVA confirmed that there was a significant difference in composition between the treatments (PERMANOVA: F=25.9, *P*=0.001), with post hoc analysis indicating that all three treatments contained significantly different compositions. Communities from the Discharge Monitoring treatment were more similar to the Downstream Discharge Monitoring treatment (58.9 %) than they were to the Reference treatment (38.0 %). Similarly, there was a relatively low similarity between the Reference and Downstream Discharge Monitoring treatments (45.7%).

OTUs indicative of each treatment at the time of sampling are presented in Figure 9. Notably, a number of OTUs from Dinophyceae, Cryptophyceae and Choanoflagellida appeared to be unique indicators of the Reference treatment, with the other treatments having diatom indicators (Bacillariophyceae).

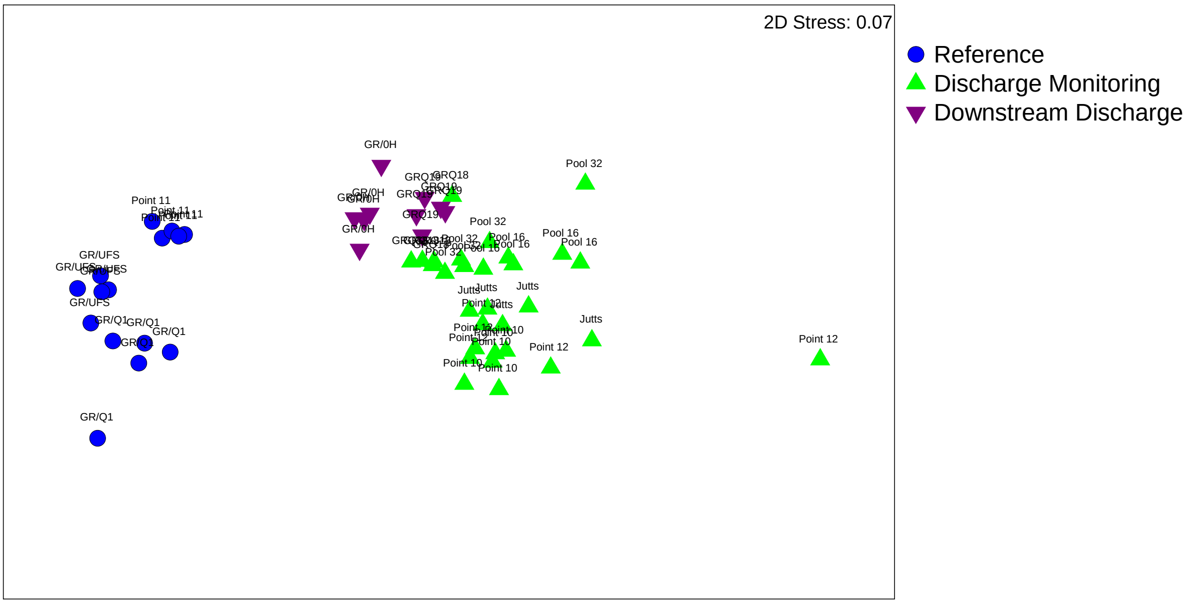

Figure 28. nMDS of the metabarcoding data. Analysis is derived from presence/absence data at the level of Operational Taxonomic Unit (OTU).

Table 9. 'Best' (based on Indicator Values >0.85) potential indicator OTUs for the Reference, Discharge Monitoring and Downstream Discharge treatments.

Treatment	OTU	Phylum	Class	Order	Family
Reference	OTU_2967	Myzozoa	Dinophyceae	Peridiniales	Glenodiniaceae
	OTU_475	Myzozoa	Dinophyceae	Syndiniales	
	OTU_334	Cryptophyta	Cryptophyceae	Cryptomonadales	Cryptomonadaceae
	OTU_2104	Cryptophyta	Cryptophyceae	Cryptomonadales	Cryptomonadaceae
	OTU_351	Choanozoa	Choanoflagellida	Acanthoecida	Codonosigidae
	OTU_297	Choanozoa	Choanoflagellida	Acanthoecida	Codonosigidae
	OTU_599	Cercozoa	Imbricatea	Euglyphida	Paulinellidae
	OTU_190	Oomycetes	Peronosporea	Pythiales	Pythiaceae
Discharge Monitoring	OTU_160	Bacillariophyta	Bacillariophyceae	Cymbellales	Cymbellaceae
	OTU_6965	Bacillariophyta	Bacillariophyceae	Rhopalodiales	Rhopalodiaceae
	OTU_108	Platyhelminthes	Turbellaria	Catenulida	Stenostomidae
	OTU_224	Chlorophyta	Chlorophyceae	Sphaeropleales	Sphaeropleaceae
	OTU_113	Gastrotricha		Chaetonotida	Chaetonotidae
	OTU_144	Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae
	OTU_57	Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae
Downstream Discharge	OTU_637	Cercozoa	Proteomyxidea	Aconchulinida	Vampyrellidae
	OTU_2888	Cnidaria	Hydrozoa	Trachymedusae	Rhopalonematidae
	OTU_627	Bacillariophyta	Bacillariophyceae	Cocconeidales	Cocconeidaceae
	OTU_235	Chlorophyta	Chlorophyceae		
	OTU_404	Chytridiomycota			
	OTU_70	Bacillariophyta	Mediophyceae	Eupodiscales	Eupodiscaceae
	OTU_86	Annelida	Clitellata	Haplotaxida	Enchytraeidae
	OTU_73	Arthropoda	Ostracoda	Podocopida	Cyprididae
	OTU_4259	Bacillariophyta	Bacillariophyceae	Naviculales	Amphipleuraceae
	OTU_119	Arthropoda	Ostracoda	Podocopida	
	OTU_143	Mollusca	Gastropoda	Sorbeoconcha	Caecidae
	OTU_205	Bacillariophyta	Bacillariophyceae	Cocconeidales	Cocconeidaceae

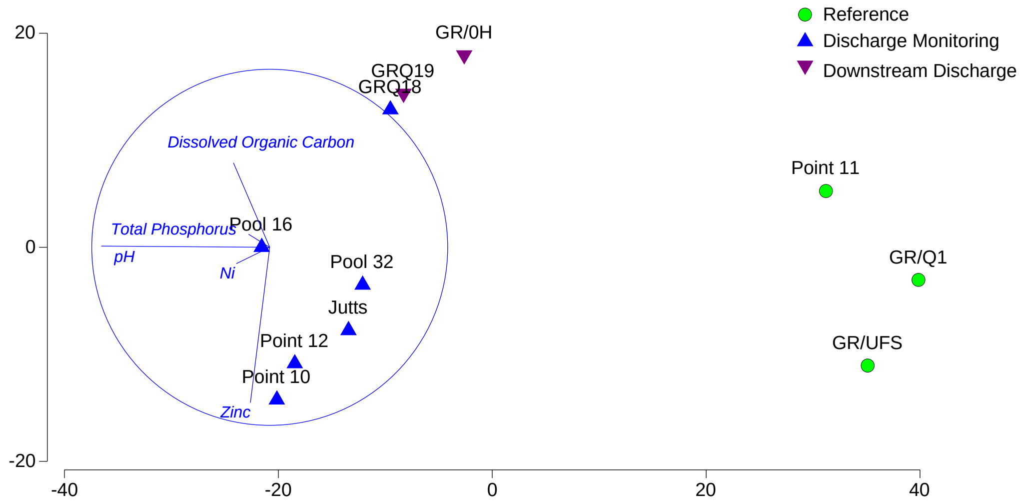

Figure 29. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and eukaryotic communities obtain by metabarcoding. The horizontal and vertical axes explain 57% and 13 % of the total variation, respectively.

As illustrated in the ordination plot (Figure 29), at the time of sampling, benthic communities from the Discharge Monitoring sites Points 10 and 12, Jutts and Pool 32 were strongly correlated with dissolved zinc. Communities from the two Downstream Discharge sites and the Discharge Monitoring site GRQ18 appeared to be driven by a suite of environment variables, however, it is less clear what specific variables were driving these patterns. Reference sites were negatively correlated with pH. Within the Discharge Monitoring treatment, eukaryote communities from the upstream sites (e.g. Point 10 and 12) were more strongly correlated with water quality than the downstream sites (e.g. Pool 16). When examined independently, pH, conductivity, nickel and total nitrogen are all significantly correlated with the metabarcoded eukaryotic communities. However, when examined collectively, only nickel, zinc and pH explained significant proportions in the variation of the biological data. Specifically, 56 % of the variation was explained by dissolved nickel, 13 % by dissolved zinc and 7% by pH. It is emphasised that given the complexity of the mixture, the strong correlations between all variables, and the need to limit the selection of variables prior to running the model; the focus of this finding should be on the composite of the discharge rather than its individual constituents.

4 Discussion

4.1 Long-term patterns in macrobenthic communities

4.1.1 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 both the Reference and Downstream Discharge Monitoring treatments, with no difference in the mean abundance of macrobenthos occurring between the Reference and Downstream Discharge treatments. However, in all sites within all treatments, abundances varied greatly across the sampling period, and there was no clear temporal trend.

Mean Family richness was similar across all treatments. As in the case of abundance, Family richness varied greatly within sites and across time. Given the unbalanced experiment design (3 Reference sites, 6 Discharge Monitoring sites and 2 Downstream Discharge Monitoring sites), these findings should not be considered to be statistically robust, but rather just as guide of the overall trends.

Community level analysis clearly showed that across all sampling events communities sampled from the Reference sites were different to those sampled from both the Discharge Monitoring and Downstream Discharge Monitoring treatments. The anomaly being two sites (Point 11 and Point 10) sampled in Autumn 2017, with both sites having very low abundances. While the Discharge Monitoring and Downstream Discharge Monitoring treatments did contain different compositions of macrobenthic communities, these treatments were more similar to each than they were to the Reference treatment. Collectively these findings suggest that different conditions are shaping each treatment. Evidence from previous surveys indicates that the compositional differences between the Reference and Discharge Monitoring treatments were strongly correlated with the physico-chemical properties associated with the discharge waters (Niche 2014, 2016).

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 had periods of no flow in the last two years (pers. obs. David Gregory, South32). Furthermore, observational evidence (pers. obs. David Gregory, South32) also suggest 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.

4.1.2 Long-term trend in EPT % and SIGNAL

As highlighted in section 4.1.1, there were distinct long-term differences in the compositions of the macrobenthic invertebrate communities between the treatments. Examination of the EPT %, derived from the relative collective abundances of Ephemeroptera (mayflies); Plecoptera (stoneflies); and Trichoptera (caddisflies), clearly showed that on average these pollution intolerant taxa make up a greater proportion of macrobenthic invertebrate communities in the Reference treatment than they did in either the Discharge Monitoring and Downstream Discharge Monitoring treatments, with the latter having the lowest mean EPT %. This suggests that conditions in the Reference sites were more favourable for these taxa. However, it important to note that EPT % varied greatly among all sites.

The SIGNAL scores, which captures a wider number of taxa, added credence to the composition and EPT % findings. The SIGNAL scores suggested that when examined collectively, the Reference sites were in better ecological condition than both the Discharge Monitoring and Downstream Discharge Monitoring treatments. In contrast to the EPT % scores, no difference in the mean SIGNAL scores were detected between the Discharge Monitoring and Downstream Discharge Monitoring treatments.

Although we have provided ecological rankings for each site based on their long-term mean SIGNAL scores (Table 4), as in the case of the other 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 sites from the other two treatments, rather than any specific ranking.

4.1.3 Leptophlebiidae genera of interest (2016-2017)

It has been suggested that specific Leptophlebiidae species are sensitive to conductivity (Cardno, 2010), leading to the recommendation by the George's River Working Group to examine this group at the species level. The analysis of the 2016-2017 data clearly showed that *Atelophlebia* spp., *Ulmerophlebia* spp., *Kooronga* spp were observed far more frequently and in higher abundances in the Reference sites. Furthermore, *Kooronga* was not in observed in either the Discharge Monitoring or Downstream Discharge Monitoring treatment. As specimens for these genera were not confirmed by a professional taxonomist, here we have restricted our interpretation to the genus level. We strongly recommend that if species level data is required in future studies, Leptophlebiidae should be identified by a professional taxonomist. However, given the inclusion of the additional indices, most notably SIGNAL 2.0, it is unclear if detailed taxonomy of this group is required for future studies.

4.2 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; with conductivity and concentrations of ammonia and metals (aluminium, nickel and zinc) generally declining. However, especially in the case of nickel, concentrations in all Discharge Monitoring sites were still several times above the guideline trigger value, and potentially pose a threat to aquatic species (ANZECC/ARMCANZ, 2000). While marked exceedances of the guidelines for aluminium and zinc were observed in most Discharge Monitoring sites, in recent years, concentrations were below in the trigger value in the most downstream site (GRQ18).

For all analysed variables, there was a general decline in concentrations with downstream distance, however, for most Discharge Monitoring sites, metal concentrations were still at concentrations 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 poor, and likely sufficient to impair biological integrity.

4.3 Ecotoxicology

Between 2013 and 2017, a suite of ecotoxicological tests were performed on the waters from the Discharge Monitoring site Point 10, with the number and frequency of the tests being reduced in April 2016. The findings clearly demonstrated the sensitivity of a number of tests to the discharge waters, e.g. *Selenastrum capricornatum*, *Lemna disperma* and the *Ceriodaphnia dubia* chronic reproduction tests. Interestingly, even with a reduction in conductivity, the toxic units for the

Selenastrum capricornatum test increased from July 2014, suggesting that possibly other constituents of the discharge waters was contributing to the sensitivity of the species. Conversely, there is evidence to suggest that the waters were becoming less toxic to fish based on the 96-hr fish imbalance tests.

There was no evidence to suggest that the toxicity of both the *Paratya australiensis* 10-day acute and *Ceriodaphnia dubia* reproduction tests declined following the reductions in conductivity. However, the waters did not appear to be particularly toxic to *Paratya australiensis*. While this species is found across a wide range of salinities (Carpenter, 1983), it is emphasised that the discharge waters contain a complex mixture of chemicals, and therefore the test is still valid. The *Ceriodaphnia dubia* reproduction tests produced markedly varied results across the study, indicating that this end-point was not being directly influenced conductivity, and was more likely responding to other constituents associated with the discharge waters. While *Ceriodaphnia dubia* reproduction was uncorrelated with conductivity, the findings suggest that the survivorship of this species increased as a response to a reduction in conductivity and its correlates.

4.4 2017 Macrobenthic invertebrate surveys

4.4.1 Autumn 2017

The total abundance of macrobenthic invertebrates sampled in autumn varied greatly between sites. In particular, in both Point 11 (Reference) and Point 10 (Discharge Monitoring) less than 10 individuals were obtained. The low abundances in Point 11 and Point is also reflected in their relatively low richness. Anecdotally, the remaining Discharge Monitoring sites had higher abundances than the other treatments, with this mirroring the long-term patterns previously discussed (section 4.1.1.). In general, richness was similar between the Reference and Discharge Monitoring treatments, whilst being marginally lower in the Downstream Discharge Monitoring treatment. As noted earlier, the unbalanced design and low number of replicates constrains the use of performing any robust statistically analysis.

The low abundances at Point 11 and Point 10 unsurprisingly also influenced the multivariate analysis, with both sites having markedly different compositions to the other sites associated with their treatments. However, for the remaining sites, it is clear that there were compositional differences between Reference and Discharge Monitoring sites. These differences were driven by a number of taxa, notably the relatively higher abundance of Leptophlebiidae in the Reference treatment. As previously discussed, this Family has been identified as a potential indicator of health for this system, with the taxon considered to be pollution intolerant (SIGNAL=8) (Chessman, 2003). Interestingly, the Ephemeroptera Caenidae were more abundant in the Discharge monitoring treatment. As this taxon is used to calculate EPT % it can be assumed that its abundance would be lower in this treatment. However, in this case, the Family is considered to be moderately insensitive to pollution (SIGNAL=4) (Chessman, 2003). Similarly, the other taxa indicative of the Discharge Monitoring treatment, the odonate Libellulidae (SIGNAL= 4) and the Coleoptera Hydrophilidae (SIGNAL=2) are also known to be pollution tolerant (Chessman, 2003).

The distance-based linear modelling (DistLM) which explored the correlations between the macrobenthic invertebrate communities and water quality indicated that the macrobenthic invertebrates, especially those from the Discharge Monitoring treatment, were being driven by the range of physico-chemical properties. 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.

4.4.2 Spring 2017

In comparison to Autumn 2016, the abundance of macroinvertebrates in all sites was far greater in Spring 2017. The only site with a very low abundance was the Downstream Discharge Monitoring site GRQ19. Again, the macrobenthic invertebrate abundance appeared to be higher in the Discharge Monitoring sites than the Reference sites, following the long-term trend earlier presented. With the exception of GRQ19, richness was similar across most sites, and fairly similar between the Reference and Discharge Monitoring treatments.

Community level analysis supported the Autumn 2017 trend, with compositions from the Reference treatment being markedly different to those from the Discharge Monitoring treatment. Given the marked difference in the two sites from the Downstream Discharge Monitoring treatment it is not possible to compare this treatment to the other two treatments. The taxa contributing to the differences between the Reference and Discharge Monitoring sites were the same as those reported in Autumn 2017. That is, a higher relative abundance of the pollution intolerant Leptophlebiidae in the Reference treatment, with corresponding higher abundances of the pollution tolerant Caenidae and Libellulidae in the Discharge Monitoring treatment.

As in the case of Spring 2017, in August 2017 strong correlations between elevated water quality parameters and macrobenthic communities were observed in the Discharge Monitoring sites, with a large amount of the variation being attributed to pH. A notable difference was that sites closer to the point source, e.g. Point 10, appeared to be equally influenced by the discharge waters as the other Discharge Monitoring sites, however, the influence of the discharge waters appeared to be less pronounced in GRQ18, the most Downstream Discharge Monitoring site. Collectively, this suggest that the influence of the discharge is markedly reduced in site GRQ18.

4.4.3 EPT % and SIGNAL for 2017

In the Reference treatment EPT % for 2017 were generally below the long-term average, however, this varied greatly within and between sites. The underpinning trend was that on average EPT % scores were higher the Reference Treatment than the other two treatments, reflecting the previously discussed long-term trend. However, no differences in EPT % were found between the six a prior selected Reference and Discharge Monitoring sites. It should be noted that no EPT taxa were detected in either Point 10 or GRQ19 during the Autumn survey.

The SIGNAL scores showed less variation within treatments than the EPT % scores. In most cases, SIGNAL scores were similar to the previously reported long-term patterns. Collectively, the findings show that the SIGNAL scores were consistently higher in the Reference sites than those obtained from the Discharge Monitoring and Downstream Discharge Monitoring treatments. Furthermore, on both occasions, SIGNAL scores within the Discharge Monitoring treatment increased with downstream distance.

4.5 2017 Metabarcoding survey

The metabarcoding (DNA-profiling of eukaryote communities) survey performed in Spring 2017 clearly demonstrated the technique's capacity to capture a diverse range of taxa, with >760 Operational Taxonomic Units (OTUs) from 36 phyla being sampled. As in the case of the 2015 metabarcoding survey (CSIRO, 2016), approximately 95% of the OTUs could be confidently assigned to at least Kingdom.

The multivariate analysis of the metabarcoding data clearly showed that that eukaryote composition of the Reference sites was markedly different to those sampled in both the Discharge Monitoring and Downstream Discharge Monitoring sites. In common with the traditional macrobenthic data, the sequenced communities from the Downstream Discharge Monitoring treatment were more similar to the Discharge Monitoring sites than they were the Reference sites. In general, samples and sites within the same treatment were tightly clustered, emphasising their similarity. The only exception was a single sample from Point 12, which appeared to contain a distinct assemblage, however, it was still more similar to other Discharge Monitoring samples than it was to samples from other treatments.

The metabarcoding survey identified a number of potential indicator OTUs indicative of each treatment at the time of sampling (Table 9). Interestingly, OTUs from a number of these Orders have been previously reported in other metabarcoding surveys of impacted environments. For example, diatoms from the Orders Cymbellales and Cocconeidales were also shown to be indicative of anthropogenically modified waters in S.E. Queensland (Chariton et al. 2015). 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 the Spring 2017 survey differed from those previously observed in 2013 and 2015 (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.

A large proportion of the variation (≈ 80%) in the metabarcoded eukaryote communities could be explained by the selected water quality parameters. When examined collectively, the strongest correlates with eukaryote composition were dissolved nickel (56 %), dissolved zinc (13 %) and pH (7 %). These findings support the water chemistry analysis, with all three variables being more elevated in the sites from the Discharge Monitoring and Downstream Discharge Monitoring treatments. As such, these findings indicate that the elevated constituents with the discharge waters were altering the composition of the Discharge Downstream sites. This is highlighted in Point 10, the site closest to the discharge, which showed the strongest correlations between eukaryote composition and metals (including their correlates). In contrasts, the communities from GRQ18 appeared to be driven by variables similar to those shaping the Downstream 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 Downstream Discharge Monitoring sites. In addition, 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 and Downstream Discharge Monitoring sites were complex, and the focus should be of the composite 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; with a different suite of variables influencing the Downstream Discharge Monitoring sites. Furthermore, the influence of the discharge waters was more pronounced in the upstream Discharge Monitoring sites.

5 Conclusions

5.1 Long-term trends

Examination of the macrobenthic data obtained between 2013 and 2017 showed that macrobenthic abundance were 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 contrasts to total abundance and richness, both EPT % and SIGNAL are designed to focus the analysis on taxa which may be influenced by the ecological condition of the stream. In the case of EPT %, the analysis is derived on the abundances of three Orders, with the findings of this report clearly showing a higher EPT % in Reference sites. Interestingly, the Downstream Discharge Monitoring sites on average had the lowest EPT %, indicating that this environment was not favourable to these taxa. While there were clear patterns in EPT %, it should be noted that Plecoptera were very rare in the system, and some Ephemeroptera families are pollution tolerant (e.g. Caenidae). Furthermore, this index appears to be very sensitive to changes in abundance and richness. Consequently, we believe that this approach is not ideal for this specific system.

Long-term patterns in SIGNAL scores added credence to the EPT %, indicating that the Reference sites were consistently in better ecological condition. The SIGNAL approach also separated the upstream and downstream sites within the Discharge Monitoring treatment, indicating that the influence of the discharge was more pronounced in the upstream sites (Point 10 and 12). Capturing a wider range of taxa than the EPT, and including a range of tolerances, the SIGNAL approach appears to be less responsive to changes in abundances and richness, especially in this case where it was derived from presence/absence data, potentially providing more robust data. Given that this approach 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 used in preference to EPT %.

Analysis of the Leptophlebiidae genera *Atelophlebia*, *Ulmerophlebia* and *Koornonga* clearly showed that between 2016 and 2017 these taxa were more abundant and more frequently observed in the Reference sites. In particular, *Koornonga* was only observed in the Reference treatment. While this information 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. However, if this approach is taken, we strongly recommend that identification to the species level is performed by a professional taxonomist.

The analysis of the macrobenthic surveys at the community level clearly demonstrated marked differences in the composition of macrobenthic invertebrates between the Reference and Discharge Monitoring sites, providing additional evidence of the influence of the discharge waters on benthic communities. Given the variability of the data, it is unclear if there have been any significant changes in the composition of macrobenthic communities since the conductivity of the discharge waters was reduced from 2500 to 2000 µS/cm. Interestingly, the Downstream Discharge Monitoring sites contained macrobenthic communities which differed from the other two treatments, suggesting that these communities were being shaped by a different suite of variables (natural or anthropogenic) to those from the Reference and Discharge Monitoring sites.

Collectively, the analysis of long-term macrobenthic data provides correlative evidence that the discharge is altering the composition of the Discharge Monitoring sites, with the influence of discharge decreasing with downstream distance. While the ecological condition of the Downstream Monitoring Discharge sites appears to be also relatively poor, there is no evidence to suggest that this is related to the discharge per se.

The long-term data highlights some limitations in the current experimental design. In particular, the patchiness in the macrobenthos. This could be potentially attenuated by increasing the number of biological replicates, e.g. from three to five replicates per site. Furthermore, as the communities in the Downstream Discharge Monitoring treatment appear to be uninfluenced and very much disconnected from the discharge, it is unclear how the future inclusion of these sites will assist the aims of the monitoring program. A possible scenario is to reallocate resources by increasing the number of biological replicates for the Reference and Discharge Monitoring sites.

However, it is noted that altering the design will limit future long-term analysis of the data. As there is anecdotal evidence to suggest that habitat is also playing a key role in shaping the benthic communities, the inclusion of some simple, but robust, measurements of habitat assessment should be included in future surveys.

5.2 Water quality

The water quality data indicates an overall improvement over the years, however, a large number of variables were still above, and in many cases, markedly exceeded water quality trigger values. Specifically, conductivity, pH and metal concentrations remained elevated in the upstream Discharge Monitoring sites. This suggest a high likelihood that the discharge waters are impairing the biological integrity of the system, most notably in the upstream Discharge Monitoring sites. 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).

5.3 Ecotoxicology

The ecotoxicological tests on the Point 10 discharge waters showed that historically the waters were toxic, as derived by a suite of end-points. Three tests appear to be particularly sensitive, the *Selenastrum capricornatum*, *Lemna disperma* and the *Ceriodaphnia dubia* chronic reproduction tests.

The more detailed analysis found that the reduction in conductivity had no significant influence on the *Paratya australiensis* 10-day acute and *Ceriodaphnia dubia* reproduction tests, with the latter being particularly sensitive. However, survivorship of *Ceriodaphnia dubia* appeared to have improved. The ecotoxicological tests suggests that the discharge waters were highly toxic to the primary producers *Lemna disperma* and *Selenastrum capricornutum*. While the impact of the waters on these taxa is important, it is unclear whether these test are suitable for this particular scenario given the high conductivity (USEPA, 2000). Consequently, we support the approach of limiting the ecotoxicological testing to the *Paratya australiensis* 10-day acute and *Ceriodaphnia* *dubia* reproduction and survivorship assays. Collectively, the ecotoxicological tests indicate that the discharge wasters from Point 10 still poses a significant risk to biota.

5.4 Weight of Evidence (2013-2017)

Table 10 provides a summary of the long-term macrobenthic community, water quality and ecotoxicological data obtained between 2013 and 2017. 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 EPT % and SIGNAL indices and the compositional data to infer that the discharge is altering communities within the Discharge Monitoring treatment. 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 suggest 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 weight of evidence framework.

Table 10. A summary of multiple lines of evidence obtained between 2013 and 2017.

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 robust measure of environmental stress.
	Richness	Richness was similar between all treatments.	Abundance is not a robust measure of environmental stress.
	EPT %	EPT % were highest in the Reference sites. The Downstream Discharge Monitoring site had the lowest EPT %.	Reference sites are in better ecological condition than the other sites.
	SIGNAL	SIGNAL scores were higher in the Reference sites.	Reference sites are in better ecological condition than the other sites.
	Leptophlebiidae	This group was far more abundance and frequent in Reference sites. *Kooronga* was only observed in the Reference treatment.	Suggest that this group is sensitive to the discharge waters. However, the Downstream Discharge Monitoring sites appear to be also unsuitable for the taxa.
	Community structure	Communities from the Reference treatment were consistently different to the Discharge Monitoring and Downstream Discharge Monitoring treatments. With the Discharge Monitoring and Downstream Discharge Monitoring treatments	Discharge is altering community structure in the Discharge Monitoring sites. Downstream Discharge Monitoring sites are being influenced by other factors.
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.
Ecotoxicology	7 tests	Point 10 waters remain toxic based a number of end-points. Due to the unreliability of several of the tests it is unclear how effective the reduction in conductivity has been on the overall toxicity.	A reduction in toxicity has occurred (e.g. *Ceriodaphina* survivorship), however, Point 10 waters still elicit a toxicological response to sub-lethal end-points (e.g. *Ceriodaphina* reproduction).

5.5 2017 Surveys

Collectively the macrobenthic and metabarcoding surveys for 2017 supported the findings of previous reports (CSIRO 2014, 2016; Niche 2014, 2016), providing strong correlative evidence that the discharge was altering the composition of macrobenthic biota within the Discharge Monitoring treatment. This is supported by multiple lines of ecological evidence, including EPT%, SIGNAL scores, macrobenthic community structure and correlative patterns between the communities and water quality measurements.

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 do not appear to be the same as those driving the communities within either the Reference or Discharge Monitoring treatments. 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 ANZECC/ARMCANZ (2000) trigger values for a range of metrics. Given the relatively brief period since the dilution, it is not currently possible to determine whether the 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 (Chariton et al., 2016).

5.6 Recommendations

- The univariate end-points of abundance and richness should be excluded from future analyses.
- SIGNAL 2.0 should be used instead EPT %.
- The need for detailed taxonomy of the Leptophlebiidae genera *Kooronga*, *Atelophlebia* and *Ulmerophlebia* should be reassessed given the sensitivity of this group at the Family level. If these taxa are deemed to be ecologically important and species level information is required, then future identifications of these taxa should be performed by a professional taxonomists.
- The experimental design of the routine monitoring programs requires re-evaluation. An increase in biological replicates (e.g. five replicates per site) may reduce within and between site variability, thereby aiding interpretation. Furthermore, it is unclear how the inclusion of the Downstream Discharge Monitoring sites assist in identifying the ecological impacts of the discharge. It is, however, noted that a change in design will limit the future interpretation of the long-term datasets.
- 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 should include downstream Discharge Monitoring sites (e.g. GRQ18) in order to gain a true understanding of the spatial extent of the discharge.

References

ANZECC/ARMCANZ (2000) Australian and New Zealand Guidelines for Freshwater and Marine Water Quality Vol.1. Australian and New Zealand Environment and Conservation Council and Agriculture and Resource Management of Australia and New Zealand, Canberra.

Barbour M. T. and Stribling B. (1991). Use of habitat assessment in evaluating the biological integrity of stream communities. Pages 25–38 in Biological criteria: Research and regulation. EPA-440/5-91/00 5. U.S. Environmental Protection Agency, Office of Water, Washington, D.C.

Batley G.E., van Dam R.A., Warne M.S., Chapman J.C., Fox D.R., Hickey C.W. and Stauber J.L. (2014). Technical Rationale for Changes to the Method for Deriving Australian and New Zealand Water Quality Guideline Values for Toxicants. CSIRO Land and Water Report Prepared for the Council of Australian Government's Standing Council on Environment and Water (SCEW), Sydney, Australia.

Cardno Ecology Lab Pty Ltd (2010) ACARP C1506 – Effects of Mine Water Salinity on Freshwater Biota, Investigations of Coal Mine Water Discharge in NSW.

Carpenter A. (1983). Population biology of the freshwater shrimp *Paratya curvirostris* (Heller, 1862) (Decapoda: Atyidae). New Zealand Journal of Marine and Freshwater Research 17, 147-158.

Chariton A. A., Stephenson S., Morgan M., Steven A., Colloff M., Court L. and Hardy, C. (2015). Metabarcoding of benthic eukaryote communities predicts the ecological condition of estuaries. Environmental Pollution 203:165-174

Chariton A., Baird D.J. and Pettigrove, V. (2016). Chapter 7: *Ecological Assessment*. In *Handbook for Sediment Quality Assessment, 2nd Edition* edited by Stuart L. Simpson and Graeme E. Batley. CSIRO, ISSN: 9781486303847, pages 151-189

Chessman, B. C. (2003). New sensitivity grades for Australian river macroinvertebrates. Marine and Freshwater Research, 54, 95-103.

Cole J. R., Wang Q., Fish J. A., Chai B., McGarrell D.M., Sun Y., Brown C.T., Porras-Alfaro A., Kuske C. R. and Tiedje J.M. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Research: 42, D633-D642

CSIRO (2014). Aquatic Monitoring Program for the Upper Georges River: Metabarcoding of the benthic eukaryotic assemblages. CSIRO Oceans and Atmosphere Report prepared for BHP Billiton/Illawara Coal.

CSIRO (2016). Aquatic Monitoring Program for the Upper Georges River/Brennans Creek: Metabarcoding of the benthic eukaryotic assemblages. Prepared for South 32.

Edgar R.C. (2013) UPARSE: Highly accurate OTU sequences from microbial amplicon reads, Nature Methods 10: 996-998.

Egge E., Bittner L., Andersen T., Audic S., de Vargas C. and Edvardsen, B. (2013). 454 Pyrosequencing to describe microbial eukaryotic community composition, diversity and relative abundance: a test for marine haptophytes. PLoS ONE 8:e74371

Legendre P. and Anderson M.J. (1999) Distance-based redundancy analysis: testing multispecies responses in multifactorial ecological experiments. Ecological Monographs 69:1-24

Niche Environmental and Heritage (2014). Westcliff Colliery – BHP Illawarra Coal: Aquatic monitoring report 2013

Niche (2016). Appin North – South 32 Pollution Reduction Program 20 – Aquatic Health Monitoring Report 2015.

Quast C., Pruesse E., Yilmaz P., Gerken J., Schweer T., Yarza P., Peplies J. and Glöckner F.O. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41, D590-D596.

South32 (2017). Georges River Environmental Improvement Program.

USEPA (2000). Method guidance and recommendations for whole effluent toxicity (WET) testing (40 CFR Part 136). EPA/821/B -00/004. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. 20460

Warne M.S., Batley G.E., van Dam R.A., Chapman J.C., Fox D.R., Hickey C.W. and Stauber J.L. (2015). Revised Method for Deriving Australian and New Zealand Water Quality Guideline Values for Toxicants. Prepared for the Council of Australian Government's Standing Council on Environment and Water (SCEW) Department of Science, Information Technology, Innovation and the Arts, Brisbane, Queensland.

Appendix A Ecotoxicological results

A.1 Summary of the ecotoxicological endpoints for the Point 10 discharge waters.

Test	Unit	Jun-13	Aug-13	Jan-14	May-14	Jul-14	Oct-14	Jan-15	Apr-15	Jul-15	Oct-15	Jan-16	Apr-16	Nov-17
96 hour fish imbalance test - Melanotaenia duboulayi	EC10/IC10	>100%	65.2%*	>100%	21.8%*	68%*	>100%	16.80%	35.40%	39.1%*	66.30%	25%*	27.7%*	
	EC25/IC25	>100%	>100%	>100%	40.50%	86.8%*	>100	36.50%	82.40%	74.6%*	>100%	39.30%	40.6%**	
	EC50	>100%	>100%	>100%	80.40%	>100%	>100%	Not reliable	>100%	>100%	>100%	>100%	>100%	
	NOEC	100%	100%	100%	50%	50%	100%	50%	25%	100%	100%	100%	50%	
	LOEC	>100%	>100%	>100%	100%	100%	>100%	100%	50%	>100%	>100%	>100%	100%	
10 day Acute Survival Test using the freshwater shrimp Paratya australiensis	EC10/IC10	60.00%	79.8%*	35.60%	23%	55.8%*	52.6%*	30.70%	53.80%	59.1%*	55.6%*	24.1%*	>100%	>100%
	EC25/IC25	92.8%*	>100%	44.30%	63%	63.40%	59.70%	41.40%	61.20%	70%	68.50%	>100%	>100%	>100%
	EC50	>100%	>100%	56.60%	93%	73.00%	72.40%	61.00%	70.70%	83.20%	86.30%	>100%	>100%	>100%
	NOEC	50%	100%	50%	50%	50%	50%	50%	50%	50%	50%	100%	100%	100%
	LOEC	100%	>100%	100%	100%	100%	100%	100%	100%	100%	100%	>100%	>100%	>100%
7-day Growth Inhibition of the freshwater aquatic duckweed Lemna disperma	EC10/IC10	13.0%*	9.5%*	9.1%*	24.2%*	Not Reliable	6.2%*	8.5%*	15.40%	<6.3%	7.20%	>96.8%	23.9%*	
	EC25/IC25	>96.8%	>96.8%	16.50%	28.20%	Not Reliable	31.6%*	12.20%	20.40%	21.8%*	10.10%	>96.8%	87.2%**	
	EC50	>96.8%	>96.8%	40.00%	35.00%	32.20%	77.9%*	21.30%	29.30%	41.10%	22.80%	>96.8%	>96.8%	
	NOEC	12.10%	96.80%	24.20%	24.20%	24.20%	24.20%	6.10%	12.10%	12.10%	6.10%	96.80%	48.40%	
	LOEC	24.20%	>96.8%	48.40%	48.40%	48.40%	48.40%	12.10%	24.20%	24.20%	12.10%	>96.8%	96.80%	
Partial life-cycle toxicity test using the freshwater cladoceran Ceriodaphnia dubia (Survival)	EC10/IC10	89.50%	50.10%	40.60%	62.20%	54%	62%	52.20%	27.40%	50%*	50%	23.7%*	41.1%*	44.80%
	EC25/IC25	97.90%	61.00%	43.80%	69.80%	65.20%	75.90%	55.60%	28.90%	76.5%*	67.10%	69.40%	>100%	49%
	EC50	>100%	76.0%*	43.50%	73.90%	75.30%	88.50%	66.00%	35.40%	>100%	87.10%	>100%	>100%	>100%
	NOEC	100%	50%	25%	50%	50%	50%	50%	25%	50%	50%	100%	100%	50%
	LOEC	>100%	100%	50%	100%	100%	100%	100%	50%	100%	100%	>100%	>100%	100%
Partial life-cycle toxicity test using the freshwater cladoceran Ceriodaphnia dubia (Reproduction)	EC10/IC10	56.60%	55.00%	8.60%	55%	55.10%	55%	32.30%	27.50%	21.70%	55.50%	35.20%	59.40%	6.70%
	EC25/IC25	66.60%	62.80%	12.00%	62.60%	62.60%	62.60%	43.30%	31.40%	34.70%	63.80%	54.40%	73.40%	9.40%
	EC50	83.20%	75.70%	31.50%	75.10%	75.30%	75.10%	62.00%	37.70%	56.23%	77.70%	72.20%	96.8%*	26.80%
	NOEC	50%	50%	6.30%	50%	50%	50%	50%	25%	25%	50%	50%	50%	6.30%
	LOEC	100%	100%	12.50%	100%	100%	100%	100%	50%	50%	100%	100%	100%	12.50%
48hr Acute Toxicity Test using the freshwater cladoceran	EC10/IC10	81.10%	91.5%*	76.60%	>100%	79.2%*	>100%	65.2%*	65.20%	>100%	>100%	71.5%*	>100%	
	EC25/IC25	87.50%	>100%	81.90%	>100%	85.0%*	>100%	67.9%*	67.90%	>100%	>100%	>100%	>100%	
	EC50	82.00%	>100%	73.50%	>100%	77.10%	>100%	70.7%*	70.70%	>100%	>100%	>100%	>100%	
	NOEC	50%	100%	50%	100%	50%	100	50%	50%	100%	100%	100%	100%	
	LOEC	100%	>100%	100%	>100%	100%	>100%	100%	100%	>100%	>100%	>100%	>100%	
72-hour microalgal growth inhibition test - Selenastrum capricornutum (green alga)	EC10/IC10	68.70%	>100%	Not Reliable	Not Reliable	<6.3%	Not Reliable	<6.3%	<6.3%	<6.3%	Not reliable	<6.3%	6.4%**	
	EC25/IC25	96.7%*	>100%	Not Reliable	Not Reliable	<6.3%	23%	6.9%*	<6.3%	<6.3%	22.80%	<6.3%	8.00%	
	EC50	>100%	>100%	>100%	>100%	<6.3%	40.40%	9.40%	7.70%	<6.3%	59.60%	<6.3%	10.60%	
	NOEC	100%	100%	100%	100%	<6.3%	25%	<6.3%	<6.3%	<6.3%	25%	<6.3%	6.30%	
	LOEC	>100%	>100%	>100%	>100%	6.30%	30%	6.30%	6.30%	6.30%	50%	6.30%	12.50%	

95th percentile limits not, reliable; **95th percentile limits not available. EC10 - Effective concentration - concentration that has a sub-lethal effect on 10% of the test organisms. EC25 - Effective concentration - concentration that has a sub-lethal effect on 25% of the test organisms. EC50 - Effective concentration - concentration that has a sub-lethal effect on 50% of the test organisms. IC10 - Inhibiting concentration - concentration that inhibits or impairs a biological function of 10% the test organisms. IC25 - Inhibiting concentration - concentration that inhibits or impairs a biological function of 25% the test organisms. IC50 - Inhibiting concentration - concentration that inhibits or impairs a biological function of 50% the test organisms. LOEC - Lowest observed effect concentration where there was an observable impact that was significantly different from control. NOEC - No observed effect concentration - concentration where there is no observable impact that is significantly different to the control. NB: APR14 - April Fish Imbalance test QA/QC failed. Additional sample collected and retested in May. JUL14 - Works were being conducted on the Dam scour line and the water was slightly more turbid than usual. NOV17 - Tests undertaken for the Georges River Environmental Improvement Program (EIP2)

Appendix B Metabarcoding

Ecological studies are an important line of evidence for assessing sediment quality. In aquatic systems, ecological data are commonly derived from the collection and enumeration of macrobenthic organisms (e.g. mayflies and caddisflies). However, macrobenthic data have significant limitations: (i) they are costly to collect; (ii) they are labour intensive; (iii) they require regionally-specific taxonomic expertise; (iv) they entail a large number of replicate samples; and (v) it is impractical to include juvenile and cryptic taxa. From a risk assessment perspective, a critical concern with macrobenthic studies is that only a small fraction of the total diversity, often less than 40 taxa, is being used to make assumptions about total ecosystem health. This is despite the fact that size, trophic position, diet, behaviour and life-stage influence the resilience and resistance of organisms to environmental disturbances.

While the inclusion of meio- and microfauna (including algae and diatoms) has been demonstrated to be of great benefit, as many of these taxa have been shown to be sensitive indicators of environmental condition (Kennedy and Jacoby, 1999), their size and taxonomic issues have made it impractical to include these organisms in routine monitoring programs. New molecular tools circumvent many of these issues, enabling ecologists to rapidly and comprehensively examine the biotic composition of sediments, regardless of organism size or taxonomy, providing a more realistic view of the ecological status of a system. Furthermore, this approach only requires a small amount of sediment, enabling sub-samples to be collected from sediments obtained for other purposes, e.g. chemical analysis.

Ecogenomics can broadly be defined as the examination of genetic materials from the environment. In the applications of environmental monitoring and assessment, ecogenomic techniques examine single or multiple genes which are present in the targeted organisms, an approach known as metabarcoding. For example, in eukaryote studies (all organisms except bacteria and archaea), a gene called 18S rRNA is often targeted to provide eukaryotic taxonomic information. The 18S rRNA gene is found in all eukaryotes, with related animals having similar genes that have slight variations in the sequences of the gene. For example, the 18S genes of two types of dragonflies will be more similar than a dragonfly and a beetle. Once the sequence of an

18S rRNA gene is known, it can be queried against extensive on-line databases such as SILVA and GenBank where the taxonomic information for the corresponding organism can be obtained.

While the application of molecular techniques to environmental research is not novel, until recently, complex mixtures of genes had to be separated into individual genes (cloning) before they could be sequenced. This biased the procedure to certain taxa, and was time-consuming, expensive and impractical for obtaining representative samples from highly diverse communities such as sediments. Recently, a technology called 'high throughput sequencing' has emerged which enables all of the targeted genes (e.g. 18S rRNA) within a complex mixture to be sequenced simultaneously, producing over 1 million sequences in a single analysis run. An additional advantage of this technique is that by placing a unique 'tag' or 'barcode' on the front of the DNA extracted from each individual sample, numerous samples (e.g. sites, plots or replicates) can be pooled for a single sequencing run, with each sequence being traceable back to its sample of origin.

This makes the procedure practical for complex experimental designs such as environmental monitoring programs. The metabarcoding approach has been applied to a range of ecological studies, including studies examining: the eukaryotic composition of estuarine sediments (Chariton et al., 2010); the effects of drought on soil communities (Baldwin et al., 2013); the effect of triclosan on estuarine biota (Chariton et al., 2014) and assessing the ecological condition of estuaries (Chariton et al. 2015).

References

Baldwin DS, Colloff MJ, Rees GN, Chariton AA, Watson GO, Court LN, Hartley DM, Morgan Mj, King AJ, Wilson JS, Hodda M, Hardy CM (2013) Impacts of inundation and drought on eukaryote biodiversity in semi-arid floodplain soils. Molecular Ecology 22:1746-1758

Bik HM, Halanych KM, Sharma J, Thomas WK (2012) Dramatic shifts in benthic microbial eukaryote communities following the deepwater horizon oil spill. PLoS ONE 7:e38550

Chariton AA, Court LN, Hartley DM, Colloff MJ, Hardy CM (2010) Ecological assessment of estuarine sediments by pyrosequencing eukaryotic ribosomal DNA. Frontiers in Ecology and the Environment 233-238

Chariton AA, Ho KT, Proestou D, Bik H, Simpson SL, Portis LM, Cantwell MG, Baguley JG, Burgess RM, Pelletier MM, Perron M, Gunsch C, Matthews RA (2014) A molecular-based approach for examining responses of eukaryotes in microcosms to contaminant-spiked estuarine sediments. Environmental Toxicology and Chemistry 33:359-369

Chariton, A. A., S. Stephenson, M. J. Morgan, A. D. L. Steven, M. J. Colloff, L. N. Court, and C. M. Hardy. 2015. Metabarcoding of benthic eukaryote communities predicts the ecological condition of estuaries. Environmental Pollution 203:165-174.

Kennedy AD, Jacoby CA (1999) Biological indicators of marine environmental health: Meiofauna - A neglected benthic component? Environmental Monitoring and Assessment 54:47-68

CONTACT US

t 1300 363 400
+61 3 9545 2176
e csiroenquiries@csiro.au
w www.csiro.au

AT CSIRO, WE DO THE EXTRAORDINARY EVERY DAY

We innovate for tomorrow and help improve today – for our customers, all Australians and the world.

Our innovations contribute billions of dollars to the Australian economy every year. As the largest patent holder in the nation, our vast wealth of intellectual property has led to more than 150 spin-off companies.

With more than 5,000 experts and a burning desire to get things done, we are Australia's catalyst for innovation.

CSIRO. WE IMAGINE. WE COLLABORATE. WE INNOVATE.

FOR FURTHER INFORMATION

Dr Anthony Chariton
anthony.chariton@mq.edu.au
+61 2 9850 6950