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2/4/2014

Mr David Gregory
BHP Billiton Illawarra Coal
Level 3, Enterprise 1
Innovation Campus
Squires Way
North Wollongong, NSW 2500

Dear David,

RE: IDENTIFICATION OF AUSTROCORDULIIDAE SPECIMENS

Niche Environment and Heritage was commissioned by BHP Billiton Illawarra Coal (BHPIC) to monitor macroinvertebrates and fish with in the Georges River and reference sites as part of mine water discharge monitoring under Pollution Reduction Program 20. The study collected specimens from the Austocorduliidae dragonfly family which potentially included (*Austrocorduliidae leonardi*), the threatened Sydney Hawk dragonfly. The report preliminarily identified some of the specimens as *Autrocorduliidae leonardi* and recommended that these specimens be identified by an aquatic invertebrate taxonomist. The specimens were identified by dragonfly expert Dr Gunther Theischinger (Office of Environment and Heritage). This letter is to inform you that all specimens collected were in fact *Austrocorduliidae refracta* and not the threatened *A.leonardi* and reference to the threatened species in the report should be discounted.

Your Sincerely,

M. Russell

Matthew Russell

Aquatic Ecologist

Niche Environment and Heritage

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Environment and Heritage

West Cliff Colliery – BHP Illawarra Coal

Pollution Reduction Program 20 – Aquatic Health Monitoring Report 2014

Fish and Macroinvertebrates

Prepared for BHP Billiton Pty Limited

Document control

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Cover photograph: Electrofishing in the Georges River.

Executive Summary

Endeavour Coal Pty Limited (the licensee), a wholly owned subsidiary of BHP Billiton Illawarra Coal BHPBIC, 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 West Cliff 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.

This report documents the outcomes of the fish and macroinvertebrate monitoring undertaken in 2013, which are the first surveys undertaken as part of the long term Aquatic Health Monitoring Plan. Definitive conclusions have not been made, however, preliminary findings based on the initial survey and literature review are:

- The fish community in the study area is low in abundance and diversity (this is likely to be natural in head water streams).
- Fish are not a reliable indicator for monitoring because of the low diversity and abundance and are hence limited in ability to detect small or gradual environmental change.
- No threatened fish were observed.
- There was no statistical difference between density and family richness between discharge monitoring and reference sites, however there was statistical difference between macroinvertebrate assemblages
- Lower densities of pollution sensitive Leptophlebiidae (SIGNAL 8) and increased densities of pollution tolerant Chironominae (SIGNAL 3) and Caenidae (SIGNAL 4) were observed in discharge monitoring groups compared to reference groups.
- It is likely that the water quality in discharges from LDP 10 are resulting in the observed difference in the distribution of macroinvertebrate assemblages, particularly the family Leptophlebiidae, however other environmental variables may also explain the difference.
- Sydney Hawk dragonfly (*Austrocordulia leonardi)* was positively identified in Cascade Creek (CC1), and Georges River (GRQ18), which is listed as a threatened species under the *Threatened Species Conservation Act 1995* and *Fisheries Management Act 1993*.

It is recommended that monitoring continue to be undertaken in accordance with the requirements of the EPL and Aquatic Health Monitoring Plan.

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1 Introduction

1.1 Project 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);
- Fish surveys;
- 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; and
- Laboratory water testing.

The full requirements of the AHMP are documented in EPL 2504. The AHMP is included as Appendix E.

1.2 Aims of the Aquatic Health Monitoring Plan

The aim of the study is to monitor the 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 through time
- 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
- Investigating whether the discharges into Brennans Creek from West Cliff colliery, under the EPL 2504; has had a demonstrable impact on fish and macroinvertebrate abundance, richness and assemblages within the study area. This will be inferred once improvements have been made to the water quality at Point 10 as water projects required by PRP 19 are commissioned.

1.3 Background

1.3.1 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 the north-east and south.

1.3.2 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. Brennans Creek and Sawpit Gully flow into the Georges River from the eastern side of the catchment. The upper reaches flow through areas subject to the effects of previous longwall mining but otherwise relatively undisturbed land from the Cataract Scout Park through to Jutt's Crossing and Marhneys Hole. The Georges River flows northward to Campbelltown, eventually flowing into Botany Bay (Gilbert and Associates 2009).

The catchment incorporates recreational areas, agricultural areas, the Appin Township, and mining and mine related infrastructure. Licensed discharges comprising treated stormwater runoff from the Appin East pit top enter the Georges River upstream of the Brennans Creek confluence. The West Cliff pit top and associated Brennans Creek Dam (BCD) are located in the Brennans Creek catchment. Licensed discharges from BCD are a major proportion of surface water flow to the Georges River during low flow periods (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 *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).

Downstream of the confluence with Brennans Creek, the sandstone bedrock provides for short, infrequent riffles, separating long reaching pools. The substratum of the pools was predominantly bedrock, soft-sediment and boulders. A number of small tributaries flow into this section of the river. These tributaries drain rural properties, urban development and native bushland (Bio-analysis 2009).

1.3.3 Water quality and hydrology

Currently 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 BCD at the West Cliff pit top and licensed discharges from the Appin East pit top. The average flow released from the BCD LDP 10 (29/6/2012-12/02/2014) was 0.23 ML/day (Table 1). The maximum flow released over this period was 0.827 ML/day.

Licensed releases from the 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. Other 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.bhpbilliton.com/home/aboutus/regulatory/Documents/_coal/illawarra/bulliseam/140213_coal_illawarra_bulliseam_14DayMonitoringReport.xlsx

Table 1 Long term water quality parameters for Georges River.

Location	pH	Conductivity EC ($\mu S.cm^{-1}$)[j]	Turbidity (NTU)	Discharge ($ML.day^{-1}$) Mean (min,max)	Total suspended solids (mg/L)
Point 10 (LDP 10)	7.9	2219	8	0.232 (0-0.827)	-
Point 11	7.0	182.9	-	-	4.16
Point 12	8.7	1824.46	-	-	9.45
Georges River @ Minto	-	-	-	234 (0.2-30,096)	-

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 from NSW Office of Water (http://realtimedata.water.nsw.gov.au/water.stm) (6/11/2012-23/02/2014).

1.3.4 Threatened macroinvertebrates and fish

Two threatened aquatic species are likely to occur in the study area, these are Macquarie Perch and Sydney Hawk dragonfly. Macquarie Perch has been found in the Georges River, near its confluence with Punchbowl Creek (Bio-analysis 2009). Previously, Macquarie Perch has only been reported from the Georges River catchment once, in 1894 (DPI 2008). Department of Primary Industries (2007) states that Sydney Hawk dragonfly is known from the Georges River catchment.

1.3.5 Relevant Previous Studies

PRP 6 – Ecological Effects of Mine Water Discharge from West Cliff Colliery into Brennans Creek (The Ecology Lab Pty Ltd, 2004)

Background and Aims:

The Ecology Lab Pty Ltd was commissioned by BHP Billiton Illawarra Coal in 2004 to investigate the effects of water discharged from West Cliff Colliery on issues relating to aquatic ecology in the upper Georges River. The overall aim of the study was to undertake a pilot investigation into the ecological effects of mine water discharged from West Cliff into the Georges River. The specific aims were to compare the Georges River near the discharge point from West Cliff Colliery with two control creeks, using as primary indicators water quality and aquatic macroinvertebrate fauna.

Conclusions:

Whilst the water quality indicators showed elevated conductivity in the Georges River consistent with the mine water discharges, it is important to recognise that this does not, in itself indicate that the biota of the river are adversely affected by this finding. Whilst the sampling of biota does show some differences consistent with the mine discharges, these differences are not large and they may be due to mine discharges or to other anthropogenic or natural influences in the Georges River.

AUSRIVAS analyses indicated slightly fewer taxa than might be expected at all sites in the Georges River and at the controls. The observed SIGNAL scores were slightly less than expected at all but one of the sites in the Georges River; at the control sites the observed scores were close to or slightly higher than expected. Similarly, analysis of the raw signal scores indicated that assemblages in the Georges River were slightly more pollution-tolerant than at the controls. AUSRIVAS habitat bands indicated that the sites within the Georges River were significantly impaired compared to the AUSRIVAS reference standard, whilst at the controls half were significantly impaired and the other half were equivalent to the reference condition. Those results suggest that macroinvertebrate assemblages in the Georges River very close to the mine discharges were impaired, but they were not severely impaired or impoverished. Several taxa that were expected by the AUSRIVAS model to occur at sites in the Georges River were not sampled by RAM but were, in fact, recorded using the more intensive quantitative sampling. Hence, it is possible that the findings of AUSRIVAS under-estimated the ecological condition the Georges River by presuming that some taxa were absent when in fact they were present.

Analysis of replicated sampling at the assemblage level (i.e. multivariate procedures) differentiated samples collected in the Georges River from the controls. It also differentiated the four locations sampled (two within each treatment). Some of the taxa that discriminated between the Georges River and the controls have been found in other studies to be salt sensitive (e.g. Baetidae and Chironomidae) but were relatively more abundant in the Georges River. Conversely, macrocrustaceans have been found to be relatively salt tolerant, yet the Atyidae (shrimps) were more common at the controls, particularly in Punchbowl Creek. Thus the patterns observed are not particularly consistent with potential impacts that might be expected due to increased conductivity from mine water discharges.

Analysis at the taxon level (i.e. univariate procedures) allowed a comparison of variability at three spatial scales, Treatment, Location and Site. Several treatment effects were identified, indicating a difference between the Georges River and controls, but abundance (including total abundance) was often greater in the Georges River. Location and site effects were also detected for some taxa, indicating variability at the smaller scales, often in both the Georges River and controls. Total biodiversity, as measured by numbers of taxa, did not vary at any scale, indicating taxon richness in the Georges River was similar to the controls.

The findings of this study did not suggest urgent remediation action was necessary at the time to modify the current mine water discharges from West Cliff and Appin Colliery.

PRP 9 - Ecological Effects of Mine Water Discharge from West Cliff Colliery into Brennans Creek (The Ecology Lab Pty Ltd, 2006)

Background and Aims:

The overall aim of this study was to determine whether the results obtained during the initial investigations (PRP 6) into the ecological effects of mine water discharge (The Ecology Lab 2004) were consistent over time. The incorporation of these studies into one sampling program provided an opportunity to obtain a more general understanding of the effects of mine water discharges.

Conclusions

Although there were consistent differences in some macroinvertebrate indicators between treatments, there was no indication that the macroinvertebrate assemblages in the vicinity of the West Cliff and Appin Collieries were impoverished or that taxon richness and abundances were very small relative to those at controls. The study indicated that the temporal variability in macroinvertebrates was not directly related to the variability in water quality indicators. It could, instead, reflect temporal differences in the quality of the edge habitat available to macroinvertebrates at individual sites brought about by changes in the volume and frequency of discharge. It was recommended that the feasibility of adapting the discharge so that it mimics natural variability in flow be considered.

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

Background and Aims:

The project was funded by the Australian Coal Association Research Program, with significant in kind contributions from several coal mining companies. The study was undertaken in the Southern and Hunter coalfields of New South Wales.

The aim of the project was to obtain information on, and develop an understanding of the effects of saline mine water discharge on aquatic biota and contribute to the development of site-specific water quality guidelines for the coal mining industry in New South Wales.
The following topics were investigated:

- Characterisation of the chemical composition of different mine waters. Relationships between the composition and abundance of invertebrate and microalgal assemblages and salinity gradients in streams receiving mine water discharge.
- Effects of desalination on invertebrate and microalgal assemblages in a stream that had previously received saline mine water
- Effects of salinity gradients on rates of decomposition of leaf litter in streams.
- Eco toxicological studies on the effects of mine water on local biota and on standard test organisms.
- Field-based studies of changes in composition and abundance of invertebrates translocated between streams of differing salinity

Conclusions

- Gradients in conductivity are readily detectable in some streams receiving mine water discharge, but conductivity can remain well above ANZECC default guidelines, even at considerable distances downstream of discharge points.
- The relationships between conductivity and abundance of aquatic biota are generally weak and vary between streams, suggesting that the default guidelines may be overly conservative and/or that other environmental factors play equal or more important roles in determining the abundance and distribution of stream biota.
- Surveys of macroinvertebrates in natural and artificial habitats have found diverse assemblages with many taxa considered to be pollution-sensitive present in areas of high salinity.

- The ecotoxicology study indicated highly variable toxicities among species and different mine waters. All mine waters were more toxic to all species than literature estimates based on pure NaCl.
- Site-specific trigger values for conductivity based on integration of laboratory and field data are less conservative than those of the ANZECC guidelines, but are still between 50 and 85% lower than the conductivity measured in receiving streams in this study.
- To identify the most important toxicants in mine water, future studies should include a toxicity identification and evaluation procedure and in situ toxicity tests. Future studies should adopt standardised approaches as far as possible to enable comparisons between studies and better prediction of toxicity in novel situations. In particular, the use of artificial habitat for microalgae and invertebrates has proved to be a valuable monitoring tool.

West Cliff Longwalls 33-38 Aquatic Ecology Monitoring 2002 – 2013 (Cardno Ecology Lab, 2014)

Background and Aims:

Cardno Ecology Lab (formerly The Ecology Lab Pty Ltd) was commissioned by BHPBIC to assess the potential impact of longwall mining-related subsidence on the aquatic ecology of the Georges River and other nearby watercourses within the West Cliff Area 5 mine area through the implementation of an aquatic ecology monitoring programme. The aims of the monitoring programme are to:

- Assess the relative abundance of fish and macroinvertebrates and condition of aquatic habitat that may be affected by subsidence related impacts; and,
- Determine whether any changes observed in aquatic habitat or biota may be linked to subsidence related impacts.

Conclusions:

The results suggested that impacts to aquatic ecology are restricted to the areas directly affected by mining impacts. Although a loss of river connectivity during low flow conditions could impact the passage of migratory fish species (e.g. eels and the Cox's gudgeon), with potential consequences for these species upstream and downstream of the affected areas, at this stage, there is no data to suggest an impact to fish has occurred outside of Site 9. The increased releases from Brennans Creek Dam appear to have been successful in temporally restoring pool water levels and flow in the affected areas to pre-mining levels. This measure will help maintain connectivity among stretches of river and pools affected by the recent mining impacts.

There is no evidence to suggest the commencement of extraction of Longwall 36 has had any impact on aquatic ecology. This is not surprising considering that no physical impacts had been observed by December 2013 and that at the time of the survey extraction of the longwall was taking place several hundred metres away from the Georges River.

1.3.6 Limitations

As the Aquatic Health Monitoring Plan has just commenced, at this stage sampling has only occurred for one season. 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 Cascade Creek and Upper Georges River above Brennans Creek confluence. It should also be noted that water quality improvement projects at Point 10 (as required by PRP 19) had not been commissioned at the time of sampling.

1.4 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 West Cliff licensed discharge Point 10. Five sites were located in pool habitats downstream of Licence discharge point 10 (Table 2). Four reference sites were also sampled, GRUFS and GRQ1 (upstream Georges River) and CC1, CC2 (Cascade Creek). Site 11 is upstream of Brennans Creek however is potentially impacted from Appin Mine East drainage. Analysis in section 2 determined the site is appropriate to use as a reference site for this report.

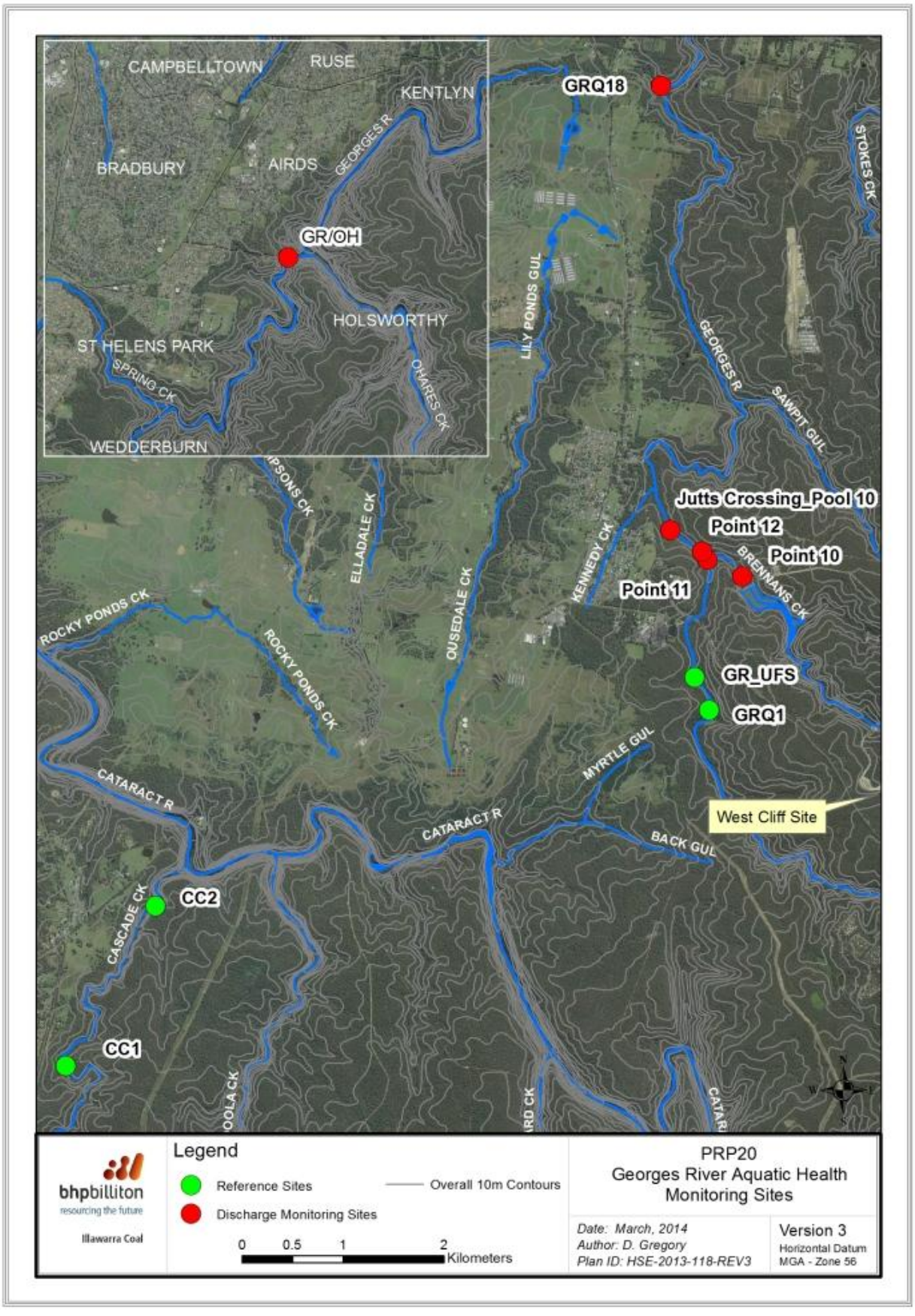
Table 2 Site location and treatment

Site Number	Stream	Location	Eastings	Northings	Treatment
Point 10	Brennans creek	Discharge point (LDP10)	297558	6212772	Discharge monitoring
Point 11	Brennans creek	U/s of Brennans Creek and Georges River confluence	297207	6212940	Reference (note: Potential impact from Appin Mine East)
Point 12	Georges River	D/s of Brennans Creek confluence	297157	6213016	Discharge monitoring
Jutts crossing	Georges River	D/s of Jutts Crossing	296844	6213232	Discharge monitoring
GRQ18	Georges River	U/s of O'Hares creek confluence	296748	6217637	Discharge monitoring
GR/OH	Georges River	U/s O'Hares creek confluence	300013	6225211	Discharge monitoring
GRUFS	Georges River	U/s of confluence	297082	6211771	Reference
GRQ1	Georges river	U/s of confluence	297225	6211446	Reference
CC1	Cascade Creek	Upper Cascade creek	290841	6207918	Reference
CC2	Cascade Creek	Lower Cascade creek	291730	6209505	Reference

1.5 Survey timing and frequency

The EPL conditions dictate that sampling must occur every two years commencing in 2013 and concluding in 2019, and that sampling must occur between 1 September and 30 November in each of the sampling years. Sampling was conducted in the period of 21st- 24th November 2013 (fish) and 28th October – 5 November 2014 (Macroinvertebrates).

Figure 1 Location of macroinvertebrate and fish sampling locations



1.6 Field methods

1.6.1 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).

Duplicate measures (shallow and deep) of the above indicators were taken if the water parameters deviated by >10%; although at the majority of sites, this was not possible due to the lack of depth of the pool.

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.

Duplicate samples (shallow and deep) were collected if the field parameters deviated by >10%; although as mentioned above, at the majority of sites, this was not possible due to the lack of depth of the pool.

1.6.2 Fish survey

The method of fish capture utilised combination of backpack electrofishing and trapping in an effort to catch a range of fish species including threatened Macquarie Perch. Electrofishing is to target fish habitat (macrophytes, overhanging banks, rocky boulders and large woody debris). Back pack electrofishing was used at each site unless the sampling at the site is unviable or unsafe and was conducted in accordance with Australian Code of Electrofishing Practice (1997). Fish were collected under Scientific Collection Permit -Section 37 *Fisheries Management Act 1994* Permit No: P13/0008-1.0

Four unbaited concertina traps where set for one hour at each site.

Fish are identified in the field using Field Guide to the Freshwater Fishes of Australia (Allen *et al.* 2002) and abundance recorded.

1.6.3 Macroinvertebrate survey

Macroinvertebrates were sampled from three random pool edges at each site. Pool-edge samples where collected from depths of 0.2-0.5m within 2m 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. Macroinvertebrates were collected under Scientific Collection Permit -Section 37 *Fisheries Management Act 1994* Permit No: P13/0008-1.0.

1.6.3.1 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.

1.6.4 Data analysis

1.6.4.1 Monitoring Design

The monitoring design will incorporate the BARI (Before After Reference Impact) monitoring approach. The design is to test whether the abundance, richness and assemblages of aquatic biota will become more similar to the reference sites. The data collected from the first monitoring occasion is part of the before component, that is before the implementation of water management measures under PRP 19. 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.

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, and Cascade Creek (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 fish and 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.

1.6.4.2 Statistics

Water quality

Water quality results of both field and laboratory processed data were tabulated. Water quality parameters were also used in a BEST procedure, (a biota environmental matching technique in Primer 6) that will be discussed under macroinvertebrate statistics. Also, water quality was examined by comparison to the limits outlined in PRP 19.

Fish

Due to the low abundance and species richness no tests of significance were conducted. The fish data were tabulated.

Macroinvertebrates

The three subsamples at each site were combined to give one sample.

Univariate data

Univariate variables density and family richness were graphed. Permutational Analysis of Variance (PERMANOVA) was performed on discharge monitoring and reference sites to test for significant differences between these groups. The results were tabulated.

Multivariate data

Multivariate data of macroinvertebrate assemblages were fourth root transformed, and resemblance matrix created with Bray Curtis similarity measure (Log x +1). Multidimensional scaling (MDS) plot was derived from the similarity matrix (Clark and Warwick 1993).

A PERMANOVA was performed on discharge monitoring and reference groups to test for significant differences between macroinvertebrate assemblages. A Similarity of Percentages (SIMPER) procedure was conducted with reference verse discharge monitoring groups to determine families that contribute most to any observed differences between these groups. Average similarity measure of discharge monitoring and reference sites were plotted showing downstream changes in similarity of macroinvertebrate assemblages. Fauna that contributed most to differences between discharge monitoring and reference groups were also graphed to show downstream changes in it density.

Macroinvertebrate assemblages were compared with water quality variables with BEST analysis (a Biota Environmental Matching Technique (Bio-Env) (see Clark *et al.* 2008), to see if water quality significantly explained macroinvertebrate communities and what variables accounted for any significant observations. Water quality variables were initially explored with a draftsman plot to determine which variables were highly skewed and/or correlated. Skewed data were log transformed and all water quality data normalised. A resemblance matrix (Euclidan distance transformed) of the water quality variables was created from which a MDS plot was made.

The BEST analysis was performed on transformed and normalised data, with highly correlated variables being represented by only one variable. A permutational test, tested for significant explanation of macroinvertebrate assemblages by water quality variables.

2 Results

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 3 Field water quality results

. 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 Appendix D. Reference sites had lower temperature, electrical conductivity and dissolved sodium, chloride, dissolved oxygen, alkalinity, nitrates, however was more acidic (Low pH), and higher in iron and manganese. Discharge monitoring sites were also higher in other metals (Table 4). A comprehensive water quality monitoring program is conducted by BHPBIC and hence will not be discussed in detail with in this report; however will be analysed further is section 2.3 in context of water quality variables relationship to aquatic ecology.

Table 3 Field water quality results

Site No.	Temperature (C°)	Conductivity (µS/cm)	Turbidity (NTU)	Dissolved Oxygen (% sat)	pH
Point 10	20.05	2700	4.54	92.3	8.88
Point 11	19.93	238	6.67	83.4	7.95
Point 12	19.1	2690	2.46	91	8.85
Jutts Crossing	20.03	2580	4.86	86.6	8.98
GRQ18	17.77	1450	26.6	68.2	7.09
GROH	18.29	955	1.17	80.5	7.88
CC1	15.8	477	6.8	47	5.72
CC2	17.89	295	3.7	60.8	6.2
GRQ1	17.93	177	2.92	73.8	6.35
GRUFS	19.45	182	1.63	90.5	6.3

2.1.1 Comparison of Point 10 water quality to limits outlined in PRP 19

Table 4 Point 10 water quality tracking table.

Pollutant	Units	100 percentile concentration limit (as defined by PRP 19)	2013 PRP 20 – Point 10 Results Sample collected on 31/10/2013 (from end of pipeline) *Red – Above PRP 19 Limit Green – Within PRP 19 Limit	2015
Oil and Grease	mg/L	10	<5	----
pH	pH Units	6.5-8.0	**9.0	----
TSS	mg/L	50	<5	----
Conductivity	µs/cm	495	**2992	----
Bicarbonate	mg/L	225	888	----
Al (dissolved)	µg/L	55	560	----
As (Dissolved)	µg/L	24	11	----
Cd (dissolved)	µg/L	0.2	0.1	----
Co (dissolved)	µg/L	30	11	----
Cu (dissolved)	µg/L	1.4	11	----
Pb (dissolved)	µg/L	3.4	4	----
Mn (dissolved)	µg/L	1900	11	----
Ni (dissolved)	µg/L	11	142	----
Zn (dissolved)	µg/L	8	41	----
COD	mg/L	50	15	----
TDS	mg/L	340	1600	----
Total N	µg/L	250	1800	----
N (ammonia)	µg/L	13	100	----
Oxides of Nitrogen	µg/L	15	950	----

*PRP 19 limits are to be achieved by 30 December 2016.

** pH and Conductivity results are taken from the Pt 10 continuous monitoring in-line instrumentation (maximum value recorded for the week 25/10/13 to 9/11/13) – See website for data:

http://www.bhpbilliton.com/home/aboutus/regulatory/Pages/default.aspx

2.2 Fish

The survey of the Georges River and reference sites (Cascade Creek) identified 7 native and 1 exotic fish species as well as two crustaceans (*Cherax sp. and Euastica sp*.) (Table 5). With the exception of Long Finned eel, the fish collected were mostly of small sized classed fish. The Georges River was commonly habited by Fire Tail gudgeon (10 individuals) and Cascade Creek being relatively depauperate consisting of only 1 Long finned eel and Paratacidae (Yabbies). Other native fish observed in the Georges River include Striped gudgeon (1 individual), Flathead gudgeon (5 individuals), Cox's gudgeon (1 individual), Long Finned eel (6 individuals), and Empire gudgeon (1 individual). Exotic species *Gumbusia holbrooki* (30 individuals) occurred at Point 12 and downstream sites GRQ18 and GROH. No threatened species, i.e. Macquarie Perch were observed from the spring survey. Fish electrofishing survey effort is shown in Appendix A.

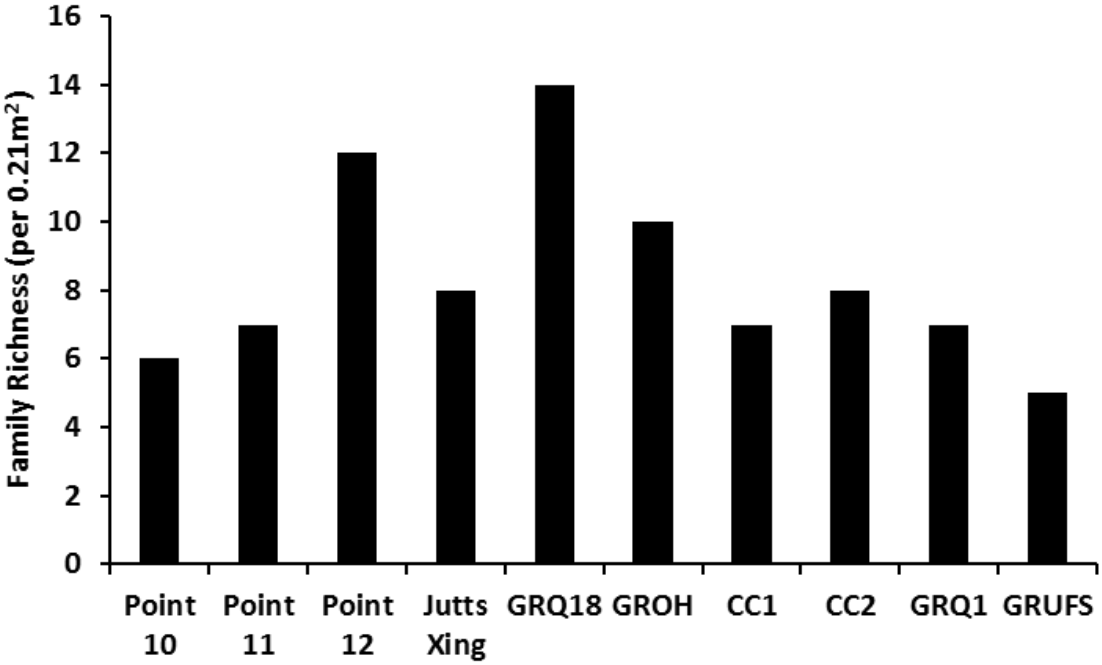
Table 5 Fish sampling results.

		Discharge monitoring Sites						Reference Sites			
		Point 10	**Point 11**	**Point 12**	**Jutts Crossing**	**GRQ18**	**GROH**	**CC1**	**CC2**	**GRQ1**	**GRUFS**
Fish species											
Native	Fire tailed gudgeon *Hypseleotris galii*	1	2		2	2	7				
	Flat head gudgeon *Philypnodon grandiceps*					4	1				
	Coxs gudgeon *Gobiomorphus coxii*									1	
	Striped gudgeon *Gobiomorphus australis*			1						1	
	Long finned eel (juvenile)(5-10cm) *Anguilla reinhardti*	5								1	
	Long finned eel adult (30-80cm) *Anguilla reinhardti*	3			1				1		
	Empire gudgeon *Hypseleotris compressa*			1							
	Euastica sp				1			5			
	Cherax sp							6	7		3
Invasive	Mosquito Fish *Gambusia Holbrooki*		1			5	24				

2.3 Macroinvertebrates

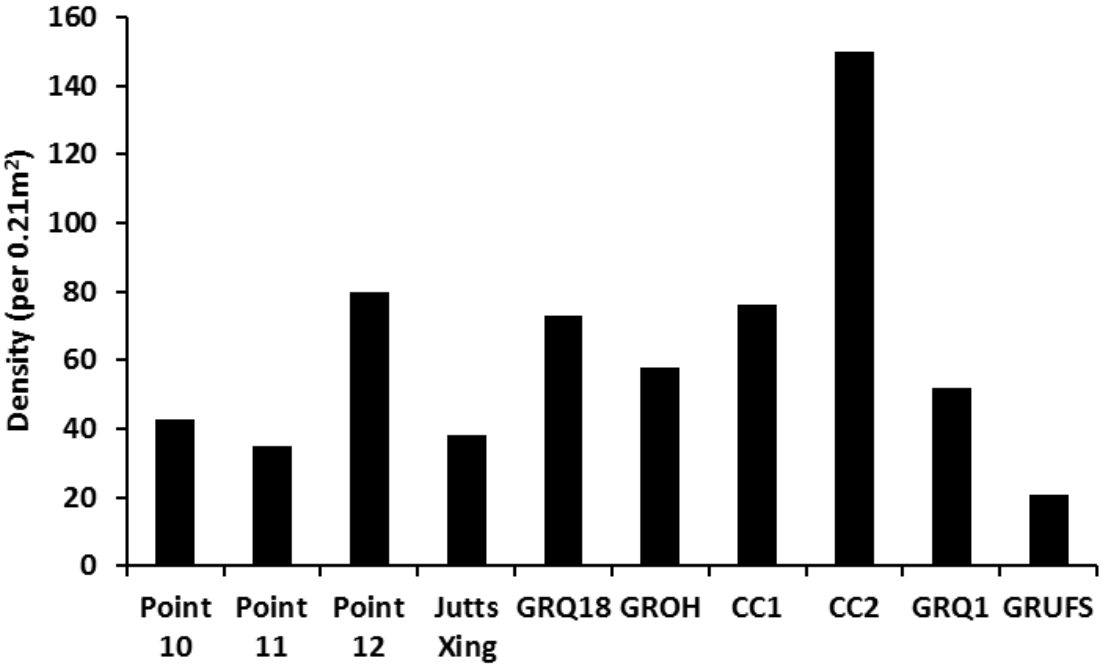
2.3.1 Univariate results

There were a total of 26 families observed across all sites (Graph 1). The highest mean family richness was recorded in Georges River (GRQ18) (14 families per 0.21m^2) and the lowest in Georges River reference site (GRUFS) (5 families per 0.21m^2). PERMANOVA results (Table 6) showed no significant difference in variation ($p<0.01$) between discharge monitoring and reference groups.



Graph 1Total family richness at each site

The total abundance over all sites sampled was 626 individuals. The highest density was recorded in Cascade Creek (CC2) (150 per 0.21m^2). The lowest density was recorded in Georges River reference site (GRUFS) (21 per 0.21m^2) (Graph 2). PERMANOVA results showed that there is no statistical difference of density between discharge monitoring and reference sites (Table 6).



Graph 2 Density at each site

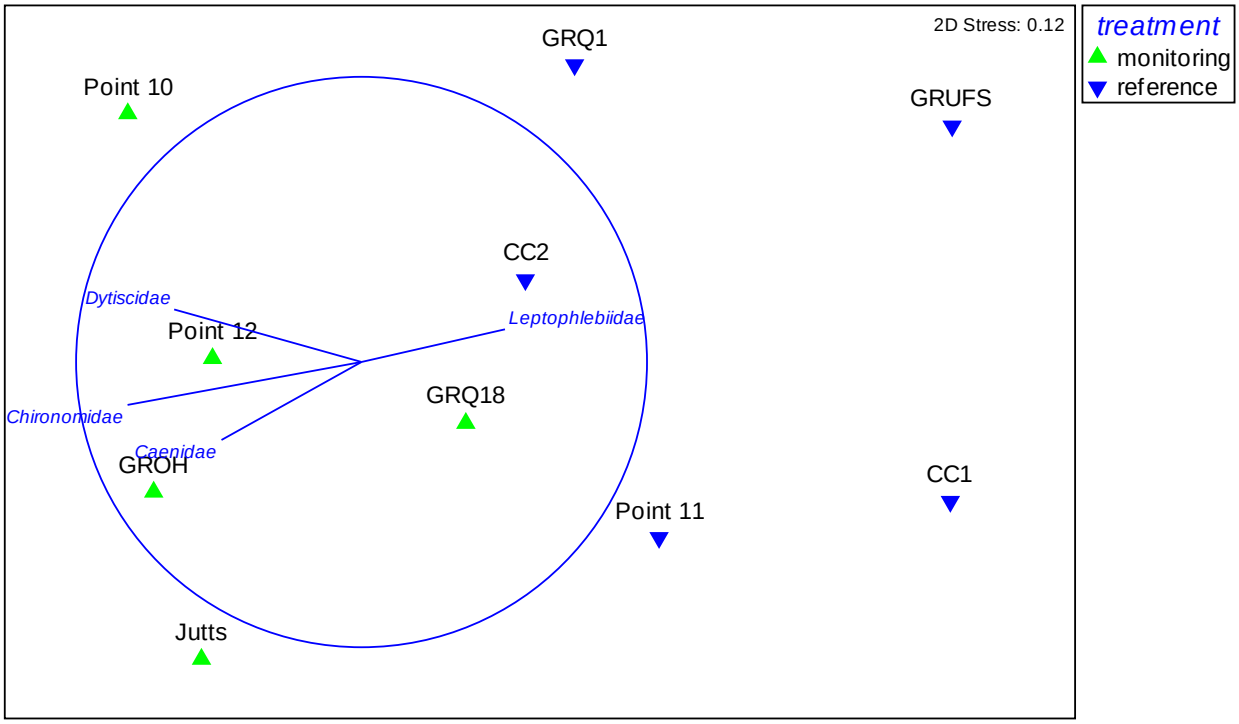
Table 6 PERMANOVA results for mean family richness and mean density

Variable	Source	Degrees of freedom	Sum of squares	Mean squares	Pseudo-F	P(perm)	Permutations	MonteCarlo (MC)
Mean family richness	Discharge monitoring/reference	1	9.6	9.6	2.0426	0.011	18	0.083
	Residual	8	37.6	4.7				
	Total	9	47.2					
Mean density	Discharge monitoring /reference	1	176.4	176.4	0.12095	0.832	84	0.736
	Residual	8	11668	148.5				
	Total	9	11844					

2.3.2 Multivariate

The MDS plot (Graph 3) shows that there are differences between discharge monitoring and reference sites; however there is considerable variation within these groups as well. Site 11 (downstream from Appin surface discharge) was more similar in macroinvertebrate assemblage (39.85% average similarity) to reference groups than discharge monitoring groups (34.59%). It is also closer (Euclidian distance) in water

quality variables to reference sites (3.5) than discharge monitoring sites (8.0) (Graph 6). For this reason the design has included Site 11 as a reference even though it is situated downstream of the Appin East mine site licenced surface water discharge point. PERMANOVA results shows significant difference between discharge monitoring and reference treatment groups (p-0.008, pseudo f-4.1324) (Table 7).



Graph 3 MDS ordination plot of macroinvertebrate assemblages at discharge monitoring and reference sites

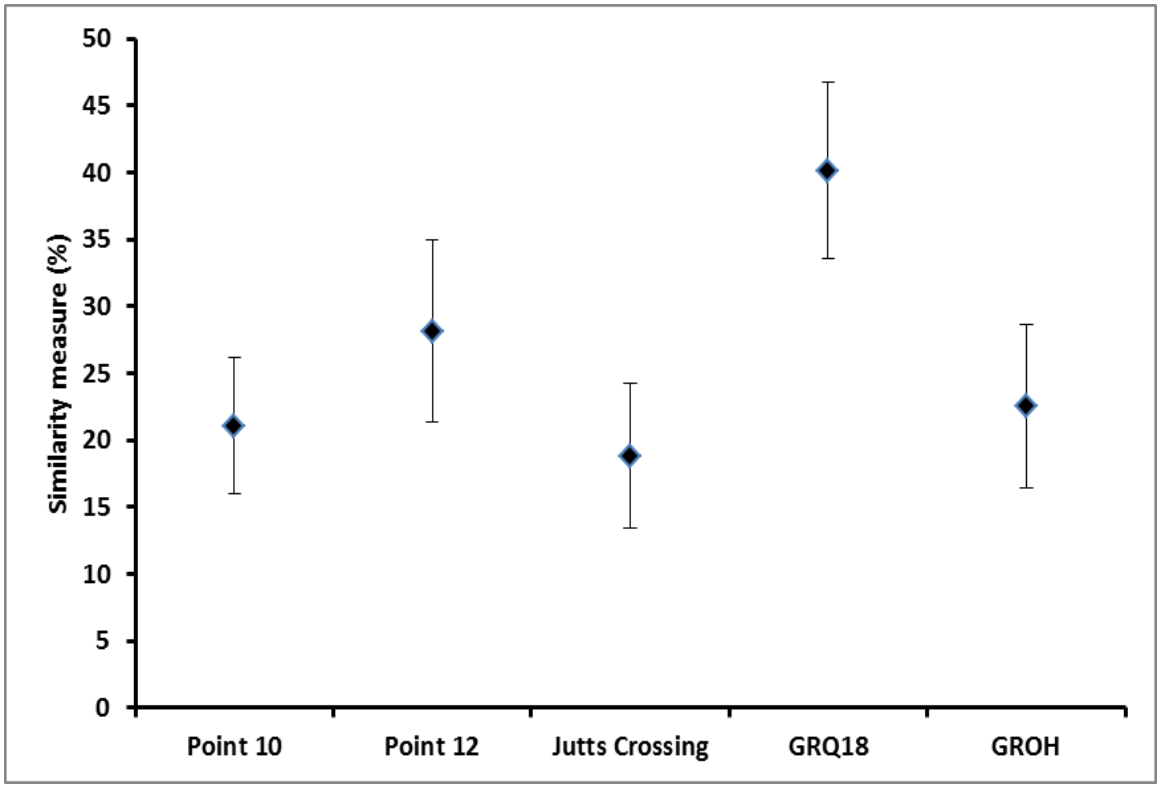
SIMPER procedure showed that within discharge monitoring groups the families Chironominae, Dytiscidae, and Caenidae contributed most to the within stream similarity. Leptophlebiidae, Chironomidae and Hemicorduliidae contributed most to reference group similarity (Appendix B). Point 10 was on average 21% similar to reference sites, Point 12 (28%), Jutts Crossing (19%), GRQ18 (40%) and GROH (23%) (Graph 4). Lower densities of Leptophlebiidae (Graph 5) and increased densities of Chironomidae, Caenidae and Dytiscidae (Graph 5) in discharge monitoring sites contributed most to the dissimilarity (overall dissimilarity 73.85%) between discharge monitoring and reference sites (Table 8, Graph 3).

Table 7 PERMANOVA results for macroinvertebrate assemblages

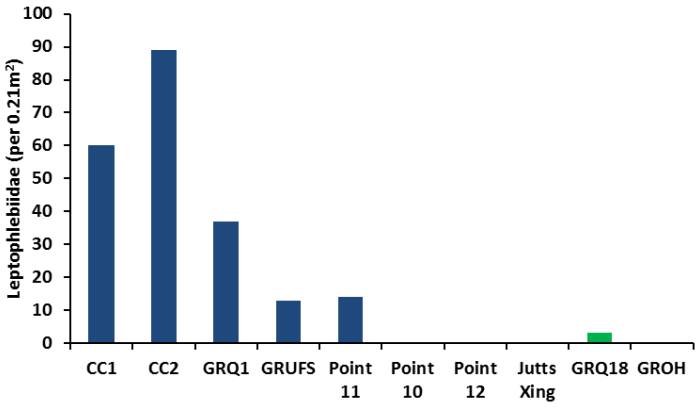
Variable	Source	Degrees of freedom	Sum of squares	Mean squares	Pseudo-F	P(perm)	Permutations	MonteCarlo (MC)
Macroinvertebrate assemblages	Discharge monitoring /reference	1	7188.8	7188.8	4.1324	0.008	125	0.005
	Residual	8	13917 8	1739.6				
	Total	9	21106					

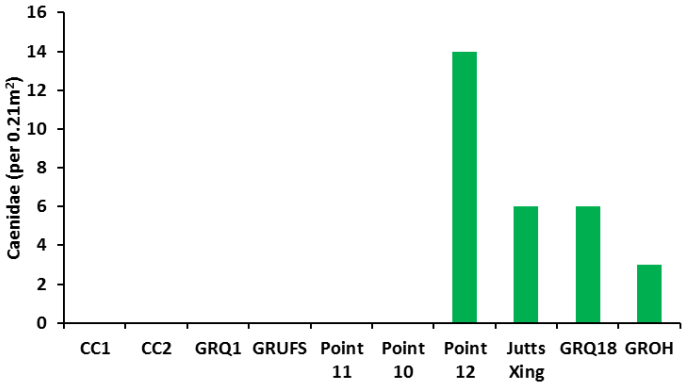
Table 8 SIMPER results of dissimilarity between discharge monitoring and reference sites

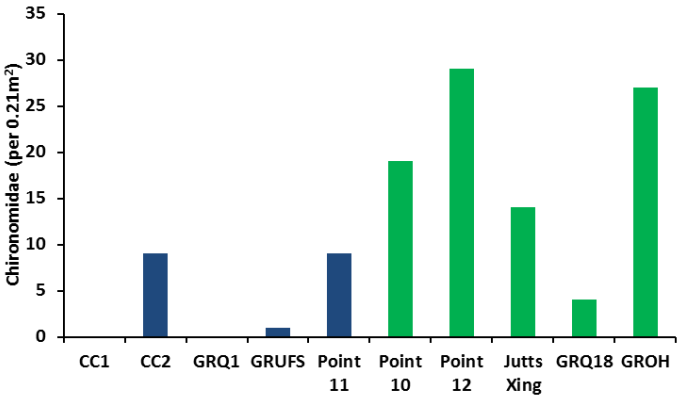
Species	Average Abundance discharge monitoring	Average abundance reference	Average Dissimilarity	Dissimilarity/Standard Deviation	Contribution%	Cumulative. %
Leptophlebiidae	0.26	2.43	9.67	2.68	13.1	13.1
Chironomidae	1.39	0.34	5.74	1.31	7.77	20.87
Dytiscidae	2.01	0.89	5.27	1.41	7.13	28
Caenidae	1.28	0	5.25	1.82	7.1	35.1

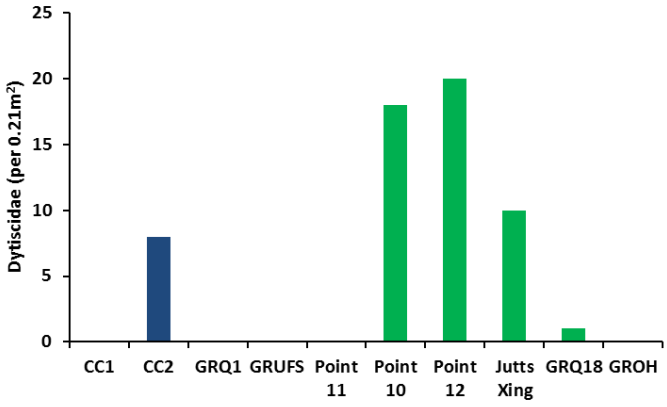


Graph 4 Average similarity measure and standard error of discharge monitoring sites and reference sites (sites arranged from upstream to downstream



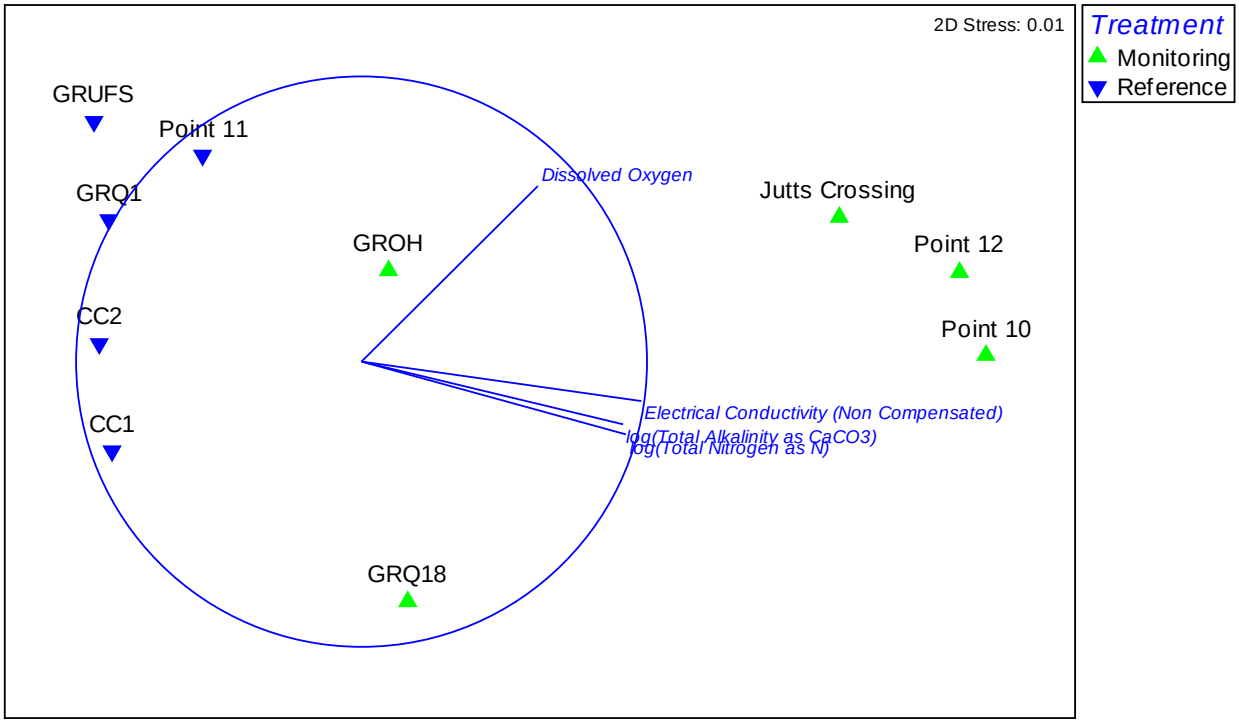






Graph 5 Density of Leptophlebiidae, Caenidae, Chironomidae and Dytiscidae at each site (sites arranged upstream to downstream). Blue-Reference sites; Green-Discharge monitoring sites.

The BEST procedure showed water quality significantly explained macroinvertebrate assemblages (P-0.001, Rho- 0.673. Total alkalinity, dissolved oxygen and total nitrogen (Graph 6, Appendix AAppendix C) variables best explained macroinvertebrates assemblages. However it must be noted that most water quality variables were correlated. This is likely to be because discharge is such a large proportion of stream flow in the upper Georges River and its associated water quality variables decreases proportionally downstream. Therefore each variable relating to discharge may exhibit similar patterns and show correlation. Thus it is likely that discharge from LDP10 (Point 10) is a key factor in the difference in distribution of macroinvertebrate assemblages.



Graph 6 MDS ordination of water quality variables at discharge monitoring and reference sites

2.4 Threatened species

No threatened fish (Macquarie Perch) were observed from the discharge monitoring; however a threatened dragonfly family (Austrocorduliidae) was observed at Cascade Creek (CC1), and Georges River (Point 11 and GRQ18). Preliminary species identification has classified Austrocordulia odonates at CC1 (1 individual) and GRQ18 (3 individuals) as the threatened (*Fisheries management Act 1994*) Sydney Hawk dragonfly (*Austrocordulia leonardi).* The Point 11 specimens (2 individuals) were difficult to separate at the species level because of inconspicuous morphological features and could be either *Austrocordulia refracta* or *A. leonardi*. It is recommended that all Austrocorduliidae specimens be confirmed for quality control.

3 Discussion

3.1 Fish

In general, the fish population was low in abundance and lacked diversity. Monitoring of the reference sites only found 2 Long Finned eels and 1 Cox's gudgeon. Other studies in the same area have found low diversity and abundance; for instance, sampling conducted by Bio-analysis (2009) found no fish in Cascade Creek; and Long Finned eel, Striped gudgeon, and Gambusia were recorded upstream of Point 11. It is likely that the Upper Georges River and Cascade creek is naturally low in abundance and diversity of fish. Because of this, the ability of the program to statistically detect differences or similarities between discharge monitoring and reference sites (spatially and temporally) is limited. Recently, BHPB IC's Aquatic Ecology monitoring for West Cliff Longwalls 33-38 in the Georges River (Cardno 2014) found five species of fish at sites downstream of the mine water discharge (Gambusia, Short finned and Long Finned eel, Cox's gudgeon and Carp gudgeon (*Hypseleotris spp*.) with introduced Gambusia being the most dominant. This is compared to this discharge monitoring program which found three species of fish, Fire Tailed gudgeon (*Hypseleotris galii*), Flat head Gudgeon (*Philypnodon grandiceps*) and *Gambusia holbrooki* at downstream sites GRQ18 and GROH. Gambusia were also the most abundant species downstream.

3.2 Macroinvertebrates

3.2.1 Macroinvertebrate communities

The 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 assemblage composition between discharge monitoring and reference sites. 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.

Similarly, previous studies on West Cliff mine water discharge (Cardno 2006) found Leptoceridae, Leptophlebiidae, Ceinidae and Atyidae were 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 leptophlebiid mayflies downstream of mine water discharge from West Cliff. This was consistent with results of the laboratory toxicity tests conducted under that study, which indicated that significant impacts on populations would be likely at conductivities above those at the study's reference sites. They also found that discharge waters from mines in the Hunter and Illawarra/Macarthur regions induced deleterious responses in a range of aquatic biota with the leptophlebiid mayfly *Atalophlebia spp.* being the most sensitive of these.

3.2.2 The relationship between macroinvertebrates and environmental variables

The monitoring program for West Cliff Colliery attempted to link macroinvertebrate assemblages to water quality variables. The BEST analysis showed that water quality variables were highly correlated and of all the analytes tested, dissolved oxygen, total alkalinity and total nitrogen best explained macroinvertebrate assemblages. However because of this correlation, other water quality variables cannot be discounted. This being said, the data does show that mine water discharge in general is likely to significantly explain macroinvertebrate assemblage composition. However, other non-measured variables may also contribute to this.

In addition to the effects of discharge water quality there are significant differences between the natural catchment water qualities expected at the discharge sites and the 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 marine 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.

Cardno (2006) concluded that differences between impact and control sites were likely to be attributable to hydrology; through temporal differences in habitat and its availability, brought about by changes in volume and frequency of discharge. Later studies (Cardno 2011) on Teatree Hollow mine water discharge at Tahmoor Colliery, found that physical conditions, such as water depth and substratum best "explained" the spatial distribution of invertebrates in Teatree Hollow. Cardno (2010) ACARP study concluded that the observed difference between sites downstream of mine water discharge and control sites could not be directly related to salinity; they found that there were increased correlations between biota and physical variables when conductivity was excluded from the analyses. The study also highlighted the need for site-specific toxicity information that takes into account the variable composition of saline mine waters, including consideration of other constituents; that the mine water discharges are complex effluents that contain potentially toxic components other than salinity. It is therefore difficult to ascertain whether the difference in faunal assemblages are the result of water quality or are the result of a constant flow of water that alters the flow dynamics, geomorphology, and thus habitat with in Brennans Creek and Upper Georges River and further studies on toxicity are required to ascertain the specific causes to potential deleterious effects on specific macroinvertebrates. It is understood that BHPB IC is currently undertaking ecotoxicity testing as required by EPL 2504. In addition, ACARP C23010 (recently commissioned) will identify salinity tolerances on freshwater organisms from the southern and western coalfields.

The results from this study suggest an absence of pollution intolerant Leptophlebiidae (SIGNAL 8) immediately downstream of the discharge (Point 10, 12 and Jutts Crossing). It is not clear whether this is caused by the influence of mine water discharge on water quality, hydrology or a natural distribution of this particular family within the catchment. However, as mentioned in Section 3.2.1, the results suggest that the mine water discharge is favouring pollution tolerant taxa given the increased densities of pollution tolerant Chironomidae (SIGNAL 3) and Caenidae (SIGNAL 4) in discharge monitoring groups compared to reference groups. This family and particularly the species *Atalophlebia spp.* could provide a useful indicator in the Southern Coal Fields in general. Recovery of this species may be an important tool to gauge stream recovery after the implementation of PRP19.

3.2.3 Longitudinal patterns

The lack of downstream site replication within this study did not allow statistical comparison between near and far sites (i.e. from the discharge point); however this will be able to be conducted following another sampling occasion. What is evident from the program is that the influence of mine water discharge on water quality decreases downstream, for example electrical conductivity is almost halved at GRQ18. GRQ18 had the highest family richness of all sites (including the reference sites). GRQ18 was most similar (40%) to reference sites in terms of macroinvertebrate assemblages. GRQ18 showed the presence of pollution sensitive Leptophlebiidae (which was absent from upstream discharge monitoring sites) and also contained the threatened dragonfly *Austrocordulia leonardi*. It appears as though by this point 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. This may be due to habitat constraints; as pool edge habitat was limited because of the steep sided banks, which may have led to reduced diversity and decreased similarity to reference sites.

3.2.4 Threatened species: Sydney Hawk Dragonfly

The Sydney Hawk dragon fly was positively identified in two locations (Cascade Creek CC1, Georges River GRQ18) and potentially Georges River Point 11. The Sydney Hawk dragonfly has a very restricted or patchy known distribution. The known distribution of the species included three locations in a small area south of Sydney, from Audley to Picton (DPI 2007). It has also been located north of the Hunter Valley extending its possible distribution (Theischinger *et al*. 2013). Despite the range extension for *A. leonardi*, extensive habitat degradation that has occurred, particularly of coastal catchments, the fragmented nature of the records, and the ongoing development of Sydney's suburbs, *A. leonardi* is still of high conservation concern (Theischinger *et al*. 2013).

The Sydney Hawk dragonfly spends most of its life underwater (1-2 years) as an aquatic larva, before metamorphosing and emerging from the water as an adult. Adults are thought to only live for a few weeks. The Sydney Hawk dragonfly has specific habitat requirements, and until recently has only been collected from deep and shady riverine pools with cooler water (DPI 2007) and rocky substrate. Theischinger (2013) suggests that the Sydney Hawk is restricted to larger streams in coastal areas. The finding of Sydney Hawk dragonfly in Cascade Creek and the Georges River is significant. Until recently the Sydney Hawk dragonfly has only been found in larger coastal streams. The finding provides evidence that *Austrocorduliidae leonardii* can also occur in semi-permanent/ephemeral/headwater streams such as Cascade Creek and Upper Georges River. It is also encouraging that despite the history of mine water discharge that this species can continue to inhabit the Georges River. Furthermore, there is potential for it to recruit back into affected areas.

4 Conclusion

The Aquatic Health Monitoring Plan is a long term monitoring program which has just commenced and definitive conclusions have not been made, however, preliminary findings based on the initial survey and literature review are:

- The fish community in the study area is low in abundance and diversity (this is likely to be natural in head water streams).
- Fish are not a reliable indicator for monitoring because of the low diversity and abundance and are hence limited in ability to detect small or gradual environmental change.
- No threatened fish were observed.
- There was no statistical difference between density and family richness between discharge monitoring and reference sites, however there was statistical difference between macroinvertebrate assemblages.
- Lower densities of pollution sensitive Leptophlebiidae (SIGNAL 8) and increased densities of pollution tolerant Chironominae (SIGNAL 3) and Caenidae (SIGNAL 4) were observed in discharge monitoring groups compared to reference groups.
- It is likely that the water quality in discharges from LDP 10 are resulting in the observed difference in the distribution of macroinvertebrate assemblages, particularly the family Leptophlebiidae, however other environmental variables may also explain the difference.
- Sydney Hawk dragonfly (*Austrocordulia leonardi)* was positively identified in Cascade Creek (CC1), and Georges River (GRQ18), which is listed as a threatened species under the *Threatened Species Conservation Act 1995* and *Fisheries Management Act 1994*.

4.1 Recommendations

It is recommended that monitoring continue to be undertaken in accordance with the requirements of the EPL and Aquatic Health Monitoring and to further investigate preliminary findings.

It is also recommended that initial identification of *A. leonardii* be confirmed by an invertebrate taxonomist.

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http://realtimedata.water.nsw.gov.au/water.stm

6 Appendix

Appendix A Survey effort for back pack electrofishing

Site No.	Frequency (Hz)	Duty Cycle (%)	Voltage (Volts)	On time (seconds)
Point 10	120	25	150	410
Point 11	120	25	350	312
Point 12	120	25	150	289
Jutts Crossing	120	25	150	383
GRQ18	120	25	150	300
GROH	120	25	350	320
CC1	120	25	350	390
CC2	120	25	400	452
GRQ1	120	25	350	348
GRUFS	120	25	350	320

Appendix B SIMPER procedure results

SIMPER
Similarity Percentages - species contributions

One-Way Analysis

Data worksheet
Name: Data2
Data type: Abundance
Sample selection: All
Variable selection: All

Parameters
Resemblance: S17 Bray Curtis similarity
Cut off for low contributions: 90.00%

Factor Groups

Sample	treatment
point 10	impact
Point 12	impact
Jutts	impact
GRQ18	impact
GROH	impact
Point 11	control
CC1	control
CC2	control
GRQ1	control
GRUFS	control

Group impact
Average similarity: 48.82

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Chironomidae	2.01	13.75	3.07	28.16	28.16
Dytiscidae	1.39	6.71	0.95	13.75	41.90
Caenidae	1.28	5.83	1.14	11.95	53.85
Gomphidae	1.18	4.73	1.11	9.69	63.54
Hydrophilidae	0.95	4.37	1.13	8.96	72.50
Gyrinidae	0.70	2.54	0.61	5.21	77.71
Hemicorduliidae	0.85	2.47	0.62	5.07	82.78
Sialidae	0.66	2.24	0.62	4.59	87.36
Leptoceridae	0.79	2.06	0.61	4.23	91.59

Group control
Average similarity: 35.58

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Leptophlebiidae	2.43	21.69	7.94	60.96	60.96
Chironomidae	0.89	3.75	0.61	10.53	71.49
Hemicorduliidae	0.78	3.08	0.60	8.67	80.16
Ceinidae	0.85	1.53	0.32	4.29	84.44
Atyidae	0.54	1.48	0.32	4.15	88.59
Leptoceridae	0.58	1.13	0.32	3.18	91.77

Groups impact & control
Average dissimilarity = 73.85

	Group impact	Group control				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Leptophlebiidae	0.26	2.43	9.67	2.68	13.10	13.10
Dytiscidae	1.39	0.34	5.74	1.31	7.77	20.87
Chironomidae	2.01	0.89	5.27	1.41	7.13	28.00
Caenidae	1.28	0.00	5.25	1.82	7.10	35.10
Gomphidae	1.18	0.20	4.42	1.44	5.98	41.09
Hydrophilidae	0.95	0.24	3.54	1.53	4.79	45.88
Leptoceridae	0.79	0.58	3.44	1.25	4.66	50.53
Ceinidae	0.00	0.85	3.39	0.78	4.59	55.13
Hemicorduliidae	0.85	0.78	3.22	1.07	4.36	59.49
Baetidae	0.82	0.44	3.11	1.15	4.21	63.70
Gyrinidae	0.70	0.20	2.99	1.11	4.05	67.75
Sialidae	0.66	0.00	2.94	1.11	3.98	71.72
Atyidae	0.20	0.54	2.69	0.82	3.65	75.37
Isostictidae	0.46	0.46	2.37	0.99	3.21	78.58
Austrocorduliidae	0.26	0.44	2.23	0.89	3.02	81.60
Corixidae	0.48	0.00	2.23	0.77	3.01	84.61
Libellulidae	0.40	0.20	1.85	0.84	2.50	87.11
Megapodagrionidae	0.24	0.20	1.57	0.68	2.13	89.24
Calamoceratidae	0.20	0.20	1.41	0.66	1.91	91.15

Appendix C BEST procedure results

BEST
Biota and/or Environment matching

Data worksheet
Name: Data7
Data type: Environmental
Sample selection: All
Variable selection: All

Resemblance worksheet
Name: Resem2
Data type: Similarity
Selection: All

Parameters
Rank correlation method: Spearman
Method: BIOENV

Maximum number of variables: 10
Resemblance:
Analyse between: Samples
Resemblance measure: D1 Euclidean distance

Variables
1 Dissolved Oxygen
2 Temperature
3 log(Carbonate Alkalinity as CaCO3)
4 log(Bicarbonate Alkalinity as CaCO3)
5 log(Total Alkalinity as CaCO3)
6 Dissolved Sulfate as SO4 - Turbidimetric
7 Chloride
8 Dissolved Calcium
9 log(Dissolved Magnesium)
10 Dissolved Sodium
11 Dissolved Potassium
12 log(Dissolved Aluminium)
13 log(Dissolved Arsenic)
14 log(Dissolved Cadmium)
15 log(Dissolved Cobalt)
16 log(Dissolved Copper)
17 log(Dissolved Manganese)
18 log(Dissolved Nickel)
19 log(Dissolved Lead)
20 log(Dissolved Zinc)
21 log(Dissolved Iron)
22 pH
23 Electrical Conductivity (Non Compensated)
24 log(Dissolved Organic Carbon)
25 log(Ammonia as N)
26 log(Nitrite + Nitrate as N)
27 log(Total Kjeldahl Nitrogen as N)
28 log(Total Nitrogen as N)
29 log(Total Phosphorus as P)

Number of variables: 1

No.Vars	Corr.	Selections
1	0.590	5
1	0.525	28
1	0.331	10
1	0.203	2
1	0.191	1
1	0.103	24
1	-0.145	21

Number of variables: 2

No.Vars	Corr.	Selections
2	0.627	1,5
2	0.625	5,28
2	0.618	1,28
2	0.602	2,5
2	0.595	2,28
2	0.540	5,10
2	0.472	1,10
2	0.457	10,28
2	0.421	5,24
2	0.414	2,10
2	0.392	5,21
2	0.347	21,28
2	0.335	1,24
2	0.330	24,28
2	0.260	2,24
2	0.248	10,24
2	0.197	10,21
2	0.172	1,2
2	0.046	21,24

```
       2    0.004 1,21
       2   -0.050 2,21
```

Number of variables: 3

```
No.Vars     Corr. Selections
       3    0.673 1,5,28
       3    0.638 2,5,28
       3    0.590 1,5,10
       3    0.585 1,2,5
       3    0.584 1,2,28
       3    0.573 1,10,28
       3    0.557 1,5,24
       3    0.550 2,5,10
       3    0.539 2,10,28
       3    0.517 5,10,28
       3    0.513 1,24,28
       3    0.499 2,5,24
       3    0.485 5,21,28
       3    0.464 5,24,28
       3    0.463 2,24,28
       3    0.451 1,2,10
       3    0.417 1,21,28
       3    0.408 2,21,28
       3    0.400 5,10,24
       3    0.396 1,10,24
       3    0.373 2,5,21
       3    0.366 1,5,21
       3    0.361 5,10,21
       3    0.353 1,2,24
       3    0.351 10,21,28
       3    0.351 2,10,24
       3    0.343 10,24,28
       3    0.335 5,21,24
       3    0.309 1,10,21
       3    0.297 21,24,28
       3    0.243 2,10,21
       3    0.199 1,21,24
       3    0.186 10,21,24
       3    0.170 2,21,24
       3    0.014 1,2,21
```

Number of variables: 4

```
No.Vars     Corr. Selections
       4    0.649 1,2,5,28
       4    0.633 1,5,10,28
       4    0.596 1,5,24,28
       4    0.584 2,5,10,28
       4    0.566 2,5,24,28
       4    0.554 1,2,10,28
       4    0.548 1,2,5,24
       4    0.545 1,2,5,10
       4    0.540 1,5,21,28
       4    0.535 1,5,10,24
       4    0.519 1,2,24,28
       4    0.517 2,5,21,28
       4    0.487 1,10,24,28
       4    0.481 2,5,10,24
       4    0.475 1,5,10,21
       4    0.459 2,10,24,28
       4    0.459 5,21,24,28
       4    0.453 5,10,21,28
       4    0.452 1,10,21,28
       4    0.426 5,10,24,28
       4    0.424 1,2,10,24
       4    0.417 1,21,24,28
       4    0.416 2,10,21,28
       4    0.407 1,5,21,24
       4    0.404 2,5,10,21
       4    0.401 2,5,21,24
       4    0.396 1,2,21,28
       4    0.389 2,21,24,28
       4    0.358 1,2,5,21
```

4	0.356	5,10,21,24
4	0.329	10,21,24,28
4	0.304	1,10,21,24
4	0.272	2,10,21,24
4	0.259	1,2,10,21
4	0.200	1,2,21,24

Number of variables: 5

No.Vars	Corr.	Selections
5	0.614	1,2,5,10,28
5	0.592	1,2,5,24,28
5	0.556	1,5,10,24,28
5	0.528	1,5,21,24,28
5	0.526	1,2,5,10,24
5	0.523	1,5,10,21,28
5	0.522	2,5,10,21,28
5	0.520	2,5,10,24,28
5	0.518	1,2,10,24,28
5	0.506	1,2,5,21,28
5	0.487	2,5,21,24,28
5	0.459	1,5,10,21,24
5	0.444	1,2,10,21,28
5	0.441	1,2,5,10,21
5	0.440	1,10,21,24,28
5	0.420	2,5,10,21,24
5	0.416	5,10,21,24,28
5	0.412	1,2,21,24,28
5	0.403	1,2,5,21,24
5	0.395	2,10,21,24,28
5	0.331	1,2,10,21,24

Number of variables: 6

No.Vars	Corr.	Selections
6	0.570	1,2,5,10,24,28
6	0.511	1,2,5,10,21,28
6	0.511	1,2,5,21,24,28
6	0.503	1,5,10,21,24,28
6	0.468	2,5,10,21,24,28
6	0.440	1,2,5,10,21,24
6	0.434	1,2,10,21,24,28

Number of variables: 7

No.Vars	Corr.	Selections
7	0.512	1,2,5,10,21,24,28

Global Test

Sample statistic (Rho): 0.673
Significance level of sample statistic: 0.1%
Number of permutations: 999 (Random sample)
Number of permuted statistics greater than or equal to Rho: 0

Best results

No.Vars	Corr.	Selections
3	0.673	1,5,28
4	0.649	1,2,5,28
3	0.638	2,5,28
4	0.633	1,5,10,28
2	0.627	1,5
2	0.625	5,28
2	0.618	1,28
5	0.614	1,2,5,10,28
2	0.602	2,5
4	0.596	1,5,24,28

Appendix D Laboratory water quality results

		Monitoring Sites								Reference Sites				
	Sample Date	29/10/2013	29/10/2013	29/10/2013	29/10/2013	30/10/2013	30/10/2013	30/10/2013	30/10/2013	29/10/2013	29/10/2013	5/11/2013	5/11/2013	5/11/2013
	Site	Point 10	Point 11	Point 12 Deep	Point 12 Shallow	JUTTS	GRQ 18	GROH Shallow	GRO H Deep	GRQ 1	GRUFS	CC1	CC2 Shallow	CC2 Deep
ED037P: Alkalinity by PC Titrator	Units													
Hydroxide Alkalinity as CaCO3	mg/L	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Carbonate Alkalinity as CaCO3	mg/L	138	<1	129	140	126	<1	<1	<1	<1	<1	<1	<1	<1
Bicarbonate Alkalinity as CaCO3	mg/L	878	12	874	919	862	329	289	286	5	3	6	10	10
Total Alkalinity as CaCO3	mg/L	1020	12	1000	1060	988	329	289	286	5	3	6	10	10
ED041G: Sulfate (Turbidimetric)														
Dissolved Sulfate as SO4 - Turbidimetric	mg/L	56	6	54	54	59	34	17	16	7	7	2	2	2
ED045G: Chloride Discrete analyser														
Chloride	mg/L	189	38	188	188	196	246	117	118	39	40	132	77	77
ED093F: Dissolved Major Cations														
Dissolved Calcium	mg/L	3	3	3	3	3	9	4	4	<1	1	6	3	3

Dissolved Magnesium	mg/L	2	2	2	2	2	14	4	4	3	3	11	7	7
Dissolved Sodium	mg/L	606	26	589	597	558	261	194	193	25	24	60	37	37
Dissolved Potassium	mg/L	6	1	6	6	6	4	4	4	<1	<1	3	3	3
EG020F: Dissolved Metals by ICP-MS														
Dissolved Aluminium	mg/L	0.57	0.04	0.48	0.5	0.49	<0.01	0.04	0.03	0.02	0.01	0.02	0.05	0.07
Dissolved Arsenic	mg/L	0.012	<0.001	0.009	0.01	0.012	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Dissolved Cadmium	mg/L	0.0001	<0.0001	0.0001	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Dissolved Cobalt	mg/L	0.011	<0.001	0.01	0.011	0.011	0.02	<0.001	<0.001	<0.001	<0.001	0.002	0.001	0.002
Dissolved Copper	mg/L	0.009	<0.001	0.007	0.008	0.007	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Dissolved Manganese	mg/L	0.019	0.019	0.018	0.018	0.017	0.406	0.014	0.013	0.103	0.124	0.386	0.264	0.262
Dissolved Nickel	mg/L	0.157	0.002	0.147	0.155	0.159	0.069	0.038	0.038	0.001	<0.001	<0.001	<0.001	<0.001
Dissolved Lead	mg/L	0.003	<0.001	0.003	0.003	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Dissolved Zinc	mg/L	0.044	0.007	0.041	0.048	0.064	0.016	0.014	0.006	0.021	0.013	0.017	<0.005	0.006
Dissolved Iron	mg/L	0.18	0.19	0.1	0.1	0.09	<0.05	0.36	0.36	0.19	0.14	1.02	1.08	1.18
EN67 PK: Field Tests														
pH	pH Unit	8.8	7	8.8	8.8	8.9	7.2	8.1	8	6	6.1	5.8	6	6
Electrical Conductivity (Non Compensated)	µS/cm	2560	197	2560	2560	2540	1410	977	969	183	181	465	294	296
EP002: Dissolved Organic														

Carbon (DOC)														
Dissolved Organic Carbon	mg/L	104	5	4	82	6	8	8	9	7	3	8	7	8
Ultra-Trace Nutrients														
Ammonia as N	mg/L	0.056	<0.005	0.035	0.04	<0.005	0.008	<0.005	<0.005	<0.005	<0.005	<0.005	0.005	0.007
Nitrite + Nitrate as N	mg/L	0.383	0.002	0.507	0.746	0.121	0.009	0.02	0.03	<0.002	<0.002	0.014	0.053	0.06
Total Kjeldahl Nitrogen as N	mg/L	1.06	0.12	0.74	0.48	0.93	0.32	0.29	0.28	0.09	0.04	0.17	0.13	0.15
Total Nitrogen as N	mg/L	1.44	0.12	1.25	1.23	1.05	0.33	0.31	0.31	0.09	0.04	0.18	0.18	0.21
Total Phosphorus as P	mg/L	0.014	0.016	0.012	0.01	0.016	0.013	0.007	0.007	0.013	0.012	0.013	0.019	0.023

Appendix E Aquatic Ecology Monitoring Program

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

Anthony Chariton and Sarah Stephenson

21st March 2014

Commercial-in-confidence

Prepared for BHP Billiton, Illawarra Coal Pty Ltd

Contents

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Tables

Executive summary

BHP Billiton Illawarra Coal (BHPBIC) 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 Sustainability, Environment, Water, Population and Communities, now known as the NSW Office of the 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 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.

CSIRO was asked by BHPBIC 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 Brennans Creek/Georges River five discharge monitoring sites with four reference sites; and by examining the relationships between the compositional data and the water quality of the sampled sites. Sequencing produced over 712,000 reads encompassing 818 unique 18S rRNA genes, here on referred to as operational taxonomic units (OTUs). More than ten percent of the OTUs were associated with Bacillariophyta (diatoms), with OTUs from 45 phyla and other coarse taxonomic groups being observed in the survey. Biological replication was sufficient to cover 80 % of the estimated OTUs from each site. Total OTU richness was substantially higher in the discharge monitoring sites, however, the ecological significance of this finding requires consideration as the assemblages would have included organisms derived from the point source and the river, as well as deceased taxa and organisms attached or retained within other organisms. Multivariate analysis clearly showed that the biological communities sampled from the discharge monitoring sites differed from those from the reference sites. Anecdotal evidence also suggests that location of the site within the catchment and its distance from the discharge point may have also contributed to composition. More than 400 OTUs aided in the characterisations of the two treatments (reference and discharge) at the time of sampling, with the literature supporting the observations for several of the 'best' indicator taxa identified for both the reference and discharge monitoring treatments.

The water chemistry from the discharge monitoring sites was complex with a number of variables exceeding ANZECC/ARMCANZ (2000) trigger values. While it was not possible to attribute changes in biological composition to any specific water quality variable, the findings strongly suggests that the discharge is altering the biological composition (as defined by the eukaryotic communities), with this being most evident at Jutts Crossing, Point 10 and Point 12.

1 Introduction

1.1 Background and objectives

BHPBIC proposes to continue its underground mining at Appin and West Cliff mines (collectively referred to as the Bulli Seam Operations), located in the Southern Coalfield of New South Wales, by extracting coal from the Bulli Seam using longwall mining techniques. Project Approval for the Bulli Seam Operations was granted by the Planning Assessment Commission on 22 December, 2011 and by the Department of Sustainability, Environment, Water, Population and Communities under the Commonwealth Environmental Protection and Biodiversity Conservation Act 1999 (EPBC Approval 2010/5350) issued in May 2012. 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 BHPBIC to perform an ecogenomic analysis of the study's eukaryotic communities as an additional line of ecological evidence.

The aim of this study was to use ecogenomics 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 five Brennans Creek/Georges River discharge monitoring sites with the four reference sites; and
- Examining the relationships between the compositional data and the water quality of the sampled sites.

1.2 Ecogenomic 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 that 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, amplicon analysis, or tagged-pyrosequencing. 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 gene can be obtained. A schematic of the workflow required for ecogenomic analysis is provided in Figure 1.

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 ‘pyrosequencing’ 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 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 the Deep Horizon oil spill on benthic communities (Bik et al., 2012).

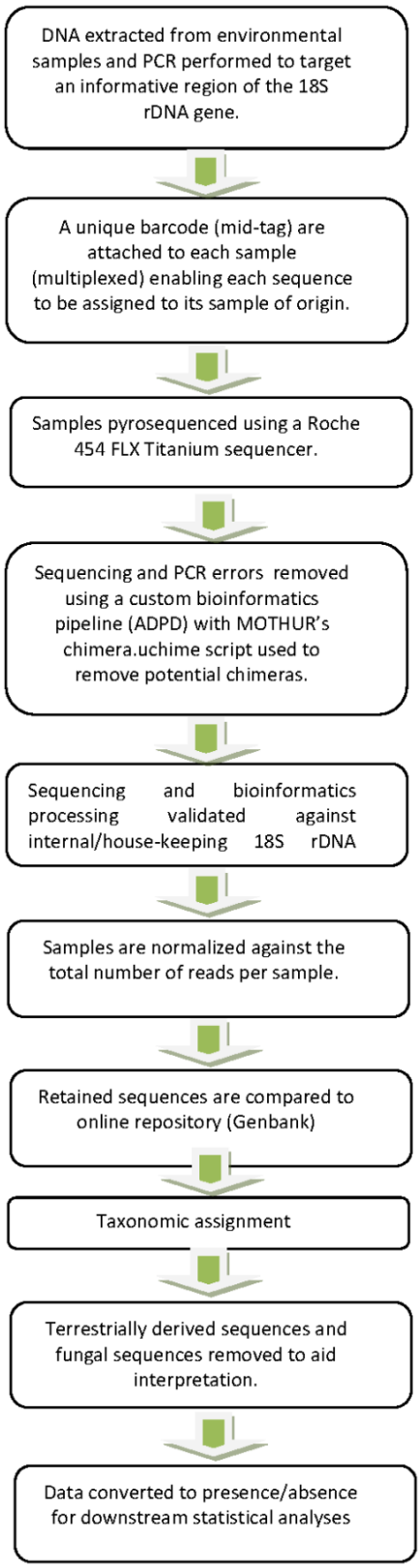

Figure 1. Framework for the collection and analysis of 18S rDNA ecogenomic data

2 Methods

2.1 Sampling design

The study area is located within the upper Georges River Catchment commencing at Site GRQ1 and runs for 21 km to Site GR/OH, just downstream of the confluence with O'Hares Creek (Figure 2). Site GR/OH is located approximately 17.5 kilometres downstream of the West Cliff licensed discharge Point 10 (Table 1). Four sites (Point 12, Jutts Crossing, GRQ18 and GR/OH) were located in pool habitats downstream of licence discharge Point 10, with these five sites referred to as 'discharge monitoring' sites (Figure 3). Four reference sites were also sampled, GRUFS and GRQ1 (upstream Georges River) and CC1, CC2 (Cascade Creek).

2.2 Water chemistry

Measurements for water quality were obtained by BHPBIC. 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 corers (diameter 10 cm). All samples were transferred into clean 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 pre-rinsed for at least 24 h in 5% sodium hypochlorite, and rinsed thoroughly five times with Milli-Q water (Millipore, Academic Water Systems, Australia).

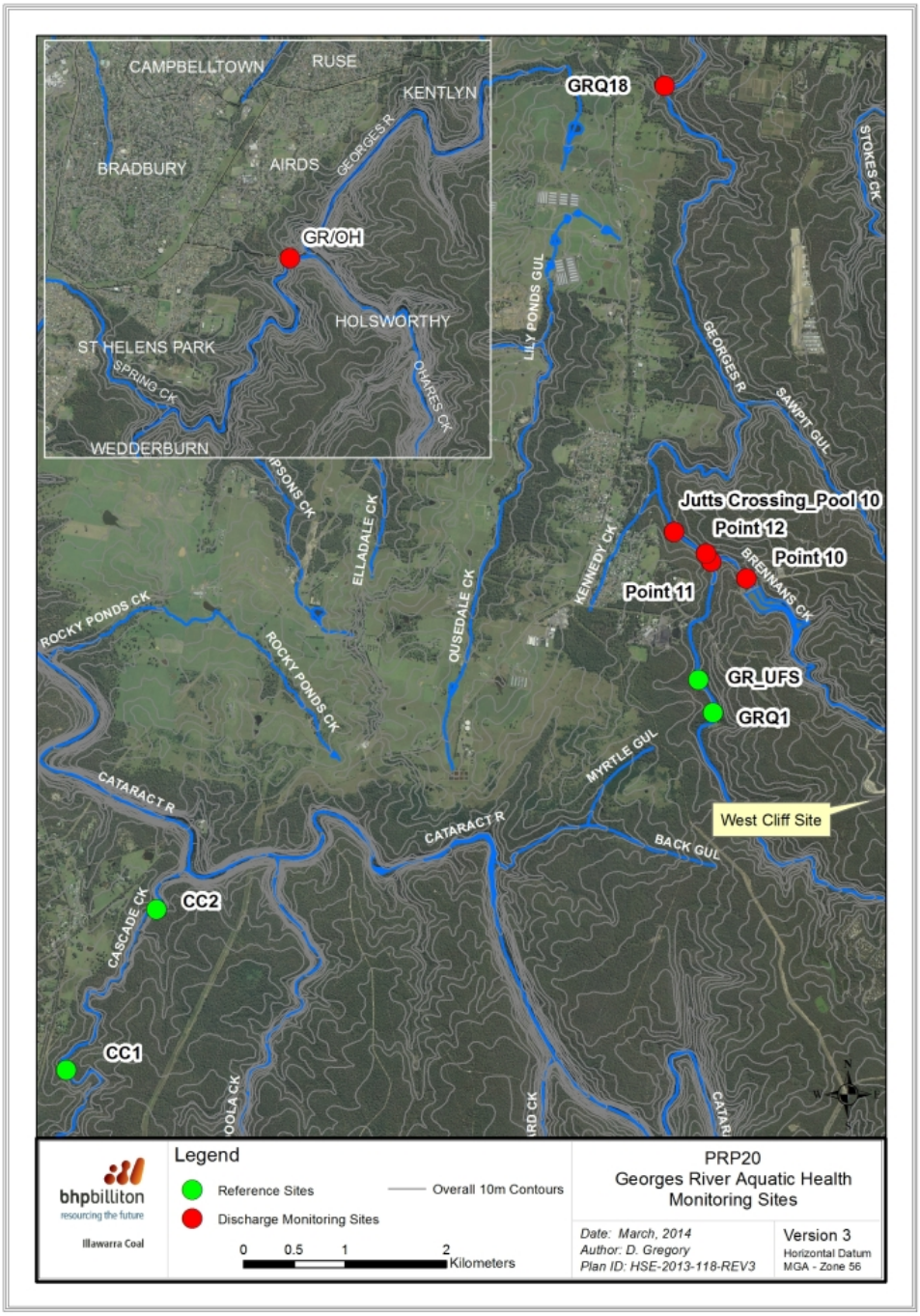

Figure 2. Location of sampling sites. Reference sites = C1, C2, GRQ1 and GR_UFS; discharge monitoring sites = Jutts Crossing, Point 10, Point 12 and GR/OH. Point 11 was not analysed as part of this study.

Table 1. Location of sampling sites and treatment allocations

SITE	STREAM	LOCATION	TREATMENT
GRUFS	Georges River	Upstream of confluence	Reference
GRQ1	Georges river	Upstream of confluence	Reference
CC1	Cascade Creek	Upstream Cascade Creek	Reference
CC2	Cascade Creek	Downstream Cascade Creek	Reference
Point 10	Brennans creek	Discharge point (LDP10)	Discharge monitoring
Point 12	Georges River	Downstream s of Brennans Creek confluence	Discharge monitoring
Jutts crossing	Georges River	At Jutts Crossing	Discharge monitoring
GRQ18	Georges River	Upstream of O'Hares Creek confluence	Discharge monitoring
GR/OH	Georges River	Downstream O'Hares Creek confluence	Discharge monitoring

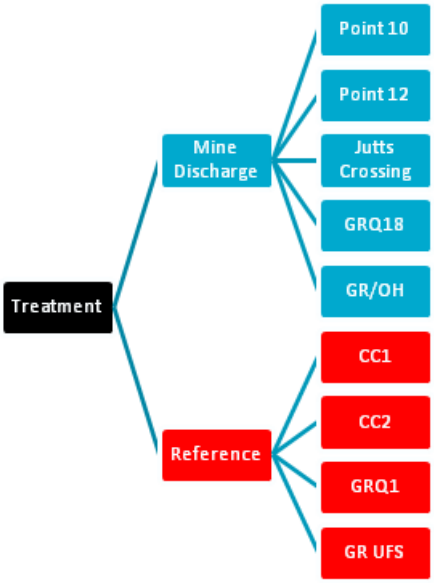

Figure 3. Sampling design: four reference sites and five discharge monitoring sites were sampled from the Upper Georges River with five ecogenomic samples collected at each site

Using 10 g of homogenised sediment, DNA was extracted and purified from each using MoBio PowerMax© Soil isolation kits (MO BIO, Carlsbad, CA) following the manufacturer's protocols. In addition to the sediment samples, five internal reference samples containing a rotifer clone were also processed. Polymerase chain reaction (PCR) amplification of a 200-500-bp fragment of the 18S rRNA gene was carried out with the 'universal' primers All18SF-TGGTGCATGGCCGTTCTTAGT and All18SR-CATCTAAGGGCATCACAGACC (Hardy et al., 2010), using the 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). The products 5'and 3' were labelled with unique 10-nucleotide tags using an addition four cycle PCR (Baldwin et al., 2013), with the labelled products cleaned-up using QIAquick PCR purification kits (Qiagen, Germantown, MD, USA). In preparation for pyrosequencing, the labelled products were mixed in equimolar concentrations, with a final clean-up performed using AMPure XP. Sequencing was performed by the Australian Genome Research Facility using one and half plates of Roche 454 GFLX Titanium (St Lucia, Queensland). Demultiplexing and the removal of potential PCR artefacts, sequencing errors and chimeras sequences were performed using the Amplicon Pyrosequence Denoising Program (ADPD) (Morgan et al., 2013). Taxon identification of each unique sequence, herein referred to as an Operational Taxonomic Unit (OTU), was inferred using the RDP classifier with the SILVA 18S rRNA database (release 113) (www.arb-silva.de/). As a means of obtaining additional taxonomic information, especially where no taxonomic similarities were observed in SILVA, the ten OTUs (which could be assigned to Phylum or below) identified as the best indicators (highest IndVal scores) for each treatment were additionally blasted using GenBank, with the OTUs assigned to their taxonomic groups following the procedures described in Baldwin et al. (2013).

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 all OTU data were expressed as presence or absence prior to computation (Chariton et al., 2014). A species accumulation curve was created to compare the number of observed and expected (Choa2) OTUs sampled across the study. Differences in total MOTU richness and the richness of the dominant taxonomic groups were examined using a two-factor nested ANOVA (treatment and 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). Because of the relatively small samples size, the level of statistical significance was set at p <0.01 for all analyses.

Using the Primer 6+ 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 streams were tested by a two-factor permutational multivariate analysis of variance (PERMANOVA), with 'sites' nested within 'treatment' (reference or mine influenced). Differences between treatments were identified by pairwise *a posteriori* tests based on 9,999 random permutations. Potential indicator OTUs for treatment (reference and discharge) 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 (see Appendix Table A.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, this included variables that represented potential gradients in acidity (pH), conductivity (conductivity), nutrients (nitrate/nitrite, total phosphorus, dissolved organic carbon) and metals (copper). It should be noted that other permutations of the analysis were performed using a range of environmental variables, with similar results occurring when key correlates were replaced, for examplec onductivity with total alkalinity. All metals and nutrients 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 and discharge monitoring sites. A summary of the water quality is provided in Table 2. The 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 and discharge monitoring sites included: total alkalinity (reference sites 6.0 mg/L ± 1.5 mg/L S.E.; monitoring sites 740 mg/L ± 180 mg/L S.E); dissolved sulfate (reference sites 4.5 mg/L ± 1.4 mg/L S.E.; monitoring sites 44 mg/L ± 8 mg/L S.E); chloride (reference sites 72 mg/L ± 22 mg/L S.E.; monitoring sites 187 mg/L ± 21 mg/L S.E); and sodium (reference sites 37 mg/L ± 8 mg/L S.E.; monitoring sites 443 mg/L ± 89 mg/L S.E). The pH at all references sites was within the range (5.8-6.1) lower than expected for lowland rivers (6.5-8.0), with all discharge monitoring sites having a pH greater than that expected for rivers from the region (7.2-8.9). Similarly, the conductivity of the reference sites (183-465 µS/cm) was generally with the expected range (200-300 µS/cm), with the exception being Site CC1, with all monitoring sites exceeding this value (977-2560 µS/cm) by at least three fold.

The default trigger values for dissolved aluminium, copper, nickel and zinc were exceeded in a majority of discharge monitoring sites (ANZECC/ARMCANZ, 2000). No exceedances of dissolved metals were observed at the reference sites. In some cases, nutrient concentrations (total nitrogen, nitrate + nitrite, and ammonia) exceeded default guideline values in the discharge monitoring sites.

Table 2. Summary of water quality measurements[a]

Variable	Default trigger values		Reference Sites				Discharge monitoring				
		Units	GRQ1	GRUFS	CC1	CC2	Point 10	Point 12	Jutts Crossing	GRQ 18	GROH
Total alkalinity as $CaCO_3$		mg/L	5	3	6	10	1020	1060	988	329	289
Dissolved sulfate as SO_4		mg/L	7	7	2	2	56	54	59	34	17
Chloride		mg/L	39	40	132	77	189	188	196	246	117
Calcium		mg/L	<1	1	6	3	3	3	3	9	4
Magnesium		mg/L	3	3	11	7	2	2	2	14	4
Sodium		mg/L	25	24	60	37	606	597	558	261	194
Potassium		mg/L	<1	<1	3	3	6	6	6	4	4
Aluminium	0.055 mg/L (pH>6.5)	mg/L	0.02	0.01	0.02	0.05	**0.57**	**0.5**	**0.49**	<0.01	0.04
Arsenic	0.024 mg/L	mg/L	<0.001	<0.001	<0.001	<0.001	0.012	0.01	0.012	<0.001	<0.001
Cadmium	0.0002 mg/L	mg/L	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	0.0001	<0.0001	<0.0001	<0.0001
Cobalt		mg/L	<0.001	<0.001	0.002	0.001	0.011	0.011	0.011	0.02	<0.001
Copper	0.0014 mg/L	mg/L	<0.001	<0.001	<0.001	<0.001	**0.009**	**0.008**	**0.007**	<0.001	<0.001
Manganese	1.9 mg/L	mg/L	0.103	0.124	0.386	0.264	0.019	0.018	0.017	0.406	0.014
Nickel	0.011 mg/L	mg/L	0.001	<0.001	<0.001	<0.001	**0.157**	**0.155**	**0.159**	**0.069**	**0.038**
Lead	0.0034 mg/L	mg/L	<0.001	<0.001	<0.001	<0.001	0.003	0.003	0.002	<0.001	<0.001
Zinc	0.008 mg/L	mg/L	0.021	0.013	0.017	<0.005	**0.044**	**0.048**	**0.064**	**0.016**	**0.014**
Iron		mg/L	0.19	0.14	1.02	1.08	0.18	0.1	0.09	<0.05	0.36
pH (6.5-8.0)	6.5-8.0 (lowland rivers)	pH Unit	6	6.1	5.8	6	**8.8**	**8.8**	**8.9**	**7.2**	**8.1**
Electrical conductivity	125-2500 (lowland rivers) NSW typically 200-300	µS/cm	183	181	**465**	294	**2560**	**2560**	**2540**	**1410**	**977**
Dissolved organic carbon		mg/L	7	3	8	7	104	82	6	8	8
Ammonia as N	0.013 mg/L	mg/L	<0.005	<0.005	<0.005	0.005	**0.056**	**0.04**	<0.005	0.008	<0.005
Nitrite + nitrate as N	0.015 mg/L	mg/L	<0.002	<0.002	0.014	0.053	**0.383**	**0.746**	**0.121**	0.009	**0.02**
Total Kjeldahl nitrogen as N		mg/L	0.09	0.04	0.17	0.13	1.06	0.48	0.93	0.32	0.29
Total nitrogen as N	0.5 mg/L	mg/L	0.09	0.04	0.18	0.18	**1.44**	**1.23**	**1.05**	0.33	0.31
Total phosphorus as P	0.05 mg/L	mg/L	0.013	0.012	0.013	0.019	0.014	0.01	0.016	0.013	0.007

Tigger values for metals were obtained from ANZECC/ARMCANZ (2000), with the values for physico-chemical stressors being the default values for lowland rivers. Highlighted values indicate measurements which exceeded the default guideline values for 95% level of protection.

3.2 Ecogenomic results

3.2.1 SEQUENCING RESULTS

After the removal of potentially erroneous sequences, the sequence data set contained 712,416 reads, encompassing 818 unique OTUs. Of the 67% of OTUs that could be confidently assigned to a kingdom, the largest proportion belonged to the Bacillariophyta (10%) (Figure 4). Chlorophyta, Ciliophora and Cercozoa each contributed 6-8% to the total taxon richness (Figure 4). As illustrated by the accumulation curve (Figure 5), the 50 replicate samples obtained in the survey were sufficient to capture estimated richness (Chao2) of the sampled sites, with the five replicates obtained at each site capturing approximately 80% of the estimated OTU richness.

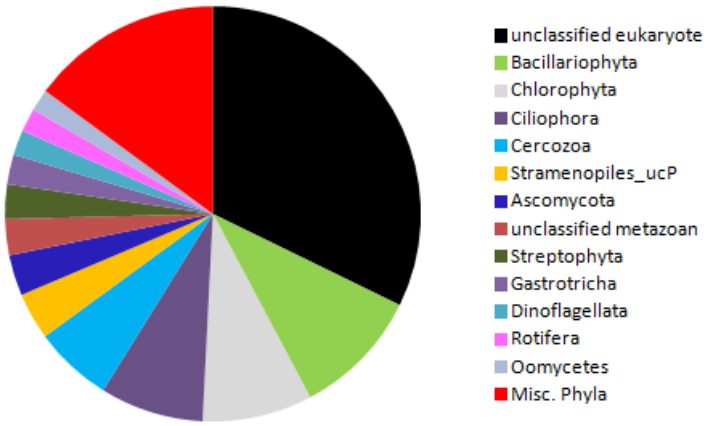

Figure 4. Summary of the OTU data (818 unique OTUs) illustrating the proportion of OTUs associated with each major taxonomic group. To aid interpretation data is aggregated at phylum and above. OTUs that could not be confidently assigned to a taxonomic group are referred to as 'unclassified eukaryotes'. Misc (miscellaneous) phyla encompass all taxonomic groups represented by a small number of OTUs. ucP indicates OTUs that could not assigned to a specific phylum.

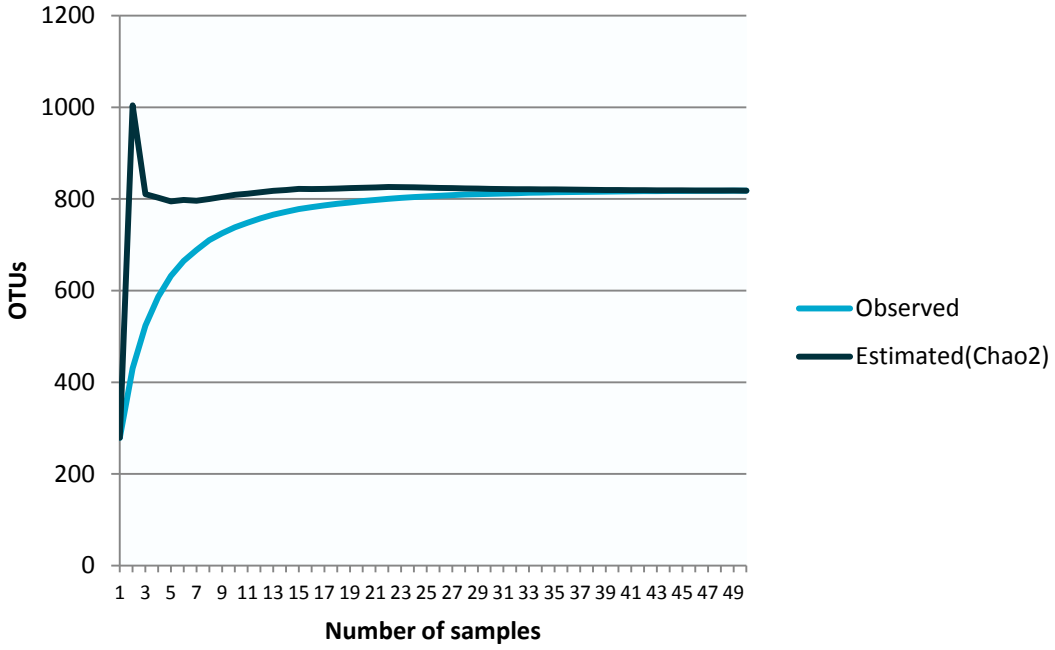

Figure 5. Species accumulation curves illustrating the relationships between the number of samples and the number of observed and estimated (Chao2) OTUs

3.2.2 UNIVARIATE COMPARISONS BETWEN REFERENCE AND MINE INFLUENCED SITES

The number OTUs sampled in each phyla (and higher) at both the reference and monitoring sites is illustrated in Figure 6. The biological communities sampled from both the reference and discharge monitoring sites contained a diverse range of organisms. The richest phyla in the reference sites were Ciliophora, Bacillariophyta and Cercozoa; with Bacillariophyta, Chlorophyta and Ciliophora being the richest in the discharge monitoring sites. Mean total richness was substantially greater in the discharge monitoring sites (339 ± 12.3 S.E.) than the reference sites (194 ± 13.8 S.E.) ($F=87.3$, $p<0.001$), with no differences in richness occurring among the sites within the treatments ($F=2.52$, $p=0.032$). A number of taxa contributed to the higher richness in the mine discharge sites, including a large proportion of unclassified OTUs (no matches in either SILVA or GenBank) ($F= 37.14$, $p<0.001$), as well as OTUs from Bacillariophyta ($F=27.28$, $p=0.001$), Chlorophyta ($F=209.5$, $p<0.001$) and Ciliphora ($F=157.3$, $p<0.001$) (Figure 7).

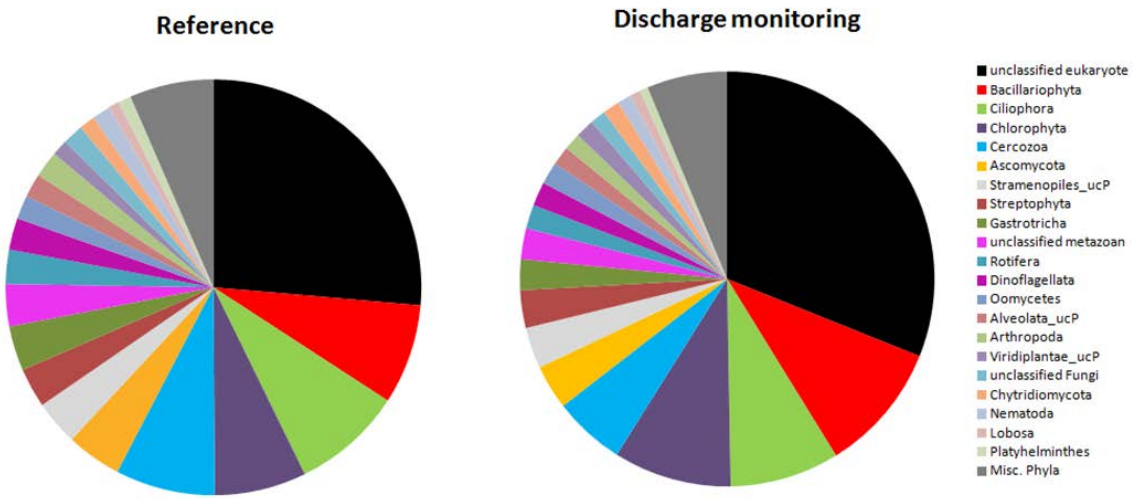

Figure 6. The proportion of OTUs associated with each major taxonomic group from samples obtained from the reference and mining discharge sites. To aid interpretation data are aggregated at phylum and above. Abbreviations are described in Figure 4.

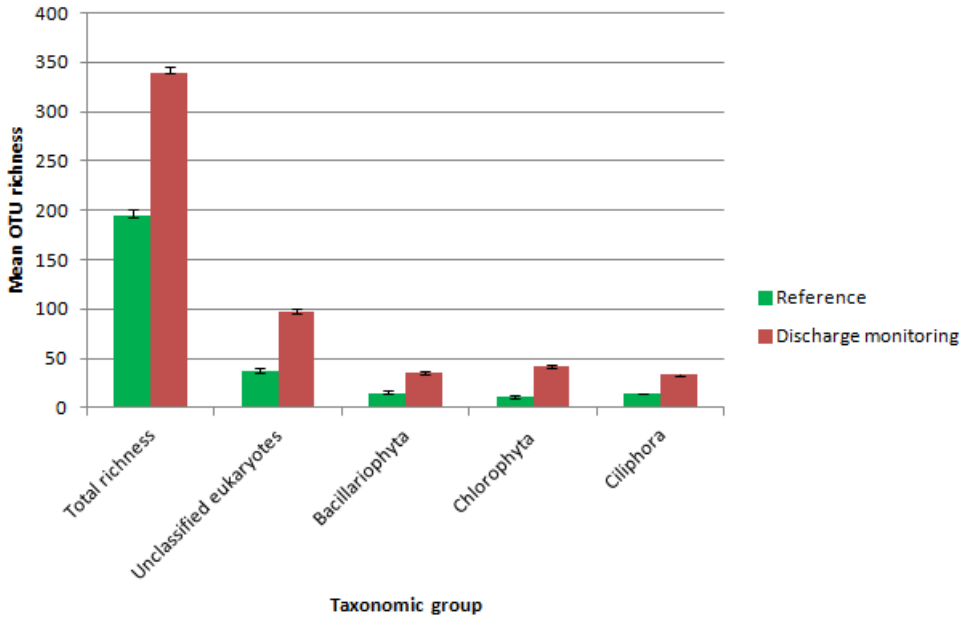

Figure 7. Differences between the reference and discharge mining sites in mean total OTU richness and the OTU richness of the dominant taxonomic groups

3.2.3 MULTIVARIATE COMPARISONS BETWEEN THE REFERENCE AND DISCHARGE SITES

As illustrated in the nMDS ordination plot (Figure 8), there were marked differences in the compositions of the eukaryotic communities sampled from the reference and discharge monitoring sites. This difference was statistically supported by the PERMANOVA (F_{pseudo} =7.65, p=0.009). PERMANOVA also identified a statistically significant difference among sites nested within each treatment (F_{pseudo} =5.13, p=0.001). Qualitative examination of the nMDS ordination plots indicates that there are two clusters of sites within the reference treatment (CC1 and CC2) and (GRQ1 and GRUFS). Similarly, the discharge treatment contained two clusters of sites (Jutts Crossing, Point 10 and Point 12) and (Sites GR/OH and GRQ18), with the dispersion of the sites being more pronounced in the latter.

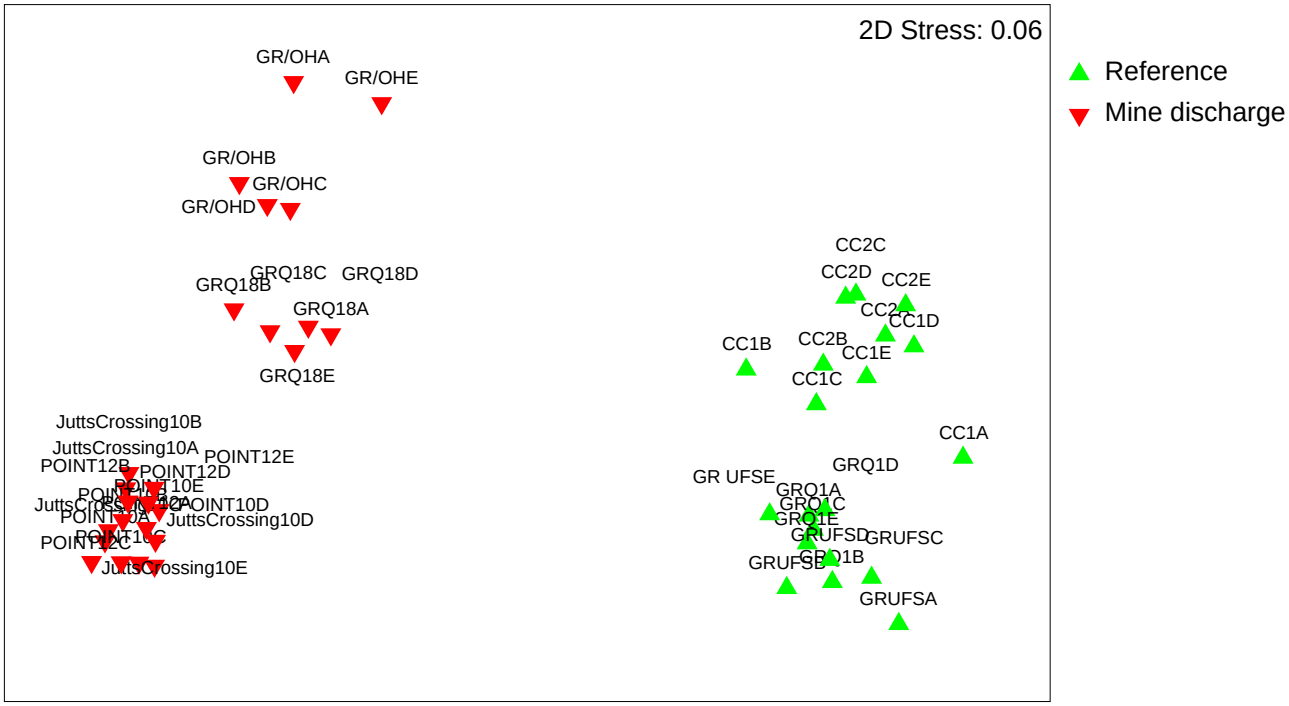

Figure 7. nMDS plot illustrating the similarities and differences in the eukaryl compositions from the reference and mine discharge treatments. Each point represents an individual biological sample.

Indicator analysis indentified 97 OTUs and 323 OTUs as being indicative of the reference and discharge monitoring treatments at the time of sampling, respectively. For both treatments a large proportion of these OTUs were unclassified eukaryotes, however, a large number of OTUs from the phyla Bacillariophyta, Cercozoa, Chlorophyta and Ciliphora were also identified (Figure 9). This was especially the case for the discharge monitoring treatment, possibly reflecting its greater number of potential indicator OTUs. A greater number of potential indicator OTUs from the phyla Arthropoda and Gastrotricha were indentified in the reference treatment than the discharge monitoring treatment.

The best indicator OTUs for the reference sites included diatoms (Bacillariophyta) from the families Eunotiaceae and Pinnulariaceae; and OTUs from Dinophyceae; Chaetonotidae (Gastrotricha), green alga (Ulotrichales), Raphidophyceae, and a foraminiferan (Allogromiidae) (Table 3). The best indicators for the

discharge monitoring sites also included different diatom families (Fragilariaceae, Catenulaceae and Bacillariaceae) from those identified in the reference sites, as well as mite (Hydrozetidae), an alveolate (Colpodellidae), a fungus (Plectosphaerellaceae) and an amoebozoan (Echinamoebidae).

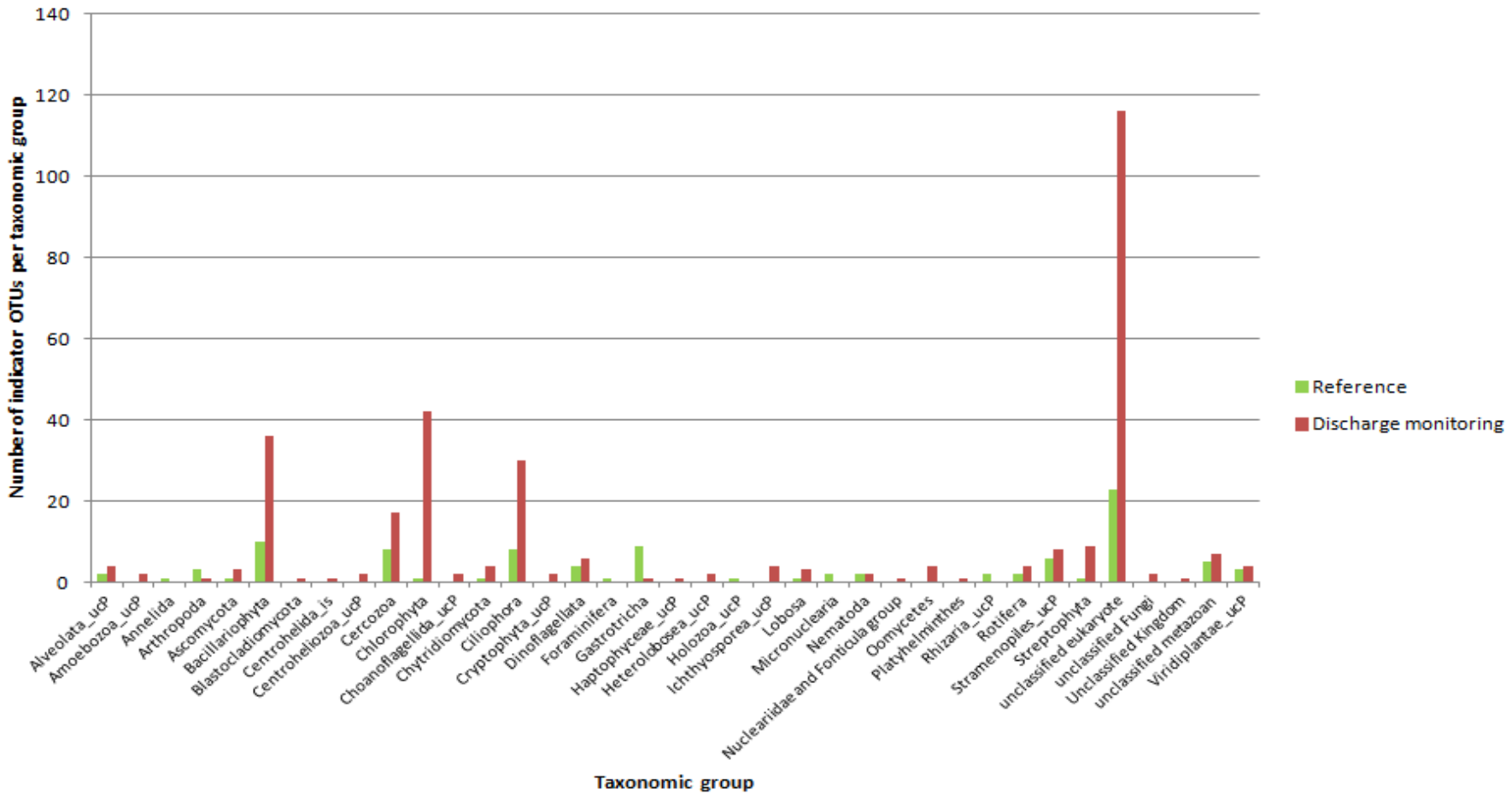

Figure 8. A summary of the Indicator Analysis illustrating the number of OTUs associated with each taxonomic group form both the reference and mine discharge treatments. To aid interpretation data are aggregated at phylum and above.

Table 3. Top ten 'best' (based on Indicator Values) potential indicator OTUs for the reference and discharge treatments [a]

Treatment	OTU	A	B	Indicator value	p.value	Kingdom	Phyla	Class	Order	Family	Genus
Reference	104390	0.96	0.95	0.955	0.001	Stramenopiles	Stramenopiles_ucP	Raphidophyceae	Chattonellales*	Chattonellaceae*	
Reference	10325	0.86	1	0.928	0.001	Rhizaria	Foraminifera	Rotaliida	Allogromida*	Allogromiidae*	
Reference	131567	1.00	0.85	0.922	0.001	Rhizaria	Cercozoa				
Reference	109531	0.83	1	0.913	0.001	Stramenopiles	Bacillariophyta	Bacillariophyceae	Eunotiales	Eunotiaceae	Eunotia
Reference	115369	0.83	1	0.913	0.001	Stramenopiles	Bacillariophyta	Bacillariophyceae	Naviculales	Pinnulariaceae	Pinnularia
Reference	141450	0.83	1	0.913	0.001	Alveolata	Dinoflagellata	Dinophyceae	Gymnodiniales		
Reference	112539	0.81	1	0.898	0.001	Stramenopiles	Bacillariophyta	Bacillariophyceae	Naviculales		
Reference	91975	1.00	0.8	0.894	0.001	Metazoa	Gastrotricha		Chaetonotida	Chaetonotidae	
Reference	173802	0.88	0.9	0.891	0.001	Viridiplantae	Chlorophyta	Ulvophyceae	Ulotrichales*		
Reference	141406	0.88	0.9	0.891	0.001	Alveolata	Dinoflagellata	Dinophyceae			
Discharge monitoring	142631	1.00	0.6	0.775	0.001	Alveolata	Alveolata_ucP			Colpodellidae	Colpodella
Discharge monitoring	10987	0.84	0.52	0.66	0.004	Amoebozoa	Amoebozoa_ucP	Tubulinea	Euamoebida	Echinamoebidae	
Discharge monitoring	90335	0.86	0.6	0.717	0.003	Metazoa	Arthropoda	Arachnida	Oribatida	Hydrozetidae	
Discharge monitoring	66438	0.80	0.6	0.693	0.008	Fungi	Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	
Discharge monitoring	113358	0.95	1	0.976	0.001	Stramenopiles	Bacillariophyta	Coscinodiscophyceae	Fragilariales	Fragilariaceae	
Discharge monitoring	111700	1.00	0.92	0.959	0.001	Stramenopiles	Bacillariophyta	Bacillariophyceae	Thalassiophysales	Catenulaceae	Amphora
Discharge monitoring	111849	1.00	0.88	0.938	0.001	Stramenopiles	Bacillariophyta	Bacillariophyceae	Thalassiophysales	Catenulaceae	Amphora
Discharge monitoring	108987	1.00	0.84	0.917	0.001	Stramenopiles	Bacillariophyta	Bacillariophyceae	Bacillariales	Bacillariaceae	Nitzschia
Discharge monitoring	118171	0.90	0.92	0.911	0.001	Stramenopiles	Bacillariophyta	Coscinodiscophyceae	Fragilariales	Fragilariaceae	Fragilaria
Discharge monitoring	112094	1.00	0.76	0.872	0.001	Stramenopiles	Bacillariophyta	Bacillariophyceae	Bacillariophycidae	Entomoneidaceae	

[a] Only OTUs that could be assigned to Phylum or lower are presented. *Additional taxonomic information was derived from GenBank, all other taxonomic information was obtained from the SILVA database. ucP indicates that the OTU could not be assigned to phylum due to taxonomic resolution or a lack of formal taxonomic classification. A = indicates how well the OTU was represented in the treatment, a value of 1 indicates that the OTU was found in all replicates of the treatment. B= indicates the fidelity of the OTU to a particular treatment, a value of 1 indicates that OTU was only observed in the treatment.

3.2.4 THE RELATIONSHIPS BETWEEN BENTHIC COMMUNITY COMPOSITION AND WATER CHEMISTRY

Constrained analysis using a distance-based linear model found that individual copper, pH and conductivity data all explained significant proportions of the variation of the biological data when examined in isolation (Table 4, marginal tests). However, when examined collectively (sequential tests), only copper and pH were shown to significantly contribute to the observed variation, contributing 43% and 28%, respectively. However, identifying specific water chemistry variables that may be driving the perceived changes in the biologically communities was difficult due to the strong correlations among water quality variables (see Appendix Table 1). For example, as copper concentrations were strongly correlated with total alkalinity, sodium, aluminium, and possibly other constituent e.g. organics that are difficult to measure, all of which were elevated in the mine discharge, it is likely that that it is a cumulative response of these and other variables that is altering composition, rather than any single stressor in isolation. Furthermore, because of the large number of variates measured (compared to sites) and the strong correlations between variables, numerous variables were excluded from the final analysis. While other permutations of the data (not presented) consistently identified pH as a key correlate with biological composition, similar contributions as that shown by copper were observed when this variable was replaced by aluminium, and conductivity with total alkalinity. Consequently, the discharge should be viewed as a mixture, with little weight placed on the ecological ramifications of a single stressor. Based on the positioning of the sites along the dbRDA1 (the horizontal axis) (Figure 10), it can be inferred that the water chemistry is having a more pronounced effect on the biological communities from the discharge sites at Jutts Crossing, Point 10 and Point 12, than those from GRQ18 and GR/OH.

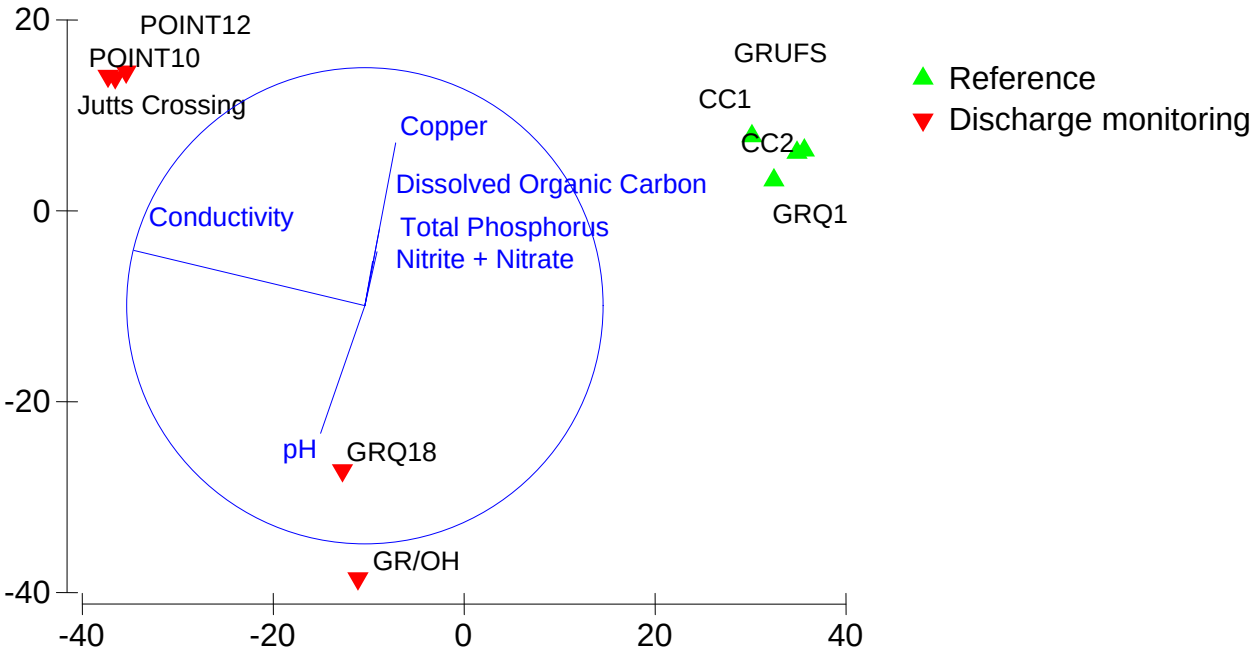

Figure 9. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and benthic composition. Sites are derived from their distances among centroids. dbRDA1 (62% of fitted, 55% of total variation); dbRDA2 (21% of fitted, 19% of total variation).

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

MARGINAL TESTS						
VARIABLE	**SS (TRACE)**	**PSEUDO-F**	**P**	**PROPORTION**		
Copper	6751	5.21	**0.018**	0.427		
pH	8376	7.87	**0.002**	0.529		
Conductivity	8359	7.84	**0.001**	0.528		
Dissolved organic carbon	3993	2.36	0.079	0.252		
Nitrite + nitrate	4287	2.60	**0.013**	0.271		
Total phosphorus	1650	0.815	0.47	0.104		
SEQUENTIAL TESTS						
VARIABLE	**R^2**	**SS (TRACE)**	**PSEUDO-F**	**P**	**PROPORTION**	**CUMULATIVE**
Copper	0.427	6751	5.21	**0.013**	0.427	0.427
pH	0.704	4397	5.64	**0.004**	0.278	0.704
Conductivity	0.781	1218	1.76	0.117	0.077	0.781
Dissolved organic carbon	0.821	628.1	0.887	0.524	0.04	0.821
Nitrite + nitrate	0.835	213.9	0.245	0.834	0.014	0.835
Total phosphorus	0.889	861.2	0.981	0.469	0.054	0.889

[a] 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; sequential 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.

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 (4 reference and 5 discharge monitoring). The following sections focus on comparing the *en masse* benthic community data obtained from reference and discharge monitoring sites, and indentifying 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 Ecogenomic comparisons between reference and monitored sites

The results of this study clearly illustrate the capacity for ecogenomics to capture a wide breadth of biota, with the study obtaining 818 OTUs (unique 18S rRNA genes) from 45 phyla and other high level taxonomic groups. Approximately thirty-three percent of the OTUs could not be confidently assigned to any taxonomic group (unclassified eukaryote). This is not surprising given that the catchment is likely to contain a large number of taxa whose sequences have yet to be deposited into the major online genomic repositories (SILVA and GenBank), with similar proportions of unclassified eukaryotes being observed in other systems, e.g. floodplain soils (Baldwin et al., 2013) and estuarine sediments (Chariton, manuscript in preparation). Species-accumulation curves indicate that there was sufficient depth of coverage, with the estimated number of taxa surveyed being comparable to that sampled. The five replicates used in each site captured approximately eighty percent of the estimated richness, with this being comparable to other ecogenomic monitoring programs (Chariton, unpublished results).

Total OTU richness was substantially greater in the monitoring discharge sites than the reference sites. Traditionally obtained macrobenthic data obtained concurrently with the ecogenomic data indicated that family richness was highest at two of the discharge monitoring sites (GRQ18 and Point 12), while being similar among the other sites (Niche Environment and Heritage, 2014). It is emphasised that the ecogenomic approach 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). The discharge monitoring sites is effectively the pooled contents of two communities, the intrinsic community which naturally resides in the river and the exogenous community derived from the discharge. As such, DNA-derived diversity is likely to be inflated. Previous ecogenomic 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, manuscript in preparation). 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 the reference from the discharge monitoring sites. The biological communities from the discharge monitoring sites clustered into two groups, with the communities located closest to discharge point containing similar compositions (Jutts Crossing, Point 10 and Point 12), while those further away (GR/OH and GRQ18) were relatively more heterogeneous, and more similar to each other than those from the other discharge monitoring sites. Proximity also appeared to influence the composition of the reference sites, with compositions from GRUFS and GRQ1 being more similar to each other than CC1 and CC2. This suggests that the ecogenomic line of evidence was not only able to discriminate the treatment, but also, the location of reference sites within the catchment.

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 phyla, Bacillariophyta (diatoms), chlorophyts (green algae) and ciliophorans (single-celled protozoans), with the largest proportion of these being indicative of the discharge monitoring sites. Some taxa indicative of the reference sites, e.g. gastrotrichs have been shown to be sensitive to environmental change (Evans et al., 1993). Similarly, diatoms from the genus *Eunotia*, while also found in a range of water types, 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. Conversely, diatoms from the genus *Nitzschia*, for which an OTU was identified as being representative of the discharge monitoring sites, has a strong affiliation for brackish or organically polluted waters that are rich in nutrients but poor in oxygen (van Dam et al., 1994).

4.2 Relationships between community structure and water quality

As previously indicated, the water chemistry from the 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 a number of key water quality variables, including those that exceeded guideline trigger values. As illustrated by the dbRDA ordination plot, it was the biological composition of the discharge monitoring sites and not the reference that were strongly correlated with increases in pH and analyte concentrations, with different permutations of environmental variables producing similar results. Collectively, this information indicates that the water quality in the discharge monitoring sites is strongly altering biological composition compared to the reference sites, with this effect being diminished in the discharge monitoring sites furthest away from the point source.

In addition to the effects of discharge water quality there are significant differences between the natural catchment water qualities expected at the discharge sites and the 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 marine 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

The described ecogenomic findings are based on a single sampling event, with this being the first time that the approach has been applied to survey the region. Consequently no definitive trends can be confirmed, however, based on this initial ecogenomic survey the following conclusions are made:

- Metabarcoding of a conserved region of the 18S rRNA gene was able to capture of wide breadth of taxa, with 818 OTUs being observed from 45 phyla and other coarse taxonomic groups.
- Biological replication was sufficient to capture eighty percent of the estimated richness of the sites.
- Total OTU richness was markedly higher in the discharge monitoring sites, however, this is likely an artefact of these sites containing biological material from both the discharge and the river.
- Bacillariophyta, Chlorophyta and Ciliophora richness was greater in the discharge treatment.
- Clear differences in eukaryote composition were observed between the samples taken from the reference and discharge monitoring sites.
- The proximity of sites was reflected in their compositional similarity, i.e. the closer the sites were to each the more similar they were.
- 410 OTUs were shown to aid in the characterisation of the treatments at the time of sampling.
- The literature supported the observations for several of the 'best' indicator taxa for both the reference and discharge monitoring treatments.
- 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).
- The influence of water chemistry on biological composition was more evident in the sites closer to the discharge (Jutts Crossing, Point 10 and Point).

5.2 Recommendations

As illustrated in this report, the ecogenomic technique of 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 the system.

In future sampling runs, we suggest the inclusion of additional genes to provide greater taxonomic depth and certainty for key taxa (e.g diatoms).

Regardless of the line of ecological evidence chosen in future surveys, some consideration is required of the experimental design of the current sampling program. Presently, comparisons are between reference and monitored sites. This approach may be improved using a gradient-based design which focuses on how the discharge alters composition as it is diluted down the system. We suggest that this approach has several advantages, including (i) the number of biological samples will be matched to the number of water quality samples;(ii) a larger number of biological samples would enable more environmental variables to be

included in the analysis (e.g. DistLM); (iii) community and taxa thresholds could be determined for the discharge as a whole; and (iv) the confounding influence of geology would be reduced.

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Appendix A

A.1 Correlation matrix derived from the water quality measurements

	Total Alkalinity (CaCO3)	Dissolved Sulfate	Chloride	Calcium	Magnesium	Sodium	Potassium	Aluminium	Arsenic	Cadmium	Cobalt	Copper	Manganese	Nickel	Lead	Zinc	Iron	pH	Conductivity	DOC	Ammonia	Nitrite + Nitrate	Kjeldahl Nitrogen	Total Nitrogen
Total Alkalinity(CaCO3)																								
Dissolved Sulfate	**0.976**																							
Chloride	0.705	0.778																						
Calcium	0.000	0.099	0.700																					
Magnesium	-0.461	-0.358	0.290	0.861																				
Sodium	**0.997**	**0.982**	0.755	0.073	-0.395																			
Potassium	0.891	0.860	0.835	0.327	-0.144	0.909																		
Aluminium	**0.958**	0.901	0.539	-0.192	-0.577	0.943	0.814																	
Arsenic	**0.955**	0.915	0.557	-0.177	-0.563	0.941	0.804	**0.993**																
Cadmium	-0.750	-0.662	-0.403	0.135	0.428	-0.749	-0.611	-0.786	-0.717															
Cobalt	0.624	0.750	0.917	0.612	0.293	0.672	0.641	0.460	0.485	-0.369														
Copper	**0.958**	0.904	0.550	-0.177	-0.561	**0.946**	0.801	**0.997**	**0.986**	-0.824	0.484													
Manganese	-0.585	-0.499	0.088	0.687	**0.951**	-0.531	-0.317	-0.625	-0.611	0.463	0.136	-0.609												
Nickel	**0.994**	**0.992**	0.755	0.063	-0.396	**0.996**	0.892	0.939	0.945	-0.710	0.694	0.940	-0.527											
Lead	0.940	0.873	0.532	-0.172	-0.547	0.929	0.781	**0.975**	0.947	-0.899	0.472	0.987	-0.593	0.914										
Zinc	0.903	0.892	0.553	-0.152	-0.526	0.885	0.718	0.904	0.938	-0.540	0.491	0.894	-0.589	0.903	0.838									
Iron	-0.522	-0.629	-0.349	0.104	0.371	-0.518	-0.207	-0.386	-0.421	0.299	-0.536	-0.415	0.496	-0.551	-0.403	-0.529								
pH	0.943	0.926	0.686	0.048	-0.460	0.941	0.881	0.844	0.842	-0.626	0.564	0.836	-0.657	0.935	0.812	0.805	-0.567							
Conductivity	**0.988**	**0.985**	0.802	0.146	-0.329	**0.996**	0.926	0.918	0.922	-0.705	0.715	0.920	-0.478	**0.995**	0.898	0.878	-0.514	0.940						
Dissolved Organic Carbor	0.739	0.658	0.416	-0.109	-0.403	0.744	0.618	0.784	0.717	**-0.989**	0.374	0.821	-0.444	0.704	0.886	0.522	-0.276	0.622	0.701					
Ammonia	0.727	0.661	0.442	-0.067	-0.355	0.737	0.612	0.766	0.703	-0.976	0.423	0.805	-0.397	0.700	0.867	0.494	-0.289	0.609	0.697	0.994				
Nitrite + Nitrate	0.780	0.682	0.422	-0.136	-0.439	0.765	0.651	0.785	0.722	-0.923	0.375	0.813	-0.475	0.735	0.888	0.611	-0.294	0.653	0.730	0.861	0.827			
Total Kjeldahl Nitrogen	0.894	0.894	0.667	0.036	-0.381	0.903	0.837	0.896	0.917	-0.583	0.565	0.884	-0.491	0.906	0.818	0.840	-0.393	0.849	0.900	0.635	0.644	0.490		
Total Nitrogen	**0.977**	0.931	0.654	-0.039	-0.466	**0.976**	0.877	**0.980**	**0.965**	-0.832	0.563	**0.984**	-0.559	**0.964**	**0.976**	0.860	-0.408	0.887	**0.958**	0.838	0.828	0.806	0.911	
Total Phosphorus	-0.068	-0.039	-0.033	-0.057	0.142	-0.069	-0.009	0.087	0.118	0.167	0.070	0.054	0.318	-0.028	-0.016	0.018	0.394	-0.260	-0.062	-0.127	-0.081	-0.187	0.141	0.005

[a] Correlations with r>0.95 are in bold.

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EPL 2504

Pollution Reduction Program 20

Aquatic Health Monitoring Program

Condition U3 – PRP 20 – AQUATIC HEALTH MONITORING PROGRAM

1) Prepare Aquatic Health Monitoring Program Plan

The licensee must provide an aquatic health monitoring program plan to the EPA for review and approval. The program must require the monitoring and assessment of the aquatic health of Brennans Creek and the Upper Georges River between 1 September and 30 November (monitoring period) in the years 2013, 2015, 2017 and 2019.
The monitoring program must include, but is not limited to, chemical analysis and in-stream biota assessment, including representative macroinvertebrate, algal and vertebrate species. The monitoring program must be carried out at five or more locations including discharge point 10, discharge point 11, discharge point 12 and the Upper Georges River to the confluence with O'Hares Creek.

5 August 2013

Table of Contents

1 INTRODUCTION

1.1 BACKGROUND

BHP Billiton Illawarra Coal (BHPBIC) proposes to continue its underground mining at Appin and West Cliff mines (referred to as the Bulli Seam Operations or BSO), located in the Southern Coalfield of New South Wales, by extracting coal from the Bulli Seam using longwall mining techniques.

An Environmental Assessment was prepared by Resource Strategies to support the Bulli Seam Operations Project application in consultation with a number of specialist consultants. Project Approval for the Bulli Seam Operations was granted by the Planning Assessment Commission on 22 December 2011 and by the Department of Sustainability, Environment, Water, Population and Communities under the Commonwealth Environmental Protection and Biodiversity Conservation Act 1999 (EPBC Approval 2010/5350) issued on 15 May 2012.

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.

In 2012, the EPA commissioned CSIRO's Dr Graeme Batley to review metal speciation issues associated with waters discharging to Brennans Creek. Batley (2012) advised of improved methods for studying the ecology of waters, rather than macroinvertebrate surveys which only target a minute fraction of the total biodiversity. Illawarra Coal has followed up with the Research Team Leader (Dr Anthony Chariton) from CSIRO regarding their Ecological assessment processes.

This Aquatic Health Monitoring Program includes the following:

- Quantitative sampling of macroinvertebrates conducted in line with previous studies undertaken in PRP6, PRP9 and ACARP C15016 (2010);
- Fish surveys;
- Ecological assessment processes using DNA extracted from sediment samples as per Baldwin *et al.* 2013;
- In-stream water quality testing; and
- Laboratory water testing.

1.2 PURPOSE

This study plan is being developed to meet the aquatic health monitoring requirements of EPL2504 Condition U3 - PRP 20 Aquatic Health Monitoring Plan.

1) Prepare Aquatic Health Monitoring Program Plan

The licensee must provide an aquatic health monitoring program plan to the EPA for review and approval. The program must require the monitoring and assessment of the aquatic health of Brennans Creek and the Upper Georges River between 1 September and 30 November (monitoring period) in the years 2013, 2015, 2017 and 2019.

The monitoring program must include, but is not limited to, chemical analysis and in-stream biota assessment, including representative macroinvertebrate, algal and

vertebrate species. The monitoring program must be carried out at five or more locations including discharge point 10, discharge point 11, discharge point 12 and the Upper Georges River to the confluence with O'Hares Creek.

1.3 AIMS

The aim of the study is to monitor the 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 Ck/Georges River site with reference sites
- Estimate changes over time in the composition and abundance of in-stream and sediment biota; and
- Assessing the downstream gradient changes in composition and abundance of in-stream and sediment biota

We hypothesise that the abundance and composition of aquatic biota will become more similar to the reference sites as Water Projects required by PRP 19 are commissioned.

2 STUDY METHODS

2.1 STUDY AREA AND SITES

The study area is located within the Upper Georges River Catchment (Figure 6), commencing at GR_UFS and runs for 21 kilometres to site GR/OH, just downstream of the confluence with O'Hares Creek. Site GR/OH is located approximately 17.5 kilometres downstream of the West Cliff licensed discharge Point 10.

The sampling design consists of the following:

- One treatment (mine discharge) (Figure 1)
    - o One location for mine discharge (5 sites);
    - o Two reference watercourses (2 sites in each watercourse or 4 replicates as the reference sites will be pooled); and
    - o Point 11 (as required by PRP) – included as a requirement of PRP20, but excluded from the Sampling design for statistical purposes.

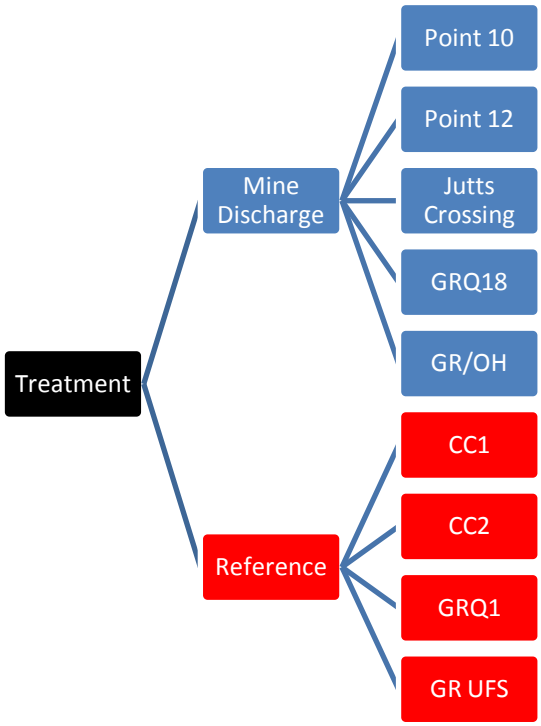

Figure 1: Sampling design.

There are other anthropogenic influences that could potentially confound the effects of mine water discharge from West Cliff Colliery including runoff from local farms and Appin Township and effects of mining subsidence (The Ecology Lab Pty Ltd, 2004 & 2006).). The aquatic monitoring program has been designed to reduce the influence of confounding effects by taking samples at several places well away from the mine discharge point (GRQ18 & GROH), sampling away from localised influences (Appin village runoff and EPA Licensed Waste disposal sites) and assessing the amount of variation between the sites in the Georges River in accordance with recommendations from Quinn and Keough, 2002.

Point 11 will also be sampled in line with the other sites (as required by PRP20); however, this site is likely to be confounded by licensed mine discharge from Appin Colliery (as it is located between the Appin discharge point and the confluence of Brennans Ck with Georges River) and hence, will be excluded from the statistical analysis.

2.1.1 Reference Sites

A number of reference sites have been selected for comparison. These include:

- Upstream Georges River (upstream of any mine water discharge)
- Cascade Ck (within SCA land); and

The two reference locations were chosen on the basis of their similarity to the Georges River in terms of geomorphology and channel form and accessibility for sampling. The sites were also selected as they contain suitable habitat for fish and contain no known barriers for fish colonisation. The reference sites represent similar geology to the Georges River Catchments i.e. a shale capped sandstone gully system (P. Mcmillan *pers.comm*).

Upstream Georges River (2 Sites): GRQ1 and Georges River Upper Flow Station (GR_UFS)

Site descriptions from *Bio-Analysis (2009):*

Both have been selected within the Upper Georges River catchment as they are located upstream of human influences from the Appin East Pit Top, West Cliff Pit Top and Appin village. The river channel at this location is characterised by pools (up to 10 metres wide and approximately 2 metres deep) interspersed by rock bars (Figure 2). Substratum of the stream channel is predominantly bedrock with deposits of sand in deeper areas. The banks of the channel are mostly soft sediment and generally well vegetated by trees (including *Eucalypt spp*. and *Acacia spp*.) and other emergent macrophyte species including Tall Spikerush (*Eleocharis sphacelata*) and *Typha orientalis*. The submerged species of macrophyte, *P. sulcatus*, is relatively abundant.

Figure 2: Site GRQ1

Cascade Creek (2 sites): CC1 & CC2

Cascade Creek is a tributary of the Cataract River, which lies mostly within the SCA area. The stream, which is classified as a 3rd order stream, originates in native bushland approximately 8.5 km south of the Cataract River and joins the river about 350 m upstream of Broughtons Pass Weir.

Site descriptions from *Bio-Analysis (2009):*

Two locations have been selected on Cascade Creek (Figure 3 & Figure 4). At the most upstream sampling location (CC1), the stream consists of a series of relatively shallow (to a depth of approximately 1 m deep) pools that are approximately 0.5 to 8 m wide. The substratum is predominantly bedrock (~ 80%) with boulders (~ 10%) and sand (10%) in areas of low flow. There is no evidence of disturbance at this location. Dominant riparian macrophytes include *L. longifolia*, *Schoenus breviculmis*, *Hypolepis muelleri*, *Juncus prismatocarpus* and *Juncus subsecundas*.

At sampling location CC2, the stream is a series of much broader (up to 55 m wide), deeper (up to approximately 1.5 m deep) pools than at the upstream location, punctuated by chokes of large boulders and, in some places, exposed bedrock. The in-stream habitat is predominantly bedrock, boulder and deposits of sand in areas of low flow. There is no evidence of disturbance at this sampling location. The dominant bank and riparian vegetation includes *L. longifolia*, *Callicoma serratifolia*, Water gum (*Tristaniopsis laurina*) and *Lepidosperma filiforme*. Water visibility is relatively clear and free of sediment.

Figure 3: Site CC1.

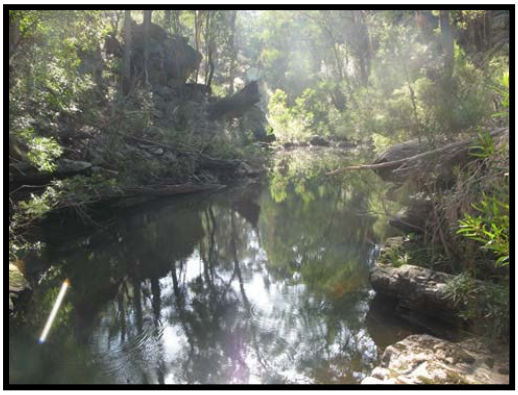

Figure 4: Site CC2.

2.1.2 Sites Potentially Influenced by Mine Discharge

Six sites have been selected within the Geroges River and Brennans Ck including:

GR/OH (Just U/S of the Confluence with O'Hares Ck)	GRQ18	Pool 10 (D/S of Jutts Crossing)
Point 10	Point 11 (PRP)	Point 12

As mentioned previously, site Point 11 will also be sampled in line with the other sites (as required by PRP20); however, this site is likely to be confounded by licensed mine discharge from Appin Colliery (as it is located between the Appin discharge point and the confluence of Brennans Ck with Georges River) and hence, will be excluded from the statistical analysis.

Refer to the table below for a comparison between impact and reference sites.

Table 1: Site comparison table

Treatment	Watercourse	Site Name	Elevation	Estimated pool depth and width	Distance D/S from LDP10 (Where applicable)	Gradient	Substrate
Impact	Brennans Ck	Point 10	To be determined in the field	Up to 0.5m depth Up to 5m width	0 km	18m/km	Predominantly bedrock, boulder and deposits of sand in areas of low flow
	Georges River	Point 11	To be determined in the field	Up to 1.5 m depth Up to 10m width	N/A	18m/km	Predominantly bedrock, boulder and deposits of sand in areas of low flow
	Georges River	Point 12	To be determined in the field	Up to 1m depth Up to 5m width	0.5 km	18m/km	Predominantly bedrock, boulder and deposits of sand in areas of low flow
	Georges River	Jutts crossing (GR 10)	To be determined in the field	Up to 2m depth Up to 20m width	0.9 km	18m/km	Predominantly bedrock with deposits of sand
	Georges River	GRQ18	To be determined in	Up to 1m	7.5 km	18m/km	Predominantly bedrock,

Treatment	Watercourse	Site Name	Elevation	Estimated pool depth and width	Distance D/S from LDP10 (Where applicable)	Gradient	Substrate
			the field	depth Up to 20m Width			boulder and deposits of sand in areas of low flow
	Georges River	GR/OH (just upstream of the confluence)	To be determined in the field	Depth to be determined in the field Up to 15m width	17.5 km	18m/km	Predominantly bedrock, boulder and deposits of sand in areas of low flow
Reference	Cascade Ck	CC 1	To be determined in the field	Up to 1m depth 0.5 to 1m wide	N/A	40m/km	Predominantly bedrock (~ 80%) with boulders (~ 10%) and sand (10%) in areas of low flow
	Cascade Ck	CC 2	To be determined in the field	Up to 1.5m depth Up to 55m width	N/A	40m/km	Predominantly bedrock, boulder and deposits of sand in areas of low flow
	Georges River	GR 1	To be determined in the field	2m depth 10m width	N/A	18m/km	Predominantly bedrock with deposits of sand
	Georges River	GR/UFS	To be determined in the field	1m depth Up to 3m width	N/A	18m/km	Predominantly bedrock with deposits of sand

2.2 SAMPLING METHODOLOGY

2.2.1 Water Quality

Field Water Quality Measurements

Duplicate measures of water quality indicators will be taken near the surface and at the bottom of the pool at each site if water parameters deviate by >10%. Field parameters will include water temperature, electrical conductivity, pH, dissolved oxygen, turbidity.

Laboratory Water Quality

The following chemical parameters have been selected to align with potential toxicants and stressors and the Environmental Protection Licence 2504 conditions. This analysis will be undertaken in conjunction with fauna and algae sampling and field water quality measurements:

- pH and electrical conductivity;
- Major cations: calcium (Ca) magnesium (Mg), potassium (K) and sodium (Na);
- Major anions: chloride (Cl), sulfate (SO4), bicarbonate alkalinity and total alkalinity (T. Alk.);
- Filtered metals: aluminium (Al), arsenic (As), cadmium (Cd), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), nickel (Ni), and zinc (Zn); and
- Filtered ultra-trace nutrients: ammonia (NH3-N), nitrate and nitrite nitrogen (NOx-N), total Kjeldahl nitrogen (TKN), total nitrogen (TN), total phosphorous and dissolved organic carbon (DOC).

As mentioned above, duplicate samples will be taken near the surface and at the bottom of each pool where field parameters deviate by >10%.

2.2.2 CSIRO Method for Invertebrates and Algae (From Baldwin *et al.* 2013)

The proposed invertebrate and algal monitoring will cover a greater range of taxa and increase our ability to assess the overall ecological condition of the environment.

CSIRO Land and Water will collect five sediment samples from each of the nominated sites using the protocol described in Baldwin *et al.* 2013, DNA will be extracted and purified from 10g of sediment, with a hypervariable region of the 18S rDNA gene amplified using PCR. All samples, plus three internal controls, will be labelled with fusion primers and MID tags for high throughput sequencing. Sequencing will be performed on ½ plate of Roche 454 FLX or using similar coverage on another platform.

Subsequent to sequencing, the data will be processed using CSIRO's custom pipeline (ADPD) (Baldwin *et al.* 2013). Follow-up analysis will be performed to examine the relationships between the ecogenomic data and the water quality data concurrently obtained.

The CSIRO conducted ecological assessments by pyrosequencing eukaryotic ribosomal DNA within Sydney Harbour, Parramatta River and Lane Cove River (Chariton et al. 2010).

2.2.3 Quantitative Macroinvertebrate Sampling

At least 3 samples will be collected from each pool to represent the different substrates. A suction sampler (Figure 5) described by Brooks (1994) will be placed over the substrate and operated for one minute at each sampling location. The sample is washed thoroughly over a 500-µm mesh sieve. All material retained on the 500-µm mesh sieve is preserved in 70% ethanol for laboratory sorting. This method has been used extensively by NSW Office of Water.

Figure 5: Suction sampler.

2.2.4 Fish

Fish assemblages in the study area will be compared to fish assemblages recorded in reference streams. The comparison to reference streams is to account for natural variation fish assemblages (e.g. changes from drought/flood). Reference sites will be surveyed simultaneously to the discharge sites.

A review of the monitoring data at the completion of the first years sampling (as per standard adaptive monitoring design) will be critical to ensure the monitoring design is 'powerful' enough to detect changes in fish assemblages over time, should those changes occur.

The method of fish capture would include a combination of backpack electrofishing and bait trapping. T Back pack electrofishing would be used at each site unless the sampling at the site is unsafe. The method would be conducted in accordance with Australian Code of Electrofishing practice (1997).

Four bait traps would be set for two hours at each site. The samples would be checked in 15 minute intervals.

2.2.5 Sampling time

Sampling will occur between 1 September and 30 November in the years 2013, 2015, 2017 and 2019 (in line with EPL 2504). Reference and mine discharge sites will be sampled during the same period.

2.2.6 Statistical Methods

Water quality will be examined by comparison to the limits outlined in PRP 19.

Providing the data meet the relevant assumptions, the study proposes the following for all of the monitoring programs described in this document:

Univariate data

Permutational ANOVAs (PERMANOVA – Primer) will be used to test for differences in total abundance and richness between treatments (mine discharge vs reference). Mean plots of univariate data will also be provided.

Multivariate data

Multivariate analyses will be used to compare taxon composition between mine discharge and reference sites. Analysis of Similarities (ANOSIM) will identify whether the overall composition between treatments is significant. Ordinations of captures using Multidimensional scaling (MDS) will provide graphical interpretation of the separation. To identify which taxon account for the observed assemblage difference, the SIMPER procedure will be used (Clarke and Gorley, 2001). This procedure examines the contribution each species makes to the average similarity within a group (Clarke and Warwick, 2001). Similar methods will be used to identify spatial differences in composition, abundance and richness between far and near sample sites in the Georges River/Brennans Ck. The relationship between environmental gradients and stream biota will be explored with BIOENV (Primer) where possible.

One-way repeated measures ANOVA will be used to test for temporal differences in abundance and taxon richness between reference and treatment at time 1 (2013 sampling period) and time 2 (2015 sampling period) etc.

The significance level of 0.05 will be used in all analyses as protection against false significant results (type one errors). A lower significance level would have increased the likelihood of making type 2 errors (Quin and Keough 2002; Pallant 2005).

3 REPORTING

For each monitoring period, a report will be prepared detailing the results of the monitoring and assessment for that period.

It is proposed that a sensitivity analysis of the data will be conducted after each monitoring period to determine its ability to detect change and provide recommendations (if required) regarding improvements/changes to the monitoring program. If such a change is detected, discussions with the Ecological consultants, BHPBIC and the EPA will occur to consider the implications of the changes and the course of action that should be taken.

4 CONSULTATION

Illawarra Coal discussed the proposed Aquatic Health Monitoring Program with the Illawarra Coal Community Consultative Committee who supported the monitoring methodology and approach.

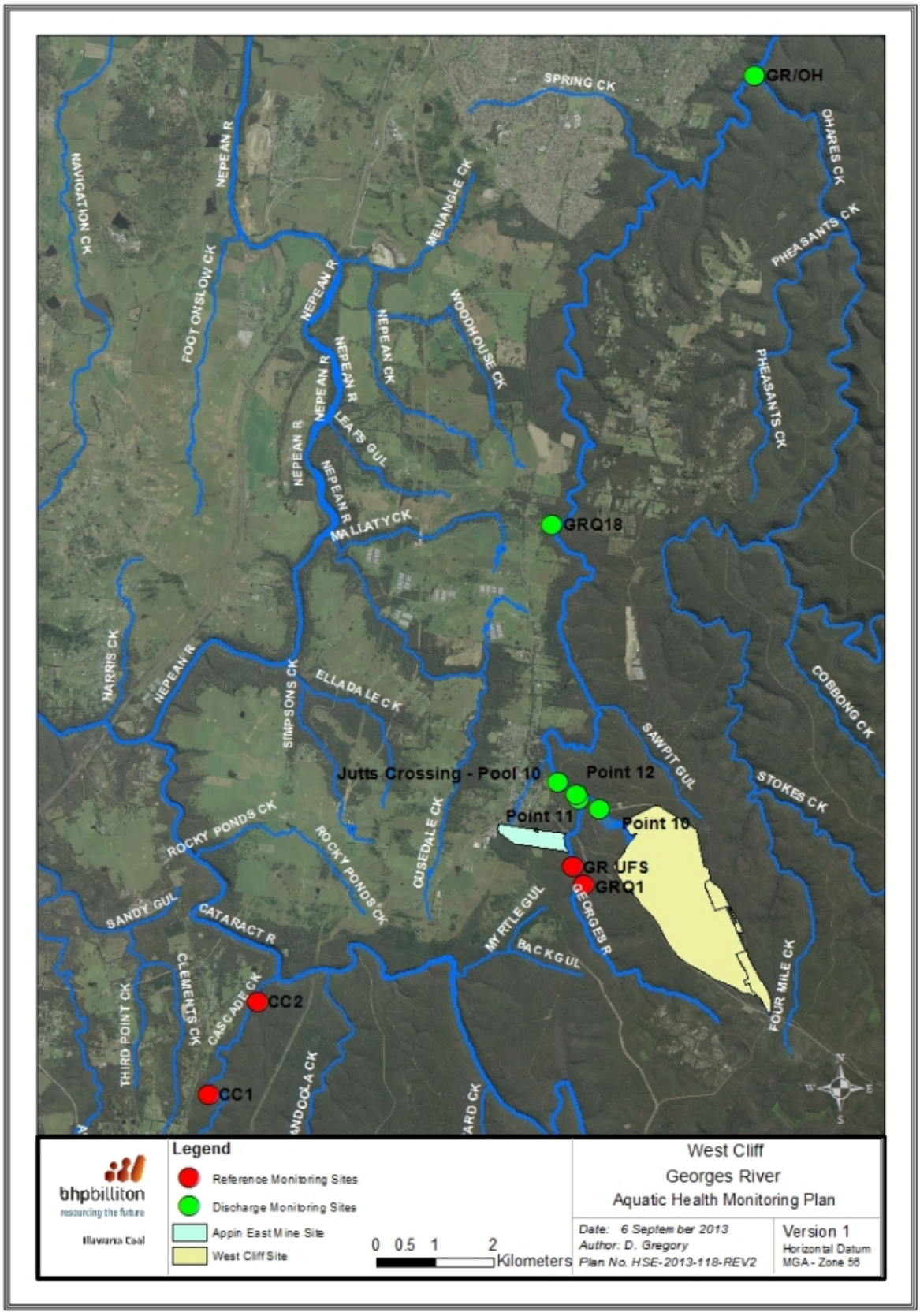

Figure 6: Georges River Aquatic Health Monitoring Locations

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