Australia's National
Science Agency

Georges River Aquatic Health Monitoring Program: Report 2

2023

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6th April 2023

Prepared for: Illawarra Metallurgical Coal /South32

Commercial-in-confidence

CSIRO

Environment Business Unit

Energy Business Unit

Citation

Stephenson SA, Binet MT & Tran-Dinh N (2023) Georges River Aquatic Health Monitoring Program report 2. CSIRO, Australia, EP2023-1066, 125pp.

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Acknowledgments

We acknowledge the Traditional Custodians whose ancestral lands we live and work upon and we pay our respects to their Elders past and present. We acknowledge and respect the deep spiritual connection and the relationship that Aboriginal and Torres Strait Islander people have to Country. We also pay our respects to the Gundangurra, Dharawal and Darug Aboriginal People and the cultural authority of Aboriginal and Torres Strait Islander people and their nations in the Georges River area, as well as those across Australia.

We thank the following: Paul Greenfield for his assistance with bioinformatics and data processing, Dr David Midgley for statistical guidance, graphical data visualisation support, Dr Graeme Batley and Sharon Hook for CSIRO internal report reviews.

Executive summary

The Georges River Aquatic Health Monitoring Program (GRAHMP) follows a multiple lines of evidence approach to assess the water quality and ecological condition of the upper Georges River. Multiple lines of evidence approaches are regarded as robust scientific monitoring programs used by environmental regulators to identify the key environmental disturbance drivers and ecological responses in an ecosystem. Several lines of evidence (LoEs) were used in this study to describe the health of the Georges River catchment downstream of South32 Illawarra Metallurgical Coal's (IMC) discharge into Brennans Creek at Licence Discharge Point 10. LoE's included those to assess for likelihood of impact (ecotoxicology and water chemistry on the discharge waters) and those to assess for observed ecological impact, i.e., changes in community structure and biodiversity (using macrobenthic/macroinvertebrate and gene loci DNA metabarcoding). These LoEs have been used yearly in previous programs (Environment Improvement Programs and Pollution Reduction Programs), since 2013 (previously BHP 2013-2015, South32 2015 - current), to assess for environmental impact of South32/IMCs discharge from LDP10. The monitoring has also been used to identify any changes/improvements to water quality and ecology of the ecosystem over time, as IMC take steps to improve the quality of the discharge into Brennans Creek.

The current GRAHMP study was initially developed to investigate water quality and ecological changes to the Georges River pre and post installation of a long-term water treatment plant (WTP) at LDP40. However, installation of the long-term Appin North WTP (ANWTP) was not complete for the 2022 data presented in this report, so instead, data from the temporary WTP which has been in operation since May 2021, was presented. Therefore, this report presents an additional year of assessment of ecosystem health pre-installation of the long-term WTP, but also includes assessments of toxicity and chemistry of the discharge from the temporary WTP as a means of forecasting water quality improvements once the long-term WTP is operating consistently.

The chemistry data reviewed in this report show that water discharged from LDP40, which was treated with reverse-osmosis (RO) and ultra-filtration had lower conductivity and reduced metal concentrations. However, LDP40 discharge was toxic to *Ceriodaphnia* (reproduction and survival) on three occasions. The LDP40 samples were consistently low in alkalinity, which could pose an ecological risk, and was likely a contributing cause, but not the sole cause of toxicity in the February, May, and November LDP40 samples. However, on occasion, toxicity of LDP40 was higher than would be expected, based on low ionic balance alone. The exact cause of toxicity in the LDP40 samples remains unknown and requires further testing (e.g., toxicity identification evaluation procedures) to identify the cause of toxicity. Overall, based on the ecotoxicity LoE, the discharge from LDP10 was unlikely to cause negative impact to receiving waters. However, LDP40, which is representative of the water quality expected for the intended large-scale WTP had the potential to negatively impact ecosystems in the receiving environment through biological effects on organisms. This is of significant concern, if the future WTP-treated water will form the bulk of the flow in the catchment.

Flow and water level data were included in this program which were used to interpret some of the changes to water quality at each site during the sampling occasions. Water flow and pool levels fluctuated over the program period and reflected the major rainfall events observed in 2022. The ranges of monthly flows through LDP10 ranged from 64 ML to 119 ML and LDP40 ranged from 3 ML to 30 ML in 2022. There was not a clear correlation between water flows, rainfall and metal concentrations observed in the system, but some improvements (reduction in metal concentrations) in the spring 2022 samples were observed.

In general, based on the parameters measured, water quality was poorer at the discharge monitoring sites than at reference sites. Water quality parameters measured at reference sites were mostly within the ANZG (2018) guideline value (GV) ranges, with some exceptions for aluminium and zinc on occasion. The pH values (8.3-8.5) of waters from Point 10 and all downstream sites (7.7-8.7) were higher than those at reference sites (6.0-7.3).

Conductivity remained higher in the discharge monitoring sites compared to the reference sites. Conductivity, pH, copper, and nickel decreased with increasing distance from the discharge source at LDP10 and LDP40. Aluminium was above the guideline value for all sites, including reference sites and on all sampling occasions in 2022. Nitrogen and zinc concentrations were variable across the sites and sampling occasions. Alkalinity was much lower (orders of magnitude) in the reference sites (4-32 mg/L) compared with the discharge monitoring sites (152-765 mg/L) in both sampling occasions. For the discharge monitoring sites, alkalinity decreased with distance from the discharge source.

All macrobenthic surveys found marked differences in community structure between reference and discharge monitoring sites, with water chemistry explaining a vast majority of the total variation in the ecological data. In particular, pH was shown to be a key correlate of macrobenthic, prokaryote, eukaryote and diatom micro-eukaryote communities. This suggests that the discharge waters may be altering the catchment's aquatic biotic communities, with this effect being more pronounced at the most upstream sites following LDP10 and LDP40 (Points 10, 12 and Jutts). The use of SIGNAL, a program designed to focus the analysis on the sensitivity of macrobenthic taxa to varying ecological conditions, was also examined. SIGNAL scores overall have improved slightly in 2022. There were differences in SIGNAL scores between the reference and discharge monitoring sites. The difference in SIGNAL scores indicate a lower level of ecological integrity at the discharge monitoring sites when compared with the reference sites.

The metabarcoding for the sampling occasions spring 2020, spring 2021 and spring 2022 all showed that at the Operational Taxonomic Unit (OTU) level, community composition differed between reference sites and discharge monitoring sites for prokaryotes, eukaryotes and diatoms. These observations were supported by statistical analyses which presented evidence of the differences between reference and discharge monitoring community structure and showed a correlation with water quality changes. The main driving water quality factors which contributed most to the variation in all the metabarcoded communities were pH and, on some occasions, also aluminium, copper, total nitrogen, and alkalinity.

The metabarcoding analysis assisted in identifying potential biological indicator taxa which were representative of the treatments, i.e., more abundant in either reference or discharge monitoring sites. From all the metabarcoded communities studied, diatoms and bacteria showed sensitivity and stronger community shifts in response to the water quality.

The data presented in this report highlight some changes to water quality and ecological community composition, which could be a result of natural variability or increased rainfall and flows through the entire catchment system. With the bulk of the discharge source through the system remaining as Brennans Creek Dam discharge, through LDP10 (79-93% of total discharge), into site Point 10, for this report, the program remains in a stage of pre-implementation of long-term larger scale WTP. The bulk of discharge from LDP10 in 2022 reflects the consistent results with previous years which show significant differences between reference sites and discharge monitoring biological communities.

1 Introduction

1.1 Program requirements

The aim of the Georges River Aquatic Health Monitoring Program (GRAHMP) is to investigate changes in water quality in the upper Georges River via an ecological and chemical evidence-based approach, specifically investigating changes with implementation of a reverse osmosis (RO) Water Treatment Plant (WTP).

In April 2019, the EPA issued Illawarra Metallurgical Coal (IMC) with a Notification of Intention (NoI) to make licence changes to provide greater certainty as to whether the desired water quality outcomes had been achieved, address the ongoing delays in environmental improvements and to provide data for greater public involvement in the regulatory decision-making process. IMC reviewed measures that could be undertaken to meet the proposed water quality concentration limits in the NoI and made a commitment to the EPA to progress the proposed improvements.

The EPA issued a Notice of Variation to Environmental Protection Licence (EPL) 2504 in March 2020. The EPA revoked the Georges River Environment Improvement Program (EIP2) and attached Special Condition E1.1 to the EPL requiring the installation and operation of a Water Treatment Plant (WTP) at Appin North by 31 March 2021 to meet revised water quality concentration limits (detailed in Condition E1.1, Table 1). Further variations to EPL 2504 were issued by the EPA, with commissioning of the long-term WTP with process water commencing in January 2023 and process proving completed in March 2023. . The EPA specified concentration limits (Table 1) that the WTP must be designed to meet, and they required the development of an aquatic health monitoring program to verify improvements to the aquatic health of the Georges River. The Notice of Variation issued in March 2021 also included the new Licenced Discharge Point,LDP40, that will be used to monitor compliance with the water quality concentration limits associated with the discharge from the upgraded, larger scale, long-term ANWTP.

1.2 EPL requirements

The Georges River Aquatic Health Monitoring Program (GRAHMP) is a requirement of EPL 2504, Special Condition E3.1 which states: The licensee must prepare an aquatic health monitoring program to verify improvements to the aquatic health of the Georges River following commissioning of the reverse osmosis water treatment plant required by condition E1.1.

The monitoring must include:

- quantitative sampling of macroinvertebrates;
- ecological assessment processed using DNA extracted from sediment (as appropriate);
- in-stream water quality assessment;
- laboratory ecotoxicology and chemistry water testing; and
- pool level and flow monitoring.

The Appin North Water Treatment Plant (ANWTP) was due to be commissioned in November 2021 but due to COVID-19 impacts and operational delays the ANWTP was delayed. A temporary WTP was commissioned in May 2021 which discharged via LDP40 into the Brennans creek adjacent to LDP10. The temporary WTP was in operation during the 2022 sampling for this report 2. It is expected that the long-term ANWTP will treat sufficient water to produce 1.5 ML of water per day on average as opposed to the current temporary WTP plant being less than 1 ML/day. The pool that both LDP40 and LDP10 discharge into is referred to as Point 10 when referring to the macroinvertebrate and eDNA monitoring within this report.

Table 1. Contaminant concentration limits for LDP40 - EPL 2504 Condition E1.1

Analyte/ contaminant	Unit of measure	100 Percentile
pH	pH	6.5-8.5
Electrical Conductivity	µS/cm	495
Bicarbonate Alkalinity (as $CaCO_3$)	mg/L	185
Aluminium	µg/L	55
Cobalt	µg/L	1.4
Copper	µg/L	1.4
Nickel	µg/L	11
Zinc	µg/L	8
Total Nitrogen	µg/L	250

1.3 Objectives of this report

The main aim of the Program is to summarize the changes in biotic (macrobenthic, DNA metabarcoding) and abiotic measurements (chemistry, physicochemical and flow) over time. The Program aims to compare the water quality in the Georges River pre-installation (2020, 2021, and 2022) and post-installation (expected autumn and spring 2023) of the long-term ANWTP. This second report includes data for nine sites within the Georges River catchment prior to installation of the long-term ANWTP and examines the quality of water from a temporary reverse osmosis WTP at Point 40 (LDP40), which mixes with water from LDP10 in the pool immediately below LDP10, at Point 10. The 2023 report examines the abiotic and biotic data obtained for the GRAHMP in two parts. Firstly, it provides focussed detail on the water level (Section 3.1) water chemistry (Section 3.2) ecotoxicology (Section 3.4) and macrobenthic surveys (Section 3.6) from autumn and spring 2022, and the spring 2020, 2021 and 2022 DNA metabarcoding surveys (Section 3.8). Secondly, it provides an overview of the long-term trends (2013-2021) in these parameters, chemistry long-term patterns (Section 3.3), long-term ecotoxicology (Section 3.5) and macrobenthic long-term patterns (Section 3.7). In addition, the report aims to summarise the long-term information within a weight of evidence framework, drawing upon the collective results of the water chemistry, physical properties, water flow, community ecology and ecotoxicological data.

The metabarcoding (DNA-profiling broad eukaryote, prokaryote communities and diatom micro-eukaryotes) survey was included in the weight of evidence program as a component of the biological community structure LoE. The metabarcoding data provides a comprehensive representation of the biological community and hence compliments the other traditional macrobenthic/microscopy – based biological approaches in the GRAHMP.

To aid comparisons, in accordance with the experimental design previously used for EIP2 (Chariton and Stephenson, 2018, 2020), the macrobenthic and metabarcoding data were examined as two statistical treatments, reference and discharge monitoring:

(i) Reference sites – 3 sites prior to the mine’s influence; and

(ii) Discharge monitoring sites – 6 sites which capture the gradient from the discharge.

The entire 4-year program (2020-2024) aims to test the hypothesis:

> *There will be an improvement to water quality and ecotoxicity in pools downstream of the discharge into Brennans Creek, following the commencement of operation of the ANWTP. There will be a gradual increase in the abundance of contaminant-sensitive taxa within pools downstream of the discharge into Brennans Creek.*

The aim of the GRAHMP is to verify changes in water quality by:

a) comparing water chemistry in the Georges River before and after commencement of the ANWTP;

b) assessing the ecotoxicity of discharge waters from LDP10 and LDP40;

c) comparing the in-stream and sediment biota of pools downstream of the discharge with reference sites (located upstream of the Brennans Creek confluence);

d) calculating changes over time in the composition of in-stream and sediment biota, particularly downstream of the discharge; and

e) assessing the downstream gradient changes in composition of the in-stream and sediment biota.

1.3.1 2022 surveys

These were examined by:

1. Summarising the water chemistry, water flow and water level measurements obtained in autumn and spring for 2022;
2. Interpreting the 2022 ecotoxicological tests data performed on waters obtained from the discharge pipes (end of pipe sampling) at LDP10 and LDP40;
3. Analysis of macroinvertebrate SIGNAL scores;
4. Exploring compositional patterns (community structure) of in-stream macrobenthic invertebrate communities sampled in autumn and spring;
5. Exploring correlative relationships between water chemistry and macrobenthic communities;
6. Exploring compositional patterns in the metabarcoding data for prokaryote and eukaryote communities; and
7. Exploring correlative relationships between the water chemistry, environmental parameters and metabarcoding data.

1.3.2 Long-term trends (2013-2022)

These were assessed by:

1. Examining long-term patterns in key water quality parameters;
2. Analysing and interpreting long-term patterns in SIGNAL scores. This approach is used to score macrobenthic samples from Australian rivers based on the known sensitivities of specific macrobenthic taxa. SIGNAL predicts that macrobenthic communities with high scores tend to be from sites with low levels of contamination (e.g., increased nutrients and changes in conductivity) and high dissolved oxygen;
3. Analysing the abundance and occurrences of three Leptophlebiidae genera (*Atelophlebia*, *Ulmerophlebia* and *Koornonga*) (2016-2022); and
4. Interpreting the long-term data from ecotoxicological tests performed on waters obtained from the discharge pipes (LDP10) at Point 10 (2013-2022).

2 Methods

2.1 Site locations

The study area is located within the upper Georges River Catchment. It commences at site GRQ1 and continues down to GRQ18 (Figure 1), a distance gradient of approximately 9.3 km. The catchment of the Georges River drains a landmass of nearly 1000 km^2, including parts of 14 local government areas (LGAs) (NSW DPE 2022). The land use in the upper reaches includes a mixture of protected areas including the Dharawal National Park, industrial land use and rural agricultural land use. The IMC Appin East colliery and West Cliff Coal Preparation Plant/Appin North colliery are located within the upper catchment of the Georges River. Water from the Appin North and West Cliff Coal Preparation Plant sites currently discharge site water into Brennans Creek Dam, which flows into Brennans Creek, before reaching the Georges River. The water that is discharged from Brennans Creek Dam consists of flows from Brennans Creek (diverted around the coal wash emplacement area), clean runoff from northern areas of the site, water from IMC site stormwater ponds, diverted water from the water treatment plant, rainfall falling on the Brennans Creek Dam surface, water entrained in coal wash emplaced or water resulting from rainfall infiltration through the coal wash emplacement area. The Georges River catchment land use becomes progressively more urbanised and industrial, moving downstream to Campbelltown and Liverpool LGAs. The entire Georges River catchment is one of Australia’s most urbanised catchments.

In total, the experimental design consisted of nine sampling sites divided into two statistical treatments, reference and discharge monitoring (Figure 1 and Table 2).

These sites are:
Reference sites (3 sites) – GRQ1, GRUFS and Point 11; and
Discharge monitoring sites (6 sites), which capture the gradient from Brennans Creek discharge - Point 10, Point 12, Jutts Crossing (here on referred to as Jutts), Pool 16, Pool 32 and GRQ18.

This report will refer to the three reference sites collectively as the reference treatment and the six discharge monitoring sites collectively as the discharge monitoring treatment.

In addition, sampling was also carried out at end of pipe at LDP40, an addition to the study, which was not initially factored into the experimental design and is a result of delays of the long-term RO WTP completion. A temporary WTP was installed in May 2021 to treat water from underground operations at Appin North using reverse osmosis (RO) and is discharged via a pipe (LDP40) that sits beside the LDP10 discharge pipe (Figure 2), such that both discharges enter and mix in the pool at site Point 10 in Brennans Creek. For the ecotoxicology and associated water chemistry, water was sampled from the end of pipe from LDP10 and LDP40. The pool that both LDP40 and LDP10 discharge into is referred to as Point 10 when referring to the macroinvertebrate and eDNA monitoring within this report. To differentiate the sample locations (end of pipe or pool sampled), herein, Point 10 refers to the pool receiving discharge from LDP10 and LDP40, whereas LDP10 refers to the discharge at end of pipe. At LDP40, water was only ever collected from end of pipe and is referred to throughout this report as LDP40.

Discharge from both LDP40 and LDP10 during the sampling period have fluctuated over time, however, a large proportion of the flow in Brennans Creek, is water from LDP10. The discharge volumes from LDP10 being typically 30-300-fold higher than that from LDP40 (based on data provided in this report). Following commissioning of the long term WTP in March 2023, the long-term WTP will process both waters from Appin North underground operations together with water from the emplacement underdrainage. For the 2022 LDP40 data presented in this report, the LDP40 was composed of water drawn from underground operations only, which was then treated via reverse osmosis and ultrafiltration to produce permeate (Chris Schultz, South32).

The Point 11 reference site may be confounded by licenced mine discharge from Appin East (Point 19) (as it is located between the Appin East discharge point and the confluence of Brennans Creek with the Georges River).

Table 2. Location of sampling sites, treatment allocation and sampling type that occurred at each location

Site code	Stream	Location	Distance from LDP10/ LDP40 (km)	Easting	Northing	Treatment/ Statistical group	Sampling activities
GRQ1	Georges R.	U/S of confluence	1.3	297082	6211446	Reference	Water chemistry, macrobenthos, metabarcoding and water flow/level of the pool ecosystem
GRUFS	Georges R.	U/S of confluence	1	297082	6211771	Reference	Water chemistry, macrobenthos, metabarcoding and water flow/level of the pool ecosystem
Point 11	Brennans Ck	U/S of Brennans and Georges confluence	0.4	297207	6212940	Reference	Water chemistry, macrobenthos, metabarcoding and water flow/level of the pool ecosystem
LDP10	Brennans Ck	Discharge point LDP10, Water from Brennans Creek Dam	0	297558	6212772	Discharge Monitoring	Ecotoxicity and associated water chemistry from water from end of pipe
LDP40	Brennans Ck	ANWTP discharge point, adjacent to LDP10	0	297558	6212772	Additional monitoring	Ecotoxicity testing and associated water chemistry from water from end of pipe, water flow
Point 10	Brennans Ck	Receiving water site which LDP10 and LDP40 flow into	0	297558	6212772	Discharge monitoring	Water chemistry, macrobenthos, metabarcoding and water flow/level of the pool ecosystem
Point 12	Georges R.	D/S of Brennans and Georges confluence	0.5	297157	6213016	Discharge monitoring	Water chemistry, macrobenthos, metabarcoding and water flow/level of the pool ecosystem
Jutts	Georges R.	D/S of Jutts Crossings	1	296844	6213232	Discharge monitoring	Water chemistry, macrobenthos, metabarcoding and water flow/level of the pool ecosystem
Pool 16	Georges R.	D/S of Kennedy Ck	2	296890	6213908	Discharge monitoring	Water chemistry, macrobenthos, metabarcoding and water flow/level of the pool ecosystem
Pool 32	Georges R.	D/S of Sawpit Gully	4	297192	6215029	Discharge monitoring	Water chemistry, macrobenthos, metabarcoding and water flow/level of the pool ecosystem
GRQ18	Georges R.	U/S of O'Hares confluence	8	296748	6217637	Discharge monitoring	Water chemistry, macrobenthos, metabarcoding and water flow/level of the pool ecosystem

U/S - upstream; D/S - downstream

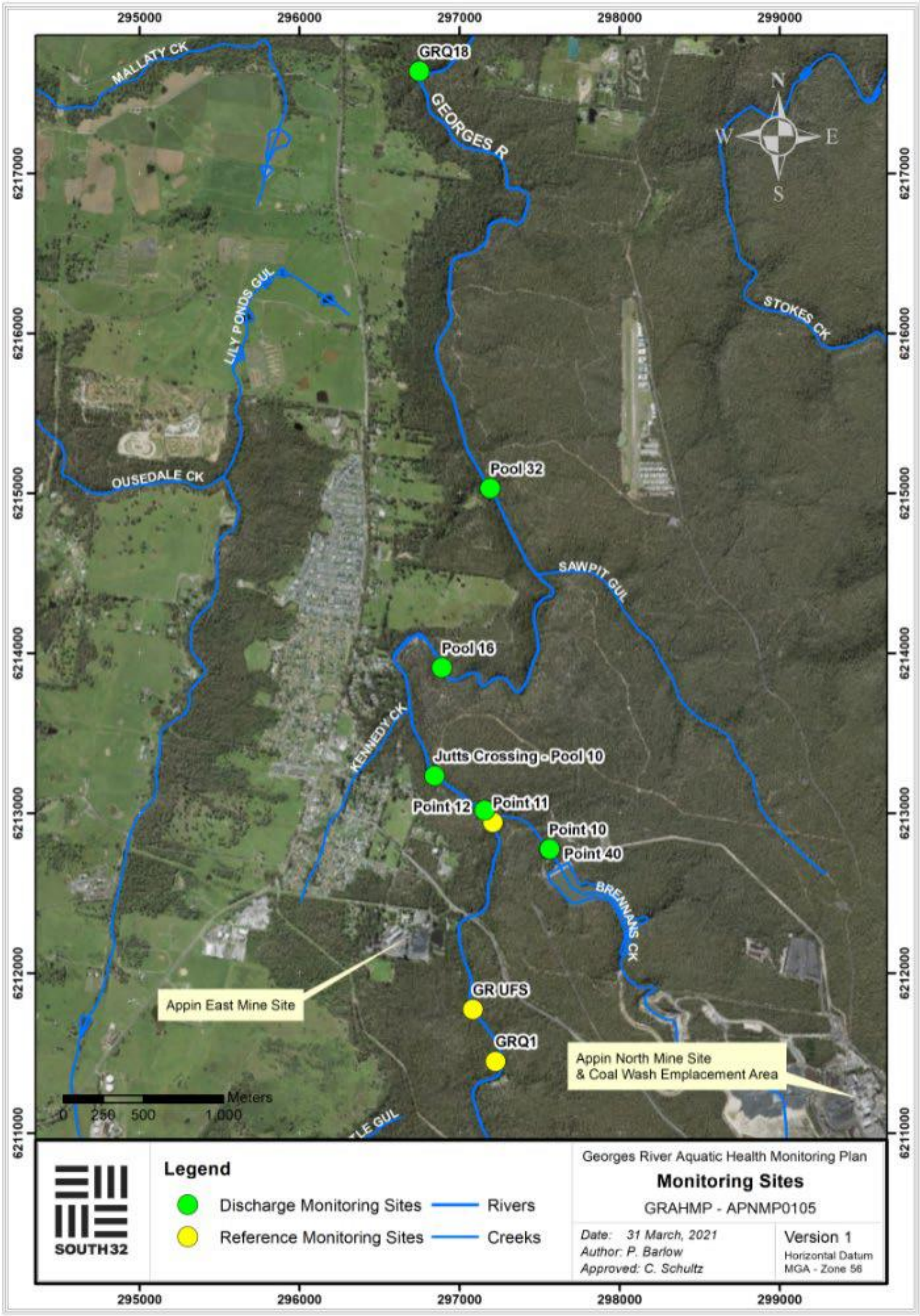

Figure 1. Location of sampling sites

Reference sites = GRQ1, GRUFS (Georges River) and Point 11 (Upstream of Brennans Ck and Georges River confluence); Discharge monitoring sites = Jutts-pool 10, Point 10, Point 12, Pool 16, Pool 32 and GRQ18

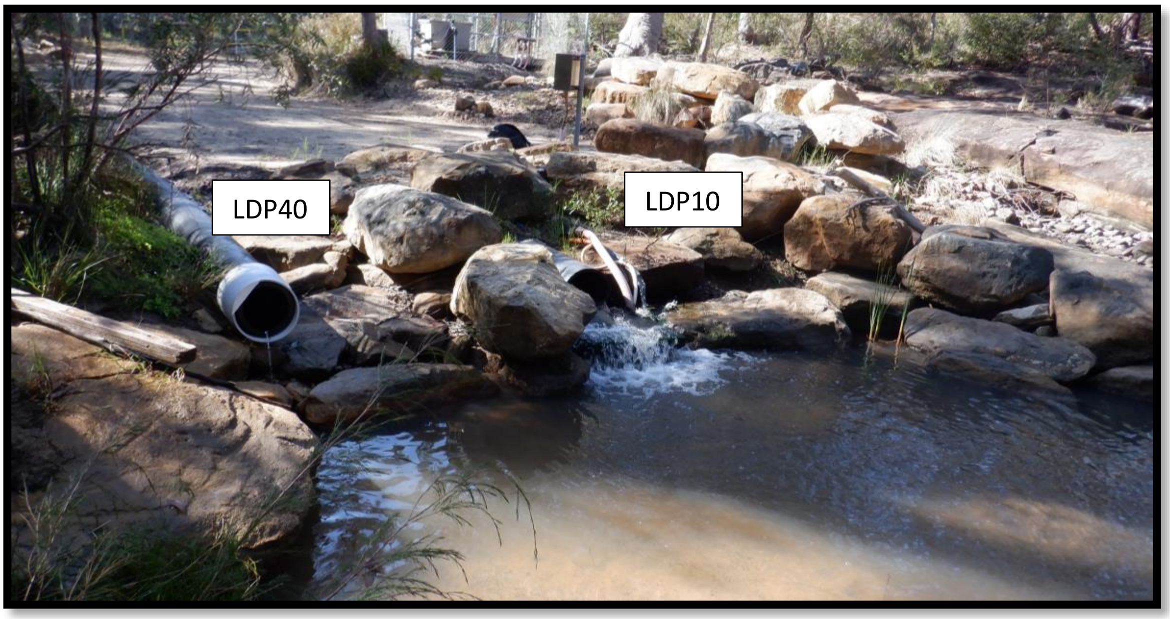

Figure 2. LDP10 and LDP40 discharge pipes flowing into Point 10 site at Brennans Creek

LDP10 and LDP40 samples for ecotoxicity testings and associated water chemistry were collected at end of pipes. Water chemistry samples for macrobenthic analysis were sampled from the pool.

Figure 3. Georges River reference sites

a) GRQ1 the most upstream site of the survey b) GRUFS Upper flow station of the Georges River, upstream of Appin North and Brennans Creek confluence. c) Point 11 reference site upstream of the Brennans Creek and Georges River confluence but downstream of GRQ1 and GRUFS. Photos taken during spring 2022 sampling.

Figure 4. Georges River discharge monitoring sites

a) Point 10: Pool at the discharge point LDP10 b) Point 12: downstream of Brennans Creek and Georges River confluence c) Jutts: downstream of Jutts Crossing d) Pool 16: downstream of Kennedy Creek e) Pool 32: downstream of Sawpit Gully f) GRQ18: furthest downstream site, upstream of O'Hares confluence. Photos taken during spring 2022 sampling except for Pool32 taken in spring 2020.

2.2 Pool water levels and flow monitoring

Flow and level monitoring at some sites was included in 2022 to help interpret ecological, chemical and ecotoxicological findings at each site. In the current study, the following measurements were included: monitoring of pool levels at each site; discharge rates at LDP10, LDP40 and flow rates at the reference site, upper flow station, GRUFS, which was frequently dry during sampling in previous years (2018, 2019).

Pool water levels were monitored at each site by South32 staff using installed pressure sensors and loggers (diver) at each of the pool monitoring sites (Figure 5). Water level data were calibrated to an installed benchmark (in this case a nail), typically a single bolt inserted to the rock-bar or bedrock step (Figure 5). Loggers were housed in PVC pipes bolted to the pool's rock-bar or step (Figure 5). Logging was set to 1-hour intervals to adequately capture fluctuating water levels across the duration of the monitoring program. Due to some extremely high flows through the sampling period in 2022, some site loggers and divers were disturbed or dislodged, and water level data were missing for these sites.

Figure 5. Water level field sampling equipment

Example of equipment at Point 10 showing nail with pink flag tape and logger casing clamped against sandstone pool banks.

Surface flows were monitored by South32 staff using spot flow gauging at GRUFS. A Pygmy flowmeter was used to calculate the discharge during inspections at the site, on a biannual basis. This flow discharge was obtained by measuring the velocity of the water at different points across a known cross-sectional area at GRUFS.

Discharge flows from the two discharge points at Point 10, from LDP10 and LDP40 were recorded over the sampling period. Total discharge including spillway values were measured for Brennans

Creek Dam, LDP10. Daily rainfall (mm) data were also downloaded from Wedderburn, NSW weather station for 2022 (Bureau of Meteorology 2022), to help interpret the pool level data.

2.3 Water chemistry methods

Samples for water quality were collected from the pools of water at each location immediately prior to macrobenthos sampling. A range of water quality parameters, listed in Table 3, were measured either *in-situ* or preserved and sent to Australian Laboratory Services (ALS) for analyses or analysed via both approaches. Field sampling and preservation was carried out as recommended by ALS using sampling containers with nitric acid preservatives provided by ALS.

The sample locations are slightly different here than those where water samples were collected for ecotoxicity testing. These samples were analysed for the same analytes, which were measured in samples collected at end of pipe for the LDP10 and LDP40 pipes listed in Table 3. Therefore, once the WTP started discharging at LDP40 in May 2021, water collected for water chemistry alongside the macrobenthic sampling (described in this section) contained contributions from both LDP10 and LDP40. To differentiate the sample locations (end of pipe or pool sampled), herein, Point 10 refers to the pool following LDP10 and LDP40, whereas LDP10 refers to the discharge at end of pipe. At LDP40, water was only ever collected from end of pipe and is referred to throughout this report as LDP40. Sampling and analysis of these waters was co-ordinated by South32 staff and results were provided to CSIRO for interpretation. Samples for metal analyses were filtered to 0.45 µm in the field by South32 staff.

In-situ measurements of temperature, conductivity, pH, dissolved oxygen, and turbidity (Table 3) were also obtained by South32 staff using a Horiba U51 water quality device. For all analyses examining the relationships between the benthic biota and water chemistry, and for trend analysis of water chemistry, measurements provided by ALS were used in preference to the in-situ measurements, with the exceptions being dissolved oxygen, temperature, conductivity, and pH, for which the in-situ measurements were used when available. Given the large number of water quality variables historically measured, analysis of long-term patterns in water quality (2013-2022) were restricted to a selection of key variables which are included in the current GRAHMP and have historically been shown to be elevated in the discharge waters. These were: conductivity, pH, aluminium, cobalt, copper, nickel, zinc, and total nitrogen.

Table 3. Water chemistry parameters analysed as part of EPL 2540

Contaminant/analyte	Unit of Measure	Analysis	
		In-situ	ALS
pH	pH units	X	X
Dissolved Oxygen	mg/L	X	
Temperature	°C	X	
Electrical Conductivity	µS/cm	X	X
Bicarbonate Alkalinity (as $CaCO_3$)	mg/L		X
Dissolved Aluminium	µg/L		X
Dissolved Cobalt	µg/L		X
Dissolved Copper	µg/L		X
Dissolved Nickel	µg/L		X
Dissolved Zinc	µg/L		X
Total Nitrogen (as N)	mg/L		X
Nitrite + Nitrate (as N)	mg/L		X
Total Kjeldahl Nitrogen (as N)	mg/L		X

Water samples were filtered in the field with 0.45µm filter. Dissolved is <0.45µm.

2.4 Ecotoxicity testing

Samples were collected for both ecotoxicity testing and water chemistry on four quarterly occasions in 2022.

2.4.1 Ecotoxicity tests

Ecotoxicity tests were carried out by Ecotox Services Australia (ESA), Sydney on four quarterly samples (February, May, August, and November) collected from: (i) end of pipe at LDP10, i.e., discharge water from Brennans Creek Dam that flows into Point 10, Brennans Creek and then into the Georges River; (ii) from the discharge pipe from a temporary RO WTP at Appin North (LDP40) that feeds into Point 10, Brennans Creek. Two ecotoxicity tests were carried out with each sample to compare the ecotoxicity of the current licence discharge point waters (LDP10) to the assessment criteria in EPL 2504 (Table 4). LDP40 will ultimately become the licence discharge point following commissioning of the long-term WTP at Appin North, however the future LDP40 will contain water from a larger WTP, processing additional and different source water than the current LDP40 reported here on 2022 data. Water quality from the current smaller volume of water discharged from LDP40 is generally indicative of expected water quality from the future LDP40, however total nitrogen which has often exceeded licence limits from the temporary water treatment plant is expected to improve with the long-term WTP to meet licence limits.

Toxicity tests included the chronic 7-d reproductive impairment test using the cladoceran *Ceriodaphnia* cf *dubia* and the acute 4-d larval imbalance test with *Melenotaenia splendida* (rainbowfish). In addition, the chronic 7-d survival using *C. dubia* was also measured as an additional test endpoint calculated from the 7-d reproductive test data. The *C. dubia* toxicity tests followed the methods of ESA Standard Operating Procedure (SOP) 102 (ESA, 2016a), based on those of the USEPA (2020b) and Bailey et al., (2000). The fish ecotoxicity tests followed the methods described in ESA SOP 117 (ESA, 2016b) and was based on USEPA (2002b) with adaptations for use with the native rainbowfish. A brief summary of the methodology utilised is provided below.

Samples were diluted to 6.3, 13, 25, 50 and 100% with diluted mineral water (DMW; pH 7.9-8.1, conductivity of 171-179 mS/cm) where 100% sample is undiluted. The samples were not filtered or adjusted in any way prior to ecotoxicity testing. For the *C. dubia* tests, ten replicates per concentration of sample were prepared and one neonate (<24 h old) added to each replicate. For the fish tests, four replicates per concentration of sample were prepared and five larval fish added to each replicate. Controls consisting of DMW were also prepared. The pH, conductivity and dissolved oxygen were measured in each dilution and control throughout each test. The test vessels were incubated at 25°C. The number of surviving (unaffected) inoculated *C. dubia* and the number of offspring (newly hatched neonates) per surviving *C. dubia* were counted daily for 7 days (allowing enough time for three broods). The number of unaffected larval fish were counted daily for 96 h. Affected fish were removed and euthanised. The fish test was carried out in compliance with the animal ethics licence (Animal Research Authority CSB V20/10359(3)[1]).

The *C. dubia* tests were renewed daily, however fish larval imbalance test solutions were not renewed, following advice from the ecotoxicity testing laboratory (ESA) that this species is particularly sensitive to handling, and that mortality in controls would occur if renewals were done. This was in line with the EPA requirements that stated fish tests could be done with or without renewals (Table 4). If renewals in this test are desired for future samplings to avoid potential degassing occurring during tests, preliminary experiments would be required to determine the acceptable renewal test conditions for this species. Routine reporting of physico-chemical properties throughout the test period in test reports has been helpful to determine if extensive degassing has occurred. This was requested by CSIRO in November 2021 and was provided by the testing lab for 2022 data.

The concentration to cause 10% effect (decrease) (EC10) on *C. dubia* 7-d reproduction and 7-d survival, and *M. splendida* 96-h larval imbalance were calculated by linear interpolation or log-logit interpolation, while the 50% effect concentrations (EC50s) were calculated using linear interpolation, log-logit interpolation or trimmed Spearman Karber methods. Hypothesis tests were also used to determine the highest concentration (lowest dilution) of sample water tested to have no significant (p£0.05) effect (NOEC) on the test species and endpoints. While hypothesis testing

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[1] Issued by the Animal Care and Ethics Committee of The Secretary, Department of Regional NSW, NSW Department of Primary Industries, valid from 11 May 2021 to 11 May 2022.

to derive NOEC values is no longer recommended (Fox, 2008; Warne and van Dam, 2008), it was included here for comparison to EC10 values that could not be calculated or were unreliable.

Table 4. Ecotoxicity tests and assessment criteria from EPL 2504 for LDP10 and LDP40

Species	Sampling frequency	Sampling method	Assessment criteria
Ceriodaphnia dubia	Quarterly (minimum of 80-day intervals)	Chronic toxicity US EPA Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th Edition (2002), EPA-821-R-02-013.	EC10 Reproduction ≥ 100% sample
Melenotaenia duboulayi or Melenotaenia splendida	Quarterly (minimum of 80-day intervals)	96-hour larval imbalance test with or without water renewal (if with renewal- daily or once at 48 hours). US EPA (2002). Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. 5 ed. EPA-821-R-02012. Washington DC, USA.	EC10 Imbalance ≥ 100% sample

2.4.2 Ecotoxicity tests: long-term assessment (2013-2022)

All ecotoxicity tests were performed by ESA. Between 2013 and 2022, a range of ecotoxicity tests using fish, shrimp, duckweed, cladocerans and microalgae (Table 5) were performed on discharge waters collected from LDP10. However, for the current GRAHMP (EPL 2504), ecotoxicological testing was reduced to two freshwater ecotoxicity tests: a 7-d chronic survival and reproduction test using the cladoceran *C. dubia* and an acute 96-h larval imbalance test with rainbowfish *M. splendida.*

Long-term ecotoxicity comparisons were made with LDP10 samples only because LDP40 was only tested in 2021 and 2022. The EC10 values, the assessment criteria of EPL 2504, were compared. Toxicity was also expressed as toxic units (TUs) for each ecotoxicity test (100 ÷ EC10) to enable direct comparisons between the ecotoxicity data and for presentation in figures. A TU of 1 indicates that the sample is not toxic (EC10 ≥ 100%) and a TU >1 indicates that the sample is toxic (EC10 <100%).

Table 5. Ecotoxicity tests performed on LDP10 waters between 2013-2017

Test organism	Test
Melanotaenia duboulayi (fish)	96-h acute fish imbalance test
Paratya australiensis (shrimp)	10-day acute survival test
Lemna disperma (duckweed)	7-day acute growth inhibition
Ceriodaphnia cf *dubia* (cladoceran)	Partial life cycle 7-day survival
Ceriodaphnia cf *dubia* (cladoceran	Partial life cycle 7-day reproduction
Ceriodaphnia cf *dubia* (cladoceran)	48-h acute survival test
Selenastrum capricornutum (microalga)	72-h chronic algal growth inhibition

2.5 Macrobenthos sampling

On all sampling occasions (spring 2013 - spring 2022) at each site, macroinvertebrates were collected from three to five random pool edges, then combined giving one sample at each site (Downs et al., 2002). The number of sample replicates for each site was increased from three to five in 2018. Pool-edge samples were collected from depths of 0.2-0.5 m within 2 m of the bank. A suction sampler described by Brooks (1994) was placed over the substrate and operated for one minute at each sampling location. The samples were 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 and identification.

Macrobenthic sorting and identification was performed by Niche Environment and Heritage and South32 and provided to CSIRO in a tabulated format. The data were presented at the taxonomic level of Family. In addition, abundances of three potential indicator taxa from *Leptophlebiidae* (*Atelophlebia*, *Ulmerophlebia* and *Koornonga*) were analysed from the data obtained between 2016 and 2022.

For the current GRAHMP report 2, sampling for the macrobenthic surveys was performed in autumn 2022 and spring 2022. Water chemistry samples were collected at the same time as the macrobenthic samples.

2.6 Collection and analysis of DNA samples for metabarcoding

2.6.1 DNA sample collection and processing

The collection of samples for the DNA-based survey (metabarcoding) was performed concurrent to the spring 2022 macrobenthic survey. At each site, five sediment samples were collected from the soft sediment located approximately 1 m from the edge of the water bodies where the water column was approximately 30 to 40 cm deep. All materials used for the collection and storage of DNA samples were soaked for at least 24 h in 1% sodium hypochlorite and rinsed thoroughly five times with Milli-Q water (Millipore, Academic Water Systems, Australia). Surficial sediment samples (top 2 cm) were obtained using a clean shallow polycarbonate shovel/corer (diameter 10 cm). All samples were transferred into DNA-free sterile 50 mL Greiner tubes and placed on ice immediately, then frozen at -80°C within 8 h of collection. Samples were thawed just prior to DNA extraction. Using 10 g of homogenised sediment, DNA was extracted and purified from each using Qiagen DNeasy PowerMax® Soil isolation kits (QIAGEN® Germany) following the manufacturer's protocols.

Three primer sets from two conserved-gene regions were targeted to capture the system's biodiversity. For bacterial communities, the V4 region of the 16SrDNA gene for prokaryotes was amplified (Caporaso et al., 2012). 18S V7 rDNA gene (Hardy et al., 2010) was amplified for broad eukaryotes monitoring; and the 18S V4 (Zimmerman et al., 2011) region was amplified for diatom (Bacillariophyceae) specific eukaryotes. Diatoms were included in the design because many of the potential indicator OTUs from previous reports associated with differences between reference and discharge monitoring sites were diatoms (Chariton and Stephenson, 2018, 2020).

For all 2022 samples, three identical polymerase chain reaction (PCR) plates were amplified for each primer set (18S V7, 18S V4, 16S V4) and the amplicons for the three PCRs were pooled into one library per target primer set. For 18S V7 PCRs, in addition to the sediment DNA samples, reference samples containing sequences of the saltwater crocodile (*Crocodylus porosus)* and a tropical marine cnidarian (*Carukia barnesi*) were also processed in three sample replicates as positive controls. For 16S V4 a synthetic chimeric bacterial control (containing fungal mycorrhizal species, *Cairneyella variabilis*), was processed alongside DNA and for 18S V4 diatoms two marine micro-eukaryote species, *Dunaliella* sp. and *Ulkenia* sp. were processed as PCR positive controls. Negative water controls were included in all PCR experiments to test for biological contamination during amplification. Amplification and purification success were interrogated on a MultiNA gel, MultiNA© (Shimadzhu, Oceania). The three pooled final amplicon library concentrations were measured on the Nanodrop® spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). Target gene libraries of DNA samples from 2020 and 2021 were then prepared with the Illumina Tru-Seq PCR-free library preparation kit and libraries were sequenced over one MiSeq run at 2x 250bp. The Illumina MiSeq sequencing was performed by the Ramaciotti Centre for Genomics, UNSW. The spring 2020 samples were sequenced in February 2021 and the spring 2021 samples were sequenced in January 2022.

2.6.2 Bioinformatics 18S and 16S rDNA

Sequenced data were processed using a custom pipeline (Greenfield Hybrid Amplicon Pipeline (GHAP) which is based around USEARCH tools V11 (Edgar, 2013). The pipeline is available at https://data.csiro.au/dap/landingpage?pid=csiro:26534. GHAP first demultiplexes the sequence reads to produce a pair of files for each sample. These paired reads were then merged, trimmed, de-replicated, and clustered at 97% similarity to generate a set of representative Operational Taxonomic Units (OTUs) sequences which were classified after clustering at 97% similarity in sequences. USearch V11 tools (fastq_mergepairs, derep_fulllength and cluster_otus) (Edgar, 2013) were used for the merging, de-replicating and clustering steps.

For 18S broad eukaryotes and diatoms, the 2022 sequencing data were processed with the previous OTU sequencing data from 2020 and 2021 to ensure consistent OTU assignments and temporal comparisons. For 18S each OTU sequence was classified in two different ways: first, by using the RDP Classifier (v2.10.2) to determine a taxonomic classification for each sequence, down at best to the level of genus; and second, by using ublast to match a representative sequence from each OTU against a curated set of 18S reference sequences derived from the SILVA v138 SSU reference set for the broad eukaryotes V7 dataset (Cole et al., 2014; Quast et al., 2013). This 18S reference set was built by taking all the eukaryote sequences from the SILVA v138 SSU dataset, and removing those sequences found to contain bacterial or chloroplast regions. The SILVA v138 SSU reference set was also used to assign taxonomy to the V4 diatoms 18S dataset. The pipeline then used usearchglobal to map the merged reads from each sample back onto the OTU sequences to obtain accurate read counts for each OTU/sample pairing. The classified OTUs and the counts for each sample were finally used to generate OTU tables in both text and BIOM (v1) file formats, complete with taxonomic classifications, species assignments and counts for each sample. All OTUs with a maximum read abundance of 50 reads, or that were only observed in less than four biological replicates were removed.

For 16S, the latest 2022 sequencing data were processed with the previous 2020 and 2021 sequencing data to ensure consistent OTU assignments. For 16S, representative sequences from each OTU were classified both by finding their closest match in a set of reference 16S sequences, and by using the RDP Naïve Bayesian Classifier. The pipeline used both the RDP 16S Training Set and the RefSeq 16S reference sequence collection for the purposes of species-level classification. The pipeline then mapped the merged reads back onto the classified OTU sequences to obtain accurate read counts for each OTU/sample pairing and generate OTU tables in both text and .biom (v1) formats, complete with taxonomic classifications and species assignments. The OTU tables were then summarised over all taxonomic levels, combining the counts for identified taxa across all OTUs. The pipeline finally classifies all the merged reads using the RDP Classifier, regardless of whether they were assigned to an OTU. This last step is done to provide confidence in the clustering and OTU formation steps by providing an independent view of the community structure.

After processing, and prior to statistical analyses, the data sets were filtered to remove potentially erroneous sequences. For all data sets, the proportion of contamination OTU reads in the positive controls (the max read count that is not the positive control divided by the positive control read count) was determined. The proportion of read counts for each OTU in each sample (the read count for each OTU divided by the total read count for that sample) was determined to identify sequencing leakage. The proportion of contamination was relatively low in all data sets and this value was set as the cut-off for filtering the dataset. If the proportion of read counts for each OTU per sample was less than the proportion of contamination, then those reads were removed from the dataset. After quality control checks were complete, controls were removed from the dataset. Any OTUs that had a match percent of <80 or appeared in less than two samples were also removed. Processed data has been archived in CSIRO’s Data Access Portal (DAP) http://data.csiro.au.

2.7 Statistical analysis

2.7.1 Macrobenthos data

Multivariate statistics on community structure were undertaken using the statistical software package Primer 7+ (Plymouth Marine Laboratory, UK). Prior to multivariate analysis, the macrobenthos data were log10 transformed. Ordinations of the data were performed by non-metric multidimensional scaling (nMDS) using the Bray-Curtis similarity coefficient. Statistical differences between sites were tested by permutational multivariate analysis of variance (PERMANOVA), with differences between sites identified by pairwise a posteriori tests based on 9999 random permutations. The key taxa contributing to significant differences between sites were identified using Primer's SIMPER function, Primer 7+ (Plymouth Marine Laboratory, UK).

The relationships between macrobenthic communities and environmental variables were examined using distance-based linear models (DISTLM) (Legendre and Anderson, 1999). In order to match the number of biological and environmental (physico-chemical) samples, i.e., one sample per site, the similarity matrix for the biological data was recalculated using the distance between centroids for each site derived from the replicate samples. The environmental variables obtained from the monitoring program were both numerous and often strongly correlated, and consequently all highly correlated variables ($r>0.95$) were removed. To reduce over-fitting and to conform to the assumptions of the analysis (number of biological samples > environmental variables), DISTLM was performed using only a limited number of environmental variables. The final variables used in the DISTLM were pH, conductivity, alkalinity, dissolved aluminium, dissolved copper, dissolved nickel, dissolved zinc and total nitrogen. The sequential DISTLM method was used to test the correlation between environmental variables with biological community structure (Plymouth Marine Laboratory, UK). Sequential testing in DISTLM involves testing each predictor variable separately in a stepwise manner to determine its contribution to the model. The analysis starts by testing the predictor variable that has the strongest correlation with the response variable. It is emphasised that these variables provide a summary of the discharge water, and it is not possible to robustly quantify the contribution of each measured variable in isolation. The dbRDA option was selected to provide an ordination of the fitted values from the model.

2.7.2 SIGNAL

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

Comparisons in mean SIGNAL scores between the three reference sites and three of the six discharge monitoring sites (Point 12, Pool 32 and GRQ18) were examined using a one-way ANOVA.

2.7.3 Metabarcoding statistics

Statistical analyses were undertaken using the statistical software package Primer 7+ (Plymouth Marine Laboratory, UK). Univariate attributes of the metabarcoded data for each primer set were obtained using Primer 7's 'Diverse' function. To investigate patterns in community composition (beta diversity) subsampled OTU abundance tables were standardised and transformed to presence and absence for broad 18S V7rDNA and 18S V4rDNA diatoms OTUs and for 16S rDNA data was normalised prior to analysis.

OTUs were assigned to Family for the 16S rDNA dataset, Family for the broad 18S rDNA dataset and Genus for the diatom 18S V4 rDNA dataset. For the 18S and 16S rDNA data, ordination of the OTU data was performed by non-metric multidimensional scaling (nMDS) using the Bray-Curtis similarity coefficient, as was the PERMANOVA analysis. Statistical differences between reference and discharge monitoring sites, and individual sites were tested by a PERMANOVA. The relationships between metabarcoded communities and environmental variables were examined using distance-based linear models (DISTLM) on centroids for sites, as previously described for macroinvertebrate statistical methods.

3 Results and Discussion

3.1 Pool water levels and flow monitoring

3.1.1 Pool water levels (relative level)

There were some issues with the diver relative water level measurements over 2022, mostly due to extremely high flood flows and the diver instruments becoming dislodged from the rocky bank substrate. Due to the variable flow and extreme rainfall events in 2022, the water level data should be interpreted with caution.

Point 12 water level diver was removed in May 2022 due to rainfall. At Point 12, the diver was displaced from a rockbar probably during high rainfall at the end of February and early March 2022 (the highest being ~150mm on the 8th of March). The Point 12 diver was damaged and not reinstalled as the rock bar was not accessible. This explains why the Point 12 data abruptly stop recording in 20th May 2022. Nail readings were not recorded for site GRQ1 for autumn and spring due to a field error. It is expected from South32 reports that water levels may have drifted slightly from true water level. Pool 32 does not contain any solid rockbars that allow the placement of a diver. South32 selected to use water level monitoring in Pool 28 as a proxy for Pool 32 water levels for the GRAHMP. The GRQ18 and GRUFS nails were underwater in autumn 2022. The GRQ18 nail was found under water for spring 2022 water sampling.

The relative water level and rainfall data at each site are shown in Figure 6 to Figure 14. Water levels are relative since they are based on differences in distance from a set point (nail) installed above the water line at each site in March 2021. In addition, 2022 daily rainfall data from Wedderburn station were overlayed onto the pool water-level data. Due to major flood waters and high flows through the catchment in 2022 many of the barometers were impacted and periods of time had missing data as a result for some sites. Despite these limitations, significant rainfall events and subsequent higher water levels were observed in February and March 2022, July 2022 and November 2022. A smaller rainfall event in July also caused a momentary increase in pool levels for most sites. In broad terms, the relative water levels at each of site follow the general pattern of the rainfall data over the time period of recording in 2022. Point 10 (Figure 10) flow showed similar trends to the surrounding sites such as Point 11 and Jutts and followed the pattern of rainfall over the sampling period.

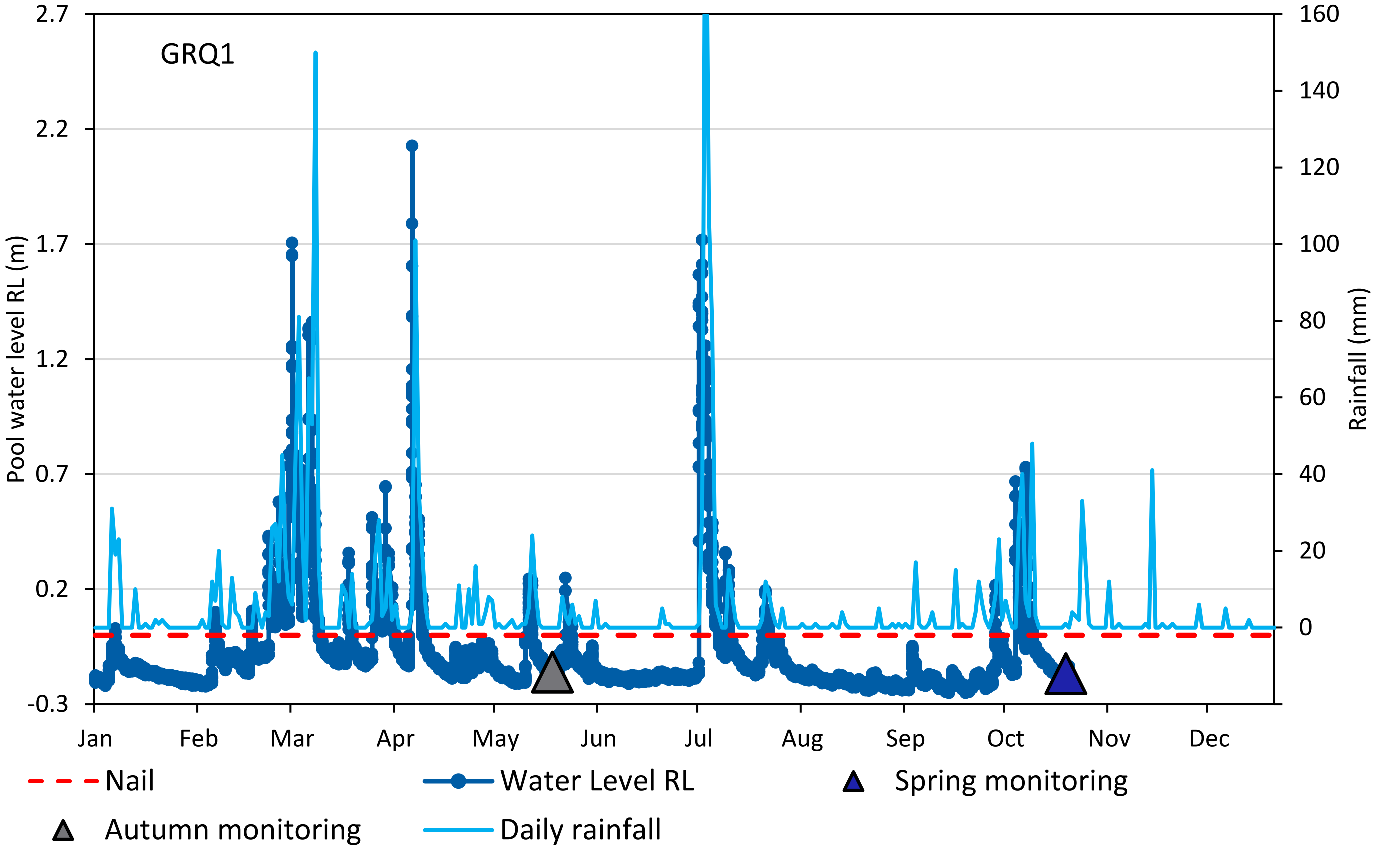


Figure 6. Rainfall and relative (RL) water levels at GRQ1 during 2022

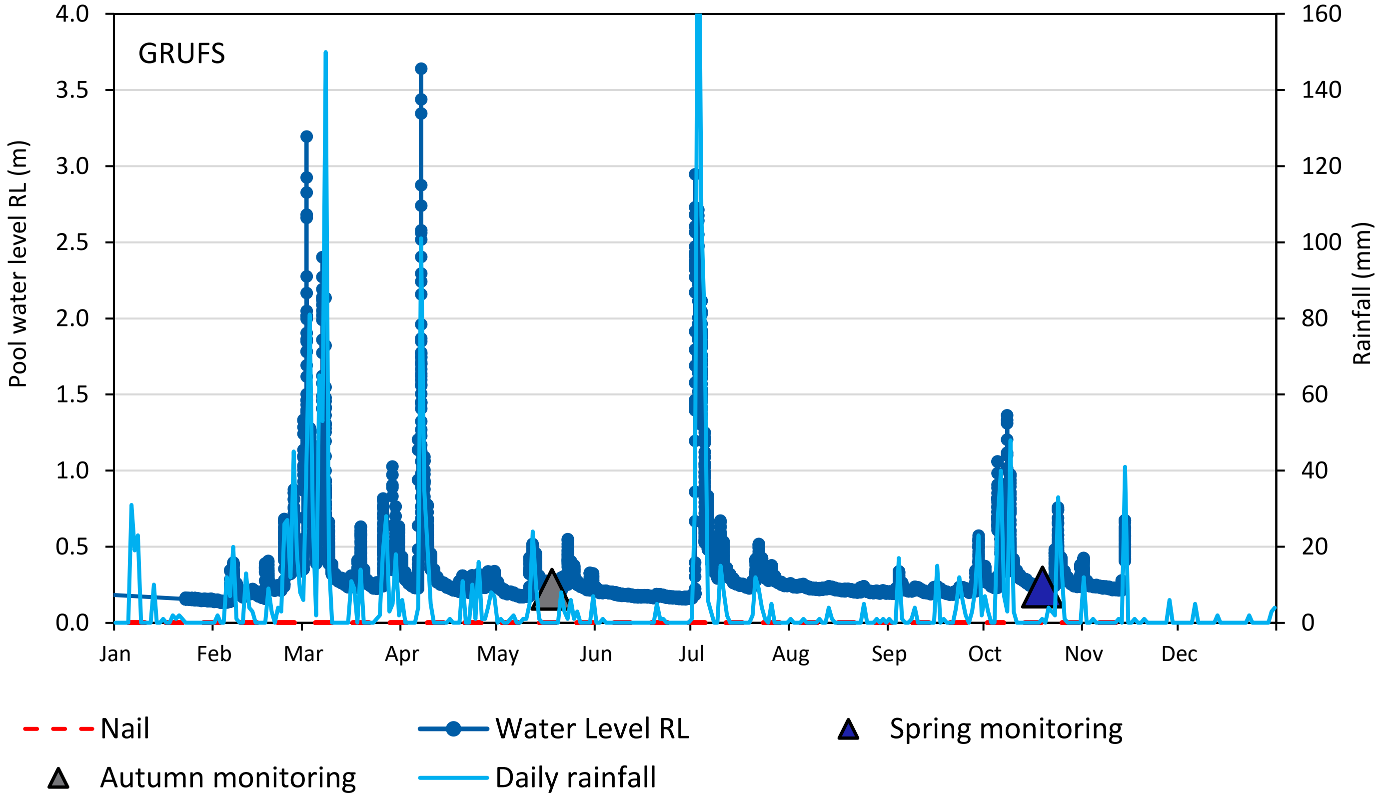


Figure 7. Rainfall and relative (RL) water levels at GRUFS during 2022

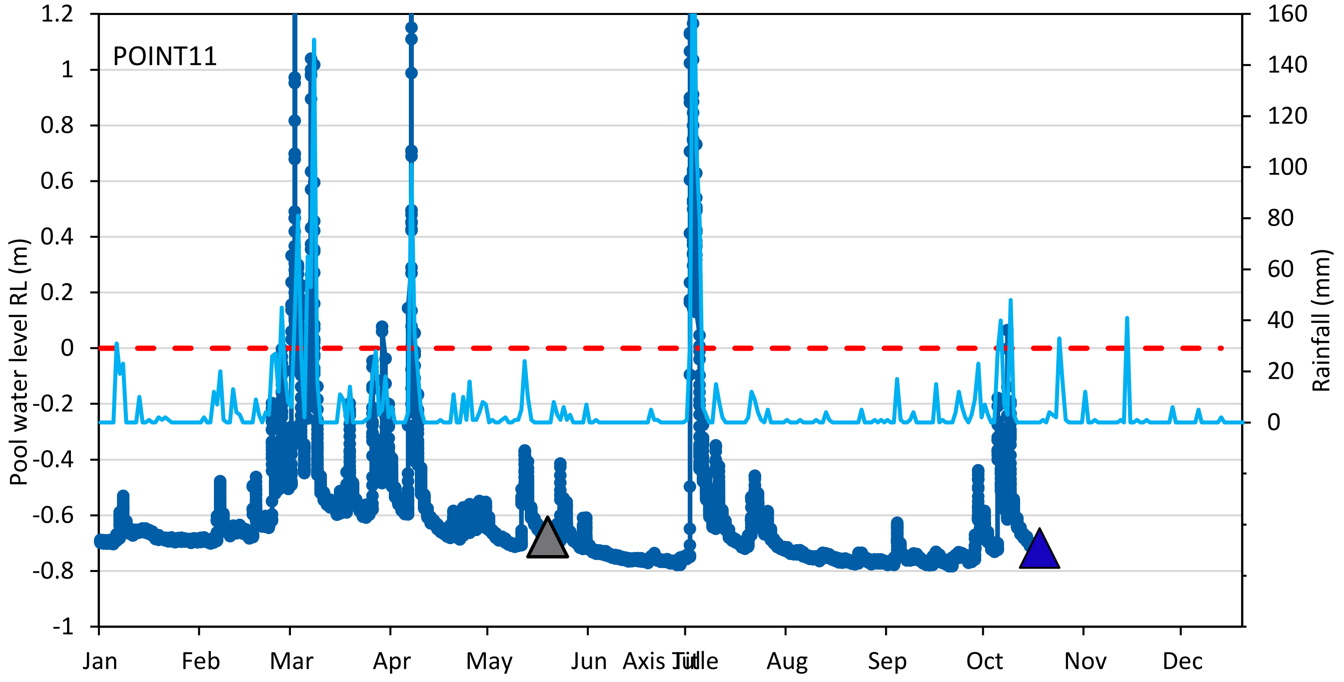


Figure 8. Rainfall and relative (RL) water levels at Point 11 during 2022

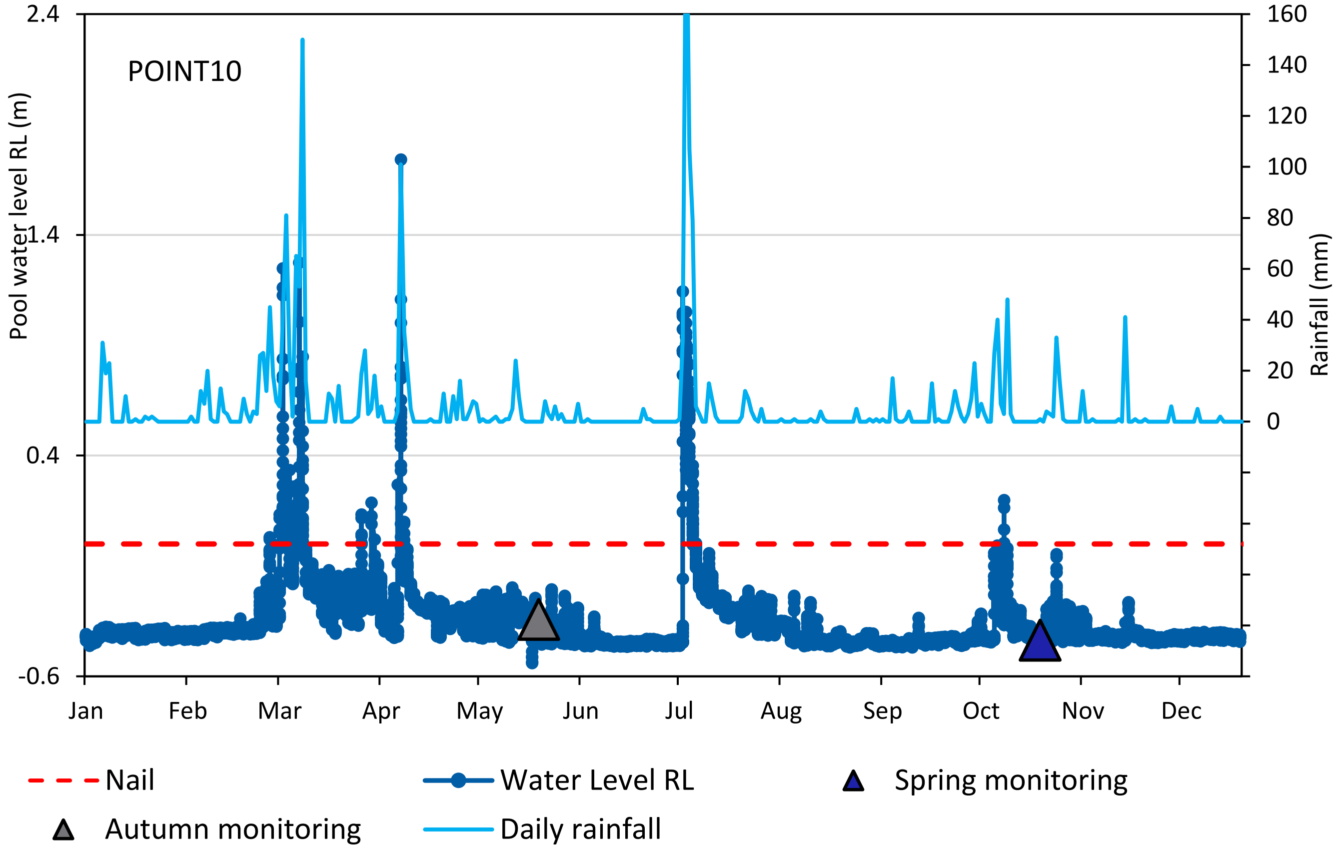


Figure 9. Rainfall and relative (RL) water levels at LDP10/Point 10 during 2022

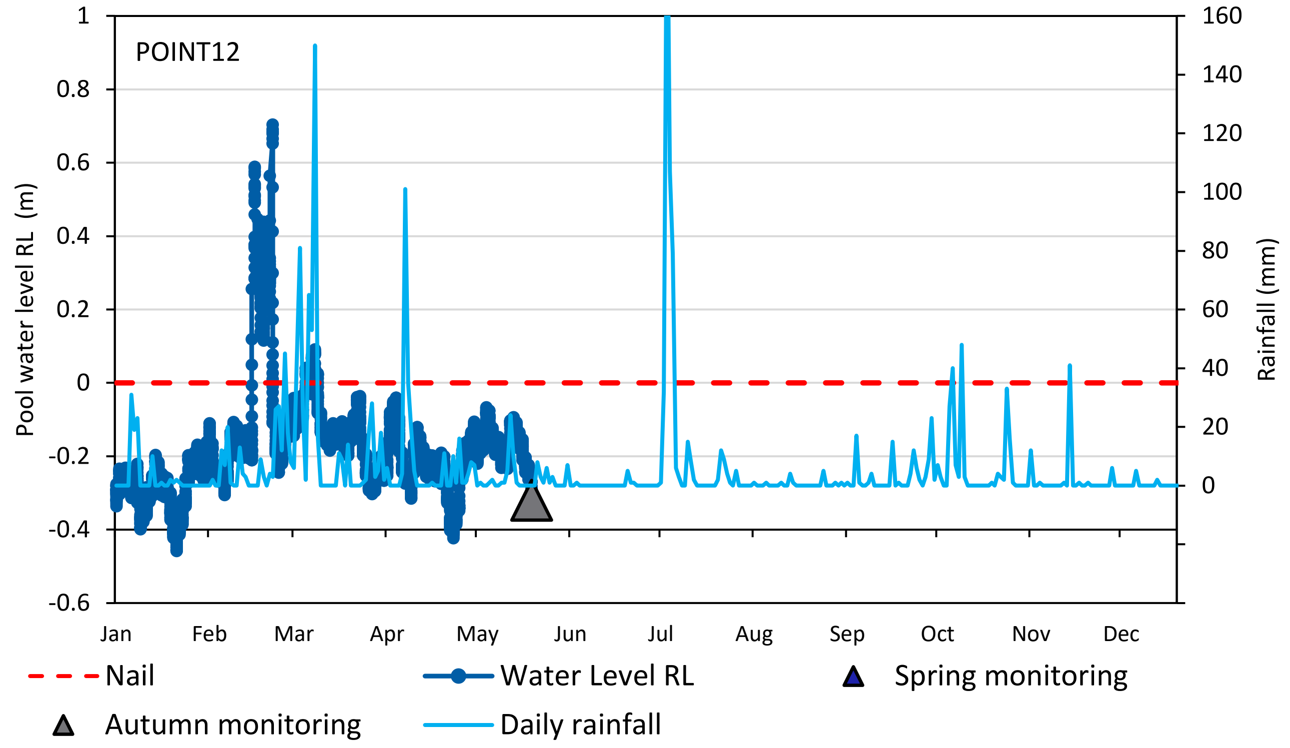


Figure 10. Rainfall and relative (RL) water levels at Point 12 during 2022

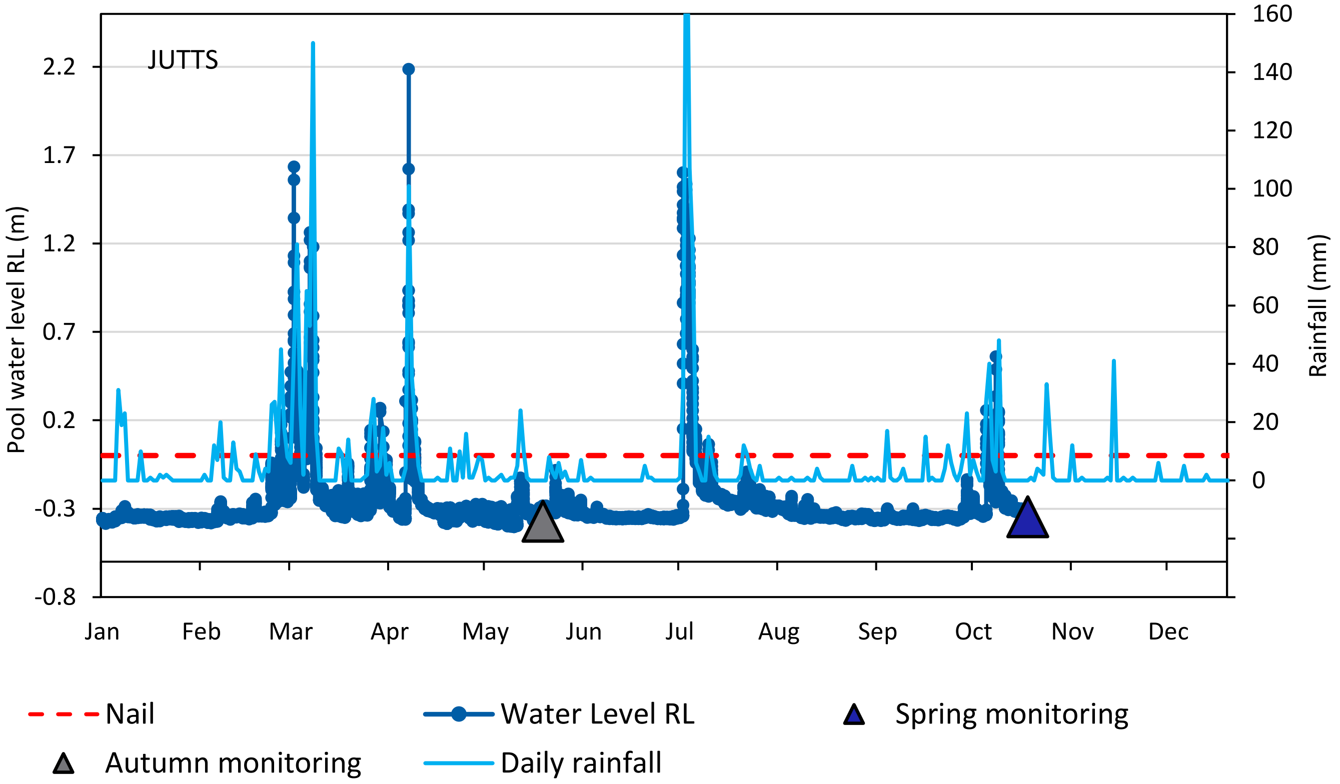


Figure 11. Rainfall and relative (RL) water levels at JUTTS during 2022

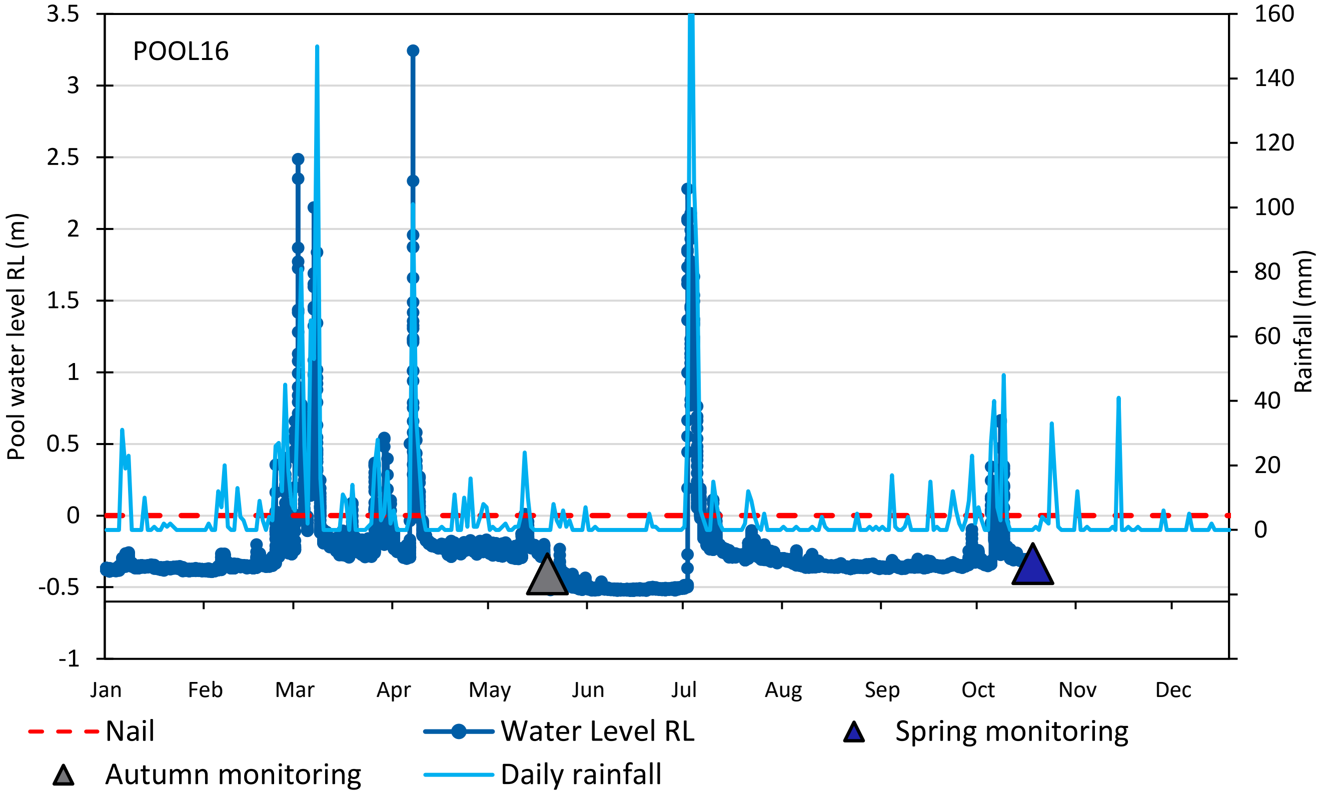


Figure 12. Rainfall and relative (RL) water levels at Pool 16 during 2022

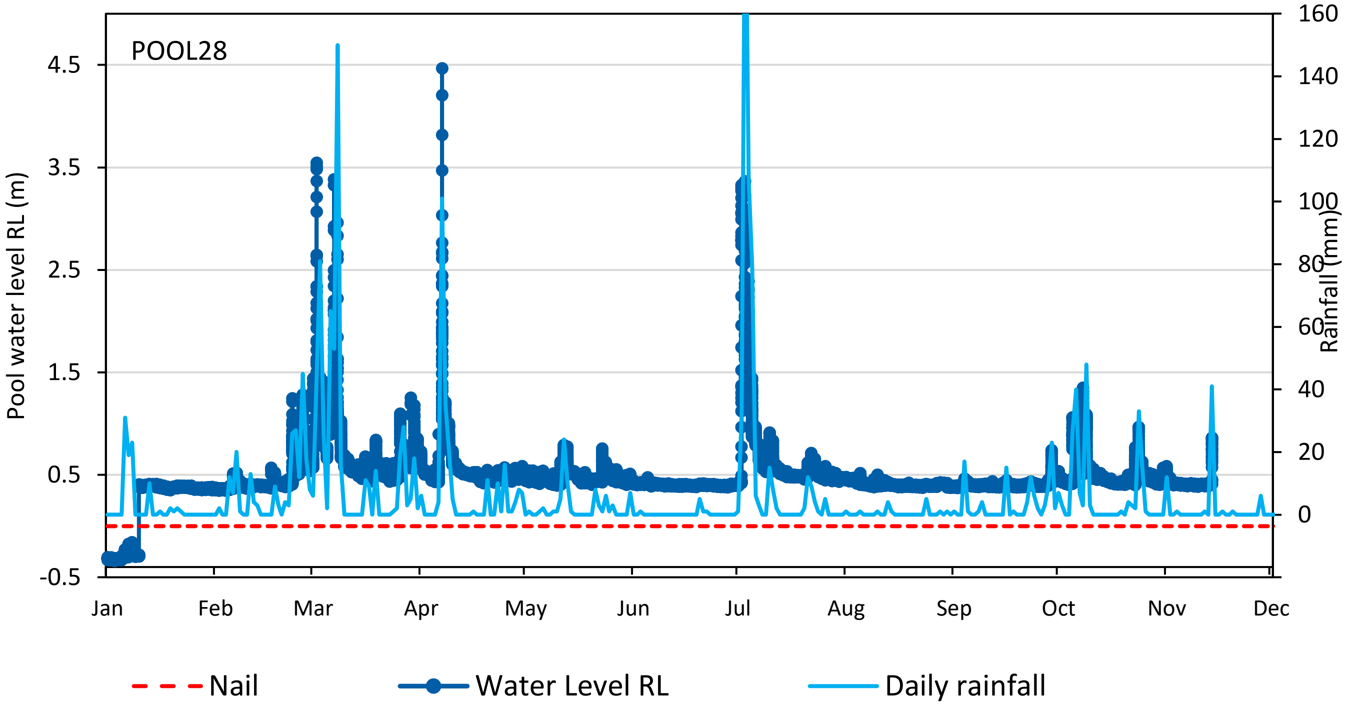


Figure 13. Rainfall and relative (RL) water levels at Pool 28 during 2022

Note: Autumn and spring 2022 sampling is not shown at Pool 28 as this site is not part of the GRAHMP.

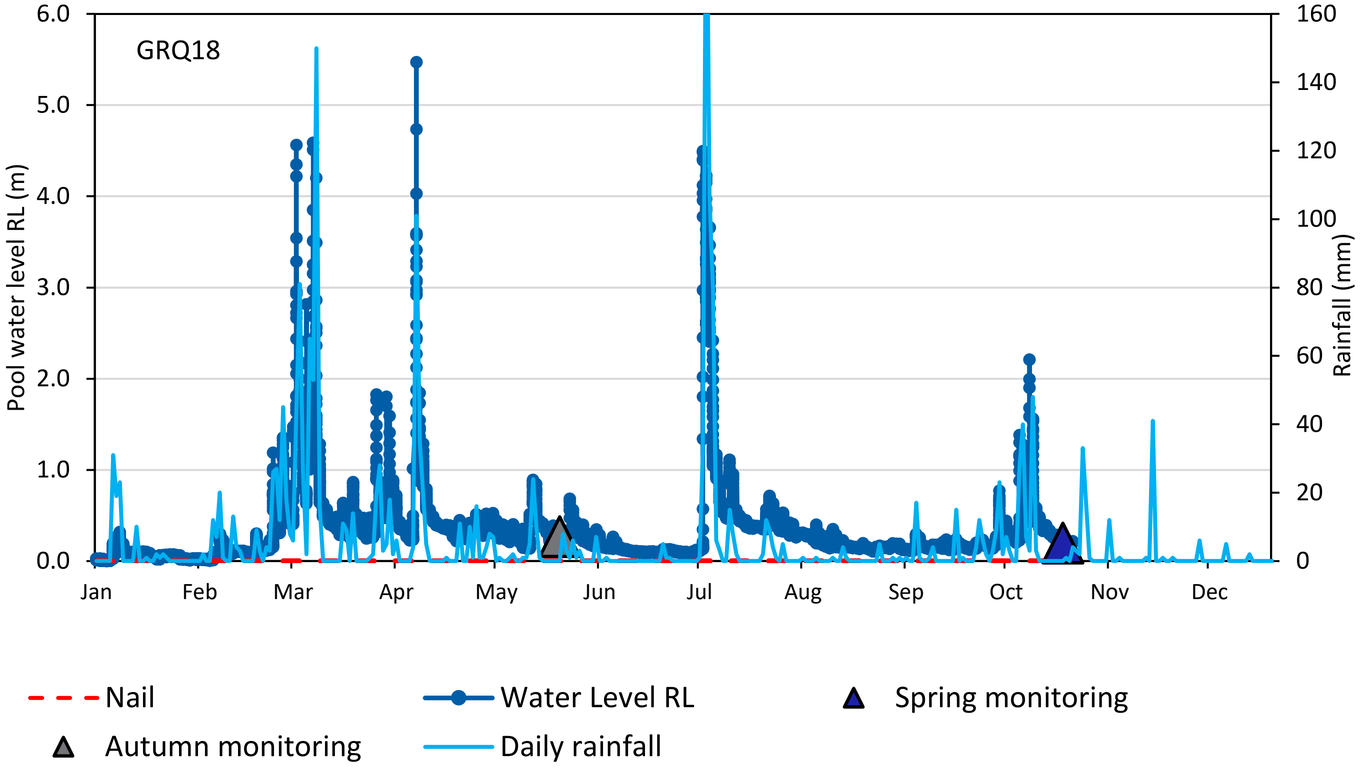


Figure 14. Rainfall and relative (RL) water levels at GRQ18 during 2022

3.1.2 Flow monitoring

During 2022, higher than average flows were observed through the Georges River catchment. Higher flows than previous years were consistently observed at GRUFS across the spot-flow sampling period of 2022. Flow peaks are presented in Figure 15 showing peak flows in February-March 2022, August 2022, and December 2022. In the period from 2020 to 2022, higher flow rates were observed than in previous years (2018-2019). Compared to more recent years, drier spells were observed during January 2021 and November 2021, with very low water flow detected during autumn 2020, spring 2020, summer 2020/21 and winter to spring 2021. Both spring sampling events in 2020 and 2021 coincided with very low flow rates at GRUFS. Autumn and spring 2022 sampling coincided with flows higher than observed in previous years, but sampling did not take place during maximum flows.

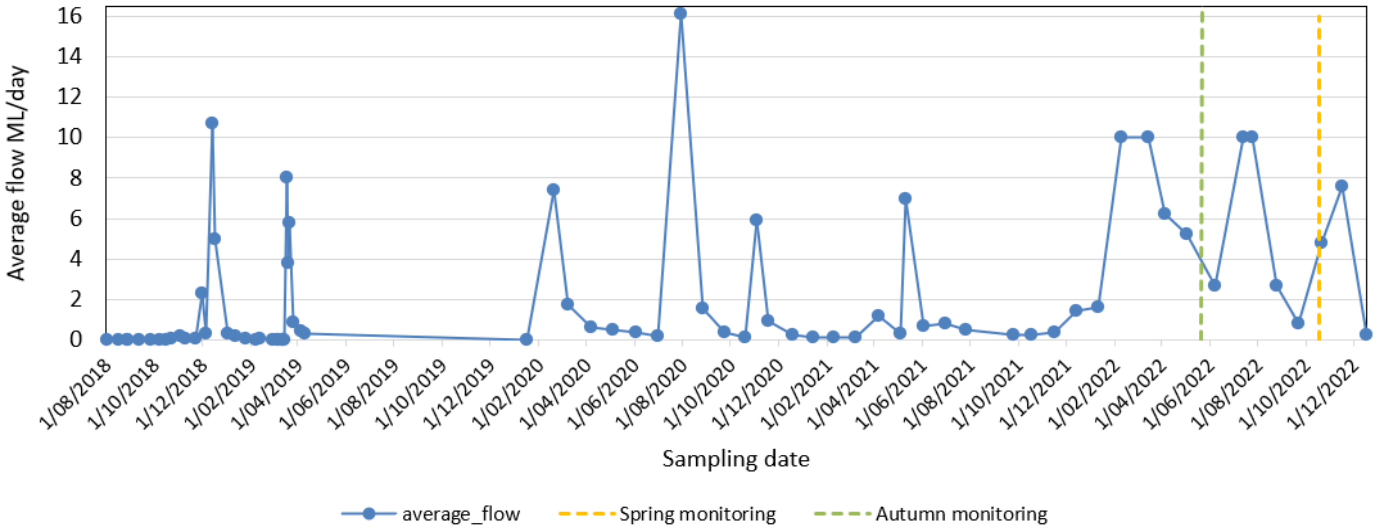


Figure 15. Average flow (ML/day) at reference site GRUFS over sampling time period July 2018 – November 2022

Discharge from LDP10 and LDP40

The volumes of the discharges from LDP10 and LDP40 to the environment in 2022 are shown in Figure 16 and Figure 17. The proportion of LDP40 water in overall discharge to the pool at Point 10 per month was low, ranging from 3-21% (Figure 16). The flow discharging from LDP40 in 2022 has increased in volume compared to 2021 flows. For example, in 2021 the LDP40 discharge per month was very low, ranging from 0.20-3.3%. Similar to 2021, the composition of LDP40 is only contributing a small fraction of the overall discharge flowing into Point 10 in Brennans Creek.

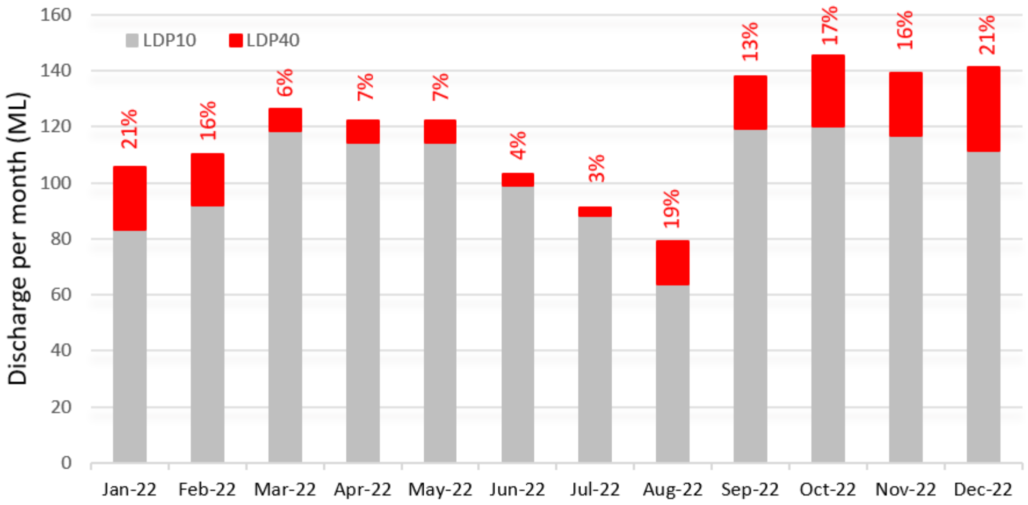


Figure 16. Total monthly discharge volumes (ML) from LDP10 and LDP40 during 2022

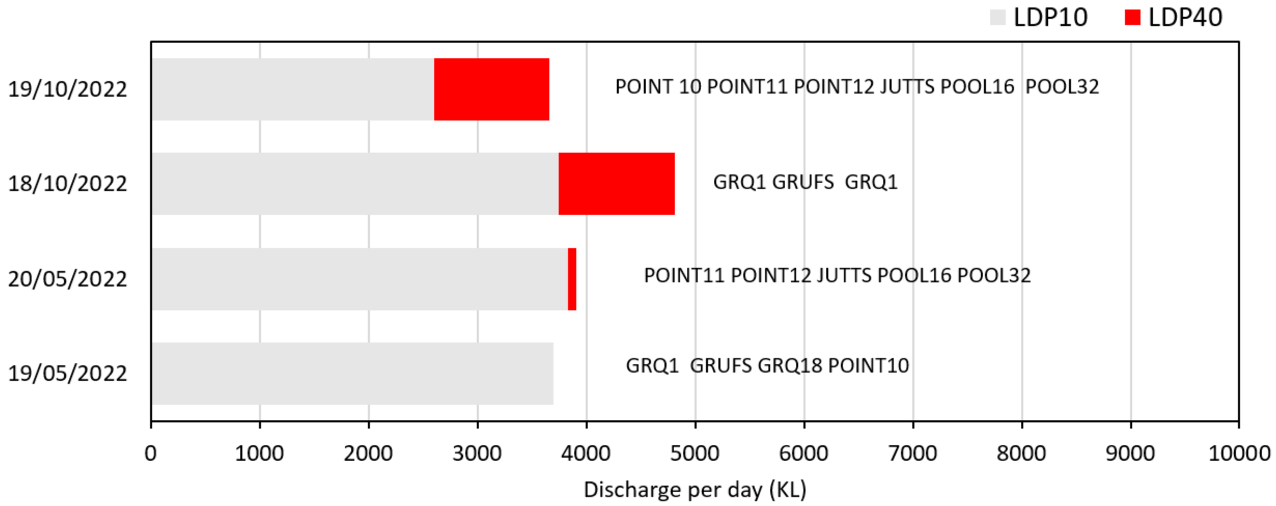


Figure 17. Discharge to the environment from LDP10 and LDP40 during sampling from each site for monitoring of water chemistry, macroinvertebrates and metabarcoding

Site names beside each bar indicate the sites sampled on those dates for macroinvertebrate and metabarcoding.

Monthly rainfall trends over the years 2018, 2019, 2020, 2021 and 2022 are shown in Figure 18. The rainfall data for 2022 (Figure 18) highlight the higher peaks of rainfall in March, July and October 2022 compared to the previous years. This rainfall leading to larger periods of flow during 2022 must be considered when interpreting the river flow and water quality data for the Georges River aquatic health as a whole.

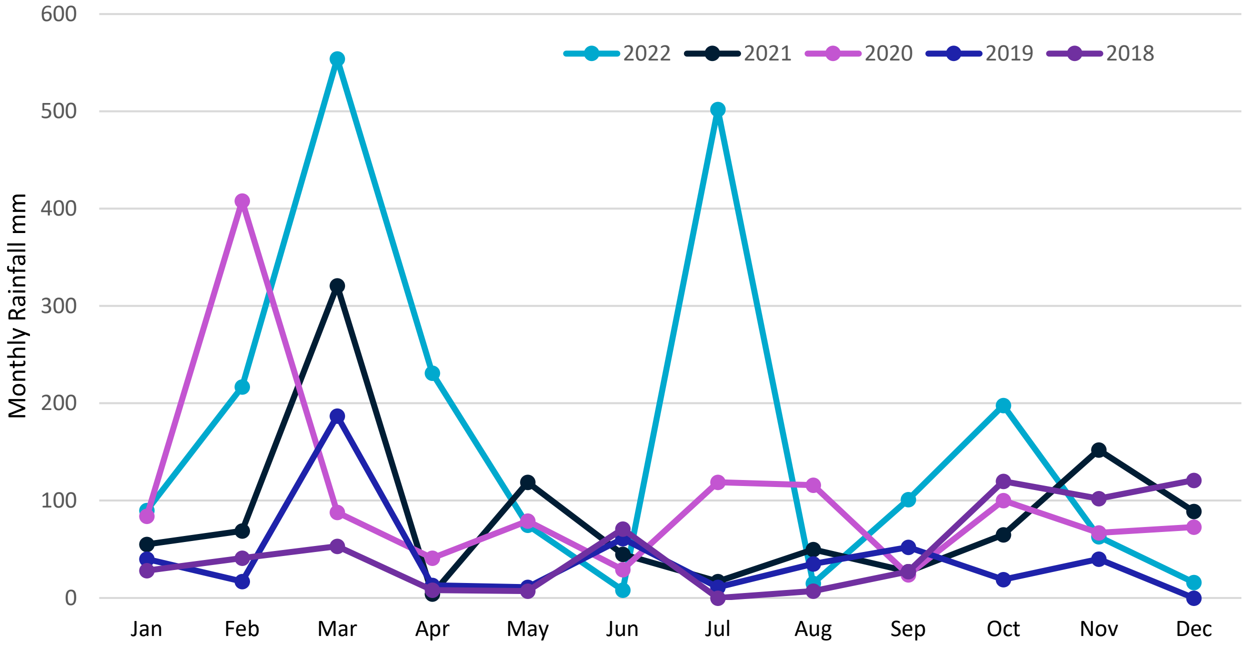


Figure 18. Monthly total rainfall trends influencing flow in Brennans Creek and the Georges River 2018-2022

3.2 Water chemistry

Analyses for water chemistry of the Georges River monitoring sites, presented in Table 6, Table 7, Figure 19 and Figure 20, were carried out on samples from each location alongside the macroinvertebrate surveys. Additional analyses for water chemistry were undertaken on samples collected from the end of pipe alongside those for ecotoxicity testing and are reported separately in Section 3.4.

In general, based on the parameters measured, water quality relative to guideline values (GVs) was poorer during 2022 at the downstream discharge monitoring sites than at reference sites. This was most evident at the sites closest to the discharge sources. Aluminium and pH values showed the values most frequently above and below the GV of the elements analysed (Table 6 and Figure 19). Aluminium exceeded the GV for both reference and discharge monitoring sites in autumn and spring 2022 (Table 6), with slightly better water quality in spring than in autumn. It is important to note that no blank contamination controls were included in the analysis of the field chemistry samples in 2022, despite CSIRO recommendations for these to be included in 2021 to help identify sources of erratic zinc concentrations in water samples.
Water quality parameters measured at reference sites were mostly within the ANZG (2018) GV ranges, with some exceptions for one or two sites on each sampling occasion.

At the reference site Point 11, the pH was within the ANZG (2018) GV range in both autumn and spring 2022 (Table 6 and Figure 19). The other reference sites had pH measurements below GV (Table 6 and Figure 19). In contrast, all downstream discharge monitoring sites had pH measurements above GV (Table 6 and Figure 19). Of the sites downstream of LDP10 and LDP40 discharges, those closest to the sources (Points 10, 12 and Jutts) generally had the highest pH measurements. For the discharge sites, pH was higher in autumn than in spring 2022. The reference sites showing low pH identifies that the upstream reference sites are characteristically more acidic, and this has been observed in previous years. The reasons for the low pH in the reference sites is unknown but these sites may be naturally low due to riverbank vegetation-derived organic acids (Holland et al., 2012). GRQ1 and GRUFS had pH lower than the freshwater GV (6.5) in autumn and spring 2022. In autumn all discharge sites were above the GV for pH except for GRQ18. In spring 2022, all discharge sites were above the GV for pH except for pool 16 and GRQ18.

Conductivity is a physical chemical stressor above and below certain levels for freshwater ecosystems. Conductivity for autumn and spring 2022 reference sites (94-170 µS/cm) was lower than discharge sites (358-1560 µS/cm). Autumn 2022 showed similar conductivity values to those from previous years. While conductivity was high at the discharge sites in autumn 2022, no sites had values above the upper GV for conductivity (125-2200 µS/cm) for lowland rivers in south-eastern Australia (Table 6). However, in spring 2022, conductivity was much lower than that recorded from previous years. In autumn and spring 2022, GRQ1 and GRUFS conductivity was lower than the lower threshold guideline value for freshwater systems. This reduction in conductivity could be a result of higher rainfall and flows through the catchment during spring 2022. There is also a possibility that flow from LDP40 could potentially be contributing to the reduction in conductivity in Spring 2022.

In 2022, for both autumn and spring, dissolved aluminium concentrations were above the GV (55 µg/L (pH>6.5)) for all sites and all sampling occasions (Table 6). Aluminium was highest in Point 10 (280 µg/L) in spring 2022. Aluminium has been increasing in the reference sites from 2020, 2021 to 2022 data. For example, in 2020 aluminium was only above the GV in Point 11 in spring 2020. In 2021, aluminium started increasing in the reference sites in both autumn and spring 2021. Aluminium was elevated for all sites and all sampling occasions in 2022. While aluminium was above GV for all sites, the values were slightly higher in the discharge monitoring sites compared with the reference sites.

Copper was below detection limits at all reference sites on all sampling occasions (Table 6). In autumn 2022, copper was measured above the GV for all discharge monitoring sites except for GRQ18, the most distant site from the discharge source. In spring 2022, copper was only above the GV at Point 10, which is closest to the discharge source and all other sites measured low concentrations of copper.

In both autumn and spring, nickel was below detection limits for all reference sites except Point 11. In autumn 2022, nickel was measured above the GV for the sites closest to the discharge source waters (Point 10, Point 12 and Jutts). In spring 2022, nickel was low in the discharge monitoring sites and was only measured above the GV at the source of the discharge at Point 10.

In 2022, zinc measurements were variable with no discernible relation to sampling occasion or distance from discharge source (Table 6). This variability in zinc measurements has been observed in datasets from previous years. The highest zinc concentration was recorded at Pool 16 (30 µg/L) in autumn 2022, however, during that same sampling occasion most of the other discharge monitoring sites recorded zinc concentrations below GV (8 µg/L). The majority of zinc concentrations at reference sites were below GV with the exception of Point 11 during autumn 2022 (19 µg/L). The source of zinc at reference sites is unknown. Zinc concentrations in spring 2022 were below the GV in most the discharge sites (Table 6) and was only measured above the GV at the source of the discharge at Point 10. Variability in zinc concentrations may be due to sample handling in the field. Incorporating field and trip blank controls in future samplings may assist with contamination issues in measurements and provide some analytical insight and explanation into the variability of metal concentration measurements observed.

Total nitrogen was below the GV for all sites for the autumn 2022 sampling occasion. For the spring 2022 sampling occasion, nitrogen was variable in the discharge sites and was above the GV at the discharge source site Point 10, but also at Jutts and Pool 16. The higher concentrations at Jutts and Pool 16 may suggest other nutrient sources inputs along the catchment in addition to discharge from LDP10 and LDP40 into the river.

The water quality parameters measured in 2022 for LDP40 are described in Table 7. The quality of the waters discharged from the temporary WTP at LDP40 was similar to that measured at the reference sites. Based on the limited measurements within this program, when comparing all discharge and downstream monitoring sites, water from LDP40 appeared to be of higher quality with lower metal concentrations, however, the pH was above the upper GV for February, May, and August sampling occasions. In 2022, LDP40 was only contributing a small proportion, ranging from 3-21% (Figure 16) of the total discharge volume to Point 10 and Brennans Creek, so the water from LDP40 with lower metals and conductivity is only contributing to a small volume of the total river flow. Therefore, overall water quality across the discharge monitoring sites continued to have elevated pH, metals, conductivity and bicarbonate in 2022 at the discharge sites compared to the reference sites.

Table 6. Summary of water quality measurements taken alongside macrobenthic surveys in 2022[a]

Values in bold exceed GV for analyte.

			Autumn 2022									Spring 2022								
		ANZG (2018) Guideline	Reference			Discharge monitoring						Reference			Discharge monitoring					
Analyte	**Units**		GRQ1	GRUFS	Point 11	Point 10	Point 12	Jutts	Pool 16	Pool 32	GRQ18	GRQ1	GRUFS	Point 11	Point 10	Point 12	Jutts	Pool 16	Pool 32	GRQ18
pH (field)	pH Unit	6.5-8	**6.0**	**6.1**	6.7	**8.5**	**8.6**	**8.7**	**8.6**	**8.6**	8	**6.3**	**6.1**	7.3	**8.3**	**8.2**	**8.1**	8	**8.2**	7.7
Conductivity (field)	µS/cm	125-2200	**103**	**105**	166	1557	1110	1040	943	1090	770	**95**	**94**	170	941	462	457	403	380	358
Bicarbonate Alkalinity	mg/L	NV[bc]	7	5	32	765	528	504	451	480	329	6	4	15	480	190	190	170	152	154
Aluminium	µg/L	55 (pH>6.5)	**110**	**110**	**100**	**160**	**150**	**150**	**220**	**180**	**150**	**80**	**80**	**60**	**280**	**140**	**130**	**100**	**140**	**150**
Cobalt	µg/L	1.4	<1	<1	<1	1	1	1	1	1	1	<1	<1	<1	<1	1	**3**	1	1	1
Copper	µg/L	1.4	<1	<1	<1	**3**	**2**	**2**	**3**	**2**	1	<1	<1	<1	**4**	1	1	1	1	1
Nickel	µg/L	11	<1	<1	2	**16**	**12**	**12**	10	10	8	<1	<1	<1	**12**	4	4	4	4	4
Zinc	µg/L	8	5	5	**19**	6	8	6	**30**	**9**	7	6	7	5	**10**	8	5	5	5	8
Nitrite + Nitrate (NOx)	µg/L	40	10	10	10	**150**	**100**	**100**	**90**	**100**	**60**	10	10	10	**150**	**80**	**120**	**50**	20	10
Total Kjeldahl Nitrogen	µg/L	NV	200	200	200	100	200	200	100	200	200	200	200	200	200	200	300	300	300	200
Total Nitrogen	µg/L	350	200	200	300	200	300	300	100	300	300	200	200	200	**400**	300	**400**	**400**	300	200

[a] pH and conductivity values are taken from field measurements, not ALS lab measurements. Exceptions to this were for autumn 2022 GRUFS and Point 11, where field data were missing.

[b] NV = No ANZG (2018) guideline value available

[c] Although no guideline value available, Vera et al. (2014) reported a bicarbonate EC10 for 7-d reproduction in the local Australian isolate of *C.* cf. *dubia* of 340 mg/L, and the Office of Environment and Heritage (2012) calculated an interim trigger value to use for bicarbonate of 225 mg/L, based on acute North America freshwater data with an acute to chronic ratio applied.

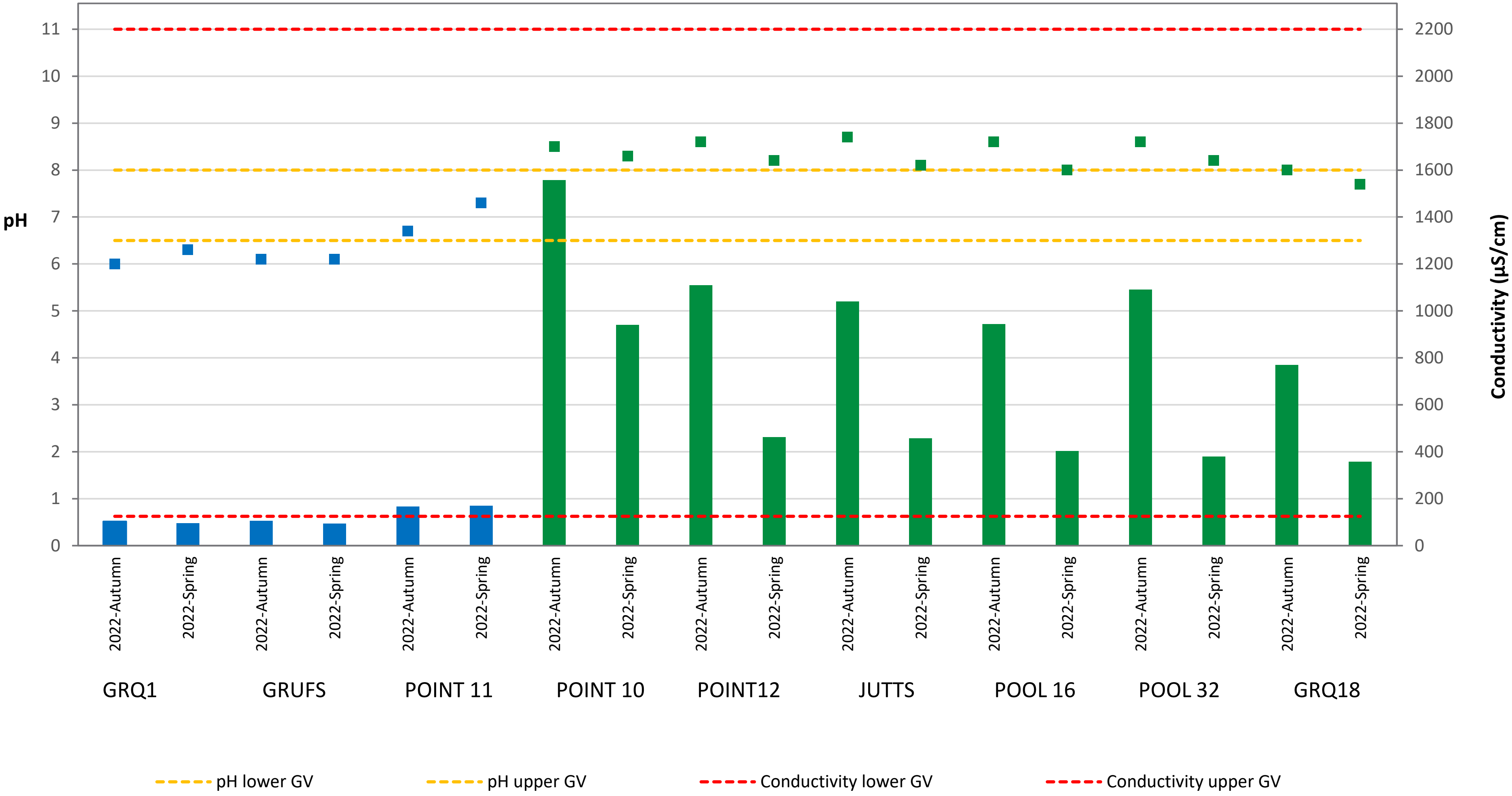


Figure 19. Field measured pH and conductivity at nine study sites

Note that field data were unavailable for GRUFS and Point 11 autumn 2022 samples so lab measurements are presented instead. Reference sites (blue) and discharge monitoring sites (green). Columns are conductivity and squares are pH. Dashed lines indicate upper and lower ANZG (2018) guideline values for pH and conductivity in lowland rivers.

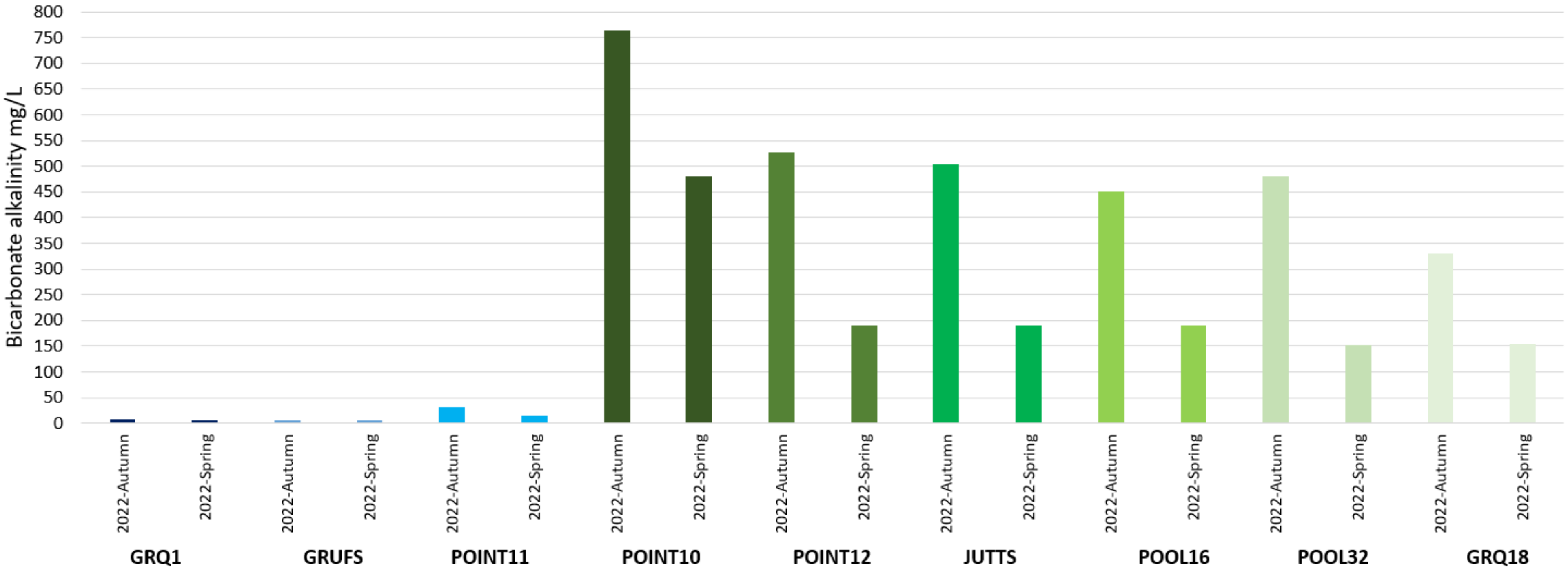


Figure 20. Lab measured bicarbonate alkalinity for all study sites sampled during autumn and spring 2022

Table 7. Summary of water quality measurements taken from LDP40 (end of pipe) in 2022 (data are from ALS reports unless otherwise specified)[a]

Values in bold exceed guideline value for analyte.

Analyte	Units	Guideline Value[b]	Feb-22	May-22	Aug-22	Nov-22
			LDP40	LDP40	LDP40	LDP40
pH	pH unit	6.5-8	7.0	8.0	7.9	**8.1**
pH (ESA)	pH unit	6.5-8	**8.3**	**8.3**	**8.1**	8.0
Conductivity	µS/cm	125-2200	137	175	182	182
Conductivity (ESA)	µS/cm	125-2200	139	172	186	182
Dissolved Oxygen (ESA)	% saturation	85-110	90	88	97	95
Carbonate Alkalinity	mg $CaCO_3$/L	NV[c]	NM	NM	NM	<1
Bicarbonate Alkalinity	mg $CaCO_3$/L	NV[d]	70	89	86	94
Total Alkalinity	mg $CaCO_3$/L	NV	NM	NM	NM	94
Aluminium	µg/L	55 (pH>6.5)	<10	<10	<1	0.9
Arsenic	µg/L	24 (III); 13 (V)	NM	NM	NM	0.2
Cadmium	µg/L	0.2	NM	NM	NM	<0.02
Cobalt	µg/L	1.4[g]	<1	<1	<1	0.04
Copper	µg/L	1.4	<1	<1	<1	0.07
Manganese	µg/L	1900	NM	NM	NM	0.16
Nickel	µg/L	11	<1	3	<1	0.3
Zinc	µg/L	8	**8**	<5	<5	0.8
Ammonia	µg/L	900	NM	NM	NM	284
Nitrite + Nitrate (NOx)	µg/L	40	20	**60**	**90**	37
Total Kjeldahl Nitrogen	µg/L	NV	400	200	400	NM
Total Nitrogen	µg/L	350	**400**	300	**500**	**370**

[a] Values outside of GV range appear in bold; metal concentrations are dissolved (0.45 µm filterable); [b] Water quality guidelines for pH, conductivity and total nitrogen for lowland east flowing NSW coastal rivers (ANZECC/ARMCANZ 2000, since there are no updated default guidelines for these in ANZG, 2018). Water quality guidelines for metals are reported as those for moderately-to-disturbed ecosystems (95% species protection values); [c] No guideline value available in ANZG (2018); [d] Vera et al. (2014) reported a bicarbonate EC10 for 7-d *C. dubia* (Australian isolate) of 340 mg/L, and the Office of Environment and Heritage (2012) calculated an interim trigger value for bicarbonate of 225 mg/L, based on acute North America freshwater data with an acute to chronic ratio applied, therefore values above 225 mg/L bicarbonate are likely to be harmful; [e] NM = not measured. [g] unknown reliability (as defined by Warne et al., 2018); [h] USEPA, 2002

3.3 Long-term trends in water chemistry (2013-2022)

The key water quality variables of pH, conductivity, aluminium, cobalt, nickel, copper, zinc, and total nitrogen have been monitored long-term (2013-2022) and are described here. Across all sites and years (2013 to 2022), these measured parameters were generally lower at reference sites than at the discharge monitoring sites. Long-term trends observed with respect to time and to distance from the discharge source at LDP10 varied for different parameters.

Waters collected from the discharge monitoring sites were consistently higher in pH than those from the reference sites (Figure 21). The reference sites GRQ1 and GRUFS continue to be more acidic, consistent with the long-term trend. The reference sites GRQ1 and GRUFS have, on occasions, been below the ANZG (2018) lower GV for pH while Point 11 has predominantly been within the range of pH GVs (6.2-8) over time. The pH of waters from discharge monitoring sites frequently fell outside the ANZG (2018) GV range over the period 2013-2022. However, the most downstream discharge monitoring site (GRQ18) generally had lower pH values than the other monitoring sites, and on eight (of seventeen) occasions (including the two most recent three sampling occasions in 2020, 2021 and 2022) were within the acceptable GV pH range. The pH of waters in pools at the source of discharge (LDP10) consistently exceeded the upper pH ANZG (2018) GV of 8, and between 2016-2018, the pH was greater than 9. Since that time, there has been a slight reduction in pH at Point 10 from 2019 to 2022 (8.3). In general, there was no clear overall decline in pH over time within the discharge monitoring sites, but certainly pH becomes more acidic with increasing distance from Point 10 on each sampling occasion.

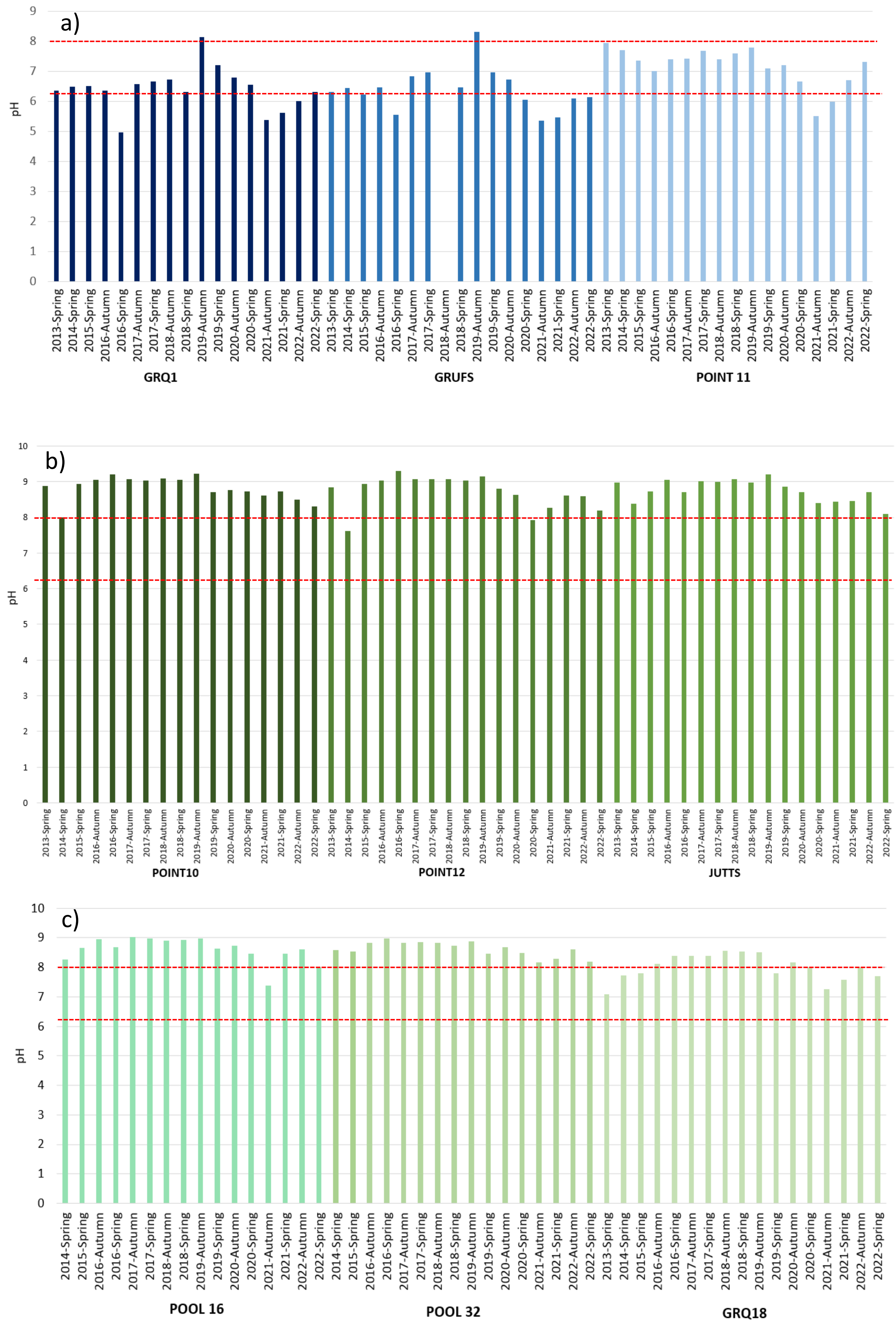


Figure 21. Long-term (2013-2022) pH (field measured) values for a) reference sites GRQ1, GRUFS and Point 11; b) discharge monitoring sites Point 10, Point 12 and Jutts; c) discharge monitoring sites Pool 16, Pool 32 and GRQ18

Dotted red lines represent the upper and lower ANZG (2018) guideline value for pH in lowland rivers for south-eastern Australia.

In recent years, the conductivity of waters at the discharge monitoring sites (Figure 22b, c) has been within the ANZG (2018) GV range for lowland east coast rivers (125-2,200 µS/cm), however, it was markedly higher in the discharge monitoring sites compared to the reference sites (Figure 22a). For GRQ1 and GRUFS there has been consistently lower conductivity measurements at these sites while Point 11 has been more variable in conductivity values over time. For the discharge monitoring sites, there is a decreasing trend in conductivity for the more recent years (Figure 22b, c). In autumn 2022, conductivity values were similar to recent years (2020-2021) while in spring 2022 conductivity showed much lower values than previous years in the discharge monitoring sites during spring. This reduction could be an associated with increased rainfall and flows through the river in 2022 as well as potentially slight increased volume of LDP40 in the river system compared with 2021.

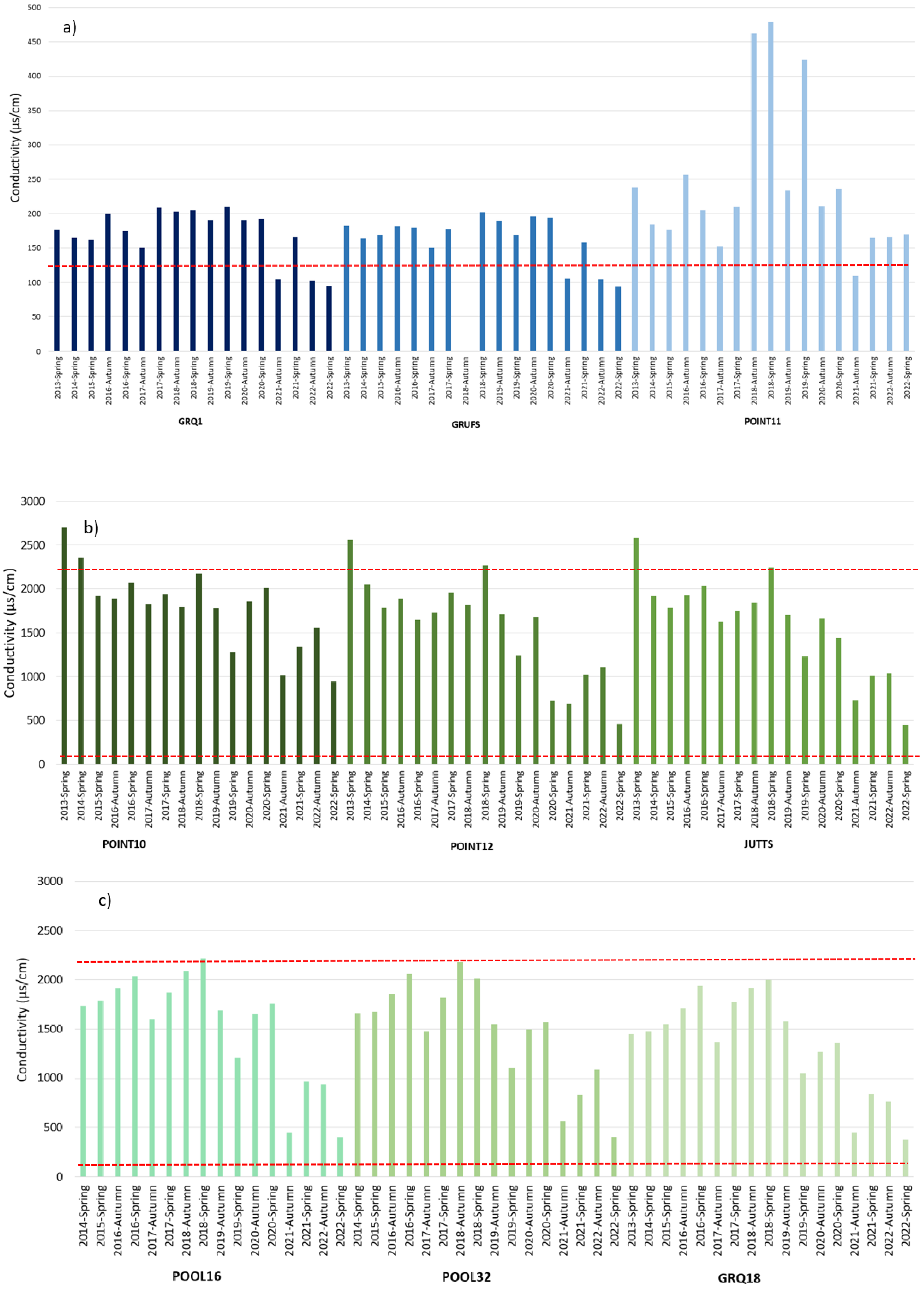


Figure 22. Long-term (2013-2022) conductivity (field measured) values for a) reference sites GRQ1, GRUFS and Point 11; b) discharge monitoring sites Point 10, Point 12 and Jutts c) discharge monitoring sites Pool 16, Pool 32 and GRQ18

Dotted red lines represent the upper and lower ANZG (2018) guideline value for conductivity in lowland rivers for south-eastern Australia.

Dissolved aluminium concentrations overall have been higher in discharge monitoring sites compared to the reference sites over the period 2013-2022 (Figure 23). Aluminium concentrations were consistently elevated above the GV concentration at all discharge monitoring sites (Figure 23b and c). While measurements varied over time, there have been consistently higher aluminium concentrations in discharge monitoring sites compared with reference sites. Across all years, aluminium has been highest in reference sites in the years 2021 and 2022. For some reference sites such as GRUFS, aluminium has increased in recent years compared to historical values of aluminium. In the discharge monitoring sites, aluminium values have been variable over the years and generally aluminium has been measured at higher values closer to the discharge point at sites Point 10, 12 and Jutts and lower at the downstream site GRQ18. The GV for aluminium in freshwaters of a pH >6.5 is 55 µg/L, the discharge monitoring sites closest to the discharge (Point 10, 12 and Jutts) have consistently been above the GV value over the period 2013-2022. On more recent sampling occasions (autumn and spring 2022) the reference sites have exceeded the GV for aluminium for pH above 6.5.

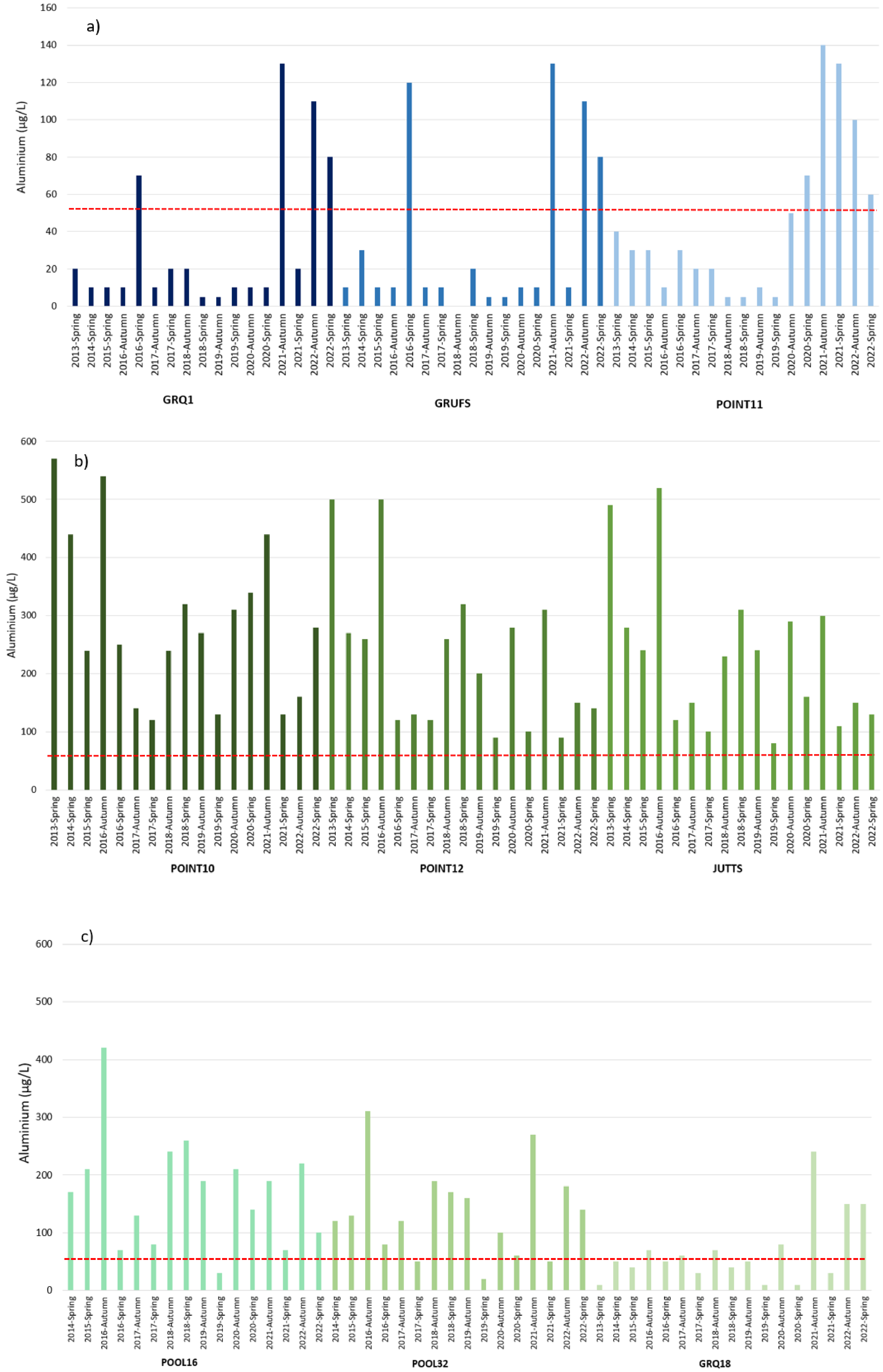


Figure 23. Long-term (2013-2022) dissolved aluminium concentrations for a) reference sites GRQ1, GRUFS and Point 11; b) discharge monitoring sites Point 10, Point 12 and Jutts; c) discharge monitoring sites Pool 16, Pool 32 and GRQ18

Dotted red lines represent the ANZG (2018) guideline value.

Cobalt concentrations have declined over time at the discharge monitoring sites. Cobalt values are generally overall lower than other metals measured, however, values are higher at sites closer to the discharge sources (Point 10, 12 and Jutts) (Figure 24b). Cobalt has been below detection for all reference sites for all years (Figure 24a). The cobalt GV of 1.4 µg/L was exceeded at most sites in 2013 and 2014, however it has declined over the years (Figure 24b and c). The GV was exceeded at Jutts in 2022. Concentrations of cobalt also declined with increasing distance from Point 10, and in earlier years (2013 to 2018) were higher than those at reference sites.

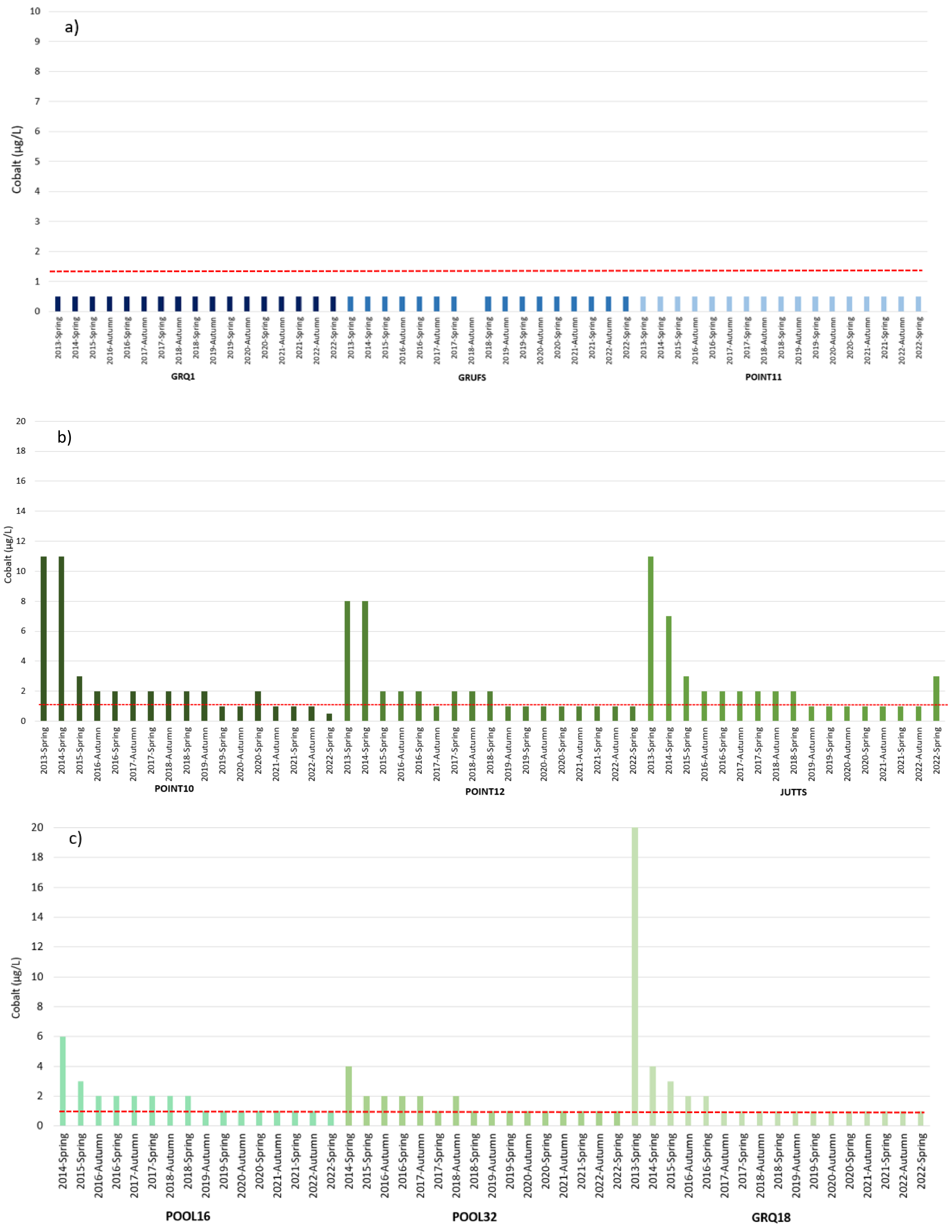


Figure 24. Long-term (2013-2022) dissolved cobalt concentrations for a) reference sites GRQ1, GRUFS and Point 11; b) discharge monitoring sites Point 10, Point 12 and Jutts; c) discharge monitoring sites Pool 16, Pool 32 and GRQ18

Dotted red lines represent the ANZG (2018) guideline value.

Dissolved copper concentrations have been consistently below detection for the reference sites across all years (Figure 25a). Copper values are generally lower than other metals, however, values are higher at sites closer to the discharge sources (Point 10, 12 and Jutts) (Figure 25b). Copper concentrations have declined over time at the discharge monitoring sites. Waters from all sites in the final sampling occasion of spring 2022, contained concentrations that were at or below GVs (Figure 25). This contrasts with samples collected in 2013 when concentrations were up to 8-fold higher (Figure 25b). It should be noted that at some sites, particularly Point 10 and Point 12, copper concentrations have been slightly erratic, but the overall long-term trend (2013-2022) has been a decline over time at these sites.

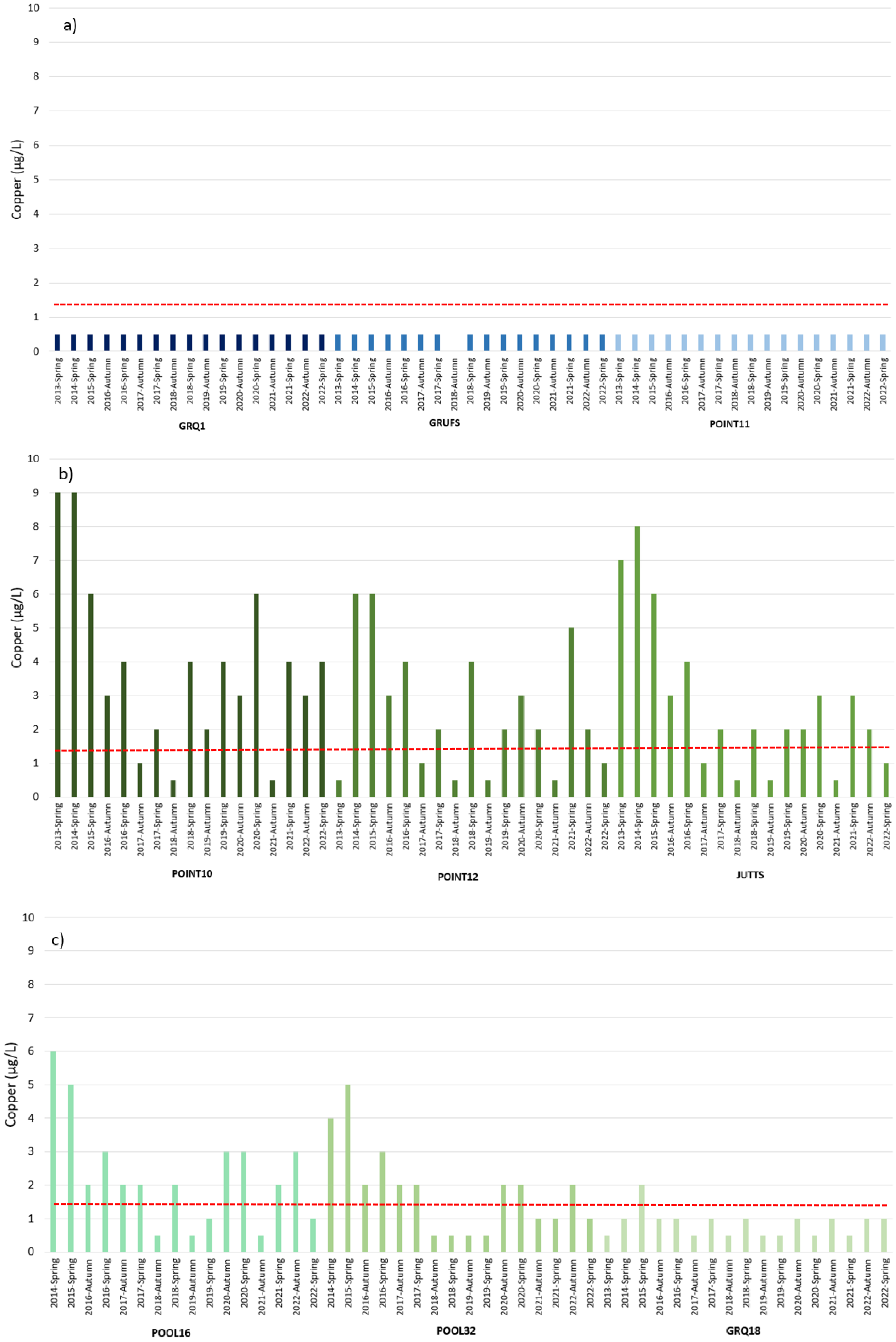


Figure 25. Long-term (2013-2022) dissolved copper concentrations for a) reference sites GRQ1, GRUFS and Point 11; b) discharge monitoring sites Point 10, Point 12 and Jutts; c) discharge monitoring sites Pool 16, Pool 32 and GRQ18

Dotted red lines represent the ANZG (2018) guideline value.

Concentrations of dissolved nickel have generally been low at the reference sites, although concentrations above the GV recorded in 2019. In autumn and spring 2019, waters sampled from the reference sites GRQ1 and GRUFS contained very high concentrations of nickel (Figure 26a) but returned to concentrations below the GV in 2020 through to 2022. For the discharge monitoring sites, the nickel concentrations were higher during the period 2013-2018 and concentrations have reduced during the period 2019-2022 (Figure 26b and c). Overall, nickel concentrations declined over time in all discharge monitoring sites, particularly those furthest from LDP10 and LDP40 discharge points.

Figure 26. Long-term (2013-2022) dissolved nickel concentrations for a) reference sites GRQ1, GRUFS and Point 11; b) discharge monitoring sites Point 10, Point 12 and Jutts c) discharge monitoring sites Pool 16, Pool 32 and GRQ18

Dotted red lines represent the ANZG (2018) guideline value.

Dissolved zinc concentrations have been erratic and variable over the years (Figure 27). The highest zinc concentrations at all discharge monitoring sites were observed in 2019 (up to 10-fold above the GV) but measurements fell in 2020 and 2021 to levels sometimes below the GV and have continued to stay lower than previous years in discharge monitoring sites in 2022. The sources of higher zinc over the long-term is inconclusive and uncertain, other metals did not increase at the same time points as elevated zinc, so it is difficult to infer the causes of elevated zinc concentrations at specific times.

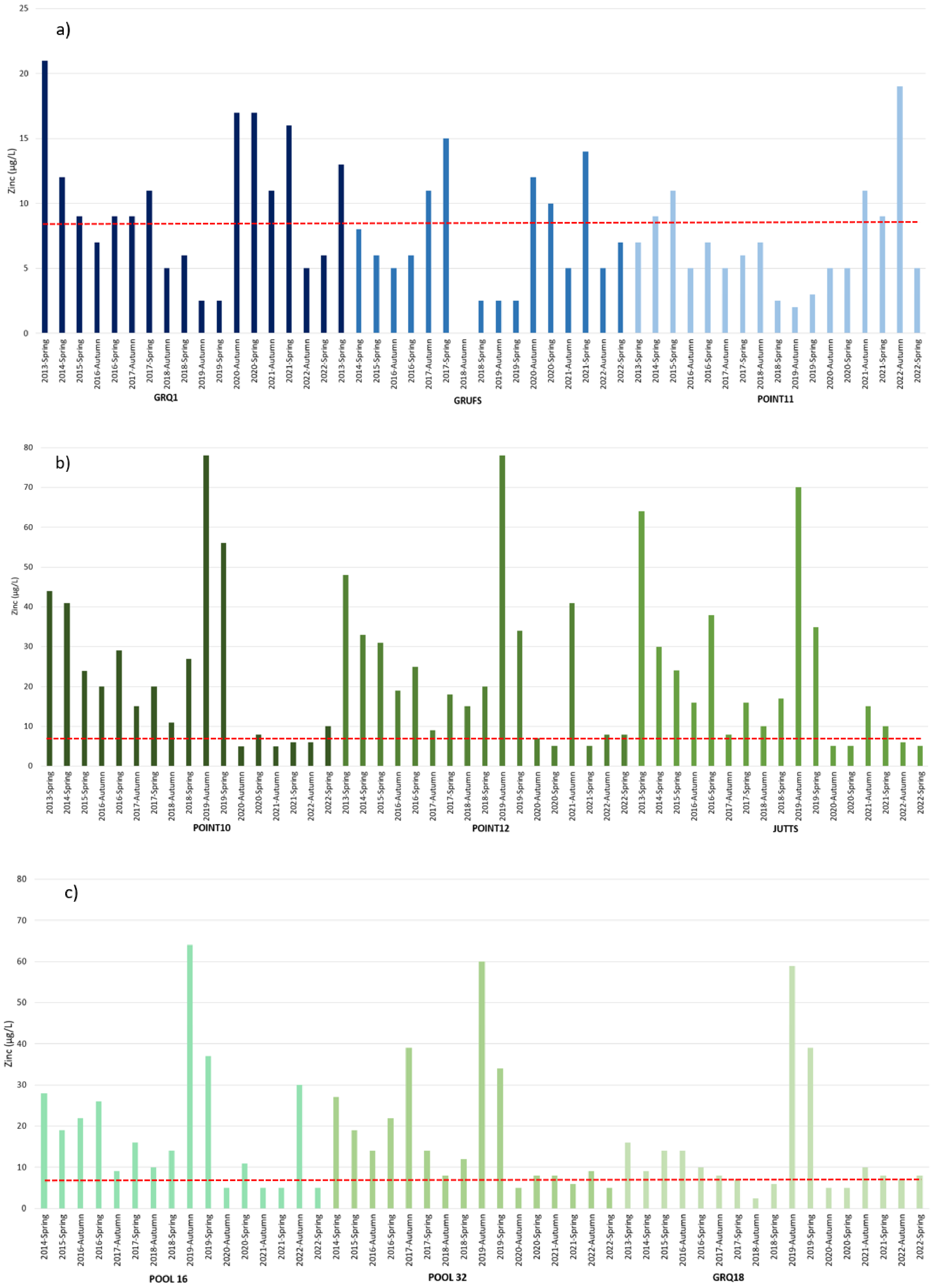


Figure 27. Long-term (2013-2022) dissolved zinc concentrations for a) reference sites GRQ1, GRUFS and Point 11; b) discharge monitoring sites Point 10, Point 12 and Jutts c) discharge monitoring sites Pool 16, Pool 32 and GRQ18

Dotted red lines represent the ANZG (2018) guideline value.

There is a clear difference in total nitrogen concentrations between the reference sites and the discharge monitoring sites across all years (Figure 28). The GV for total nitrogen is 350 µg/L and historically the reference sites have been below the GV value. In recent years (2019-2021), total nitrogen concentrations for all sites were generally below the GV. Some spikes of total nitrogen were observed in 2022. At reference sites (e.g., Point 11), values were measured above the GV for total nitrogen in spring 2022. For the discharge monitoring sites, there was a general decline in total nitrogen with distance from the discharge source, with Point 10, Point 12 and Jutts generally containing higher concentrations of total nitrogen compared to GRQ18 (8 km from Point 10). The sites closer to the discharge source have measured concentrations exceeding the GV for total nitrogen over the period 2013-2022.

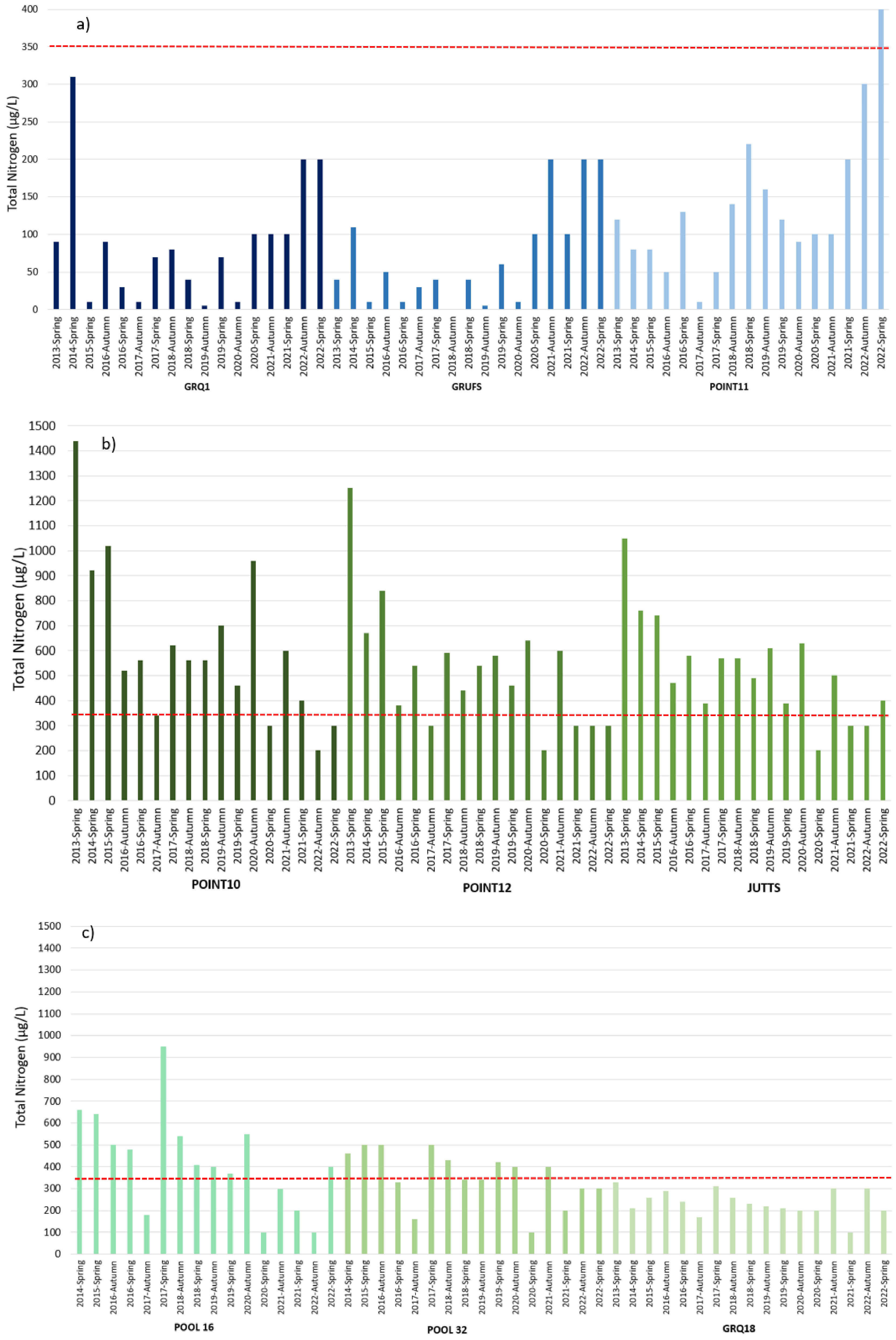


Figure 28. Long-term (2013-2022) total nitrogen concentrations for a) reference sites GRQ1, GRUFS and Point 11; b) discharge monitoring sites Point 10, Point 12 and Jutts; c) discharge monitoring sites Pool 16, Pool 32 and GRQ18

Dotted red lines represent the ANZG (2018) guideline value.

The long-term data for the analytes measured highlight the differences in water quality between the reference and the discharge monitoring sites. There has been some improvement to water quality over time at discharge monitoring sites, with conductivity, and concentrations of copper, cobalt and nickel generally decreasing over time. There is also some evidence of a decrease in aluminium in recent years. For pH, and the analytes zinc and total nitrogen, however, there were no clear trends with respect to time, and these parameters continue to exceed the respective GVs indicating that the overall water quality at the discharge monitoring sites (particularly those closest to the source) is poor and has the potential to cause biological and ecological impacts. Aside from increased rainfall and flows in 2022, there have not been any other major additional factors to contribute to changes in water quality, and the overall trends in water chemistry remain similar to those reported in 2021. It will be interesting to observe the chemistry data once the long-term, larger scale ANWTP starts running and contributing a greater volume to the discharging flows. This monitoring of chemistry data will be a critical LoE in predicting any impacts to the environment as the discharge source changes in the future.

3.4 Ecotoxicology

3.4.1 Ecotoxicology tests

Results of the chronic 7-d *Ceriodaphnia dubia* reproduction and survival tests, and acute 96-h larval imbalance tests with the fish *Melenotaenia splendida* are presented in Table 8 and Appendix A.

Table 8. Ecotoxicity of waters from LDP10 and LDP40 in 2022

Month	**Site:**	**LDP10**			**LDP40**		
	Species:	***C. dubia***		***M. splendida***	***C. dubia***		***M. splendida***
	Endpoint:	**Reproduction**	**Survival**	**Imbalance**	**Reproduction**	**Survival**	**Imbalance**
February	**Toxic/Not toxic**	Not toxic	Not toxic	Not toxic	Toxic	Toxic	Not toxic
	EC10 (%)	>100	>100	>100	34 (8.1-48)[a]	53 (50-65)	>100
May	**Toxic/Not toxic**	Not toxic	Not toxic	Not toxic	Toxic	Toxic	Not toxic
	EC10 (%)	>100	>100	>100	6.9[b]	8.2[b]	>100
August	**Toxic/Not toxic**	Not toxic	Not toxic	Not toxic	Not toxic	Not toxic	Not toxic
	EC10 (%)	>100	>100	>100	>100	>100	>100
November	**Toxic/Not toxic**	Not toxic	Not toxic	Not toxic	Toxic	Toxic	Toxic
	EC10 (%)	>100	>100	>100	14 (8.9-17)	23 (6.9-32)	79 (69-90)

[a] 95% confidence limits in parentheses

[b] 95% confidence limits were not calculable

All ecotoxicity tests met their respective quality assurance and quality control criteria. Where samples showed no or low toxicity, concentration-response curves were poor with no or one partial response, resulting in potentially unreliable EC10 values. However, the similar trend in NOECs and EC10 values in this study (Appendix A) provided confidence that the EC10 values quoted here are sufficiently reliable to enable informed water management decisions. In future testing, modification of ecotoxicity test design (i.e., modifying the concentration series tested) could improve the reliability of the EC10 values.

Toxicity tests with rainbowfish larvae were done without water renewal, based on advice from ESA that this species cannot tolerate excessive handling. There is a concern that without water renewals, excessive degassing can occur (for LDP10), altering the toxicity of the sample. Degassing would be indicated by an increase in pH of the sample during the test exposure period. The pH of LDP10 was relatively stable, varying by no more than 0.3 pH units between Day 0 and Day 4 of the rainbowfish test for any sample tested, suggesting that degassing was unlikely to have occurred during testing.

Water from LDP10 was not toxic to *C. dubia* reproduction or survival, nor was it toxic to *M. splendida* larval imbalance throughout all quarters of 2022, with NOEC and EC10 values >100%.

As found in 2021, the cladoceran tests were more sensitive than rainbowfish, with LDP40 samples causing toxicity to *C. dubia* reproduction and survival in three samples (February, May and November), whereas toxicity to *M. splendida* larval imbalance was only observed once (in November). The order of toxicity of LDP40 samples to *C. dubia* based on EC10 values was: May > November > February.

Waters collected from LDP40 in May and November caused acute toxicity to *C. dubia* within 24-48 h. A very high level of acute toxicity was observed in response to the May sample, whereby no cladocerans in treatments >6.3% LDP40 survived the first 24 h of exposure. For the November sample, within 48 h there were no surviving cladocerans in undiluted (100%) LDP40. Acute and chronic toxicity was observed at a similar concentration of LDP40 for the May sample, suggesting that, for this sample, all the toxicity observed was acute (i.e., there were no surviving animals to have produce offspring). For the November and February samples, chronic toxicity was observed at LDP40 concentrations that were around half those where acute toxicity was observed, i.e., those cladocerans that survived exposure had lower reproductive outputs.

The assessment criteria with respect to ecotoxicity at LDP10 were not exceeded, with no toxicity observed to any species, however exceedances of the license requirement occurred for LDP40 for the February, May and November samples.

The high levels of acute toxicity to *C. dubia* observed for LDP40 in May 2022 and to a lesser extent in November are of significant concern. LDP40 water is most representative of the expected water quality from the anticipated large-scale water treatment plant. If water was discharged from the new WTP with the levels of toxicity observed in May, there would likely be significant detrimental impact on the organisms in receiving ecosystems, particularly if the WTP-treated water was the bulk of the water flow in the catchment.

3.4.2 Water quality parameters and comparison to ecotoxicology test results

The water quality parameters measured in the LDP10 and LDP40 water samples used for ecotoxicity testing (and collected from end of pipe) are shown in Table 9 and Appendix A.

There was a large difference in pH measurements determined by ALS on LDP10 and LDP40 collected in February (8.4 and 7.0, respectively), compared to those measured in the same samples at the time of toxicity test commencement (9.0 and 8.3, respectively). This very large discrepancy is unusual and suggests either error in one of the laboratory's' measurements, or that the samples analysed by ALS on those occasions were not representative of those used for ecotoxicity tests and warrants further investigation. CSIRO advised South32 of this at the time, however, it is not known if any investigations were done.

For LDP10, the pH values (using either ALS or ESA measurements) were always above the ANZG GV range for lowland rivers of 6.5-8.0 and were also higher than those measured for the control DMW water in toxicity tests. Concentrations of dissolved aluminium and nickel were consistently at or above the ANZG GV for moderately disturbed ecosystems, while concentrations of copper, NOx and total nitrogen were above the GVs on three of the four sampling occasions.

The pH of LDP40 was usually closer to that measured for DMW, close to or above the upper GV for lowland rivers of 8.0. One on occasion (February) dissolved zinc was detected in LDP40 above the GV for moderately disturbed systems. Total nitrogen (in February and August) and NOx (in May and August) were elevated above their respective GVs.

LDP10 which had higher concentrations of most analytes than LDP40, was not toxic to cladocerans or rainbowfish larvae, whereas LDP40 which had relatively lower concentrations of most analytes caused toxicity on three occasions. LDP40 water is RO-treated and is therefore likely to be low in major ions. This not only causes potential ionic imbalance-related toxicity but can also make low concentrations of some metals more bioavailable (i.e., more toxic) than they may be to organisms in an ion-rich water base, such as LDP10.

The causative toxic agent(s) of toxicity in LDP40 could not be identified based on the chemical analyses provided by South32 in Table 7 (also see Appendix A). It is highly likely that observed toxicity was caused by a toxicant currently not measured as part of the usual suite of analyses, and/or by ionic imbalance in the RO-treated water. Further investigation is needed to identify the cause of toxicity.

At the time of receiving toxicity test reports for review (September 2022), we strongly recommended actions be taken to identify the cause of toxicity. However, to our knowledge these actions of identifying toxicity have not been done. Note, however, that in September 2022, CSIRO was provided with the toxicity data for February, May and August to review. By that time the samples would have aged significantly, during which time sample chemistry may have changed. It is of utmost importance, if causative agent(s) are to be identified, that action be taken as soon as toxicity is observed in a given sample.

It is therefore strongly recommended that an action plan be developed to respond to toxic events in future. If South32 cannot explain the likely cause of toxicity (e.g., based on operational or other information that CSIRO are not aware of, e.g., other chemical use for dosing against biofouling etc), a Toxicity Identification Evaluation (TIE) approach could be considered to identify the toxicant(s) responsible for toxicity. Noting however, that TIE approaches must be done as soon as possible after toxicity is detected, to avoid significant aging of the sample. In addition, ionic investigations should be carried out to determine if toxicity is related to a lack of particular ions in

LDP40 water. It is possible that additional salts are required to be added back to the RO-treated water prior to discharge. The add-back of salts has improved water quality of RO-treated water for other Clients and Water Authorities. Alternatively, the TIE process may identify a toxicant of concern that is currently not analysed in the usual suite, in which case, South32 may need to consider the source of the new toxicant, whether this will be a likely contaminant in the large scale WTP treated water, and what steps might be needed to remove the toxicant or mitigate its impact. The option of pre-diluting LDP40 in LDP10 water has been suggested by South32 as a way of reducing LDP40 toxicity, however this requires additional testing, and will only be effective if LDP10 is not toxic in its own right, and if dilution is based on a worst case scenario toxic LDP40 sample (e.g., the May 2022 sample). It is recommended that the cause of toxicity be first identified, as there may be other strategies that can be used to mitigate LDP40 toxicity.

It is also worth noting that the standard ANZECC/ARMCANZ approach to determining an appropriate dilution required to protect a nominated percentage of the organisms in the receiving environment is to use toxicity data from 8 species to generate a species sensitivity distribution and generate statistically derived dilution values. It is not known whether data from 2 species in dilution scenario testing is sufficient for estimating true dilutions needed to protect the receiving environment.

Overall, based on the ecotoxicity LoE, the discharge from LDP10 was unlikely to cause negative impact to receiving waters. However, LDP40, which is representative of the water quality expected for the intended large-scale WTP had the potential to negatively impact ecosystems in the receiving environment through biological effects on organisms. From the measurements undertaken and the analyses conducted it is still not obvious what is causing toxicity for the LDP40 sample. This is of significant concern, if the future WTP treated water will form the bulk of the flow in the catchment.

Table 9. Water quality parameters for LDP10 and LDP40 samples used in ecotoxicity testing (data are from ALS reports unless otherwise specified)[a]

Analyte	Units	Guideline Value[b]	Feb-22	May-22	Aug-22	Nov-22	Feb-22	May-22	Aug-22	Nov-22	Ecotoxicity Test Control Water
			LDP10	LDP10	LDP10	LDP10	LDP40	LDP40	LDP40	LDP40	DMW[f]
pH	pH unit	6.5-8	8.4	8.6	8.8	8.7	7.0	8.0	7.9	8.1	
pH (ESA)	pH unit	6.5-8	9.0	8.6	8.6	8.5	8.3	8.3	8.1	8.0	7.9-8.1
Conductivity	µS/cm	125-2200	1120	1610	1570	1090	137	175	182	182	
Conductivity (ESA)	µS/cm	125-2200	1113	1616	1579	1070	139	172	186	182	171-179
Dissolved Oxygen (ESA)	% saturation	85-110	81	83	106	91	90	88	97	95	98-100
Carbonate Alkalinity	mg $CaCO_3$/L	NV[c]	30	NM[e]	NM	57	NM	NM	NM	<1	
Bicarbonate Alkalinity	mg $CaCO_3$/L	NV[d]	436	738	685	510	70	89	86	94	
Total Alkalinity	mg $CaCO_3$/L	NV	467	NM	NM	567	NM	NM	NM	94	57-64[h]
Aluminium	µg/L	55 (pH>6.5)	600	140	100	250	<10	<10	<1	0.9	
Arsenic	µg/L	24 (III); 13 (V)	3	NM	NM	2	NM	NM	NM	0.2	
Cadmium	µg/L	0.2	<0.1	NM	NM	<0.1	NM	NM	NM	<0.02	
Cobalt	µg/L	1.4[g]	1	<1	<1	<1	<1	<1	<1	0.04	
Copper	µg/L	1.4	2	3	3	1	<1	<1	<1	0.07	
Manganese	µg/L	1900	5	NM	NM	<1	NM	NM	NM	0.16	
Nickel	µg/L	11	16	14	13	11	<1	3	<1	0.3	
Zinc	µg/L	8	<5	<5	<5	<5	8	<5	<5	0.8	
Ammonia	µg/L	900	30	NM	NM	20	NM	NM	NM	284	
Nitrite + Nitrate (NOx)	µg/L	40	180	150	30	120	20	60	90	37	
Total Kjeldahl Nitrogen	µg/L	NV	400	200	200	400	400	200	400	NM	
Total Nitrogen	µg/L	350	600	400	200	500	400	300	500	370	
Toxic to *C. dubia*			Not toxic	Not toxic	Not toxic	Not toxic	**Toxic**	**Toxic**	Not toxic	**Toxic**	
Toxic to *M. splendida*			Not toxic	Not toxic	Not toxic	Not toxic	Not toxic	Not toxic	Not toxic	**Toxic**	

[a] Values outside of GV range appear in red; metal concentrations are dissolved (0.45 µm filterable); [b] Water quality guidelines for pH, conductivity and total nitrogen for lowland east-flowing NSW coastal rivers (ANZECC/ARMCANZ 2000, since there are no updated default guidelines for these in ANZG, 2018). Water quality guidelines for metals are reported as those for moderately-to-disturbed ecosystems (95% species protection values); [c] No guideline value available in ANZG (2018); [d] Vera et al. (2014) reported a bicarbonate EC10 for 7-d *C. dubia* (Australian isolate) of 340 mg/L, and the Office of Environment and Heritage (2012) calculated an interim GV for bicarbonate of 225 mg/L, based on acute North America freshwater data with an acute to chronic ratio applied, therefore values above 225 mg/L bicarbonate are likely to be harmful; [e] NM = not measured.
[f] Dilute mineral water; [g] unknown reliability (as defined by Warne et al., 2018); [h] USEPA, 2002

3.5 Long-term ecotoxicity (LDP10 waters collected 2013 – 2022)

The ecotoxicity of LDP10 water was measured using the chronic *C. dubia* reproduction and survival test and the acute *M. splendida* larval imbalance test from June 2013 to the most recent sampling even in November 2022 (Figure 29), with the exception that fish testing was not carried out during 2019. Also note that testing with other species in previous years are not captured on the graph in Figure 29.

Water from LDP10 was not toxic to rainbowfish larval imbalance in 2022, compared to previous years when of the twelve samples collected across 2013 to 2016, nine were toxic to larval imbalance. Waters from LDP10 in 2022 were also not toxic to *C. dubia* survival and reproduction. This is an improvement compared to previous years where some level of toxicity was usually observed to this species. It is not possible to determine whether previous events of elevated toxicity were related to any specific toxicant or stress, since water quality parameters were not measured in the same samples collected for ecotoxicity testing, i.e., water quality data for those years were from samples taken at other times during the year for the macrobenthic analysis. The alignment of sampling for water quality and ecotoxicity in 2021 has been an improvement to the program, allowing better cross comparison of key drivers to toxicity. The continuation of this approach will allow for longer-term trends to be identified.

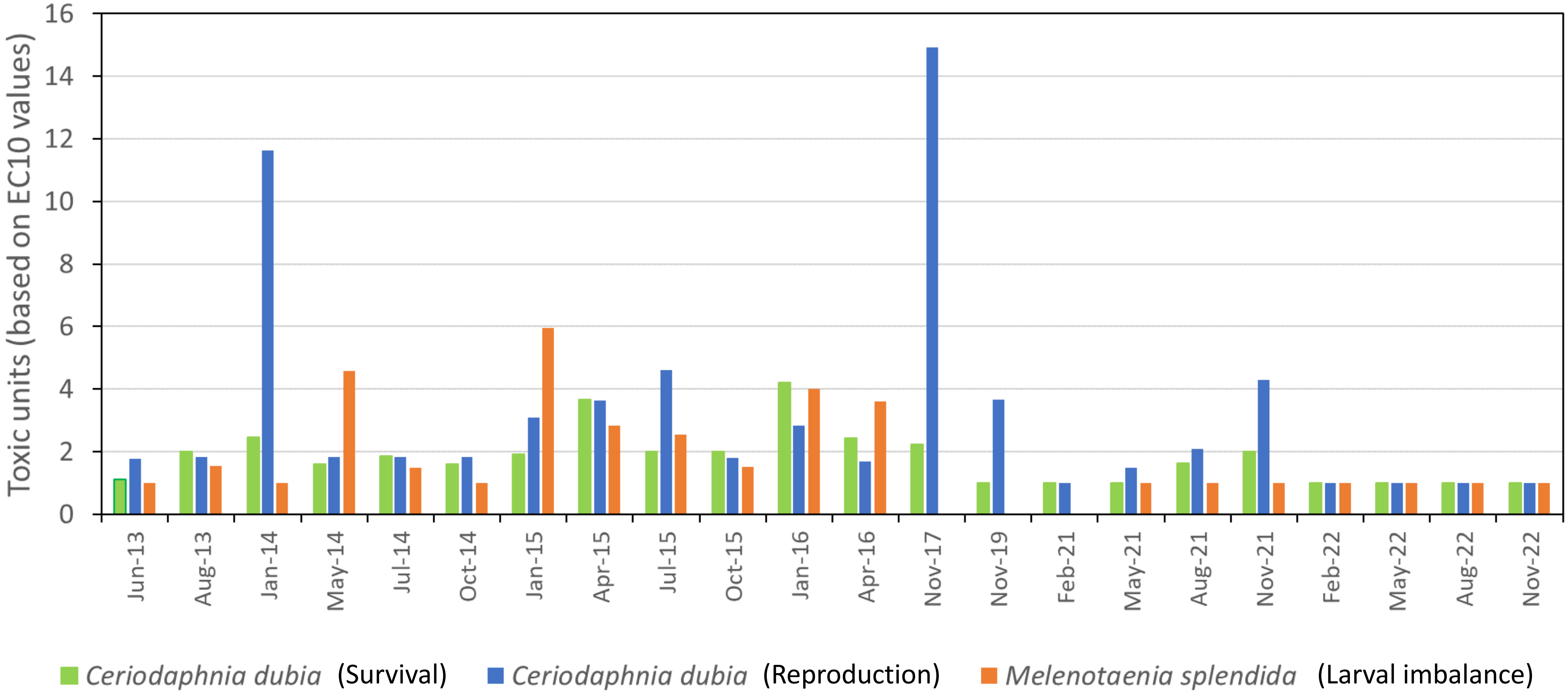


Figure 29. Ecotoxicity of water from LDP10 to *Ceriodaphnia dubia* survival and reproduction and *Melenotaenia splendida* (rainbowfish) larval imbalance from June 2013 to November 2022

3.6 Macrobenthic results

The description of macrobenthic macroinvertebrate community structure for autumn and spring 2022 is consistent with previous reports. Macrobenthic community composition is considered an acceptable water quality indicator LoE for river and stream health commonly used in freshwater river health assessment. Based on the diversity and composition of the invertebrates in the system the community structure can indicate stress or levels of stream health. The SIGNAL scores contribute to the health assessment and here we also describe the interactions of the macroinvertebrate communities on the sampling occasions with the environmental variables measured.

3.6.1 Macrobenthic composition

The macrobenthic community structure was investigated to compare reference and discharge monitoring sites. On both sampling occasions, the macrobenthic community structures in reference sites were different to those in the discharge monitoring sites. The similarities/differences between macroinvertebrate assemblages sampled in autumn and spring in 2022 are presented in the ordination plots in Figure 30 and Figure 31. The ordination plots (Figure 32 and Figure 33) show the aggregation of the reference sites in blue and the aggregation of the separate discharge monitoring sites in green. For both spring and autumn, the ordination plots highlight that the discharge monitoring sites were more closely clustered together than the reference sites, indicating that the discharge monitoring macrobenthic communities were more similar to each other. In autumn 2022, two distinct macrobenthic communities for the treatments (reference and discharge monitoring) were clearly separated from each other in the ordinations.

PERMANOVA tests were undertaken to investigate macrobenthic community structure differences in treatments and season. The results of the PERMANOVAs testing for differences in macroinvertebrate community composition between sampling timepoints (autumn and spring 2022) and treatments are presented in Table 10. On both occasions, there were significant differences in the composition in macrobenthic communities between the reference and discharge monitoring treatments: autumn 2022 (PERMANOVA: Pseudo-F=12.5, p=0.0001); spring 2022 (PERMANOVA: Pseudo-F=9.4, p= 0.001). Significant differences in community composition were found with respect to time (season) (PERMANOVA: Pseudo-F=4.7, p=0.0001) and treatment (reference or discharge monitoring) (PERMANOVA: Pseudo-F=19.3 p=0.0001), when tested individually. In addition, there was a significant interaction between season and treatment (PERMANOVA: Pseudo-F=2.4, p=0.009). The PERMANOVA results confirm that the macrobenthic communities in the reference treatment are different to those in the discharge monitoring treatment and that the communities are different in autumn compared with spring 2022. Consistent with previous years (2018-2021), there were marked differences in the macrobenthic community composition between the reference and discharge monitoring treatments shown in the nMDS ordinations for autumn and spring 2022 the differences in composition were confirmed by the PERMANOVA tests on the macrobenthic data.

Table 10. Results of PERMANOVA testing for variation in all macrobenthic community composition data (2022) between sampling timepoints (season), sites (reference vs discharge monitoring)

Factor: source of variation	***df***	***MS***	***Pseudo-F***	***P(perm)***	***Unique perms***
Autumn 2022 treatment	1	22484	12.5	**0.001**	9920
Spring 2022 treatment	1	16207	9.4	**0.001**	9930
Season	1	8542	3.8	**0.0006**	9930
Treatment (both seasons)	1	35066	19.3	**0.0001**	9932
Season*Treatment	1	4418	2.4	**0.0092**	9926
Res	86	1817			
Total	89				

Df: degrees of freedom; MS: mean squares; Pseudo-F: multivariate analogue to Fisher's F test statistic of the null hypothesis; P(perm): probability by permutations; Unique Perms: number of unique permutations. Treatment refers to reference and discharge monitoring.
Bold values denote significance at p< 0.05.

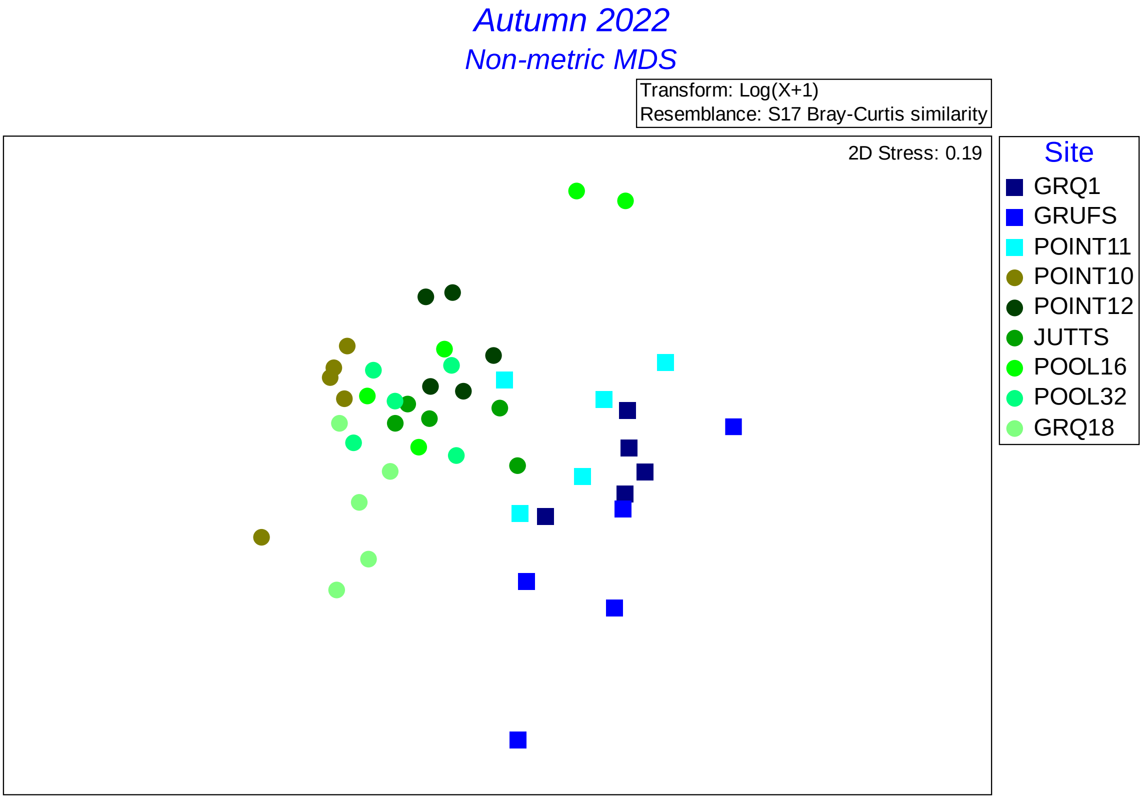


Figure 30. non-metric Multidimensional Scaling (nMDS) of macrobenthic communities autumn (2022)

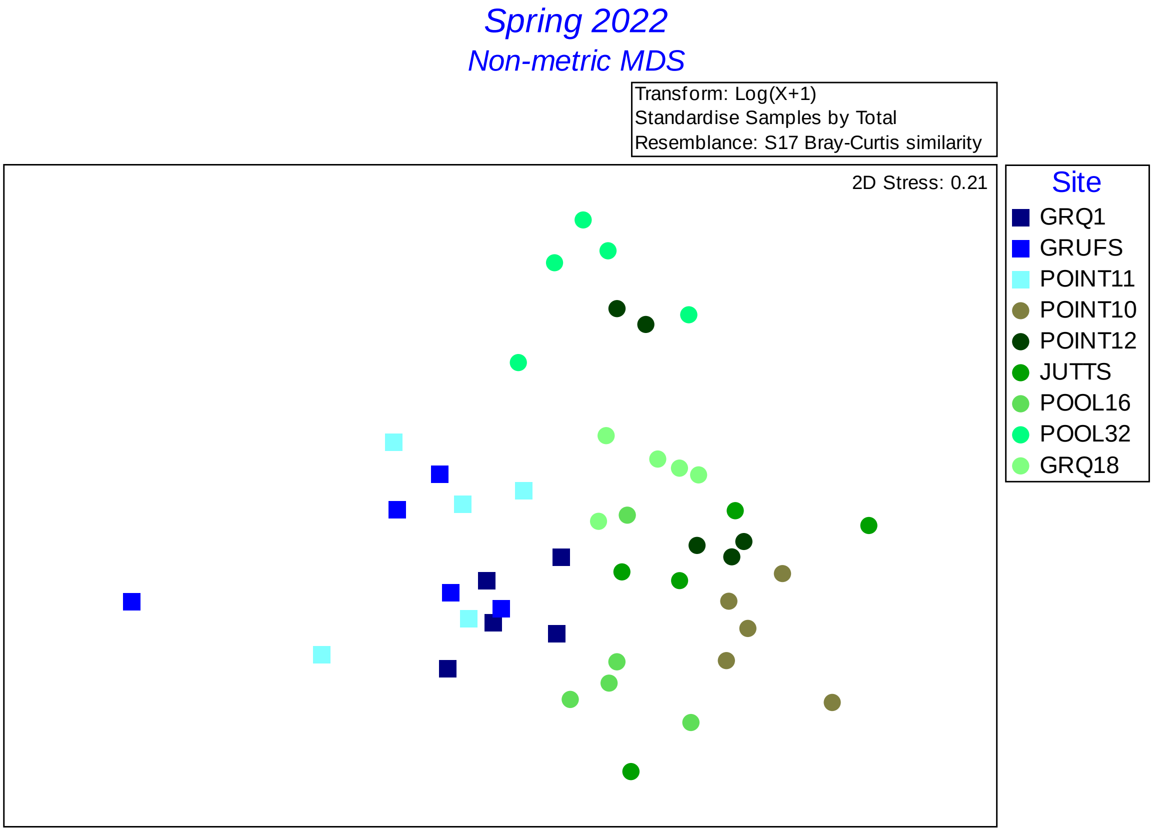


Figure 31. non-metric Multidimensional Scaling (nMDS) of macrobenthic communities spring (2022)

SIMPER analysis of macrobenthic communities

The key taxa contributing to significant differences between treatments were identified using Primer's SIMPER function, Primer 7+ (Plymouth Marine Laboratory, UK). The top five taxa which discriminated between the reference and discharge monitoring treatments on each occasion are shown in Table 11**.** In autumn 2022, key taxa which contributed most to the observed differences in compositions in the discharge monitoring treatment were: Caenidae, Chironominae and Tanypodinae (Table 11). In autumn 2022, the taxa which were higher in abundance in the discharge monitoring sites and had a positive relationship with discharge monitoring sites included Caenidae, Chironominae, Tanypodinae and Oligochaeta. In spring 2022, the reference treatment had higher abundances of Leptophlebiidae, and there was a positive relationship with reference treatments and abundances of Leptophlebiidae in spring 2022. Chironominae, Tanypodinae, Dytiscidae and Caeindae were more abundant in the discharge monitoring sites than the reference sites in spring 2022. The SIMPER analysis, which explains the dissimilarity between treatment composition, found the reference sites to contain more organisms from the family Leptophlebiidae while the discharge monitoring sites contained organisms from the families Caenidae and Tanypodinae, which are regarded as tolerant invertebrate taxa (Chessman, 2003; Walsh 2006). Leptophlebiidae have been identified as a potential indicator of health for this system, with this taxon considered to be sensitive to contamination (SIGNAL=8) (Chessman, 2003). In general, the discharge monitoring sites had macrobenthic communities composed of more tolerant invertebrate taxa while those at reference sites included more sensitive taxa. There could be multiple factors explaining the different taxa present in the reference and discharge monitoring sites including differences in habitat, pool depth, turbidity, and substrate type.

Table 11. SIMPER results illustrating the top 5 taxa which contributed to differences between the reference and discharge monitoring sites in autumn and spring 2022

Year	Season	Family	Reference Average abundance	Discharge Monitoring Average abundance	(%) contribution of total dissimilarity
2022	Autumn	Caenidae	0.27	21.4	16.3
		Chironominae	2.13	14.9	12
		Tanypodinae	2.27	11	9
		Leptophlebiidae	4.9	5.7	9
		Oligochaeta	0.13	10.3	9
2022	Spring	Chironominae	1.6	2.4	12
		Leptophlebiidae	1.8	0.6	10
		Dytiscidae	0.3	1.6	10
		Caenidae	0	1.3	8
		Tanypodinae	1.2	1.4	7

3.6.2 Relationships between macrobenthic communities and water quality

Multivariate correlative statistics were undertaken to understand how the macroinvertebrate communities were responding to the water quality variables measured. Correlative patterns were studied to identify which environmental factors were driving the macrobenthic community composition and to identify key relationships between the macroinvertebrate communities and the measured water quality variables. Distance-based linear modelling (DistLM) was used to correlate environmental variables to the composition of the macrobenthic community composition for each sampling occasion and the correlative relationships between the macrobenthic communities and water quality for each sampling are presented in Figure 32 and Figure 33.

Autumn 2022

The distance-based analysis of the autumn 2022 data is presented in Figure 32. The fitted DistLM was visualised using a distance-based redundancy analysis (dbRDA) constrained ordination demonstrating the correlation of significant variables on the autumn 2022 macrobenthic community. Approximately 80% of the variation of the macrobenthic data sampled in autumn 2022 could be explained by the measured environmental variables conductivity, alkalinity, pH and aluminium (Table 12). When examined collectively, conductivity was the only variable which was significantly correlated ($p<0.05$), explaining approximately 48% of the total variation of the macrobenthic community structure. The dbRDA (Figure 32) shows that axis 1 (dbRDA 1), (which corresponds to conductivity,) is explaining approximately 50.3% of the total variation and axis 2 (dbRDA 2) (corresponding to pH and alkalinity) is explaining approximately 14.7% of the total macrobenthic variation. Figure 32, clearly shows the separation of the blue reference sites to the right of the ordination and the green discharge monitoring sites to the left of the ordination, mostly driven by conductivity.

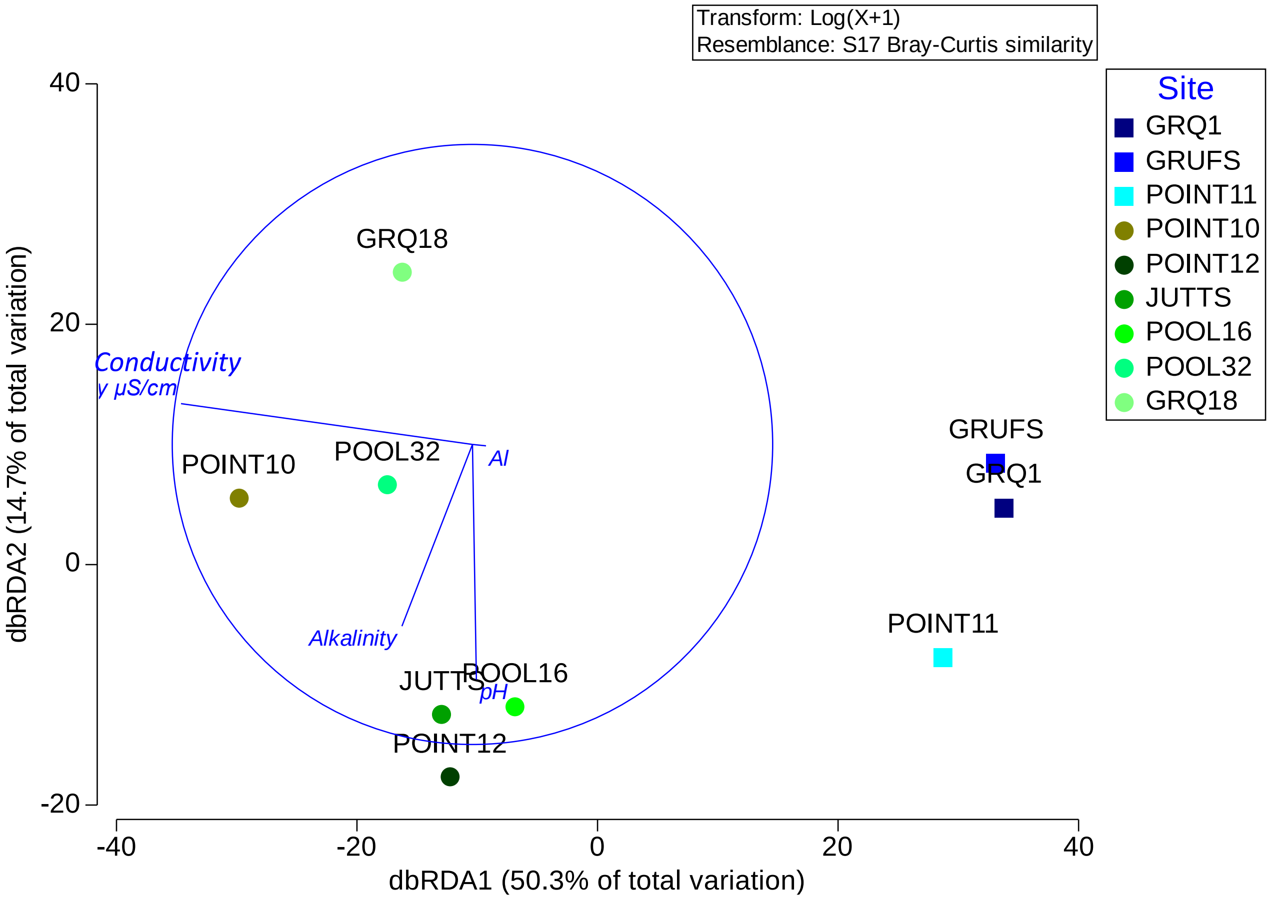


Figure 32. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and macrobenthic composition from autumn 2022

Table 12. Sequential test results of distance-based linear model (DistLM) autumn 2022

Variable	Adj R^2	SS(trace)	Pseudo-F	P	Prop	Cumulative contribution	res.df
+Conductivity µS/cm	0.41	4696	6.55	**0.0018**	0.48	0.48	7
+Alkalinity	0.47	1126	1.74	0.0983	0.12	0.60	6
+pH	0.55	1143	2.08	0.0673	0.12	0.71	5
+Aluminium	0.60	782	1.59	0.1895	0.08	0.79	4

SS (trace): sum of squares; Pseudo-F: multivariate analogue to Fisher's F test statistic of the null hypothesis; P: probability; Prop: the proportion (%) of variation; res.df: residual degrees of freedom.

Bold values denote significance at p< 0.05.

Spring 2022

The distance-based analysis of the spring 2022 data found that the measured water quality variables of pH, alkalinity, aluminium, conductivity, nickel, zinc and cobalt combined explained 97% of the total variation in the macrobenthic data. When examined collectively, only pH (30%) was shown to significantly contribute to a proportion of the variation in the data (Table 13). The fitted DistLM was visualised using a distance-based redundancy analysis (dbRDA) constrained ordination (Figure 33) demonstrating the correlation of significant variables on the spring 2022 macrobenthic community. The dbRDA (Figure 33) shows that dbRDA 1 (pH, conductivity) is explaining 34.6% of the total variation and dbRDA 2 is explaining 32% (metals and alkalinity) of the total variation (Figure 33). The composition of the water quality variables driving Point 10, Point 12, and Jutts differed to those for Point 11, GRUFS and GRQ1. The ordination dbRDA (Figure 33) shows GRQ18, Pool 16 and Pool 32 were separated from the upper discharge sites (Point 10, Point 12, Jutts) in spring 2022.

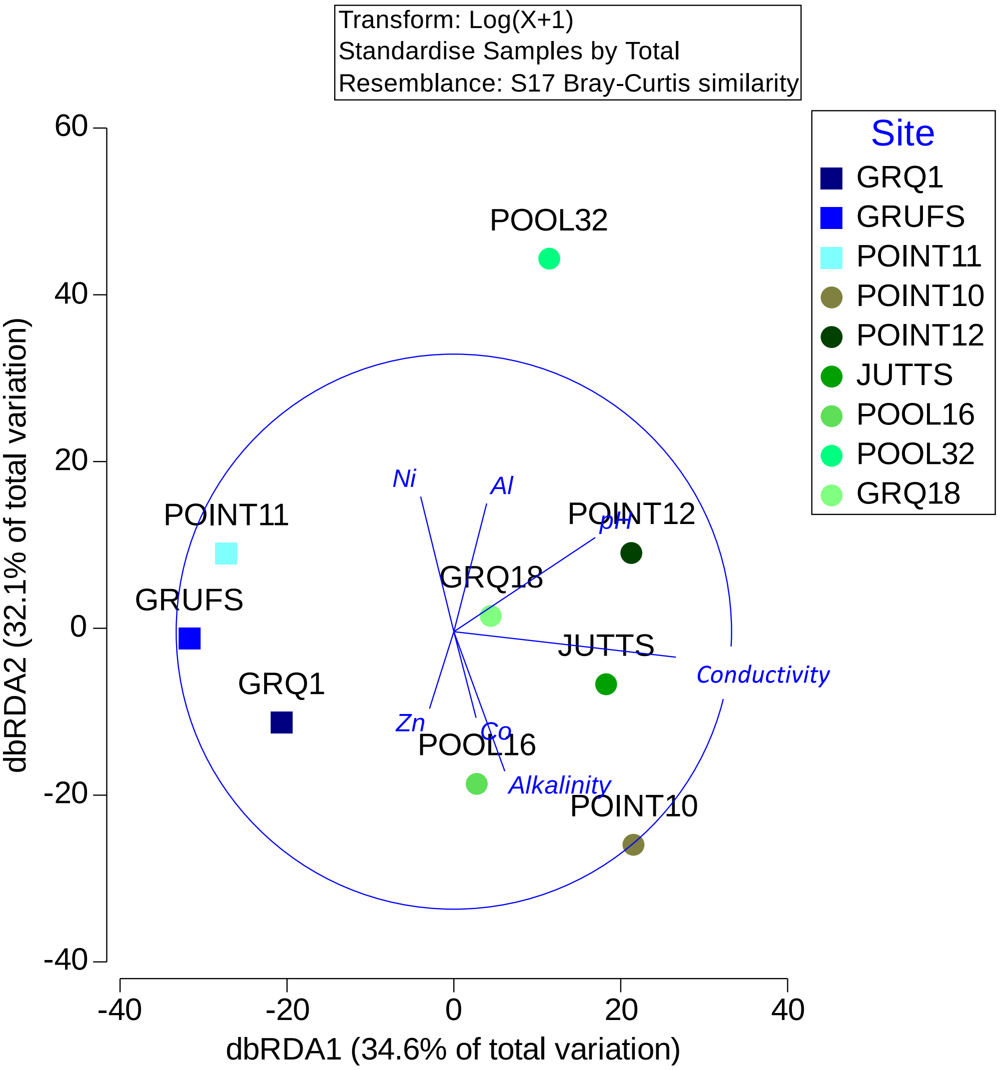


Figure 33. Ordination plot derived from the distance-based model illustrating the relationships between environmental variables and macrobenthic composition from spring 2022

Table 13. Sequential test results of distance-based linear model (DistLM) spring 2022

Variable	Adj R^2	SS(trace)	Pseudo-F	P	Prop	Cumulative contribution	res.df
+pH	0.20	3131.2	3.03	**0.0045**	0.30	0.30	7
+Alkalinity	0.32	1925.3	2.18	0.1124	0.19	0.49	6
+Aluminium	0.45	1715.9	2.39	0.0816	0.17	0.65	5
+Conductivity µS/cm	0.48	895.56	1.33	0.2739	0.09	0.74	4
+Nickel	0.64	1296.1	2.78	0.1268	0.13	0.86	3
+Zinc	0.75	754.98	2.34	0.193	0.07	0.94	2
+Cobalt	0.79	370.36	1.35	0.4301	0.04	0.97	1

SS(trace): sum of squares; Pseudo-F: multivariate analogue to Fisher's F test statistic of the null hypothesis; P: probability; Prop: the proportion (%) of variation; res.df: residual degrees of freedom.

Bold values denote significance at p< 0.05.

3.6.3 SIGNAL scores

The SIGNAL scores from the autumn and spring macrobenthic surveys performed in 2022 are presented in Figure 34 and Table 15. Reference sites had variable, but overall higher SIGNAL scores compared to the long-term historical mean (2013-2021) in autumn and spring in 2022 (Figure 34). The only reference site and sampling occasion where the SIGNAL score was below the historical mean was GRQ1 in spring 2022, all remaining reference sites and occasions were at or above the historical mean SIGNAL score. The SIGNAL scores for the discharge sites varied and improvements were observed in GRUFS, Jutts and Pool32 sites compared to the historical mean scores. Mean SIGNAL scores, standard errors and One-Way ANOVA results for the reference and discharge monitoring treatments are presented in Table 14. Mean SIGNAL (±S.E.) scores for the reference treatment in autumn 2022 (4.8±0.24) and spring 2022 (4.6±0.17) were significantly greater than those for the discharge monitoring treatment on each sampling occasion (autumn 2022,3.9 ± 0.14 and spring 2022, 3.8 ±0.16 based on One-Way ANOVA analyses (autumn 2022: F=11.7, p=0.01; spring 2022: F=12.2, p=0.01). The 2022 SIGNAL scores and potential chessman ranking (Table 15) were mostly consistent with previous years, however there were improvements observed in GRUFS, improving to probably doubtful water quality, compared with previous years (moderate contamination). Pool 32 also improved SIGNAL ranking from the 2021 ranking of probable severe contamination, in 2022, the improved SIGNAL score changed the Pool 32 ranking to probable moderate contamination (Table 15).

Table 14. One-way ANOVA results on macrobenthic SIGNAL values for reference and discharge monitoring treatments

SIGNAL	Reference	Reference	Discharge monitoring	Discharge monitoring	One-Way ANOVA	
Sampling time	**mean**	**± S.E.**	**mean**	**± S.E.**	***F***	***P-value***
Autumn 2022	4.8	0.24	3.9	0.14	11.7	**0.01**
Spring 2022	4.6	0.17	3.8	0.16	12.2	**0.01**

Bold values denote significance at p< 0.05.

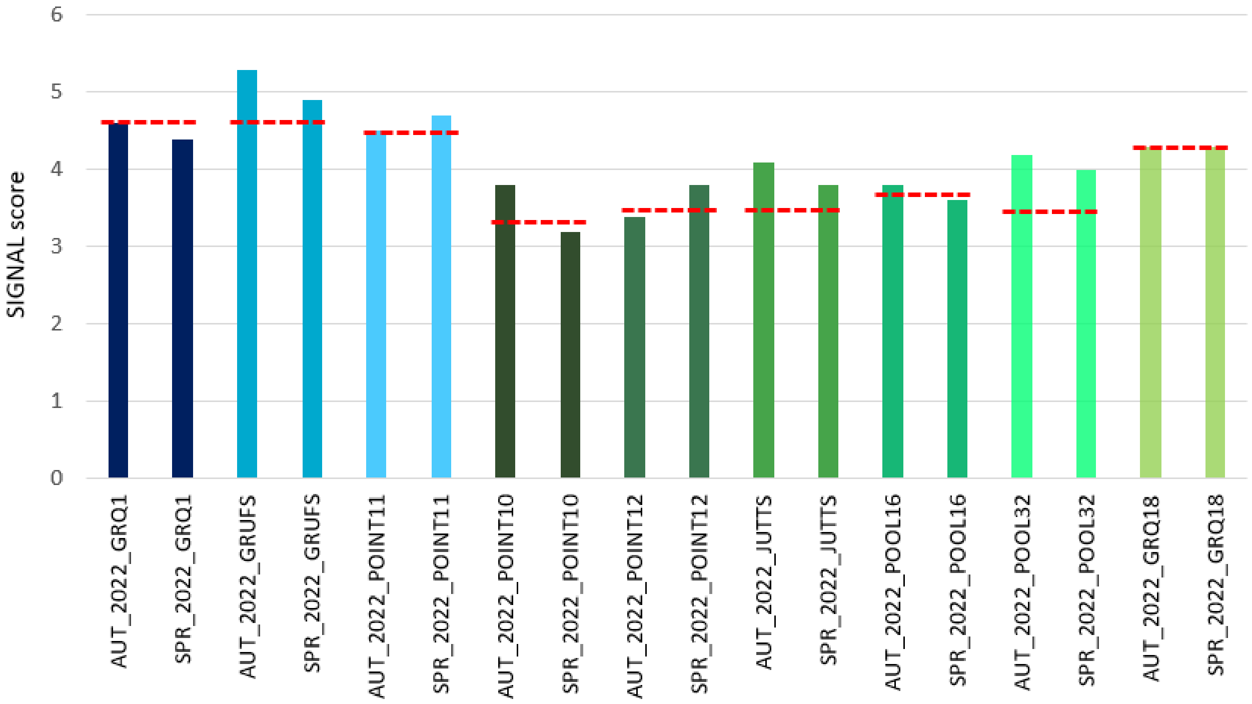


Figure 34. Signal scores from autumn and spring 2022

Reference sites (blue) and discharge monitoring sites (green). Red dashed line indicates mean SIGNAL scores from historical data 2013-2021.

Table 15. SIGNAL scores and rankings for each site (2022)

Treatment	Year	Season	Site	Potential ranking*	SIGNAL
Reference	2022	Autumn	GRQ1	Probable moderate contamination	4.6
	2022	Spring	GRQ1	Probable moderate contamination	4.3
Reference	2022	Autumn	GRUFS	Probable doubtful water quality	5.3
	2022	Spring	GRUFS	Probable moderate contamination	4.9
Reference	2022	Autumn	Point 11	Probable moderate contamination	4.5
	2022	Spring	Point 11	Probable moderate contamination	4.7
Discharge monitoring	2022	Autumn	Point 10	Probable severe contamination	3.8
	2022	Spring	Point 10	Probable severe contamination	3.2
Discharge monitoring	2022	Autumn	Point 12	Probable severe contamination	3.4
	2022	Spring	Point 12	Probable severe contamination	3.8
Discharge monitoring	2022	Autumn	Jutts	Probable moderate contamination	4.1
	2022	Spring	Jutts	Probable severe contamination	3.8
Discharge monitoring	2022	Autumn	Pool 16	Probable severe contamination	3.8
	2022	Spring	Pool 16	Probable severe contamination	3.6
Discharge monitoring	2022	Autumn	Pool 32	Probable moderate contamination	4.2
	2022	Spring	Pool 32	Probable moderate contamination	4.0
Discharge monitoring	2022	Autumn	GRQ18	Probable moderate contamination	4.3
	2022	Spring	GRQ18	Probable moderate contamination	4.3

*Potential rankings based on Chessman (1995)

3.7 Long-term patterns in macrobenthic community attributes

3.7.1 SIGNAL (2013-2022)

Long-term SIGNAL scores for all sites sampled between 2013 and 2022 are illustrated in Figure 35. Based on the classifications for SIGNAL by Chessman (1995), on average, at the times of sampling, the reference sites can be considered of ‘probable moderate contamination’ and the discharge monitoring sites of ‘probable severe contamination’ (Table 16). The exception being the most distant discharge monitoring site (GRQ18) which was classified as ‘probable moderate contamination’. The long-term mean (±S.E.) SIGNAL scores for the reference sites (4.6±0.12) were greater than the discharge monitoring sites (3.7±0.14). The long-term mean SIGNAL scores for the reference and discharge monitoring were significantly different between the two treatments (ANOVA: F=13.5, p=0.007) (Figure 35).

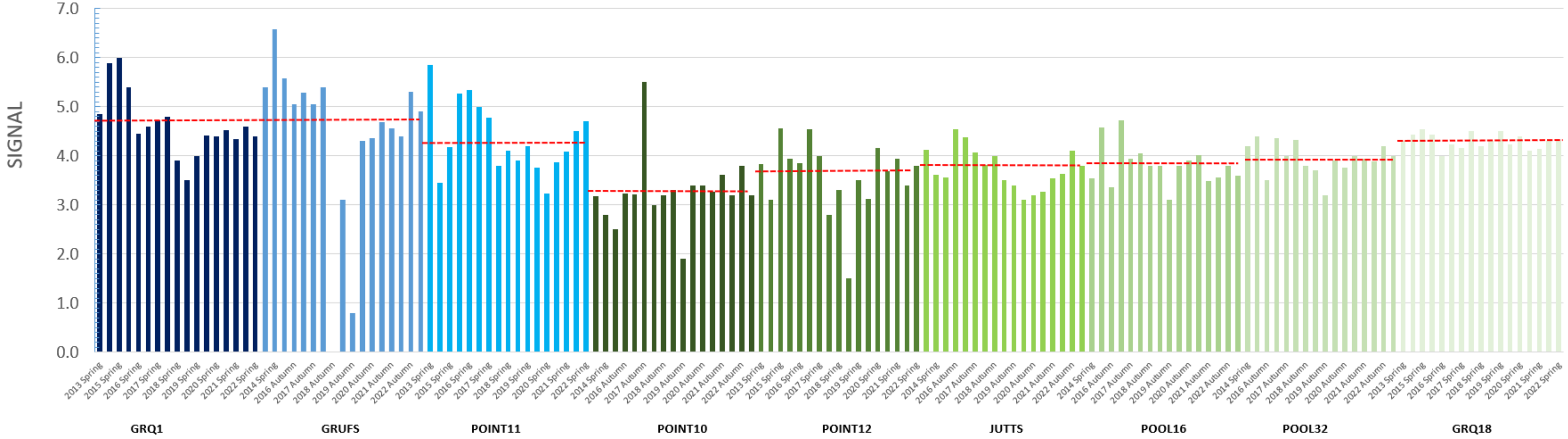


Figure 35. Long-term SIGNAL scores for sites (2013-2022)

Reference sites (blue) and discharge monitoring sites (green). Dotted red lines represent the historical mean value for each site for the period 2013- 2022.

Table 16. Mean SIGNAL scores for each site (2013-2022)

Treatment	Site	Potential ranking*	Mean SIGNAL	Minimum	Maximum
Reference	GRQ1	Probable moderate contamination	4.7	3.5	6
Reference	GRUFS	Probable moderate contamination	4.7	0.8	6.6
Reference	Point 11	Probable moderate contamination	4.4	3.2	5.9
Discharge monitoring	Point 10	Probable severe contamination	3.3	1.9	5.5
Discharge monitoring	Point 12	Probable severe contamination	3.6	1.5	4.6
Discharge monitoring	Jutts	Probable severe contamination	3.7	3.1	4.5
Discharge monitoring	Pool 16	Probable severe contamination	3.8	3.1	4.7
Discharge monitoring	Pool 32	Probable severe contamination	3.9	3.2	4.4
Discharge monitoring	GRQ18	Probable moderate contamination	4.3	4.0	4.5

*Potential rankings based on Chessman (1995).

Some small changes to the SIGNAL scores have occurred over time. When observing the SIGNAL scores from 2013 to 2022 there was a significant difference between those observed for the two treatments. The scores were on average higher in the reference sites. The improved SIGNAL scores also place the reference sites in a better Chessman SIGNAL classification. On average, at the times of sampling, the reference sites can be considered: of 'probable moderate contamination' and the discharge monitoring sites of 'probable severe contamination' the exception being the most distant discharge monitoring site (GRQ18) which was classified as 'probable moderate contamination'. Although we have provided ecological rankings for each site based on their long-term mean SIGNAL scores (Table 16), these scores varied widely within sites. Consequently, these rankings should be limited to emphasising that based on the SIGNAL approach, the reference sites showed better ecological condition than discharge monitoring sites Point 10, Point 12, Jutts, Pool 16 and Pool 32, rather than any specific gradient ranking.

3.7.2 Leptophlebiidae genera of interest (2016-2022)

Leptophlebiidae are recognised as sensitive macroinvertebrate taxa (Chessman, 1995). It has been suggested that specific Leptophlebiidae species are sensitive to conductivity and ionic balance (Cardno, 2010), leading to the recommendation by the Georges River Working Group to examine this group at the genus level. The abundances of the three sensitive Leptophlebiidae taxa of interest: *Atelophlebia* spp*, Ulmerophlebia* spp and *Thraulophlebia* spp for the nine sites from 2016 through to 2022 are presented in Figure 36. As indicated in Figure 36, both the abundance and the occurrence of all three Leptophlebiidae genera were higher in the reference treatment than the discharge monitoring treatment. It should be noted that Leptophlebiidae abundance was greatest at GRQ1 in spring 2021 and spring 2022. Figure 36 shows there is clearly a greater abundance of Leptophlebiidae in reference sites compared with discharge sites. There has generally been an increase in Leptophlebiidae abundance in the Point 11 reference site with the highest abundance observed in spring 2019. The results for Leptophlebiidae abundance across seasons were variable over the years without a clear trend for genera associated with seasons. One difference observed in 2022 was that in autumn 2022, GRQ18 had the highest abundance of Leptophlebidae across all sites while in spring 2022 GRQ1 had the greatest Leptophlebiidae abundance across all sites. Of the discharge sites, GRQ18 shows the highest frequency of Leptophlebiidae taxa and, of the three taxa, GRQ18 is dominated by *Atelophlebia* spp. This differs from other reference sites, which generally have higher abundances of *Ulmerophlebia* spp and *Thraulophlebia* spp than *Atelophlebia* spp. This suggested different habitat and environmental preferences between the Leptophlebiidae species.

Leptophlebiidae taxa were rare in the discharge monitoring sites across both seasons, in autumn they were completely absent from sites Point 12 and Pool 32 and in very low frequencies in Pool 16 and Jutts. In spring 2022, no Leptophlebiidae were recorded for sites Point 12, Pool 16 and Pool 32 and only one individual was recorded at sites point 10 and Jutts. *Atelophlebia* spp were detected in the discharge monitoring sites Point 12, Jutts and Pool 16 in spring 2021 and this is a change in detection from recent years with similar flows*.* *Ulmerophlebia* spp were most abundant in reference sites GRQ1 and GRUFS across all years*.* *Ulmerophlebia* spp were rarely observed in the discharge monitoring sites, historically being recorded at Jutts and GRQ18 in 2016. *Atelophlebia* in 2018 – 2022 have been more abundant compared to earlier years 2016-2017. *Thraulophlebia* spp was predominantly observed in the reference treatment but was also present at downstream site GRQ18. The analysis of the 2016-2022 data also showed that *Atelophlebia* spp*,* *Ulmerophlebia* spp and *Thraulophlebia* spp were observed far more frequently and in higher abundances in the reference sites. *Atelophlebia* spp were more abundant at GRQ18 in 2020, 2021 and in 2022 in GRQ18. *Atelophlebia* spp remained rare however across all remaining discharge monitoring sites in 2022. Leptophlebiidae have been reported to have ecological habitat preferences including riparian vegetation shade cover, low turbidity, flowing water and riffle habitats (Corbin and Goonan 2010). Leptophlebiidae are known to have physical habitat preferences and it is there for important to consider the physical habitat features of the sites, in addition to water flow and water quality where Leptophlebiidae are present rather than water chemistry alone. The physical habitats and riparian substrates are very different in the reference sites compared with the discharge monitoring sites. For example, Point 11 is considered an ephemeral water body. Observational evidence (pers. Obs David Gregory, South32, 2019) also suggests that the structural complexity of the sites varies greatly between the reference sites and the discharge monitoring sites, with the former containing more complex habitats, including structures such as log jams and greater riparian pool vegetation. Some of the discharge monitoring sites appear to also be unsuitable habitat for Leptophlebidae taxa. Consequently, the observed differences between the two treatments are likely due to a combination of the discharge waters and macroinvertebrate habitat condition.

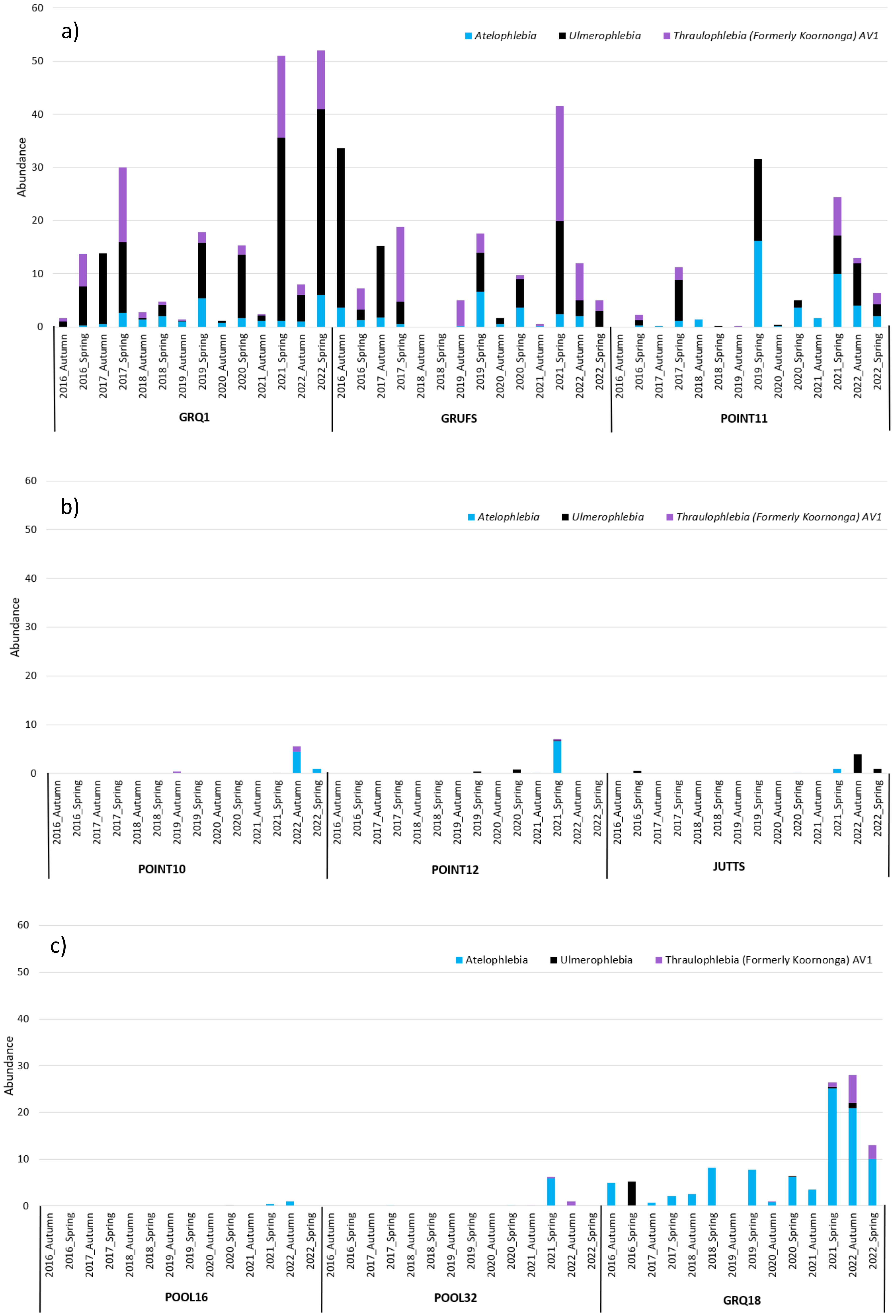


Figure 36. Abundances of *Atelophlebia* spp, *Ulmerophlebia* spp and *Thraulophlebia* (formerly *Koornonga*) spp (2016-2021) for a) reference sites GRQ1, GRUFS and Point 11; b) discharge monitoring sites Point 10, Point 12 and Jutts; c) discharge monitoring sites Pool 16, Pool 32 and GRQ18

3.8 Metabarcoding results

Similar to that done in 2021 (Stephenson et al., 2022), the prokaryote, eukaryote and diatom metabarcoding diversity and community structure for the 2022 sampling was described and used to assess for differences in biological communities in sediments between reference and discharge monitoring sites. The correlative patterns of the prokaryotic, eukaryotic and diatom metabarcoding community structures with the water quality parameters measured are also presented here.

3.8.1 16S rDNA metabarcoding (prokaryotes)

Across the nine sites surveyed, a total of 13,814 OTUs were detected in 2022. The top 10 most abundant prokaryotic phyla detected at each site for 2022 are shown in (Figure 37). Proteobacteria were the most abundant prokaryotic phylum in the sediments at all sites surveyed. Proteobacteria are a bacterial phylum consisting of a phenotypically diverse phylogenetic lineage. Representative bacteria from this phylum are important ecologically because they play key roles in nutrient cycling (carbon, sulfur and nitrogen) in several ecosystems, including freshwater sediments (Kersters et al., 2006), and play a vital role in contaminant degradation (Feng et al., 2022). Proteobacteria have been previously reported to be the dominant phylum from microbial communities in freshwater sediments (Li et al 2019, Zhang et al 2020, Feng et al 2022). In 2022, the other abundantly found phyla were Acidobacteria, Planctomycetes and bacteria from unknown phyla. The phylum Bacteriodetes was relatively less abundant in 2022 than in previous years (Stephenson et al., 2022).

The top 20 most abundant families are shown in Figure 38 as bubble plots and have been ordered by phylum. Prokaryotic families from the phylum Proteobacteria accounted for at eight of the top 20 families detected in 2022. A large number of unknown (novel) bacteria were found in all sites examined. There are several prokaryotic families that change in abundance at the discharge monitoring sites relative to the reference sites. In the 2022 data (Figure 38), there are several prokaryotic families that increased in abundance at discharge monitoring sites relative to the reference sites, including Cyanobacteria, Comamonadaceae, Rhodobacteracea, and Verrucomicrobiacea. In 2021, (Stephenson et al., 2021), increases in relative abundance had been reported for Rhodobacteracea and Verrucomicrobiacea families. Conversely, there are also families that decreased in relative abundance at the discharge monitoring sites, including the Bradyrhizobiaceae, Isosphaeraceae, Nitrososphaeraceae, and to a lesser extent the Gemmataceae and Acetobacteraceae. In 2021, the Bradyrhizobiaceae were also reported to decrease in relative abundance (Stephenson et al., 2021). Biodegradation of xenobiotic organic substrates has been observed in taxa from the family Rhodobacteracea (Pujalte et al., 2014; Siddavattam et al., 2011; Strnad et al., 2010) and may potentially explain the increase in relative abundance of this family in discharge monitoring sites. Taxa from the Verrucomicrobiacea family are widely distributed and are a major phylogenetic grouping found in many different habitats, including soils and sediments (Hugenholtz et al., 1998; Rappe et al., 2003; Sangwan et al., 2005; Wise et al., 1997), however, this family is poorly characterised due to the relatively few species that have been isolated in pure culture (Yoon 2014). Members of the family Verrucomicrobiaceae have been identified as methanotrophs (Guerrero-Cruz et al., 2021) and have been observed from heavily polluted habitats (Dojka et al., 1998; Cho and Kim, 2000; Juretschko et al., 2002). Cyanobacteria or blue-green algae are naturally found in freshwater ecosystems and are an essential part of healthy water systems. Increases in relative abundance of Cyanobacteria may be in response to nutrient fluctuations (Lurling et al., 2018) or water level rises (Bakker and Hilt, 2015). The family Comamonadaceae contain taxa that are mostly environmental bacteria in water and soil habitats that are pristine as well as polluted (Willems, 2014). Taxa from the family Comamonadaceae have been isolated from various industrial settings including coal-tar contaminated aquatic sediments (Jeon et al., 2004), activated sludge for coke plant effluent (Felfoldi et al., 2011) and industrial wastewater (Zhang, 2013). Taxa from the Bradyrhizobiaceae family are metabolically versatile in nitrogen assimilation (Marcondes De Souza et al., 2014), playing a key role in biogeochemical nitrogen cycling and their relative abundance may change in response to disturbances in nitrogen levels. Members of the Isosphaeraceae family are chemoorganotophic aerobes that are common inhabitants of soils, wetlands, and freshwater habitats (Dedysh and Ivanova, 2020). Isosphaeraceae belong to the relatively unexplored bacterial phylum of Planctomyces and little is known about their potential functions in the environment (Ivanova et al., 2017). The Nitrososphaeraceae family belong to the archaeal phylum Thaumarchaeota and are ammonia-oxidising archaea (Konneke et al., 2005), which have been isolated from a diverse range of habitats (Hatzenpichler 2012), including freshwater sediments (French et al., 2012). Members of the Nitrososphaeraceae play a key role in biogeochemical nitrogen cycling. Some taxa from this family are obligate acidophilic ammonia oxidisers (Lehtovira-Morley et al., 2011) which may explain the decrease in relative abundance between the acidic reference sites and the alkaline conditions of the discharge monitoring sites. Nitrososphaeraceae are also chemoautotrophic, fixing inorganic carbon to produce biomass, and their ecological niche would presumably be in low organic carbon environments (Wu et al., 2022; Berg et al., 2015).

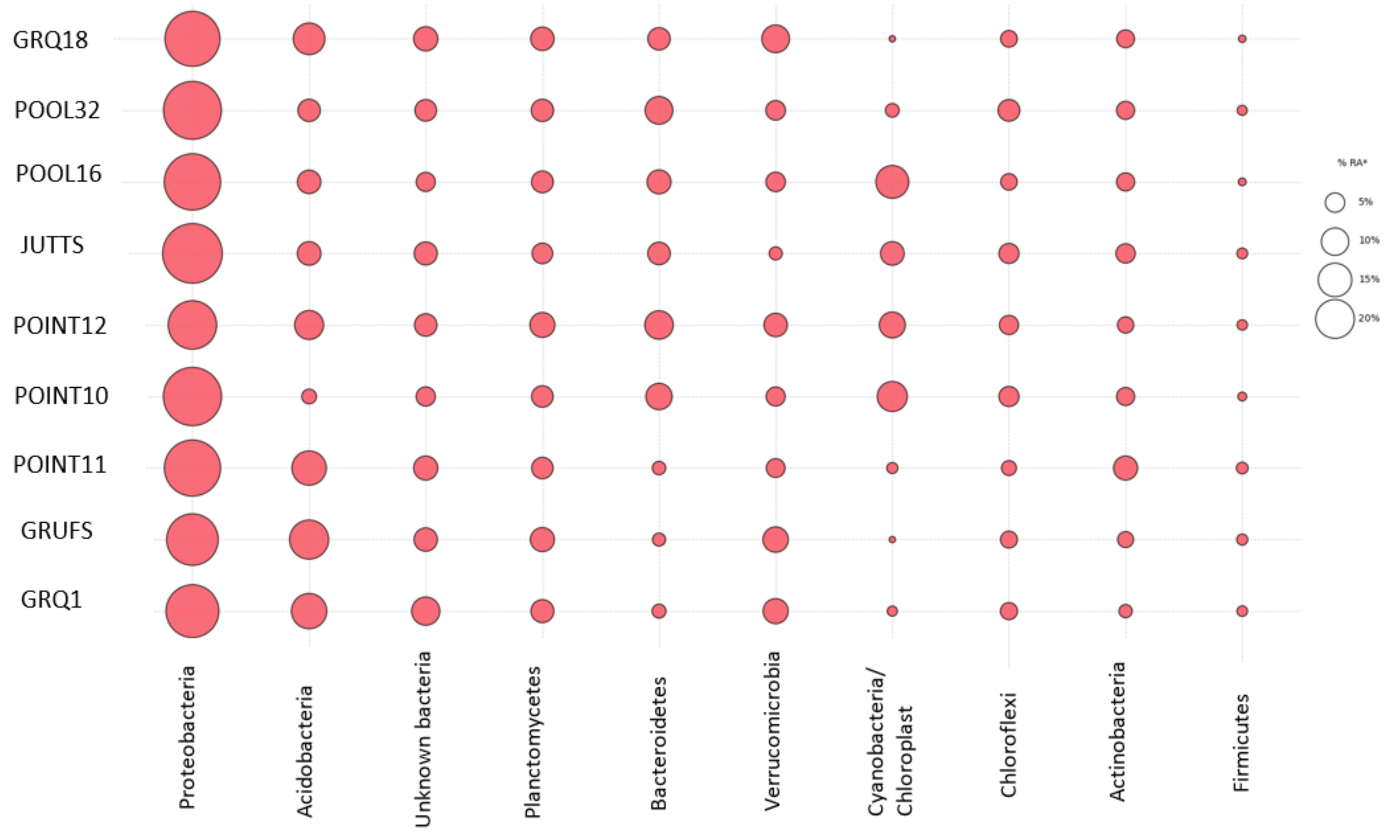


Figure 37. Bubble plot of the top 10 most abundant prokaryotic phyla (on average across all sites) for 2022

% RA is % relative abundance shown as bubble size.

Figure 38. Bubble plot of the top 20 most abundant prokaryotic families (on average across all sites) for 2022

Prokaryote richness

Prokaryote richness for the nine sites over 2020, 2021 and 2022 is presented in Figure 39. Prokaryote richness exhibited a range of values across most sites, treatments, and sampling occasions (Figure 39). Overall, across the sites, richness was higher in 2021 and 2022 compared with 2020. Prokaryote OTU richness was significantly different between the treatments in 2020 (Table 17). However, in 2021 and 2022, generally, OTU richness was variable across the treatments with no significant difference between treatments (Table 17). For all years Point 12 consistently had the highest prokaryote richness across the sites, potentially suggesting some enrichment at Point 12 which the bacteria communities are responding positively to.

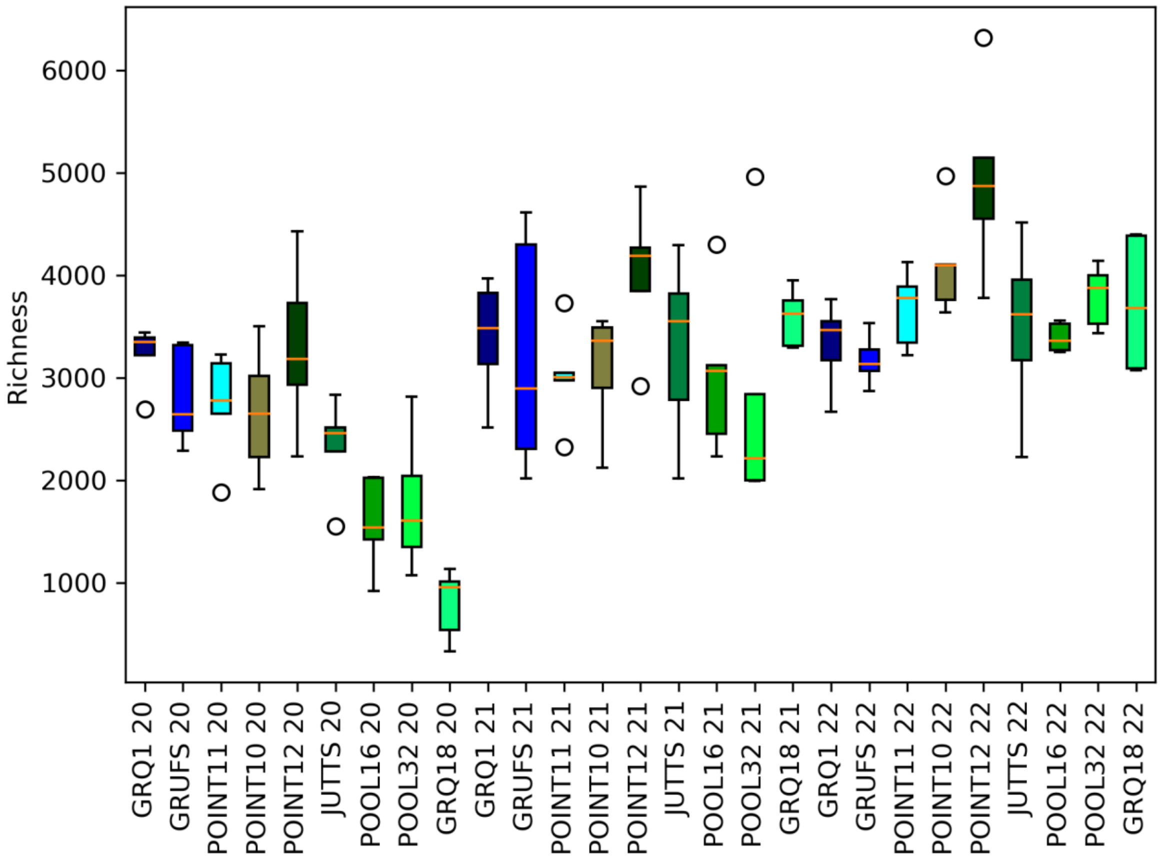


Figure 39. 16S prokaryote OTU richness from 2020, 2021 and 2022 OTUs

The interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively) are represented by the boxes and the line inside the box is the median. The whiskers represent the highest and lowest values within 1.5 times the IQR from the first and third quartiles, respectively. Outliers exceeding these values are represented as points. Reference sites (blue) and discharge monitoring sites (green).

Table 17. One-Way ANOVA results on 16S prokaryote richness for reference and discharge monitoring treatments

16S OTU Richness	Reference	Reference	Discharge monitoring	Discharge monitoring	One-Way ANOVA	
	mean	**S.E.**	**mean**	**S.E.**	***F***	***p-value***
Spring 2020	907	52.3	672	61.5	5.9	**0.019**
Spring 2021	603	54.2	553	42.2	0.5	0.48
Spring 2022	711	22.7	737	31.6	0.3	0.59

Bold values represent p<0.05

Prokaryote community composition

The prokaryote community compositions, at the OTU level, from the reference sites were markedly different to those from the discharge monitoring sites for 2020, 2021 and 2022. The separation of reference from discharge monitoring site prokaryote communities for 2020, 2021 and 2022 is visualised in the nMDS ordination plots in Figure 40. There was a clear clustering of the reference sites together, separated from the discharge monitoring sites (Figure 40). The prokaryotic communities observed at the discharge monitoring sites are more broadly spread across the nMDS compared to those of the reference sites which appear more tightly clustered across all years. The reference sites GRUFS and GRQ1 cluster closer together in the nMDS in 2020 and 2021, while Point 11 is more separated and spread, however in 2022 Point 11 clusters closer to the other two reference sites. In general, however, the discharge monitoring sites are clearly separated from the reference sites and show greater variability in prokaryote community structure.

PERMANOVA tests were undertaken to investigate prokaryote OTU community structure differences in treatments and time. The results of the PERMANOVAs testing for differences in 16SrDNA community composition between sampling timepoints (spring 2020, 2021 and 2022) and treatments are presented in Table 18. The visual separation of treatments observed in the nMDS (Figure 40) is confirmed by the PERMANOVA which found a significant difference in composition between the two treatments in spring 2020 (PERMANOVA: F=18.9, p=0.0001), spring 2021 (PERMANOVA: F=20.7 p= 0.0001) and in spring 2022 (PERMANOVA: F=22.2, p=0.0001). For each year, the prokaryote community composition was significantly different between reference and discharge monitoring sites. This did not change over time between 2020 and 2022, significant differences in community composition were also found with respect to time (year) (PERMANOVA: F=46.1, p=0.0001) and treatment (reference or discharge monitoring), when tested individually (Table 18). In addition, the PERMANOVA found there was a significant interaction between time and treatment for prokaryote composition (PERMANOVA: F=7, p=0.0001) (Table 18).

Table 18. Results of PERMANOVA testing for variation in broad prokaryotic community composition (2020, 2021 and 2022) between timepoints (years), and treatments (reference vs discharge monitoring)

Factor: source of variation	df	MS	Pseudo-F	P(perm)	Unique perms
Treatment (2020)	1	29733	18.9	**0.0001**	9914
Treatment (2021)	1	31092	20.7	**0.0001**	9927
Treatment (2022)	1	21576	22.2	**0.0001**	9918
Time (year)	2	13902	7.0	**0.0001**	9910
Treatment	1	73212	46.1	**0.0001**	9913
Time*Treatment	4	27272	7.0	**0.0001**	9922
Res	108	827			
Total	134				

Df: degrees of freedom; MS: mean squares; Pseudo-F: multivariate analogue to Fisher's F test statistic of the null hypothesis; P(perm): probability by permutations; Unique Perms: number of unique permutations.
Bold values denote significance at p< 0.05.

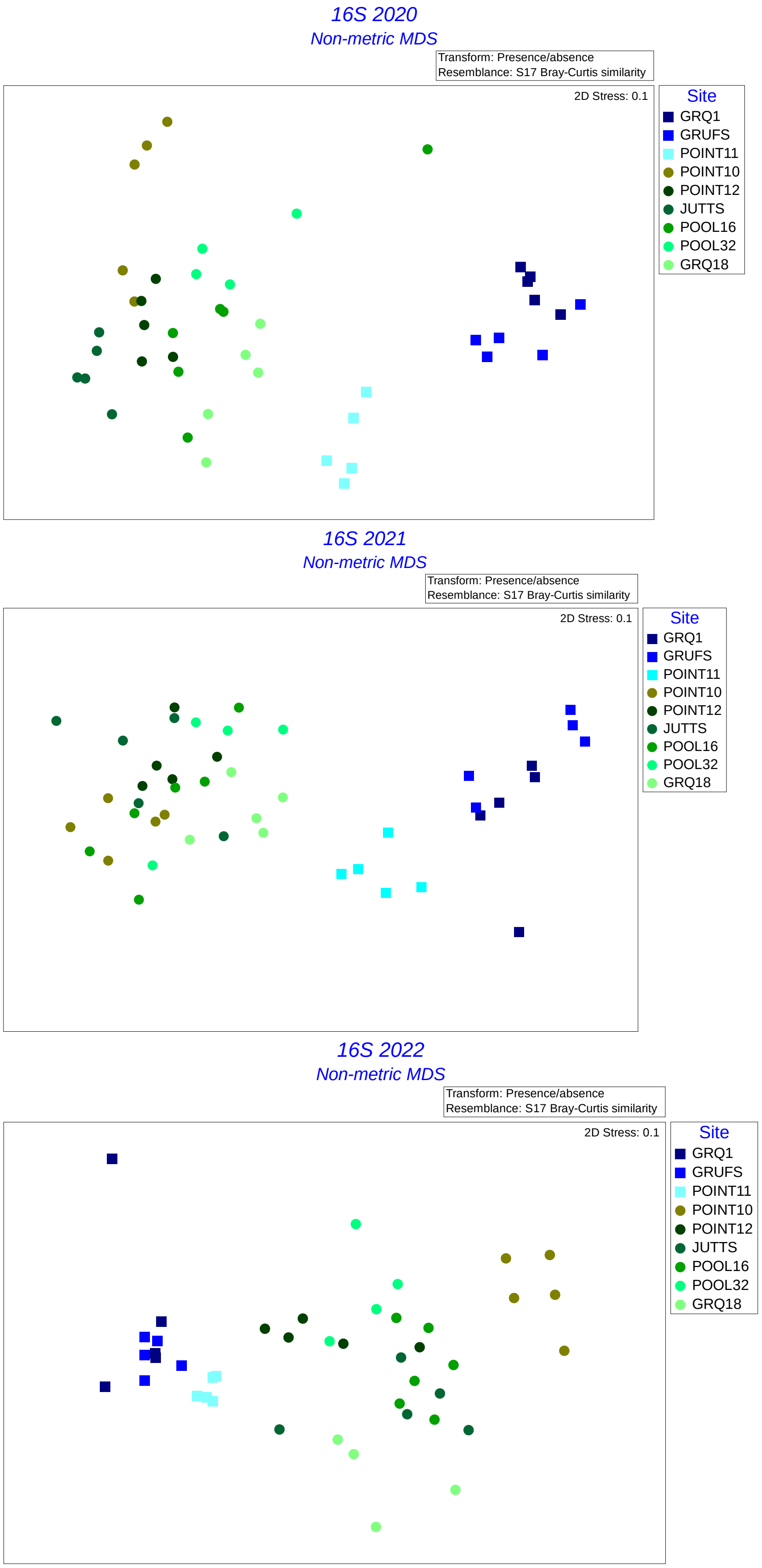


Figure 40. nMDS of 16S OTU prokaryote communities (2020, 2021 and 2022)

a) Spring 2020; b) Spring 2021 c) Spring 2022. Analysis is derived from normalised abundance data at the level of Operational Taxonomic Unit (OTU).

3.8.2 Relationships between prokaryotic community and water quality

Prokaryotic community relationships with water quality

The fitted DistLM was visualised using a dbRDA constrained ordination (Figure 41), demonstrating the correlations of significant variables on the prokaryote community for the 2022 data. The distance-based analysis of the prokaryotic community data for 2022 found the measured variables including pH, alkalinity, conductivity, copper, zinc, nickel, and aluminium explained 97% of the total 2022 prokaryotic community variation. The DISTLM shows that 97% of the biological variation was explained by the physical-environmental parameters measured.

The distance-based analysis investigating the relationships of the prokaryotic community data with measured environmental variables in 2022, is shown in Table 19. When examined collectively, the variables which significantly explained the variation in the prokaryotic community in 2022 were pH (54%) and alkalinity (15%). Figure 41 shows that dbRDA1 is explaining 59.6% of the total variation, mostly driven by conductivity and pH and dbRDA2 is explaining 16.1% of the total prokaryote variation, driven by alkalinity. Figure 41 also shows the discharge monitoring sites at the right of the dbRDA1 correlated with conductivity and pH while a small proportion is contributed by dbRDA2 which is shown to be driven by alkalinity. Consistent with 2020 and 2021 data (Stephenson et al., 2022), the strongest variable which explained most of the prokaryotic community variation in 2022 was pH, explaining 54% of the total prokaryotic community variation. In 2022, the results show alkalinity (15%) highlighted as a significant variable explaining prokaryote community variation, alkalinity was not identified as significant in 2020 or 2021 for prokaryotes (Stephenson et al., 2022). This highlights a change in the drivers of variation for prokaryote communities. In 2020, 2021 and in 2022 pH was the dominant, strongest, driver explaining variation in the prokaryotic community composition. In 2021, the DISTLM found that pH was the main driver of the biological variation for prokaryotes, to a lesser extent, the elements aluminium (11%) and total nitrogen (10%) also significantly explained the prokaryote variation in 2021, but only contributed to a small proportion of the variation (Stephenson et al., 2022). Aluminium and total nitrogen did not show any significant contribution to the prokaryote communities in 2022. Across the years 2020 to 2022 the DISTLM has identified pH as the strongest variable (of the variables measured) contributing to variation in the prokaryote community.

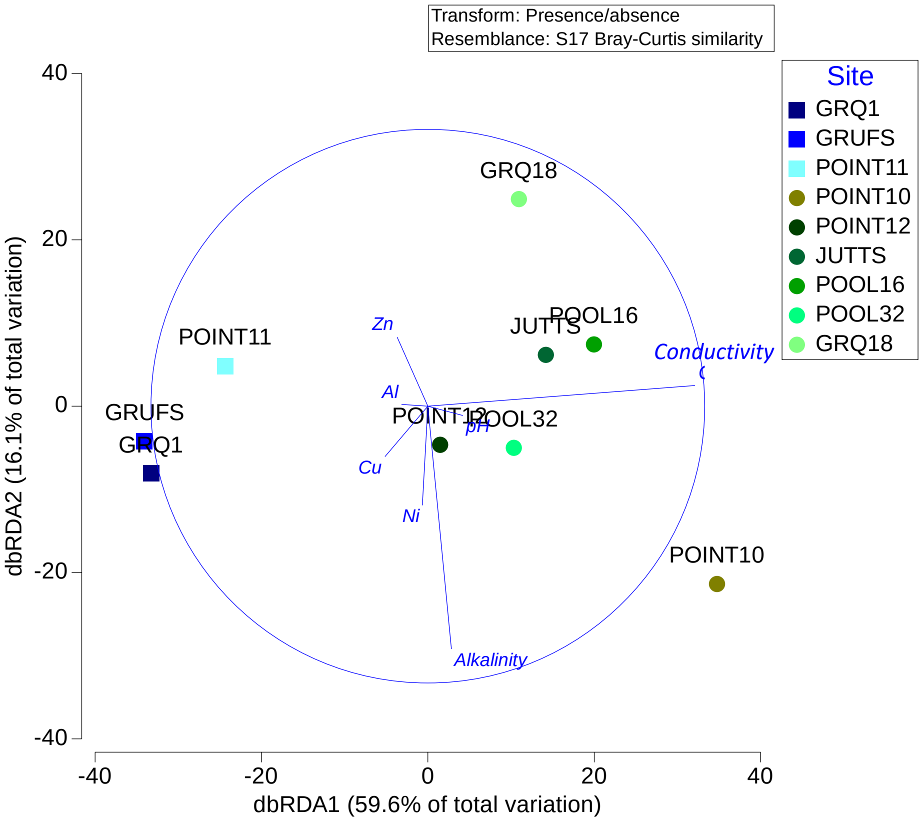


Figure 41. Ordination plot derived from the distance-based model illustrating the relationships between key environmental variables and metabarcoded prokaryotes composition from spring 2022

Table 19. Sequential test results of distance-based linear model (DistLM) for prokaryote 2022 data

Variable	Adj R^2	SS(trace)	Pseudo-F	P	Prop.	Cumulative contribution	res.df
+pH	0.47	4434	8.21	**0.0008**	0.54	0.54	7
+Alkalinity	0.59	1248	2.96	**0.0047**	0.15	0.69	6
+Conductivity	0.63	613	1.60	0.1606	0.07	0.77	5
+Copper	0.65	480	1.33	0.291	0.06	0.82	4
+Zinc	0.68	461	1.41	0.299	0.06	0.88	3
+Nickel	0.7	366	1.19	0.371	0.04	0.93	2
+Aluminium	0.81	413	2.08	0.328	0.05	0.98	1

SS (trace): sum of squares; Pseudo-F: multivariate analogue to Fisher's F test statistic of the null hypothesis; P: probability; Prop: the proportion (%) of variation; res.df: residual degrees of freedom

Bold values denote significance at $p < 0.05$.

3.8.3 18S rDNA metabarcoding (eukaryotes)

Sequencing data from the eukaryotic 18S V7 rDNA in 2022 were used to analyse broad eukaryote OTU compositions at the study sites and treatments over time. Across the nine sites surveyed, the broad eukaryotes dataset contained reads encompassing 6063 OTUs in 2022 from 49 phyla and 761 unique families.

The 18S V7 rDNA broad eukaryote marker provided comprehensive coverage of eukaryotes for the sampling time points. The top phyla which made up the bulk of the broad eukaryote community across the whole 18S V7 rDNA data set were Ochrophyta (photosynthetic algae) (6.5%), Ciliophora (protists) (6%) and Cercozoa (amoeba and flagellate microeukaryotes) (6%). It should be noted that some OTUs could not be assigned taxonomy to family, genus or species level from the GenBank reference database, in these cases the higher taxonomic level assignment was used to describe OTUs. Unknown (novel) eukaryotes (45%) made up a large proportion of the OTUs in the dataset, the SILVA 138 database could not assign taxa to these OTUs.

The main phyla present in 2022 across the sites and the relative read abundances of taxonomic groups for each site are shown in Figure 42. The bubble plot shows the main eukaryote phyla on the x-axis of the plot. Ochrophyta (photosynthetic algae), Chlorophyta (photosynthetic green algae) and Arthropoda (insect invertebrates) were common across sites for 2022.

In 2022, the taxa composition for relative abundances showed more consistency of taxa across the sites sampled. The relative abundance bubble plots show Arthopoda, Ochrophyta, Chlorophyta, Ascomycota and Ciliophora were present at similar relative abundances across all sites. Miozoa were higher at the downstream discharge monitoring sites such as Pool 16, Pool 32 and GRQ18. Miozoa are a group of unicellular eukaryotic organisms that includes various types of protozoa. Miozoa are characterized by the presence of a distinct organelle called the myoneme which is a contractile filament used for movement. Gastroricha taxa (aquatic slugs) were highest at GRQ18 in 2022. Platyhelminthes (flat worms) were low at most sites with the exception of Jutts in 2022. Platyhelminthes are known as the scavengers and important decomposers in aquatic ecosystems. Nematoda (roundworms) relative abundances were variable across both reference and discharge monitoring sites but were highest at GRUFS, in 2022.

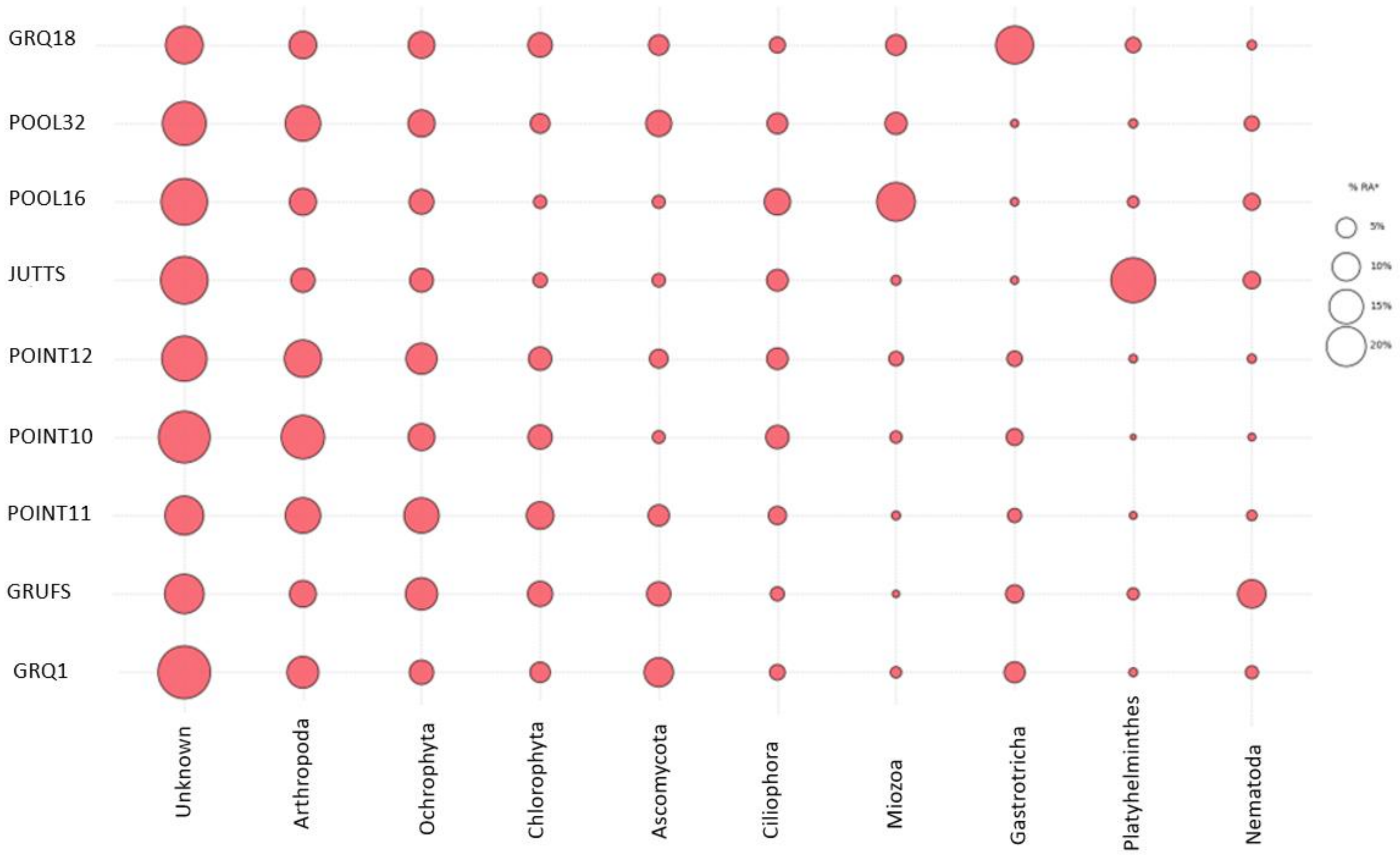


Figure 42. Bubble plot of the top 10 most abundant eukaryote phyla (on average across all sites) for 2022

Eukaryote richness

Eukaryote richness exhibited a range of values across most sites, treatments, and sampling occasions (Figure 43). For eukaryotes, richness has been greater in the reference sites compared with the discharge monitoring sites in 2021 and in 2022, while in 2020 discharge monitoring sites were richer in OTUs. Richness in 2022 across the dataset sites was higher compared with 2020 and 2021. In 2020, there was no significant difference between reference and discharge monitoring treatment richness (Table 20) while in 2021 and 2022 significant differences in treatment richness were observed (Table 20). Point 11 in 2022 had the highest richness of all sites and all years however, GRQ18 was also one of the richest sites in 2022.

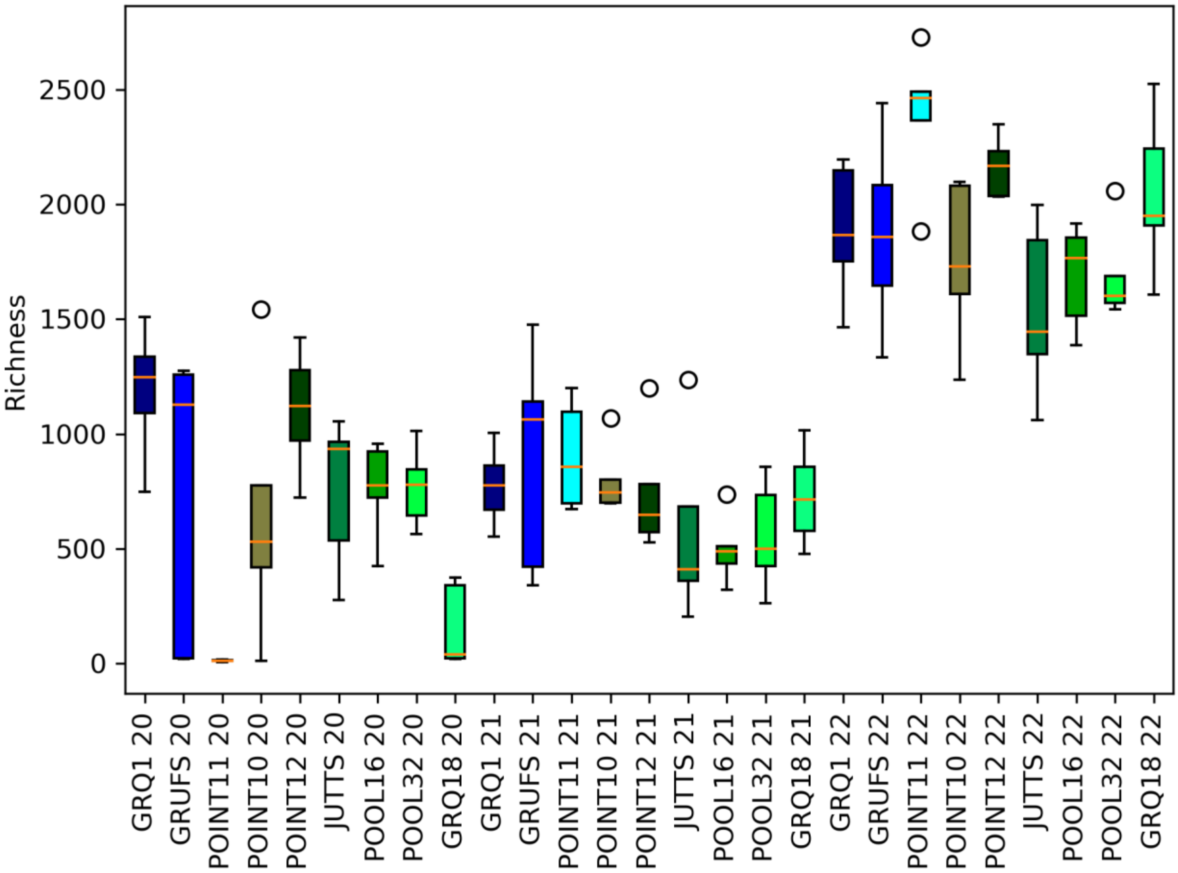


Figure 43. Broad eukaryote OTU richness from 2020, 2021 and 2022 OTU

Reference sites (blue) and discharge monitoring sites (green).

Table 20. One-Way ANOVA results on eukaryote richness for reference and discharge monitoring treatment

Eukaryote OTU Richness	Reference	Reference	Discharge monitoring	Discharge monitoring	One-Way ANOVA	
	mean	**± S.E.**	**mean**	**± S.E.**	***F***	***P-value***
Spring 2020	647	163	700	74.2	4.0	0.729
Spring 2021	856	80.2	652	47.1	5.4	**0.025**
Spring 2022	2048	104.9	1813	63	4.1	**0.049**

Bold values denote significance at p< 0.05.

Eukaryote community composition

Eukaryote community compositions in the reference sites were markedly different to those from the discharge monitoring sites for 2020, 2021 and 2022 at the OTU level. The separation of reference from discharge monitoring site eukaryote communities for 2020, 2021 and 2022 is visualised in the nMDS ordination plots in Figure 44. The nMDS for years 2021 and 2022 shows a clear clustering of the reference sites GRQ1, GRUFS and Point 11 together in blue, separated away from the discharge monitoring sites in green (Figure 44). The reference sites GRUFS and GRQ1 cluster closer together, across all years, but particularly in 2020 and 2021. Point 11 is more variable in 2020 and spread across the ordination slightly closer to the discharge monitoring sites in 2020. This may indicate the influence of other discharge inputs from Appin East but may also be indicative of geographical differences at Point 11 leading to unique community structure. In 2021, the eukaryote communities observed at the discharge monitoring sites are more broadly spread across the nMDS compared to those of the reference sites which appear more tightly clustered. While the reference sites are separated in space from the green discharge sites in 2021 there is less nMDS spatial separation visually for reference in 2022. In general, however, the discharge monitoring sites for all years, showed a broadly similar eukaryote composition that was separate from the reference sites. PERMANOVA verified the visual differences observed in the nMDS plots for the years studied.

PERMANOVA tests were undertaken to investigate eukaryote community structure differences in treatments and time. The results of the PERMANOVAs testing for differences in eukaryote community composition between sampling timepoints (spring 2020, 2021 and spring 2022) and treatments are presented in Table 21. The visual separation of treatments is confirmed by the PERMANOVA which found a significant difference in composition between the two treatments in spring 2020 (PERMANOVA: F=24.7, p=0.0001), spring 2021 (PERMANOVA: F=15.5 p=0.0001) and in spring 2022 (PERMANOVA: F=14.9, p=0.0001). Significant differences in community composition were found with respect to treatment (discharge monitoring or reference) and time (year) (PERMANOVA: F=10.3, p=0.0001) when tested individually (Table 21). In addition, there was a significant interaction between time and treatment (PERMANOVA: F=3.5, p=0.0001) (Table 21).

Table 21. Results of PERMANOVA testing for variation in broad eukaryote community composition (2020, 2021 and 2022) between timepoints (years), and treatments (reference vs discharge monitoring)

Factor: source of variation	df	MS	Pseudo-F	P(perm)	Unique perms
Treatment (2020)	1	57915	24.7	**0.0001**	9903
Treatment (2021)	1	26794	15.5	**0.0001**	9911
Treatment (2022)	1	22227	14.9	**0.0001**	9918
Time (year)	2	24928	10.3	**0.0001**	9892
Treatment	1	57501	3.9	**0.008**	9935
Time*Treatment	4	99566	3.5	**0.0001**	9857
Res	107	4741			
Total	133				

Df: degrees of freedom; MS: mean squares; Pseudo-F: multivariate analogue to Fisher's F test statistic of the null hypothesis; P(perm): probability by permutations; Unique Perms: number of unique permutations.
Bold values denote significance at p< 0.05.

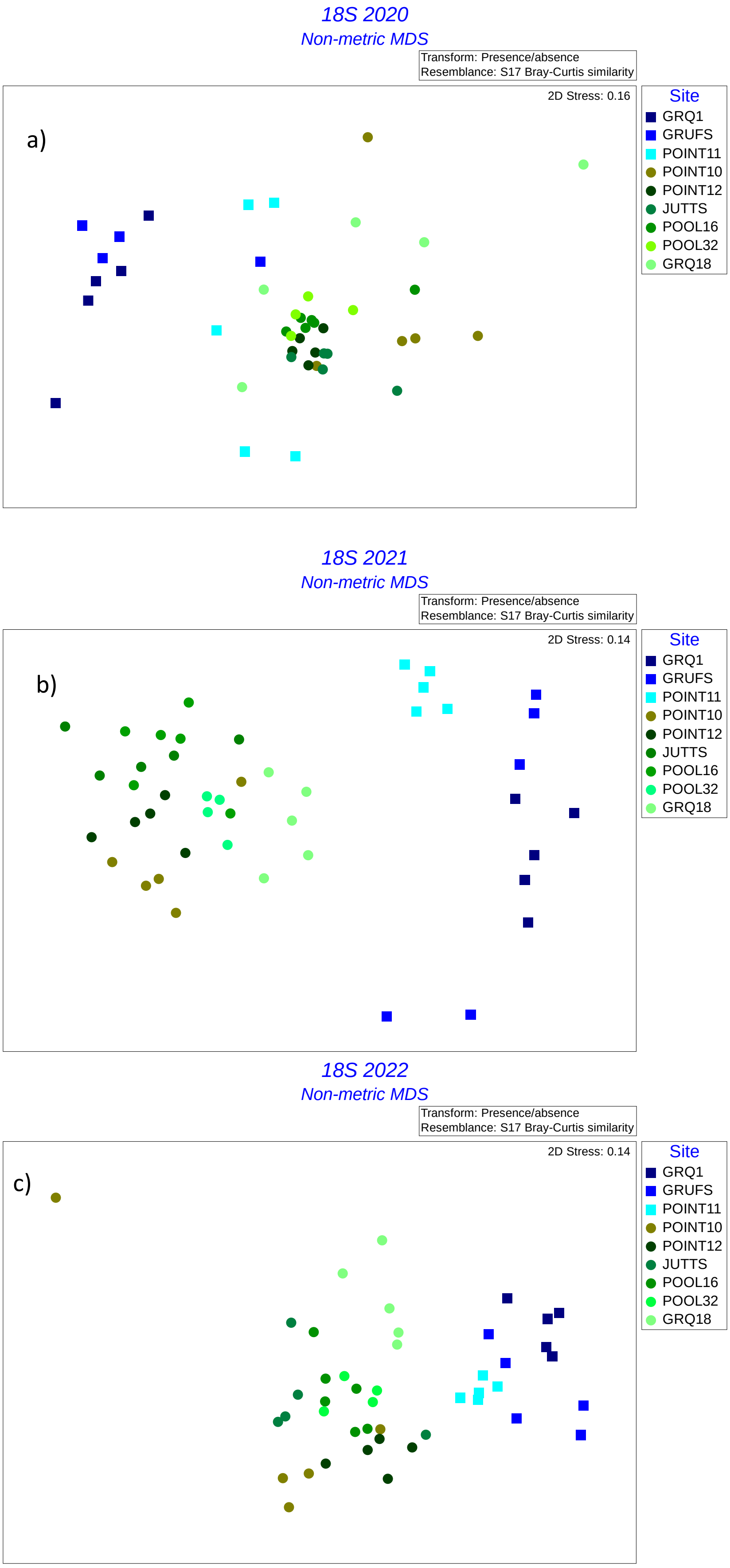


Figure 44. nMDS of 18S OTU eukaryote communities (2020, 2021 and 2022)

a) Spring 2020; b) Spring 2021 c) Spring 2022. Analysis is derived from normalised abundance data at the level of Operational Taxonomic Unit (OTU).

3.8.4 Relationships between eukaryotic community and water quality

Broad eukaryotes relationships with water quality

The fitted DistLM was visualised using a dbRDA constrained ordination (Figure 45), demonstrating the influence of significant variables on the eukaryote community for 2022. The correlative patterns of the eukaryote OTU community data with measured environmental variables in 2022 were analysed by DistLM and results are shown in Table 22. Approximately 90% of the total eukaryote OTU community variation was explained by the measured variables including pH, alkalinity, conductivity, zinc, copper and total nitrogen in spring 2022. When the measured variables were examined collectively, the variables which explained the most eukaryote community variation was pH (45%). Consistent with 2020 and 2021 the strongest variable which explained most of the eukaryotic community variation in 2022 was pH, explaining 45% of the total eukaryotic community variation.

The ordination (Figure 45) shows the discharge monitoring sites positioned to the right of the dbRDA 1 axis, driven by conductivity and pH, while the reference sites remain on the left of the dbRDA1 axis showing a negative relationship with pH for the reference sites. The ordination shows pH and conductivity driving the separation of the green discharge monitoring sites away from GRUFS and GRQ1 in 2022 and this is consistent with results from 2020 and 2021. Point 11 appears to be its own unique community sitting between the other two upper reference sites and the discharge monitoring sites for eukaryotes in 2022. In 2022, dbRDA1 explained 47% of the total eukaryote variation, largely driven by pH and conductivity, while dbRDA2 explained 15% of the total eukaryote variation, driven by alkalinity.

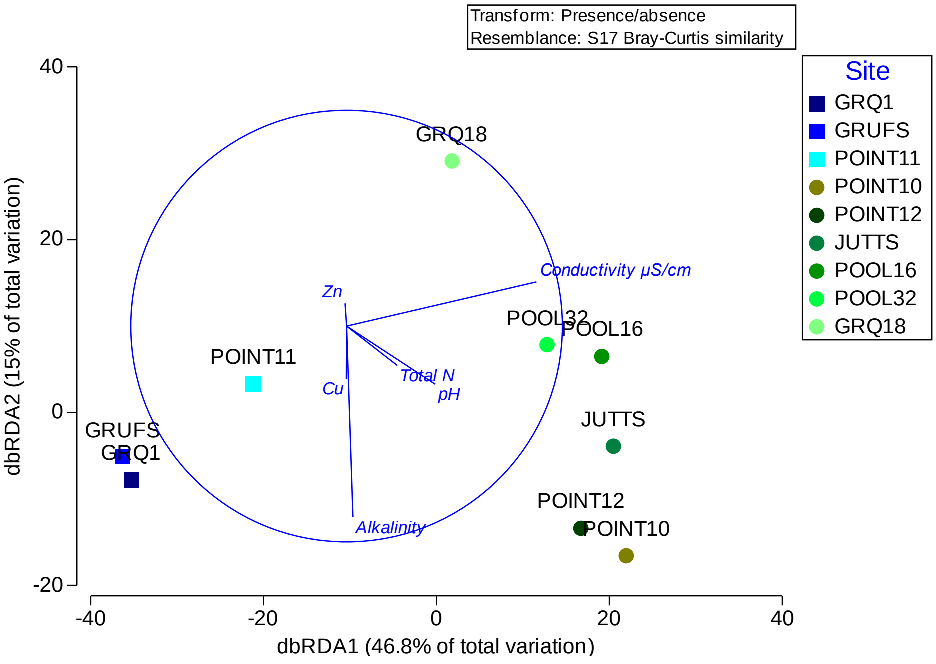


Figure 45. Ordination plot derived from the distance-based model illustrating the relationships between key environmental variables and metabarcoded eukaryotes composition from spring 2022

Table 22. Sequential test results of distance-based linear model (DistLM) for broad eukaryotes for 2022 data

Variable	Adj R^2	SS(trace)	Pseudo-F	P	Prop.	Cumulative contribution	res.df
+pH	0.37	4506	5.6	**0.001**	0.45	0.45	7
+Alkalinity	0.41	1155	1.56	0.133	0.11	0.56	6
+Conductivity	0.48	1179	1.81	0.099	0.12	0.68	5
+Zinc	0.52	876	1.47	0.226	0.09	0.76	4
+Copper	0.56	723	1.31	0.339	0.07	0.84	3
+Total nitrogen	0.58	618	1.18	0.396	0.06	0.90	2

SS (trace): sum of squares; Pseudo-F: multivariate analogue to Fisher's F test statistic of the null hypothesis; P: probability; Prop%: the proportion of variation; res.df: residual degrees of freedom
Bold values denote significance at $p< 0.05$.

3.8.5 18S rDNA V4 metabarcoding Bacillariophyceae (diatoms)

Across the nine sites surveyed, a total of 640 diatom OTUs were detected in 2022. The order of diatoms which made up the bulk of the diatoms across all sites included Naviculales (25.6%), Bacillariales (9.4%) and Cymbellales (6.0%). Unknown (novel) diatom taxa made up 41.6% of OTUs. The 2022 diatom dataset was composed of 32 different family and 65 unique genera. The main diatom genera 2022 across sites and the relative read abundances of taxonomic groups for each site are shown in Figure 46. The bubble plots show the main Bacillariophyceae genus on the x-axis of the plot. The relative abundance bubble plots show, a high proportion of unknown diatom OTUs across all sites, this is due to the SILVA 138 database lacking the local taxonomic information required to assign taxonomy for these specific OTUs.

Similar to previous years, *Eunotia* were highest at the three reference sites. *Eunotia* are characteristic from acidic and electrolyte poor waters, often correlated with low pH and low electrical conductivity (Hofmann et al., 2013; Vouilloud et al., 2014). The presence of *Eunotia* in high abundances in the reference sites correlates with the physical chemical characteristics of the acidic, low conductivity reference sites GRQ1, GRUFS and Point 11. Unknown diatoms were consistent across all sites in 2022. *Nitszchia* were present across the study sites but were in highest abundances at the discharge monitoring sites and highest at Pool32. *Nitszchia* are a genera of diatom present in freshwater, estuarine and marine ecosystems. Species of *Nitzchia* have been associated with high nutrients and are regarded as pollution tolerant in the literature (Hill et al., 2001). *Navicula* had higher relative abundances in the discharge monitoring sites with highest abundances observed at GRQ18. *Entomoneis* had highest abundances at the three sites closest to the discharge source including Point 10, Point 12 and Jutts. *Gomphonema* were present in higher abundances at Point 10 compared with the other sites. *Gomphonema* taxa are associated with alkaline conditions and respond to nutrients in aquatic systems. *Diploneis* were higher at the furthest downstream, discharge monitoring sites, including Pool 16, Pool 32 and GRQ18. *Pinnularia* abundances were variable across sites but were generally they were more common in the reference sites. *Pinnularia* are classed as acidobiontic diatoms and are characteristic in acidic freshwater systems (Hill et al., 2001). In 2022, the presence and relative abundances of some diatoms were very site specific for example, *Fragilaria* were found in high abundances in Jutts and Pool 16 only. High abundances of *Pseudostaurosira* were specific to Point 10.

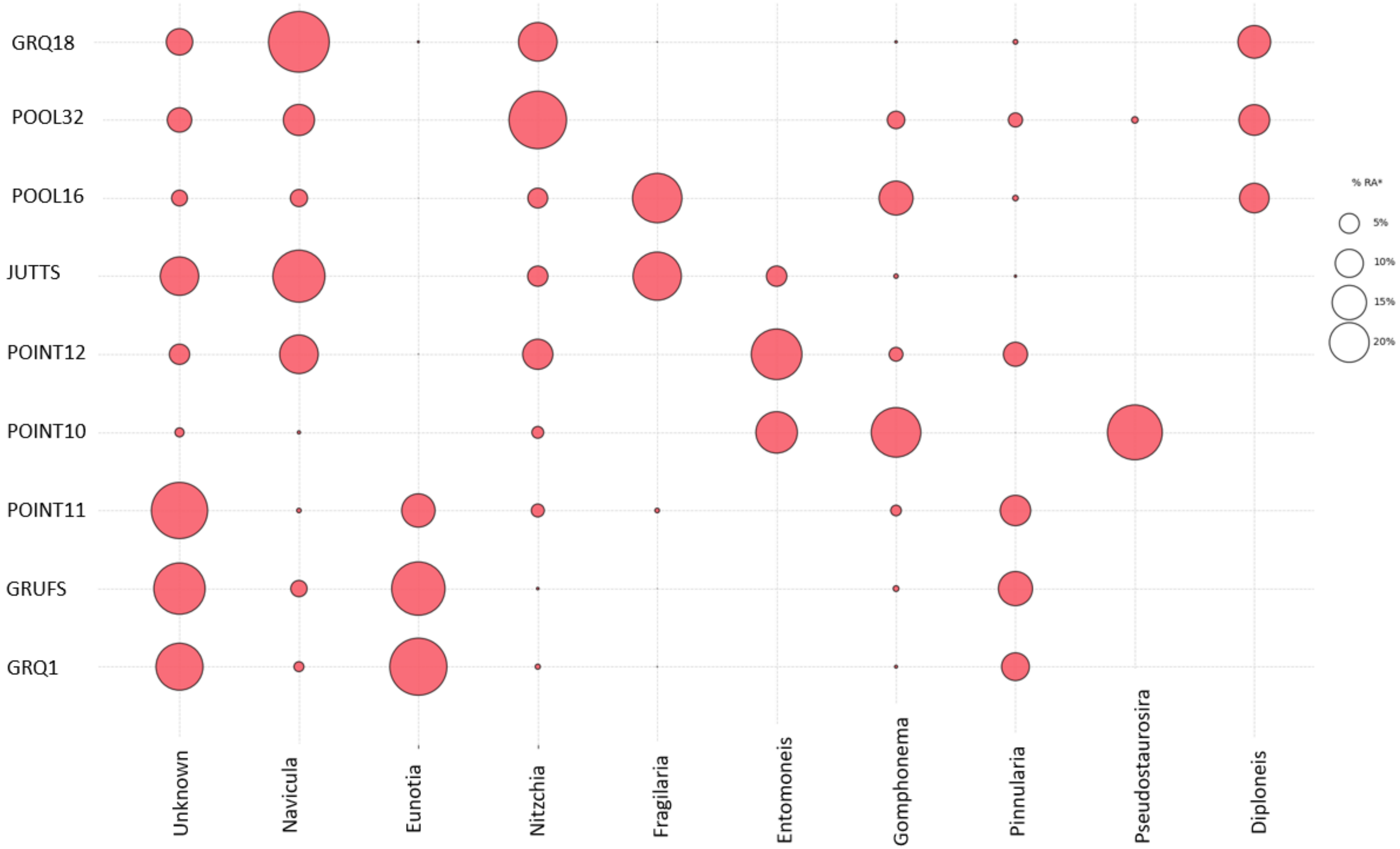


Figure 46. Bubble plot of the main diatom taxa groups (genus) across all sites in 2022

Diatom richness

A summary of family richness (the number of OTUs) from the diatom data collected in 2020, 2021 and 2022 is provided in Figure 47. Overall diatom OTU richness fluctuated over the monitoring period for both treatments. Univariate statistical results including means, standard errors and One-Way ANOVA results for diatom richness are presented in Table 23.

There were significant differences in richness for the diatom OTUs in spring 2020, 2021 and 2022. (One-Way ANOVA detected significant differences for all years) Table 23. For diatom richness in 2020 and 2021 the reference sites richness was significantly different compared to the discharge monitoring sites. In 2022 mean diatom richness (± S.E.) was much higher in reference (132 ± 10.5) than the discharge monitoring treatments (68 ± 6.6) and the difference between treatments was highly significant (p=0.00001, F=30.7). In 2020 and 2021 the diatom OTU richness was high in site GRQ18 with a similar richness to the reference sites.

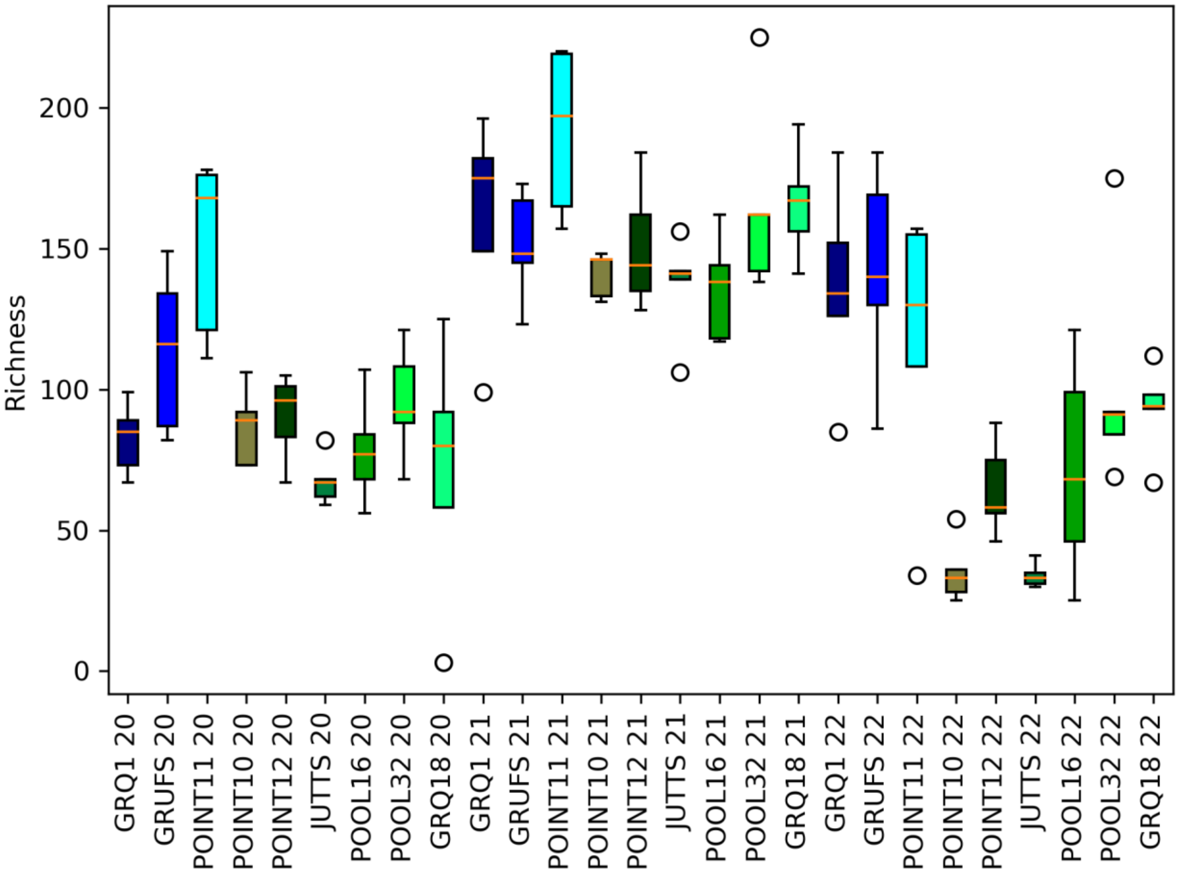


Figure 47. Diatom OTU richness from 2020, 2021 and 2022

Reference sites (blue) and discharge monitoring sites (green).

Table 23. One-Way ANOVA results on 18S v4 diatoms richness for reference and discharge monitoring treatments

Diatom OTU Richness	Reference	Reference	Discharge monitoring	Discharge monitoring	One-Way ANOVA	
	mean	**± S.E.**	**mean**	**± S.E.**	***F***	***P-value***
Spring 2020	116	9.7	82	3.5	13.7	**0.0006**
Spring 2021	168	8.5	149	4.2	4.6	**0.04**
Spring 2022	132	10.5	68	6.6	30.7	**0.00001**

Bold values denote significance at p< 0.05.

Diatom community composition

The similarities and differences in the diatom community compositions in 2020, 2021 and 2022 are presented in the ordination plot (nMDS) (Figure 48). The diatom communities from the reference sites were markedly different to those from the discharge monitoring sites. Across all three years, the diatom communities from the reference sites GRQ1 and GRUFS were markedly different to Point 11 reference site, with Point 11 clustering away from the other two reference sites for all years (Figure 48). The discharge sites have become more distinctly separated in 2021 and 2022 for diatom communities. For each year, the diatom community composition was significantly different between reference and discharge monitoring sites. This did not change over time between 2020 and 2022. This difference between reference treatments and discharge monitoring treatments is confirmed by the PERMANOVA, (Table 24) which found a significant difference in composition between the two treatments in spring 2020 (PERMANOVA: F=39.3, p=0.0001), spring 2021 (PERMANOVA: F=46.4, p=0.0001) and spring 2022 (PERMANOVA: F=59.4, p=0.0001). When looking at the diatom communities over time independently, there was no significant difference between the diatom communities between spring 2020, 2021 and 2022 (PERMANOVA: F=19.1, p=0.0001). When analysing the contribution of treatment and sampling time to the diatom community, there was a significant difference in diatom treatments (reference and discharge monitoring) with time (2020, 2021 and 2022) (PERMANOVA: F=112.3, p=0.0001).

Table 24. Results of PERMANOVA testing for variation in diatom community composition (2020, 2021 and 2022) between sampling timepoints (years) and treatments (reference vs discharge monitoring)

Factor: source of variation	df	MS	Pseudo-F	P(perm)	Unique perms
Treatment 2020	1	31460	39.3	**0.0001**	9934
Treatment 2021	1	43570	46.4	**0.0001**	9941
Treatment 2022	1	36982	59.4	**0.0001**	9948
Time (year)	1	8743	5.4	**0.0002**	9937
Treatment (all years)	1	71591	70.7	**0.0001**	9945
Time*Treatment	1	3289	2.1	**0.045**	9937
Res	86	951.3			
Total	89				

Df: degrees of freedom; MS: mean squares; Pseudo-F: multivariate analogue to Fisher's F test statistic of the null hypothesis; P(perm): probability by permutations; Unique Perms: number of unique permutations.

Bold values represent $p<0.05$

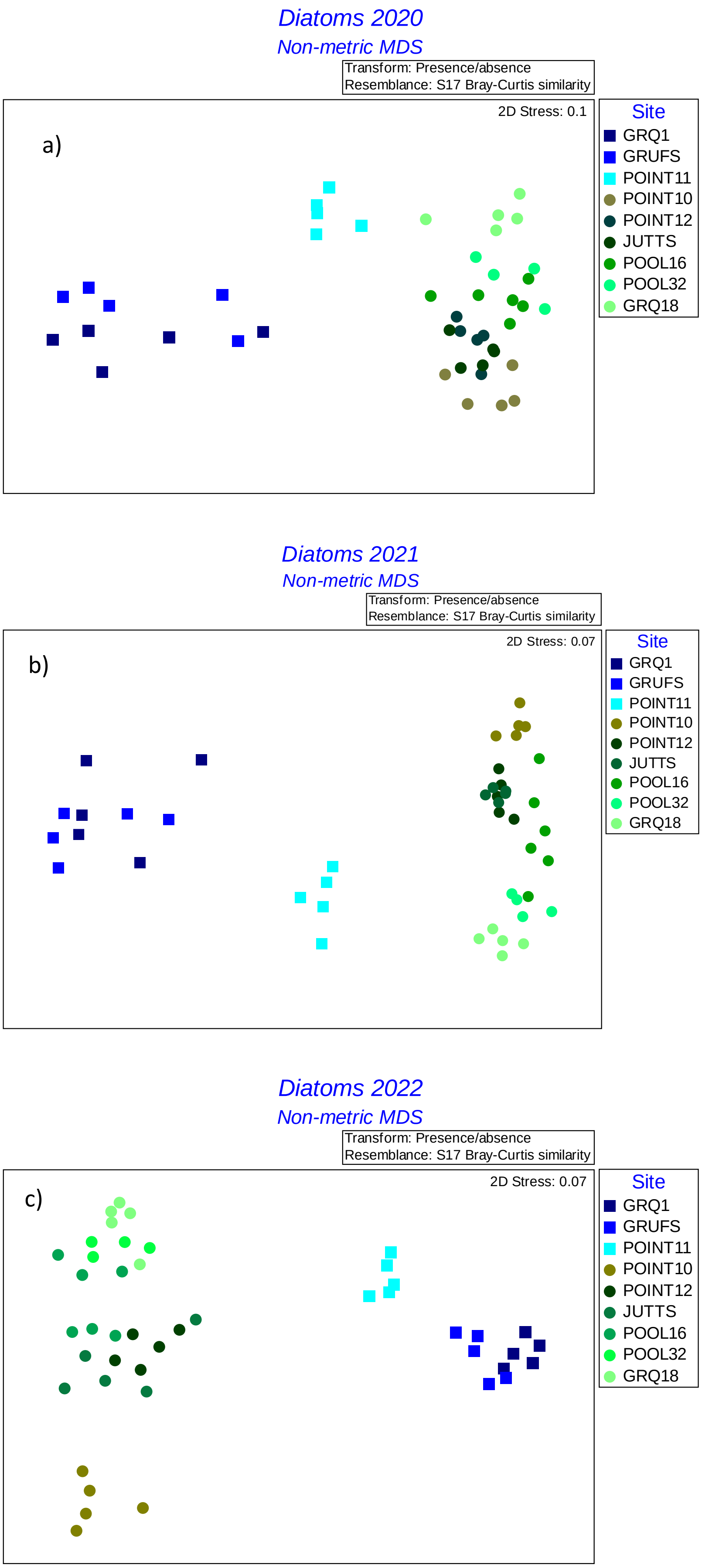


Figure 48. nMDS of 18S diatom communities (2020, 2021 and 2022)

a) Spring 2020; b) Spring 2021 c) Spring 2022. Analysis is derived from normalised abundance data at the level of Operational Taxonomic Unit (OTU).

3.8.6 Relationships between diatom community and water quality

Diatoms relationships with water quality

The fitted DistLM (Table 25) was visualised using a dbRDA constrained ordination (Figure 49), demonstrating the influence of significant variables on the eukaryote community for 2022. The correlative patterns of the diatom community data with measured environmental variables in 2022 were analysed by DistLM and results are shown in Table 25. Approximately 99% of the total diatom OTU community variation was explained by the measured variables including pH, alkalinity, nickel, copper, total nitrogen, aluminium and conductivity in spring 2022. When the measured variables were examined collectively, the significant variables which explained the most eukaryote community variation were pH (68%) and to a lesser extent alkalinity (16%).

The ordination shows dbRDA1 explained 69% of the diatom variation largely driven by pH and conductivity, while dbRDA2 explained 18.3% of the total diatom variation driven by alkalinity. The sites furthest from the discharge (Pool32 and GRQ18) are in the top left corner of the ordination while Point 10 is in the bottom left -20 position highlighting dbRDA2 and alkalinity is driving variation in these discharge sites. The ordination (Figure 49) shows the discharge monitoring sites positioned to the left of the dbRDA 1 axis, with a positive relationship with conductivity and pH, while the reference sites remain on the far right of the dbRDA1 axis showing a negative relationship with pH and conductivity for the reference sites. Consistent with previous years, 2020 and 2021, pH is the strongest, dominant variable driving the diatom biological variation. This is also consistent with the other 2022 metabarcoded communities, broad eukaryotes and prokaryotes. In 2020 and 2021 some additional elements were shown to be contributing to diatom variation including copper in 2020 and aluminium and total nitrogen in 2021. The DISTLM for 2022 did not find these water quality elements (predictor variables) contributing significantly to the diatom variation. While alkalinity has shown significance as a driver of diatom biological variation, pH still remains the dominant, strongest driver in 2022.

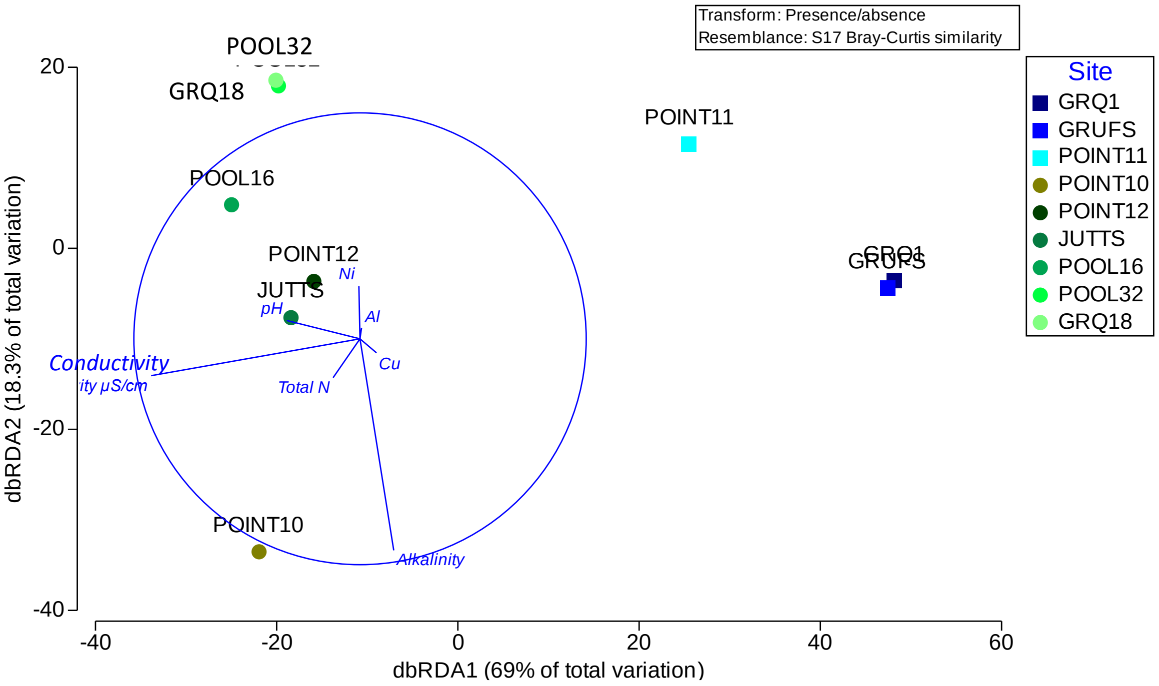


Figure 49. Ordination plot derived from the distance-based model illustrating the relationships between key environmental variables and metabarcoded diatom composition from spring 2022

Table 25. Sequential test results of distance-based linear model (DistLM) for diatoms OTUs 2022

Variable	Adj R^2	SS (trace)	Pseudo-F	P	Prop.	Cumulative contribution	res.df
+pH	0.63	7553	14.6	**0.002**	0.68	0.68	7
+Alkalinity	0.78	1749	5.6	**0.003**	0.16	0.83	6
+Nickel	0.80	529	1.97	0.09	0.05	0.88	5
+Copper	0.84	487	2.3	0.12	0.04	0.92	4
+Total nitrogen	0.88	355	2.1	0.12	0.03	0.95	3
+Aluminium	0.89	190	1.2	0.38	0.02	0.97	2
+Conductivity µS/cm	0.97	268	6.1	0.16	0.02	0.99	1

SS (trace): sum of squares; Pseudo-F: multivariate analogue to Fisher's F test statistic of the null hypothesis; P: probability(<0.01 significant); Prop: the proportion (%) of variation; res.df: residual degrees of freedom

Bold values represent p<0.05

3.8.7 Summary of metabarcoding results

The prokaryote, eukaryote and diatom metabarcoding for the sampling occasions spring 2020, spring 2021 and spring 2022 all showed that at the OTU level, community composition, for prokaryotes, eukaryotes and diatoms, differed between reference sites and discharge monitoring sites (Figure 40, Figure 44 and Figure 48, respectively). For most years and all three primer sets, OTU richness was higher in the reference sites compared with the discharge monitoring sites. These observations were supported by statistical analyses (PERMANOVA) for prokaryotes (Table 18), eukaryotes (Table 21) and diatoms (Table 24) which presented statistical evidence of the differences between reference and discharge treatment community structure and correlated with water quality changes. Of the water quality factors measured, the main driver of variation in all the metabarcoded communities was pH. To a lesser extent, alkalinity, aluminium, and total nitrogen, on some occasions, were also contributing to the prokaryote, eukaryote, and diatom community variation.

The metabarcoding data assisted in identifying potential key biological indicators which were representative of the treatments, i.e., more abundant in either reference or discharge monitoring sites. A large number of prokaryote taxa were positively affected and increased in abundance at downstream monitoring sites. Several prokaryotic families increased in abundance at the discharge monitoring sites relative to the reference sites including Rhodobacteracea and Verrucomicrobiacea. Verrucomicrobiacea have been reported in other heavily disturbed habitats (Cho and Kim, 2000; Juretschko et al., 2002). This trend of Rhodobacteracea and Verrucomicrobiacea taxa increasing in discharge monitoring sites is consistent with previous years findings. Families that decreased in abundance relative to the reference sites in 2022 included Bradyrhizobiaceae and Nitrososphaeraceae. Bradyrhizobiaceae were also reported to decrease in relative abundance in the previous GRAHMP report (Stephenson et al., 2022). These taxa are associated with nitrogen cycling and acidic conditions. Nitrososphaeraceae have been associated with acid habitat conditions and the higher abundance in GRUFS and GRQ1 is consistent with the characteristic acid conditions (pH 6-6.3) of the reference sites GRUFS and GRQ1. For the broad eukaryotes, Ascomycota were higher in the reference sites than the discharge monitoring sites in 2022. Protists from the family Miozoa were present in higher abundances in the discharge monitoring site Pool 16 in 2022. Miozoa may be a potential indicator of disturbance at the discharge monitoring sites. Miozoa have been associated in other aquatic systems with high nitrogen and phosphorus conditions (Huang et al 2022).

Diatoms are an important component of the Georges River ecological community. Diatoms overall can be considered biological indicators of water quality, as diatom abundances and composition can be sensitive to changes in water quality such as pH, conductivity, nutrient and metal concentrations (Hill et al., 2001; Leland, 2011; Round, 1991; Van Dam, 1982). Diatoms broadly, play important ecological roles in aquatic ecosystems, contributing to primary production and the cycling of nutrients such as carbon and nitrogen. Some diatom genera were negatively affected by the discharge and decreased in abundance at discharge monitoring sites relative to the reference sites. Key diatom indicator taxa identified in 2022, which distinctly decreased in abundance at the discharge monitoring sites were *Eunotia* genera which had distinct higher abundances and greater presence in the reference sites. *Eunotia* are accepted a sensitive taxa of diatom (Keck et al., 2016). *Eunotia* were absent from most discharge monitoring sites and were only present with very small abundance at GRQ18. Given that *Eunotia* are characteristic of acidic conditions, it was expected to observe these taxa in higher abundances at the more acidic and low conductivity reference sites. This result of *Eunotia* with higher abundances at reference sites is consistent with results from 2020 and 2021 (Stephenson et al., 2022).

A number of diatom taxa were positively affected by the discharge and increased in abundances at the discharge monitoring sites, these included *Entomoneis* and *Fragilaria. Entomoneis* and *Fragilaria* appear to be responding positively to changes in water quality (potentially nutrients) at the discharge monitoring sites. *Entomoneis* are more common in estuarine and brackish sediments but are also found in freshwater ecosystems (Liu et al., 2018).

The metabarcoded data for microbial communities (prokaryotes and diatoms) shows that microbial communities are responding to discharge water quality and the habitat structure at the different sites and taxa are shifting in response to the environmental factors, particular for the sites Point 10, Point 12 and Jutts. The metabarcoding results reveal the dynamic nature of the upper reaches of the Georges River and provide some insight into the changes in biological communities, highlighting that there are complex ecological interactions taking place with the biological communities and the physical and chemical properties of the system.

4 Conclusions

- Water from LDP10 continued to be the major discharge into Brennans Creek in 2022. The temporary WTP was in operation throughout 2022, discharging through LDP40. While flows of LDP40 from the temporary WTP have slightly increased in spring 2022, LDP40 discharge is currently contributing <20% of total flow to Brennans Creek.
- Water flow through each site peaked on multiple occasions during 2022 including March, April, July, and October, and this aligned with high rainfall.
- Water from LDP10 was not toxic to *C. dubia* (reproduction or survival) or *M. splendida* (imbalance), however, water from LDP40 was toxic on three occasions to *C. dubia* (reproduction and survival) and on one occasion to *M. splendida* (imbalance). In May 2022, water from LDP40 showed high acute toxicity to *C. dubia*, with no surviving animals in concentrations greater than 6% within 24h. The cause of these incidents of toxicity are not known and require further investigation. Low ionic balance is assumed to be contributing to toxicity but is not likely to be the sole cause of toxicity when high level of toxicity was observed (e.g., May 2022).
- Aluminium was measured above the GV for all sites and all sampling occasions in 2022. Zinc measurements were variable in autumn 2022 and spring 2022.
- Similar to previous years the macrobenthic community structure was different in reference sites compared with the discharge monitoring communities in the 2022 study. In autumn 2022, the discharge monitoring sites, macrobenthic communities were more similar in structure to each other while in spring 2022 the data show less similarity and more heterogeneity in communities across the discharge monitoring sites. Generally though, in 2022 the sites showed more variation between discharge monitoring sites in biological communities than previous years.
- While the DISTLM statistics identified pH as a key correlate of the differences in the macrobenthic communities and metabarcoded communities, it seems likely that pH is affecting the bioavailability of metals and resulting in some potential toxicity which then translate into ecological impacts. In some seasons and years, pH and metals (aluminium and copper) were highlighted as potential drivers of biological change. The water chemistry of the study area is complex and pH in combination with other water quality variables such as alkalinity, dissolved organic carbon (DOC) and metal interactions should be considered in relation to metals and other stressors. Metal bioavailability is influenced by many aspects of water chemistry such as major ions, pH, hardness, alkalinity, and dissolved organic matter. An important characteristic for most metals is pH because many metals will have differing speciation across a pH range, which in turn can lead to differences in toxicity (Price et al., 2021).
- We recommend adding DOC to the suite of analyses currently carried out seasonally.

- SIGNAL scores were higher in reference sites compared with discharge monitoring sites. Little change from previous years SIGNAL scores and rankings were observed in 2022.
- pH continues to be a main driver of macrobenthic, prokaryote, eukaryote and diatom communities. In spring 2022, alkalinity became a significant driver of variation in the prokaryote and diatom community structure.
- It is acknowledged that GRQ1 and GRUFS are not the optimal reference sites but the only available upstream options with Point 11. The reference sites were selected for the EIP2 with knowledge of this limiting effect however, the project team were unable to find more suitable reference sites for the GRAHMP in the local area without this limiting experimental design effect. The acidic nature of these reference sites is highlighted in this report.

Table 26. A summary of multiple lines of evidence obtained between 2013 and 2022

Evidence	Attributes	Evidence	Summary
Water chemistry	Conductivity, pH, metals, and nutrients	pH remains different and lower at reference sites compared with the discharge monitoring sites. Reference sites are acidic and discharge monitoring sites are more alkaline. Conductivity below GV for all discharge monitoring sites in 2022. Aluminium measured high across all sites. Zinc and nutrients were variable across time and sites.	Water quality at sites closest to the discharge remain contaminated and the discharge remains an ecological and ecotoxicity concern.
Ecotoxicology	7 tests, 6 species (2013-2019), reduced to 2 tests, 2 species (2020-2022)	LDP40 was toxic on more occasions in 2022 than LDP10. High level acute toxicity was observed on one occasion from LDP40.	Investigation into cause of toxicity for LDP40 is critical for improving water quality and understanding the ecological risk of LDP40 discharge.
Macrobenthic communities	Community structure	In 2022, the sites showed more variation in biological communities between treatments and within treatments. Reference sites had a greater abundance and frequency of sensitive taxa such as Leptophlebiidae while discharge monitoring sites were composed of mostly tolerant taxa such as Chironominae and Caenidae.	Discharge monitoring showed that macrobenthic communities are being affected by water quality and flows. Sites closest to the discharge source are most affected by the water quality. The furthest discharge monitoring site GRQ18 is being influenced by other catchment factors.
	SIGNAL	SIGNAL scores higher in reference sites compared with	Reference sites have more sensitive taxa suggesting

		discharge monitoring sites. Little change measured in SIGNAL scores in 2022 compared to previous years but some small improvements observed for GRUFS and Pool 32.	better ecological condition than the discharge monitoring sites.
	Leptophlebiidae	Greater abundance of the three main Leptophlebiidae taxa of interest*; Atelophlebia* spp*,* *Ulmerophlebia* spp and *Thraulophlebia* spp in the reference sites than the discharge monitoring sites. Some taxa observed at furthest GRQ18 site.	Leptophlebiidae are sensitive to ionic balance changes, high pH and high conductivity. Water quality may be contributing to the presence of Leptophlebiidae at reference sites but also flows and habitat must be considered.
Metabarcoding	Metabarcoding	For the prokaryotes the community composition differed between the reference treatments compared to the discharge monitoring treatment sites. Eukaryote communities from the reference treatment were consistently different to the discharge monitoring treatments. Similarly, diatom communities are different in the reference treatments compared to the discharge monitoring treatment sites. Diatom abundance appears to correlate with water quality parameters.	pH, aluminium and alkalinity were contributing to the biological variation in the metabarcoded communities in 2022.

5 Recommendations

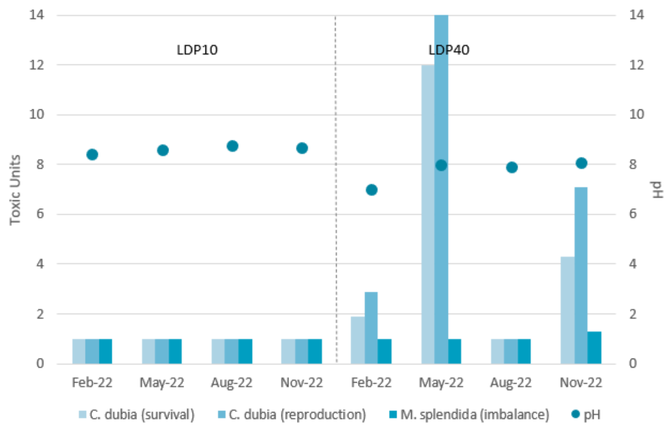
- It is strongly recommended that an action plan be developed to respond to toxic events in future. If South32 cannot explain the likely cause of toxicity (e.g., based on operational or other information that CSIRO is not aware of, such as additional chemicals or treatments done to pipes prior to discharge), a Toxicity Identification Evaluation (TIE) approach could be considered to identify the toxicant(s) responsible for toxicity. Noting however, that TIE approaches must be done as soon as possible after toxicity is detected, to avoid significant aging of the sample. The TIE process may identify a toxicant of concern that is currently not analysed in the usual suite, in which case, South32 may need to consider the source of the identified toxicant, identify whether this will be a likely contaminant in the large-scale WTP treated water, and what steps are needed to remove the toxicant or mitigate its impact.
- Investigations should be carried out to determine if the toxicity of LDP40 is related to a lack of particular ions in the WTP-treated water. It is possible that additional salts are required to be added back to the RO-treated water prior to discharge. The add-back of salts has improved water quality of RO-treated water for other clients and water authorities.
- Consider incorporating total organic carbon and water hardness (as measured by Ca^{2+} and Mg^{2+}) in the key parameters measured in the water samples. Revised ANZG GVs for copper, nickel and potentially zinc will be based on modifying factors depending on pH, water hardness and total organic carbon. These revised ANZG GVs can be applied to the GRAHMP water chemistry metal analysis if total organic carbon and water hardness measurements are collected and analysed. Potential to investigate site specific water quality GVs as the ANZG GVs are updated.
- Inclusion of field blanks for chemical analysis is strongly recommended to enable identification of any possible contamination sources during sampling, particularly in light of the erratic zinc concentrations reported for 2020, 2021 and 2022 in both reference and discharge monitoring sites.
- We recommend including DOC in seasonal analysis as an additional environmental variable to test.

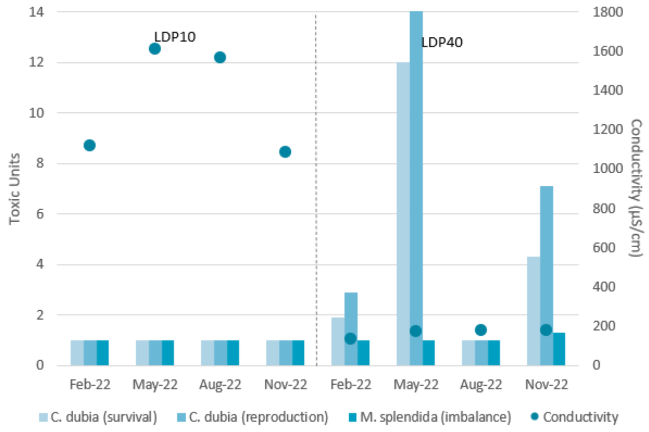
Appendix A Ecotoxicity Results

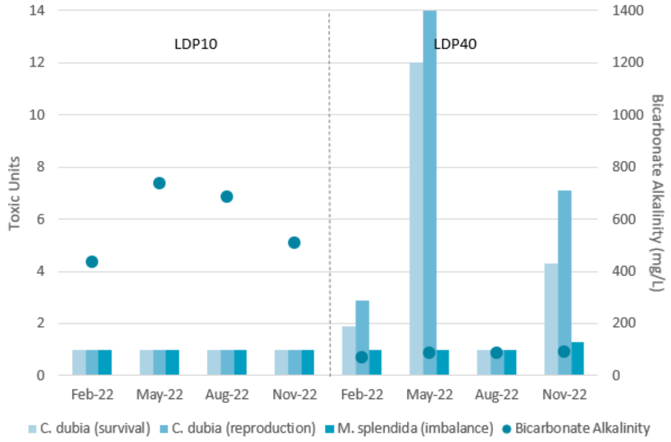
		Feb-22			May-22		
Point 10	**ecotox results - Point 10 (Brennans Creek)**	**EC50 (%)**	**EC10 (%)**	**NOEC (%)**	**EC50 (%)**	**EC10 (%)**	**NOEC (%)**
	Ceriodaphnia dubia- survival	>100	>100	100	>100	>100	100
	Ceriodaphnia dubia- reproduction	>100	>100	100	>100	>100	100
	M.Splendida fish imbalance over 96h	>100	>100	100	>100	>100	100
Point 40	**ecotox results - Point 40 (WTP)**	**EC50 (%)**	**EC10 (%)**	**NOEC (%)**	**EC50 (%)**	**EC10 (%)**	**NOEC (%)**
	Ceriodaphnia dubia- survival	60 (57-71)	53 (50-65)	50	8.9*	8.2*	6.3
	Ceriodaphnia dubia - reproduction	68 (64-72)	34 (8.1-48)	25	9.4*	6.9*	6.3
	M.Splendida fish imbalance over 96h	>100	>100	100	>100	>100	100

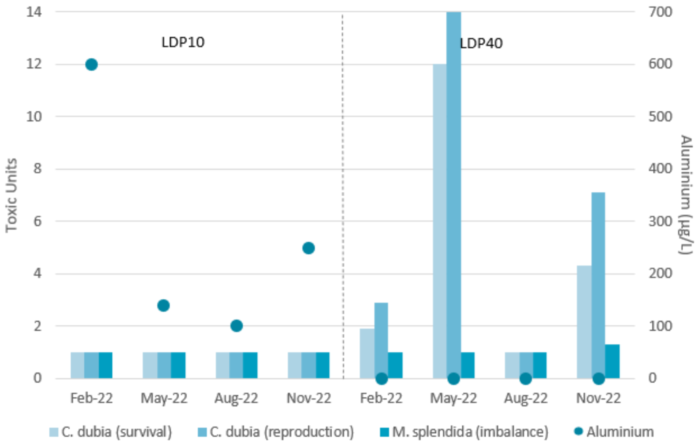
* 95% CLs not determinable

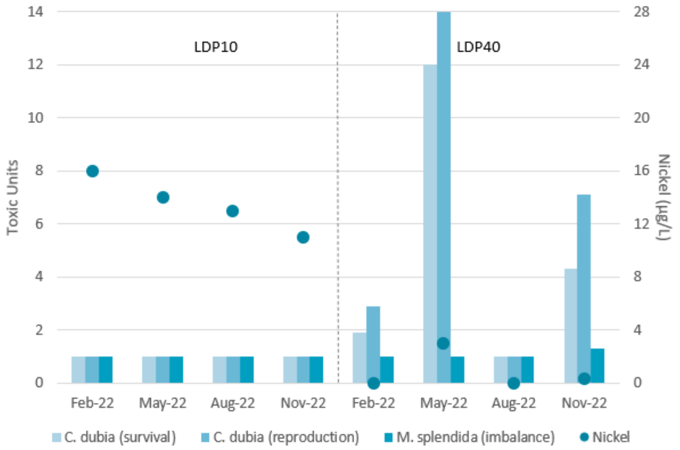
		Aug-22			Nov-22		
Point 10	**ecotox results - Point 10 (Brennans Creek)**	**EC50 (%)**	**EC10 (%)**	**NOEC (%)**	**EC50 (%)**	**EC10 (%)**	**NOEC (%)**
	Ceriodaphnia dubia- survival	>100	>100	100	>100	>100	100
	Ceriodaphnia dubia- reproduction	>100	>100	100	>100	>100	100
	M.Splendida fish imbalance over 96h	>100	>100	100	>100	>100	100
Point 40	**ecotox results - Point 40 (WTP)**	**EC50 (%)**	**EC10 (%)**	**NOEC (%)**	**EC50 (%)**	**EC10 (%)**	**NOEC (%)**
	Ceriodaphnia dubia- survival	>100	>100	100	39 (26-53)	23 (6.9-32)	25
	Ceriodaphnia dubia - reproduction	>100	>100	100	27 (21-34)	14 (8.9-17)	13
	M.Splendida fish imbalance over 96h	>100	>100	100	>100	79 (69-90)	100

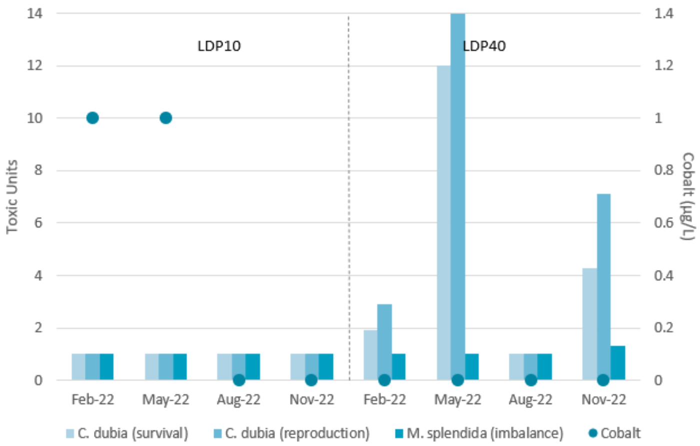

Toxic Units
pH
14
12
10
8
6
4
2
0
LDP10
LDP40
Feb-22
May-22
Aug-22
Nov-22
Feb-22
May-22
Aug-22
Nov-22
C. dubia (survival)
C. dubia (reproduction)
M. splendida (imbalance)
pH

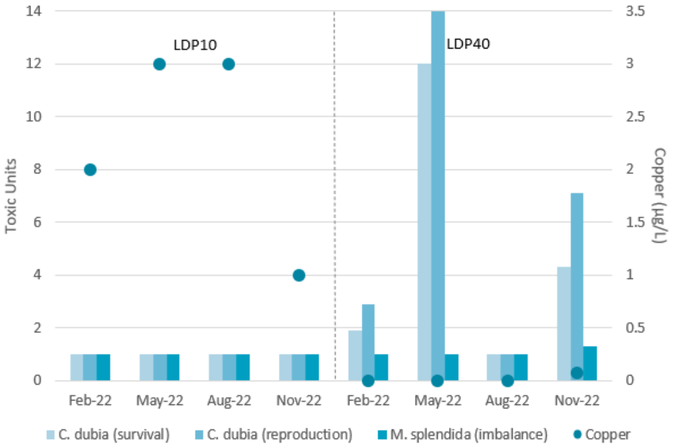

Toxic Units
Conductivity (µS/cm)
14
12
10
8
6
4
2
0
1800
1600
1400
1200
1000
800
600
400
200
0
LDP10
LDP40
Feb-22
May-22
Aug-22
Nov-22
Feb-22
May-22
Aug-22
Nov-22
C. dubia (survival)
C. dubia (reproduction)
M. splendida (imbalance)
Conductivity

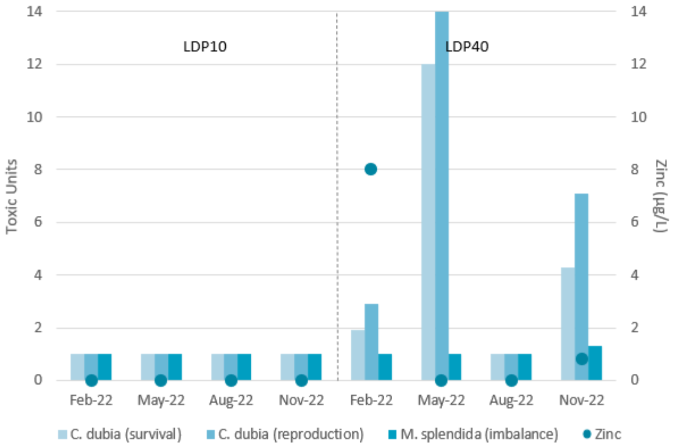

Toxic Units
Bicarbonate Alkalinity (mg/L)
14
12
10
8
6
4
2
0
1400
1200
1000
800
600
400
200
0
LDP10
LDP40
Feb-22
May-22
Aug-22
Nov-22
Feb-22
May-22
Aug-22
Nov-22
C. dubia (survival)
C. dubia (reproduction)
M. splendida (imbalance)
Bicarbonate Alkalinity


Toxic Units
Aluminium (µg/L)
14
12
10
8
6
4
2
0
700
600
500
400
300
200
100
0
LDP10
LDP40
Feb-22
May-22
Aug-22
Nov-22
Feb-22
May-22
Aug-22
Nov-22
C. dubia (survival)
C. dubia (reproduction)
M. splendida (imbalance)
Aluminium


Toxic Units
Nickel (µg/L)
14
12
10
8
6
4
2
0
28
24
20
16
12
8
4
0
LDP10
LDP40
Feb-22
May-22
Aug-22
Nov-22
Feb-22
May-22
Aug-22
Nov-22
C. dubia (survival)
C. dubia (reproduction)
M. splendida (imbalance)
Nickel


Toxic Units
Cobalt (µg/L)
14
12
10
8
6
4
2
0
1.4
1.2
1
0.8
0.6
0.4
0.2
0
LDP10
LDP40
Feb-22
May-22
Aug-22
Nov-22
Feb-22
May-22
Aug-22
Nov-22
C. dubia (survival)
C. dubia (reproduction)
M. splendida (imbalance)
Cobalt


Toxic Units
Copper (µg/L)
14
12
10
8
6
4
2
0
3.5
3
2.5
2
1.5
1
0.5
0
LDP10
LDP40
Feb-22
May-22
Aug-22
Nov-22
Feb-22
May-22
Aug-22
Nov-22
C. dubia (survival)
C. dubia (reproduction)
M. splendida (imbalance)
Copper


Toxic Units
Zinc (µg/L)
14
12
10
8
6
4
2
0
LDP10
LDP40
Feb-22
May-22
Aug-22
Nov-22
Feb-22
May-22
Aug-22
Nov-22
C. dubia (survival)
C. dubia (reproduction)
M. splendida (imbalance)
Zinc

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