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Appin North – South 32

Pollution Reduction Program 20 – Aquatic Health Monitoring Report 2015

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Cover photograph: Georges River

Executive summary

Project outline

Endeavour Coal Pty Limited (the licensee), a wholly owned subsidiary of South 32 Illawarra Coal, is the holder of Environment Protection Licence (EPL) No. 2504 issued under the Protection of the Environment Operations Act 1997. The licence authorises, among other things, the carrying out of coal works and mining for coal at Appin North Colliery. On 24 April 2013 the EPA issued a notice of variation of EPL 2504, which included a requirement (Pollution Reduction Program 20) to implement an Aquatic Health Monitoring Plan. The Aquatic Health Monitoring Plan monitors and assesses the aquatic health of Brennans Creek and Upper Georges River, with surveys to be undertaken between 1st September -30th November in the years 2013, 2015, 2017 and 2019. The monitoring must include chemical analysis and in-stream biota assessment, including representative macroinvertebrate, algal and vertebrate species. The monitoring must be carried out in five or more locations including licence discharge point, Point 10, Point 11, Point 12 and Upper Georges River to the confluence of O'Hares Creek. The full requirements of the Aquatic Health Monitoring Plan are documented within EPL 2504.

Results and conclusions

This report documents the outcomes of macroinvertebrate monitoring undertaken in Spring 2015, which is the 2nd survey undertaken as part of the long term Aquatic Health Monitoring Plan. This data however still represents the 'before' component of the monitoring design as the full implementation of Pollution Reduction Program (PRP) 19 is not yet complete. Findings based on the survey are:

- There was no statistical difference between density and family richness through time, between discharge monitoring and reference sites and between sites (Treatment).
- There was no statistical difference between multivariate assemblages through time, however there were statistical differences between monitoring and reference sites and sites (Treatment).
- Higher densities of pollution tolerant Chironominae (SIGNAL 3,) Caenidae (SIGNAL 4), Dyticidae (SIGNAL 2) and Hydrophilidae (SIGNAL 2) were observed in discharge monitoring groups compared to reference groups.
- There has been a slight increase in similarity to reference sites at Point 12 and Jutts Crossing and is thought to be primarily the result of the observed Leptophlebiidae at these sites, however whether this is the result of any management action is unclear.
- Given the absence of Leptophlebiidae at Point 10 in 2013 and 2015 and its known sensitivity to changes in water quality, this family can be a useful indicator for detecting improvements in stream ecological health.
- Macroinvertebrate assemblages in 2015 did not show as clear separation between the discharge sites and reference sites compared to 2013 monitoring results, however the discharge and reference site were still statistically different.

Recommendations

It is recommended to separate the subsamples at each site to allow within site replication. This will permit statistical testing at the site level; so that individual sites (rather than grouped sites) can be statistically compared to reference sites. This is required to further address one of the key aims to assess the downstream gradient changes in composition and abundance of in-stream and sediment biota.

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1. Introduction

1.1 Program requirements

The monitoring discussed in this report was 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. 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.

The EPA approved a request by Illawarra Coal to extend the above reporting deadlines to 31 March each year.

The AHMP includes the following:

- Quantitative sampling of macroinvertebrates conducted in line with previous studies undertaken in PRP6, PRP9 and ACARP C15016 (2010).
- Ecological assessment processes using DNA extracted from sediment samples as per Baldwin et al. 2013 (these works undertaken by CSIRO and to be reported separately).
- In-stream water quality testing.
- Laboratory water testing.

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

1.1.1 Changes to Monitoring Program

Changes made to Version 3 of the AHMP following a review of the findings from the Year 1 (2013) campaign include:

- Removal of Cascade Creek reference sites.
- Moved monitoring site GR_OH slightly downstream to a more accessible site (now situated downstream of the O'Hares confluence).
- Added an additional three Georges River sites downstream of the Brennan's Creek confluence i.e. Pool 16, Pool 32 and GRQ19.
- Removed fish monitoring from the program.
- Removed the need to take duplicate water quality samples following a review of the water quality results from the year 1 (2013) campaign. All of the above changes were approved by the EPA in November 2014.

1.2 Aims of the Aquatic Health Monitoring Plan

The aim of the study is to monitor changes to biota in-stream and within the sediment within the upper Georges River as Water Projects required by PRP 19 are commissioned.

The aim will be achieved by:

- Comparing the Brennans Creek/Georges River sites with reference sites (upstream of the Brennan's Creek Confluence).
- Estimate changes over time in the composition and abundance of in-stream and sediment biota.

- Assessing the downstream gradient changes in composition and abundance of in-stream and sediment biota.

1.3 Background

1.3.2 Update on PRP19 Water Quality Improvements

Illawarra Coal provides six monthly progress reports against PRP 19 (30 June and 31 December) to the EPA. Details of PRP19 can be found in EPL 2504 on the EPA website
http://www.epa.nsw.gov.au/prpoeoapp/ViewPOEOLicence.aspx?DOCID=45231&SYSUID=1&LICID=2504

The table below provides a summary of the works undertaken within the six month period to 31 Dec 2015.

Table 1: PRP19 Works to improve discharge water quality (Dec 2015)

Works	Purpose	Status
Stage 1. Transfer of minewater from underground directly to the West Cliff Coal Preparation Plant (Washery)	Reduce Brennans Creek dam (BCD) water taken for process water in the Washery. Reduce higher salinity water diverted into BCD by reducing minewater diverted into BCD.	Stage 1 of the project was completed in December 2012.
Flocculant review. Trial of non-aluminium based flocculants at West Cliff Coal Preparation Plant.	Reduction of the aluminium concentration in water treatment chemicals to reduce aluminium levels within treatment ponds and therefore Brennans Creek dam (BCD).	The original scope of the flocculant review was to replace all aluminium based flocculants with one alternative flocculant (One product for all ponds as per our current water treatment processes).The iinitial trial of non-aluminium based flocculants did not meet water quality and / or operational parameters. The scope of the flocculant trial was been modified based upon the results of the initial trial and the outcomes of the semi-closed loop at the Washery (i.e. treatment of Washery process water is now out of scope). Illawarra Coal and several flocculant suppliers are undertaking targeted jar tests of the different waters (emplacement ponds, stockpile ponds and washery water / semi-closed loop system). The aim of the tests is to tailor water treatment systems and flocculants for the different waters, their potential pollutants (eg clays, coalwash, etc) and to reduce excess flocculant carry-over
Modification to the Washery water management system to create a 'semi-closed loop'. Includes installation of a slurry pipeline.	Reduce BCD water taken for process water in the Washery. Reduce diversion of Washery waters into BCD Slurry pipeline will improve Washery solids management and increase potential for further water reuse.	Infrastructure was installed and the 'semi-closed loop' commissioned in early June 2015. This project has resulted in a reduction of Brennans Creek dam water used by the Washery by approximately 1 ML/day, which has increased the volume of water available for environmental flow. Construction of the slurry pipeline has been completed.
Water treatment technology review	Review of water treatment technologies to increase the capacity of the current Water	Technologies selected and funding approved for the upgrade of the water treatment plant which

Works	Purpose	Status
	Filtration Plant and reduce potable water usage.	is planned to deliver an additional 2.4 ML/day of treated water. Construction has commenced on the pre-treatment system and water storage (to reduce reliance on potable water).
Water supply line from Appin East pit top to West Cliff.	This project allows salinity levels to be met to maintain Environmental Protection Licence and development consent conditions.	The under-bore and pipework from the Appin East pit top to Appin North Mine was installed and commissioned in April 2015. The supply line is currently being used to maintain salinity levels in line with EPL.

Not all improvements (as required by PRP 19) had been commissioned at the time of sampling, and as such the 2015 survey is considered a 'before' component of the monitoring design.

1.3.3 Climate

The region experiences a wet temperate climate. Average monthly maximum temperatures vary from 17 degrees Celsius (^{0}C) in July to 29^{0}C in January. Average monthly minimum temperatures vary from 1.7 ^{0}C in July to 15.2 ^{0}C in January. The dominant wind direction is from the south and south-east in January, February and March and from the west and south-west in June, July, August and September. The dominant wind directions in November and December are from both the north-east and south.

1.3.4 Catchment characteristics

The Georges River rises in the Hawkesbury Sandstone plateau approximately 5 km south-east of the Appin township (

Figure 1). The Georges River has formed in typical Hawkesbury Sandstone terrain. The catchment in its upper reaches has a long narrow shape. It flows predominantly northward before making its way east to Botany Bay. Brennans Creek and Sawpit Gully flow into the Georges River from the eastern side of the catchment. The upper reaches flow through areas of previous longwall mining, the Cataract Scout Park Appin Village and the Wedderburn Airport. The Georges River flows northward to Wedderburn and Campbelltown, eventually flowing into Botany Bay (Gilbert and Associates 2009).

Long, deep pools with frequent short riffles flowing over sandstone bedrock define the channel in the Upper Georges River catchment. The channel gradually widens from 6 m to 20 m and deepens from 0.2 m to 2 m (Bio-analysis 2009). Substratum of the channel is predominantly bedrock with deposits of sand in deeper areas. Sandstone boulders and logs occur throughout the channel. The banks of the channel are mostly soft sediment and are generally well vegetated by trees (including Eucalyptus spp. and Acacia spp.), ferns (i.e. *Gleichenia sp.* and *Sticherus flabellatus*), emergent macrophyte species (including *Eleocharis sphacelata*, *Juncus spp.*) and the reed *Typha orientalis* (Bio-analysis 2009). Some species of weeds (i.e. *Cynodon dactylon* and *Hypochaeris radicata*) were recorded near the town of Appin. The submerged species of macrophyte, *P. sulcatus*, was present in some of the pools (Bio-analysis 2009).

1.3.5 Water quality and hydrology

There is no continuously recorded flow monitoring data along the Georges River within the study area. Low, dry weather flows in the Georges River are predominantly derived from licensed discharges from Brennans Creek Dam (BCD) at the Appin North site and licensed discharges from the Appin Central pit top. The average flow released from BCD (Point 10) (1st February 2012-29th February 2016) was 2.5 ML/day (Table 2).

Licensed releases from BCD to the Georges River were generally elevated in aluminium, copper, nickel and zinc (Gilbert and Associates 2009). Elevated total iron concentrations in the Georges River may be due to increased groundwater interaction from earlier mining. Water quality studies in the region concluded that changes in stream waters result from the dissolution of marcasite under reducing conditions (Low Oxidation – Reduction Potential) of water saturation, transfer into stream water and precipitation on a change to oxidising conditions as an orange-brown hydroxide, ferrihydrite, which contributes to high iron, manganese, nickel and zinc (Geoterra 2006). The elevated levels observed for these parameters in the Georges River and its tributaries indicate the influence of urban area runoff, agricultural, industrial and mining activities in the Georges River catchment (Gilbert and Associates 2009). Water quality monitoring of major cations, nutrients and metal can be found at:

http://www.south32.net/our-operations/australia/illawarra-coal/regulatory-document

Table 2 Long term water quality parameters for Georges River

Location	pH	Conductivity EC ($\mu S.cm^{-1}$))	Turbidity (NTU)	Discharge ($ML.day^{-1}$) Mean (min,max)	Total suspended solids (mg/L)
Point 10	8.5	2342	20	2.5 (0-9.5)	7.8
Point 11	7.5	260	-	-	6.7
Point 12	8.8	1741	-	-	8.3
Georges River @ Minto	-	-	-	554 (2.307-107,960)	-

Averaged data of sites Point 10, 11 and 12 includes sampling data available at:

http://www.bhpbilliton.com/home/aboutus/regulatory/Documents/_coal/illawarra/bulliseam/140213_coal_illawarra_bulliseam_14DayMonitoringReport.xlsx.

Hydrology data for Georges River at Minto was provided by NSW Office of Water (http://realtimedata.water.nsw.gov.au/water.stm) (1st February 2014-31st August 2015).

1.3.6 Limitations

No sampling was conducted prior to discharge to establish pre-discharge baseline condition, as mine water discharge has been occurring from LDP10 for many years prior. Inference to changes in stream health is based upon the current condition of discharge monitoring sites compared to reference locations in Upper Georges River above Brennans Creek confluence. Sampling is also conducted every two years in spring only which may limit the interpretation of stream fauna temporal variability.

2. Methods

2.1 Site Locations

The study area is located within the Upper Georges River catchment commencing at GRQ1 and runs for 21 kilometres to site GROH, just upstream of the confluence with O'Hares Creek. Site GROH is located approximately 17.5 kilometres downstream of the Appin North LDP 10. Eight sites were located in pool habitats downstream of LDP 10 (Table 3). Three sites were added to the 2015 monitoring, Pool 16, Pool 32, GRQ 19 to strengthen the representation of Georges River ecology downstream of Point 10. Three reference sites were also sampled, GRUFS, GRQ1 and Point 11 (upstream Georges River). Cascade Creek (CC1, CC2) was not sampled in 2015 for logistical reasons.

Table 3 Site location and treatment

Site Number	Stream	Location	Easting	Northing	Treatment	Sampling
Point 10	Brennans creek	Discharge point (LDP10)	297558	6212772	Discharge monitoring	2013, 2015
Point 11	Brennans creek	U/s of Brennans Creek and Georges River confluence	297207	6212940	Reference	2013, 2015
Point 12	Georges River	D/s of Brennans Creek confluence	297157	6213016	Discharge monitoring	2013, 2015
Jutts crossing	Georges River	D/s of Jutts Crossing	296844	6213232	Discharge monitoring	2013, 2015
Pool 16	Georges River	D/s of Kennedy Creek	296890	6213908	Discharge monitoring	2015
Pool 32	Georges River	D/s of Sawpit Gully	297192	6215029	Discharge monitoring	2015
GRQ18	Georges River	U/s of O'Hares Creek confluence	296748	6217637	Discharge monitoring	2013, 2015
GRQ19	Georges River	U/s of Spring Creek	298747	6223615	Discharge monitoring	2015
GR/OH	Georges River	D/S O'Hares Creek confluence	300156	6225390	Discharge monitoring	2015
GR/OH (Removed)	Georges River	U/s O'Hares Creek confluence	300013	6225211	Discharge monitoring	2013
GRUFS	Georges River	U/s of confluence	297082	6211771	Reference	2013, 2015
GRQ1	Georges river	U/s of confluence	297225	6211446	Reference	2013, 2015
CC1 (Removed)	Cascade Creek	Upper Cascade Creek	290841	6207918	Reference	2013
CC2 (Removed)	Cascade Creek	Lower Cascade Creek	291730	6209505	Reference	2013

2.2 Survey timing and frequency

The EPL conditions require sampling every two years commencing in 2013 and concluding in 2019, and that sampling occur between 1st September and 30th November in each of the sampling years. Sampling was conducted in the period of 12th October – 15th October 2015 (Macroinvertebrates).

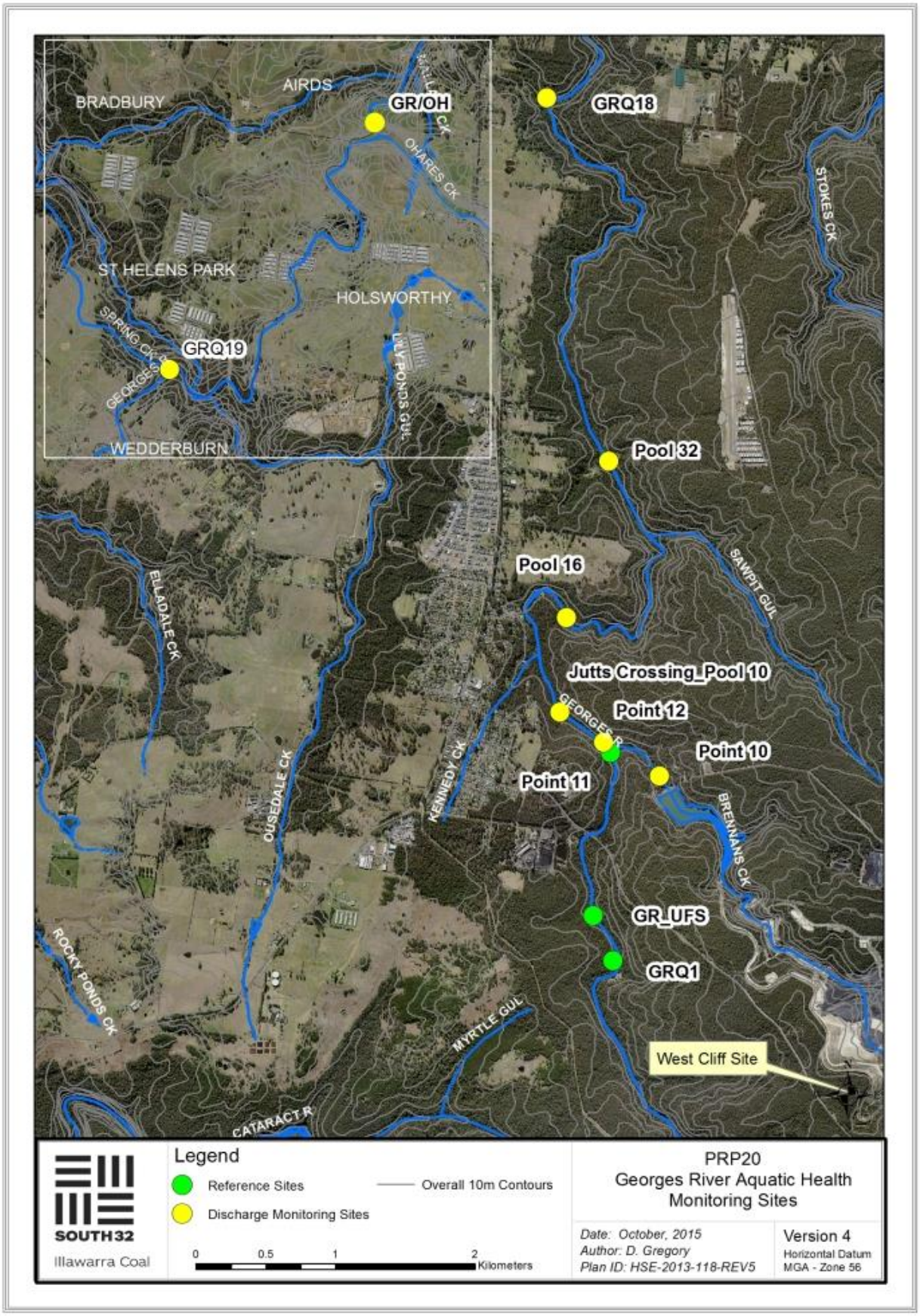


Figure 1 Location of macroinvertebrate 2015 sampling sites.

2.3 Field methods

2.3.1 Water quality

Surface water quality was measured *in situ* using a Horiba U51 water quality probe at each site. The following variables were recorded:

- Temperature (°C)
- Conductivity (µS/cm)
- pH
- Dissolved Oxygen (% saturation) and
- Turbidity (NTU).

Grab samples were also taken at each sampling location. The following analytes were tested:

- Alkalinity.
- Dissolved Sulfate.
- Chloride.
- Dissolved Major Cations.
- Dissolved Metals.
- Dissolved Organic Carbon DOC.
- Ultra trace nutrients.

2.3.2 Macroinvertebrate survey

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.

Laboratory Identification

Macroinvertebrates were sorted from the organic matter. All macroinvertebrates (except for segmented and unsegmented worms) were identified to family level. The segmented worms were identified to class (Oligochaeta) and unsegmented worms to phylum, except for flatworms which were identified to order (Tricladida). Acarina are identified to order. Small crustaceans Ostrocoda, Copapoda and Cladocera were not identified.

Leptophlebiidae was also identified to species where possible.

2.4 Analysis of data

To meet licensing requirements descriptive statistics were used to report on data collected at each monitoring site.

2.4.3 Water quality

Water quality results of both field and laboratory processed data were tabulated. Water quality was examined by comparison to the limits outlined in PRP 19.

2.4.4 Macroinvertebrates

Macroinvertebrate data taxa were tabulated. The following parameters were calculated and presented for the assemblages:

- Family Richness
- Density
- Any other taxa identified to be driving differences.

A control chart showing average similarity measure of discharge monitoring and reference sites was also plotted to show downstream changes in similarity of macroinvertebrate assemblages of both 2013 and 2015 data and its comparison to 95% confidence limits of similarity among all reference samples. This was conducted to compare the range of similarities of monitoring/reference to the similarities that would occur naturally between sites.

2.5 Statistical Analysis

The design is to test whether the abundance, richness and assemblages of aquatic biota will become more similar to the reference sites. The design is aimed to detect improvement of stream health. Conversely the design can also permit the assessment of future negative impacts if aquatic biota becomes more dissimilar to the reference sites. The data collected is before the implementation of water management measures under PRP 19. Therefore the data presented in this report is exploratory and examines current differences and patterns in macroinvertebrates and water quality.

Faunal assemblages in the study area are compared to those recorded in non-affected streams above the confluence of Brennans Creek in the Georges River (Figure 1). These comparisons infer whether the monitored sites within the study area differs from reference sites and subsequently whether the aquatic fauna of the study area is continuing to change relative to reference sites. The comparison to reference streams is to account for natural changes to macroinvertebrate assemblages (e.g. changes from drought/flood), as well also provide a reference condition; representing what the stream fauna will be like in the absence of mine water discharge.

Statistical analysis of data was undertaken to identify large scale changes between surveys (Years) and upstream and downstream stretches of the river (Treatment) in the macroinvertebrate assemblage. Permutational Multivariate Analysis Of Variance (PERMANOVA) using the PRIMER v6 Statistical Package was used to test for significant differences between the upstream and downstream parameters and the assemblage in entirety (Anderson *et al.* 2008). At each site and within each habitat, the macroinvertebrate data from the three subsamples were combined, giving a representative sample for each site for each sampling occasion (Downes et al. 2002 and Downes 2010).

Transformation (log10) of the univariate density-based response variables was required to ensure data normality. A resemblance matrix was created based on Euclidean distances for both density and richness. For the calculation of multivariate resemblance matrix using Bray-Curtis similarity measure, densities of all families were 4^{th} root transformed to reduce differences in scale among variables, but still retain information regarding relative abundances.

Differences in the assemblages were also visualised using non-metric Multi-Dimensional Scaling (nMDS) and taxa driving differences were identified through investigating Similarity of Percentages (SIMPER) (Warwick 1993).

The following experimental design was applied:

- Year: 2013 vs. 2015
- Treatment: Discharge vs. Reference
- Site (Treatment): 13 sites

As the program does not have replication at the site level the highest order interaction term has been excluded from the analysis (Anderson *et al*. 2008).

A one-way ANOSIM was also performed using 2015 data only. This was conducted to determine if differences between discharge monitoring and reference sites were still apparent with 2013 data omitted from the statistical testing.

2.1 Water Quality

The field water quality results of temperature (°C), Conductivity (µS/cm), pH; Dissolved Oxygen (% saturation); Turbidity (NTU) are shown in Table 4.

The results of water samples taken: alkalinity, dissolved sulfate, chloride, dissolved major cations, dissolved metals, dissolved organic carbon and ultra-trace nutrients are shown in **Error! Reference source not ound.**. Reference sites had lower temperature, electrical conductivity, major cations, alkalinity, nitrates, however was more acidic (Low pH), and higher in iron and manganese. Discharge monitoring sites were also higher in all other metals (Annex 2). A comprehensive water quality monitoring program is conducted by South 32 and hence will not be discussed in detail within this report.

Table 4 Field water quality results.

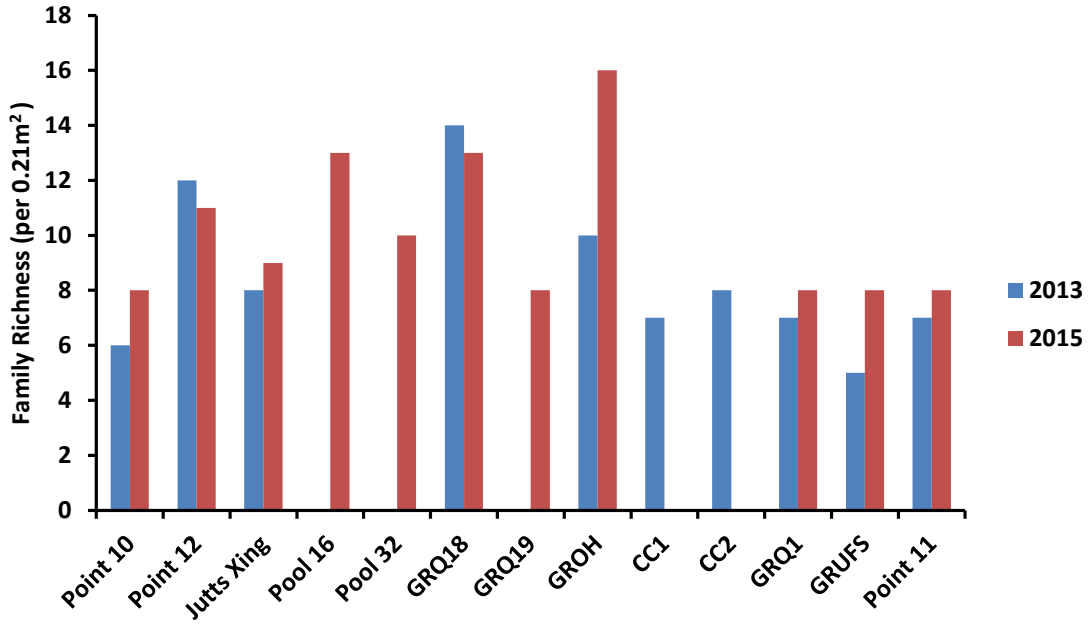
Site No.	Temperature (C°)	Conductivity (µS/cm)	Turbidity (NTU)	Dissolved Oxygen (% sat)	pH
GRQ1	17.25	162	3.4	35.5	6.5
GR_UFS	17.74	169	6.3	51.3	6.23
Point 11	18.82	177	3.8	21	7.36
Point 10	18.95	1920	16.4	18	8.94
Point 12	20.46	1790	10.7	33	8.94
Jutts Crosssing	18.28	1790	9.7	28.6	8.72
Pool 16	18.48	1790	20.7	20.7	8.65
Pool 32	18.37	1680	6.1	32.8	8.53
GRQ18	16.58	1550	2.8	50.7	7.8
GRQ19	18.32	1440	10.2	76.4	8.23
GR/OH	20.32	900	21	37.3	7.97

http://www.south32.net/our-operations/australia/illawarra-coal/regulatory-document

2.2 Macroinvertebrates

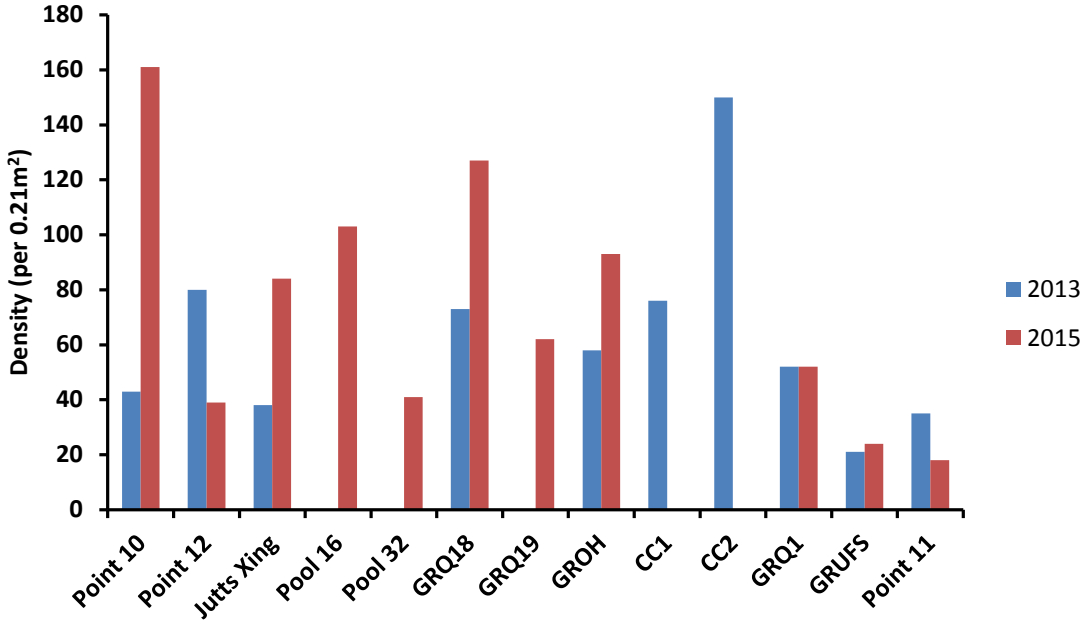
2.2.1 Density and family richness

There were a total of 28 families observed across all sites. The highest mean family richness was recorded at the Georges River discharge monitoring site GR0H (16 families per 0.21m^2) and the lowest at the Georges River reference site GRUFS (5 families per 0.21m^2) (Graph 1). However, these differences were not statistically different for both factors of Year (2013 vs. 2015) and Treatment (discharge vs. reference) (Table 5).



Graph 1 Family richness at each site (2013 and 2015).

The total density over all sites sampled was 804 individuals. The highest density was recorded at the discharge location, Point 10 (161 per 0.21m^2), consisting mostly of dytiscid beetles. The lowest density was recorded in Georges River reference site Point 11 (18 per 0.21m^2) (Graph 2). These differences in density were not found to be statistical different for both factors of Year (2013 and 2015) and Treatment (discharge vs. reference) (Table 5).



Graph 2 Density at each site (2013 and 2015).

Table 5 PERMANOVA results for mean family richness and mean density.

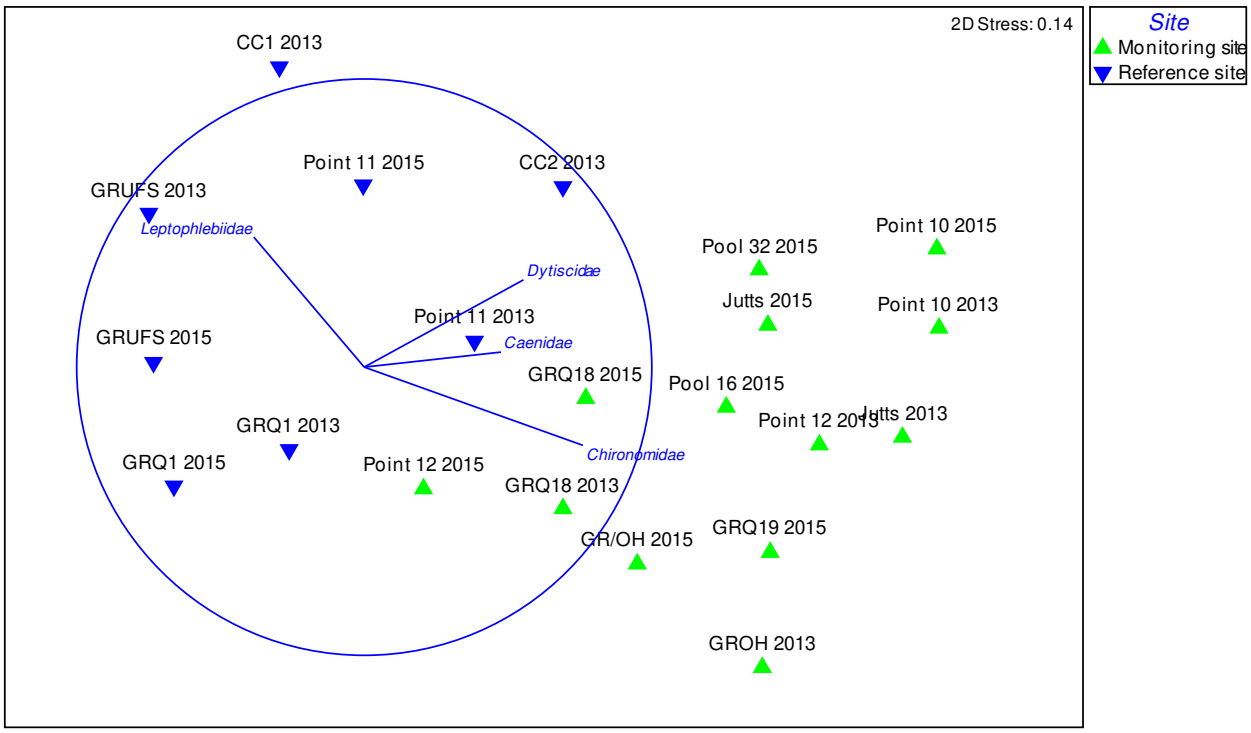
Variable	Source / factor	Degrees of freedom	Sum of squares	Mean squares	Pseudo-F	P(perm)	Permutations
Mean family richness	Year	1	8.8167	8.8167	2.9498	0.1378	4807
	Treatment	1	26.667	26.667	4.8313	0.0494	9962
	Site (Treatment)	11	82.867	7.5333	2.5204	0.1351	9739
	Year x Treatment	1	6.6667E-2	6.6667E-2	2.2305E-2	0.8834	4773
	Residual	6	17.933	2.9889			
	Total	20	162.67				
Mean density	Year	1	0.06675	0.06675	0.6208	0.4619	9849
	Treatment	1	0.066945	0.066945	0.45145	0.7787	9961
	Sites (Treatment)	11	1.8396	0.16724	1.5553	0.2947	9948
	Year x Treatment	1	0.20424	0.20424	1.8994	0.2179	9811

	Residual	6	0.64516	0.10753			
	Total	20	3.5546				

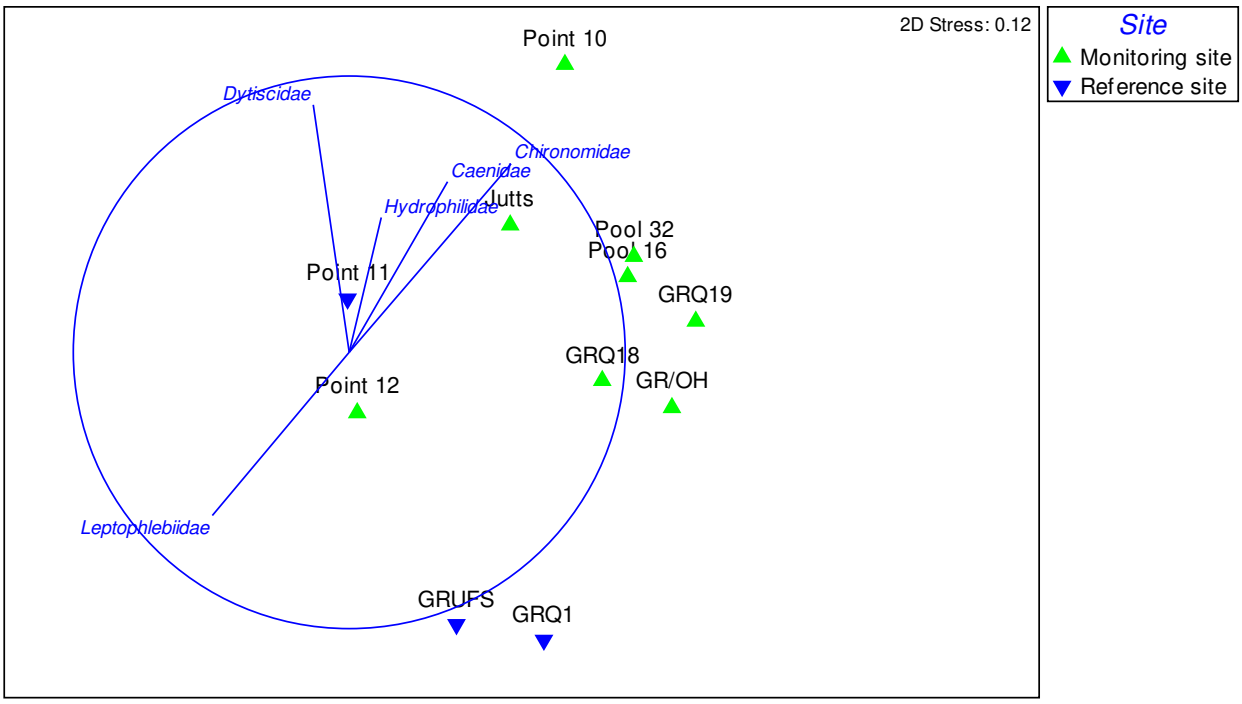
2.2.2 Macroinvertebrate assemblages

PERMANOVA detected significant difference between Treatment (discharge vs. reference groups) and Sites (Treatment) (Table 6). The MDS plot (Graph 3) illustrates differences between discharge and reference sites. Interestingly the MDS plot using 2015 data only (Graph 4) does not show as clear separation of discharge and reference sites.

A one-way ANOSIM performed using 2015 data only showed significant difference between monitoring and reference sites (significance level of statistic -0.006, global R- 0.745), indicating that despite this variation significant differences between monitoring and reference sites are still evident.



Graph 3 MDS plot of monitoring and reference sites (2013 and 2015). Vectors show macroinvertebrate families that contributed 50% of monitoring and reference sites dissimilarity.



Graph 4 MDS plot of monitoring and reference sites (2015 only).

SIMPER procedure of 2015 data showed that within the discharge Treatment the families Chironominae, Dytiscidae, Coengrionidae, and Hydrophilidae contributed most to the within stream similarity (51%). Leptophlebiidae, Atyidae contributed most to reference Treatment similarity (50.36%). Point 10 was on average 10.82% similar to reference sites, Point 12 (36.85%), Jutts Crossing (29.6%), Pool 16 (28.8%), Pool 32 (29.1 %), GRQ18 (41.3%), GRQ 19 (22.5%) and GROH (32.7%) (Graph 5).

Overall (including both 2013 and 2015 data), lower densities of Leptophlebiidae and higher densities of Chironomidae, Caenidae, Dytiscidae contributed most significantly to dissimilarities between discharge and reference Treatments (Table 8). However in 2015, higher densities of Chironomidae, Caenidae, Dytiscidae, and Hydrophilidae (Graph 5) for the discharge Treatment contributed most to the dissimilarity (overall dissimilarity 71.11%) between Treatments (Table 8, Graph 5).

This indicated Leptophlebiidae is not contributing as significantly to dissimilarity between monitoring and reference sites compared to 2013. Sites particularly those close to the discharge point, Point 12 and Jutts Crossing, have increased in similarity to reference sites compared to 2013 (Graph 5), with Point 12 being marginally within the 95% confidence limits of similarity among reference sites. This increase in similarity is likely the result of Leptophlebiidae occurring at these sites compared to previous sampling in 2013 when the mayfly family was absent from sites immediately downstream of the discharge point. Despite this, there is still statistically significant differences in macroinvertebrate assemblages, however as mentioned, these differences are driven primarily by Chironomidae, Caenidae and Dytiscidae within monitoring sites (Table 8, Graph 6). Point 10 showed a decrease in similarity to reference sites and is likely to be influenced by low family richness, lack of Leptophlebiidae and a high proportion of dytiscid beetles (Graph 6).

Table 6 PERMANOVA results for macroinvertebrate assemblages

Variable	Source	Degrees of freedom	Sum of squares	Mean squares	Pseudo-F	P(perm)	Permutations
Macroinvertebrate assemblages	Year	1	2798.5	2798.5	3.1093	0.0127	9948
	Treatment	1	9862.3	9862.3	4.3476	**0.0003**	9917
	Site (Treatment)	11	21315	1937.7	2.153	**0.0022**	9901
	Year x Treatment	1	903.58	903.58	1.0039	0.4377	9944
	Residual	6	5400.3	900.04			
	Total	20	43039				

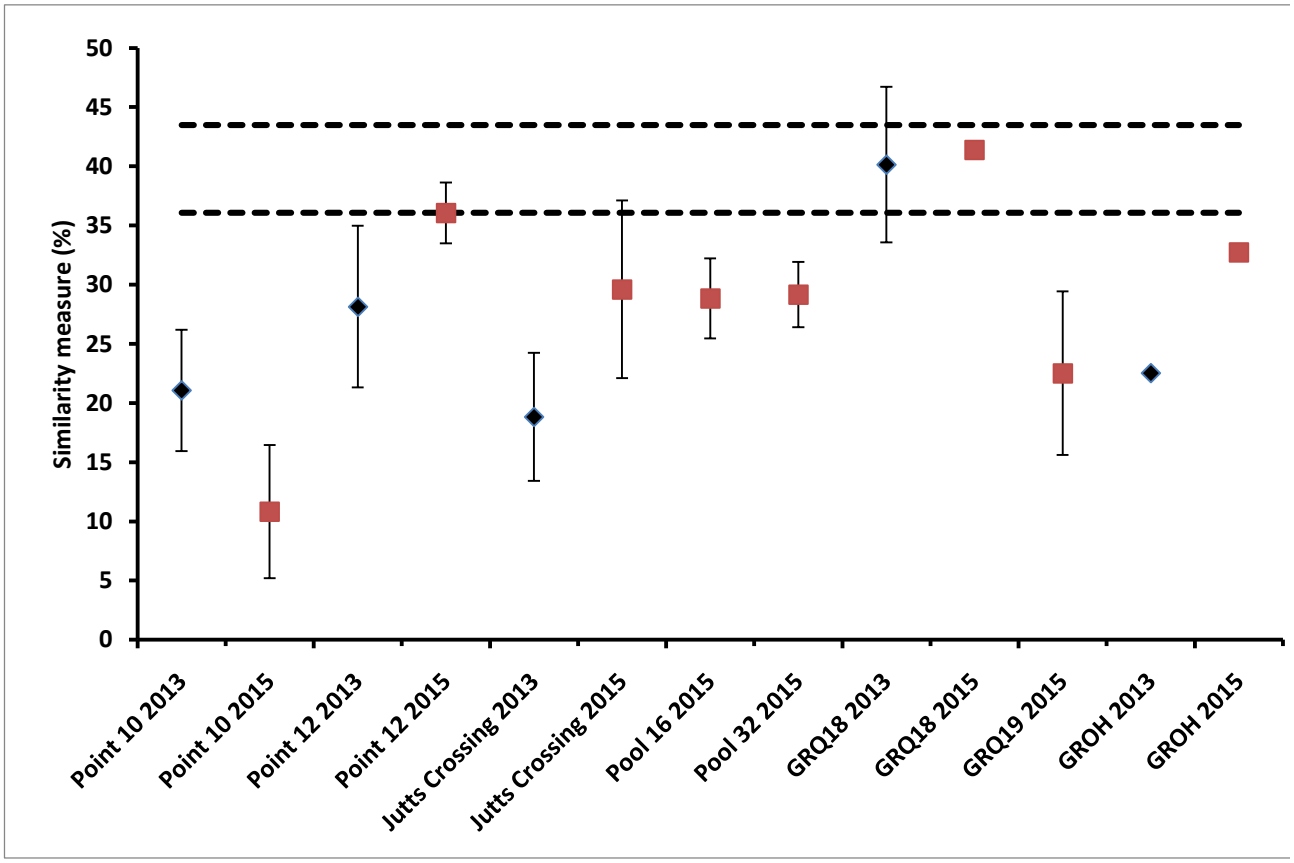
Table 7 SIMPER results of dissimilarity between discharge monitoring and reference sites overall (2013 and 2015)

Species	Average Abundance discharge monitoring	Average abundance reference	Average Dissimilarity	Dissimilarity/Standard Deviation	Contribution%	Cumulative. %
Leptophlebiidae	0.86	3.17	8.94	1.85	11.63	11.63
Chironomidae	2.54	0.75	7.42	1.69	9.65	21.27
Dytiscidae	1.84	0.36	6.49	1.29	8.44	29.72
Caenidae	1.75	0.00	6.09	1.45	7.91	37.63

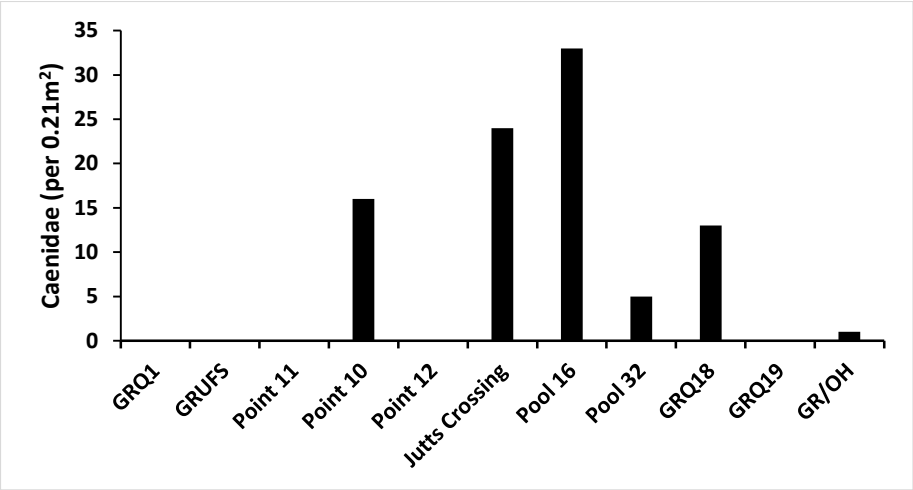
Table 8 SIMPER results of dissimilarity between discharge monitoring and reference sites 2015

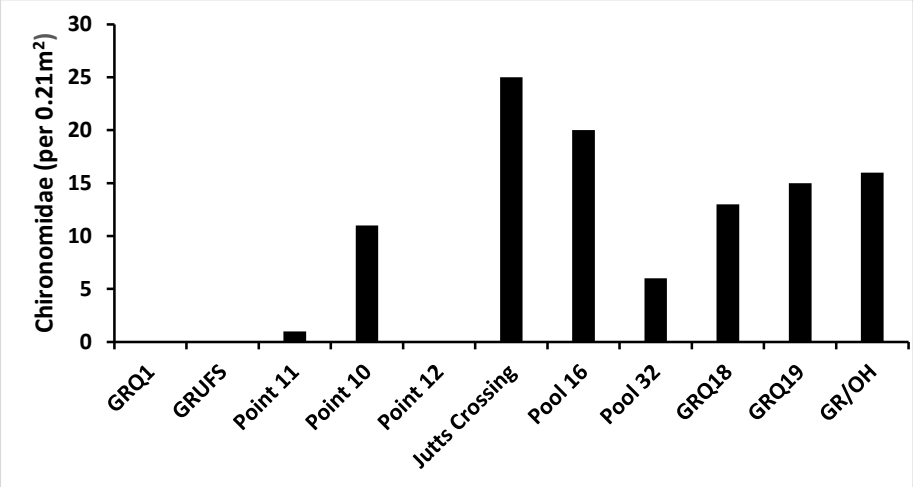
Species	Average Abundance discharge monitoring	Average abundance reference	Average Dissimilarity	Dissimilarity/Standard Deviation	Contribution%	Cumulative. %
Chironomidae	1.70	0.33	5.51	2.16	7.75	7.75

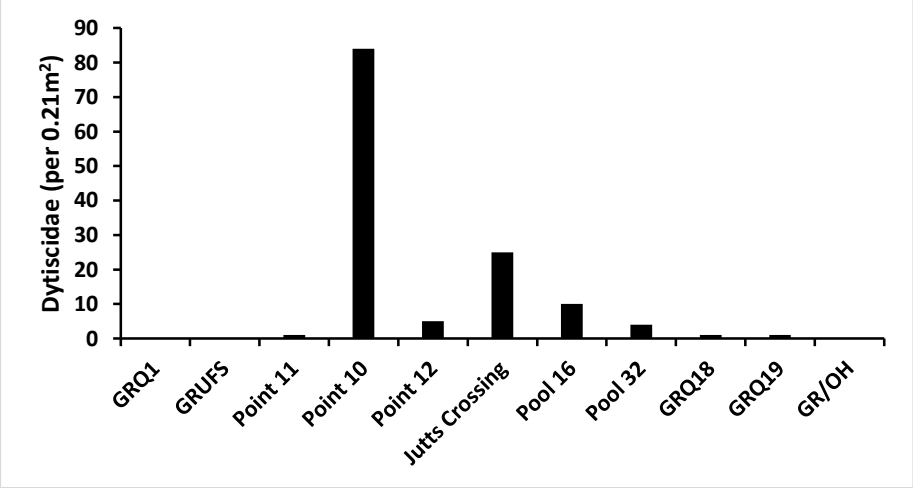
Caenidae	1.38	0.00	5.13	1.47	7.21	14.96
Dytiscidae	1.49	0.33	4.84	1.42	6.80	21.76
Hydrophilidae	1.14	0.00	4.40	1.89	6.19	27.96

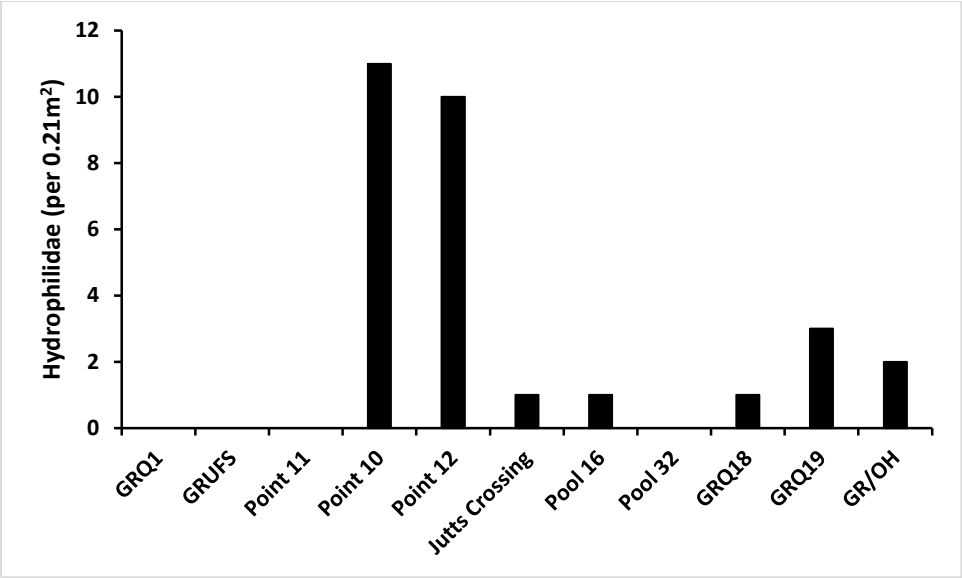


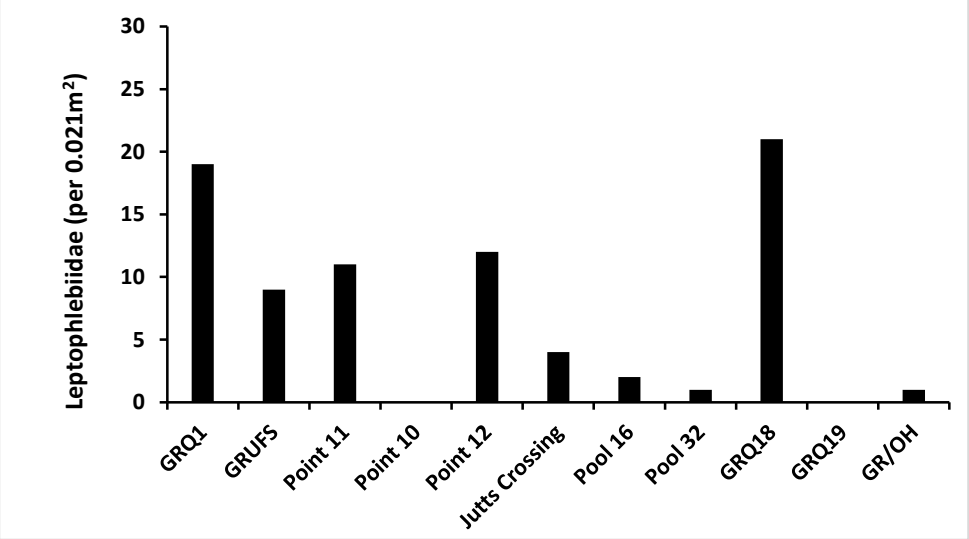
Graph 5 Average similarity measure and standard error of monitoring to reference sites (2013 and 2015). Note that Cascade Creek reference sites (CC1 and CC2) were removed to allow comparison between 2013 and 2015 sampling occasions. The dotted lines represent the 95% confidence limits of similarity among reference sites.


Caenidae (per 0.21m²)
35
30
25
20
15
10
5
0
GRQ1
GRUFS
Point 11
Point 10
Point 12
Jutts Crossing
Pool 16
Pool 32
GRQ18
GRQ19
GR/OH


Chironomidae (per 0.21m²)
30
25
20
15
10
5
0
GRQ1
GRUFS
Point 11
Point 10
Point 12
Jutts Crossing
Pool 16
Pool 32
GRQ18
GRQ19
GR/OH


Dytiscidae (per 0.21m²)
90
80
70
60
50
40
30
20
10
0
GRQ1
GRUFS
Point 11
Point 10
Point 12
Jutts Crossing
Pool 16
Pool 32
GRQ18
GRQ19
GR/OH





Graph 6 Density of Caenidae, Chironomidae, Dytiscidae, Hydrophilidae and Leptophlebiidae at each site (sites arranged upstream to downstream).

2.3 Leptophlebiidae species

Species identification of Leptophlebiidae families (Table 9) found three species of three different genera, including *Atalophlebia*, *Ulmerophlebia* and *Koornonga*. Off these, *Atalophlebia sp. AV13* was the most common with *Koornonga sp AV2* only being found in the reference streams.

Table 9 Leptophlebiidae species at each site

Leptophlebiidae species	*Atalophlebia sp.AV 13*	*Ulmerophlebia sp. AV2*	*Koorrnonga sp. AV3*	Very small Not identified
Point 10				
Point 12	5	2		3

Jutts Crossing	4			
Pool 16	2			
Pool 32				
GRQ18	8			
GRQ19				
GR/OH				
GRQ1	7	2	6	
GRUFS	1	1	7	
Point 11	9	3		

3. Discussion

3.1 Macroinvertebrates assemblages –discharge monitoring and reference sites

Univariate data showed no significant difference between discharge monitoring and reference sites in density and family richness, however multivariate data showed there was significant difference in assemblages between years and discharge monitoring and reference sites. In 2013 (Niche 2014) and overall (2013/2015 data), the difference was attributed to lower densities of pollution sensitive Leptophlebiidae (SIGNAL 8) and increased densities of pollution tolerant Chironomidae (SIGNAL 3) and Caenidae (SIGNAL 4) in discharge monitoring groups compared to reference groups.

However in 2015, Leptophlebiidae was observed in sites immediately downstream of the mine water discharge (Point 12 and Jutts Crossing), and thus contributed less to observed differences between the monitoring and reference sites, the average similarity increasing (7.9% and 10.8% respectively). Despite this, there was still statistical difference between monitoring and reference sites and as shown in Graph 5 the average similarity of discharge monitoring sites to control sites remain outside the 95% confidence limits of similarities observed between reference sites. This difference is primarily driven by pollution tolerant species: true fly (Chironomidae), mayfly (Caenidae), and beetles (Dytiscidae and Hydrophilidae) at discharge sites. It is difficult to relate this change (increase in similarity) to any management action such as dilution of mine water discharge which has seen electrical conductivity reduce by approximately 15% as well as reduction in concentration of most water quality analytes.

It appears that mine water quality may be influencing macroinvertebrate assemblages at the site closest to the discharge point (Point 10). This is consistent with the results from Point 10 in 2013, particularly with absence of pollution sensitive family Leptophlebiidae at this site.

3.2 Leptophlebiidae as an indicator

3.2.1 Previous studies

Previous studies on Appin North mine water discharge (Cardno 2006) found Leptophlebiidae more abundant in the control treatment, whereas Chironomidae and Baetidae were more abundant in the mine discharge treatment. Cardno (2010) also found macroinvertebrate edge samples contained relatively few Leptophlebiidae mayflies downstream of mine water discharge from Appin North. The PRP20 survey in 2013 (Niche 2014) found no Leptophlebiidae mayflies immediately downstream of mine water discharge, however Leptophlebiidae mayflies were common in upstream reference sites. Niche (2014) recommended that Leptophlebiidae could be used as an indicator to monitor stream health and ecological changes as a result of the implementation of PRP19. This family has been shown to be sensitive to pollution (Cardno 2010) and overall appears to be affected immediately downstream (i.e. Point 10) of the discharge point.

While Leptophlebiidae is known to be sensitive to high conductivity, it is recognised that other minewater constituents may contribute to its ecotoxicity (Cardno 2010, 2014). Studies by Ecoengineers (2012) have found that factors other than conductivity may be affecting macroinvertebrates as discharge water was found not to be ecotoxic to water flies (*Ceriodaphnia sp*.) and freshwater shrimp (*Parataya sp.).* However, untreated, mine process water was found to be ecotoxic to these species.

3.2.2 Species

Identification of Leptophlebiidae found three species:

- *Atalophlebia sp. AV 13*

- *Ulmerophlebia sp. AV2*
- *Koorrnonga sp. AV3*

Of these *Koornanga sp AV2* was only found in the reference sites. It is recommended that species identification of Leptophlebiidae be conducted in future monitoring programs to determine if specific species are more affected by mine water discharge.

3.2.3 Predicted changes to Leptophlebiidae

It is hypothesised that with significant improvements from PRP19 that Leptophlebiidae will commonly inhabit sites downstream of the mine water discharge point, indicating a return of assemblages to pre-disturbance composition. This is likely to be more evident at sites immediately downstream of the mine water discharge compared to sites further downstream. Sites further downstream receive generally more diluted mine water and their assemblages are likely shaped by a different selection of environmental variables (Vannote et al. 1980).

3.3 Longitudinal patterns

It is evident from the program that the influence of mine water discharge on water quality decreases downstream, for example electrical conductivity is almost halved at GRQ18.

GROH had the highest family richness of all sites (including the reference sites). GRQ18 was most similar (40%) (2013 and 2015) to reference sites in terms of macroinvertebrate assemblages and has a similarity that is within the similarity experienced between reference sites (Graph 5). It appears the magnitude of influence from mine water discharge likely to affect macroinvertebrate assemblages is less evident than sites closer to the discharge point. At site GROH however, the similarity to reference sites decreases, although it did increase in 2015 compared to 2013. Site GROH site has been relocated downstream of the confluence of O'Hares Creek and as such the changes observed in GROH macroinvertebrate assemblages could be the result of this change in site location.

Interestingly, the families that contributed to difference between monitoring and reference sites, Caenidae, Dytiscidae, and Hydrophilidae appear to decrease downstream (Graph 6), however whether this pattern is related to mine water discharge is unclear. Longitudinal change in faunal assemblages are difficult to relate to mine water discharge particularly as: the influence of mine water is reduced downstream (therefore faunal changes are likely to be smaller and more difficult to detect); and there are more confounding factors that influence macroinvertebrate assemblages (such as changed geomorphology and increased flow (Vannote et al. 1980).

4. Conclusion

The Aquatic Health Monitoring under PRP20 is a long term study. Findings based on the 2013 and 2015 are:

- There was no statistical difference between density and family richness through time, between discharge monitoring and reference sites and between sites (Treatment).
- There was no statistical difference between macroinvertebrate assemblages through time, however there was statistical difference between discharge monitoring and reference sites and between sites (Treatment).
- Higher densities of pollution tolerant Chironominae (SIGNAL 3,) Caenidae (SIGNAL 4), Dytiscidae (SIGNAL 2) and Hydrophilidae (SIGNAL 2) were observed in discharge monitoring groups compared to reference groups.
- There has been a slight increase in similarity at Point 12 and Jutts Crossing and this is thought to be primarily the result of Leptophlebiidae at these sites, however whether this is the result of any management action at the mine is unclear.
- Macroinvertebrate assemblages in 2015 did not show as clear separation between the discharge sites and reference sites compared to 2013 monitoring results, however the discharge and reference site were still statistically different.

4.1 Recommendations

It is recommended to separate the subsamples at each site to allow within site replication. This will permit statistical testing at the site level; so that individual sites (rather than grouped sites) can be statistically compared to reference sites. This is required to further address one of the key aims to assess the downstream gradient changes in composition and abundance of in-stream and sediment biota.

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Annex 1 Macroinvertebrates

		Impact								Reference		
Family	**Sensitivity Grade**	**Point 10**	**Point 12**	**Jutts**	**Pool 16**	**Pool 32**	**GRQ18**	**GRQ19**	**GR/OH**	**GRQ1**	**GRUFS**	**Point 11**
Austrocorduliidae	10		2				1				2	1
Leptophlebiidae	8		12	4	2	1	21		1	19	9	11
Oniscigastridae	8											
Gripopterygidae	8									2	1	
Elmidae	7											
Calamoceratidae	7								1			
Odontoceridae	7											
Leptoceridae	6				1	1	5		15	11	3	
Arrenuridae (Acarina)	6				10	2	20	33	40	2		
Oxidae (Acarina)	6				1							
Baetidae	5				6	12	35		2			
Megapodagrionidae	5		2				6		1	2	3	

Corduliphyidae							1			7		
Corduliidae	5											
Hemicorduliidae	5		2	1	6	1	5	1	5			
Gomphidae	5		1	1				1	2	2		1
Sialidae	5								1			
Gyrinidae	4	5										
Ecnomidae	4				1						2	
Caenidae	4	16		24	33	5	13		1			
Aeshnidae	4											
Ceratopogonidae	4		1									
Aeshnidae	4											
Libullidae	4		1									
Isostictidae	3		1		5			6	3			
Chironominae (chironomidae)	3	11		25	20	6	13	15	16			1
paratya	3									7	3	
Oligochaeta	2											
Dytiscidae	2	84	5	25	10	4	1	1				1

Hydrophilidae	2	11	10	1	1		1	3	2			
Coenagrionidae	2	4		2	6	8	5	2	3		1	1
Corixidae	2	14	2									
Sythemistidae	2											1
Notonectidae	1	16		1		1						
Culicidae	1							1				
Gastropoda	1								1			
Telephlebiidae	9				1							1
Ceratopogonidae	2		1									
Cladocera (Daphniidae)	4				206							

Species	**Point 10**	**Point 12**	**Jutts**	**Pool 16**	**Pool 32**	**GRQ18**	**GRQ19**	**GR/OH**	**GRQ1**	**GRUFS**	**Point 11**
Atalophlebia sp.AV 13		5	4	2		8			7	1	9
Ulmerophlebia sp. AV2		2							2	1	3
Koorrnonga sp. AV3									6	7	
Unidentified-too small		3									

Annex 2 Water quality

Water chemistry results

			Discharge Sites								Reference Sites		
		Sample date:	12/10/2015	12/10/2015	13/10/2015	13/10/2015	13/10/2015	14/10/2015	14/10/2015	14/10/2015	15/10/2015	15/10/2015	12/10/2015
		Site	Point 10	Point 12	Jutts Crossing - Pool 10	Pool 16	Pool 32	GRQ18	GRQ19	GR/0H	GR/UFS	GR/Q1	Point 11
Analyte grouping Analyte	**Units**												
EA005FD: Field pH													
pH Value	**pH Unit**		9	9	9	8.9	8.7	7.9	8.4	8	6.1	5.7	6.8
EA010FD: Field Conductivity													
Electrical Conductivity (Non Compensated)	**MicroSiemens/cm**		2200	1820	1800	1780	1670	1550	1520	914	170	165	184
ED037P: Alkalinity by PC Titrator													
Hydroxide Alkalinity as CaCO3	**mg/L**		<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

Carbonate Alkalinity as CaCO3	**mg/L**		177	138	124	105	77	24	24	13	<1	<1	<1
Bicarbonate Alkalinity as CaCO3	**mg/L**		671	516	545	554	543	538	496	275	5	4	10
Total Alkalinity as CaCO3	**mg/L**		849	653	669	659	620	562	520	289	5	4	10
ED041G: Sulfate (Turbidimetric) as SO4 2- by DA													
Sulfate as SO4 - Turbidimetric	**mg/L**		42	36	37	36	35	34	34	22	7	6	7
ED045G: Chloride by Discrete Analyser													
Chloride	**mg/L**		161	131	133	136	128	141	143	90	39	39	40
ED093F: Dissolved Major Cations													
Dissolved Calcium	**mg/L**		8	8	8	7	7	8	8	7	1	1	3
Dissolved Magnesium	**mg/L**		2	2	2	3	3	6	6	5	4	4	3
Dissolved Sodium	**mg/L**		497	446	400	407	397	354	307	178	20	20	20
Dissolved Potassium	**mg/L**		3	3	2	2	2	3	3	2	<1	<1	<1

EG020F: Dissolved Metals by ICP-MS													
Dissolved Aluminium	**mg/L**		0.31	0.26	0.24	0.21	0.13	0.04	0.05	0.04	0.01	0.01	0.03
Dissolved Arsenic	**mg/L**		0.007	0.009	0.007	0.006	0.004	0.002	0.002	<0.001	<0.001	<0.001	<0.001
Dissolved Cadmium	**mg/L**		<0.0001	<0.0001	<000.1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Dissolved Cobalt	**mg/L**		0.003	0.002	0.003	0.003	0.002	0.003	0.001	<0.001	<0.001	<0.001	<0.001
Dissolved Copper	**mg/L**		0.006	0.006	0.006	0.005	0.005	0.002	0.002	<0.001	<0.001	<0.001	<0.001
Dissolved Lead	**mg/L**		0.002	0.002	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Dissolved Manganese	**mg/L**		0.008	0.007	0.005	0.008	0.008	0.03	0.009	0.015	0.11	0.089	0.022
Dissolved Nickel	**mg/L**		0.093	0.087	0.082	0.083	0.082	0.071	0.062	0.034	0.001	<0.001	<0.001
Dissolved Zinc	**mg/L**		0.032	0.031	0.024	0.019	0.019	0.014	0.006	0.005	0.006	0.009	0.011
Dissolved Iron	**mg/L**		0.06	0.06	0.07	0.2	0.18	0.54	0.18	0.27	0.32	0.14	0.28
EK255A: Ammonia													
Ammonia as N	**mg/L**		0.029	0.023	0.023	0.007	0.038	0.011	<0.005	<0.005	0.017	0.012	0.01
EK259A: Nitrite and Nitrate (NOx)													
Nitrite + Nitrate as N	**mg/L**		0.545	0.448	0.424	0.344	0.237	0.112	0.014	0.23	0.009	0.003	0.014

EK261A: Total Kjeldahl Nitrogen												
Total Kjeldahl Nitrogen as N	**mg/L**		0.48	0.39	0.32	0.3	0.26	0.15	0.26	<0.01	<0.01	<0.01	0.07
EK262A: Total Nitrogen												
Total Nitrogen as N	**mg/L**		1.02	0.84	0.74	0.64	0.5	0.26	0.27	0.23	<0.01	0.01	0.08
EK267A: Total Phosphorus (Persulfate Digestion)												
Total Phosphorus as P	**mg/L**		0.023	0.016	0.014	0.016	0.012	0.007	0.019	0.013	0.012	0.01	0.007
EN055: Ionic Balance												
Total Anions	**meq/L**		22.4	17.5	17.9	17.8	16.7	15.9	15.1	8.77	1.34	1.3	1.47
Total Cations	**meq/L**		22.2	20	18	18.4	17.9	16.4	14.3	8.55	1.25	1.25	1.27
EP002: Dissolved Organic Carbon (DOC)												
Dissolved Organic Carbon	**mg/L**		11	5	4	3	3	5	4	4	22	21	5

Physiochemical water quality results

	Temperature	Conductivity (us/cm)	DO%	pH	Turbidity (NTU)

GRQ1	17.25	162	35.5	6.5	3.4
GR_UFS	17.74	169	51.3	6.23	6.3
Point 11	18.82	177	2.1mg/L	7.36	3.8
Point 10	18.95	1920	18	8.94	16.4
Point 12	20.46	1790	3.87mg/L	8.94	10.7
Pool 10_Jutts	18.28	1790	28.6	8.72	9.7
Pool 16	18.48	1790	20.7	8.65	20.7
Pool 32	18.37	1680	32.8	8.53	6.1
GRQ18	16.58	1550	50.7	7.8	2.8
GRQ19	18.32	1440	76.4	8.23	10.2
GR/OH	20.32	900	37.3	7.97	21

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Aquatic Monitoring Program for the Upper Georges River/Brennans Creek: Metabarcoding of the benthic eukaryotic assemblages

Anthony Chariton, Sarah Stephenson, Francesca Gissi and Paul Greenfield

April 2016

Commercial-in-confidence

Prepared for South32, Illawarra Coal Pty Ltd

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Executive summary

South32 Illawarra Coal proposes to continue its underground mining at Appin and West Cliff mines 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 Planning Assessment Commission in December 2011 and 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. Following an OEH-commissioned review into metal speciation issues pertaining to Brennans Creek, it was recommended that an ecogenomic approach (also known as metabarcoding) be included into the biological monitoring program as a means of assisting in examining the relationships between discharge water quality and biological composition. Metabarcoding is a relatively new DNA-based approach which examines community structure by high-throughput sequencing targeted genes from bulk DNA extracts. A number of studies have demonstrated the capacity of metabarcoding to cover a far wider range of organisms than can be obtained using traditional techniques.

In 2013, CSIRO was asked by the former owners of the mine (BHP) to perform a metabarcoding analysis of the study's eukaryotic communities as an additional line of ecological evidence. This component aimed to survey the composition of the stream's benthic eukaryotes by comparing the five Brennans Creek/Georges River, Discharge Monitoring sites with four reference sites; and by examining the relationships between the compositional data and the water quality of the sampled sites. The main findings of this sampling program were: (i) that metabarcoding captured a wide breadth of taxa; (ii) total OTU (operational taxonomic unit) richness was markedly higher in the discharge monitoring sites, however, this was likely an artefact of these sites containing biological material from both the discharge and the river; (iii) clear differences in eukaryote composition were observed between the Reference and Discharge Monitoring sites; (iv) 410 OTUs were shown to aid in the characterisation of the treatments at the time of sampling; (v) the water chemistry from the discharge sites was complex, and as such it is not possible to attribute any perceived patterns to a single environmental variable, however there was strong evidence to indicate that

the water quality from the discharge sites was altering eukaryote composition; and the influence of water chemistry on biological composition was more evident for the sites closer to the discharge (Jutts Crossing, Point 10 and Point 12).

In this study, metabarcoding was again used to examine the benthic composition of sampling sites associated with the Upper Georges and Brennans Creeks. The sampling program slightly varied from that used in 2013, with the current program designed to better capture the environmental gradient associated with the discharge. Briefly, this involved sampling three treatments: Reference sites, Discharge Monitoring Sites, and Downstream Discharge Monitoring sites. The Discharge Monitoring Sites and Downstream Discharge Monitoring treatments were separated as it was envisaged that their compositions would differ due to their surrounding environments, with the Downstream Discharge Monitoring sites not being directly associated with the environmental gradient from which the Discharge Monitoring sites were sampled.

After the removal of potentially erroneous sequences, the sequencing dataset contained 9,918,815 reads, encompassing 11,231 unique OTUs. Of these, 95% of OTUs could be confidently assigned to a Kingdom, with the largest proportion belonging to Ciliophora (10%). Chytridiomycota, Cercozoa and Ascomycota each contributed to 5-6% of the total taxon richness. Mean total OTU richness was markedly higher in the Downstream Discharge Monitoring sites, however, the ecological significance of this finding requires consideration as this is likely an artefact of these sites containing biological material from a number of tributaries. Multivariate analysis clearly showed differences in eukaryote composition between the samples taken from the Reference and Discharge Monitoring sites. The compositions of samples obtained from Downstream Discharge Monitoring sites were different to those from the other two treatments, but more similar to the Discharge Monitoring treatment. The composition of samples from the Point 11 Reference site were different to those from the other two Reference sites. More than 4,200 potential indicator OTUs were shown to aid in the characterisation of the treatments at the time of sampling. In 2015 as in the case of the 2013 sampling event, an OTU from the genus *Eunotia*, was shown to be a strong indicator of the Reference treatment, with many of the potential indicator OTUs associated with the Discharge Monitoring and Downstream Discharge Monitoring sites also being diatoms, but from different taxonomic groups. This finding supports that of Chariton et al. (2015) who showed diatoms to be excellent indicators of environmental condition, especially in cases where nutrients are elevated.

The water chemistry from the discharge sites was complex, and as such, it was not possible to attribute any perceived patterns to a single environmental variable. When examined in the context of a mixture, there was strong evidence to indicate that the water quality from the discharge sites was altering eukaryote composition, with this being primarily driven by pH, conductivity, and their co-variates. Furthermore, there was a strong environmental gradient and a corresponding change in community structure from the upstream to downstream Discharge Monitoring sites.

Collectively, the findings from the metabarcoding survey illustrated the technique's capacity to identify composition changes between the three treatments and show changes along the observed environmental gradient. It is recommended that a custom database be established using morphologically identified specimens collected from the concurrent traditional macrobenthic survey system, to aid future studies and to produce higher resolution taxonomic data.

1 Introduction

1.1 Background and objectives

South32 Illawarra Coal proposes to continue its underground mining at Appin and West Cliff mines 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.

A 2012, OEH-commissioned review into metal speciation issues associated with Brennans Creek, recommended that an ecogenomic approach (also known as metabarcoding) may aid in examining the relationships between the study region's biota and water quality. With the approach providing a more holistic view of biodiversity than can be obtained using traditionally applied approaches. To address this need, CSIRO was asked by BHP to perform an ecogenomic analysis of the study's eukaryotic communities as an additional line of ecological evidence. Given the success of the 2013 metabarcoding survey, CSIRO was again requested by the new owners, South32, to survey the system.

The aim of this study was to use metabarcoding (DNA composition profiling) to survey the composition of the eukaryotic (i.e. does not include bacteria or archaea) benthic communities within the upper Georges River. Specifically, this entailed:

- Comparing the benthic compositions from sites assigned to three treatments: Reference, Discharge Monitoring and Downstream Discharge Monitoring; and
- Examine the relationships between the compositional data and the water quality of the sampled sites.

Due to advancements in metabarcoding and changes in methodology it is emphasised that the focus of this report is on the current study (2015) and no direct comparisons, with the exception of broad pattern trends, are made between the 2015 and 2013 metabarcoding surveys.

1.2 Metabarcode monitoring

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 meta-barcoding. 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 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 the impact of the Deep Horizon oil spill on benthic communities (Bik et al., 2012).

2 Methods

2.1 Sampling design

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; Pool 16, Pool 32 and GRQ18; and
- **Downstream Discharge Monitoring (2 sites)**, these sites are not directly associated with the Discharge Monitoring sites gradient– GRQ19 and GR/OH.

2.2 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).

2.3 Collection and analysis of DNA samples

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 polycarbonate corer (diameter 10 cm). All samples were transferred into DNA-free sterile 50 mL Greiner tubes and placed on ice immediately, then frozen within 8 h of collection and 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).

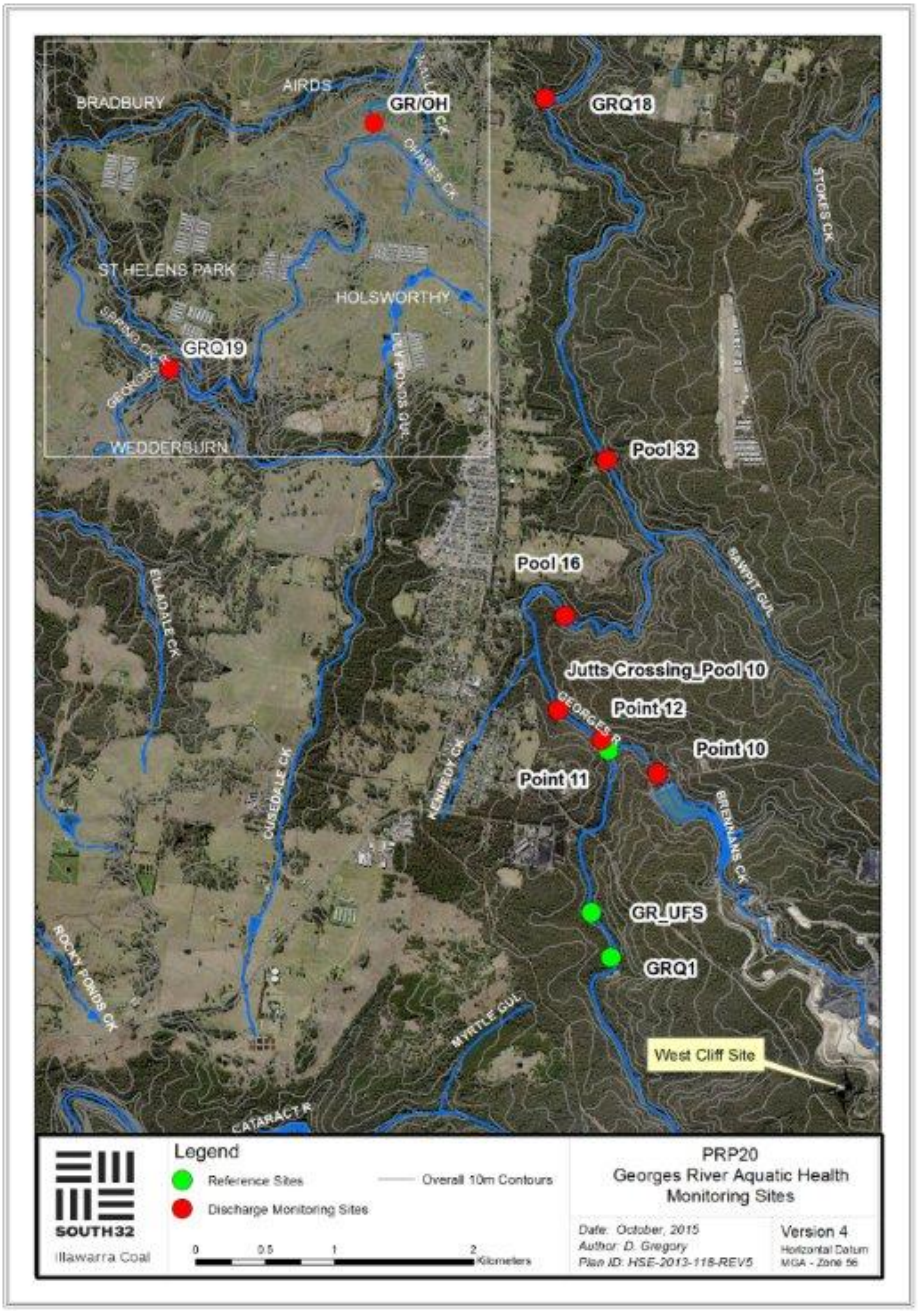

Figure 1. Location of sampling sites. Reference sites = GR/Q1, GR_UFS and Point 11; Discharge Monitoring sites = Jutts Crossing_Pool10, Point 10, Point 12, Pool 16, Pool 32, GRQ18; Downstream Discharge Monitoring sites = GRQ19 and GR/OH.

Table 1. Location of sampling sites and treatment allocations

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		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

Using 10 g of homogenised sediment, DNA was extracted and purified from each using Mo Bio PowerMax© Soil isolation kits (MO BIO, Carlsbad, CA) following the manufacturer's protocols. In addition to the sediment samples, two internal reference samples containing crocodile (*Crocodylus*) and mussel (*Mytillus*) 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 200-500-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, PCR products were purified using an AMPure XP PCR purification system (Agencourt Biosciences,

Beverely, MA, USA). Amplification and purification success was interrogated on a MultiNA gel. PCR product concentrations were measured on the Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). The Nanodrop nucleic acid concentrations for each amplicon sample were measured in preparation of pooling the final three libraries of samples for sequencing. The three pooled libraries of 62 samples were prepared with the Illumina Tru-seq library preparation kit.

In preparation for sequencing, the labelled products were mixed in equimolar concentrations, with a final clean-up performed using AMPure XP. The Illumina MiSeq sequencing was performed by the Ramaciotti Centre, UNSW. Illumina data was processed using an in-house custom pipeline, GHAP, based on USearch (Edgar 2013). The GHAP hybrid pipeline takes files of reads and produces tables of classified OTUs and their associated read counts across all samples. The raw Miseq Illumina data was de-multiplexed, de-replicated and then clustered at 97% similarity to generate OTUs. A representative sequence from each OTU was then classified by BLASTing it against a curated set of 18S reference sequences derived from the SILVA v123 SSU reference set. The pipeline then mapped the starting reads back onto these OTU representative sequences to get accurate read counts for each OTU/sample pairing, and then generated OTU tables in both text and .biom (v1) formats, complete with taxonomic classifications and species assignments. OTU summaries were also produced at various taxonomic levels.

2.4 Statistical analysis

As there is a weak statistical relationship between the number of sequence reads and organism biomass or abundance (Egge et al., 2013), occurrence data only were used, and after cut-off was applied to discard the lowest count (and least reliable) OTUs, all OTU data were expressed as presence or absence prior to computation (Chariton et al., 2014). Differences in total OTU richness and the richness of the dominant taxonomic groups were examined using a three-factor nested ANOVA (treatment, site(treatment) and PCR replicate(site)). Residuals were assessed using D'Agostino's tests for skewness, kurtosis, and omnibus normality (D'Agostino et al., 1990) with homogeneity of variances examined using a modified Levene equal variance test (Levene, 1960).

When assumptions of homogeneity were violated, appropriate transformations were performed (Sokal and Rolf, 1995).

Using the Primer 7+ statistical package (Plymouth Marine Laboratory, UK), ordination of OTU data was performed by non-metric multidimensional scaling (nMDS) using the Jaccard similarity coefficient. Statistical differences between treatments were tested by a three-factor 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. Potential indicator OTUs for each treatment (Reference, Discharge Monitoring and Downstream Discharge Monitoring) and a combination of Discharge Monitoring and Downstream Discharge Monitoring were identified using the R package *Indispecies*. In addition to the package's *multipatt* function, the *signassoc* function was used to determine whether the occurrences of each potential indicator OTU was random and to correct for multiple testing.

The relationships between eukaryote 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. The environmental variables obtained from the monitoring program were both numerous and often strongly correlated (Appendix 1). 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. 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.

3 Results

3.1 Water chemistry

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 is provided in Table 2. The Discharge Monitoring sites contained a complex mixture of analytes, with many of the variables being strongly correlated (Appendix Table 1). Notable differences between the water chemistry of the Reference, Discharge Monitoring and Downstream Discharge Monitoring sites included: pH (reference 6.2 ± 0.3 standard error; Discharge Monitoring 8.6 ± 0.2 S.E; Downstream Discharge Monitoring 8.2 ± 0.2 S.E); conductivity (reference 173 µS/cm ± 5.68 S.E; Discharge Monitoring 1657 µS/cm ± 129.5 S.E; Downstream Discharge Monitoring 1217 µS/cm ± 303 S.E); dissolved nickel (reference 0.0008 mg/L ± 0.0002 S.E; Discharge Monitoring 0.0743 ± 0.0067 S.E.; Downstream Discharge Monitoring 0.048 ± S.E 0.014) and total nitrogen (reference 0.032 mg/L ± 0.024 S.E.; Discharge Monitoring 0.563 mg/L ± 0.105 S.E.; Downstream Discharge Monitoring 0.250 mg/L ± 0.020 S.E.).

The default trigger values for pH, dissolved aluminium, nickel, zinc, ammonia, nitrite/nitrate and total nitrogen were exceeded in a majority of Discharge Monitoring sites and to a lesser extent the Downstream Discharge Monitoring sites (ANZECC/ARMCANZ, 2000). Exceedances in dissolved zinc, and ammonia were observed in the references sites, with two of the reference sites (GR/Q1 and GR/UFS) also having a pH lower than the default values for lowland streams (ANZECC/ARMCANZ, 2000) (Table 2).

Table 2. Summary of water quality measurements[a]

			Reference			Discharge monitoring						Downstream discharge monitoring	
	Default trigger values	Units	GR/Q1	GR/UFS	Point 11	GRQ18	Jutts Crossing	Point 10	Point 12	Pool 16	Pool 32	GR/OH	GRQ19
pH	6.5-8.0 (lowland rivers)	pH units	**5.7**	**6.1**	6.8	7.9	**9**	**9**	**9**	**8.9**	**8.7**	8	**8.4**
Conductivity	125-2500 (lowland rivers)	µS/cm	165	170	184	1550	1800	2200	1820	1780	1670	914	1520
Carbonate Alkalinity		mg/L	0.05	0.05	0.05	24	124	177	138	105	77	13	24
Bicarbonate Alkalinity		mg/L	4	5	10	538	545	671	516	554	543	275	496
Total Alkalinity		mg/L	4	5	10	562	669	849	653	659	620	289	520
Sulfate as SO4		mg/L	6	7	7	34	37	42	36	36	35	22	34
Chloride		mg/L	39	39	40	141	133	161	131	136	128	90	143
Dissolved Calcium		mg/L	1	1	3	8	8	8	8	7	7	7	8
Dissolved Magnesium		mg/L	4	4	3	6	2	2	2	3	3	5	6
Dissolved Sodium		mg/L	20	20	20	354	400	497	446	407	397	178	307
Dissolved Potassium		mg/L	0.05	0.05	0.05	3	2	3	3	2	2	2	3
Dissolved Aluminium	0.055 (pH>6.5)	mg/L	0.01	0.01	0.03	0.04	**0.24**	**0.31**	**0.26**	**0.21**	**0.13**	0.04	0.05
Dissolved Arsenic	0.024	mg/L	0.0005	0.0005	0.0005	0.002	0.007	0.007	0.009	0.006	0.004	0.0005	0.002
Dissolved Cobalt		mg/L	0.0005	0.0005	0.0005	0.003	0.003	0.003	0.002	0.003	0.002	0.0005	0.001
Dissolved Copper	0.0014	mg/L	0.0005	0.0005	0.0005	0.002	0.006	0.006	0.006	0.005	0.005	0.0005	0.002
Dissolved Manganese	1.9	mg/L	0.089	0.11	0.022	0.03	0.005	0.008	0.007	0.008	0.008	0.015	0.009
Dissolved Nickel	0.011	mg/L	0.0005	0.001	0.0005	**0.071**	**0.082**	**0.093**	**0.087**	**0.083**	**0.082**	**0.034**	**0.062**
Dissolved Zinc	0.008	mg/L	**0.009**	0.006	**0.011**	**0.014**	**0.024**	**0.032**	**0.031**	**0.019**	**0.019**	0.005	0.006
Dissolved Iron		mg/L	0.14	0.32	0.28	0.54	0.07	0.06	0.06	0.2	0.18	0.27	0.18
Ammonia as N	0.013	mg/L	0.012	**0.017**	0.01	0.011	**0.023**	**0.029**	**0.023**	0.007	**0.038**	0.0025	0.0025
Nitrite + Nitrate	0.015	mg/L	0.003	0.009	0.014	**0.112**	**0.424**	**0.545**	**0.448**	**0.344**	**0.237**	**0.23**	0.014
Total Kjeldahl Nitrogen		mg/L	0.005	0.005	0.07	0.15	0.32	0.48	0.39	0.3	0.26	0.005	0.26
Total Nitrogen	0.5	mg/L	0.01	0.005	0.08	0.26	**0.74**	**1.02**	**0.84**	**0.64**	0.5	0.23	0.27
Total Phosphorus	0.05	mg/L	0.01	0.012	0.007	0.007	0.014	0.023	0.016	0.016	0.012	0.013	0.019
Total Anions		meq/L	1.3	1.34	1.47	15.9	17.9	22.4	17.5	17.8	16.7	8.77	15.1
Total Cations		meq/L	1.25	1.25	1.27	16.4	18	22.2	20	18.4	17.9	8.55	14.3
Dissolved Organic Carbon		mg/L	21	22	5	5	4	11	5	3	3	4	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. Variables in bold text were included in the DSTLM analysis.

3.2 Metabarcoding results

3.2.1 Sequencing results

After the removal of potentially erroneous sequences, the sequenced data set contained 9,918,815 reads, encompassing 11,231 unique OTUs. Of the 95% of OTUs that could be confidently assigned to a Kingdom, the largest proportion belonged to the Ciliophora (10%) (Figure 2). Chytridiomycota, Cercozoa and Ascomycota each contributed 5-6% to the total taxon richness (Figure 2).

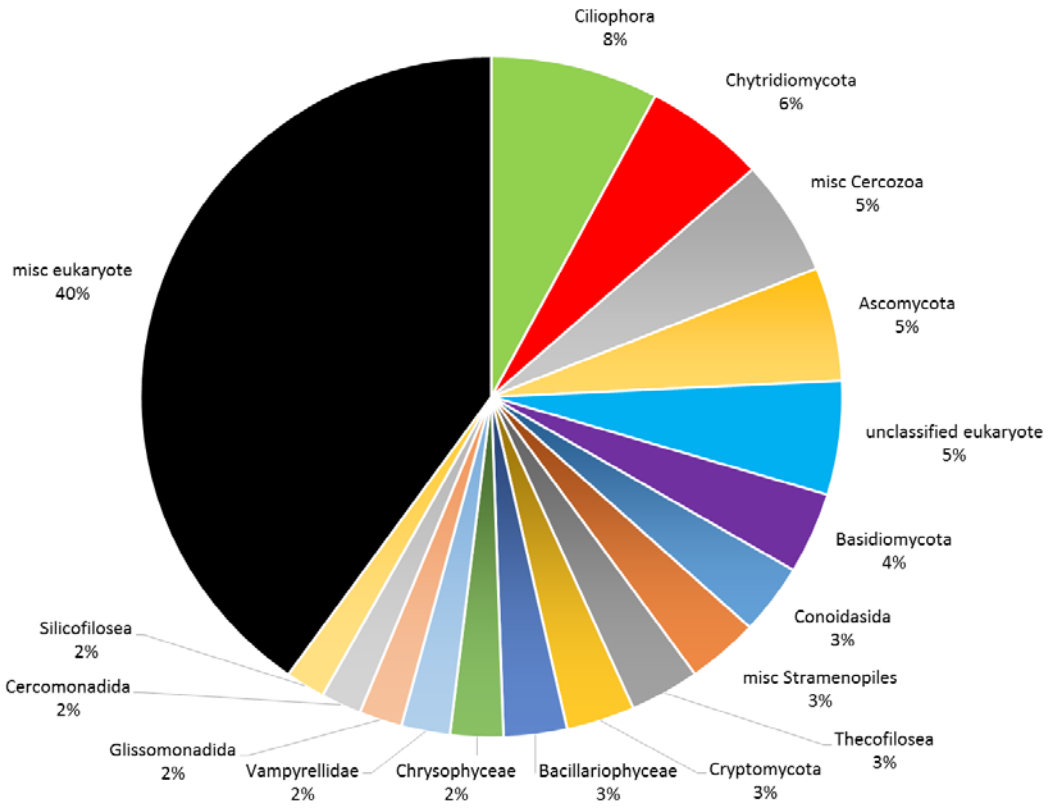

Figure 2. Summary of the OTU data (11,231 unique OTUs) illustrating the proportion of OTUs associated with each major taxonomic group. To aid interpretation data is aggregated at phylum and above. OTUs that could not be confidently assigned to a taxonomic group are referred to as 'unclassified eukaryotes'. Misc (miscellaneous) phyla encompass all taxonomic groups represented by a small number of OTUs.

3.2.2 Univariate comparisons between reference and mine influenced sites

The number of OTUs sampled in the dominant taxonomic groups for the three treatments are illustrated in Figure 3. The biological communities sampled from all treatments contained a diverse range of organisms. There was a significant difference in mean total OTU richness between the three treatments (ANOVA: $F=9.11$, $p<0.001$); with the Reference (2196 ± 177 S.E.) and Discharge Monitoring (1981 ± 125 S.E.) treatments having a lower mean total OTU richness than the Downstream Discharge Monitoring treatment (3045 ± 216 S.E.). The proportion of reads associated with each broad taxonomic group for each treatment is illustrated in Figure 3, with Ciliophora, Chytridiomycota, Ascomycota and Cercozoans being the richest taxonomic groups in all three treatments. Figure 4 shows the OTU richness for the richest taxonomic groups in all three

treatments. As indicated in this figure, some taxonomic groups, e.g. diatoms (Bacillariophyceae) and the fungi Cryptomycota, while golden algae (Chrysophyceae) were richer in the reference sites.

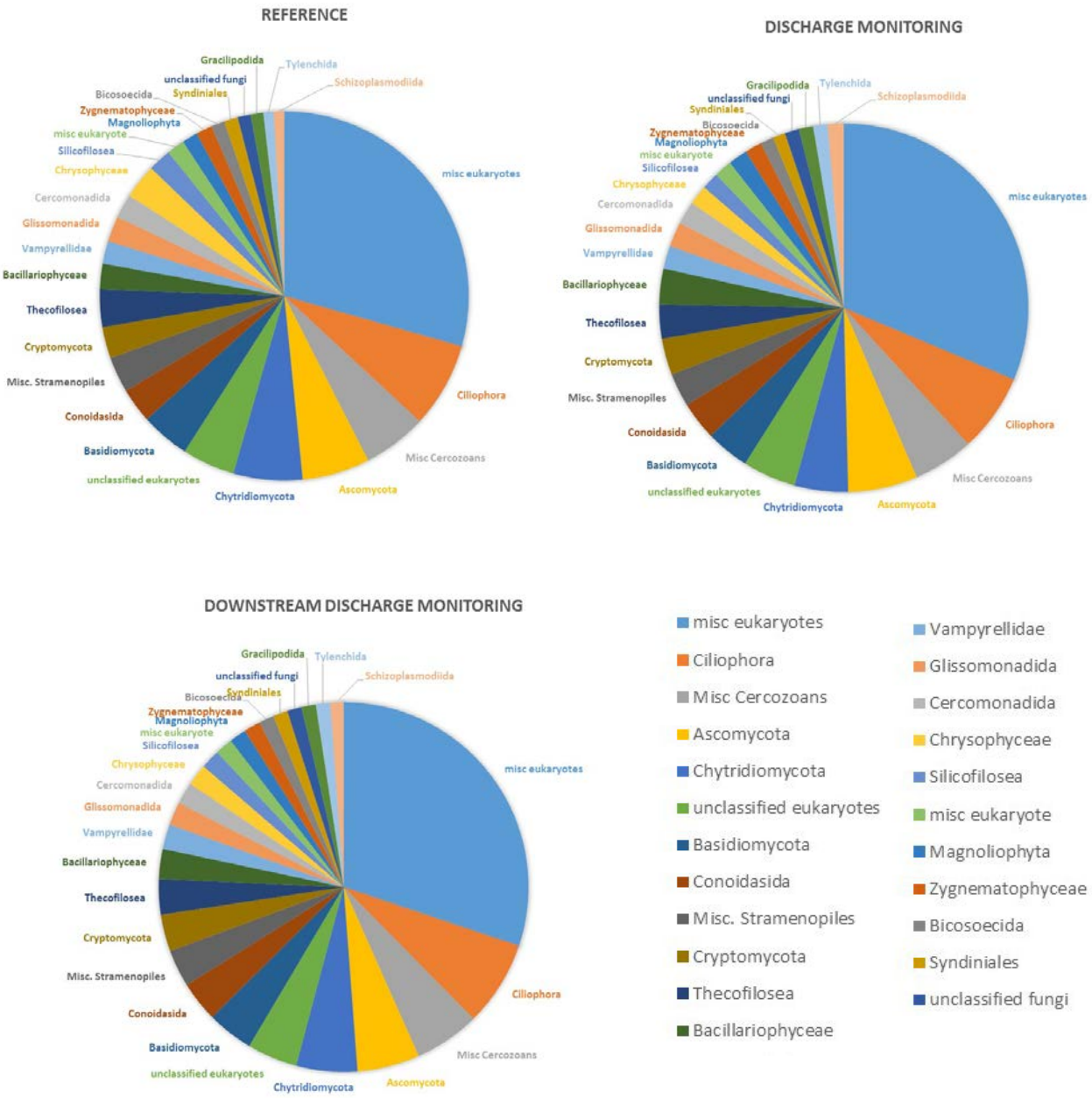

Figure 3. Proportion of OTUs associated with each major taxonomic group from samples obtained from the Reference, Discharge Monitoring and Downstream Discharge Monitoring treatments.

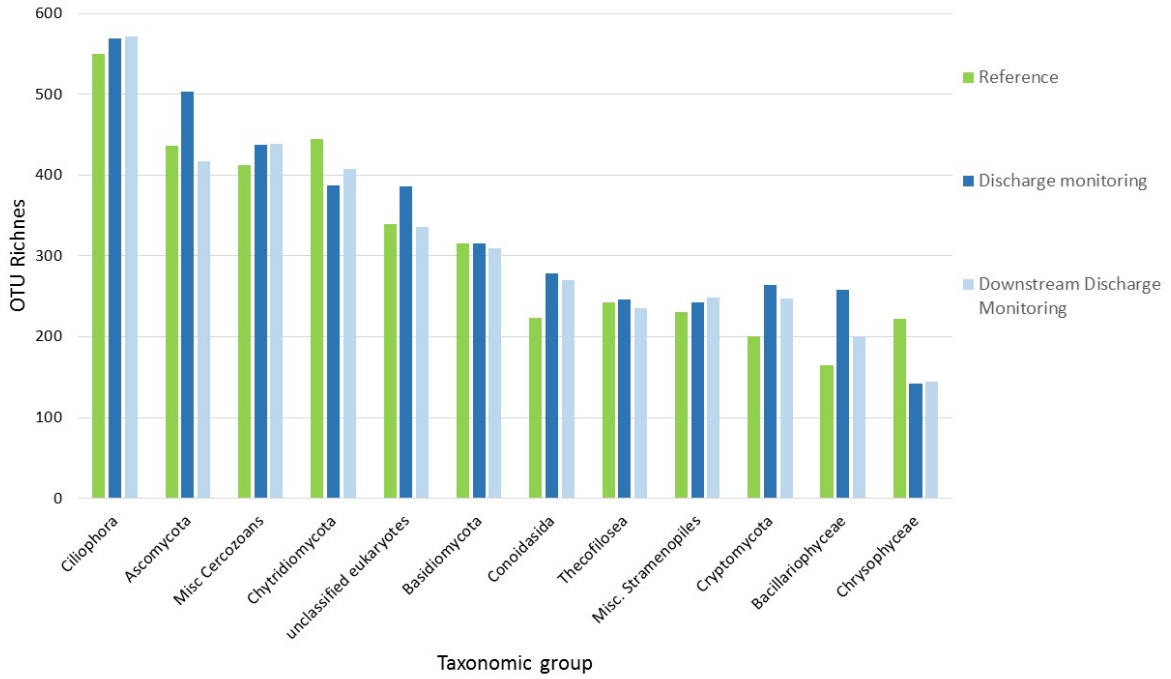

Figure 4. Differences between treatments in the OTU richness of the dominant taxonomic groups

3.2.3 Multivariate comparisons between treatments

As illustrated in the nMDS ordination plot (Figure 5), the composition of the References sites was markedly different to that obtained from the Discharge Monitoring sites, with the Downstream Discharge sites sitting in between the other two treatments. Statistical analysis of these trends using PERMANOVA detected a difference between the three treatments (F_{pseudo} =3.32, p=0.001), with post hoc analysis confirming that all three treatments contained significantly different compositions. However, the Reference treatment was more similar to the Downstream Discharge treatment (17.7) than it was with the Discharge Monitoring treatment (13.9). The Downstream Discharge treatment was also more similar to the Discharge Monitoring treatment (20.3) than it was with the Reference Treatment.

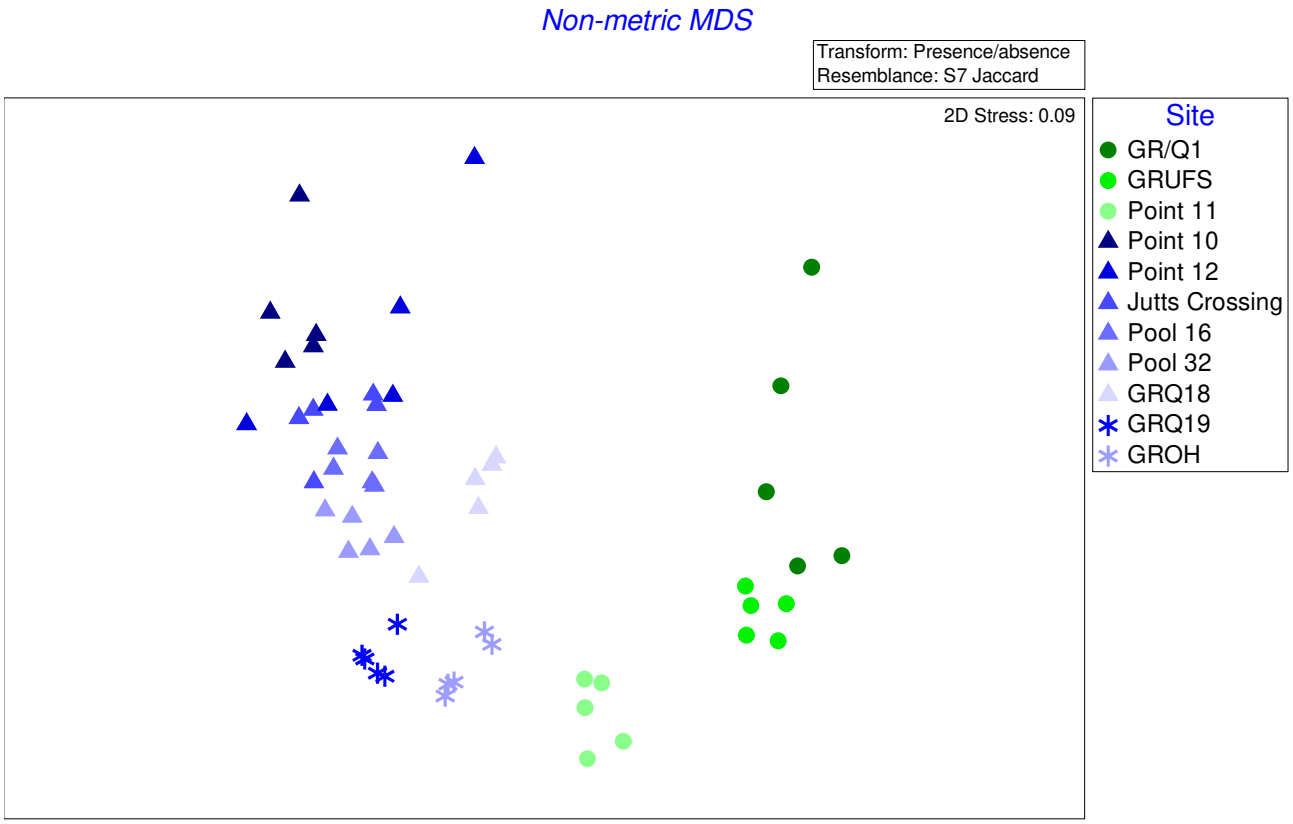

Figure 5. nMDS plot illustrating the similarities and differences in the eukaryl compositions between treatments. Reference (green circles), Discharge Monitoring (blue triangles) and Downstream Discharge Monitoring (blue asterisks). Colour indicates position of site from upstream (darker) to downstream (lighter). Points represent the centroid based on three PCR replicates per sample.

Indicator analysis identified 1,842 OTUs, 733 OTUs and 1363 OTUs as being indicative of the Reference, Discharge Monitoring and Downstream Discharge Monitoring treatments at the time of sampling, respectively. Another 563 OTUs were indicative of both the Discharge Monitoring and Downstream Discharge Monitoring treatments. A summary of potential indicator OTUs is provided in Figure 6. As indicated in this figure, many of the OTUs for both Reference and Downstream Discharge Monitoring treatments were from the fungal Phyla Chytridiomycota, Ciliophora, Cercozoa, Thecofilosea and Vampyrellidae. A number of diatoms within the class Bacillariophyceae, were indicative of the Discharge Monitoring treatment. Indicator OTUs for both the Discharge Monitoring and Downstream Discharge Monitoring treatments were from a range of taxonomic groups.

The top 10 best indicator OTUs for each treatment and combination of discharge treatments is provided in Table 3. The best indicator OTUs for the Reference treatment were mainly from the Phylum Chytridiomycota, but also included a diatom (Bacillariophyceae) and two arachnids. When

examined in isolation and collectively, the best indicator taxa for the Discharge Monitoring treatment and Downstream Discharge Monitoring treatments included a diverse array of taxa, most notably a range of diatoms and other Bacillariophyta.

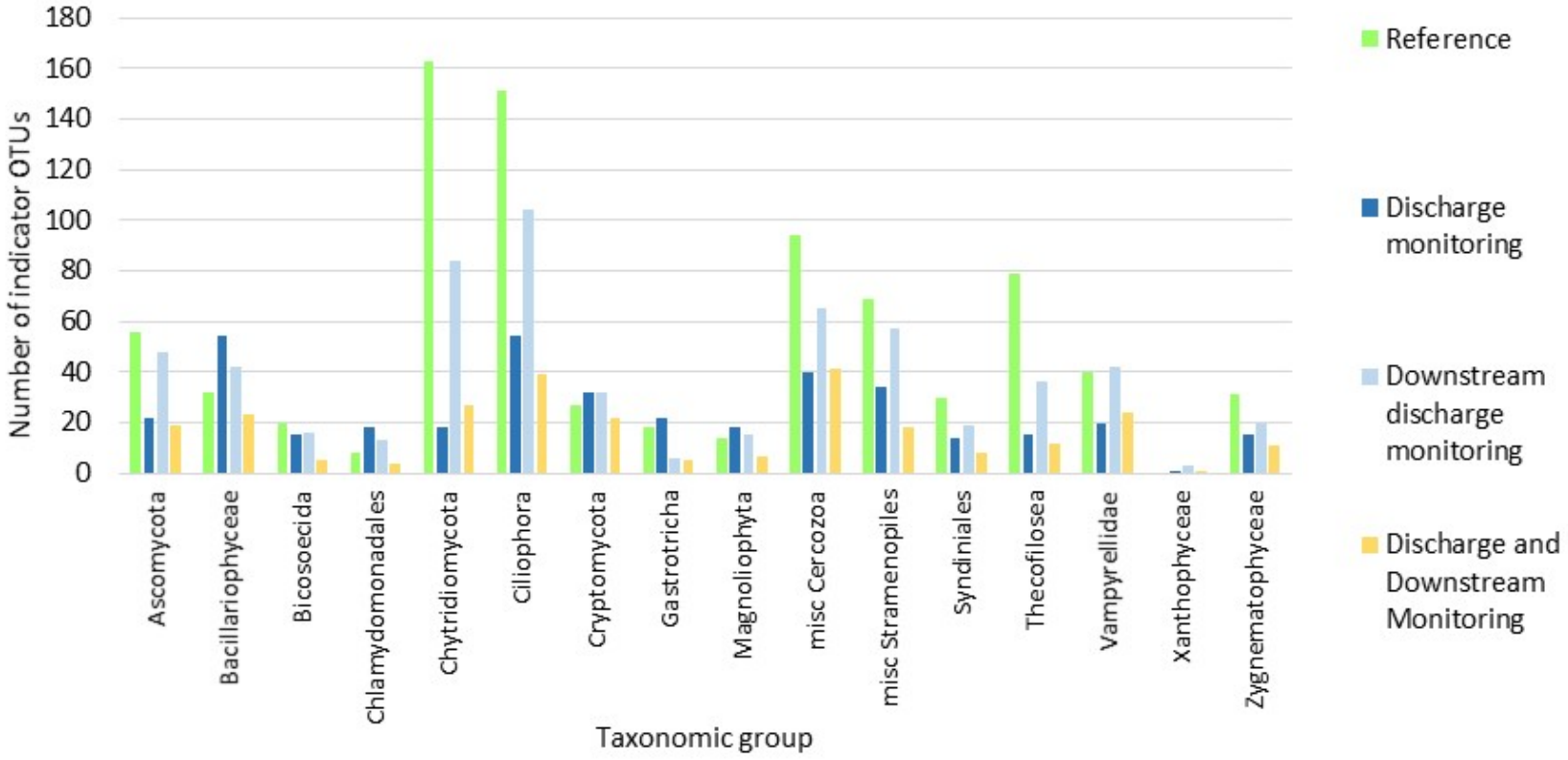

Figure 6. A summary of the Indicator Analysis illustrating the number of OTUs associated with the key taxonomic groups from the Reference, Discharge Monitoring, Downstream Discharge Monitor treatments, and indicative of both the Discharge Monitoring, Downstream Discharge Monitor treatments. To aid interpretation data are aggregated at phylum and above.

Table 3. Top ten 'best' (based on Indicator Values, IV) potential indicator OTUs for the Reference, Discharge Monitoring and Downstream Discharge treatments.

OTU	Indicator Value	Treatment	Taxonomy Level 1	Taxonomy Level 2	Taxonomy Level 3	Taxonomy Level 4	Taxonomy Level 5	Taxonomy Level 6
193	0.98	Ref	Fungi	Ascomycota	Schizosaccharomycetes	Schizosaccharomycetales	Schizosaccharomycetaceae	Schizosaccharomyces
179	0.98	Ref	Fungi	Ascomycota	Pezizomycetes	Pezizales	Pezizaceae	Peziza
123	0.98	Ref	Metazoa	Arthropoda	Chelicerata	Arachnida	Pseudoscorpiones	
408	0.94	Ref	SAR	Stramenopiles	Diatomea	Bacillariophytina	Bacillariophyceae	Pinnularia
241	0.83	Ref	Fungi	Ascomycota	Lichinomycetes	Lichinales	Lichinaceae	Paulia
280	0.82	Ref	Fungi	Ascomycota	Lecanoromycetes	Baeomycetales	Baeomycetaceae	Baeomyces
2374	0.81	Ref	Fungi	Ascomycota	Dothideomycetes	Pleosporales		
58	0.81	Ref	SAR	Stramenopiles	Diatomea	Bacillariophytina	Bacillariophyceae	Eunotia
7512	0.26	Ref	Metazoa	Arthropoda	Chelicerata	Arachnida	Acari	
11051	0.26	Ref	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Thysanophora
95	0.97	Dis. mon	SAR	Rhizaria	Cercozoa	uncultured		
17	0.96	Dis. mon	SAR	Stramenopiles	Diatomea	Bacillariophytina	Bacillariophyceae	
217	0.93	Dis. mon	SAR	Rhizaria	Labyrinthulomycetes	Vampyrellidae	uncultured	
1612	0.85	Dis. mon	SAR	Alveolata	Cercozoa	Perkinsidae	Perkinsus	
1009	0.84	Dis. mon	Amoebozoa	Discosea	Flabellinia	Stygamoebida	Stygamoeba	
414	0.82	Dis. mon	SAR	Stramenopiles	Diatomea	LG21-05		
1478	0.79	Dis. mon	Fungi	Chytridiomycota	Chytridiomycetes	Lobulomycetales	Lobulomycetaceae	Lobulomyces
763	0.79	Dis. mon	Archaeplastida	Rhodophyceae	Cyanidiales	Cyanidium		
1242	0.26	Dis. mon	SAR	Stramenopiles	Diatomea	Bacillariophytina	Bacillariophyceae	Amphora
11165	0.26	Dis. mon	SAR	Stramenopiles	Diatomea	Bacillariophytina	Bacillariophyceae	Stauroneis
86	0.96	D/Strm	SAR	Stramenopiles	Diatomea	Bacillariophytina	Bacillariophyceae	Encyonema
174	0.93	D/Strm	Archaeplastida	Chloroplastida	Chlorophyta	Ulvophyceae	Scotinosphaerales	Scotinosphaera
516	0.87	D/Strm	SAR	Stramenopiles	Diatomea	Bacillariophytina	Bacillariophyceae	Cocconeis
2308	0.86	D/Strm	SAR	Stramenopiles	Peronosporomycetes	Coscinodiscophytina	Melosirids	Melosira
896	0.86	D/Strm						
906	0.85	D/Strm	SAR	Rhizaria	Cercozoa	Vampyrellidae	Leptophrys	
267	0.84	D/Strm	Fungi	Basidiomycota	Wallemiomycetes	Wallemiales	Incertae Sedis	Wallemia
522	0.84	D/Strm	SAR	Stramenopiles	Diatomea	Bacillariophytina	Bacillariophyceae	Nitzschia
542	0.83	D/Strm	Fungi	Chytridiomycota	Chytridiomycetes	Rhizophydiales	uncultured	
828	0.80	D/Strm	unclass eukaryote					
278	0.84	Dis. D/strm	Archaeplastida	Chloroplastida	Chlorophyta	Trebouxiophyceae	Incertae Sedis	Choricystis
826	0.83	Dis. D/strm	Metazoa	Gastrotricha	Chaetonotida			
14945	0.26	Dis. D/strm	Metazoa	Nematoda	Chromadorea	Monhysterida		
13233	0.26	Dis. D/strm	SAR	Rhizaria	Cercozoa	Thecofilosea		
10488	0.26	Dis. D/strm	SAR	Stramenopiles	Chrysophyceae	Thraustochytriaceae	Aplanochytrium	
6323	0.26	Dis. D/strm	SAR	Stramenopiles	Diatomea	Bacillariophytina	Bacillariophyceae	Fistulifera
10147	0.26	Dis. D/strm	SAR	Stramenopiles	Diatomea	Bacillariophytina	Mediophyceae	Thalassiosira
7375	0.26	Dis. D/strm	SAR	Stramenopiles	Diatomea	Coscinodiscophytina	Fragilariales	

3.2.4 The relationships between benthic community composition and water chemistry

Constrained analysis using a distance-based linear model (DISTLM) found that the environmental variables used in the analysis explained approximately 84% of the variation in the biological data. When examined individually, all *a prior* selected variables, with the exception of total phosphorus, were significantly correlated with metabarcoded composition (Table 4, marginal tests). However, when examined collectively (Table 4, sequential tests), only pH and conductivity were shown to significantly contribute to the observed variation, contributing 29% and 12%, respectively. However, identifying specific water chemistry variables that may be driving the perceived changes in the biological communities was difficult due to the strong correlations among water quality variables (see Appendix Table 1). For example, pH was strongly correlated with nickel and sulfate. Similarly, conductivity was strongly correlated with alkalinity, sulfate, chloride, nickel, sodium, total cations and anions. Consequently, the discharge should be viewed as a mixture, with little weight placed on the ecological ramifications of a single stressor.

The dbRDA ordination map derived from the DISTLM analysis (Figure 6), clearly illustrates that the primary correlate for the reference sites was dissolved organic carbon (DOC), reflecting the relatively higher concentrations of DOC at GR/Q1 and GR/UFS. Another notable feature of the analysis is the correlation between site positioning along the discharge gradient (nutrients, metals, pH and conductivity) and composition, with the correlations between the gradient and composition of Discharge Monitoring sites increasing from downstream (light blue triangles) to upstream (dark blue triangles). Interestingly, there appears to be a weak correlative relationship between the discharge gradient and the sites from the Downstream Discharge Monitoring treatment, as reflected by their position in the ordination plot.

Table 4. Results of distance-based linear model (DISTLM)

MARGINAL TESTS					
VARIABLE	**SS (TRACE)**	**PSEUDO-F**	**P**	**PROPORTION**	
pH	4388	3.730	**0.001**	0.293	
Conductivity	4502	3.868	**0.001**	0.301	
Nickel	4156	3.457	**0.003**	0.278	
Zinc	3841	3.104	**0.002**	0.256	
Total nitrogen	4290	3.614	**0.001**	0.287	
Total phosphorus	2238	1.582	0.081	0.149	
Dissolved organic carbon	3394	2.638	**0.007**	0.227	
SEQUENTIAL TESTS					
VARIABLE	**RP^2**	**SS (TRACE)**	**PSEUDO-F**	**P**	**PROPORTION**	**CUMULATIVE**
pH	0.293	4388	3.730	**0.001**	0.293	0.293
Conductivity	0.415	1829	1.671	**0.05**	0.122	0.415
Nickel	0.530	1725	1.717	0.07	0.115	0.530
Zinc	0.617	1299	1.360	0.168	0.087	0.617
Total nitrogen	0.713	1439	1.675	0.082	0.096	0.713
Total phosphorus	0.771	868.9	1.014	0.466	0.058	0.771
Dissolved organic carbon	0.835	959.7	1.167	0.379	0.064	0.835

Marginal tests indicate the relationships between the environmental variables and the composition of the biota when the variables were examined individually, ignoring all other variables. **Sequentia**l tests examined relationships between the environmental variables and the composition of the biota when the variables were examined in a specific order. Bold p-values indicate significant relationships.

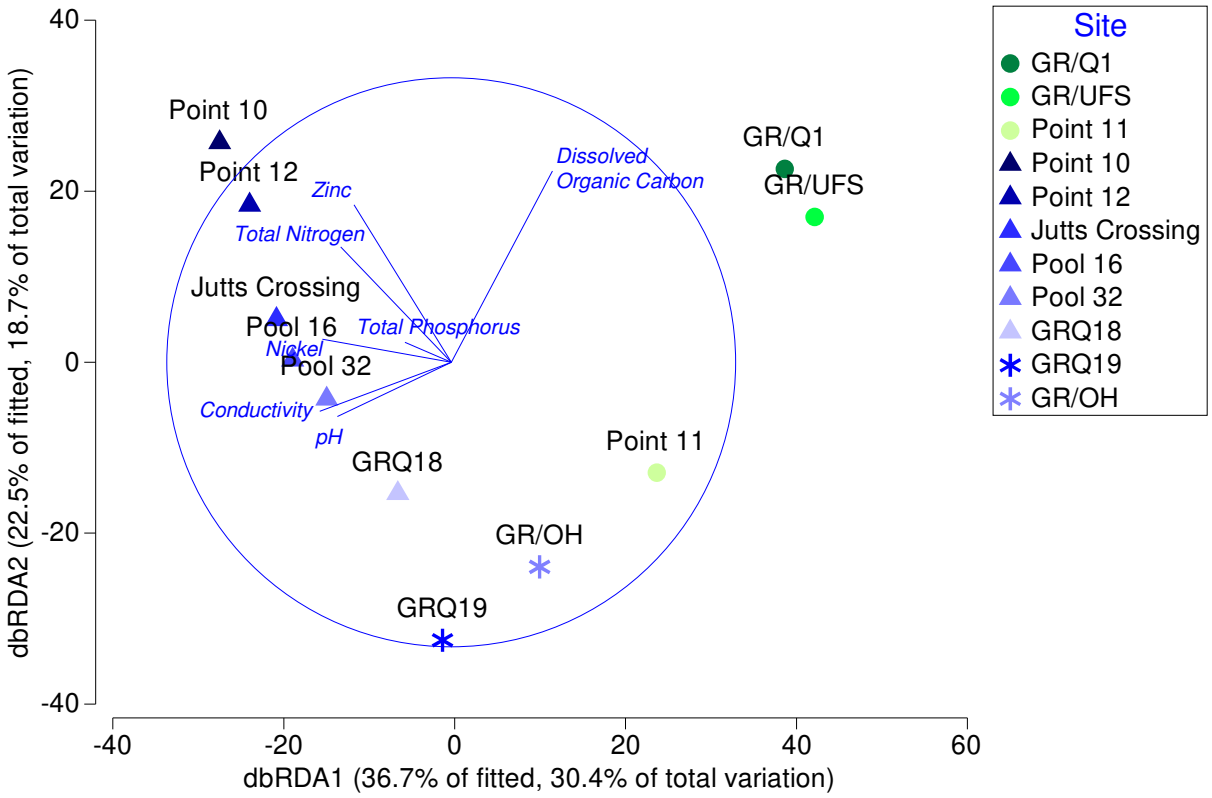

Figure 7. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and benthic composition. Sites are derived from their distances among centroids obtained from site replicates.

4 Discussion

It is important to note that the results from this survey reflect a single sampling occasion containing a limited number of sampling sites (3 Reference, 5 Discharge Monitoring and 2 Downstream Discharge Monitoring). The following sections focus on comparing the *en masse* benthic community data obtained from Reference, Discharge Monitoring and Downstream Discharge Monitoring treatments, and identifying key differences in their composition. While water quality is described in the results, this description is purely to provide a summary of the data used to explore the relationships between the benthos and water quality. Consequently, discussions regarding water quality are limited to this context.

4.1 Metabarcoding comparisons between treatments

The results of this study clearly illustrates the capacity for metabarcoding to capture a wide breadth of biota, with the study obtaining 11,231 OTUs (unique clusters of 18S rRNA genes) from more than 130 phyla and other high level taxonomic groups. It is emphasised that the greater number of OTUs found in this study when compared to 818 OTUs sampled in the 2013 survey (Chariton and Stephenson, 2014) reflects both the changes in bioinformatic approaches and the greater sequencing power (an order of magnitude greater) associated with the Illumina MiSeq platform used in the current study. Approximately only five percent of the OTUs could not be confidently assigned to any taxonomic group (unclassified eukaryotes), with this being substantially lower than the 33% reported in the 2013 survey (Chariton and Stephenson, 2014).

Total OTU richness was greater in the Downstream Discharge Monitoring treatment than either the Reference or Downstream Discharge treatments. It is emphasised that metabarcoding provides a different view of richness and composition than that traditionally obtained, capturing not only the biota residing within the sediment, but also, organisms adhered to or retained within the guts of other organisms, as well as deceased and partially degraded individuals (Chariton et al., 2010; Baird and Hajibabaei, 2012). As such, total OTU richness reflects both changes in the sampled benthos as well as changes in the surrounding catchment (organic material deposited in the stream via run-off). As both Downstream Discharge Monitoring sites are situated below the

confluence of several creeks, their OTU richness can be expected to be inflated as samples from these sites would capture the biological material associated with all the tributaries. Previous metabarcoding work has suggested that total richness may be a poor indicator of ecological condition (Chariton et al., 2014), and can be elevated in regions where there are multiple point sources of water (Chariton et al., 2015). Consequently, this information cannot be viewed in the same context as traditionally obtained data where an increase in endemic species richness often reflects an improvement in ecological condition (Lenat, 1988; Kerans and Karr, 1994).

Multivariate analysis of the community data clearly differentiated sites associated with the Reference and Discharge Monitoring treatments. While the composition from the Downstream Discharge Monitoring sites were shown to be different to those from either the Reference or Discharge Monitoring treatments, the communities were more similar to those from the Discharge Monitoring treatment. However, as indicated in the ordination plot (Figure 5), there were some similarities between communities sampled from the Downstream Discharge Monitoring treatment and the Reference site Point 11, with the biological communities from the Reference treatment clustering into two groups (GR/Q1 and GR/UFS and Point 11 by itself). Closer examination of the sites from the Discharge Monitoring treatment shows that there was a clear transition in composition from upstream (dark blue triangles) to downstream (light blue triangles), with downstream sites becoming more similar to those sampled in the Downstream Discharge treatment. The separation of sites from the Discharge and Downstream Discharge Monitoring treatments emphasises the capacity for metabarcoding to distinguish between communities from different catchments, and suggest that these two treatments should be viewed independently, enabling the Discharge Monitoring treatment to capture the sampled environmental gradient (Table 2).

It is emphasised that OTUs identified from the indicator analysis relate to the system only at the time of sampling, with a suitable spatio-temporal sampling program required to identify and validate robust and reliable candidate OTUs (De Cáceres et al. 2010). A majority of the potential OTUs were associated with the richest taxonomic groups, e.g. Chytridiomycota (fungi), ciliophorans (single-celled protozoans) and Thecofilosea (protist). In contrast to previous findings (Chariton and Stephenson, 2014), the largest proportion of these OTUs were indicative of the Reference treatment. For the most part the indicator OTUs differed from the previous sampling occasion, however, one notable exception was an OTU from the diatom genus *Eunotia*, which was a strong indicator of the Reference treatment. This taxon is found in a range of water types, and

typically thrives in well-oxygenated acidic waters (below pH 8) with low organic nitrogen (Van Dam et al., 1994), reflecting the conditions of the Reference sites. When examined separately and collectively, many of the indicator OTUs from the Discharge Monitoring and Downstream Discharge Monitoring treatments were diatoms (Bacillariophyceae). Recent work by Chariton et al. (2015) has shown this group to be highly responsive to environmental change, especially to increases in nutrients, as observed in both discharge monitoring treatments in the current study.

4.2 Relationships between community structure and water quality

As previously indicated, the water chemistry from the Discharge Monitoring and Downstream Discharge Monitoring sites was complex, with most of the variables being highly correlated. As such it is more prudent to view the discharge as a mixture rather than focus on the ecotoxicological aspects of a single analyte. A large proportion of the variation in the biological data could be explained by two environmental variables (pH and conductivity) (Table 4), however, it is emphasised that both variables had several co-variates (see Appendix 1). Most notably in the Discharge Monitoring sites, pH frequently exceeded guideline values (ANZECC/ARMCANZ, 2000). While conductivity was not above the guideline value for lowland streams, it was in the order of a magnitude greater in the Discharge Monitoring and Downstream Discharge Monitoring sites than the Reference sites.

As illustrated by the dbRDA ordination plot (Figure 7), it was the biological composition of the Discharge Monitoring sites and not the Reference sites that were strongly correlated with increases in pH and analyte concentrations. The analysis clearly showed a gradient in the increasing influence (correlations) of the water chemistry on biological communities as the sites transitioned from upstream at GRQ18 to the discharge point (Point 10). The gradient pattern is far less marked for the Downstream Discharge Monitoring sites, with the analysis indicating some correlations between pH and conductivity and benthic composition. The abiotic driver for the biota from the Reference sites GR/Q1 and GR/UFS was the relatively higher concentrations of dissolved organic carbon. Interestingly, the constrained analysis, emphasised the commonalities between the Reference site Point 11 and the Downstream Discharge Monitoring sites, with this

possibly being driven by the difference in water quality and composition between these and the other Reference sites.

In addition to the effects of discharge water quality there were significant differences between the natural catchment water qualities expected at the Discharge Monitoring, Downstream Discharge Monitoring and Reference sites. This relates to the Hawkesbury Sandstone (a freshwater sediment) dominated geology of the upstream Georges River and Cascade Creek sites compared to the Wianamatta Shale (a shale group with subsections of marine and alluvial sediment) geology of the Georges River downstream from approximately the confluence with Brennans Creek. The Reference sites were selected with knowledge of this confounding effect, however the project team were unable to find more suitable reference sites in the local area without this confounding effect.

5 Conclusions

5.1 Conclusions

As illustrated in this report, metabarcoding has the capacity to provide ecological data encompassing a wide range of taxa. Even when the data were reduced to presence/absence, clear patterns between and within the two treatments were evident, as were the correlative relationships between the biota and water chemistry. Collectively, these findings highlight the utility of the approach and its suitability as a continuing line of ecological evidence in the future monitoring of this system.

The key outputs and finding from the 2015 metabarcoding survey of the Georges River and Brennan Creek sites for South32 are:

- Metabarcoding of a conserved region of the 18S rRNA gene was able to capture a wide breadth of taxa, with 11,231 OTUs (unique clusters of 18S rRNA genes) observed from more than 130 phyla and other high level taxonomic groups.
- Mean total OTU richness was markedly higher in the Downstream Discharge Monitoring sites, however, this is likely an artefact of these sites containing biological material from a number of tributaries.
- Clear differences in eukaryote composition were observed between the samples taken from the Reference and Discharge Monitoring sites. The compositions of samples obtained from Downstream Discharge Monitoring sites were different to the other two treatments, but more similar to the Discharge Monitoring treatment.
- The composition of samples from the Point 11 Reference site were different to those from the other two Reference sites.
- 4211 OTUs were shown to aid in the characterisation of the treatments at the time of sampling.
- As was the case of the 2015 sampling event, an OTU from the diatom genus *Eunotia* was a strong indicator of the Reference treatment.

- Many of the potential indicator OTUs associated with the Discharge Monitoring and Downstream Discharge Monitoring sites were diatoms.
- The water chemistry from the discharge sites was complex, and as such it is not possible to attribute any perceived patterns to a single environmental variable. When examined in the context of a mixture, there was strong evidence to indicate that the water quality from the discharge sites was altering eukaryote composition (as defined by the eukaryotic communities), with this being primarily driven by pH and conductivity, and their co-variates.
- There was a strong environmental gradient and corresponding changes in community structure from the upstream to downstream Discharge Monitoring sites.

5.2 Recommendations

In future sampling runs, the inclusion of additional genes is suggested to provide greater taxonomic depth and certainty for key taxa (e.g. diatoms), and the formation of a custom data-base derived from specimens collected from the concurrent traditional macrobenthic survey.

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Appendix A

A.1 Correlation matrix derived from the water quality measurements

	pH	Conductivity	Carbonate Alkalinity	Bicarbonate Alkalinity	Total Alkalinity	Sulfate (SO4)	Chloride	Calcium	Magnesium	Sodium	Potassium	Aluminium	Arsenic	Cobalt	Copper	Manganese	Nickel	Zinc	Iron	Ammonia	Nitrite	TKN	TN	TP	Total Anions	Total Cations
pH Value																										
Conductivity	**0.949**																									
Carbonate_Alkalinity	0.796	0.839																								
Bicarbonate_Alkalinity	0.936	**0.993**	0.775																							
Total Alkalinity	0.942	**0.999**	0.852	**0.991**																						
Sulfate(SO4)	0.951	**0.995**	0.786	**0.998**	**0.992**																					
Chloride	0.917	**0.980**	0.726	**0.992**	**0.974**	**0.991**																				
Calcium	0.942	0.918	0.632	0.931	0.905	0.941	0.941																			
Magnesium	-0.328	-0.282	-0.725	-0.186	-0.306	-0.204	-0.095	-0.047																		
Sodium	0.941	**0.995**	0.856	**0.985**	**0.995**	**0.986**	**0.964**	0.898	-0.327																	
Potassium	0.853	0.902	0.602	0.916	0.886	0.922	**0.946**	**0.948**	0.069	0.885																
Aluminium	0.790	0.811	**0.995**	0.741	0.823	0.757	0.691	0.614	-0.756	0.828	0.565															
Arsenic	0.796	0.818	0.957	0.750	0.822	0.772	0.703	0.635	-0.706	0.846	0.603	**0.963**														
Cobalt	0.742	0.852	0.772	0.852	0.868	0.835	0.815	0.700	-0.353	0.864	0.657	0.752	0.755													
Copper	0.841	0.873	**0.957**	0.823	0.882	0.834	0.765	0.674	-0.686	0.897	0.615	0.949	**0.963**	0.815												
Manganese	-0.889	-0.767	-0.584	-0.766	-0.757	-0.779	-0.759	-0.877	0.235	-0.751	-0.728	-0.595	-0.581	-0.545	-0.626											
Nickel	**0.946**	**0.993**	0.832	0.987	**0.992**	**0.989**	**0.965**	0.905	-0.304	**0.998**	0.881	0.805	0.833	0.865	0.891	-0.758										
Zinc	0.680	0.742	**0.948**	0.674	0.757	0.681	0.617	0.531	-0.774	0.781	0.514	0.942	0.935	0.750	0.916	-0.513	0.753									
Iron	-0.376	-0.344	-0.648	-0.254	-0.346	-0.289	-0.219	-0.165	0.708	-0.346	-0.124	-0.668	-0.628	-0.122	-0.602	0.291	-0.327	-0.566								
Ammonia	0.319	0.394	0.593	0.357	0.420	0.349	0.271	0.153	-0.660	0.445	0.136	0.547	0.534	0.409	0.647	-0.130	0.433	0.677	-0.415							
Nitrite	0.801	0.799	**0.954**	0.736	0.810	0.751	0.680	0.660	-0.691	0.815	0.595	0.956	0.901	0.727	0.887	-0.611	0.792	0.889	-0.578	0.511						
TKN	0.850	0.905	0.932	0.861	0.909	0.870	0.843	0.732	-0.545	0.910	0.731	0.918	0.910	0.768	0.929	-0.682	0.892	0.877	-0.578	0.513	0.816					
TN	0.864	0.889	**0.990**	0.832	0.897	0.845	0.791	0.727	-0.656	0.901	0.690	**0.985**	**0.951**	0.783	**0.952**	-0.676	0.880	0.929	-0.608	0.540	**0.961**	0.944				
TP	0.599	0.639	0.695	0.591	0.635	0.611	0.612	0.490	-0.300	0.605	0.563	0.681	0.594	0.335	0.584	-0.388	0.576	0.488	-0.665	0.193	0.619	0.732	0.700			
Total Anions	0.942	**0.999**	0.832	**0.995**	**0.999**	**0.995**	**0.983**	0.915	-0.269	0.993	0.900	0.802	0.804	0.862	0.864	-0.761	**0.991**	0.734	-0.324	0.393	0.790	0.900	0.882	0.633		
Total Cations	0.943	**0.996**	0.845	**0.988**	**0.996**	**0.989**	**0.969**	0.906	-0.306	1.000	0.893	0.817	0.836	0.862	0.887	-0.754	0.998	0.768	-0.331	0.430	0.806	0.903	0.893	0.601	**0.995**	
DOC	-0.771	-0.607	-0.317	-0.632	-0.590	-0.644	-0.628	-0.801	0.025	-0.594	-0.622	-0.336	-0.375	-0.435	-0.426	0.927	-0.621	-0.267	0.007	0.035	-0.373	-0.440	-0.424	-0.098	-0.600	-0.602

[a] Correlations with r>0.95 are in bold. TKN= Total Kjeldahl Nitrogen; TN= Total Nitrogen; TP= Total Phosphorus; DOC= Dissolved Organic Carbon

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