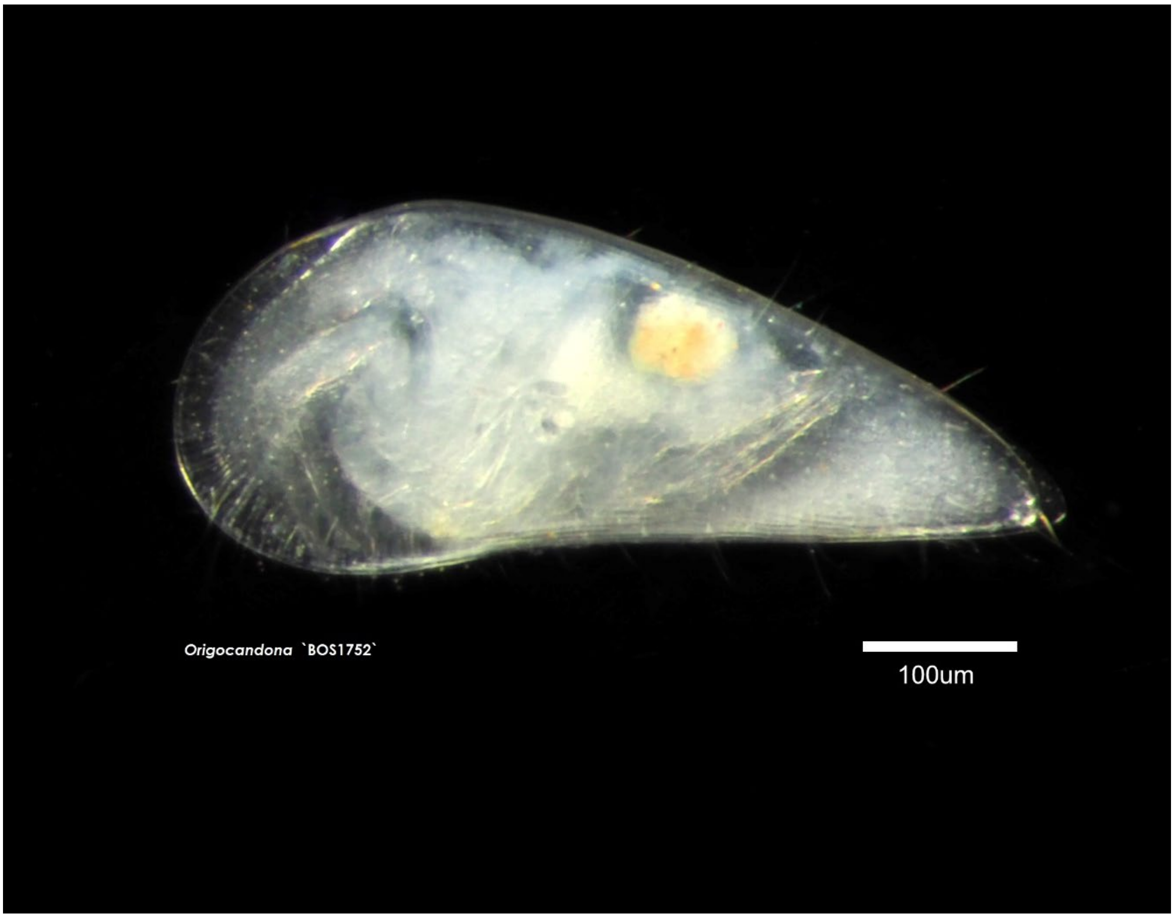
BHIO_SF_22

Ethel Gorge TEC Stygofauna Monitoring 2022/2023



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Revision Schedule

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Quality Statement

Executive Summary

Introduction

BHP Western Australia Iron Ore (BHP WAIO) commissioned Stantec Australia Pty Ltd (Stantec), to complete the 2022/2023 Ethel Gorge Aquifer Stygobiont Threatened Ecological Community (TEC) compliance monitoring program (the Program). The Program aligns with conditions outlined in Ministerial Statements (MS) 1126, 478, 1037 and 1021, established for mining below the water table for the Eastern Ridge Orebody (OB) 23, 24, and 25, OB 31 and Jimblebar deposits, located within approximately 40 km of the town of Newman. The objective of the Program was to monitor the Ethel Gorge Aquifer Stygobiont TEC, in relation to potential impacts from BHP WAIO mining operations, which include mine dewatering, groundwater extraction, mine pit salinisation and surplus water discharge.

Survey Effort

The survey effort for the Program targeted several monitoring zones (MZs) in the area, with monitoring zone 1 representing the Ethel Gorge Aquifer Stygobiont TEC and core habitat. A total of 80 samples were collected from 47 bores to assess groundwater quality and stygofauna abundance. This comprised two surveys, with 40 bores sampled in 2022 Dry (17th – 23rd November 2022) and 40 bores in 2023 Wet (11th – 17th May 2023) with 34 of the bores being sampled in both seasons and 12 bores sampled in only one season each. Rainfall during the survey period was very similar to the long term 12 month average, however it was atypically distributed with heavy unseasonal falls in September 2022.

Groundwater quality measurements were recorded *in situ* from the bores, along with standing water levels (SWLs). Samples were also collected from the bores for chemical analysis, which was completed by a NATA-accredited laboratory. The analytical suite comprised pH, salinity, ionic composition, nutrients and metals. Stygofauna sampling aligned with regulatory technical guidance using net hauling. Styogfauna samples were subsequently sorted and identified in the laboratory. A number of stygofauna specimens were also genetically sequenced to verify taxonomy, including amphipods, isopods and oligochaetes. eDNA methods were implemented for the first time as part of this Program and 20 samples were collected and analysed.

The groundwater and stygofauna data were analysed to assess spatial and temporal. Comparison to previous monitoring rounds, associated with environmental conditions and/or mining operations was undertaken. Statistical analysis was performed on stygofauna species richness and survey effort.

Groundwater Properties

Groundwater SWLs were all within published GTVs and most bores recorded only small variances from the previous survey round. The pH, salinity and nutrients were all mostly below GTVs or within the GTV range. Some individual bores displayed values outside the defined GTVs, however these were consistent with historical records, where available, or were considered to represent spatial variability associated with the introduction of additional bores.

With the transition to ultra-trace metals analysis, preliminary program-specific GTVs were able to be developed for more than half of the metals. Concentrations of all metals were low, and all records except for two for barium were below published ANZECC freshwater thresholds. Recorded concentrations of metals in the Ethel Gorge region were typically less than ten times lower than the published thresholds. There is no perceived metal toxicity risk to the Ethel Gorge Aquifer Stygobiont TEC.

Stygofauna

A total of 35 stygofauna taxa were recorded during the Program from seven higher level taxonomic groups; Acarina, Amphipoda, Bathynellacea, Copepoda, Isopoda, Ostracoda and Annelida. There were two new taxa identified; Enchytraeidae sp. nov. and *Origocandona* `BOS1752`.

Average abundance and diversity during 2022 Dry was relatively low compared to historical records, however both measures had increased considerably by 2023 Wet which had the highest average abundance per bore since 2015 and the highest average species per bore since 2017. All interannual changes observed in the abundance and number of species were within the variability documented for the TEC.

Consistent with previous surveys, the most abundant group overall was Copepoda, with considerable contributions of Amphipoda, Ostracoda and Annelida. A few rarely-documented taxa were also detected, including *Peza* sp. OB, *Coxicerberus* ISO019 and Aeolosomatidae. eDNA methods yielded considerable additional information and provided a complementary tool to traditional sampling and morphological assessment.

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1. Introduction

1.1 Background

BHP Western Australia Iron Ore (BHP WAIO) commissioned Stantec Australia Pty Ltd (Stantec), to complete the 2022/2023 Ethel Gorge Aquifer Stygobiont Threatened Ecological Community (TEC) compliance monitoring program (the Program). The Program aligned with regulatory compliance conditions in Ministerial Statements (MS) 857 (Jimblebar), 478 (OB 23), 1037 (OB 24/25), and 1021 (OB 31). It also considered the BHP WAIO's Eastern Pilbara Water Resource Management Plan (EPWRMP), appended update (BHP 2022a; Douglas and Pickard 2014) and the associated management plan for mining below the water table at the Eastern Ridge Orebody (OB) 23, 24, 25 and 31 and Jimblebar deposits (Douglas and Pickard 2014). These deposits are located within 40 km of the town of Newman, in the Pilbara bioregion of Western Australia (**Figure 1-1**).

The Ethel Gorge Stygobiont TEC (Ethel Gorge TEC) is recognised as an important environmental receptor, with stygofauna monitoring required to investigate potential impacts on the TEC, from dewatering at Jimblebar and OB 31, and the subsequent discharge of excess groundwater into Ophthalmia Dam. The monitoring also extends to Eastern Ridge, to assess local stygofauna communities in relation to dewatering at OB 23 and OB 24/25. The EPWRMP documents support adaptive management of the TEC, with the monitoring assessed against outcomes-based objectives and early warning triggers and thresholds for groundwater levels and salinity.

The Ethel Gorge TEC is located on the Fortescue River and Ophthalmia floodplain, at the confluence of several Pilbara riverine systems including Whaleback, Shovellana, and Homestead creeks as they enter the Fortescue River. The TEC, was first detected in 1997 and comprises a diverse stygofauna assemblage (Eberhard and Humphreys 1999). It occurs within a shallow, alluvium aquifer, in association with a calcrete deposit up to 40 m thick (**Figure 1-2**). The proximal Ophthalmia Dam provides substantial groundwater recharge and hydraulic loading to the alluvial aquifer (RPS 2013). Stygofauna monitoring at Ethel Gorge has been ongoing since 2003, with surveys conducted by several consultants, incorporating the core TEC area and surrounding prospective habitats. A review of the consolidated historical data series is presented in Stantec (2023b).

1.2 Objective and Scope

The overarching objective of the Program was to monitor the Ethel Gorge Stygofauna TEC, in relation to potential impacts from BHP WAIO mining operations which include mine dewatering, groundwater extraction and surplus water discharge. To address the objective, field surveys (surveys) were undertaken in the 2022 post-dry season (2022 Dry) and 2023 post-wet season (2023 Wet), with the scope of the Program comprising the following:

Groundwater in bores at which stygofauna were sampled:

- Monitoring of groundwater levels and field physico-chemistry;
- NATA-accredited analyses of groundwater including basic water quality parameters, major ions, nutrients and metals;
- Analyse water quality data and report on elevated trends of ions and nutrients that are approaching or above guideline levels;
- Develop a statically adequate baseline data set to calculate and refine water quality trigger levels (20th, 50th and 80th percentiles) for metals, following ANZECC & ARMCANZ (2000) procedures; and
- Refinement and continued development of site-specific water quality triggers, where sufficient data exists.

Stygofauna

- Monitor stygofauna abundance and species richness and map relevant species records;
- Identify stygofauna specimens to the lowest taxonomic unit possible using a combination of morphological and molecular methods, where required;
- Upload DNA sequences to GenBank, as appropriate;
- Undertake eDNA sampling at a subset of bores and provide a comparison of eDNA vs traditional sampling; and
- Investigate the suitability and efficacy of the assay suite for the eDNA analyses and report any seasonal or spatial differences detectable among samples.

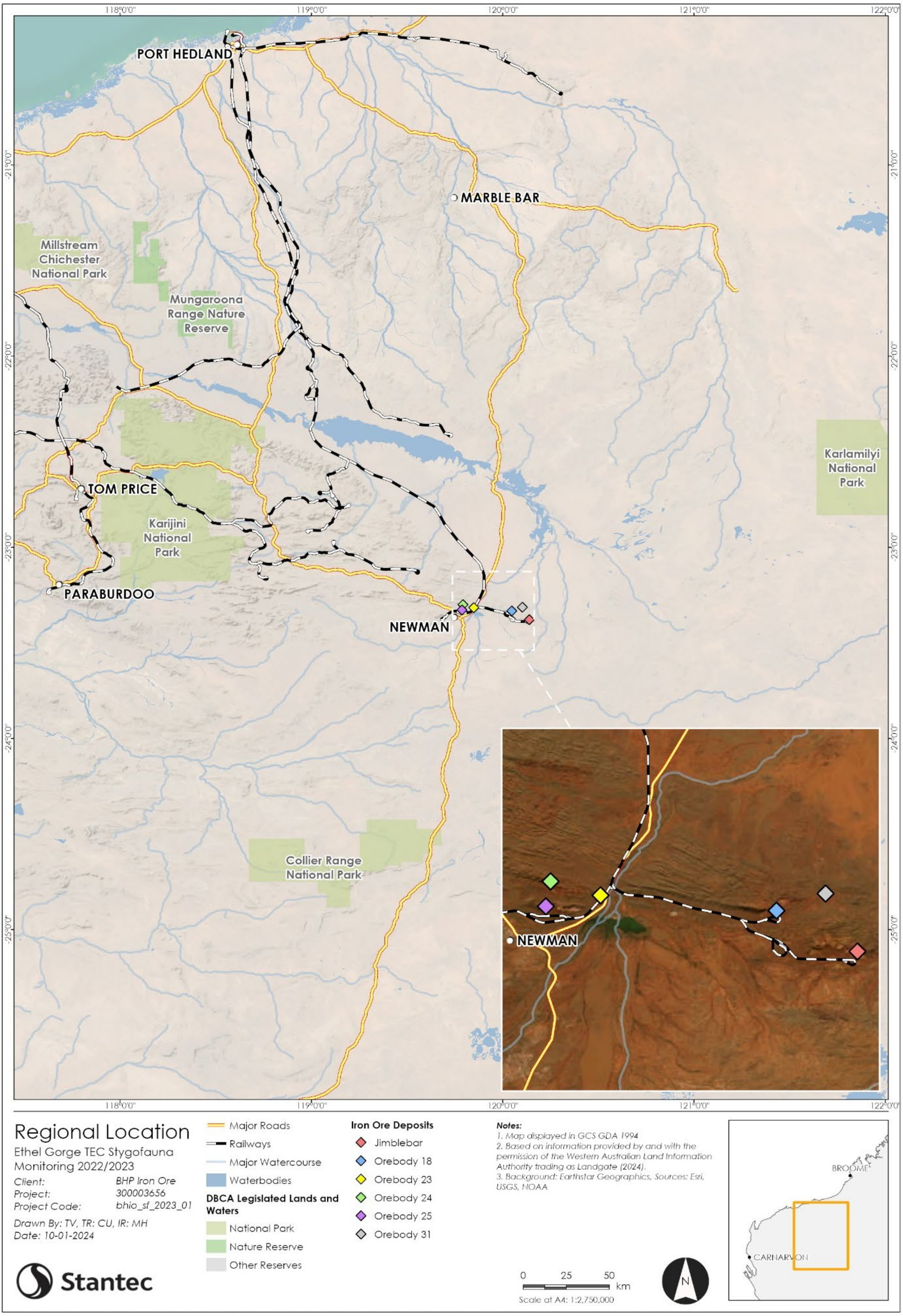

Figure 1-1 Regional location of the BHPIO WAIO deposits relevant to the Program.

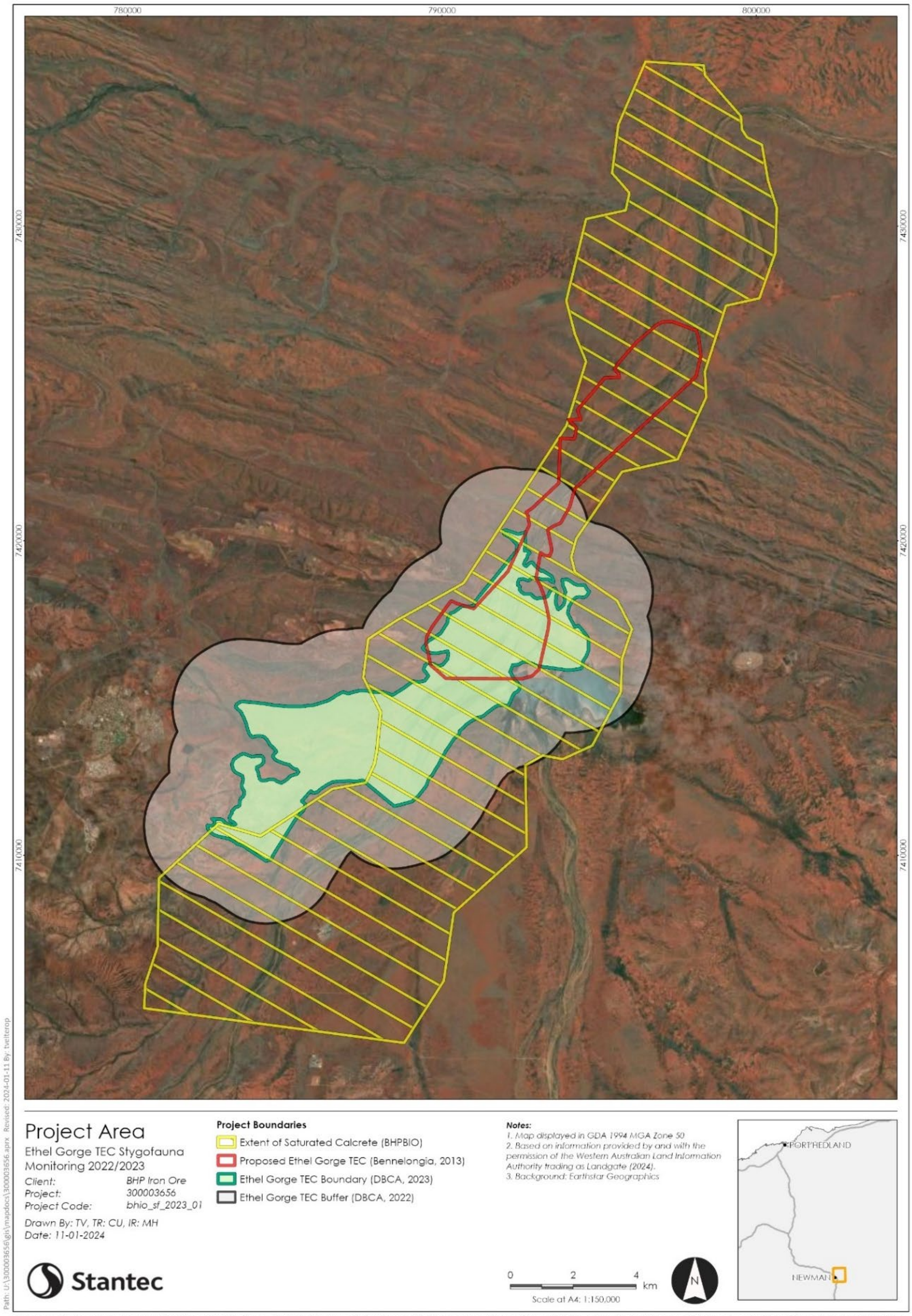

Figure 1-2 Boundaries for the TEC, TEC buffer (ammended from 5 kms to 2 kms) and extent of saturated calcrete in the Ethel Gorge area.

1.3 Ethel Gorge Stygobiont TEC

The Ethel Gorge TEC was previously designated as Endangered, defined as an ecological community that has been adequately surveyed and/or has a limited distribution, with few isolated occurrences that are very vulnerable to known threatening processes (DBCA 2018). In May 2023, this listing was amended to Critically Endangered (Minister for Environment 2023).

Stygofauna of the Ethel Gorge TEC and surrounding areas have been surveyed for almost 20 years (**Table 1-1),** with 82 stygal species recorded. As part of mapping and characterisation of the TEC (Bennelongia 2013) (**Figure 1-2**) core habitat was defined and up to 50 “core species” of stygofauna were documented, comprising taxa known only from the wider Newman area. A recent review of taxa, taxonomic consolidation and regional consideration of species’ current known ranges reduced the number of taxa that currently meet the criteria of “core species” to 39 (Stantec 2023b).

Stygal groups commonly represented in the TEC include Amphipoda, Bathynellacea, Copepoda, Isopoda, Ostracoda and Oligochaeta. While copepods have been numerically abundant, ostracods, amphipods and bathynellaceans have contributed considerably to the taxonomic diversity of the assemblage (Stantec 2023b).

The TEC boundary encompasses Ophthalmia Dam and extends approximately four kilometres to the north and 10 km to the south of the primary dam walls. The shallow alluvial and calcrete aquifers that host the highest species richness extend beyond the core area of the TEC, reaching approximately 20 km upstream and downstream of Opththalmia Dam (Bennelongia 2014; MWH 2016; RPS 2013).

To facilitate monitoring and management of sensitive receptors in the area, monitoring zones were established, based on hydrological and hydrogeological conditions. Monitoring Zone 1 (MZ1) predominantly corresponds to the northern portion of the TEC and the areas of high aquifer recharge below Ophthalmia Dam. This zone has also been used as a focus area for the assessment of groundwater levels and groundwater quality (Douglas and Pickard 2014).

1.4 Climate

The climate of the Pilbara bioregion is classified as semi-arid with very hot summers and mild winters. Rainfall typically occurs during the wet season (December to April), in response to ex-tropical cyclones or isolated storm activity. However, evaporation rates are high, and temperatures often exceed 38°C in summer. The nearest Bureau of Meteorology (BOM) weather station to the Survey Area, is Newman Aero (station number 007176), located at the southern end of the Survey Area. The mean long-term annual rainfall in the area was 323.8 mm (1971 – 2022) (BoM 2023). **Figure 1-3** presents long-term annual rainfall between 2003 to 2022, highlighting the peak period of rainfall in 2006 (545.6 mm) and a drought period in 2019 (115.8 mm).

Monthly rainfall recorded between June 2022 and May 2023 was 323.4 mm, reflecting the long-term annual average for the area (**Figure 1-4**). Rainfall recorded in September 2022 (57.2 mm) was roughly 12 times greater than the long-term monthly average (4.7 mm). Rainfall in March 2023 (118.4 mm) was substantially greater than the monthly average (42.3 mm), with a significant rainfall event on the 30th of the month contributing 85.6 mm of the monthly total and causing a flooding event.

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Table 1-1 Historic Stygofauna surveys in the wider Ethel Gorge area, including the current Program.

Year	Survey Timing	Sampler/Author	Reference
2003	Dry Season	Not Available	Not Available
2007	Dry Season	Biota Environmental Sciences	Not Available
2008	Dry Season	Ecowise Environmental	Not Available
2009	Wet and Dry Season	Ecowise Environmental, Subterranean Ecology	Not Available
2010	Wet and Dry Season	Subterranean Ecology	Not Available
2011	Wet Season	Subterranean Ecology	Not Available
2012	Wet Season	Subterranean Ecology	(Subterranean Ecology 2012)
2013	Dry Season	Subterranean Ecology	(Subterranean Ecology 2014)
2014	Wet and Dry Season	Subterranean Ecology, Stantec (MWH)	(Subterranean Ecology 2014), (MWH 2015)
2015	Wet Season	Stantec (MWH)	(MWH 2015)
2016	Wet Season	Stantec (MWH)	MWH (2016) (MWH 2016)
2017	Wet Season	Stantec	(Stantec 2017)
2019	Dry Season	Stantec	(Stantec 2020)
2020	Wet and Dry Season	Stantec	(Stantec 2020;2021)
2020	Dry Season	Bennelongia	Unpublished (East Ophthalmia)
2021	Wet and Dry Season	Stantec	(Stantec 2021;2022)
2021	Wet Season	Bennelongia	Unpublished (East Ophthalmia)
2022	Wet and Dry Season	Stantec	(Stantec 2023a) Stantec (current Program)
2023	Wet and Dry Season	Stantec	Stantec (current Program) , Stantec (In progress)

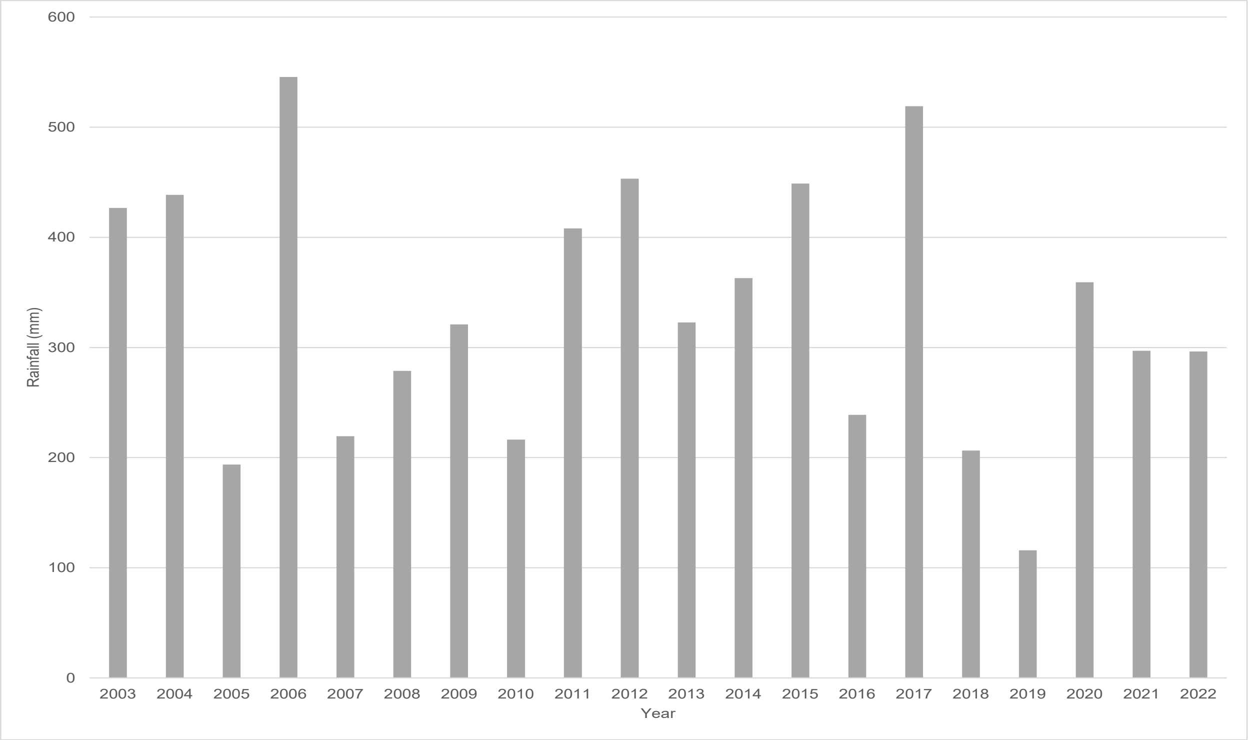


Figure 1-3 Long-term total annual rainfall (2003-2022) at Neman Aero Weather Station (007176) (BoM, 2023).

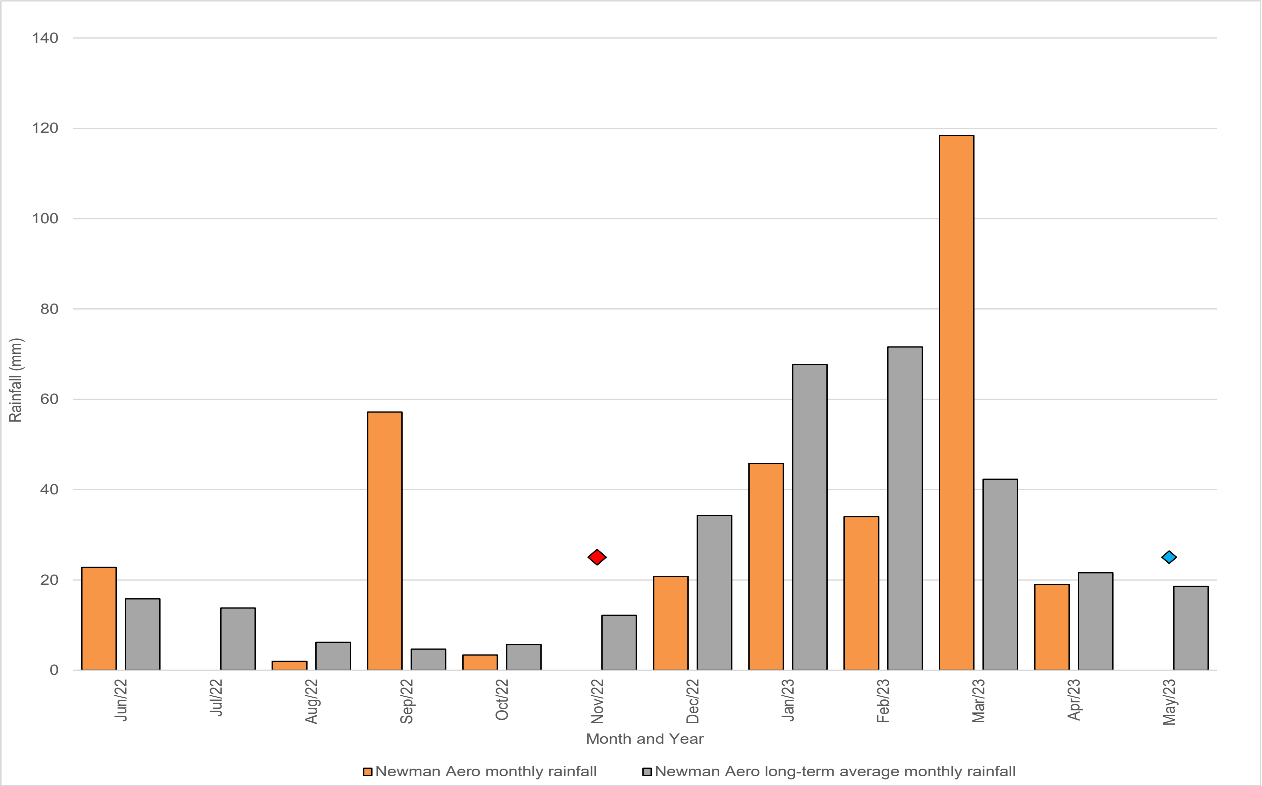


Figure 1-4 Monthly rainfall at Newman Aero (007176), compared to the long-term average monthly rainfall. Dry (◆) and wet (◆) season surveys of the Program indicated (BoM 2023) .

1.5 Hydrogeology

A highly permeable alluvial aquifer comprising an upper unit of sandy-alluvium and calcrete and a lower unit of gravelly-alluvium forms the key feature of the groundwater system in the Ethel Gorge area. The units are separated by an extensive low permeability clay sequence (RPS 2013).

Recharge to the upper alluvial aquifer is primarily as seepage from Ophthalmia Dam - a managed aquifer recharge structure that has been constructed on the Fortescue River flood plain. The upper unit, which is shallow and unconfined, also receives direct infiltrations from streamflow events along the Fortescue channel and several creeks. The lower aquifer, confined by the overlying clay, is predominantly subject to hydraulic loading from the dam (RPS 2013).The Ethel Gorge stygofauna TEC occurs in the shallow alluvial aquifer, spanning from an area on the Fortescue River floodplain approximately 2 km upstream of the gorge to approximately 4 km downstream of the gorge entrance. This coincides with a thick accumulation of calcrete (>20 m in thickness) occurring at less than 20 mbgl, commonly as outcrop.

As reported by RPS (2013), groundwater levels across the area range between 0 metres below ground level (mbgl) and 10 mbgl. While declines have been noted in response to pit dewatering within adjacent mining operations, and low rainfall periods (2018/2019), groundwater levels have remained within the range documented since Ophthalmia Dam was commissioned. Considerable recharge of the groundwaters was observed in January 2020, linked to substantial rainfall from tropical cyclone Blake (BHP 2022b; EMM 2020).

2. Methods

2.1 Survey design and sampling effort

The survey design for the Program was developed in accordance with the Ministerial conditions and the EPWRMP, with the purpose of providing information relevant to the management of the Ethel Gorge TEC. Stygofauna haul net sampling and collection of groundwater for water quality analyses aligned with previous surveys. An additional component, environmental DNA (eDNA) sampling, was integrated into the Program for a subset of bores. eDNA bores were chosen to provide a wide spatial representation of bores throughout the survey area, and to include bores that were likely to produce a high diversity of organisms to thoroughly test the capability of the eDNA methods.

A total of 80 stygofauna samples were collected during the Program using haul net sampling; 40 in 2022 Dry (17th – 23rd November 2022) and 2023 Wet respectively (11th – 17th May 2023)(**Appendix A, Figure 2-1**), which is an increase on previous survey rounds where only 25 bores were sampled in each season. Thirty-four bores were sampled in both seasons (n= 68 samples), with the remaining bores (n=12 samples) sampled in one season only. Amendments to bores sampled between seasons were made due to access restrictions. The Monitoring Zone (MZ) classifications assigned to bores were consistent with their classification in previous surveys to ensure continuity for data interpretation (**Table 2-1**). The cumulative survey locations in the area for stygofauna are shown in **Figure 2-2**, representing the consolidated effort from the surveys detailed in **Table 1-1**.

A Fauna taking (biological assessment) licence (Regulation 27, Biodiversity Conservation Regulations 2018), was obtained from the Department of Biodiversity, Conservation and Attractions (DBCA) prior to the commencement of the surveys (Licence number BA27000112). The 2022 Dry and 2023 Wet surveys were both led by Dr Mathew Hourston (**Table 2-2**).

Table 2-1 Monitoring zone descriptions for the Program.

Monitoring Zone	Description
1. Ethel Gorge habitat stygofauna TEC	Core habitat area of TEC comprised of main calcrete body within confluence zone of Fortescue River with both Homestead Creek and Shovelanna Creek.
1 B. Early Warning	Downstream of Ophthalmia Dam between MZ4 and MZ1.
2. Shovelanna Creek*	Aquifer system associated with creek prior to entering confluence zone with Fortescue River.
3. Homestead Creek	Aquifer system associated with creek prior to entering confluence zone with Fortescue River.
4. Ophthalmia Dam	Aquifer system associated with Ophthalmia Dam and Fortescue River and Warrawanda Creek catchments south of dam.
5. Fortescue River	Aquifer system associated with Fortescue River North of Ethel Gorge stygofauna TEC within MZ1.
6. Whaleback Creek	Aquifer system associated with Whaleback Creek prior to entering confluence zone with Fortescue River (MZ1B) and MZ4.

Table 2-2 Stantec personnel involved in the surveys for the Program.

Name	Qualifications and Experience	Survey
Mathew Hourston (Senior Scientist)	BSc Marine and Environ. Biology (10 yrs exp.)	2022 Dry / 2023 Wet
Joey Laugharne (Intermediate Scientist)	BSc Marine Science and Zoology (4 yrs exp.)	2022 Dry
Liam Gasbarro (Graduate Scientist)	BSc (2 yrs exp.)	2023 Wet

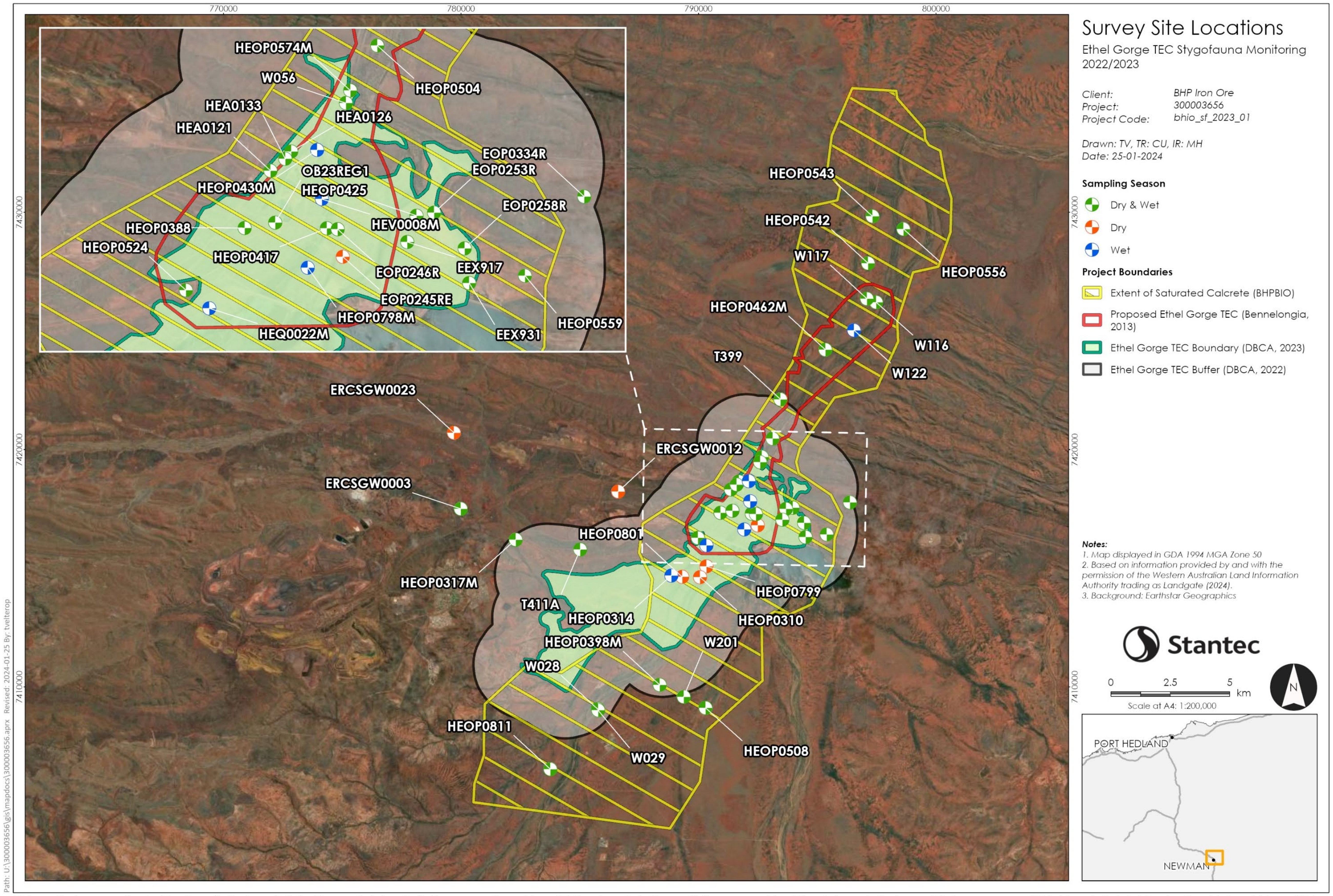
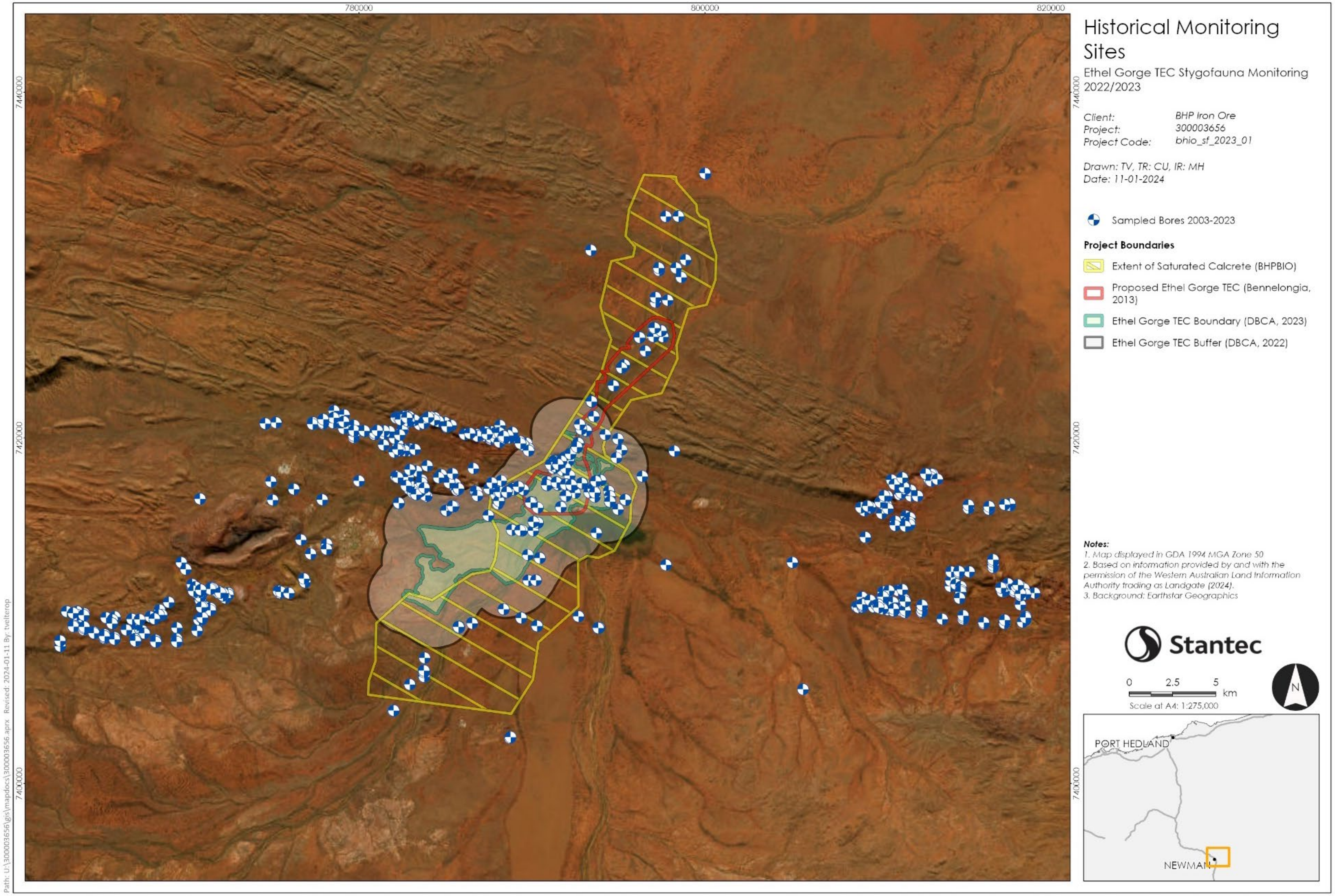


Figure 2-1 Bore locations at which stygofauna were sampled for the Program in the 2022 wet season survey and 2023 dry season survey.



Notes:
1. Map displayed in GDA 1994 MGA Zone 50
2. Based on information provided by and with the permission of the Western Australian Land Information Authority trading as Landgate (2024).
3. Background: Earthstar Geographics

Figure 2-2 Bore locations at which stygofauna were sampled for in the broader area from 2003 to 2023 during previous and current surveys.

2.2 Groundwater Assessment

2.2.1 Groundwater Properties

During the 2022 Dry and 2023 Wet surveys, the SWLs were measured at each bore using a Solinst 101 water level meter. The end of hole (EoH) was estimated using the number of rotations of the sampling winch reel, while conducting stygofauna sampling.

Basic groundwater physico-chemical parameters were measured *in-situ* including pH, water temperature, dissolved oxygen (DO), electrical conductivity; (EC), total dissolved solids (TDS) and reduction-oxidation (Redox) potential. Groundwater was collected using a bailer from the upper surface of the bore column, with water quality measurements recorded using a YSI water quality meter.

Additional groundwater samples collected using the bailer were placed into appropriate sterilised bottles provided by the NATA-accredited Australian Laboratory Services (ALS), containing preservative where required. Bottles were filled to the top with the sample and sealed, excluding any air from the water. Following collection, the samples were couriered to ALS in Perth.

The analytical suite for groundwater comprised pH, TDS, EC, alkalinity, major ions (calcium, chloride, potassium, magnesium, sodium, and sulphate), nutrients (nitrate+nitrite, nitrogen, and phosphorus) and metals (aluminium, arsenic, barium, boron, cadmium, chromium, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, and zinc). Holding times were met for all parameters except for analytical pH across the program. Results for that parameter should be considered indicative.

2.2.2 Groundwater Trigger Values

Low-risk early warning trigger values have previously been derived for groundwater quality for the Ethel Gorge stygofauna TEC, aligning with the EPWRMP, and referred to as groundwater trigger values (GTVs). The GTVs for each monitoring zone, based on the EPWRMP are presented in **Table 2-3** with a focus on changes in groundwater levels and/or salinity (EC/TDS) variances (Douglas and Pickard 2014). The groundwater levels and salinity recorded as part of the Program are used to inform the interpretation of trends in the stygofauna assemblage. While the recorded groundwater levels and salinities are compared to the published GTVs, they do not form part of the reporting and management of the groundwater for the boarder operations in the area.

Project specific GTVs have also been developed for groundwater quality variables by Stantec, following methodology outlined in ANZECC (2000), where an appropriate number of records exist (n>30). Low risk trigger values comprise the 80th percentile (and 20th percentile for pH only) of the amalgamated time series data for each parameter. Separate GTVs are calculated for MZ1 only (Ethel Gorge TEC) and for all other areas together, with the latter to provide regional context (**Table 2-4**). Pre-treatment of the data involved the removal of outliers for each parameter; values +4 standard deviations from the mean. Separate GTVs were established for the dry and wet seasons. As above, are used to inform the interpretation of trends in the stygofauna assemblage and do not form part of the reporting and management of the groundwater for the boarder operations in the area.

At present, GTVs have only been developed for some of the metals, linked to the number of records above the limit of reporting of the testing protocols. GTVs were developed for Barium and Boron in the 2021/2022 survey (Stantec 2022).The introduction of ultra-trace analyses during the current Program, has provided additional records, allowing for development of GTVs for Arsenic, Copper, Iron, Manganese, Molybdenum, Nickel and Zinc. However, data remains insufficient for Mercury, Aluminium, Selenium, Cadmium, Chromium and Lead.

The low-risk, early warning GTVs for measured parameters are an early warning system only and have been developed for the interpretation of trends in the stygofauna assemblage, not as part of the formal groundwater management plan of the area. Exceedance of any of these GTVs may indicate that further investigation is required but they should be considered in the context of historic spatial and temporal trends environmental and in an operational context prior to investigation of potential management options.

Table 2-3 Groundwater trigger values (GTVs) for standing water level (SWL) and total dissolved solids (TDS) in each of the monitoring zones for the Program, developed as part of the EPWRMP (Douglas and Pickard 2014).

Monitoring Zone	Assessment Component	Groundwater Trigger Value (GTV)
1. Ethel Gorge habitat stygofauna TEC	SWL	Lower SWL +/- >5 m or at rate >4 m/year
		Upper SWL >2 mbgl
	Historic variance in groundwater salinity (TDS/EC)	TDS >3,000 mg/L
		EC >4,545 µS/cm
1B. Early Warning	Upper SWL	>2 mbgl
	Groundwater quality (TDS)	>20 % variance from interpreted seasonal baseline
2. Shovelanna Creek	Groundwater quality (TDS)	>20% variance from interpreted seasonal average
3. Homestead Creek	SWL	+/->6 m or at rate of change >4 m/year
	Groundwater quality (TDS)	>20 % variance from interpreted seasonal baseline
4. Ophthalmia Dam	SWL	Not defined
	Groundwater quality (TDS)	>20 % variance from interpreted seasonal baseline
5. Fortescue River	SWL	Not defined
	Groundwater quality (TDS)	Not defined
6. Whaleback Creek	SWL	Not defined
	Groundwater quality (TDS)	Not defined

Table 2-4 Groundwater Trigger Values (GTVs) derived for water quality for the Program. Note, only those GTVs marked with * were developed as part of the EPWRMP (Douglas and Pickard 2014). All others have been developed subsequent to that report.

Parameter		Groundwater Trigger Values (GTVs)		
		20th percentile	80th percentile	20% Variance*
Basic	pH	✓	✓	-
	TDS	-	✓	✓ *
	EC	-	✓	✓ *
Major Ions	Sodium	-	✓	-
	Magnesium	-	✓	-
	Potassium	-	✓	-
	Calcium	-	✓	-
	Chloride	-	✓	-
	Sulphate	-	✓	-
	Total Alkalinity	-	✓	-
	Total Cations	-	✓	-
	Total Anions	-	✓	-
Nutrients	Total Nitrogen	-	✓	-
	Nitrate + Nitrite	-	✓	-
	Total Phosphorus	-	✓	-
Metals	Barium	-	✓	-
	Boron	-	✓	-
	Iron	-	✓	-
	Arsenic	-	✓	-
	Copper	-	✓	-
	Manganese	-	✓	-
	Molybdenum	-	✓	-
	Nickel	-	✓	-
	Zinc	-	✓	-

2.3 Stygofauna Assessment

2.3.1 eDNA sampling and analyses

A total of 20 samples were analysed for eDNA, nine from 2022 Dry and eleven from 2023 Wet. Sampling was undertaken by Stantec personnel as per Standard Operating Procedure provided by the molecular service provider, eDNA Frontiers, as follows:

- A 1L stainless steel bailer was used to retrieve samples from bores. The bailer had been decontaminated between bores using a 10% bleach solution and well rinsed with fresh water prior to use.
- Prior to any other disturbance of the water column in the bore, the bailer was lowered into the bore and used to gently agitate the sediments in the water column;
- The bailer was immediately retrieved, and the contents emptied into a 1L sterile sample container.
- Three replicate samples were retrieved using the above method and the samples immediately stored on ice, until filtration;
- Within 24 hours of retrieval, each replicate 1L sample was filtered onto an 0.45 µm micron filter paper using Sentino vacuum filtration units; and
- The filter papers with sample eDNA were then folded and inserted into a 2ml microvial containing an appropriate preservative liquid, which stabilised the eDNA for ambient temperature travel.
- At the conclusion of each survey, samples were couriered to the eDNA Frontiers laboratories in Perth.

Upon receipt, the samples were analysed using assays for CO1 (fwh2) and 18S.The full report from eDNA Frontiers is included in **Appendix C** (eDNA Frontiers 2023).

2.3.2 Haul Net sampling

Stygofauna were sampled using haul nets during both 2022 Dry and 2023 Wet. This method is widely considered the most efficient method to retrieve stygofauna from bores (Allford *et al.* 2008). Sampling was consistent with the procedures outlined in the Environmental Protection Authority (EPA) Technical Guidance Sampling Methods for Subterranean Fauna Survey (EPA 2021). The sampling method was as follows:

- Samples were collected using two weighted haul nets with mesh sizes of 150 µm and 50 µm, with each net fitted with a 70 ml plastic collection vial;
- The 150 µm net was lowered first, to the base of the bore;
- Once at the base of the bore, the net was gently raised up and down to agitate the sediments;
- The net was then raised slowly, to minimise the 'bow wave' effect that may result in the loss of specimens, filtering the stygofauna from the water column on retrieval;
- Once retrieved, the collection vial and net were rinsed into a collection pail;
- This process was repeated three times alternating with three hauls using the 50 µm mesh net;
- Following the final haul, the contents were filtered through a wide aperture 50 µm mesh net, rinsed with 100% undenatured ethanol and transferred to a 250 mL polypropylene vial for storage in 100% ethanol;
- To prevent cross-contamination, all sampling equipment was washed thoroughly with Decon 90 (2 to 5% concentration) and rinsed with potable water after sampling was completed at each bore;
- Samples were placed into eskies with ice bricks in the field, prior to being transferred into a refrigerated environment on-site at the end of each survey day; and
- At the end of each survey, the samples were couriered to the Stantec laboratory in Perth.

2.3.3 Sorting and Identification of Specimens

Preserved stygofauna samples were sorted manually under Leica MZ6, MZ7.5 and M80 stereomicroscopes. Sorting was conducted by suitably qualified scientists in the Stantec laboratory. Sorted specimens were preserved in 100% ethanol and were refrigerated at approximately -20°C to ensure viability for DNA analysis, should it be required.

Identification was carried out to species or morphospecies level for most stygofauna taxa, using published literature, unpublished keys and taxon descriptions. Identification was undertaken by Drs Erin Thomas and Mathew Hourston of

Stantec. Copepods and ostracods were identified by specialist taxonomists Jane McRae and Dr Stuart Halse, respectively. DNA analysis was also undertaken on a number of specimens. Genetic characterisation was completed by Dr Remko Leijs of the South Australian Museum (SAM). Haplotype characterisation of the sequenced specimens was also investigated to determine the intraspecific diversity of certain taxa. The full report for the DNA identifications is provided in **Appendix B,** (Lejis 2023).

2.3.4 Data Analysis

Interrogation of stygofauna species richness and abundance was undertaken for the Program and available historic data. Due to differences in survey effort, including sample locations and seasonality, mean species richness and abundance was calculated per bore to standardise data for comparison.

The EstimateS software package version 9.1.0 (Colwell 2013) was used to investigate species richness and survey effort for the core stygofauna species, based on monitoring data from November 2009 to May 2023. The analysis used species accumulation rarefaction and extrapolation curves, and species richness estimators using incidence and abundance data. The species richness analyses provide a statistical estimation of the proportion of the stygofauna assemblage that had been detected to date as a function of the total numbers of species estimated to occur in the area. A range in the number of species predicted to form the core assemblage was provided using seven estimators (ACE, Bootstrap, Chao1, Chao2, ICE, Jack 1 and Jack 2), which is statistically more robust than using a single estimator (Hortal *et al.* 2006).

Two data sets were used for the analyses; the first included taxa that occur in the wider Newman area, (including the Ethel Gorge TEC), while the second data set encompassed core species for MZ 1 only.

2.4 Limitations of Assessment

2.4.1 Survey

Limitations for the 2022/2023 Program included access to certain sites and lack of historical data in some newly included sites. The surveys included 34 bores sampled in both 2022 Dry and 2023 Wet. In each of seasons, a further six bores were sampled, totaling 40 bores per season. While the intention was to sample the same suite of 40 in both seasons, six of the bores sampled in 2022 Dry were inaccessible in 2023 Wet and were replaced with contingency bores. The bores that were seasonally inaccessible will be removed from future surveys to maximise consistency across seasons.

Due to the expansion of the Program to include 40 bores per season (increased form 25), some of the bores had not been previously surveyed, or had not been surveyed for many years. As a result, there are some bores for which comparative historical data on the groundwater and stygofauna is unavailable.

2.4.2 Groundwater quality

While the collation of a statistically robust baseline data set and subsequent development of GTVs for metals forms an objective of the Program, not all metals have sufficient records above the Limit of Reporting to construct robust GTVs. The introduction of ultra-trace analyses in the 2022/2023 Program provided a lower limit of reporting, increasing the number of detections. However, additional records are still required to develop GTVs for some metals.

2.4.3 Specimen identification, assessment and taxonomic resolution

Stygofauna are inherently difficult to assess, owing to their inhabitation of cryptic, concealed habitats. Although such fauna are becoming increasingly well understood, there remains a large degree of uncertainty surrounding the taxonomy and ecological preferences of many taxa, with taxonomic frameworks poorly developed or even absent for many groups. For this Program, specimens were identified to the lowest possible taxonomic level. However, specimens were not always be identified to the level of species or morphospecies due to:

- loss or damage of important taxonomic features during collection of specimens;
- lack of adult specimens;
- lack of specimens of the correct sex for identification;
- taxonomy, where the current taxonomic resolution for a particular group is insufficiently advanced, and/or relevant taxonomic keys and descriptions are lacking; or
- contamination or failure of DNA sequencing during genetic analysis.

Every effort has been made to assess the taxonomy and distribution of the stygofauna collected using historical client data, in-house data collections, publications, publicly available reports, and information provided by specialist taxonomists.

2.4.4 eDNA

The primary limitation to the eDNA component of the survey was the paucity of suitable reference material to match species' detections to. This limitation will be alleviated through further surveys, as further detections are made and as the taxon eDNA reference library is expanded. Additionally, the specificity of the 18S assay is quite low, being able to reliably discriminate detections at the family level. This is an inherent limitation of the 18S assay and is partially alleviated by the inclusion of the CO1 assay, which can achieve a much lower level of specificity. The comparative utility of traditional morphological analysis, CO1 and 18S assays is discussed in greater detail in Section 3.2.3.

3. Results and Discussion

3.1 Groundwater Properties

3.1.1 Standing Water Levels

The mean standing water levels (SWL) recorded in each monitoring zone during 2022 Dry and 2023 Wet were compared to historical means, previous years values and any established GTVs. The results are summarised below.

- The SWL in bores throughout the survey area decreased between 2021 Dry and 2022 Dry on average by 0.06 m, and increased by an average of 0.1 m between 2022 Wet and 2023 Wet.
- The maximum variation from between comparable dry and wet seasons respectively was a 3.3 m increase at HEOP0425 from 2021 Dry to 2022 Dry, and a 2.73 m decrease at HEOP0504 from 2021 Dry to 2022 Dry. This variation is less than the GTV of +/- 5 m for MZ1, in which both of these sites are located.
- All mean SWLs for the monitoring zones during 2022 Dry and 2023 Wet were within the bounds of historical ranges, and in most cases were similar to, or greater than, their corresponding long-term average. Only MZ3 in 2023 Wet was appreciably below the long term mean however was still within historical ranges **(Figure 3-1)**.
- Monitoring Zone 1
    - The mean SWL recorded in 2022 Dry (497.84 m AHD) was 0.13 m lower than the average SWL in 2021 Dry.
    - A slight decrease was also recorded from 2022 Wet to 2023 Wet, where SWL decreased by 0.02 m to 497.90 m AHD.
    - The SWL in individual bores was variable throughout the zone with both increases and decreases observed throughout that period. Most variances were <0.5 m, with the exception of HEOP0504 and HEOP0425 noted above.
    - There was an increase of 0.06 m in the mean SWL from 2022 Dry to 2023 Wet.
- For Monitoring Zone 1B, comparative SWL data was limited to a single bore; EEX931. SWL at this site increased by 0.24 m between 2021 Dry and 2022 Dry to 507.45 m AHD, and by a further 0.28 m between 2022 Dry and 2023 Wet to 507.73 m AHD.

- Monitoring Zone 3 had an average SWL of 500.17 m AHD during 2022 Dry, which was a slight (0.10 m) decrease from the previous year. In contrast, the SWL increased between 2022 Wet and 2023 Wet by 0.5 m, to 500.43 m AHD. As noted above, the 2023 Wet mean SWL is below the long-term mean of 504.43 for this MZ, however this value is still within the range of historical variability **(Figure 3-1)**.

- Monitoring Zone 4 had an average SWL of 515.87 m AHD in 2022 Dry, and 516.10 in 2023 Wet. These values represent decreases of 0.06 m and 0.03 m decreases respectively, relative to the previous dry and wet seasons. The SWL increased by 0.23 m between 2022 Dry and 2023 Wet.

- Monitoring Zone 5 had a single bore with comparative historical SWL data available. The SWL at MZ5 increased by 0.83 m to 484.61 m AHD between 2021 Dry and 2022 Dry and decreased by 0.01 m to 483.77 m AHD between 2022 Wet and 2023 Wet. Between 2022 Dry and 2023 Wet, SWL decreased by 0.84 m.

- Monitoring Zone 6 also had a single bore with comparative historical SWL data available. SWL at MZ6 decreased by 0.17 m to 515.92 m AHD between 2021 Dry and 2022 Dry and increased by 0.12 m to 516.1 m AHD between 2022 Wet and 2023 Wet. Between 2022 Dry and 2023 Wet, AHD increased by 0.18 m.

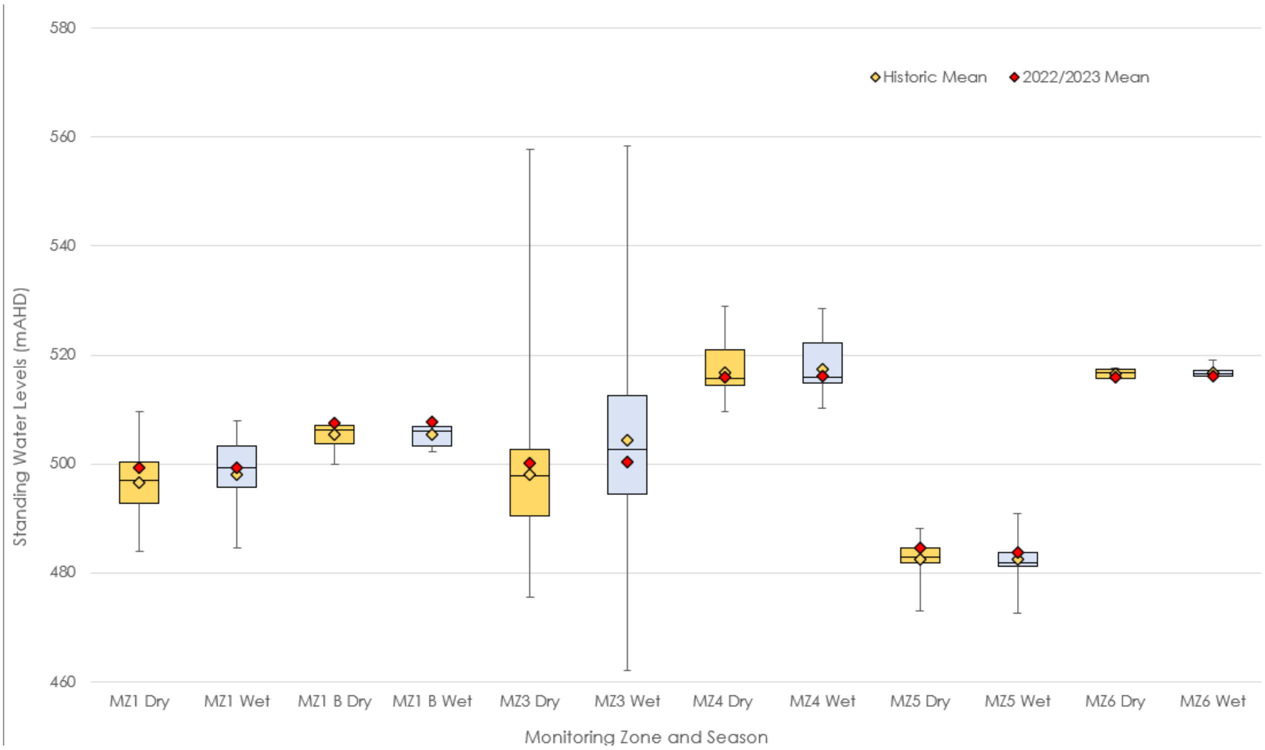


Figure 3-1 Mean SWL for each monitoring zone for 2022 Dry and 2023 Wet, compared to summary statistics for the full historical SWL data set (2009-2022).

3.1.2 Groundwater Quality

3.1.2.1 Basic Parameters

Ground water pH ranged from 4.82 to 9.09 in 2022 Dry and from 5.35 to 9.07 in 2023 Wet, corresponding to acidic to alkaline conditions (Foged 1978). The pH tended to vary more among bores and monitoring zones rather than between seasons at the same location (**Figure 3-2, Table 3-1, Table 3-2**). In line with in the previous survey (Stantec 2022), bore W201 in MZ4 had the lowest pH, with values of 4.82 and 5.35 in 2022 Dry and 2023 Wet respectively. The highest pH was 9.09 at HEOP0310 in 2022 Dry and 9.07 at HEOP0798M in 2023 Wet. The former bore does not have any recent records for comparison, while relatively high pH has been recorded in previous years at the latter bore. Overall, mean pH values in each monitoring zone were within the recorded historical variability. However, some exceedances of the 80% and 20% GTVs were recorded at individual bores. The mean pH values in monitoring zones 1, 1B and 6 were above their historical means, consistent with the previous year's data. Despite deviations from the GTVs, pH remained within the recorded variability of the Ethel Gorge TEC area and were within the known tolerance limits of stygofauna (Glanville *et al.* 2016; Reeves *et al.* 2007; Schulz *et al.* 2013).

Groundwater salinity, measured as Electrical Conductivity (EC), ranged from 467 to 11,300 µS/cm in 2022 Dry and from 437 to 1,660 µS/cm in the 2023 Wet (**Figure 3-3, Table 3-1, Table 3-2**). The minimum salinity was recorded at HEOP0811 in Dry 2022 while maxima were recorded at EOP253R for both 2022 Dry and 2023 Wet. The mean salinity for each of the MZs were within the historical range and below the relevant 80% GTVs for all except MZ2. There is little historical water quality data available for MZ2 for comparison against baseline conditions.

Values at some bores exceeded the 80% GTVs for salinity (as EC), however, these bores tended to be new inclusions into the Program or bores that had not been surveyed for several years. (e.g., EOP0253R and W247). The salinity values at

EOP253R were the highest recorded for the TEC and exceeded the EC GTV set by the EPWRMP (Douglas and Pickard 2014). However, the consistency of the salinity at this bore between the dry and wet seasons suggests the comparatively higher salinity represents a spatial difference rather than a temporal change in the overall salinity of the TEC.

The established GTV developed during the EPWRMP for Total Dissolved Solids (TDS) is 3,000mg/L. The majority of bores were below this threshold during the 2022 Dry and 2023 Wet (**Table 3-1, Table 3-2**), with the exception EOP0253R in both seasons, As noted for the EC, these values likely reflected spatial differences rather than a temporal change in the overall TDS of the TEC.

Major ion concentrations in groundwater systems, such as the Ethel Gorge Stygobiont TEC, are influenced by the inherent hydrogeological features of the area including groundwater residence and flow rates (Bakalowicz 1994). These long-term base conditions can be overlayed with seasonally variability from climatic sources such as rainfall and subsequent aquifer recharge.

The means within MZs for the individual ions concentrations (Na, Mg, K, Ca, Cl and S) were largely within the 80% GTVs and historical variability for all monitoring zones (**Figure 3-4, Figure 3-5, Table 3-1, Table 3-2**). Exceptions included elevated Na, K, Cl and S in MZ1 and MZ2, likely attributable to the inclusion of additional sites with comparatively higher values, as noted for EC and TDS. The recorded values of ion concentrations are not expected to have implications for the stygofauna organisms or assemblages as they are within the range of values that host high abundance and species richness in historical samples (Stantec 2023b).

3.1.2.2 Nutrients

Mean concentrations for nutrients tended to be below the 80% GTVs and within historical ranges in all monitoring zones in both 2022 Dry and 2023 Wet (**Figure 3-6**). Nitrogen concentrations in MZ2 were a notable exception to this trend, exhibiting mean values that exceeded the project specific GTVs. As noted above, two of the MZ2 sites surveyed during the current round are new inclusions; EOP0258R and W247, both of which show elevated nitrogen levels (**Table 3-1, Table 3-2**). Stantec (2023b) presented an analysis of nitrogen concentrations in the Ethel Gorge TEC in relation to fauna composition and abundance and determined that while most samples with high stygofauna abundances had nitrogen concentrations below 5mg/L, sites with concentrations above 20mg/L still supported abundant stygal communities, particularly of copepods. Mean phosphorus concentrations in each MZ were below the 80% GTVs and within the ranges known to support stygofauna in the Ethel Gorge TEC (Stantec 2023b). Some individual bores were above the relevant 80% GTVs however this is consistent with historical data from those bores, where available.

3.1.2.3 Metals

While ultra-trace metals analysis has increased the number of records (Table 2-4, Table 3-1, Table 3-2), the project-specific GTVs for metals are still based on limited numbers of samples and should be interpreted as preliminary values only. The ultra trace analyses are also suitable to compare concentrations against the most relevant published guidelines (ANZECC & ARMCANZ 2000). GTVs have now been developed for Barium, Boron, Manganese, Molybdenum and Zinc in both Wet and Dry seasons, as well as for Arsenic and Nickel in the Dry season, and Iron and Copper in the Wet season.

Barium concentrations at all bores apart from W201 and HEOP0556 were below the published 99% protection trigger value for barium in surface freshwater (0.09mg/L) (ANZECC & ARMCANZ 2000) (Table 3-1, Table 3-2). Mean barium concentrations in the monitoring zones were generally below the project specific 80% GTVs and within the range established for the Ethel Gorge region so far (Figure 3-7A). The exceptions to this were MZ4 and MZ5 which had means higher than the 80%GTV but still within the range established. These elevated means are due mostly to values recorded at W201 in MZ4 and HEOP0556 in MZ5 in both seasons. Site W201 showed similar concentrations in previous surveys (Stantec 2022), while HEOP0556 is a new inclusion in the current Program and has no historic data for barium.

All boron concentrations measured in Ethel Gorge samples were between 50-100x below the published 99% protection trigger value for boron (90 µg/L) (ANZECC & ARMCANZ 2000) (Table 3-1, Table 3-2). There were minor exceedances of the project specific 80% GTVs (0.768 or 0.71 µg/L) for the mean concentrations in MZ1 2022 Dry and MZ2 2022 Dry (Figure 3-7B), with the mean values of other zones below or close to the GTVs. Eight individual sites exceeded the GTVs in each season, with EOP0253R showing three times higher concentration at 2.2 µg/L, and HEOP0425 at 1.98 µg/L. However, they were considerably lower than the published 99% protection trigger value for boron (90 µg/L) (ANZECC & ARMCANZ 2000).

The maximum concentration of manganese in Ethel Gorge samples was 2 µg/L, 600x below the published 99% protection trigger value of 1200 µg/L (ANZECC & ARMCANZ 2000) (**Table 3-1, Table 3-2**). Mean manganese concentrations in the monitoring zones were generally below the project specific 80% GTV of 0.28 µg/L and within the range established for the

Ethel Gorge region to date (**Figure 3-8A**). Only MZ4 in Wet 2023 had a mean value above the project specific 80% GTV. The extremely low concentration of manganese compared to the published 99% protection trigger value indicates that the utility of the GTV for this metal is likely to be limited.

Molybdenum does not have a published 99% protection trigger value for surface water (ANZECC & ARMCANZ 2000). The project specific 80% GTV is set at 0.0043 µg/L (**Table 3-1, Table 3-2**). Mean concentrations of molybdenum were below the GTV in both seasons for MZ3, MZ4, MZ5 and MZ6, while those in MZ1 and MZ2 were relatively high but within the range of the recorded data for this metal (**Figure 3-8B**).

The maximum concentration of zinc in Ethel Gorge samples was 3.4 µg/L, which is between the published 99% and 95% protection trigger values (2.4 µg/L - 8.0 µg/L) (ANZECC & ARMCANZ 2000) (**Table 3-1, Table 3-2**). All other values in the Ethel Gorge samples were less than 0.18 µg/L. Mean Zinc concentrations in the monitoring zones were generally well below the project specific 80% GTV of 0.039 µg/L, except for MZ1 and MZ4 during 2023 Wet. However, the latter were still within the range established for the Ethel Gorge region to date (**Figure 3-8D**).

The maximum concentration of Arsenic in Ethel Gorge samples was 0.0297 µg/L, 30x below the published 99% protection trigger value of 1 µg/L (ANZECC & ARMCANZ 2000). The project specific 80% GTV has been set at 0.0018 µg/L and all except four individual samples returned values below that concentration (**Table 3-1, e**). Bore EEX931 contained the highest concentration of arsenic in both Dry 2022 and Wet 2023.

The maximum concentration of nickel in Ethel Gorge samples was 0.0098 µg/L, 800x below the published 99% protection trigger value of 8 µg/L (ANZECC & ARMCANZ 2000) (**Figure 3-8C, Table 3-1, Table 3-2**). The project specific 80% GTV has been set at 0.0030 µg/L and all except five individual samples returned values below that concentration. As for manganese, this site specific GTV may have limited utility, owing to the low concentration of nickel compared to the published 99% protection trigger value.

Iron does not have a published 99% protection trigger value for surface water (ANZECC & ARMCANZ 2000). The project specific 80%GTV has been set at 0.110 µg/L (**Table 3-1, Table 3-2**). While the majority of samples were below this GTV, and most were below the LoR of 0.002 µg/L. Bore W201 was an exception, with values of 18.8 µg/L in 2022 Dry and 30.70 µg/L in 2023 Wet, contributing to the high means for MZ4 (**Figure 3-7C**).

The maximum concentration of copper in Ethel Gorge samples was 0.006 µg/L, 150x below the published 99% protection trigger value of 1 µg/L (ANZECC & ARMCANZ 2000) (**Table 3-1, Table 3-2**). The project specific 80% GTV has been set at 0.0024 µg/L and all except one individual sample returned values below that concentration. The majority of samples were below the LoR despite the application of ultra trace analysis. While there are sufficient records to develop a project specific GTV , the low concentration of copper compared to the published 99% protection trigger value indicates that its practicality and usefulness is likely to be limited.

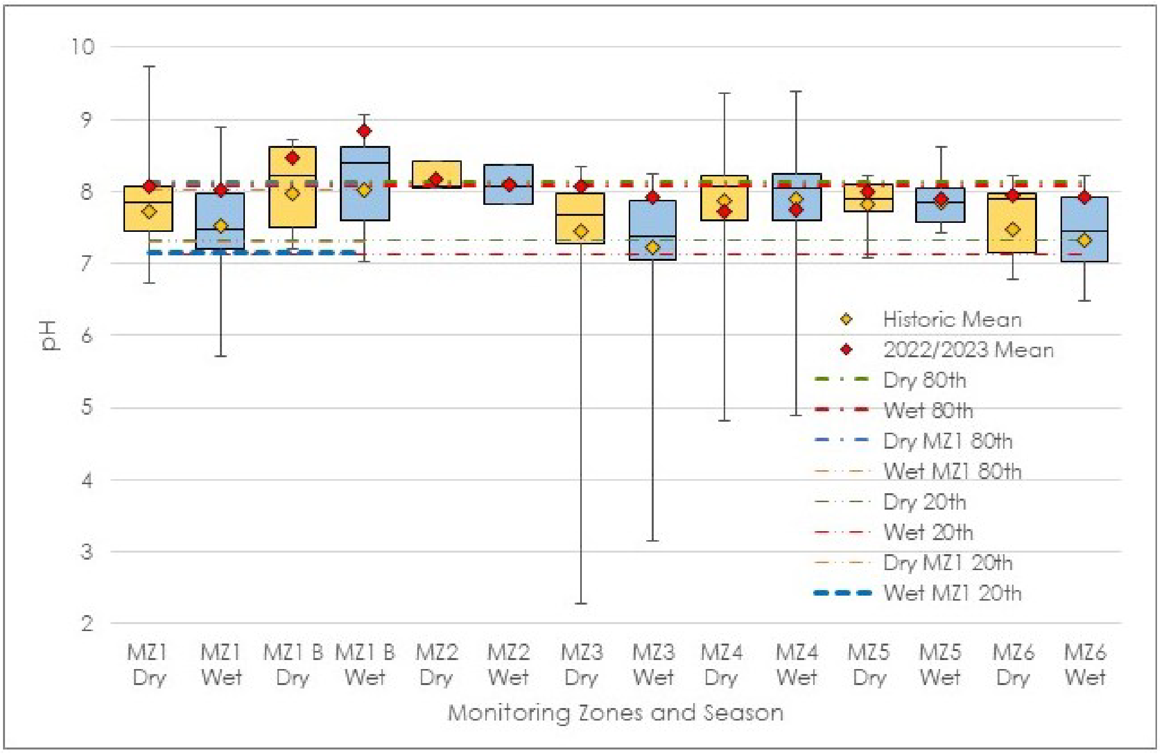


Figure 3-2 Comparison of pH for each monitoring zone and across dry and wet seasons (2009 to 2023), compared to the GTVs and current seasonal means.

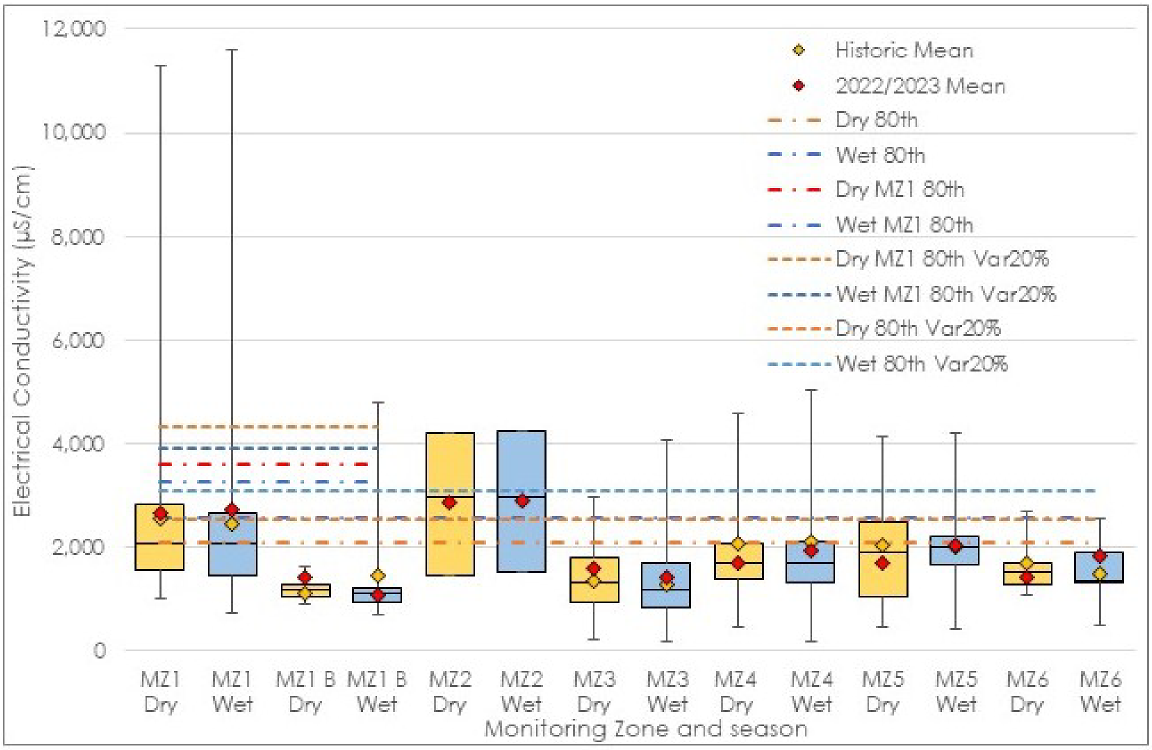


Figure 3-3 Comparison of salinity (EC) for each monitoring zone and across dry and wet seasons (2009 to 2023), compared to the GTVs and current seasonal means.

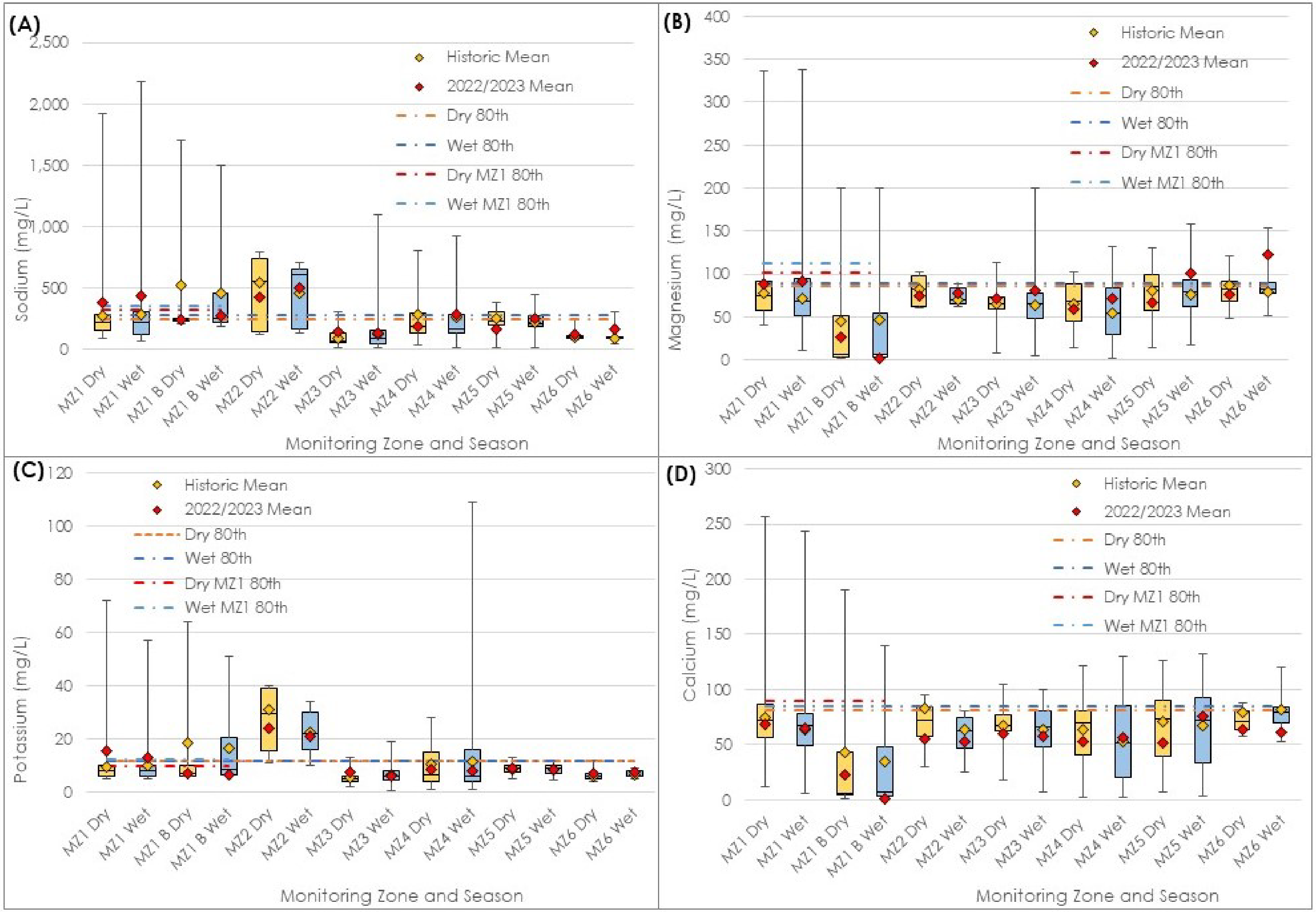


Figure 3-4 Comparison of major ions for each monitoring zone and across dry and wet seasons, compared to the GTVs and seasonal means (2012 to 2023) (A) sodium, (B) magnesium, (C) potassium, and (D) calcium.

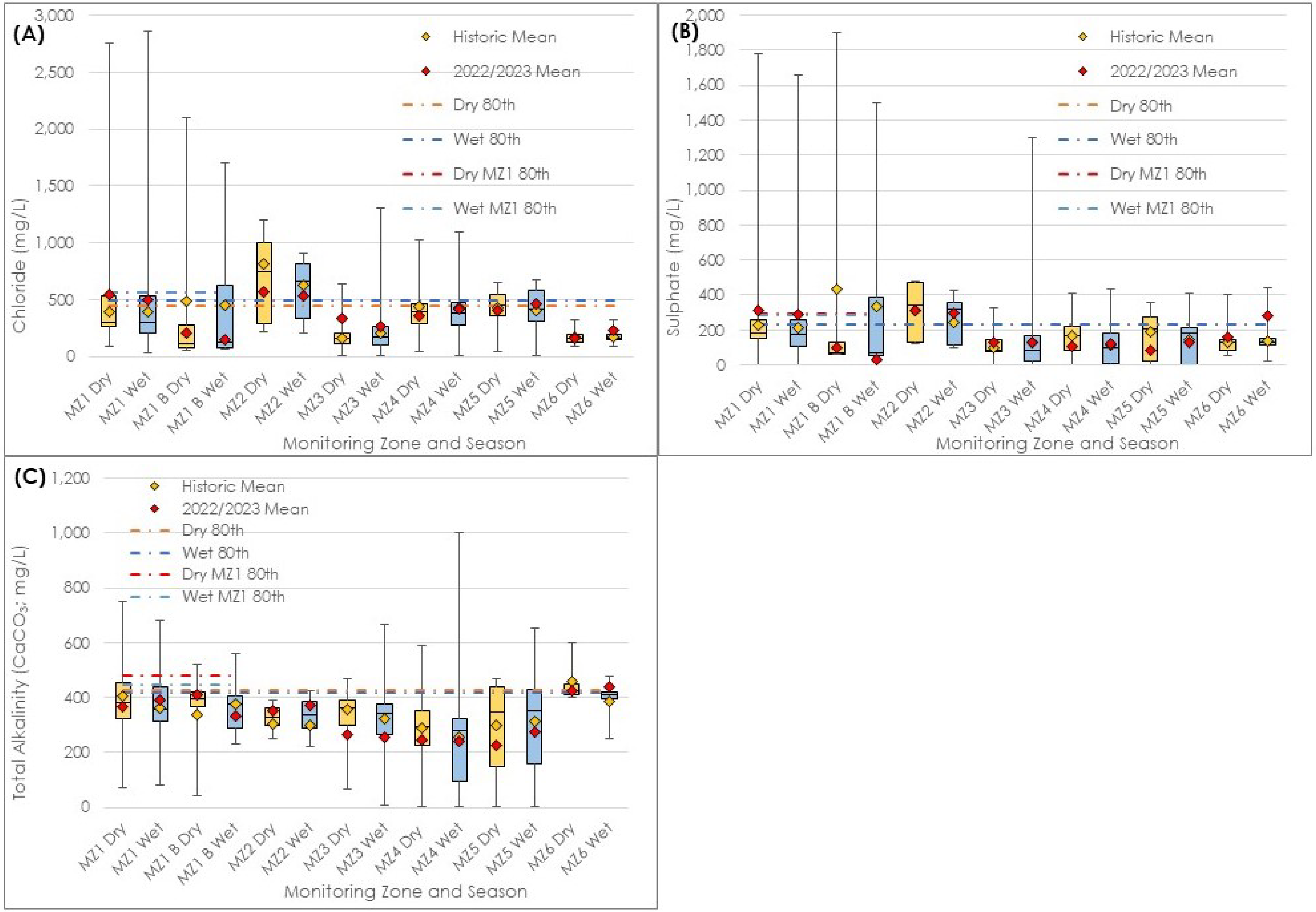


Figure 3-5 Comparison of major ions for each monitoring zone and across dry and wet seasons, compared to the GTVs and seasonal means (2012 to 2023), (A) chloride, (B)sulphate, and (C) alkalinity.

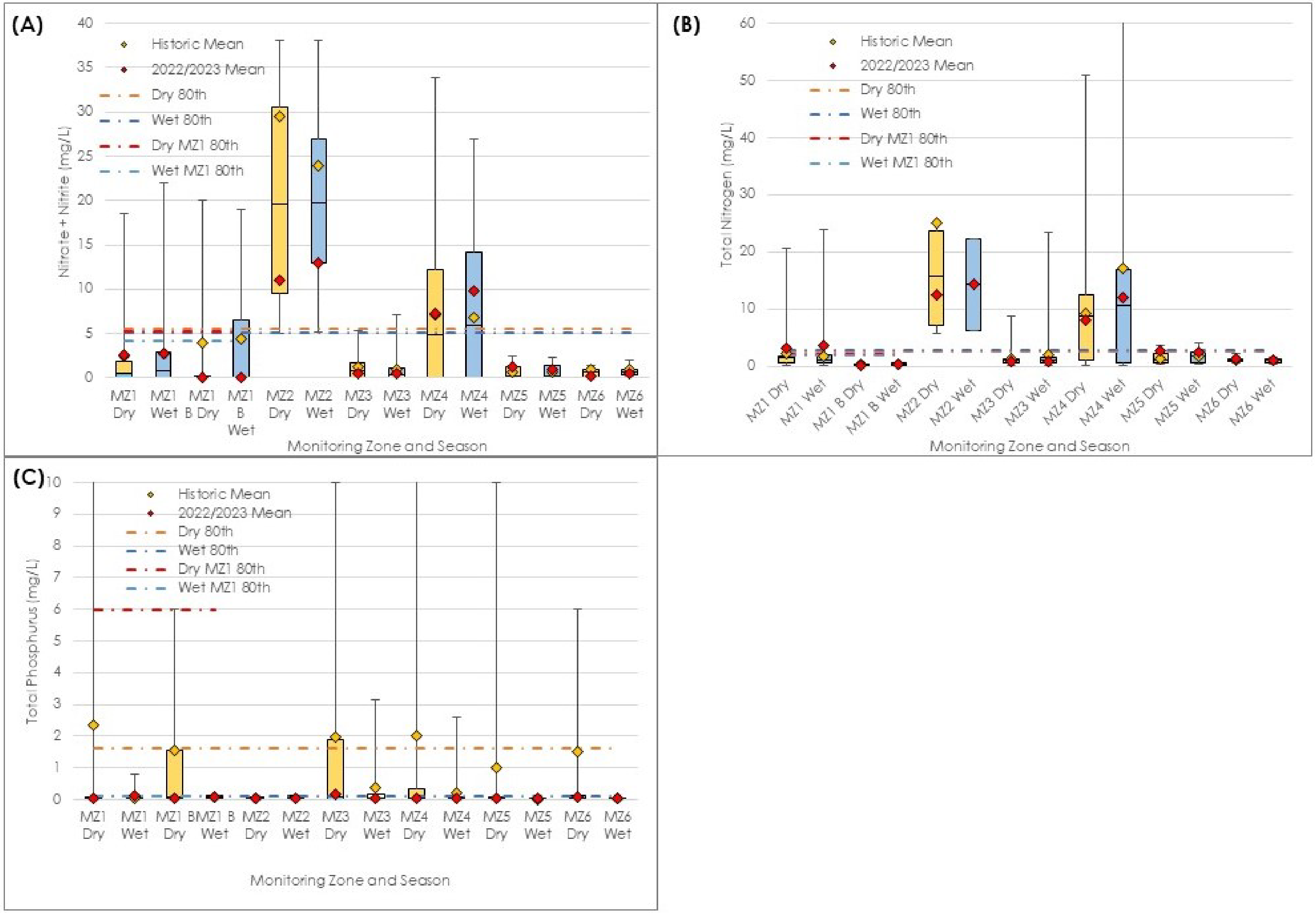


Figure 3-6 Comparison of nutrients for each monitoring zone and across dry and wet seasons, compared to the GTVs and seasonal means (2012 to 2023), (A) nitrate + nitrite, (B) total nitrogen, and (C) total phosphorus.

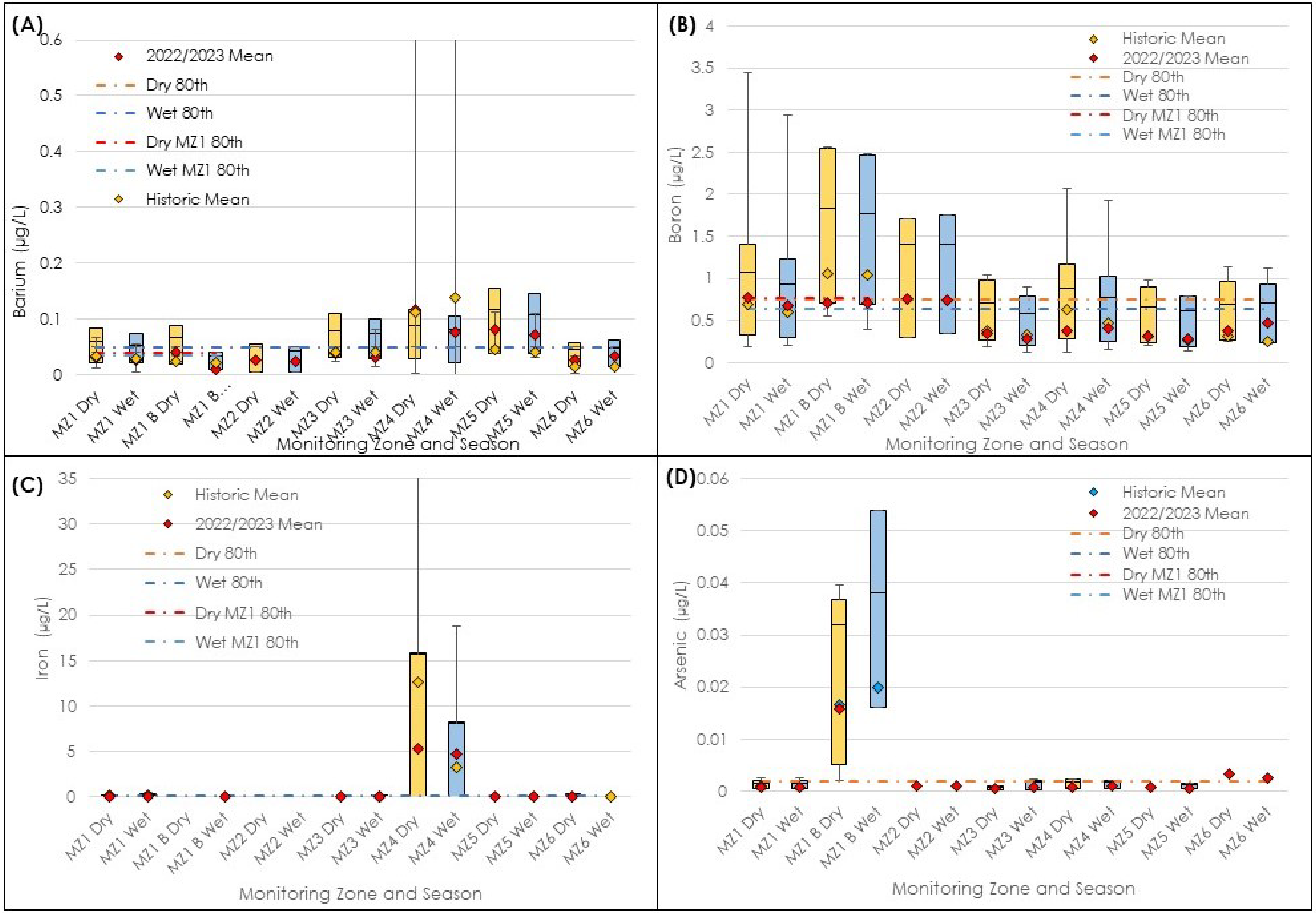


Figure 3-7 Comparison of metals for each monitoring zone and across dry and wet seasons, compared to the GTVs and seasonal means (2019 to 2023), (A) barium, (B) boron, (C) iron and (D) arsenic.

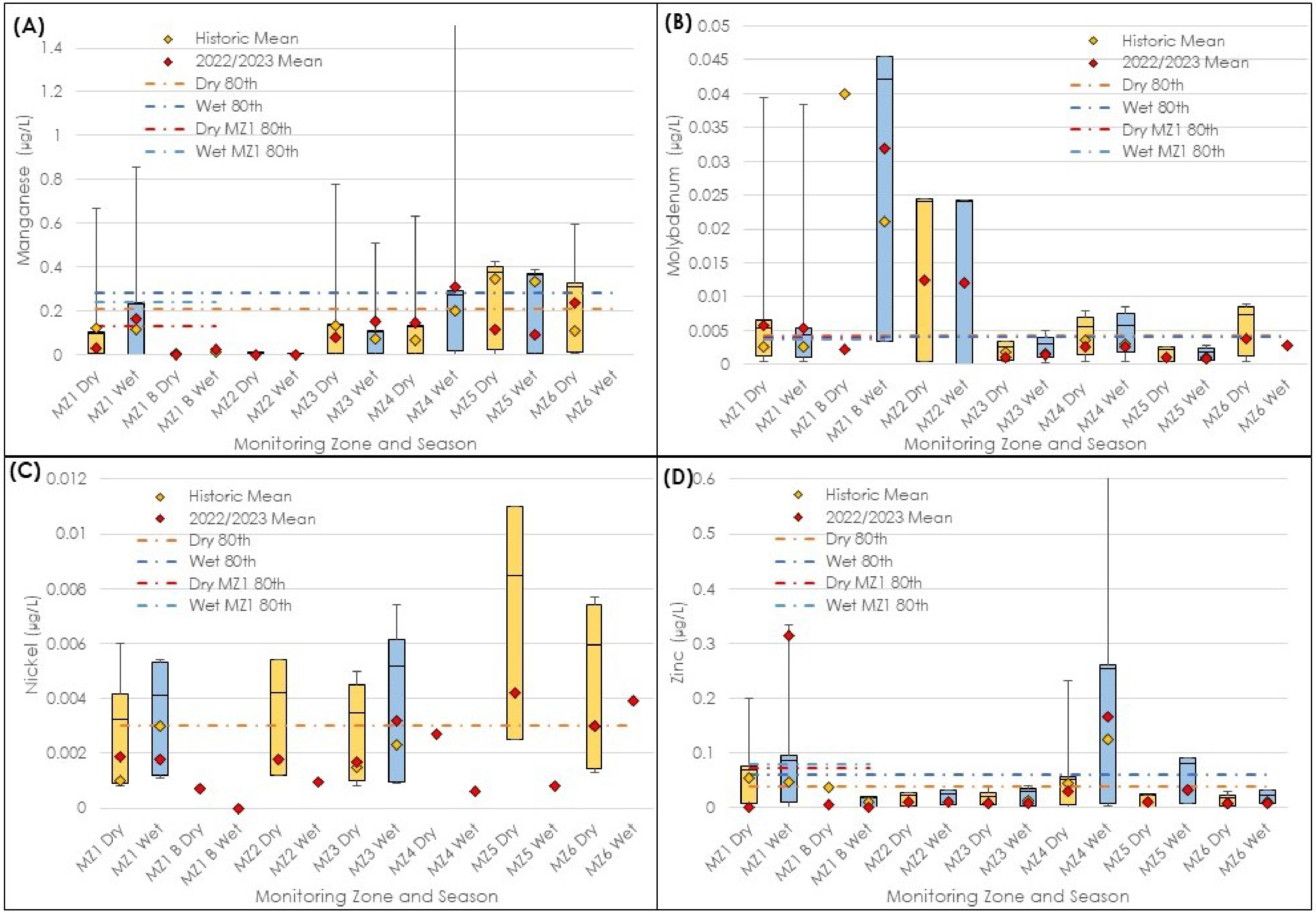


Figure 3-8 Comparison of metals for each monitoring zone and across dry and wet seasons, compared to the GTVs and seasonal means (2019 to 2023), (A) manganese, (B) molybdenum, (C) nickel and (D) zinc.

Table 3-1a Groundwater quality during the 2022 Dry survey of the Program. Basic parameter and ions

	Bore Name	pH	Total Dissolved Solids	Total Suspended Solids	Electrical Conductivity	Sodium	Magnesium	Potassium	Calcium	Chloride	Sulphate	Carbonate Alkalinity as CaCO3	Bicarbonate Alkalinity as CaCO3	Total Alkalinity as CaCO3	Total Anions	Total Cations	Ionic Balance	Total Hardness as CaCO3
MZ1	EOP0253R	7.71	8030	<5	11300	1920	337	72	257	2760	1780	<1	273	273	120	126	2.25	2030
	HEOP0388	7.67	977	10	1520	152	76	8	66	224	183	<1	392	392	18	16.4	4.65	478
	HEOP0417	8.12	856	6	1420	181	49	9	42	247	127	<1	336	336	16.3	14.2	6.85	307
	HEOP0425	8.07	2930	<5	4430	804	82	30	59	829	609	<1	570	570	47.4	45.4	2.18	485
	HEOP0504	8.15	898	<5	1430	158	61	7	16	460	1	<1	78	78	14.6	12.9	6.14	291
	HEOP0574M	8.04	813	<5	1280	95	72	7	63	217	98	<1	324	324	14.6	13.4	4.48	454
	OB23REG1	8.09	632	<5	1010	90	69	8	56	141	105	<1	276	276	11.7	12.6	3.77	424
	T399	8.11	1300	<5	2120	254	86	12	64	397	206	<1	460	460	24.7	21.6	6.59	514
	W056	8.06	852	<5	1370	98	74	8	64	240	98	<1	325	325	15.3	13.8	5.34	464
	W116	8.01	999	<5	1620	160	58	7	77	291	180	<1	290	290	17.8	15.8	5.96	431
	W117	8.22	1620	<5	2600	304	106	10	72	535	286	<1	367	367	28.4	25.8	4.77	616
	HEOP0462M	8.4	1060	<5	1850	211	85	10	46	351	130	16	418	434	21.3	18.7	6.38	465
	EEX917	8.27	2260	<5	3460	654	46	24	32	584	438	<1	593	593	37.4	34.4	4.17	269
	EOP0246R	8.24	1030	5	1670	223	44	10	37	274	165	<1	379	379	18.7	15.4	9.7	274
MZ1B	EEX931	8.72	765	23	1190	248	2	7	1	143	70	37	361	398	13.4	11.2	9.19	11
	EOP0245RE	8.22	1020	<5	1640	226	52	7	43	274	128	<1	422	422	18.8	16.4	6.78	322
MZ2	EOP0334R	8.04	904	<5	1460	147	63	11	66	222	123	<1	391	391	16.6	15.2	4.66	424
	W247	8.08	2560	<5	4190	652	97	40	70	899	467	<1	349	349	42	40.8	1.44	574
	EOP0258R	8.41	1820	<5	2980	466	62	22	30	584	359	16	300	316	30.3	27.4	4.9	330
MZ3	HEA0121	8.09	1150	<5	1790	139	92	10	78	366	176	<1	299	299	20	17.8	5.82	574
	HEA0126	8.1	797	<5	1430	126	68	10	65	219	121	<1	382	382	16.3	14.6	5.67	442
	HEA0133	8.11	1800	<5	2910	312	113	13	100	641	330	<1	313	313	31.2	28.2	5.07	715
	HEOP0524	7.97	794	6	1320	138	50	4	27	414	2	<1	66	66	13	11.6	5.98	273
	T411A	8.1	251	21	478	12	33	2	27	14	6	<1	254	254	5.59	4.64	9.37	203
MZ4	HEOP0398M	7.93	639	<5	885	76	32	12	36	72	61	<1	150	150	8.72	8.04	4.05	222
	HEOP0508	8.67	2180	7	4120	757	51	28	7	967	266	38	330	368	40.2	38.2	2.52	227
	HEOP0799	8.11	788	<5	1240	112	48	5	63	201	120	<1	265	265	13.5	12.1	5.36	355
	HEOP0801	8.12	876	13	1400	74	91	4	77	246	108	<1	362	362	16.4	14.6	5.69	567
	HEOP0811	6.6	338	45	467	35	15	2	24	143	1	<1	12	12	4.29	4	3.48	122
	W028	8.07	1080	<5	1670	127	86	4	76	290	169	<1	299	299	17.7	16.5	3.44	544
	W029	8.06	1090	<5	1710	142	86	4	69	283	197	<1	320	320	18.5	16.8	4.76	526
	W201	4.82	790	33	1370	65	26	2	122	457	1	<1	<1	<1	12.9	11.1	7.52	412
	HEOP0310	9.09	1180	536	2360	310	95	19	2	583	18	158	276	434	25.5	21.9	7.6	396
MZ5	HEOP0543	7.91	1100	<5	1820	226	51	13	19	566	<1	<1	48	48	16.9	15.3	5.02	257
	HEOP0556	8.04	768	<5	1160	83	52	5	62	216	56	<1	266	266	12.6	11.1	6.17	369
	HEOP0542	8.02	1340	<5	2120	195	97	10	75	426	194	<1	358	358	23.2	20.5	6.28	587
MZ6	ERCSGW0003	8.04	1460	<5	2260	243	121	12	60	322	403	<1	405	405	25.6	23.8	3.51	648
	ERCSGW0012	7.98	619	<5	1080	86	49	4	68	91	51	<1	425	425	12.1	11.3	3.64	372
	ERCSGW0023	7.86	668	817	1100	86	60	5	57	95	60	<1	443	443	12.8	11.6	4.62	389
	HEOP0317M	7.95	774	8	1300	88	77	7	71	144	120	<1	430	430	15.2	13.9	4.36	494
Summary																		
	min	4.82	251	<5	467	12	2	2	1	14	1	<1	66	<1	4.29	4	1.44	11
	max	9.09	8030	817	11300	1920	337	72	257	2760	1780	158	593	570	120	45.4	9.7	2030
	LoR	0.01	10	5	1	1	1	1	1	1	1	1	1	1	0.01	0.01	0.01	1
Groundwater Trigger Values (GTVs)																		
MZ 1	n=	98	75	-	95	91	91	91	91	91	89	-	-	81	56	56	63	-
	20th	7.308	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	80th	8.102	1500	-	3598.4	319	102	10	89	561	290	-	-	480	30.4	30.9	-	-
	20% Var.	-	1800	-	4318.08	-	-	-	-	-	-	-	-	-	-	-	-	-
All Zones	n=	218	274	-	122	347	347	347	347	346	345	-	-	256	144	144	-	-
	20th	7.328	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	80th	8.142	1270	-	2104	253.2	87	12	81	440	231.6	-	-	425	25.02	23.8	-	-
	20% Var.	-	1524	-	2524.8	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3-1b Groundwater quality during the 2022 Dry survey of the Program. Nutrients and metals

	Bore Name	Ammonia	Total Nitrogen	Total Kjeldahl Nitrogen as N	Nitrite + Nitrate	Total Phosphorus	Barium	Boron	Mercury	Aluminium	Iron	Selenium	Arsenic	Cadmium	Chromium	Copper	Lead	Manganese	Molybdenum	Nickel	Zinc
MZ1	EOP0253R	0.02	20.6	2.1	18.5	0.09	0.0278	2.27	<0.00004	<0.005	<0.002	0.0337	0.0011	<0.00005	0.0126	0.0007	<0.0001	0.0026	0.0059	0.0021	0.024
	HEOP0388	0.02	0.4	0.1	0.31	0.03	0.0234	0.596	<0.00004	<0.005	<0.002	0.0006	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.0046	0.0013	0.0008	0.027
	HEOP0417	0.04	0.2	0.2	<0.01	0.02	0.0475	0.601	<0.00004	<0.005	0.017	<0.0002	0.0004	<0.00005	<0.0002	0.0008	<0.0001	0.229	0.0034	0.0037	0.002
	HEOP0425	0.02	8.2	0.9	7.26	<0.02	0.0216	1.98	<0.00004	<0.005	0.004	0.0046	0.0011	<0.00005	<0.0002	<0.0005	<0.0001	0.0161	0.0108	0.0018	0.183
	HEOP0504	0.31	0.4	0.4	<0.01	<0.01	0.0199	0.297	<0.00004	<0.005	0.006	<0.0002	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.0685	0.0005	<0.0005	<0.001
	HEOP0574M	0.01	0.5	0.1		0.01	0.0277	0.278	<0.00004	<0.005	<0.002	0.0018	0.0005	<0.00005	<0.0002	<0.0005	<0.0001	0.003	0.0028	0.0012	0.009
	OB23REG1	0.02	0.4	0.1	0.35	<0.01	0.0248	0.254	<0.00004	<0.005	<0.002	0.0005	0.0006	<0.00005	<0.0002	<0.0005	<0.0001	0.0012	0.0054	0.001	0.011
	T399	0.02	0.3	0.1	0.18	0.02	0.0271	0.588	<0.00004	<0.005	0.004	0.0008	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.0616	0.0012	0.0024	0.004
	W056	0.02	0.5	0.2	0.28	0.02	0.0355	0.249	<0.00004	<0.005	<0.002	0.001	0.0005	<0.00005	0.0003	0.0022	<0.0001	0.0134	0.0026	0.0098	0.02
	W116	0.01	0.6	0.2	0.42	0.01	0.082	0.24	<0.00004	<0.005	0.002	0.0003	0.0005	<0.00005	0.0007	<0.0005	<0.0001	0.0041	0.0007	0.0042	0.007
	W117	0.02	2.2	0.3	1.87	0.02	0.0388	0.705	<0.00004	<0.005	<0.002	0.0024	0.0012	<0.00005	0.0009	<0.0005	<0.0001	0.0022	0.0017	0.0023	0.006
	HEOP0462M	0.1	0.1	0.1	0.01	0.03	0.0347	0.462	<0.00004	<0.005	0.022	<0.0002	0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.0501	0.0016	0.0015	0.002
	EEX917	0.01	8.3	0.9	7.4	0.02	0.0117	1.56	<0.00004	<0.005	<0.002	0.0046	0.0018	<0.00005	0.0007	<0.0005	<0.0001	0.0066	0.0368	0.0008	0.018
	EOP0246R	2.34	2.7	2.7	0.02	0.23	0.0335	0.783	<0.00004	<0.005	0.006	<0.0002	0.0002	<0.00005	0.0002	<0.0005	<0.0001	0.0079	0.0047	0.0008	0.035
MZ1B	EEX931	0.02	<0.1	<0.1	<0.01	0.08	0.019	0.878	<0.00004	<0.005	<0.002	<0.0002	0.0297	<0.00005	<0.0002	<0.0005	<0.0001	<0.0005	0.042	<0.0005	<0.001
	EOP0245RE	0.02	0.1	0.1	0.02	<0.01	0.0637	0.548	<0.00004	<0.005	<0.002	<0.0002	0.002	<0.00005	<0.0002	0.0006	<0.0001	0.0017	0.0021	0.0007	0.005
MZ2	EOP0334R	0.02	5.6	0.6	4.98	0.04	0.0327	0.301	<0.00004	<0.005	<0.002	0.0058	<0.0002	<0.00005	0.0008	<0.0005	<0.0001	0.0052	0.0004	0.003	0.019
	W247	0.02	19.5	2.7	16.8	0.06	0.0452	1.11	<0.00004	<0.005	<0.002	0.0137	0.0017	<0.00005	0.0019	0.0006	<0.0001	0.0033	0.0131	0.0012	0.01
	EOP0258R	0.09	12	0.9	11.1	<0.01	0.0048	0.872	<0.00004	<0.005	<0.002	0.0079	0.0003	<0.00005	0.0004	0.0006	<0.0001	0.0011	0.0237	0.0012	0.004
MZ3	HEA0121	0.05	0.9	0.2	0.7	<0.01	0.0308	0.44	<0.00004	<0.005	<0.002	0.001	0.0004	<0.00005	<0.0002	<0.0005	<0.0001	0.0028	0.0012	0.001	0.006
	HEA0126	0.05	1	0.2	0.75	0.72	0.0443	0.397	<0.00004	<0.005	<0.002	0.0009	0.0006	<0.00005	0.0004	0.0007	<0.0001	0.0013	0.002	0.003	0.026
	HEA0133	0.15	0.4	0.3	0.06	0.08	0.0536	0.426	<0.00004	<0.005	<0.002	<0.0002	0.0002	<0.00005	<0.0002	0.0006	<0.0001	0.0214	0.0006	0.0011	0.003
	HEOP0524	0.72	0.9	0.9	0.03	0.01	0.032	0.263	<0.00004	<0.005	0.014	<0.0002	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.333	0.0007	0.0008	0.002
	T411A	<0.01	0.9	0.1	0.77	<0.01	0.0383	0.222	<0.00004	<0.005	0.005	0.0008	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.0293	0.0005	0.0025	0.011
MZ4	HEOP0398M	0.74	36.7	2.8	33.9	0.34	0.0573	0.228	<0.00004	<0.005	<0.002	0.0021	0.0012	0.00021	0.0006	0.0013	0.0006	0.048	0.0014	0.0026	3.46
	HEOP0508	0.23	0.5	0.5	<0.01	0.01	0.0076	0.953	<0.00004	<0.005	0.004	<0.0002	<0.0002	<0.00005	0.0002	0.001	<0.0001	0.128	0.0039	0.0009	0.002
	HEOP0799	0.02	3	0.4	2.59	0.02	0.0592	0.353	<0.00004	<0.005	0.005	0.0016	0.0013	<0.00005	0.0013	<0.0005	<0.0001	0.0031	0.0015	0.0045	0.013
	HEOP0801	0.08	0.2	0.2	0.02	0.02	0.24	0.176	<0.00004	<0.005	0.053	<0.0002	0.0005	<0.00005	<0.0002	0.0008	<0.0001	0.342	0.0026	0.0016	0.007
	HEOP0811	0.04	0.4	0.4	0.01	<0.01	0.0906	0.129	<0.00004	<0.005	0.758	<0.0002	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.619	<0.0001	0.0008	0.005
	W028	<0.01	17.3	1.4	15.9	<0.02	0.0606	0.397	<0.00004	<0.005	<0.002	0.0028	0.0006	<0.00005	0.0003	0.0015	<0.0001	0.0038	0.0036	0.001	0.036
	W029	0.02	14.2	1.4	12.8	<0.02	0.0553	0.484	<0.00004	<0.005	<0.002	0.0028	0.0006	<0.00005	0.0003	0.0007	<0.0001	0.0238	0.005	0.0014	0.117
	W201	0.1	0.3	0.3	0.01	<0.01	0.49	0.219	<0.00004	<0.005	30.7	<0.0002	<0.0002	<0.00005	0.0002	0.0009	<0.0001	2.01	<0.0001	0.0038	0.026
	HEOP0310	0.55	0.6	0.6	0.03	0.07	0.0024	0.432	<0.00004	<0.005	0.011	<0.0002	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.0107	0.0005	<0.0005	<0.001
MZ5	HEOP0543	3.07	3.5	3.5	0.01	0.02	0.038	0.24	<0.00004	<0.005	0.003	<0.0002	<0.0002	<0.00005	<0.0002	0.0009	<0.0001	0.311	0.0005	0.0025	0.001
	HEOP0556	0.02	2.3	0.4	1.88	0.01	0.153	0.206	<0.00004	<0.005	<0.002	0.0006	0.0008	<0.00005	0.0006	0.0008	<0.0001	0.0018	0.0008	0.0041	0.009
	HEOP0542	0.04	2	0.3	1.73	0.1	0.0536	0.507	<0.00004	<0.005	<0.002	0.0021	0.0006	<0.00005	0.0005	0.001	<0.0001	0.0305	0.0016	0.006	0.022
MZ6	ERCSGW0003	0.35	1.3	1.3	<0.01	0.03	0.0512	0.584	<0.00004	0.014	0.187	<0.0002	0.0056	<0.00005	<0.0002	<0.0005	<0.0001	0.564	0.0035	0.0048	0.005
	ERCSGW0012	1.74	2.1	2.1	0.01	0.07	0.0308	0.415	<0.00004	<0.005	0.003	<0.0002	0.0011	<0.00005	<0.0002	<0.0005	<0.0001	0.254	0.0064	0.0013	0.007
	ERCSGW0023	0.02	0.5	0.4	0.06	0.16	0.0036	0.255	<0.00004	<0.005	0.004	<0.0002	<0.0002	<0.00005	0.0032	0.004	<0.0001	0.107	0.0049	0.0042	0.002
	HEOP0317M	0.02	1	0.4	0.65	0.01	0.0189	0.272	<0.00004	<0.005	<0.002	0.0006	<0.0002	<0.00005	0.0003	0.0007	<0.0001	0.0197	0.0005	0.0016	0.021
Summary																					
	min	<0.01	<0.1	<0.1	0.01	<0.01	0.0024	0.129	<0.00004	<0.005	<0.002	<0.0002	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	<0.0005	<0.0001	<0.0005	<0.001
	max	3.07	36.7	3.5	33.9	0.72	0.49	2.27	<0.00004	0.014	30.7	0.0337	0.0297	0.00021	0.0126	0.004	0.0006	2.01	0.042	0.0098	3.46
	LoR	0.01	0.1	0.1	0.01	0.01	0.0005	0.005	0.00004	0.005	0.002	0.0002	0.0002	0.00005	0.0002	0.0005	0.0001	0.0005	0.0001	0.0005	0.001
Groundwater Trigger Values (GTVs)																					
MZ 1	n=	-	93	-	55	45	47	47	-	-	-	-	-	-	-	-	-	41	37	-	28
	20th	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	80th	-	2.016	-	5.164	6	0.3896	0.768	-	-	-	-	-	-	-	-	-	0.132	0.00388	-	0.0728
	20% Var.	-	-	-	-	-	0.46752	0.9216	-	-	-	-	-	-	-	-	-	0.1584	0.004656	-	0.08736
All Zones	n=	-	373	-	175	133	105	105	-	-	-	-	32	-	-	-	-	95	84	44	65
	20th	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	80th	-	2.6	-	5.452	1.552	0.05024	0.712	-	-	-	-	0.00178	-	-	-	-	0.208	0.00428	0.003	0.0388
	20% Var.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3-2a Groundwater quality during the 2023 Wet survey of the Program. Basic parameter and ions.

	Bore Name	pH	Total Dissolved Solids	Total Suspended Solids	Electrical Conductivity	Sodium	Magnesium	Potassium	Calcium	Chloride	Sulphate	Carbonate Alkalinity as CaCO3	Bicarbonate Alkalinity as CaCO3	Total Alkalinity as $CaCO_3$	Total Anions	Total Cations	Ionic Balance	Total Hardness as $CaCO_3$
MZ1	EEX917	8.09	2180.00	13	3490	860	31	20	18	547	427	<1	655	655	37.4	41.4	5.03	173
	EOP0253R	7.86	7550.00	<5	11600	2180.00	338	57	244	2860	1660	<1	290	290	121	136	5.92	2000
	HEOP0388	7.89	997	22	1730	188	95	6	72	235	183	<1	459	459	19.6	19.7	0.34	571
	HEOP0417	8.14	898	6	1610	264	56	8	35	254	160	<1	339	339	17.3	18	2.19	318
	HEOP0425	8.30	2440.00	6	4130	934	66	26	33	771	512	<1	526	526	42.9	48.4	5.97	354
	HEOP0504	8.42	685	<5	1460	260	54	6	6	351	35	8	206	214	14.9	16.2	4.18	237
	HEOP0574M	7.87	552	32	978	76	60	7	49	116	70	<1	288	288	10.5	10.9	1.8	369
	HEV0008M	7.99	650	<5	1080	86	62	6	68	119	121	<1	281	281	11.5	12.4	3.77	425
	EOP0246R	8.07	966	<5	1630	286	48	10	35	255	128	<1	365	365	17.2	18.4	3.49	285
	OB23REG1	8.03	692	<5	1220	103	81	7	57	155	102	<1	351	351	13.5	14.2	2.39	476
	T399	7.81	1400.00	27	2400	355	108	12	78	391	214	<1	567	567	26.8	28.5	3.1	640
	W056	7.89	834	<5	1490	125	91	7	69	242	104	<1	369	369	16.4	16.5	0.56	547
	W116	7.89	825	7	1400	192	51	6	60	203	147	<1	313	313	15	15.7	2.13	360
	W117	8.03	1590.00	22	2650	375	125	9	77	535	280	<1	391	391	28.7	30.7	3.26	707
	HEOP0462M	8.16	1160.00	6	2070	261	108	9	67	349	194	<1	458	458	23	23.8	1.66	612
MZ1B	EEX931	8.64	732	134	1230	322	2	7	2	127	56	34	385	419	13.1	14.4	4.82	13
	HEOP0798M	9.07	457	<5	939	227	3	6	<1	167	3	42	202	244	9.65	10.3	3.14	12
MZ2	W247	8.08	2510.00	5	4230	704	89	32	64	826	426	<1	374	374	39.6	42	2.84	526
	EOP0258R	8.36	1760.00	5	2980	629	71	21	25	557	359	7	311	319	29.6	35	8.41	355
	EOP0334R	7.82	866	11	1530	185	74	10	68	205	116	<1	425	425	16.7	17.8	3.18	474
MZ3	HEA0121	7.87	1070.00	<5	1910	172	111	8	86	372	193	<1	312	312	20.7	21.1	0.88	672
	HEA0126	7.87	1150.00	<5	2040	230	105	9	80	413	229	<1	255	255	21.5	22.9	3.05	632
	HEA0133	7.85	1240.00	73	2180	236	109	8	87	439	232	<1	281	281	22.8	23.8	2.05	666
	HEOP0314	7.72	468	20	975	61	66	3	18	282	<1	<1	54	54	9.03	9.06	0.15	317
	HEQ0022M	8.24	520	<5	920	89	53	5	42	117	43	<1	295	295	10.1	10.4	1.79	323
	HEOP0430M	7.85	1180.00	26	2050	208	115	8	98	405	223	<1	315	315	22.4	23.6	2.71	718
	HEOP0524	8.00	200	21	437	52	14	3	9	79	3	<1	90	90	4.09	3.94	1.86	80
	T411A	8.03	380	10	774	25	71	5	44	22	14	<1	427	427	9.44	9.25	1.01	402
MZ4	HEOP0508	8.81	2240.00	<5	4110	922	56	24	4	994	266	63	344	408	41.7	45.5	4.35	240
	HEOP0811	8.12	658	12	1310	173	55	4	20	381	<1	<1	78	78	12.3	13.2	3.32	276
	HEOP0398M	8.01	645	<5	1030	102	48	9	48	97	68	<1	259	259	9.33	11	8.29	318
	W028	8.14	1030.00	<5	1700	169	106	4	82	273	162	<1	327	327	17.6	20.3	7.03	641
	W029	8.03	1290.00	<5	2120	240	132	4	94	358	246	<1	373	373	22.7	26.1	7.02	778
	W201	5.35	810	40	1290	94	32	3	88	400	<1	<1	<1	<1	11.3	11.2	0.41	352
MZ5	HEOP0542	7.90	1260.00	<5	2170	264	120	9	84	413	192	<1	406	406	23.8	25.8	4.08	704
	HEOP0543	7.88	937	<5	1860	300	61	12	18	566	<1	<1	50	50	17	19.3	6.37	296
	HEOP0556	8.00	635	21	1140	96	63	5	67	186	48	<1	290	290	12	12.8	3.18	427
	W122	7.86	1770.00	40	3050	353	158	9	132	674	279	<1	357	357	32	35.2	4.8	980
MZ6	HEOP0317M	7.87	756	89	1360	99	90	6	70	135	117	<1	456	456	15.4	15.4	0.02	545
	ERCSGW0003	7.96	1460.00	8	2320	303	154	9	53	319	442	<1	422	422	26.6	28.7	3.79	766
Summary																		
	LoR	0.01	10	5	1	1	1	1	1	1	1	1	1	1	0.01	0.01	0.01	1
	min	5.35	200	5	437	25	2	3	2	22	3	7	50	50	4.09	3.94	0.02	12
	max	9.07	7550	134	11600	2180	338	57	244	2860	1660	63	655	655	121	136	8.41	2000
Groundwater Trigger Values (GTVs)																		
MZ1	n=	142.00	75.00	-	141	84.00	84.00	84.00	84.00	84	84	-	-	78	51.00	51.00	73.00	-
	20th	7.16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	80th	8.02	1518.00	-	3274	390.40	98.80	14.40	87.40	627	306	-	-	450	28.10	27.90	-	-
	20% Var.	-	1821.60	-	3928	-	-	-	-	-	-	-	-	-	-	-	-	-
All Zones	n=	321.00	218.00	-	318	305.00	305.00	305.00	305.00	305	305	-	-	274	189.00	189.00	257.00	-
	20th	7.14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	80th	8.09	1300.00	-	2588	278.00	89.00	12.00	84.20	490	234	-	-	418	22.60	21.62	-	-
	20% Var.	-	1560.00	-	3106	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3-3a Groundwater quality during the 2023 Wet survey of the Program. Nutrients and metals.

	Bore Name	Ammonia	Total Nitrogen	Total Kjeldahl Nitrogen as N	Nitrite + Nitrate	Total Phosphorus	Barium	Boron	Mercury	Aluminium	Iron	Selenium	Arsenic	Cadmium	Chromium	Copper	Lead	Manganese	Molybdenum	Nickel	Zinc
MZ1	EEX917	0.09	8.5	0.8	7.72	0.05	0.0047	1.71	<0.00004	<0.005	<0.002	0.0042	0.0018	<0.00005	0.0003	0.0007	<0.0001	<0.0005	0.0365	<0.0005	0.029
	EOP0253R	<0.01	23.9	1.9	22	0.04	0.0308	2.20	<0.00004	<0.005	<0.002	0.0314	0.0009	<0.00005	0.0118	0.0024	0.0002	0.0014	0.0069	<0.0005	0.012
	HEOP0388	0.2	0.6	0.5	0.06	0.03	0.0222	0.46	<0.00004	<0.005	0.066	0.0003	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.2800	0.0009	<0.0005	0.086
	HEOP0417	0.3	0.5	0.5	0.04	0.03	0.0214	0.61	<0.00004	<0.005	0.054	<0.0002	<0.0002	<0.00005	<0.0002	0.0008	<0.0001	0.8490	0.0026	<0.0005	<0.001
	HEOP0425	0.24	4.2	1	3.2	0.23	0.0128	1.78	<0.00004	<0.005	0.011	0.0015	0.0011	<0.00005	<0.0002	0.0010	<0.0001	0.1020	0.0098	<0.0005	0.151
	HEOP0504	0.2	0.4	0.4	<0.01	0.04	0.0076	0.39	<0.00004	<0.005	0.011	0.0003	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.0588	0.0021	<0.0005	<0.001
	HEOP0574M	0.08	2.7	1	1.74	0.07	0.0401	0.21	<0.00004	<0.005	<0.002	0.0017	0.0006	<0.00005	0.0003	0.0059	<0.0001	<0.0005	0.0026	0.0015	0.163
	HEV0008M	0.04	2.9	0.9	1.98	0.2	0.0355	0.20	<0.00004	<0.005	<0.002	0.0024	0.0010	<0.00005	0.0003	0.0018	<0.0001	0.0007	0.0031	0.0027	0.010
	EOP0246R	1.76	4.2	3	1.22	0.79	0.0324	0.53	<0.00004	<0.005	0.010	<0.0002	0.0013	<0.00005	0.0004	<0.0005	<0.0001	0.0155	0.0037	<0.0005	0.043
	OB23REG1	0.03	0.3	0.1	0.23	0.05	0.0203	0.26	<0.00004	<0.005	<0.002	0.0004	0.0004	<0.00005	<0.0002	0.0006	<0.0001	<0.0005	0.0046	<0.0005	0.006
	T399	1.21	2.2	2.2	0.05	0.08	0.0276	0.48	<0.00004	<0.005	0.073	0.0003	<0.0002	<0.00005	0.0002	<0.0005	<0.0001	0.3500	0.0014	<0.0005	0.002
	W056	0.18	1	0.4	0.6	0.01	0.0289	0.22	<0.00004	<0.005	<0.002	0.0018	0.0004	<0.00005	0.0003	0.0012	<0.0001	0.0005	0.0023	0.0011	0.008
	W116	0.02	0.6	0.3	0.34	0.02	0.0601	0.20	<0.00004	<0.005	<0.002	<0.0002	0.0003	<0.00005	0.0008	<0.0005	<0.0001	0.0013	0.0005	<0.0005	0.008
	W117	0.03	2.2	0.5	1.72	0.05	0.0331	0.48	<0.00004	<0.005	<0.002	0.0021	0.0008	<0.00005	0.0009	<0.0005	<0.0001	0.0008	0.0013	<0.0005	0.014
	HEOP0462M	0.13	0.7	0.6	0.14	0.08	0.0295	0.36	<0.00004	<0.005	0.026	0.0003	<0.0002	0.0001	<0.0002	<0.0005	<0.0001	0.2970	0.0014	<0.0005	0.314
MZ1B	EEX931	0.09	0.3	0.3	<0.01	0.12	0.0194	1.01	<0.00004	<0.005	<0.002	<0.0002	0.0257	<0.00005	<0.0002	0.0008	<0.0001	<0.0005	0.0387	<0.0005	0.002
	HEOP0798M	0.14	0.5	0.4	0.1	0.05	0.0006	0.40	<0.00004	<0.005	0.035	<0.0002	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.0261	0.0034	<0.0005	<0.001
MZ2	W247	0.15	22.3	1.8	20.5	0.06	0.0394	1.05	<0.00004	<0.005	<0.002	0.0129	0.0015	<0.00005	0.0019	0.0024	<0.0001	0.0011	0.0123	0.0010	0.019
	EOP0258R	0.21	14.4	1.4	13	0.09	0.0052	0.82	<0.00004	<0.005	<0.002	0.0077	0.0003	<0.00005	0.0003	0.0015	<0.0001	0.0024	0.0240	0.0009	0.007
	EOP0334R	0.42	6.2	1	5.24	0.02	0.0309	0.35	<0.00004	<0.005	<0.002	0.0058	<0.0002	<0.00005	0.0007	<0.0005	<0.0001	0.0008	0.0001	<0.0005	0.009
MZ3	HEA0121	0.02	1	0.3	0.68	0.02	0.0290	0.44	<0.00004	<0.005	<0.002	0.0008	0.0003	<0.00005	<0.0002	<0.0005	<0.0001	<0.0005	0.0011	<0.0005	0.002
	HEA0126	0.03	1.1	0.2	0.91	0.05	0.0320	0.38	<0.00004	<0.005	<0.002	0.0009	0.0004	<0.00005	<0.0002	<0.0005	<0.0001	<0.0005	0.0026	<0.0005	<0.001
	HEA0133	0.28	0.8	0.5	0.26	0.01	0.0351	0.36	<0.00004	<0.005	<0.002	0.0003	0.0004	<0.00005	<0.0002	0.0011	<0.0001	<0.0005	0.0012	<0.0005	0.003
	HEOP0314	0.54	0.7	0.7	<0.01	<0.01	0.0176	0.17	<0.00004	<0.005	0.023	<0.0002	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	1.8000	0.0002	<0.0005	<0.001
	HEQ0022M	0.03	0.9	0.3	0.63	0.11	0.0431	0.21	<0.00004	<0.005	<0.002	<0.0002	0.0017	<0.00005	0.0004	0.0012	<0.0001	0.0007	0.0030	0.0009	0.008
	HEOP0430M	0.03	0.9	0.3	0.6	0.03	0.0499	0.35	<0.00004	<0.005	<0.002	0.0006	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.0110	0.0021	<0.0005	0.004
	HEOP0524	0.31	0.4	0.4	<0.01	0.04	0.0140	0.13	<0.00004	<0.005	0.006	<0.0002	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.4980	0.0002	<0.0005	0.002
	T411A	0.04	1.2	0.5	0.66	0.03	0.0247	0.19	<0.00004	<0.005	0.002	0.0006	<0.0002	<0.00005	<0.0002	0.0016	<0.0001	0.0976	0.0006	0.0055	0.033
MZ4	HEOP0508	0.03	0.2	0.2	<0.01	0.02	0.0046	0.96	<0.00004	<0.005	0.002	<0.0002	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.0718	0.0035	<0.0005	<0.001
	HEOP0811	0.56	0.6	0.6	0.04	0.02	0.0209	0.28	<0.00004	<0.005	0.031	<0.0002	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.2280	0.0004	<0.0005	<0.001
	HEOP0398M	3.39	34.3	7.7	26.6	0.18	0.0521	0.27	<0.00004	<0.005	<0.002	0.0017	0.0013	0.0001	0.0007	0.0016	0.0013	0.0211	0.0015	<0.0005	0.643
	W028	0.03	16.5	1.4	15.1	0.05	0.0491	0.32	<0.00004	<0.005	<0.002	0.0024	0.0004	<0.00005	0.0002	0.0023	<0.0001	0.0086	0.0035	<0.0005	0.011
	W029	0.04	19.2	1.6	17.6	0.02	0.0591	0.44	<0.00004	<0.005	0.017	0.0030	0.0012	<0.00005	0.0002	<0.0005	<0.0001	0.0045	0.0043	<0.0005	0.004
	W201	0.65	1.2	1.2	0.01	0.03	0.2760	0.17	<0.00004	<0.005	18.800	<0.0002	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	1.5200	<0.0001	0.0006	0.012
MZ5	HEOP0542	0.52	2.2	0.8	1.42	0.03	0.0471	0.36	<0.00004	<0.005	<0.002	0.0021	0.0004	0.0001	0.0007	0.0018	<0.0001	0.0064	0.0015	0.0008	0.074
	HEOP0543	3.43	3.5	3.5	0.04	0.02	0.0314	0.19	<0.00004	<0.005	0.008	<0.0002	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.3580	0.0004	<0.0005	<0.001
	HEOP0556	0.03	2.4	0.5	1.92	0.02	0.1400	0.15	<0.00004	<0.005	<0.002	0.0006	0.0008	<0.00005	0.0007	<0.0005	<0.0001	0.0007	0.0008	<0.0005	0.008
	W122	0.16	1.3	0.7	0.6	0.05	0.0685	0.43	<0.00004	<0.005	0.003	0.0014	0.0006	<0.00005	<0.0002	<0.0005	0.0001	0.0084	0.0009	<0.0005	0.016
MZ6	HEOP0317M	0.02	1.1	0.4	0.68	0.04	0.0171	0.28	<0.00004	<0.005	<0.002	0.0006	<0.0002	<0.00005	<0.0002	<0.0005	<0.0001	0.0392	0.0004	<0.0005	0.009
	ERCSGW0003	0.1	0.8	0.5	0.3	0.05	0.0527	0.66	<0.00004	<0.005	0.010	<0.0002	0.0026	<0.00005	<0.0002	0.0008	<0.0001	0.7890	0.0050	0.0039	0.010
Summary																					
	LoR	0.01	0.1	0.1	0.01	0.01	0.0005	0.005	0.00004	0.005	0.002	0.0002	0.0002	0.00005	0.0002	0.0005	0.0001	0.0005	0.0001	0.0005	0.001
	min	0.02	0.2	0.1	0.01	0.01	0.0006	0.13	<0.00004	<0.005	0.002	0.0003	0.0003	0.0001	0.0002	0.0006	0.0001	0.0005	0.0001	0.0006	0.002
	max	3.43	34.3	7.7	26.6	0.79	0.2760	2.20	<0.00004	<0.005	18.800	0.0314	0.0257	0.0001	0.0118	0.0059	0.0013	1.8000	0.0387	0.0055	0.643
Groundwater Trigger Values (GTVs)																					
MZ1	n=	-	70.00	-	73.00	51.00	48.0000	48.00	-	-	-	-	-	-	-	-	-	39.00	40.0000	-	34.000
	20th	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	80th	-	1.94	-	6.46	0.08	0.0350	0.64	-	-	-	-	-	-	-	-	-	0.24	0.0038	-	0.079
	20% Var.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
All Zones	n=	-	274.00	-	258.00	189.00	108.0000	110.00	-	-	31.00	-	-	-	-	32.00	-	93.00	87.0000	-	70.000
	20th	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	80th	-	2.74	-	5.06	0.09	0.0500	0.614	-	-	0.110	-	-	-	-	0.0024	-	0.2818	0.0040	-	0.060
	20% Var.	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-

3.2 Stygofauna Results

3.2.1 Overview

In total, 1,591 stygofauna specimens were collected during the Program, representing 35 taxa from seven high-level taxonomic groups; Acarina, Amphipoda, Copepoda, Isopoda, Ostracoda, Bathynellacea and Annelida (**Table 3-3, Table 3-4, Appendix D**). Twenty of those taxa were considered 'core taxa' for the Ethel Gorge Stygobiont TEC according to the consolidated taxon list in Stantec (2023b). A total of 171 stygofauna specimens were collected during 2022 Dry, with amphipods and copepods making up 85% of the specimens (**Table 3-3**). Considerably more specimens were recorded during 2023 Wet (1,420 stygofauna specimens, 29 taxa) than 2022 Dry (171 stygofauna specimens, 23 taxa) (**Table 3-4**). More than half the specimens recorded during 2023 Wet were copepods (**Figure 3-9, Table 3-3, Table 3-4**), but annelids, ostracods, and amphipods were also well represented.

The increased sampling effort during the current Program is reflected in the total number of organisms collected, relative to previous monitoring rounds (Stantec 2023a). Two of the taxa recorded, Enchytraeidae sp. nov. and *Origocandona* \`BOS1752\` had not been recognised previously and are likely to represent new species.

3.2.2 Taxa

3.2.2.1 Acarina

Three aquatic mite specimens were collected from samples within the TEC during the Program. All occurred as single individuals in a sample and all were collected in 2023 Wet (**Table 3-4, Appendix D**). *Peza* sp OB was recorded from bores HEOP0425 and HEOP0430, both within the TEC. *Peza* sp. OB is a small but distinctive component of the stygofauna in Ethel Gorge region, with 60 records of the taxon in the historical data set. The majority of records have been from HEOP0425, but it has been collected from other bores within the TEC, as well as in the surrounding buffer and regional areas. A singleton aquatic Acarina sp. indet. was recorded from reference site HEOP0543 approximately 8.5 km north-east of the TEC buffer during 2023 Wet, this specimen could not be identified further due to damage to the diagnostic characters.

3.2.2.2 Amphipoda

Six different species of amphipods were collected during the Program, with three species contributing more than 60 individuals each. Amphipods were encountered in 31 of the 80 samples, making this taxon the most widespread during the Program (**Appendix D**).

The most abundant species was Paramelitidae gen. nov. 1 \`AMP002\` with 101 specimens occurring across six samples from five bores (W116, HEOP0556, W117, HEOP0574 and HEOP0811) (**Table 3-3, Table 3-4**). The majority of samples were from northern regional bores, outside the TEC. Those that were CO1 sequenced mostly belonged to the same haplotype (Hap1), with the exception of a single individual of a newly sequenced haplotype (Hap4). A single specimen was collected from the southern-most reference site (HEOP0811) which belonged to Hap3.

Paramelitidae gen. nov. 2 \`AMP003\`, was also relatively abundant, with 77 specimens collected (**Table 3-3, Table 3-4**). This taxon is considered a core species (Stantec 2023b). Most specimens of this taxon were collected from the northern part of the TEC close to Ore Body 23, primarily from bores HEOP0121 and HEOP0574, and in lower numbers from nearby bores. This species was present in samples collected during both 2022 Dry and 2023 Wet. The distribution of Paramelitidae gen. nov. 2 \`AMP003\` appears to be associated with the TEC, with almost all samples in the historical data set being collected from within the TEC and Buffer areas.

Chydaekata acuminata was represented by 61 individuals collected across 14 samples and 11 bores, and in both 2022 Dry and 2023 Wet (**Table 3-3, Table 3-4**). Abundances were greatest outside the TEC, in several northern regional bores (W117, T399, HEOP0462), although substantial numbers were also collected inside the TEC at OB23REG1. One specimen of *C. acuminata* was sequenced from each of OB23REG1 and EOP0246R and both were found to belong to the same haplotype (Hap1).

Lower numbers of *Chydaekata* sp. \`AMP005\` (8 specimens), *Maarrka etheli* (3 specimens) and Paramelitidae gen. nov. 1 \`AMP001\` (1 specimen) were collected from across survey area in 2022 Dry and 2023 Wet (**Table 3-3, Table 3-4**). *Chydaekata* sp. \`AMP005\` is typically associated with the southern regional bores W028 and W029, while the distribution of *Maarrka etheli* is consistently in the northern part of the TEC. Paramelitidae gen. nov. 1 \`AMP001 has been collected from the northern part of the TEC as well as the buffer, and from reference bores of the East Ophthalmia Borefield. All three of these species are currently considered core taxa for the Ethel Gorge Aquifer Stygobiont (Stantec 2023b). The single specimen of Paramelitidae gen. nov. 1 `AMP001 was sequenced and identified as belonging to Hap5, while the sequenced *M. etheli* specimen belonged to Hap1.

A further six amphipod records could not be assigned to a taxon as the specimens were of too poor quality to identify using morphological or molecular techniques.

3.2.2.3 Bathynellacea

A total of eleven individuals across four species of Bathynellacea were collected during the Program (**Table 3-3, Table 3-4, Appendix D**). The most common species was *Brevisomabathynella pilbaraensis*, (8 specimens), which occurred in bores HEOP556 in 2022 Dry and in HEV0008, HEOP0574, HEOP0556 in 2023 Wet. This species' distribution within the TEC and into the northern reference bores is consistent with historical records.

Three different species of *Pilbaranella* were collected, each as a singleton (**Table 3-3, Table 3-4**). *Pilbaranella ethelensis* was found within the TEC at W056 in 2023 Wet. A singleton of *Pilbaranella* sp. A was collected approximately 200 m inside the TEC buffer at HEOP0317 in 2023 Wet and CO1 sequencing confirming it's identification.

The singleton of *Pilbaranella* sp. at T0411A in 2022 Dry was CO1 sequenced, which provided a 99.58% match to the published sequence MK546338 (WAMC57187) which is identified only as *Pilbaranella* sp. in Genbank and represents a different species to the above two taxa. Matthews *et al.* (2020) designates this species "Parabathynellidae sp 15". The historic specimen used to generate the published sequence was collected from the same bore (T411A) in 2013, but was sequenced at a later date after lodgment with the Western Australian Museum (WAM). As such, the current record of this species does not represent a new taxon for the Ethel Gorge region. In contrast to *Brevisomabathynella*, records of *Pilbaranella* were distributed from the centre to the west of the TEC, consistent with the historical distribution of *Pilbaranella* in the Ethel Gorge Aquifer Stygobiont TEC.

3.2.2.4 Copepoda

Copepods were numerically the most abundant group (796 specimens), occurring in 27 of samples (**Table 3-3, Table 3-4, Appendix D**). As for most other taxa, copepods were more abundant in Wet 2023 (731 specimens) than in Dry 2022 (59 specimens) (**Figure 3-9**). While they were collected in relatively low abundances in most bores, abundances of greater than 100 individuals per sample were documented for several bores. This variability has previously been noted for copepods in the Ethel Gorge Aquifer Stygobiont TEC (Stantec 2022;2023b).

Diacyclops humphreysi s.l. was the most abundant copepod species (519 specimens.) occurring in 24 samples across both 2022 Dry and 2023 Wet (**Table 3-3, Table 3-4**). During 2023 Wet this species reached abundances over 100 individuals in two bores (W116 and HEOP0430). Distribution was primarily in the northern part of the TEC north of Ophthalmia dam (including HEOP0430), and at five northern reference sites (including W116).

Diacyclops cockingi was less abundant and widely distributed than *D. humphreysi* s.l., being recorded from only four samples in 2023 Wet (**Table 3-4**). In three of those samples abundances were below 5 individuals, while 75 individuals were collected from bore HEV0008 in the northern part of the TEC.

Other cyclopoid copepod species included *Microcyclops varicans* and *Pilbaracyclops supersensus*, the latter of which is considered core species (Stantec 2023b). Five individuals of *M. varicans* were collected from a single bore (HEOP0574) in 2023 Wet. This species is widespread throughout northwestern Australia. *Pilbaracyclops supersensus* was collected in similar numbers from two bores (HEOP0388 and HEV0008) in that same season, located in the northern part of the TEC, north of Ophthalmia Dam. Both of these species have been recorded infrequently in the Ethel Gorge TEC and buffer areas and typically in low numbers.

Archinitocrella newmanensis was the only species of harpacticoid copepod collected during the Program, occurring in a total of eight samples across eight separate bores. In 2022 Dry, this species was recorded from two bores near Ore Body 23, while in 2023 Wet it was more broadly distributed in the northern part of the TEC and in one northern reference bore. This included a comparatively high abundance (170 individuals) at HEV0008 in the northern TEC.

3.2.2.5 Isopoda

Two species of Isopoda were collected during the current project, *Pygolabis humphreysi* and *Coxicerberus* ISO019. The former species was more abundant, with a total of 34 individuals collected, than the latter species which was recorded as a singleton.

Pygolabis humphreysi is often collected in low numbers per bore as a part of the Ethel Gorge Stygobiont assemblage, which is consistent with the patterns of abundance in this Program. One or two individuals were collected from bores HEOP0425, HEOP0417, HEOP0388 and HEOP0121 in both 2022 Dry and 2023 Wet (**Table 3-3, Table 3-4, Appendix D**). Additionally in 2023 Wet, it was collected from bores HEV0008 and T399. The collection at HEV0008, in the northern part of the TEC, was unusual, being the highest abundance recorded since 2014 (20 individuals) and fifth highest on record for this species. However, as HEV0008 has not been previously sampled as part of the long-term stygofauna monitoring program there is no historical data for comparison (Stantec 2023b). Two individuals of *P. humphreysi* were sequenced, returning a haplotype of Hap2 from HEQ0022M, and Hap26 from HEV0008.

The singleton of *Coxicerberus* ISO019 was collected from HEOP0559, which is outside of the TEC but inside the buffer, in 2022 Dry (**Table 3-3**)*. Coxicerberidae* is an infrequently encountered family, with only 11 individuals being recorded in the consolidated data set since 2008. Records of the family are typically associated with the area of the TEC immediately north of Ophthalmia Dam, including HEOP0559.

3.2.2.6 Ostracoda

A total of 204 Ostracoda were collected during the Program, 11 in 2022 Dry, and 193 in 2023 Wet (**Table 3-3, Table 3-4, Appendix D**). This greater abundance in the wet season corresponds with the patterns noted for most other taxa. Eight species were collected, one of which has not been collected from the Ethel Gorge Stygobiont TEC previously and represents a new species.

The most abundant ostracod species was *Origocandona inanitas*, with two individuals in 2022 Dry and 90 in 2023 Wet (**Table 3-3, Table 3-4**). While abundant, it was restricted to one bore within the TEC in both seasons (HEOP0388). This species has been most often collected from HEOP0388 with a few historical records to the north within the TEC at HEOP0574 and in the buffer at T399. *Origocandona* `BOS1752` was the next most abundant species with 54 individuals recorded, which was only recorded in 2023 Wet, but from three different bores (HEV0008, HEOP0121 and HEOP0314), all within the TEC boundary. This species has not been recorded from the Ethel Gorge TEC previously and may meet the criteria to be a novel species and a Core Species. A third species of *Origocandona* was collected, *O.* `BOS099`, from two bores during 2023 Wet; HEOP0388, in the northern part of the TEC, and HEOP0388 to the south outside of the TEC. This species was first recorded in 2020 at HEOP0388 and a nearby bore, and then again in 2021 at HEOP0417, in the northern part of the TEC close to where it was detected during this Program.

Three species of *Pilbaracandona* were collected, all of which have been collected previously, two of which are considered core species. Four individuals of the widespread species *Pilbaracandona colonia* were collected from W056 in 2022 Dry and a further eight were collected across four bores in 2023 Wet (**Table 3-3, Table 3-4**). A singleton of the core species *Pilbaracandona eberhardi* was collected from the northern reference bore, W116, where it has been collected in low abundances previously. *Pilbaracandona kosmos* was detected at bores T399, HEOP0388, HEQ0022, OB23REG1, with all but two of the 16 individuals collected during 2023 Wet. This distribution is consistent with historical records where it has been recorded from T399 outside the TEC and HEOP0388 inside the TEC, amongst other locations.

A total of seven specimens of *Gomphodella hirsuta* were collected from three samples across three different bores, with the majority of individuals occurring in 2023 Wet (**Table 3-3, Table 3-4**). This species was present in both TEC and regional bores T399, HEOP0574, OB23REG1, which is consistent with the patterns of distribution in previous surveys.

3.2.2.7 Annelida

Naidid oligochaetes were the most abundant annelids collected in the current survey, which is in contrast to the previous survey (Stantec 2023a) where phreodrilid oligochaetes were the most abundant. *Pristina* OB was collected from three bores (two reference bores and one within the TEC), however, 250 individuals of the total 253 were collected in just one sample (HEOP0462 in 2023 Wet). The remaining three individuals were collected in 2022 Dry from HEOP0388 and T0399. CO1 sequencing determined that the individuals in HEOP0462 belonged to Hap6. *Pristina* OB from HEOP0388 belonged to Hap9, which matches the haplotype collected from that same location during 2022 Wet in the previous survey (Stantec 2023a) (**Appendix D**). CO1 sequences for Naididae OB match those of specimens identified as Naididae sp.13 in

Brown *et al.* (2015). Five additional naidid specimens were collected during 2023 Wet, which could not be definitively assigned to a known morphospecies.

A total of 23 phreodrilid oligochaetes were collected in six samples. Eight specimens of Phreodrilidae sp. OP1 were collected in a single sample from reference bore HEOP0811 in 2022 Dry and their identification was confirmed with molecular sequencing. This species is typically recorded in regional bores to the north, south and west of the TEC, and only rarely within the actual TEC boundary. A total of 14 specimens designated as Phreodrilidae sp. 'dissimilar ventral chaetae' were collected outside the TEC to the north and south from HEOP0504, HEOP0811, T399 and W122 during 2023 Wet, and a singleton from HEOP0556 during 2022 Dry. These specimens were not identified further.

Three enchytraeid oligochaetes (Enchytraeidae sp. nov.) were collected from bore T0399 in 2022 Dry (**Table 3-3**). These individuals were identified using molecular sequencing, which determined that they were a species that had not been sequenced previously (**Appendix B**). Enchytraeid oligochaetes are common within the Ethel Gorge Stygobiont TEC, with almost 700 records present in the historical data set. Although the current records are the first time this species has been sequenced, it is possible that specimens of this taxon have been encountered in previous surveys.

A single individual of the polychaete family Aeolosomatidae was collected from reference bore W117 in 2022 Dry (**Table 3-3**). This taxon has been collected during early survey rounds from the Ethel Gorge Stygobiont TEC between 2012 and 2014. During that period, it was most abundant at bores near Jimblebar, however those bores are no longer accessible. It was also found in bores W116 and W117 which is consistent with its detection in the current Program.

3.2.2.8 Summary

Overall, the stygofauna assemblage documented during the Program was largely consistent with that observed in previous surveys with comparable species composition and diversity. Abundances in 2022 Dry were lower than recent years, while the abundances in 2023 Wet were the highest recorded since 2015. Several taxa that had not been detected in recent years were collected, including Aleosomatidae and *Coxicerberus*, which may be due to the increased survey intensity from 50 samples in the previous Program up to 80 in the current Program. The dominant species of amphipods was also somewhat different from previous years with the prevalence of Paramelitidae gen. nov. 1 `AMP002`and Paramelitidae gen. nov. 2 `AMP003` over *Chydaekata acuminata*.

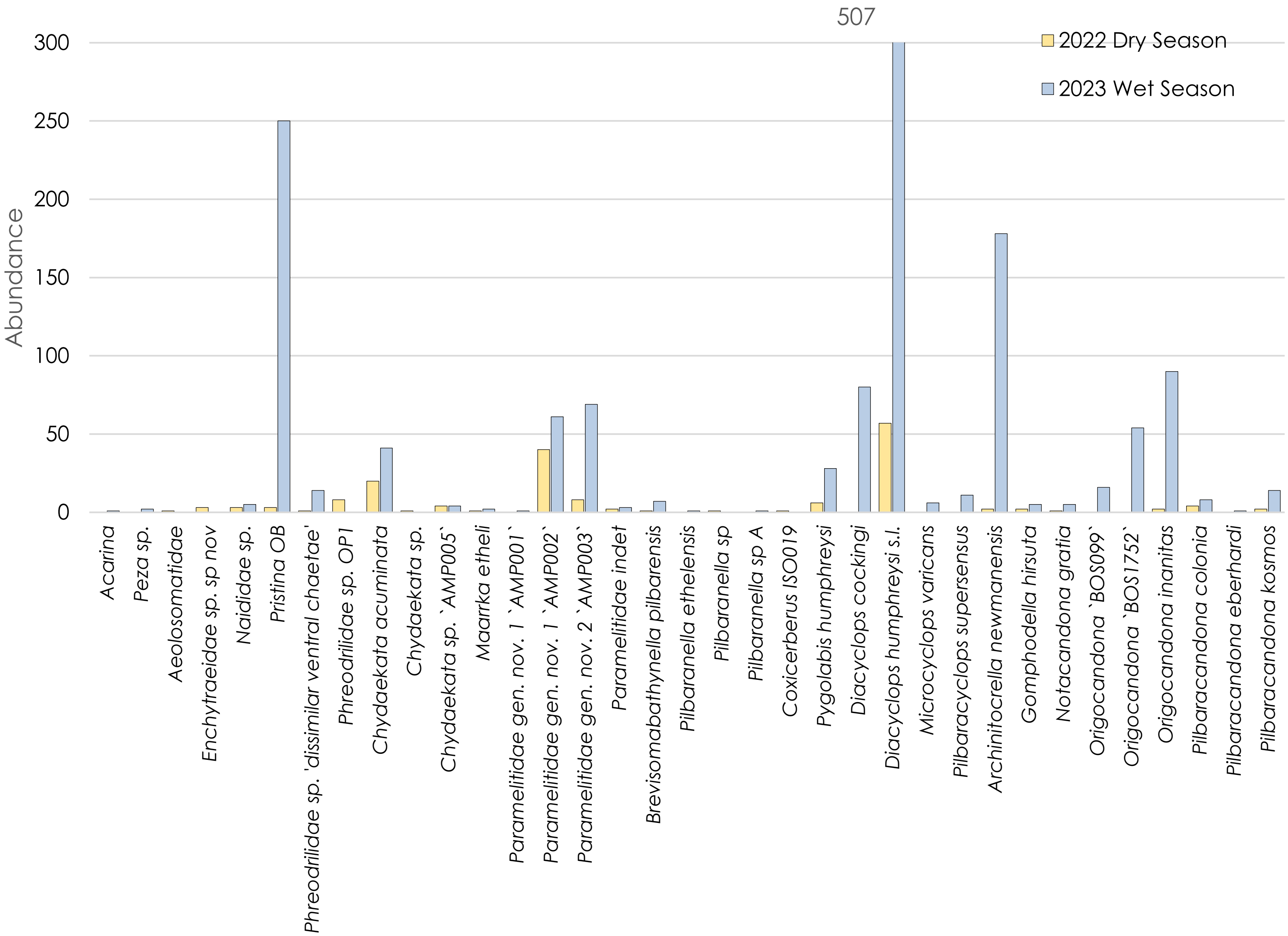


Figure 3-9 Total abundance of each stygofauna taxon in 2022 Dry (Orange) and 2023 Wet (Blue).

Table 3-4 Abundance of stygofauna in individual bores and monitoring zones in the 2022 Dry survey.

	EOP0245RE	EOP0246R	HEA0121	HEA0126	HEA0133	HEOP0388	HEOP0417	HEOP0425	HEOP0462M	HEOP0542	HEOP0556	HEOP0559	HEOP0574M	HEOP0811	OB23REG1	T399	T411A	W056	W116	W117
Annelida																				
Aeolosomatidae																				
Aeolosomatidae																				1
Enchytraeidae																				
Enchytraeidae sp. sp nov																3				
Naididae																				
Pristina OB						2										1				
Phreodrilidae																				
Phreodrilidae sp. 'dissimilar ventral chaetae'											1									
Phreodrilidae sp. OP1														8						
Amphipoda																				
Chydaekata acuminata		1				1			3				1		12				1	1
Chydaekata sp.																1				
Chydaekata sp. \`AMP005\`				4																
Maarrka etheli						1														
Paramelitidae gen. nov. 1 \`AMP002\`											37		1	1						1
Paramelitidae gen. nov. 2 \`AMP003\`			5										2					1		
Paramelitidae indet						2														
Bathynellacea																				
Brevisomabathynella pilbarensis											1									
Pilbaranella sp "Parabathynellidae sp. 15" (sensu Matthews *et al.* (2020))																	1			
Isopoda																				
Coxicerberus ISO019												1								
Pygolabis humphreysi			1			1	2	2												
Copepoda																				
Cyclopoida																				
Diacyclops humphreysi s.l.	11			1	1				2	2		10				9		3	14	4
Harpacticoida																				
Archinitocrella newmanensis					1										1					
Ostracoda																				
Gomphodella hirsuta																2				
Notacandona gratia																				1
Origocandona inanitas						2														
Pilbaracandona colonia																		4		
Pilbaracandona kosmos																2				

Table 3-5 Abundance of stygofauna in individual bores and monitoring zones in the 2023 Wet survey.

	EEX917	ERCSGW0003	HEA0121	HEOP0314	HEOP0317M	W028	W029	HEOP0388	HEOP0417	HEOP0425	HEOP0430M	HEOP0462M	HEOP0504	HEOP0542	HEOP0543	HEOP0556	HEOP0574M	HEOP0798M	HEOP0811	HEQ0022M	HEV0008M	OB23REG1	T399	T411A	W056	W116	W117	W122
Acarina																												
Acarina sp.															1													
Peza sp. OB										1	1																	
Oligochaeta																												
Naididae																												
Naididae sp.													1										4					
Pristina OB												250																
Phreodrilidae																												
Phreodrilidae sp. 'dissimilar ventral chaetae'													1						6				3					4
Amphipoda																												
Chydaekata acuminata		3						1				11								3			13				8	2
Chydaekata sp. \`AMP005\`							1														3							
Maarrka etheli																					2							
Paramelitidae gen. nov. 1 \`AMP001\`																								1				
Paramelitidae gen. nov. 1 \`AMP002\`																1										1		
Paramelitidae gen. nov. 2 \`AMP003\`			35					6			10						12				6							
Paramelitidae indet					1											59			1						1			
Bathynellacea																												
Brevisomabathynella pilbarensis																1	4				2							
Pilbaranella ethelensis																									1			
Pilbaranella sp. A					1																							
Isopoda																												
Pygolabis humphreysi			1					2	1	1	1									1	20		1					
Copepoda																												
Cyclopoida																												
Diacyclops cockingi								1										1			75				3			
Diacyclops humphreysi s.l.	15		8			12	45	16			196	2		1								6	1		1	100	10	49
Microcyclops varicans																	6											
Pilbaracyclops supersensus								6													5							
Harpacticoida																												
Archinitocrella newmanensis	1		1								4										170				1			1
Ostracoda																												
Gomphodella hirsuta																	4					1						
Notacandona gratia														5														
Origocandona \`BOS099\`							12	4																				
Origocandona \`BOS1752\`			48	3																	3							
Origocandona inanitas								90																				
Pilbaracandona colonia											2						4				2							
Pilbaracandona eberhardi																										1		
Pilbaracandona kosmos								6												7		1						

3.2.3 Environmental DNA (eDNA)

The CO1 assay detected a total of nine stygal taxa, belonging to groups Oligochaeta, Amphipoda and Syncarida. The 18S assay is coarser in taxonomic resolution comparative to CO1, providing detections at the family rather than species level. Including results from both assays, five families with stygal representatives were detected; annelids from the families Naididae and Phreodrilidae, and arthropods including Cyclopidae, Tainisopidae and Limnocytheridae (**Table 3-5, Table 3-6**).

While both assays detected Phreodrilidae and Naididae oligochaetes, each detected a different suite of arthropods. The CO1 assay detected two species each of Amphipoda and Syncarida, while the 18S detected Copepoda, Isopoda and Ostracoda. This highlights the difference in the capacity of each assay to detect certain taxa.

In addition to the nine confirmed stygal species above, the CO1 assay detected the epigean aquatic species *Peza ops* in one sample (**Table 3-5**). The Ethel Gorge TEC is known to include a stygal species of *Peza* (*Peza* "sp. OB") however there are no published reference CO1 sequences for that species. Without a published reference sequence of *Peza* "sp. OB", it is possible that the detection represents that stygal species but has been allocated to *P. ops* as the only published species of Pezidae.

Across both assays, overall number of detections of stygal taxa, and of each individual taxon, were greater in 2023 Wet than 2022 Dry. This trend was also apparent when comparing dry and wet season samples at individual bores (**Table 3-5, Table 3-6**).

Comparison of the direct detections based on traditional sampling and morphological methods[1] with the eDNA detections (CO1 and 18S) highlights the complementary nature of the two approaches. Of the 20 samples for which comparable morphological and eDNA samples were collected, six samples yielded no specimens for morphological analysis, while the eDNA method produced positive detections for five of those six. Only one sample yielded a nil result for both morphological and eDNA samples (HEOP504M, 2022 Dry).

While the 18S assay was less specific than the CO1 assay for eDNA or morphological methods, it appeared to detect the presence of certain taxa more effectively. For instance, cyclopoid copepods are known to be an abundant and relatively widely distributed component of the TEC. Morphological detections of cyclopoid copepods were made in eight of the 20 samples and the 18S assay for eDNA detected cyclopoid copepods in all but one sample, while the CO1 assay was unsuccessful in detecting any cyclopoids (**Table 3-5, Table 3-6**). This suggests that cyclopoid copepods are even more ubiquitous than is typically recorded; and indicates that the 18S assay is far more effective than the CO1 assay for detecting and classifying the diversity of copepods. It also highlights the ongoing importance of traditional sampling and morphological analyses for understanding the species-level diversity of this group. This is also the case for ostracods, which represent a similarly diverse taxon for the area.

In contrast, the CO1 assay was more effective at detecting and classifying oligochaetes and to a lesser extent amphipods. The CO1 and 18S assays provided a higher number of detections than morphological samples. The specificity of CO1 allowed several species of naidids and phreodrilids to be identified to the species-level. The ability to discern species is a product of the inherent specificity of the assay used, and also relies on the relative maturity of the reference library of comparative material. As further molecular reference material is added to the library of known taxa, the ability to assign species will be enhanced.

Overall, the eDNA methods detected certain taxa in more samples than morphological methods. Conversely, certain taxa were present in morphological samples but not detected by eDNA methods. While harpacticoid copepods were absent from eDNA detections, their presence was confirmed in high numbers at least one sample (HEV0008M, 2023 Wet), based on traditional sampling and morphological methods (**Table 3-6**). The isopod *Pygolabis humphreysi* was known to be present in five samples, but only one high-level detection of Tainisopidae was provided by the 18S assay (**Table 3-6**). The lack of detection for these taxa in particular is relevant given that Stantec (2023b) identified *Pygolabis humphreysi* and *Archinitocrella newmanensis* as two species which show potential to be indicators for the ecosystem health of the TEC.

A further difference between the morphological and eDNA methods is the ability to provide a measure of abundance. The current molecular assays used provide only presence of absence of a taxon while the morphological analyses are able to provide measures of abundance. The relevance of this difference is informed by the project aims and performance metrics.

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[1] supported by genetic sequencing

Table 3-6. eDNA detections from the CO1 (fwh2) and 18S assays compared to morphological results during 2022 Dry.

Taxa	HEOP0425			HEOP0462M			HEOP0574M			OB23REG1			T399			HEOP0504M			HEOP0338			EOP0334R			EOP0253R		
	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S
Acarina																											
Peza sp. OB		*																									
Oligochaeta																											
Enchytraeidae																											
Enchytraeidae sp. sp nov													3														
Naididae						*									*												
Pristina OB													1														
Phreodrilidae																											
Phreodrilidae sp. P10					*																						
Amphipoda																											
Chydaekata acuminata				3			1			12	*																
Chydaekata sp.													1														
Paramelitidae gen. nov. 1 `AMP002`							1																				
Paramelitidae gen. nov. 2 `AMP003`							2																				
Bathynellacea																											
Pilbaranella sp A																							*				
Tainisopidae																											
Pygolabis humphreysi	2																										
Copepoda																											
Cyclopoida			*			*			*			*			*						*			*			*
Diacyclops humphreysi s.l.				2									9														
Harpacticoida																											
Archinitocrella newmanensis										1																	
Ostracoda									*																		
Gomphodella hirsuta													2														
Pilbaracandona kosmos													2														

Table 3-7. eDNA detections from the CO1 (fwh2) and 18S assays compared to morphological results during 2023 Wet.

Taxa	HEOP0338			HEOP0334			HEOP0253R			HEOP0425			HEOP0462M			HEOP0504			HEOP0574M			HEQ0022M			HEV0008M			OB23REG1			T399		
	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S	Mor	CO1	18S
Acarina																																	
Peza sp.										1																							
Oligochaeta																																	
Enchytraeidae																																	
Enchytraeidae sp. Biologic-OLIG024		*																															
Naididae			*			*									*			*			*						*			*			*
Naididae sp.																1															4		
Pristina OB (Naididae sp. N3 from (Brown *et al.* 2015)		*			*								250	*			*						*			*			*			*	
Phreodrilidae															*			*						*			*			*			
Phreodrilidae sp. P10 (Brown *et al.* 2015)								*						*			*																
Phreodrilidae sp. P11 (Brown *et al.* 2015)		*												*			*						*			*			*				
Phreodrilidae sp. P15 (Brown *et al.* 2015)					*																												
Phreodrilidae sp. 'dissimilar ventral chaetae'																1															3		
Amphipoda																																	
Chydaekata acuminata													11	*			*			*		3	*			*			*		13		
Chydaekata sp. \`AMP005\`	1																								3								
Maarrka etheli																									2								
Paramelitidae gen. nov. 2 \`AMP003\`																			12	*			*		6	*							
Bathynellacea																																	
Brevisomabathynella pilbarensis																			4						2								
Pilbaranella ethelensis		*																															
Tainisopidae																											*						
Pygolabis humphreysi										1												1			20						1		
Copepoda																																	
Cyclopoida			*			*			*			*			*			*			*			*			*			*			*
Diacyclops cockingi																									75								
Diacyclops humphreysi s.l.	45												2															6			1		
Microcyclops varicans																			6														
Pilbaracyclops supersensus																									5								
Harpacticoida																																	
Archinitocrella newmanensis																									170								
Ostracoda															*						*						*						
Gomphodella hirsuta																			4									1					
Origocandona \`BOS099\`	12																																
Origocandona \`BOS1752\`																									3								
Pilbaracandona colonia																			4						2								
Pilbaracandona kosmos																						7						1					

3.2.4 Species Richness and Abundance

3.2.4.1 Tends over time

Mean abundance per bore in the 2022 Dry showed a decrease relative to the previous year, with an increase observed for 2022 Wet (**Figure 3-10A-B**). During both 2022 Dry and 2023 Wet there was an increase from the previous year in the mean number of species per bore (**Figure 3-10C-D**). A slight negative trend between 2013 and the current Program is observable in both wet and dry seasons for abundance and number of species (**Figure 3-10A-D**). However the variability within each year was typically greater than the mean value, highlighting the inherant spatial and temporal variability in the stygal assemblage.

Given this variability, caution should be taken when determining the validity of the observed trends. It also highlights the limitations of simple univariate diversity measures for detecting changes in the Ethel Gorge Stygobiont TEC, as noted in the recent desktop review (Stantec 2023b).

Consistent with previous years, the high variability in the abundance is likely to be attributable to taxa such as copepods and ostracods, where abundance can vary by two orders of magnitude between adjacent bores, and between seasons at the same bore.

3.2.4.2 Estimators

The stygofauna monitoring of the Ethel Gorge region (including all monitoring zones) has produced a total of 54 core species between November 2009 and May 2023 (**Table 3-7**). The current Program's sampling has added two species to the core species list (*Origocandona* \`BOS1752\` and Enchytraeidae sp. nov.) (**Table 3-3, Table 3-4**). Diversity estimators suggest that not all the species present have been collected, and that there may be up to 16 additional species that may be detected if the current sampling effort was doubled. This is demonstrate by the rarefaction curves based on those indicators which are all still generally trending upwards (**Figure 3-11**).

For MZ 1 only, 40 core species have been detected, which represents 82% of the predicted number, based on extrapolation (49 species) (**Table 3-8**). The diversity estimators in **Table 3-8** indicate that the current total of 40 species represents between 65.1% and 86.5% of the core species present in the TEC. As for the broader spatial range of samples explored in **Figure 3-11,** the rarefaction curves in **Figure 3-12** indicate that there are core species within MZ1 that are yet to be detected.

Overall, these analyses suggest that further sampling would be required to fully represent the diversity of species in the Ethel Gorge Aquifer Stygobiont TEC and surrounding area. However, the percentage of stygal species that remain undetected is decreasing.

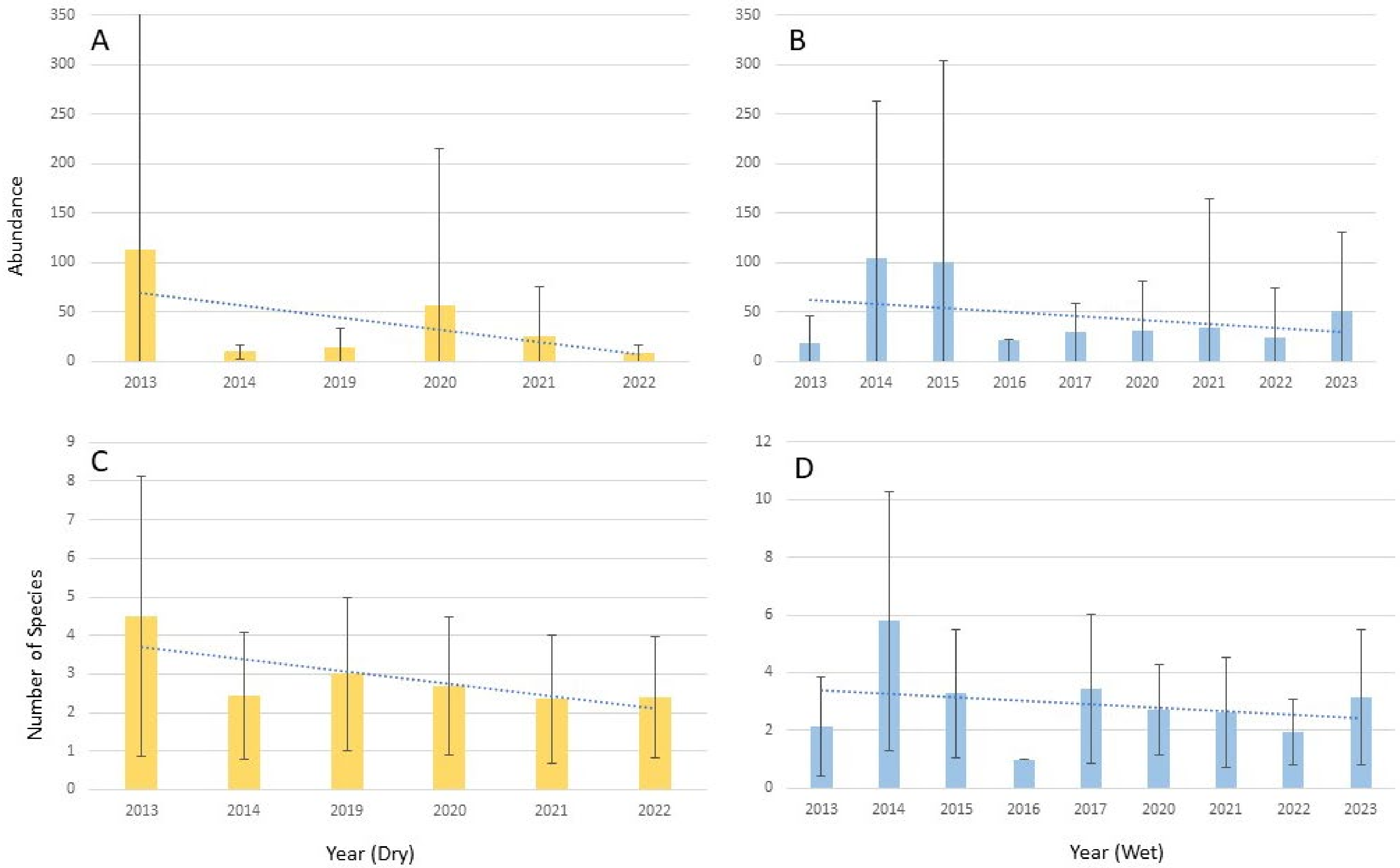

Figure 3-10 Mean abundance (with StDev) in bores during (A) Dry and (B) Wet seasons. Mean number of species (with StDev) during (C) Dry and (D) Wet seasons.

Table 3-8 Observed and extrapolated stygofauna species richness for all monitoring zones between 2009 and May 2023 compared to seven alternate diversity estimators.

Observed vs Estimated		Obs. & Pred. spp richness	% Predicted collected
Obs.	Sobs	54	
	Extrapolated (1130 samples)	60.63	89.1
Diversity estimators	Chao 1 Mean	57.1	94.6
	ACE Mean	58.4	92.4
	Bootstrap Mean	60.4	89.4
	Jack 1 Mean	67.0	80.5
	ICE Mean	64.3	83.9
	Chao 2 Mean	63.0	85.6
	Jack 2 Mean	71.5	75.5
Range		57.05 - 71.48	75.5 - 94.6

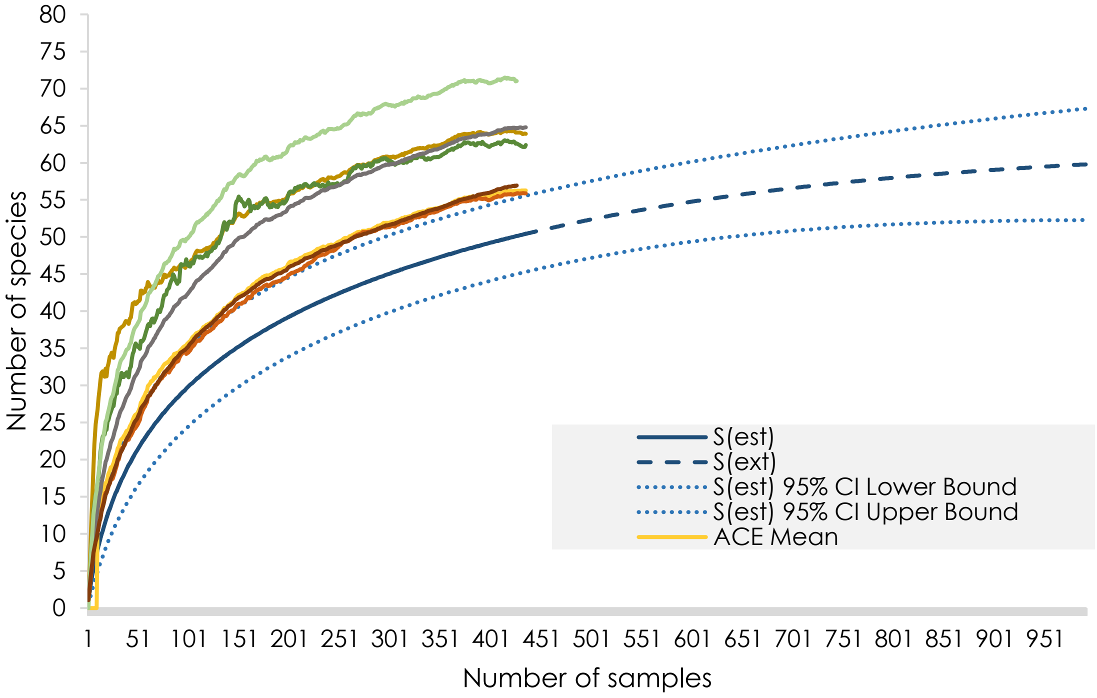

Figure 3-11 Stygofauna core species accumulation curves for observed (s(est), extrapolated (s(ext) and various species richness estimators (EstimateS (Colwell, 2013)) for the broader ethel gorge area, i.e. all monitoring zones sampled from 2009 to May 2023.

Table 3-9 Observed and extrapolated stygofauna species richness for Monitoring Zone 1 only between 2009 and May 2022 compared to seven alternate diversity estimators.

Observed vs Estimated		Obs. & Pred. spp richness	% Predicted collected
Obs.	Sobs	40	
	Extrapolated (970 samples)	48.83	81.9
Diversity estimators	Chao 1 Mean	52.6	76.0
	ACE Mean	50.4	79.4
	Bootstrap Mean	46.2	86.5
	Jack 1 Mean	54.0	74.0
	ICE Mean	58.8	68.0
	Chao 2 Mean	52.8	75.8
	Jack 2 Mean	61.4	65.1
Range		46.21 - 61.37	65.1 - 86.5

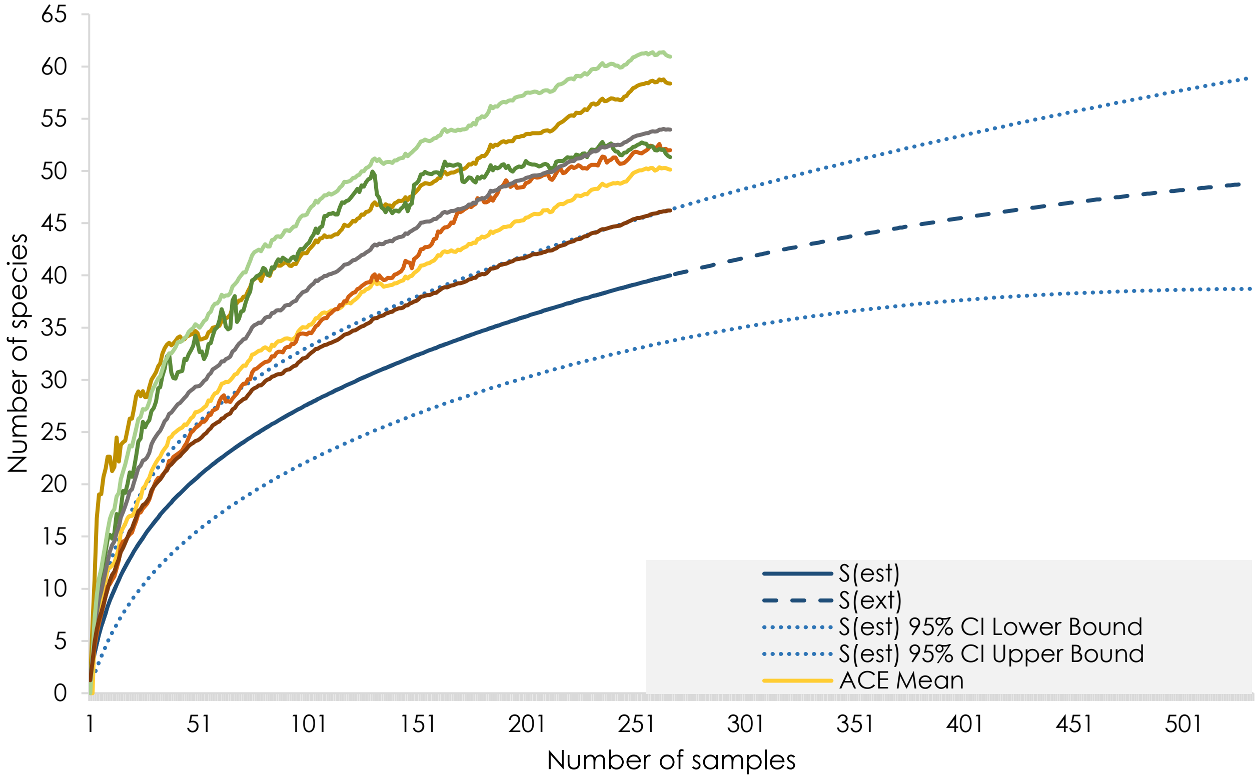

Figure 3-12 Stygofauna core species accumulation curves for observed (s(est), extrapolated (s(ext) and various species richness estimators (EstimateS (Colwell, 2013)) for Monitoring Zone 1 only in the Ethel Gorge TEC, sampled from 2009 to May 2022.

4. Conclusion

4.1 Groundwater

Groundwater SWLs were all within the GTVs defined by Douglas and Pickard (2014) and the majority of bores recorded only small variances from the previous survey round. The pH, salinity and nutrients were all mostly below GTVs or within the GTV range. Some individual bores displayed values outside the defined GTVs, however these were consistent with historical records, where available, or were considered to represent spatial variability associated with the introduction of additional bores.

The use of ultra-trace metals analysis in the Program increased the capacity to develop program-specific GTVs, with more than half of the metals having sufficient records to construct preliminary GTVs. Concentrations of all metals were low, and all but two records for barium are below published ANZECC freshwater thresholds. Recorded concentrations of metals in the Ethel Gorge area were typically less than ten times lower than the published thresholds. There is no perceived metal toxicity risk to the Ethel Gorge Aquifer Stygobiont TEC.

4.2 Stygofauna

A total of 35 stygal taxa were recorded during the Program from across seven high-level taxonomic groups including Acarina, Amphipoda, Bathynellacea, Isopoda, Copepoda, Ostracoda and Annelida. Two of the taxa recorded, Enchytraeidae sp. nov. and *Origocandona* \`BOS1752\` had not been recognised previously.

Consistent with historical records, cyclopoid copepods were numerically dominant. Other relatively abundant taxa recorded were the amphipods Paramelitidae gen. nov. 1 \`AMP002\`, and Paramelitidae gen. nov. 2 \`AMP003\`, the ostracod *Origocandona inanitas* and the annelid *Pristina* OB. In addition to the more common taxa, a few rarely documented taxa were detected, including *Peza* sp. OB, *Coxicerberus* ISO019 and Aeolosomatidae.

Average abundance during 2022 Dry was relatively low compared to historical records, however had increased considerably by 2023 Wet which had the highest average abundance per bore since 2015. All interannual changes observed in the number of species and abundance are within the variability documented for the TEC. Diversity estimators indicated that further sampling would be required to fully document the diversity of species in the Ethel Gorge Aquifer Stygobiont TEC and surrounding area. However, the percentage of stygal species that remain undetected is decreasing.

eDNA methods were implemented for the first time during this Program and provided insight into the stygofauna community as well as the relative strengths of the morphological and eDNA methods for detecting stygofauna. eDNA methods yielded considerable additional information and provide a complementary tool to traditional sampling and morphological assessment.

4.3 Summary

There were no detectable changes the physico-chemical parameters of groundwater in relation to mining activities during the Program, based on SWLs and groundwater quality. Metal concentrations were typically below detection or within published limits for aquatic biota and did not represent a risk to the Ethel Gorge Aquifer Stygobiont TEC. The stygofauna community was recorded in low abundances in 2022 Dry but demonstrated a considerable increase by 2023 Wet with the highest abundances since 2015. There has been a slight trend towards lower diversity and abundance over time. However, given the inherent variability within the system, it is difficult to distinguish temporal patterns from the spatial variability.

The findings of the Program along with previous surveys indicate that current groundwater management practices have been appropriate to prevent potential impacts to the Ethel Gorge stygofauna TEC from BHP WAIO operations. It is also considered that adequate saturation of the core habitat has been maintained, enabling the persistence of stygofauna.

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Appendices

Appendix A Program Sampling Effort

Bore Code	Alternate Name	2022 Dry	2023 Wet	Within TEC	MZ	Easting	Northing
EEX917		*	*	Y	1	793530	7417045
EEX931		*	*	Y	1B	794508	7416312
EOP0245RE		*		Y	1B	792494	7416811
EOP0246R	noID	*	*	Y	1	792422	7417287
EOP0253R		*	*	Y	1	793971	7417546
EOP0258R		*	*	Y	2	794446	7416916
EOP0334R		*	*	N	2	796378	7417776
ERCSGW0003		*	*	N	6	779991	7417505
ERCSGW0012		*		N	6	786616	7418230
ERCSGW0023		*		N	6	779703	7420708
HEA0121		*	*	Y	3	791366	7418324
HEA0126		*	*	Y	3	791704	7418651
HEA0133		*	*	Y	3	791606	7418525
HEOP0310	W7B	*		Y	4	790068	7414623
HEOP0314			*	Y	3	788854	7414695
HEOP0317M		*	*	N	6	782304	7416206
HEOP0388		*	*	Y	1	790932	7417338
HEOP0398M		*	*	N	4	788367	7410085
HEOP0417		*	*	Y	1	792248	7417308
HEOP0425		*	*	Y	1	793688	7417498
HEOP0430M			*	Y	3	792121	7418670
HEOP0462M	W152	*	*	N	1	795341	7424209
HEOP0504	HEOP0504M	*	*	N	1	793117	7420461
HEOP0508	EA0285R	*	*	N	4	790299	7409116
HEOP0524	HEOP0524 NEW	*	*	N	3	789972	7416279
HEOP0542	w230	*	*	N	5	797139	7427853
HEOP0543	W231	*	*	N	5	797323	7429830
HEOP0556	W244	*	*	N	5	798631	7429292
HEOP0559	W247 / HEOP0559	*	*	N	2	795402	7416424
HEOP0574M		*	*	N	1	792671	7419690
HEOP0798M			*	Y	1B	791928	7416632
HEOP0799	F3 / HEOP0399	*		Y	4	790336	7415083
HEOP0801	F5 / HEOP0425	*		Y	4	789311	7414640
HEOP0811	HEOP0524 (Unknown 3)	*	*	N	4	783763	7406537
HEQ0022M	HEO0022M		*	Y	3	790338	7415958
HEV0008M			*	Y	1	792178	7417817
OB23REG1		*	*	Y	1	791423	7417425
T399		*	*	N	1	793453	7422117
T411A		*	*	N	3	785020	7415786
W028		*	*	N	4	785757	7409065
W029		*	*	N	4	785772	7409018
W056		*	*	Y	1	792595	7419473
W116		*	*	N	1	797483	7426207
W117		*	*	N	1	797093	7426354
W122	WP122		*	N	5	796548	7425029
W201		*	*	N	4	789376	7409586

Appendix B Molecular identification for the 2022 Dry and 2023 Wet surveys (Lejis, 2023)

Report prepared for Stantec, 31 October 2023

by Dr Remko Leijs, Ecosystem and Biological Services

Molecular identification of stygofauna (Paramelitidae, Phreodrilidae, Naididae, Parabathynellidae, Bathynellidae and Tainisopidae) from Ethel Gorge, Western Australia

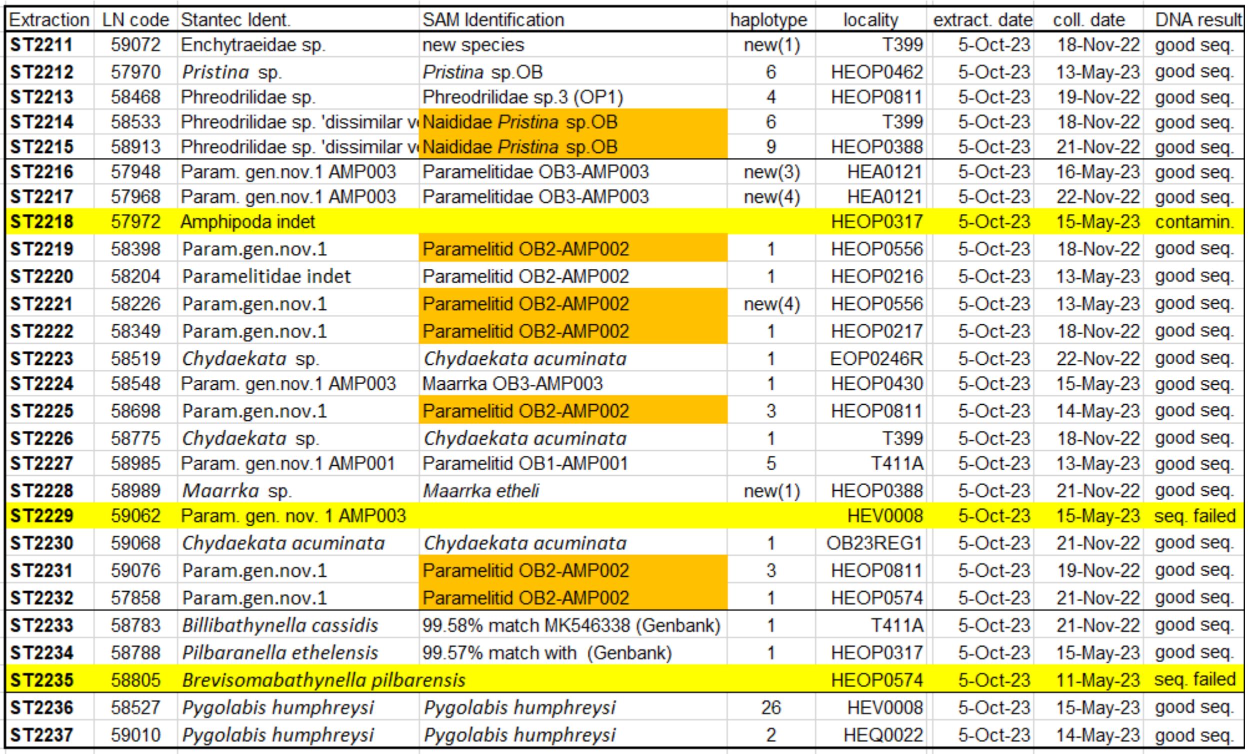
Summary

- Five species of Paramelitid amphipods were identified, which matched species and haplotypes already known from the area, and four additional haplotypes were found.
- One species of Phreodrilid oligochaete and one species of Naidid oligochaete were found, both matching species identified in earlier work. Both species had haplotypes found before. Additionally, one oligochaete species was sequenced belonging to a lineage not identified before.
- Two Syncarida species were sequenced, with high identity matches on GenBank.
- Two specimens of *Pygolabis humphreysi* were sequenced, of which both had haplotypes sampled before.

Methods

Biodiversity assessment of the collected fauna (Table 1) was performed using PCR amplification and sequencing in both directions of a 648 bp fragment of CO1, commonly used for DNA barcoding (Hebert et al. 2003). The sequences were added to large datasets that consists of related taxa from other areas complemented with published data from Genbank and unpublished sequence data at the South Australian Museum and the Western Australian Museum.

Phylogenetic analyses using neighbour joining of uncorrected sequence distances in PAUP* (Swofford 1998) were used to match the received specimens with previously identified analysed specimens. Results of phylogenetic analyses are presented as partial phylogenetic trees showing the target species with some closest related species. Results were compared with and build on to earlier reports, particularly with species naming codes and haplotype diversity.



Extraction	LN code	Stantec Ident.	SAM Identification	haplotype	locality	extract. date	coll. date	DNA result
ST2211	59072	Enchytraeidae sp.	new species	new(1)	T399	5-Oct-23	18-Nov-22	good seq.
ST2212	57970	*Pristina* sp.	*Pristina* sp.OB	6	HEOP0462	5-Oct-23	13-May-23	good seq.
ST2213	58468	Phreodrilidae sp.	Phreodrilidae sp.3 (OP1)	4	HEOP0811	5-Oct-23	19-Nov-22	good seq.
ST2214	58533	Phreodrilidae sp. 'dissimilar v	Naididae *Pristina* sp.OB	6	T399	5-Oct-23	18-Nov-22	good seq.
ST2215	58913	Phreodrilidae sp. 'dissimilar v	Naididae *Pristina* sp.OB	9	HEOP0388	5-Oct-23	21-Nov-22	good seq.
ST2216	57948	Param. gen.nov.1 AMP003	Paramelitidae OB3-AMP003	new(3)	HEA0121	5-Oct-23	16-May-23	good seq.
ST2217	57968	Param. gen.nov.1 AMP003	Paramelitidae OB3-AMP003	new(4)	HEA0121	5-Oct-23	22-Nov-22	good seq.
ST2218	57972	Amphipoda indet			HEOP0317	5-Oct-23	15-May-23	contamin.
ST2219	58398	Param.gen.nov.1	Paramelitid OB2-AMP002	1	HEOP0556	5-Oct-23	18-Nov-22	good seq.
ST2220	58204	Paramelitidae indet	Paramelitid OB2-AMP002	1	HEOP0216	5-Oct-23	13-May-23	good seq.
ST2221	58226	Param.gen.nov.1	Paramelitid OB2-AMP002	new(4)	HEOP0556	5-Oct-23	13-May-23	good seq.
ST2222	58349	Param.gen.nov.1	Paramelitid OB2-AMP002	1	HEOP0217	5-Oct-23	18-Nov-22	good seq.
ST2223	58519	*Chydaekata* sp.	*Chydaekata acuminata*	1	EOP0246R	5-Oct-23	22-Nov-22	good seq.
ST2224	58548	Param. gen.nov.1 AMP003	Maarrka OB3-AMP003	1	HEOP0430	5-Oct-23	15-May-23	good seq.
ST2225	58698	Param.gen.nov.1	Paramelitid OB2-AMP002	3	HEOP0811	5-Oct-23	14-May-23	good seq.
ST2226	58775	*Chydaekata* sp.	*Chydaekata acuminata*	1	T399	5-Oct-23	18-Nov-22	good seq.
ST2227	58985	Param. gen.nov.1 AMP001	Paramelitid OB1-AMP001	5	T411A	5-Oct-23	13-May-23	good seq.
ST2228	58989	*Maarrka* sp.	*Maarrka etheli*	new(1)	HEOP0388	5-Oct-23	21-Nov-22	good seq.
ST2229	59062	Param. gen. nov. 1 AMP003			HEV0008	5-Oct-23	15-May-23	seq. failed
ST2230	59068	*Chydaekata acuminata*	*Chydaekata acuminata*	1	OB23REG1	5-Oct-23	21-Nov-22	good seq.
ST2231	59076	Param.gen.nov.1	Paramelitid OB2-AMP002	3	HEOP0811	5-Oct-23	19-Nov-22	good seq.
ST2232	57858	Param.gen.nov.1	Paramelitid OB2-AMP002	1	HEOP0574	5-Oct-23	21-Nov-22	good seq.
ST2233	58783	*Billibathynella cassidis*	99.58% match MK546338 (Genbank)	1	T411A	5-Oct-23	21-Nov-22	good seq.
ST2234	58788	*Pilbaranella ethelensis*	99.57% match with (Genbank)	1	HEOP0317	5-Oct-23	15-May-23	good seq.
ST2235	58805	*Brevisomabathynella pilbarensis*			HEOP0574	5-Oct-23	11-May-23	seq. failed
ST2236	58527	*Pygolabis humphreysi*	*Pygolabis humphreysi*	26	HEV0008	5-Oct-23	15-May-23	good seq.
ST2237	59010	*Pygolabis humphreysi*	*Pygolabis humphreysi*	2	HEQ0022	5-Oct-23	14-May-23	good seq.

Table 1. Overview of the specimens analysed from Ethel Gorge, WA. The first column gives the DNA extraction number, the last column indicates whether the DNA sequencing was successful. The yellow highlighted specimens had weak PCR's and did not result in a DNA sequences. The orange highlighted fields show updated identifications.

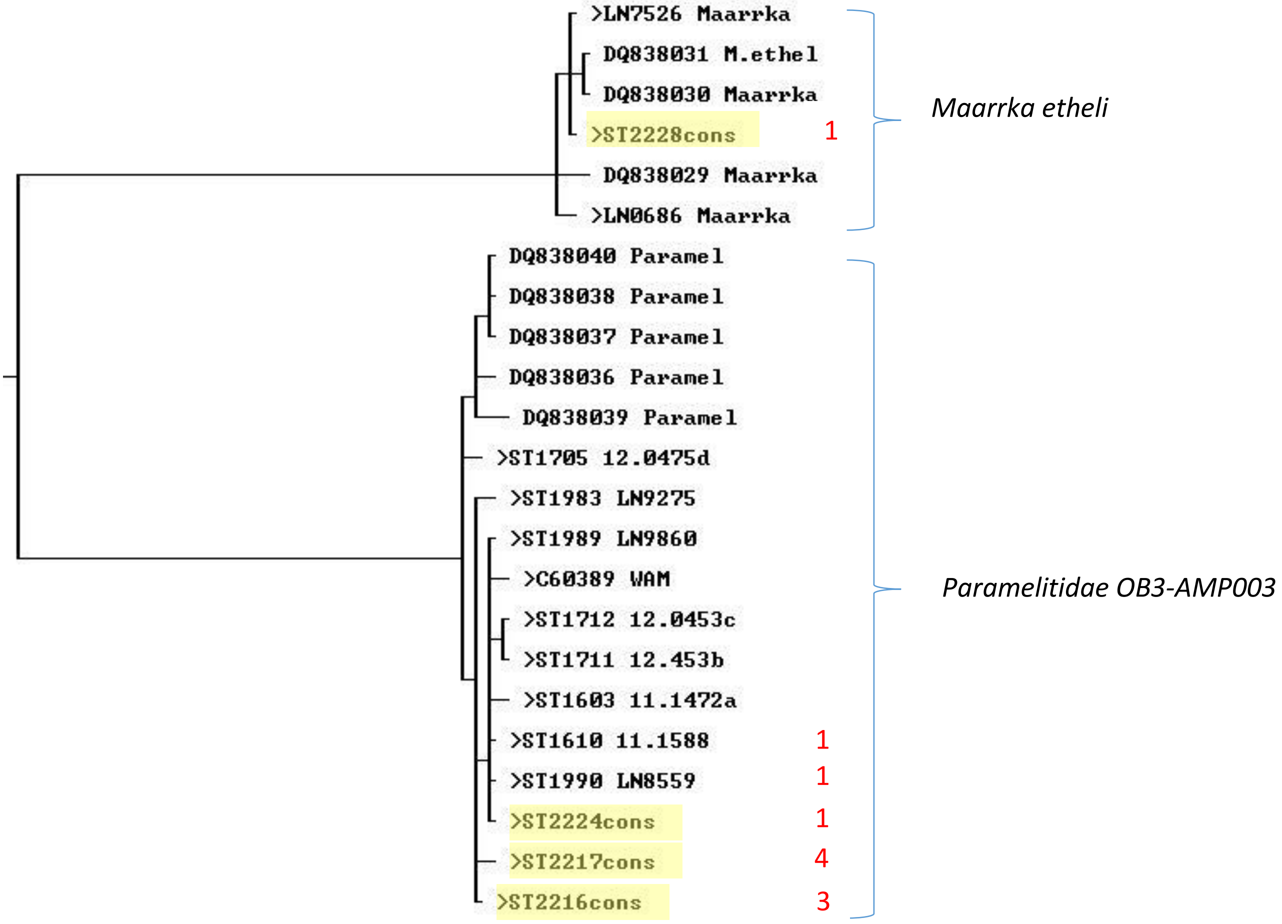


Figure 1. Partial neighbour joining cladogram of *Maarrka*. Indicated in yellow are the newly sequenced specimens. Haplotypes are indicated in the numbers in red.

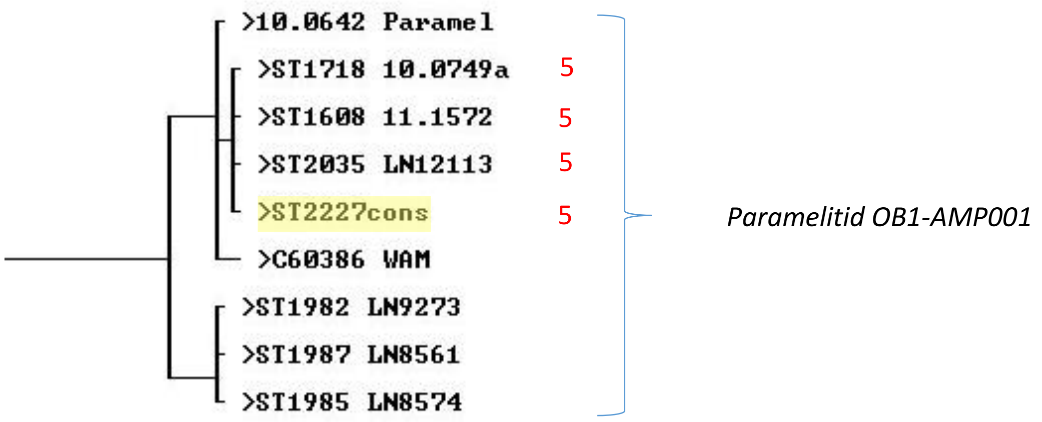


Figure 2. Partial neighbour joining cladogram of *Paramelitid OB1-AMP001.* Indicated in yellow is the newly sequenced specimen. Haplotypes are indicated in the numbers in red.

Naididae

Three Naididae specimens were sequenced (Table 1, ST2212, ST2214 and ST2215) and matched previously sequenced specimens belonging to *Pristina sp. OB.* The haplotype diversity in this species is relatively high, but no new haplotypes were found.

Phreodrilidae

Two Phreodrilidae specimens were sequenced (Table 1, ST2211 and ST2213). ST2211 belonged to a species and Oligochaete lineage not encountered before. ST2213 matched specimens of Phreodrilidae sp.3 (OP1) with haplotype # 4.

Syncarida

Two Syncarida specimens were sequenced (Table1, ST2233 and ST2234). ST2233 did not occur in the sequence database but a BLAST search on GenBank showed high identity (99.58%) with Parabathynellidae sp. (Pilbara) 15 EM-2019. (GenBank Accession # MK546338), which indicates that these are conspecific.
ST2234 shared haplotype #1 with Bathynellidae specimen #EO1 Pilbaranella sp. A GP-2018.

Taenisopidae

Two specimens of *Pygolabis humphreysi* were sequenced (Table 1), of which ST2236 had a haplotype sampled before (haplotype # 26), and ST2237 shared haplotype #2 with specimens sequenced before.

Sequences

Pygolabis humpreysi

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>ST2237cons LN59010 haplotype #2
AACGACGGCCAGTGGTCAACAAATCATAAAGATATTGGTACTTTGTATTTTATTTTCGGTGCTTGGGCTG
GTGCGGTTGGTACAGGTCTTAGTATAATTATTCGATCGGAGTTGGGTCAACCTGGCAGTTTTATCGGGGA
TGATCAAATTTATAACGTAGTCGTAACTGCTCACGCGTTTGTAATGATTTTTTTTATAGTTATGCCTGTT
ATAATTGGTGGTTTCGGGAATTGATTGGTACCGTTGATGTTGGGAGCTCCTGATATAGCTTTTCCTCGCA
TAAATAATATGAGCTTTTGATTACTTCCACCATCTTTGACTCTGTTATTAAGGAGTGGGTTAGTGGAGAG
GGGTGTTGGTACTGGTTGGACGGTTTATCCGCCATTAGCTGCTGGAATCGCGCACAGTGGAGCTTCTGTG
GATTTGGGGATTTTTTCTTTGCATTTAGCTGGGGCTTCTTCTATTTTGAGGGCTATCAATTTTATCACCA
CTTTTATTAATATGCGAGCGAGGGGGATGAGACTGGATCGTGTTCCTTTATTTGTGTGGTCGGTAGTTAT
TACGGCGGTACTTTTACTATTGTCGTTACCTGTACTCGCTGGGGCGATTACGATGCTATTAACGGATCGT
AATTTAAATACTTCTTTTTTTGACCCCAGAGGAGGTGGAGATCCTATTTTATTTCAACATTTATTTTGAT
TTTTTGGTCACCCTGAAGTTTAGT
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>ST2236cons LN58527 haplotype #26
AACGACGGCCAGTGGTCAACAAATCATAAAGATATTGGTACTTTGTATTTTATTTTCGGTGCTTGGGCTG
GTGCGGTTGGTACAGGTCTTAGTATAATTATTCGATCGGAGTTGGGTCAACCTGGCAGTTTTATCGGGGA
TGATCAAATTTATAACGTAGTCGTAACTGCTCACGCGTTTGTAATGATTTTTTTTATAGTTATGCCTGTT
ATAATTGGTGGTTTCGGGAATTGATTGGTACCGTTGATGTTGGGAGCTCCTGATATAGCTTTTCCTCGCA
TAAATAATATGAGCTTTTGATTACTTCCACCATCTTTGACTCTGTTATTAAGGAGTGGGTTAGTGGAGAG
GGGTGTTGGTACTGGTTGGACGGTTTATCCGCCATTAGCTGCTGGAATCGCGCACAGTGGAGCTTCTGTG
GATTTGGGGATTTTTTCTTTGCATTTAGCTGGGGCTTCTTCTATTTTGAGGGCTATCAATTTTATCACCA
CTTTTATTAATATGCGAGCGAGGGGGATGAGACTGGATCGTGTTCCTTTATTCGTGTGGTCGGTAGTTAT
TACGGCGGTACTTTTGCTATTGTCGTTACCTGTACTCGCTGGGGCGATTACGATGTTATTAACGGATCGT
AATTTAAATACTTCTTTTTTTGACCCCAGAGGAGGTGGAGATCCTATTTTATTTCAACATTTATTTTGAT
TTTTTGGTC
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Syncarida

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>ST2234cons LN58788 haplotype #1
AACGACGGCCAGTGGTCAACAAATCATAAAGATATTGGTACGTTATATTTAATTTTAGGAGCCTGGGCCG
GGATATTAGGAACCGGGATAAGAGTAATTATTCGTATAGAGCTTGGGCAAGCAGGAACTTTAATTGGAGA
TGACCAAATTTATAATACTATTGTAACTGCTCATGCATTCATTATAATCTTCTTTATAGTTATGCCTTTA
ATAATAGGAGGATTTGGAAATTGACTGGTACCAATAATAATTAGAGCTCCTGATATAGCTTTCCCTCGAA
TAAATAATTTAAGATTCTGGTTGCTTATTCCTTCACTATTGCTTTTAATTTGTAGAAGTATTGTAGAAAG
AGGGGTAGGAACAGGATGGACAGTTTATCCTCCTCTCGCAAGAAATATTTCACATGGGGGAGTATCCGTA
GATTTGGCAATCTTTTCTCTTCATCTAGCAGGTGCTTCTTCTATTCTTGGAGCAATCAACTTTCTTAGAA
CTATATGAAATATACGAACAATAGGGTTACTAATAGAGCTTCTTCCTTTATTTGCATGGTCTGTTTTAAT
TACTGCAGTTTTATTACTTTTATCCTTGCCTGTTTTAGCAGGAGGAATCACTATATTATTGACAGACCGT
AATATTAATACGTCTTTTTTTGATCCTGCTGGAGGAGGTGACCCTATTTTATATCAACATTTATTTTGAT
TTTTTGGTCACCC
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>ST2233cons LN58783 haplotype #1
AACGACGGCCAGTGGTCAACAAATCATAAAGATATTGGAACTATATATTTGATTTTTGGGTCATGAGCTG
GGATTTTAGGAATAGGTTTGAGAATAATTATCCGTATTGAATTAGGTCAACCTGGTAGATTTATTGGAGA
TGAACAAATCTATAACACTATTGTTACAGCTCACGCTTTCGTAATAATTTTTTTTATAGTTATACCTGTA
ATAATTGGAGGATTTGGAAACTGAATAATCCCACTAATAATTAATTCTCCTGATATAGCTTTTCCACGAA
TAAACAATATGAGATTCTGAATTTTATTACCCTCATTGACTTTACTATTAATAAGAAGTTTAATTGAAAG
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GATTTAGCCATTTTCTCTCTTCATTTAGCTGGGGCTTCTTCTATTTTAGGAGCTGTAAATTTTATTTCTA
CAGTATTAAATATACGTAGATATGAAATAAAATTAGATCGTCTTCCCCTATTTGTATGATCTGTATTTAT
TACAGCATTTTTATTATTACTAGCTTTACCTGTCTTAGCCGGAAGTATTACTATGTTGTTGACAGATCGA
AATCTTAATACCTCTTTTTTTGATCCAGCCGGAGGTGGAGATCCTATTCTATATCAACATTTATTTTGAT
TTTTTGGTCACCCTGA
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Amphipods

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>ST2232cons LN57858 haplotype #1
ATCGACGGCCAGTGGTCAACAAATCATAAAGATATTGGAACTCTCTATTTTATTCTTGGAGCCTGATCAA
GTATATTGGGTACATCCATAAGAATTATTATTCGATCAGAATTAAGATCCCCTAGCAGTCTAATCGAAAA
TGATCAACTATATAATGTACTAGTTACTGCCCACGCATTTATCATAATCTTCTTCATAGTTATACCTATT
ATAATTGGTGGATTCGGAAATTGATTAGTTCCGCTAATATTAGGTGCACCTGATATAGCATTTCCACGAA
TAAATAATATAAGATTCTGACTACTTCCACCATCATTATCATTACTTCTAATAAGAGGAATAGTAGAAAG
TGGTGTCGGTACAGGATGAACAGTTTATCCACCTTTATCCTCTAATCTATATCATTCAGGAAGAAGTGTT
GATTTTGCTATCTTTTCACTCCATTTAGCCGGAGCTAGATCTATTTTAGGAGCCATCAATTTTATTTCAA
CAGTTATAAATATACGACCAAACAAAATATCCATAGATTCTATACCTCTATTCACATGATCTGTATTTAT
TACTGCTATTCTTTTACTTTTATCTCTTCCTGTTCTTGCAGGAGCAATTACTATACTTCTCACTGACCGT
AATATTAATACATCATTTTTTGATCCTATAGGAGGAGGAGATCCAATTCTTTACCAACACTTATTTTGAT
TTTTTGGTCACCCTGAAGTTTAGTCAT

>ST2231cons LN59076 haplotype #3
AACGACGGCCAGTGGTCAACAAATCATAAAGATATTGGAACTCTCTATTTTATTCTTGGAGCCTGATCAA
GTATATTGGGTACATCCATAAGAATTATTATTCGATCAGAATTAAGATCCCCTAGCAGTCTAATCGAAAA
TGATCAACTATATAATGTACTAGTTACTGCCCACGCATTCATCATAATCTTCTTCATAGTTATACCTATT
ATAATTGGTGGATTCGGAAATTGATTAGTTCCACTAATATTAGGTGCACCTGATATAGCATTTCCACGAA
TAAATAATATAAGATTCTGACTACTTCCACCATCATTATCATTACTTCTAATAAGAGGAATAGTAGAAAG
TGGTGTCGGTACAGGATGAACAGTTTATCCACCTTTATCCTCTAATCTATATCATTCAGGAAGAAGTGTT
GATTTTGCTATCTTTTCACTCCATTTAGCCGGAGCTAGATCTATTTTAGGAGCTATCAATTTTATTTCAA
CAGTTATAAATATACGACCAAACAAAATATCCATAGATTCTATACCTCTATTCACATGATCTGTATTTAT
TACTGCTATTCTTTTACTTTTATCTCTTCCTGTTCTTGCAGGAGCAATTACTATACTTCTCACTGACCGT
AATATTAATACATCATTTTTCGATCCTATAGGAGGAGGAGATCCAATTCTTTACCAACATTTATTTTGAT
TTTTTGGTCACCCTGAAGTTTAGTCA

>ST2230cons LN59068 haplotype #1
AACGACGGCCAGTGGTCAACAAATCATAAAGATATTGGAACACTATATTTAATCCTTGGTGCATGAGCGA
GTATATTAGGTACCGCTATAAGAGTAATTATTCGCTCAGAACTAAGAGCTCCAGGAAATCTAATCGGCAA
TGACCAATTATATAATGTTATAGTTACTGCCCACGCTTTCATTATAATCTTCTTTATAGTTATACCTATT
ATAATTGGAGGATTCGGAAATTGACTTGTCCCTTTAATATTAGGCTCCCCAGATATAGCGTTTCCTCGAA
TAAATAACATAAGATTCTGATTACTACCTCCTTCATTAACATTACTATTAATAAGAGGTATAGTGGAAAG
AGGAGTAGGAACTGGTTGAACAGTCTACCCACCTTTATCCTCAACTATTTCTCATAGAGGAAGAAGAGTA
GATATAGCTATTTTCTCGTTACATCTAGCAGGTGCTAGATCAATCTTAGGAGCTATTAACTTCATCTCCA
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Oligocheata

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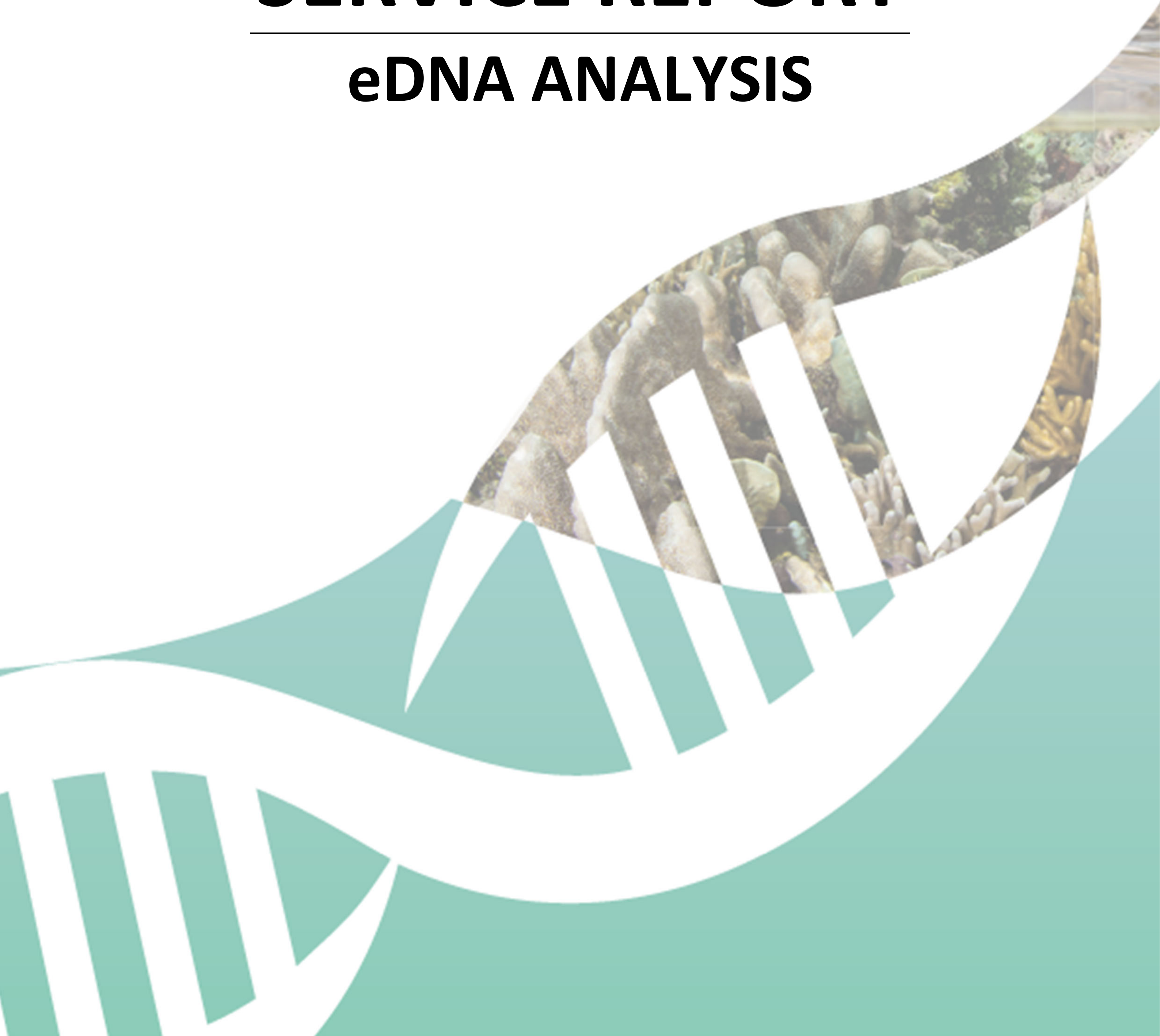
Appendix C eDNA results from the 2022 dry and 2023 wet surveys (eDNA Frontiers, 2023)

eDNA FRONTIERS

Curtin University

SERVICE REPORT

eDNA ANALYSIS



ASSAYS

- Universal
- Fish
- Sharks & Rays
- Corals
- Crustaceans
- Bacteria
- Plants & Algae
- Mammals
- Insects
- Vertebrates
- Molluscs
- Reptiles
- Birds
- Fungi

SAMPLES

- Water
- Plankton tows
- Sediment
- Deposition arrays
- Biofoul
- Bore water
- Scats
- Tissue
- Plants
- Fossils
- Pollen
- Stomach contents

DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
NCBI	National Centre for Biotechnology Information
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
ZOTU	Zero-radius operational taxonomic unit
AIS	Alien Invasive Species
LULU	A post-clustering algorithm for curation of DNA amplicon data
18S	The nuclear gene region, 18S
COI	The mitochondrial gene region, cytochrome c oxidase I
16S	The mitochondrial subunit ribosomal RNA gene region, 16S
12S	The mitochondrial gene region, 12S

DISCLAIMER

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Project Details

Scope of Work: EF256

Project Title: Biodiversity audit of stygofauna in groundwaters taken from bores in WA, using eDNA metabarcoding.

Client Details

Client: Stantec Australia Pty Ltd (ABN: 17 007 820 322)
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Report Details

Report reference: EF256_Stantec_RevB_v2

Report issue date: 10/07/2023

(Part 1) Laboratory start date: 17/01/2023 Laboratory end date: 14/03/2023
(Part 2) Laboratory start date: 25/05/2023 Laboratory end date: 30/06/2023

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(Author)

Dr Tina Berry
(Reviewer)

1.0 OBJECTIVE

The objective of this study was to assess the presence of stygofauna from groundwater samples collected from bores in Western Australia using eDNA metabarcoding.

1.1 Study Scope

Using environmental DNA (eDNA) testing, eDNA frontiers was tasked with analysing water samples for the presence of stygofauna. The client provided a total of 64 samples (including four controls) consisting of water filtrate suspended on filter membranes and preserved in ATL buffer (Tables 1 and 2).

2.0 SAMPLE DETAILS

Table 1. Sample receipt details.

Date received:	29/11/2022 & 25/05/2023
Transport temp:	Ambient – ATL buffer (540µL & 810µL)
Number of samples:	64
Storage:	All samples were stored at -20°C prior to analysis.

Table 2. Supplied sample details.

eDNA Frontiers ID	Client Sample ID	Collection Location	Sample Type	Collection Date
E-256-001	W152	Ethel Gorge, Pilbara	Water	18/11/2022
E-256-002	W152	Ethel Gorge, Pilbara	Water	18/11/2022
E-256-003	W152	Ethel Gorge, Pilbara	Water	18/11/2022
E-256-004	T399	Ethel Gorge, Pilbara	Water	18/11/2022
E-256-005	T399	Ethel Gorge, Pilbara	Water	18/11/2022
E-256-006	T399	Ethel Gorge, Pilbara	Water	18/11/2022
E-256-007	HEOP0504M	Ethel Gorge, Pilbara	Water	18/11/2022
E-256-008	HEOP0504M	Ethel Gorge, Pilbara	Water	18/11/2022
E-256-009	HEOP0504M	Ethel Gorge, Pilbara	Water	18/11/2022
E-256-010	W029	Ethel Gorge, Pilbara	Water	19/11/2022
E-256-011	W029	Ethel Gorge, Pilbara	Water	19/11/2022
E-256-012	W029	Ethel Gorge, Pilbara	Water	19/11/2022
E-256-013	EOP0334R	Ethel Gorge, Pilbara	Water	20/11/2022
E-256-014	EOP0334R	Ethel Gorge, Pilbara	Water	20/11/2022
E-256-015	EOP0334R	Ethel Gorge, Pilbara	Water	20/11/2022
E-256-016	HEOP0425	Ethel Gorge, Pilbara	Water	20/11/2022
E-256-017	HEOP0425	Ethel Gorge, Pilbara	Water	20/11/2022
E-256-018	HEOP0425	Ethel Gorge, Pilbara	Water	20/11/2022
E-256-019	EOP0253R	Ethel Gorge, Pilbara	Water	20/11/2022
E-256-020	EOP0253R	Ethel Gorge, Pilbara	Water	20/11/2022
E-256-021	EOP0253R	Ethel Gorge, Pilbara	Water	20/11/2022
E-256-022	HEOP0574M	Ethel Gorge, Pilbara	Water	21/11/2022
E-256-023	HEOP0574M	Ethel Gorge, Pilbara	Water	21/11/2022
E-256-024	HEOP0574M	Ethel Gorge, Pilbara	Water	21/11/2022
E-256-025	OB23REG1	Ethel Gorge, Pilbara	Water	21/11/2022
E-256-026	OB23REG1	Ethel Gorge, Pilbara	Water	21/11/2022
E-256-027	OB23REG1	Ethel Gorge, Pilbara	Water	21/11/2022
E-256-028	Control	Ethel Gorge, Pilbara	Water	21/11/2022
E-256-029	Control	Ethel Gorge, Pilbara	Water	21/11/2022
E-256-030	Control	Ethel Gorge, Pilbara	Water	21/11/2022
E-256-031	HEOP0425	Ethel Gorge, Pilbara	Water	12/05/2023

eDNA Frontiers ID	Client Sample ID	Collection Location	Sample Type	Collection Date
E-256-032	HEOP0425	Ethel Gorge, Pilbara	Water	12/05/2023
E-256-033	HEOP0425	Ethel Gorge, Pilbara	Water	12/05/2023
E-256-034	EOP0253R	Ethel Gorge, Pilbara	Water	12/05/2023
E-256-035	EOP0253R	Ethel Gorge, Pilbara	Water	12/05/2023
E-256-036	EOP0253R	Ethel Gorge, Pilbara	Water	12/05/2023
E-256-037	HEOP0574M	Ethel Gorge, Pilbara	Water	11/05/2023
E-256-038	HEOP0574M	Ethel Gorge, Pilbara	Water	11/05/2023
E-256-039	HEOP0574M	Ethel Gorge, Pilbara	Water	11/05/2023
E-256-040	HEOP504M	Ethel Gorge, Pilbara	Water	13/05/2023
E-256-041	HEOP504M	Ethel Gorge, Pilbara	Water	13/05/2023
E-256-042	HEOP504M	Ethel Gorge, Pilbara	Water	13/05/2023
E-256-043	W152	Ethel Gorge, Pilbara	Water	13/05/2023
E-256-044	W152	Ethel Gorge, Pilbara	Water	13/05/2023
E-256-045	W152	Ethel Gorge, Pilbara	Water	13/05/2023
E-256-046	OB23REG1	Ethel Gorge, Pilbara	Water	15/05/2023
E-256-047	OB23REG1	Ethel Gorge, Pilbara	Water	15/05/2023
E-256-048	OB23REG1	Ethel Gorge, Pilbara	Water	15/05/2023
E-256-049	HEV0008M	Ethel Gorge, Pilbara	Water	15/05/2023
E-256-050	HEV0008M	Ethel Gorge, Pilbara	Water	15/05/2023
E-256-051	HEV0008M	Ethel Gorge, Pilbara	Water	15/05/2023
E-256-052	HEO0022M	Ethel Gorge, Pilbara	Water	14/05/2023
E-256-053	HEO0022M	Ethel Gorge, Pilbara	Water	14/05/2023
E-256-054	HEO0022M	Ethel Gorge, Pilbara	Water	14/05/2023
E-256-055	W029	Ethel Gorge, Pilbara	Water	14/05/2023
E-256-056	W029	Ethel Gorge, Pilbara	Water	14/05/2023
E-256-057	W029	Ethel Gorge, Pilbara	Water	14/05/2023
E-256-058	T399	Ethel Gorge, Pilbara	Water	16/05/2023
E-256-059	T399	Ethel Gorge, Pilbara	Water	16/05/2023
E-256-060	T399	Ethel Gorge, Pilbara	Water	16/05/2023
E-256-061	EOP0334R	Ethel Gorge, Pilbara	Water	16/05/2023
E-256-062	EOP0334R	Ethel Gorge, Pilbara	Water	16/05/2023
E-256-063	EOP0334R	Ethel Gorge, Pilbara	Water	16/05/2023
E-256-064	Control	Ethel Gorge, Pilbara	Water	16/05/2023

3.0 METHODS

3.1 Sample Collection

Groundwater samples were collected at nine locations between 18th – 21st November 2022, and these same locations plus an additional two (HEV008M and HEO0022M) between 11th – 16th May 2022. Three replicates were collected at each sampling point and filtered using 0.45µM mixed cellulose ester (MCE) with a peristaltic Sentino pump to capture eDNA present in the water. Three control samples of water were supplied for the first round of sampling, and an addition control for the second round. All samples were transported at ambient temperature while preserved in ATL buffer to eDNA Frontiers' laboratories where they were stored at -20°C until scheduled for DNA extraction.

3.2 eDNA Extraction and Analysis

DNA digestion was performed on each filter paper and either the entire aliquot (November samples) or half the aliquot (May samples) taken through for DNA extraction using a Qiagen DNeasy blood and tissue kit, following the eDNA Frontiers lab's SOPs and detailed in Koziol *et al.,* (2018), Stat *et al.,* (2017), and Stat *et al.,* (2018). Differing volumes were extracted as the amount of preservative used for each sample differed between the two sampling periods. Once extracted, each sample was assigned an individual combination of index tags and amplified by PCR using two assays: an 18S assay targeting a broad array of organisms, and a COI assay targeting stygofauna/invertebrates. Libraries were generated and sequenced using the Illumina MiSeq. Laboratory extraction and PCR controls were included to test for contamination.

3.3 Bioinformatics and Taxonomic Assignments

Bioinformatic tools were used to analyse raw sequence data (Mousavi-Derazmahalleh *et al*., 2021) generated from the metabarcoding. The sequencing results were demultiplexed and trimmed using Obitools and quality filtered with Usearch v11 for sequencing errors (maxee=1) with a minimum length of 300 (18S) or 150 (COI) used. Sequences were then dereplicated and unique sequences were transformed into zero radius operational taxonomic units (ZOTUs) to provide sensitive taxonomic resolution (Usearch v11) (Edgar, 2018). ZOTUs, in contrast to OTUs, are a more exact sequence variant, clustering at 99% to improve taxonomic resolution. Generated ZOTUs were queried against the nucleotide database NCBI (GenBank) and assigned to the family- (18S) and species-level (COI) where possible. ZOTUs generated using the COI assays were also queried against the custom database BRL (Guzik *et al.* in prep). Taxonomic assignments were based on an in-house Python script which further filters the Blast results (evalue ≤1e-5, %identity ≥90 (18S) or ≥95 (COI), qCov =100 (relaxed to 95 for custom db), LULU minMatch =97%), combines them with the ZOTU table results and produces a table containing the taxonomic information available from Blast and custom taxonomy databases (accessed February/July 2023).

It is important to note that while sequences recovered are converted to the lowest possible taxon based on similarities and differences to a DNA database (NCBI's GenBank), this database, and the taxonomic framework that underpins it, may contain errors. Accordingly, the DNA taxon identifications should be interpreted as the best available assignment based on currently available information and that errors are possible.

4.0 RESULTS

4.1 Taxonomic Diversity

Using the 18S assay, detections were made across a number of families, including those in phyla Annelida, Arthropoda, Nematoda, and Platyhelminthes (Table 3 and 4). Prior to analysis of the May samples data returned for the November samples was interrogated, with the absence of both amphipods and isopods noted. Subsequent analysis of the May samples returned a detection within the Order Isopoda, indicating that the 18S assay can detect this group. Although the 18S data is only reported to the family level as taxonomic resolution using this assay is low, further investigation of this detection showed it was to the species *Pygolabis humphreysi*, which is known to occur in the study area. Non-stygofauna detections were also made using this assay, with the majority of these in the fungi group.

Initial testing targeting the COI gene region for the samples collected in November was performed using the Ar5 assay. While this assay targets invertebrates and detection were made in phyla typically associated with stygofauna, many detections were assigned to Bacteria and detected invertebrate diversity was not high (Appendix One). Additionally, expected detections of isopods and amphipods did not occur using the Ar5 assay, despite *in silico* testing suggesting this assay should amplify these organisms.

As such, further analysis was performed by targeting an alternative region of the COI gene region using the assay fwh2, with this assay applied to both November and May samples. Stygofauna diversity detected using this assay was higher than those previously obtained from the Ar5 assay and included detections within Order Amphipoda (species *Chydaekata acuminata* and *Paramelitidae sp. 3 TLF-2008*). Other detections were made in the phyla Annelida, Arthropoda, and Nematoda (Table 5 and 6). Detections for non-target groups were also made, but far fewer than those observed in the 18S data.

Laboratory extraction controls were negative for all assays, and positive controls amplified successfully. Some detections were made in the field control samples using the 18S and fwh2 assays (November samples); this has been indicated in the relevant tables.

Table 3. Diversity detected from water samples collected in November using a broad 18S assay. Presence of the family at each site is indicated by the symbol *. Taxonomy was assigned as per NCBI and classifications were standardised according to the Global Biodiversity Information Facility (accessed February 2023). Blank cells indicate where taxa could not be resolved to a lower taxonomic level.

Phylum	Class	Order	Family	W152	T399	HEOP0504M	W029	EOP0334R	HEOP0425	EOP0253R	HEOP0574M	OB23REG1	Control
(Eukaryota)				*	*	*	*	*	*	*	*	*	*
Amoebozoa	Discosea	Dactylopodida	Vexilliferidae	*	*			*	*			*	
		Longamoebia										*	
		Vannellida	Vannellidae			*	*			*			
	Lobosa	Amoebida	Vahlkampfiidae			*		*	*	*			
Annelida	Clitellata	Tubificida	Naididae	*	*								
Apicomplexa				*								*	
	Conoidasida	Eucoccidiorida	Eimeriidae			*	*	*	*	*		*	
			Sarcocystidae									*	
		Eugregarinorida	Stylocephalidae									*	
Arthropoda							*				*	*	
	Arachnida	Araneae				*		*	*	*			
			Dictynidae			*		*	*				
			Oonopidae			*							
		Mesostigmata	Parasitidae									*	
		Palpigradi	Eukoeneniidae									*	
		Pseudoscorpiones					*						
			Olpiidae				*						
		Sarcoptiformes									*	*	
			Acaridae									*	
			Grandjeanicidae			*							
			Histiostomatidae									*	
			Malaconothridae									*	
		Trombidiformes	Bogatiidae								*		
			Erythraeidae				*		*				
			Eupodidae				*						
			Stigmaeidae							*			
	Chilopoda	Scolopendromorpha	Cryptopidae			*							
	Collembola					*	*	*	*			*	
		Entomobryomorpha				*			*	*			
			Entomobryidae				*						
			Isotomidae				*					*	
	Copepoda	Cyclopoida	Cyclopidae	*	*		*	*	*	*	*	*	*
	Diplopoda	Polyxenida	Lophoproctidae									*	
	Insecta	Blattodea	Blattidae		*								
			Termitidae	*	*		*		*				
		Coleoptera				*							*
			Chrysomelidae			*							
			Scarabaeidae								*		

Phylum	Class	Order	Family	W152	T399	HEOP0504M	W029	EOP0334R	HEOP0425	EOP0253R	HEOP0574M	OB23REG1	Control
		Ephemeroptera	Baetidae							*			
		Hemiptera										*	
		Hymenoptera	Formicidae						*	*	*		
		Lepidoptera							*				
		Neuroptera	Chrysopidae					*	*				
		Orthoptera	Morabidae									*	
		Psocoptera	Lepidopsocidae				*						
		Thysanoptera	Phlaeothripidae			*		*	*	*			
			Thripidae				*						
		Zygentoma	Nicoletiidae				*				*		
	Ostracoda	Podocopida	Limnocytheridae								*		*
Ascomycota				*		*	*	*	*	*	*	*	*
	Arthoniomycetes	Lichenostigmatales						*	*				
	Dothideomycetes					*	*	*	*	*		*	
		Botryosphaeriales		*	*	*		*	*	*	*	*	
			Botryosphaeriaceae	*		*		*					
		Capnodiales		*				*					
		Dothideales				*		*					
			Saccotheciaceae	*	*	*	*	*	*	*	*	*	*
		Mycosphaerellales	Mycosphaerellaceae			*							
			Teratosphaeriaceae					*	*	*		*	
		Pleosporales				*		*	*	*		*	*
			Pleosporaceae					*	*	*	*	*	*
			Sporormiaceae					*					
			Zopfiaceae			*		*	*				
	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae			*		*					
		Eurotiales				*		*	*			*	
			Aspergillaceae	*		*	*	*	*	*	*	*	*
			Trichocomaceae					*	*	*		*	
		Onygenales									*	*	
			Ajellomycetaceae					*				*	
			Spiromastigoidaceae									*	
	Leotiomycetes							*	*		*		*
		Erysiphales	Erysiphaceae						*				
	Pezizomycetes	Pezizales	Pezizaceae									*	
	Saccharomycetes	Saccharomycetales	Debaryomycetaceae							*			
			Saccharomycetaceae				*				*		
			Trichomonascaceae						*			*	
	Sordariomycetes											*	
		Chaetosphaeriales	Chaetosphaeriaceae		*								
		Coniochaetales	Coniochaetaceae							*			
		Diaporthales										*	

Phylum	Class	Order	Family	W152	T399	HEOP0504M	W029	EOP0334R	HEOP0425	EOP0253R	HEOP0574M	OB23REG1	Control
		Glomerellales	Plectosphaerellaceae				*						
		Hypocreales				*	*	*	*	*	*	*	
			Cordycipitaceae								*		
			Hypocreaceae									*	
			Nectriaceae			*		*	*	*	*	*	
			Niessliaceae				*						
		Lulworthiales	Lulworthiaceae									*	
		Microascales		*				*		*			
			Halosphaeriaceae	*	*								
			Microascaceae									*	
		Phomatosporales	Phomatosporaceae				*						
		Sordariales	Cephalothecaceae									*	
		Xylariales				*		*		*			
Basidiomycota							*	*	*	*			
	Agaricomycetes				*		*		*	*		*	
		Agaricales										*	
		Boletales				*		*		*			
			Boletaceae					*	*	*			
	Malasseziomycetes					*	*	*	*	*			
		Malasseziales	Malasseziaceae					*	*	*			
	Tremellomycetes				*								
		Tremellales	Bulleribasidiaceae						*	*			
			Cryptococcaceae									*	
			Rhynchogastremataceae				*						
Bigyra									*	*			
	-					*		*					
	-	Bicosoecida	Cafeteriaceae			*	*	*			*		
			Siluaniidae						*				
	-	Opalinata	Blastocystidae	*	*		*		*	*	*	*	
-	Centroplasthelida	Pterocystida	Heterophryidae								*		
			Pterocystidae		*	*	*	*	*			*	
Cercozoa				*	*	*	*	*	*	*	*	*	
	-	Glissomonadida	Allapsidae			*		*					
			Viridiraptoridae						*			*	
	Imbricatea	Euglyphida	Euglyphidae									*	
Chlorophyta	Trebouxiophyceae									*			
		Chlorellales	Chlorellaceae							*			
Choanozoa	Choanoflagellatea				*						*		
		Craspedida	Salpingoecidae		*				*	*		*	
	Cristidiscoidea	Nucleariida	Nucleariidae			*	*		*		*	*	
	Filasterea									*			
Chordata	Lepidosauria	Squamata							*				

Phylum	Class	Order	Family	W152	T399	HEOP0504M	W029	EOP0334R	HEOP0425	EOP0253R	HEOP0574M	OB23REG1	Control
			Gekkonidae						*	*			
Chytridiomycota					*		*		*	*			
	Chytridiomycetes	Cladochytriales				*		*					
		Spizellomycetales	Powellomycetaceae					*		*			
			Spizellomycetaceae							*			
	Neocallimastigomycetes	Neocallimastigales	Neocallimastigaceae						*				
Ciliophora				*			*		*	*			
	Armophorea	Metopida	Metopidae	*								*	
	Colpodea				*								
		Bryometopida	Bryometopidae									*	
		Colpodida					*					*	
			Colpodidae			*	*	*		*	*		
		Cyrtolophosidida	Cyrtolophosididae		*		*		*			*	
	Hypotrichea	Euplotida	Euplotidae		*								
			Uronychiidae								*		
		Urostylida	Pseudourostylidae								*		
	Nassophorea	Microthoracida	Microthoracidae							*	*		
	Odontostomatea	Odontostomatida	Epalxellidae	*	*		*	*	*			*	
	Oligohymenophorea			*	*		*		*	*	*	*	
		Peniculida					*						
		Philasterida	Cinetochilidae	*	*		*	*	*		*	*	
			Orchitophryidae						*				
			Philasteridae			*	*	*					
		Pleuronematida			*						*		
			Cyclidiidae	*							*	*	*
	Plagiopylea			*									
	Prostomatea	Prorodontida	Colepidae								*		*
			Placidae				*		*	*		*	
	Spirotrichea				*	*	*		*	*	*		
		Sporadotrichida										*	
Cryptophyta	Cryptophyceae	Cyathomonadacea	Goniomonadaceae		*					*			
		Kathablepharidacea	Katablepharidaceae						*				
Evosea	Eumycetozoa	Dictyosteliales	Dictyosteliaceae									*	
	Variosea				*								
		Varipodida	Flamellidae					*	*	*			
Gastrotricha	-	Chaetonotida	Chaetonotidae								*		
Loukozoa	Jakobea	Jakobida				*							
	Malawimonadea	Malawimonadida	Malawimonadidae								*		
Mucoromycota	Glomeromycetes	Diversisporales	Diversisporaceae									*	
	Mucoromycetes	Mucorales						*				*	
			Cunninghamellaceae					*					
			Mucoraceae					*					

Phylum	Class	Order	Family	W152	T399	HEOP0504M	W029	EOP0334R	HEOP0425	EOP0253R	HEOP0574M	OB23REG1	Control
			Radiomycetaceae					*	*				
Myzozoa	Dinophyceae	Peridiniales	Glenodiniaceae				*						
Nematoda				*								*	
	Chromadorea	Plectida	Plectidae	*							*	*	
		Rhabditida	Thelastomatidae									*	
Nibbleridia	Nibblerideа	Nibbleridida	Nibbleridae		*								
Ochrophyta	Chrysophyceae			*	*		*	*	*	*	*		
		Chromulinales	Chromulinaceae		*	*	*	*	*		*		
			Chrysolepidomonadaceae	*	*				*				
			Dinobryaceae				*		*				
			Paraphysomonadaceae							*			
		Ochromonadales	Ochromonadaceae							*			
		Synurales	Mallomonadaceae						*				
	Eustigmatophyceae									*			
Oomycota												*	
	Hyphochytrea				*	*	*	*					
		Hyphochytriales	Hyphochytriaceae					*					
	Peronosporea	Peronosporales	Pythiaceae		*	*	*	*		*	*	*	*
		Saprolegniales	Saprolegniaceae									*	
Platyhelminthes	Cestoda								*				
	Rhabditophora	Macrostomida	Macrostomidae								*		
		Tricladida	Dugesiidae								*		
	Turbellaria	-	Catenulidae					*		*			
		-	Stenostomidae								*		
Sarcomastigophora	Phytomastigophora	Ebriida				*		*			*		
			Cercomonadidae		*	*		*	*	*	*		
			Heteromitidae					*		*			
Streptophyta													*
	Magnoliopsida			*	*	*		*	*	*		*	
		Asparagales	Amaryllidaceae							*			
		Brassicales	Brassicaceae					*	*	*			
		Caryophyllales	Chenopodiaceae						*				
		Cucurbitales	Coriariaceae	*		*		*		*		*	
			Cucurbitaceae	*		*			*	*		*	
		Fabales	Fabaceae	*	*	*	*	*	*	*	*	*	
		Myrtales		*	*			*					
			Myrtaceae	*	*	*	*	*	*	*	*	*	
		Poales						*	*	*		*	
			Poaceae	*			*	*	*	*		*	
			Restionaceae						*				
			Typhaceae						*	*		*	
			Xyridaceae						*	*			

Phylum	Class	Order	Family	W152	T399	HEOP0504M	W029	EOP0334R	HEOP0425	EOP0253R	HEOP0574M	OB23REG1	Control
	Pinopsida	Pinales	Pinaceae			*							
Tardigrada				*	*	*		*	*	*			
Tubulinea						*	*	*		*		*	
	Echinamoebida					*	*		*	*	*		
	Elardia	Arcellinida	Centropyxidae	*				*					
Zoopagomycota	Basidiobolomycetes	Basidiobolales	Basidiobolaceae	*				*	*	*		*	
	Entomophthoromycetes	Entomophthorales	Ancylistaceae			*		*			*	*	*
	Zoopagomycetes	Zoopagales	Cochlonemataceae		*								

Table 4. Diversity detected from water samples collected in May using a broad 18S assay. Presence of the family at each site is indicated by the symbol *. Taxonomy was assigned as per NCBI and classifications were standardised according to the Global Biodiversity Information Facility (accessed July 2023). Blank cells indicate where taxa could not be resolved to a lower taxonomic level.

Phylum	Class	Order	Family	HEOP0425	EOP0253R	HEOP0574M	HEOP504M	W152	OB23REG1	HEV0008M	HEO0022M	W029	T399	EOP0334R
(Bacteria)					*		*	*		*	*	*		
(Eukaryota)				*	*	*	*	*	*	*	*	*	*	*
Amoebozoa	Discosea	Dactylopodida	Vexilliferidae					*						*
		Longamoebia							*					
		Vannellida	Vannellidae		*		*	*	*		*	*		*
	Lobosa	Amoebida	Paramoebidae		*									
			Vahlkampfiidae			*						*		*
		Arcellinida	Pseudodifflugiidae							*				
Annelida	Clitellata	Tubificida	Naididae			*	*	*	*	*		*	*	*
			Phreodrilidae				*	*	*	*	*			
Apicomplexa							*		*	*	*			
	Conoidasida	Eucoccidiorida	Eimeriidae		*	*	*	*	*		*	*		*
Arthropoda								*	*	*		*		
	Arachnida				*		*		*	*				*
		Araneae			*				*	*	*	*		*
			Araneidae								*			
			Ctenidae						*	*				
			Oonopidae				*		*					
			Thomisidae				*				*	*		
		Mesostigmata	Laelapidae		*									
			Macrochelidae							*				
		Palpigradi	Eukoeneniidae					*						
		Sarcoptiformes				*	*	*	*	*			*	*
			Acaridae		*			*					*	
			Astegistidae			*								
			Grandjeanicidae				*		*	*				*
			Haplozetidae				*							
			Hemileiidae						*					
			Histiostomatidae										*	
			Oppiidae		*		*							*
			Oribatulidae								*			
			Terpnacaridae											*
		Trombidiformes							*	*				
			Erythraeidae								*			
			Eupodidae		*	*	*		*	*	*	*		
			Smarididae								*			
	Chilopoda	Scolopendromorpha	Cryptopidae									*		*
	Collembola				*	*	*	*	*	*	*	*		*
		Entomobryomorpha			*	*	*	*		*		*		*
			Entomobryidae			*		*						

Phylum	Class	Order	Family	HEOP0425	EOP0253R	HEOP0574M	HEOP504M	W152	OB23REG1	HEV0008M	HEO0022M	W029	T399	EOP0334R
			Isotomidae			*	*	*	*	*	*			*
		Symphypleona	Sminthuridae			*	*		*	*	*			
	Copepoda	Cyclopoida	Cyclopidae	*	*	*	*	*	*	*	*	*	*	*
	Diplopoda	Polyxenida							*					
	Insecta													*
		Blattodea	Termitidae			*	*	*	*			*	*	*
		Coleoptera							*		*			*
			Hydraenidae		*									*
			Scarabaeidae											*
		Diptera						*			*			*
			Calliphoridae						*					
			Drosophilidae								*			
			Muscidae				*			*				
			Sarcophagidae									*		
			Sciaridae		*									
			Sphaeroceridae						*					
			Syrphidae						*					
		Ephemeroptera	Baetidae							*				
		Hemiptera					*		*	*	*	*		
			Dictyopharidae						*					
			Membracidae								*			
			Miridae								*			
			Nabidae											*
			Scutelleridae									*		
		Hymenoptera							*	*	*			
			Formicidae		*						*			
		Lepidoptera	Limacodidae		*						*			
		Neuroptera							*					
			Chrysopidae		*						*	*		
		Orthoptera									*			
			Acrididae							*	*			
			Tettigoniidae						*					
		Psocoptera	Psocidae		*						*			
		Thysanoptera								*	*			
			Phlaeothripidae		*									*
			Thripidae				*		*	*	*	*		*
		Zygentoma	Nicoletiidae						*	*	*	*		
	Malacostraca	Isopoda	Tainisopidae							*				
	Ostracoda	Podocopida	Limnocytheridae			*		*		*				
Ascomycota				*	*	*	*	*	*	*	*	*	*	*
	Arthoniomycetes	Lichenostigmatales			*		*	*	*	*	*	*		*
	Dothideomycetes				*	*	*	*	*	*	*	*		*

Phylum	Class	Order	Family	HEOP0425	EOP0253R	HEOP0574M	HEOP504M	W152	OB23REG1	HEV0008M	HEO0022M	W029	T399	EOP0334R
		Botryosphaeriales			*	*	*	*	*	*	*	*		*
			Phyllostictaceae		*				*	*	*	*		*
		Cladosporiales	Cladosporiaceae		*			*	*	*	*	*		*
		Dothideales	Saccotheciaceae		*	*	*	*	*	*	*	*	*	*
		Hysteriales	Hysteriaceae						*			*		
		Lichenotheliales	Lichenotheliaceae		*	*	*	*	*	*	*	*		*
		Myriangiales									*			
			Myriangiaceae			*								
		Pleosporales			*	*	*	*	*	*	*	*		*
			Phaeosphaeriaceae		*							*		
			Pleosporaceae	*	*	*	*	*	*	*	*	*		*
			Zopfiaceae		*		*		*		*			
	Eurotiomycetes									*				
		Chaetothyriales			*				*	*	*			
			Herpotrichiellaceae		*		*	*			*	*	*	*
		Eurotiales		*	*	*	*	*	*	*	*			
			Aspergillaceae	*	*	*	*	*	*	*	*	*	*	
			Trichocomaceae		*	*	*	*	*	*	*		*	
		Onygenales												*
			Ajellomycetaceae			*			*				*	
			Onygenaceae		*			*			*			
			Spiromastigoidaceae		*				*	*		*		
	Lecanoromycetes	Lecanorales	Parmeliaceae								*			
	Leotiomycetes				*	*			*	*	*			
	Lichinomycetes	Lichinales	Lichinaceae						*					
	Pezizomycetes	Pezizales			*						*			
			Ascobolaceae			*								
			Pezizaceae		*	*			*	*	*	*		*
			Sarcosomataceae								*	*		
	Saccharomycetes	Saccharomycetales	Trichomonascaceae		*	*			*	*		*		
	Sordariomycetes				*	*	*		*	*	*			*
		Cephalothecales	Cephalothecaceae						*	*				
		Chaetosphaeriales	Chaetosphaeriaceae				*							
		Coniochaetales	Coniochaetaceae		*	*	*	*	*	*	*		*	
		Coronophorales	Ceratostomataceae								*	*		
		Diaporthales				*			*	*	*	*		*
		Glomerellales	Plectosphaerellaceae				*	*			*			
		Hypocreales			*	*	*	*	*	*	*	*	*	*
			Nectriaceae		*	*	*		*	*		*		
			Stachybotryaceae		*	*			*		*	*		
		Magnaporthales				*				*				
			Pyriculariaceae			*								

Phylum	Class	Order	Family	HEOP0425	EOP0253R	HEOP0574M	HEOP504M	W152	OB23REG1	HEV0008M	HEO0022M	W029	T399	EOP0334R
		Microascales		*	*	*	*	*	*	*	*	*	*	*
			Halosphaeriaceae					*					*	
			Microascaceae						*	*				
		Ophiostomatales	Ophiostomataceae			*				*	*		*	
		Sordariales			*		*	*						
		Xylariales			*	*			*	*	*	*		*
			Coniocessiaceae		*				*	*				
Basidiomycota					*	*	*	*	*	*	*	*	*	*
	Agaricomycetes				*	*		*	*	*	*	*		
		Agaricales			*	*			*		*			*
		Boletales				*	*	*			*			
			Boletaceae		*		*							
		Cantharellales	Ceratobasidiaceae						*					
		Polyporales				*			*					
			Laetiporaceae											*
	Malasseziomycetes				*	*	*	*	*	*	*	*		*
		Malasseziales	Malasseziaceae		*	*		*	*	*	*	*		*
	Tremellomycetes				*		*		*	*	*	*		*
		Tremellales					*					*		*
			Trimorphomycetaceae		*									
	Ustilaginomycetes	Ustilaginales	Ustilaginaceae			*	*	*	*		*			
Bigyra	Bigyra				*					*		*	*	
		Bicosoecida										*		
			Siluaniidae			*		*		*	*			
		-	Cafeteriaceae	*	*	*	*	*	*			*		
		Opalinata	Blastocystidae		*		*	*	*	*		*		
		Placidida	Placidiaceae		*							*		
	Bikosea	Pirsoniales				*	*							
Blastocladiomycota	Physodermatomycetes	Physodermatales	Physodermataceae									*		
-	Centroplasthelida									*				
		Pterocystida	Heterophryidae							*				
			Pterocystidae	*		*		*	*		*	*	*	*
Cercozoa				*	*	*	*	*	*	*	*	*		*
	-	Glissomonadida									*			
			Viridiraptoridae			*								
	Imbricatea	Euglyphida	Euglyphidae								*			
		Thaumatomonadida				*				*	*		*	
Charophyta	Klebsormidiophyceae				*									
Chlorophyta					*			*	*		*			
	Chlorophyceae				*	*	*			*	*	*		*
		Chlamydomonadales			*	*		*	*	*	*			*
			Volvocaceae		*							*		

Phylum	Class	Order	Family	HEOP0425	EOP0253R	HEOP0574M	HEOP504M	W152	OB23REG1	HEV0008M	HEO0022M	W029	T399	EOP0334R
		Chlorosarcinales	Chlorosarcinaceae			*			*					
		Sphaeropleales	Bracteacoccaceae		*	*	*		*	*		*		
		Volvocales	Chlamydomonadaceae		*									*
	Trebouxiophyceae						*		*		*			
		Chlorellales	Chlorellaceae			*					*	*		
	Ulvophyceae	Chlorocystidales	Chlorocystidaceae			*								
		Cladophorales	Pithophoraceae											*
Choanozoa	Choanoflagellatea					*	*	*	*	*	*	*		
		Craspedida	Salpingoecidae	*			*	*	*	*				*
	Cristidiscoidea	Nucleariida	Nucleariidae			*	*	*	*	*	*	*		
	Filasterea				*									
Chordata	Aves	Galliformes	Phasianidae					*						
	-	Squamata		*	*					*				
			Gekkonidae							*				
Chytridiomycota					*	*	*		*	*	*	*	*	*
	Chytridiomycetes				*	*			*	*		*		
		Chytridiales			*									
			Chytridiaceae						*					
		Cladochytriales	Nowakowskiellaceae									*		
		Lobulomycetales							*					
		Rhizophydiales	Angulomycetaceae			*								
		Spizellomycetales	Powellomycetaceae		*	*		*	*					*
			Spizellomycetaceae						*					
	Monoblepharidomycetes	Monoblepharidales				*								
Ciliophora					*			*						*
	Armophorea	Metopida	Metopidae				*	*	*	*				*
	Colpodea				*	*	*		*	*	*			
		Bryometopida	Kreyellidae			*		*	*	*		*		
		Colpodida			*	*	*	*	*	*	*	*		
			Colpodidae		*	*	*	*	*	*	*	*		*
			Hausmanniellidae		*									
			Marynidae			*			*		*			
		Cyrtolophosidida	Cyrtolophosididae		*		*	*	*			*		
			Woodruffiidae		*	*								
		Grossglockneriida	Grossglockneriidae		*	*			*	*	*	*		
	Heterotrichea	Heterotrichida	Spirostomidae						*					
	Hypotrichea	Euplotida	Euplotidae			*								
			Uronychiidae			*				*				
		Urostylida	Pseudourostylidae			*								
			Urostylidae		*									
	Litostomatea					*			*		*	*		
		Haptorida				*	*	*	*	*	*	*		

Phylum	Class	Order	Family	HEOP0425	EOP0253R	HEOP0574M	HEOP504M	W152	OB23REG1	HEV0008M	HEO0022M	W029	T399	EOP0334R
			Acropisthiidae											*
			Dileptidae						*					
			Dimacrocaryonidae			*								
			Enchelyidae		*						*			
			Lacrymariidae										*	
		Pleurostomatida	Litonotidae			*		*	*	*		*		
	Nassophorea	Microthoracida	Microthoracidae			*	*		*	*	*	*		
	Odontostomatea	Odontostomatida	Epalxellidae		*			*			*	*		
	Oligohymenophorea			*	*	*		*	*	*	*	*		
		Peniculida				*						*		
			Frontoniidae									*		
		Philasterida	Cinetochilidae	*		*	*	*	*	*	*	*	*	
			Cohnilembidae				*							
			Orchitophryidae			*					*			
			Philasteridae									*		*
		Pleuronematida			*	*						*		
			Cyclidiidae				*	*						
			Peniculistomatidae									*		
		Thigmotrichida	Ancistridae							*				
	Plagiopylea							*				*		
	Prostomatea	Prorodontida										*		
			Colepidae		*									
			Placidae						*			*		
			Urotrichidae				*					*		
	Spirotrichea			*	*	*	*	*	*	*	*	*		*
		Sporadotrichida			*		*		*			*		
Cryptophyta	Cryptophyceae					*								
		Cryptomonadales	Cryptomonadaceae			*		*			*			
		Cyathomonadacea	Goniomonadaceae		*			*				*		
		Kathablepharidacea	Katablepharidaceae						*			*		
Evosea	Variosea									*	*	*		*
		Varipodida	Flamellidae		*		*		*	*	*			
Gastrotricha	-	Chaetonotida	Chaetonotidae			*								
Heterolobosea								*	*	*				
Loukozoa	Jakobea	Jakobida								*	*	*		
			Stygiellidae				*							
Mucoromycota	Mortierellomycetes	Mortierellales	Mortierellaceae				*		*	*	*			
	Mucoromycetes	Mucorales			*				*			*		*
			Lichtheimiaceae			*			*	*	*			
			Mucoraceae							*				
Myzozoa	Apicomonadea	Colpodellida	Colpodellidae					*						
	Dinophyceae											*		

Phylum	Class	Order	Family	HEOP0425	EOP0253R	HEOP0574M	HEOP504M	W152	OB23REG1	HEV0008M	HEO0022M	W029	T399	EOP0334R
		Gymnodiniales	Gymnodiniaceae			*								
		Peridiniales	Glenodiniaceae									*		
			Heterocapsaceae			*								
Nematoda	Chromadorea	Monhysterida	Xyalidae			*								
		Plectida	Plectidae					*	*				*	*
	Enoplea	Dorylaimida						*						
			Tylencholaimidae						*					
		Enoplida	Rhabdolaimidae				*							
Ochrophyta					*									
	Bacillariophyceae	Bacillariales	Bacillariaceae		*	*	*	*	*		*			
		Fragilariales	Fragilariaceae					*		*	*			
		Naviculales	Diadesmidaceae		*									
	Chrysophyceae				*	*	*	*	*	*	*	*		
		Chromulinales	Chromulinaceae		*	*		*		*	*	*		*
			Chrysoamoebidaceae			*								
			Dinobryaceae	*	*							*		
			Paraphysomonadaceae			*						*		
		Synurales	Mallomonadaceae		*			*						
	Dictyochophyceae	Pedinellales			*									
Oomycota						*	*		*					
	Hyphochytrea					*	*	*	*		*	*		
		Hyphochytriales	Rhizidiomycetaceae									*		
	Peronosporea	Peronosporales	Pythiaceae		*	*	*	*	*	*	*	*	*	
Platyhelminthes						*				*				
	Rhabditophora	Macrostomida	Macrostomidae			*	*	*			*	*	*	*
	Turbellaria	-	Catenulidae											*
		-	Stenostomidae						*		*	*		*
Preaxostyla	-	-	Paratrimastigidae					*			*			
Sarcomastigophora	Phytomastigophora	Ebriida				*	*		*	*		*		
			Cercomonadidae		*		*	*	*			*		
			Heteromitidae		*	*				*	*		*	*
Sulcozoa	Thecomonadea	Apusomonadida	Apusomonadidae					*				*		
Tracheophyta	Liliopsida	Poales			*		*	*	*		*			
			Cyperaceae		*		*							
			Poaceae	*	*	*	*	*	*	*	*	*	*	*
			Restionaceae		*	*	*	*	*	*	*	*		*
	Magnoliopsida				*	*	*	*	*	*	*	*		*
		Apiales					*							
		Brassicales	Brassicaceae				*		*	*	*	*		
		Caryophyllales			*		*				*			
			Cactaceae		*					*				*
			Chenopodiaceae		*		*	*	*	*	*	*	*	*

Phylum	Class	Order	Family	HEOP0425	EOP0253R	HEOP0574M	HEOP504M	W152	OB23REG1	HEV0008M	HEO0022M	W029	T399	EOP0334R
			Frankeniaceae		*									
			Tamaricaceae				*							
		Cucurbitales	Coriariaceae		*	*			*	*	*	*	*	*
			Cucurbitaceae		*		*	*	*	*	*	*		*
		Fabales	Fabaceae	*	*	*	*	*	*	*	*	*	*	*
		Fagales	Fagaceae		*	*		*	*	*	*	*	*	*
		Lamiales			*				*	*	*	*		*
		Laurales	Lauraceae				*	*						
		Malpighiales							*		*			*
		Malvales	Malvaceae		*					*	*			
		Myrtales	Myrtaceae		*	*	*	*	*	*	*			
		Rosales	Rosaceae		*		*	*	*	*	*	*		
		Santalales	Santalaceae									*		
		Sapindales	Sapindaceae		*	*					*			
Tubulinea					*		*							
	Echinamoebida				*	*		*	*	*	*			
	Elardia	Arcellinida	Centropyxidae											*
			Cryptodifflugiidae							*				
		Leptomyxida	Flabellulidae		*	*								
Zoopagomycota	Basidiobolomycetes	Basidiobolales	Basidiobolaceae	*	*		*	*	*	*	*	*		
	Entomophthoromycetes	Entomophthorales	Ancylistaceae		*	*	*	*	*	*	*	*	*	
	-	Ramicandelaberales	Ramicandelaberaceae										*	
	Zoopagomycetes	Zoopagales	Zoopagaceae								*			

Table 5. Diversity detected from water samples collected in November using a COI assay targeting stygofauna. Presence of the species at each site is indicated by the symbol *. Taxonomy was assigned as per NCBI and classifications were standardised according to the Global Biodiversity Information Facility (accessed July 2023). Blank cells indicate where taxa could not be resolved to a lower taxonomic level.

Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**W152**	**T399**	**HEOP0504M**	**W029**	**EOP0334R**	**HEOP0425**	**EOP0253R**	**HEOP0574M**	**OB23REG1**	**Control**
(Bacteria)												*		*	
(Eukaryota)								*			*	*	*		
Amoebozoa	Discosea	Vannellida	Vannellidae	*Paravannella*	*Paravannella minima*				*		*			*	
	Lobosa	Amoebida	Acanthamoebidae	*Acanthamoeba*						*					
					Acanthamoeba byersi								*		
					Acanthamoeba comandoni			*		*	*	*	*	*	
					Acanthamoeba hatchetti								*		
					Acanthamoeba sp.			*	*		*				
			Paramoebidae	*Korotnevella*	*Korotnevella limbata*									*	
			Vahlkampfiidae	*Naegleria*	*Naegleria tenerifensis*									*	
					Naegleria tihangensis					*	*				
Annelida	Clitellata	Tubificida	Phreodrilidae	-	*Phreodrilidae sp. P10*							*			
Arthropoda										*		*			
	Arachnida	-	-	-	*Acari sp. TB-2009*			*							
		Trombidiformes	Pezidae	*Peza*	*Peza ops*						*				
	Diplopoda	Polyxenida						*						*	
	Insecta	Blattodea	Termitidae	*Drepanotermes*						*					
		Coleoptera	Dermestidae	*Anthrenus*	*Anthrenus verbasci*					*					
				Orphinus	*Orphinus sp.*				*						
			Scarabaeidae	*Mimeoma*	*Mimeoma maculata*									*	
		Diptera	Chironomidae					*							
				Larsia	*Larsia sp. WA17.2.3*				*						
				Polypedilum	*Polypedilum nubifer*			*	*						
				Procladius						*					
				Tanytarsus	*Tanytarsus bispinosus*					*					
			Drosophilidae	*Drosophila*	*Drosophila busckii*									*	
			Sarcophagidae	*Sarcophaga*	*Sarcophaga bidentata*					*					
			Sciaridae											*	
		Ephemeroptera	Baetidae	*Cloeon*						*					
		Hemiptera	Aleyrodidae	*Neomaskellia*	*Neomaskellia bergii*					*		*		*	
			Aphididae	*Hysteroneura*	*Hysteroneura setariae*							*	*		
				Myzus	*Myzus persicae*							*			
		Hymenoptera	Formicidae	*Camponotus*										*	
		Lepidoptera									*				
			Cosmopterigidae	*Macrobathra*	*Macrobathra diplochrysa*						*				

Phylum	Class	Order	Family	Genus	Species	W152	T399	HEOP0504M	W029	EOP0334R	HEOP0425	EOP0253R	HEOP0574M	OB23REG1	Control
			Erebidae	*Diatenes*	*Diatenes gerula*						*	*			
				Eudesmeola	*Eudesmeola lawsoni*						*				
				Pandesma	*Pandesma submurina*						*			*	
				Praxis	*Praxis marmarinopa*						*				
			Geometridae	*Prasinocyma*	*Prasinocyma ocyptera*									*	
			Pyralidae	*Faveria*	*Faveria tritalis*					*					
				Meyrickiella	*Meyrickiella homosema*							*			
		Neuroptera	Chrysopidae	*Mallada*						*		*		*	
		Orthoptera	Acrididae	*Austracris*	*Austracris guttulosa*								*		
		Psocoptera	Liposcelididae	*Liposcelis*	*Liposcelis bostrychophila*							*			
					Liposcelis brunnea				*						*
					Liposcelis rufa									*	
	Malacostraca	Amphipoda	Paramelitidae	*Chydaekata*	*Chydaekata acuminata*									*	
		Bathynellacea	Bathynellidae	*Pilbaranella*	*Pilbaranella sp. A GP-2018*					*					
	Pauropoda	Tetramerocerata	Pauropodidae	*Decapauropus*	*Decapauropus sp. WAM PAUD005*							*			
Ascomycota	Dothideomycetes	Cladosporiales	Cladosporiaceae	*Cladosporium*					*	*	*	*			
					Cladosporium sphaerospermum					*	*			*	
		-	-	-	*Dothideomycetes sp. NU30*				*						
		Pleosporales									*				
			Didymellaceae								*	*		*	
				Phoma	*Phoma sp. 1 OB-2014*			*	*	*	*	*		*	
			Pleosporaceae	*Bipolaris*	*Bipolaris sorokiniana*			*			*				
	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Cladophialophora*	*Cladophialophora bantiana*						*				
			Trichomeriaceae	*Arthrocladium*	*Arthrocladium fulminans*								*		
		Eurotiales	Aspergillaceae	*Aspergillus*						*	*	*		*	
					Aspergillus puulaauensis					*	*				
					Aspergillus terreus	*						*	*	*	
				Penicillium					*	*	*	*		*	
	Leotiomycetes									*					
		Erysiphales	Erysiphaceae	*Blumeria*	*Blumeria graminis f. sp. tritici*							*			
	Sordariomycetes	Glomerellales	Glomerellaceae	*Colletotrichum*	*Colletotrichum coccodes*					*					
		Hypocreales	Bionectriaceae	*Gliomastix*	*Gliomastix murorum var. felina*			*							
			Cordycipitaceae	*Cordyceps*	*Cordyceps pruinosa*									*	
				Parengyodontium	*Parengyodontium album*				*	*	*	*			
			Hypocreaceae	*Trichoderma*										*	
			Nectriaceae	*Fusarium*				*			*	*			
			Stachybotryaceae	*Memnoniella*	*Memnoniella echinata*										*
Basidiomycota	Agaricomycetes	Agaricales	Agaricaceae	*Macrolepiota*	*Macrolepiota fuliginosa*									*	

Phylum	Class	Order	Family	Genus	Species	W152	T399	HEOP0504M	W029	EOP0334R	HEOP0425	EOP0253R	HEOP0574M	OB23REG1	Control
	Cystobasidiomycetes	Cystobasidiales	Cystobasidiaceae	*Cystobasidium*	*Cystobasidium sp.*						*			*	
	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Filobasidium*	*Filobasidium floriforme*						*				
Loukozoa	Malawimonadea	Malawimonadida	Malawimonadidae	*Malawimonas*	*Malawimonas jakobiformis*								*		
Mucoromycota	Mucoromycetes	Mucorales						*							
			Lichtheimiaceae	*Lichtheimia*	*Lichtheimia ramosa*								*	*	
Nematoda	Chromadorea	Rhabditida	Rhabditidae	*Diploscapter*	*Diploscapter sp.*									*	
	Enoplea	Dorylaimida	Longidoridae	*Xiphinema*	*Xiphinema brevicolle*			*							
Oomycota	Peronosporea	Peronosporales	Peronosporaceae	*Phytophthora*					*						
			Pythiaceae					*	*	*			*	*	
				Pythium								*			
		Saprolegniales	Saprolegniaceae	*Achlya*	*Achlya bisexualis*									*	
Porifera	Demospongiae	Spongillida													*

Table 6. Diversity detected from water samples collected in May using a COI assay targeting stygofauna. Presence of the species at each site is indicated by the symbol *. Taxonomy was assigned as per NCBI and classifications were standardised according to the Global Biodiversity Information Facility (accessed July 2023). Blank cells indicate where taxa could not be resolved to a lower taxonomic level.

Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**HEOP0425**	**EOP0253R**	**HEOP0574M**	**HEOP504M**	**W152**	**OB23REG1**	**HEV0008M**	**HEO0022M**	**W029**	**T399**	**EOP0334R**
(Bacteria)						*	*	*	*	*			*	*		
Pseudomonadota	Alphaproteobacteria	Rhodobacterales	Paracoccaceae	*Frigidibacter*	*Frigidibacter mobilis*	*										
		Rickettsiales	Rickettsiaceae	*Candidatus Megaira*	*Candidatus Megaira endosymbiont of Mesostigma viride*							*				
	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Enterobacter*		*										
(Eukaryota)						*	*	*	*	*	*	*	*	*		*
Amoebozoa	Discosea	Vannellida	Vannellidae	*Paravannella*	*Paravannella minima*			*		*	*	*	*	*		*
	Lobosa	Amoebida	Acanthamoebidae	*Acanthamoeba*			*	*	*		*	*	*	*		*
					Acanthamoeba byersi			*	*			*	*			
					Acanthamoeba castellanii			*	*		*		*	*		*
					Acanthamoeba comandoni	*		*	*	*	*	*	*	*		*
					Acanthamoeba hatchetti	*	*	*		*	*	*				
					Acanthamoeba jacobsi	*		*	*	*	*	*	*	*		*
					Acanthamoeba sp.	*	*	*	*		*	*	*	*		*
			Paramoebidae	*Korotnevella*	*Korotnevella limbata*						*					
			Vahlkampfiidae	*Naegleria*	*Naegleria lovaniensis*					*						
					Naegleria tenerifensis						*					
					Naegleria tihangensis	*			*	*		*	*	*		*
Annelida	Clitellata	Enchytraeida	Enchytraeidae	-	*Enchytraeidae sp. Biologic-OLIG024*									*		
		Tubificida	Naididae	-	*Naididae sp. N3*				*	*	*	*	*	*	*	*
			Phreodrilidae	-	*Phreodrilidae sp. P10*		*		*	*						
				-	*Phreodrilidae sp. P11*				*	*	*	*	*	*		
				-	*Phreodrilidae sp. P15*											*
	Polychaeta	-	Capitellidae	*Dasybranchus*	*Dasybranchus sp. DH1*									*		
Arthropoda									*	*	*		*	*		*
	Arachnida	-	-	-	*Acari sp. TB-2009*				*						*	*
		Araneae	-	-	*Araneae sp. Biologic-ARAN028*						*	*				
			Araneidae	*Backobourkia*	*Backobourkia collina*						*		*			
			Oxyopidae	*Oxyopes*									*	*		
					Oxyopes hindostanicus						*					
		Trombidiformes	Tetranychidae					*								
				Tetranychus	*Tetranychus lambi*			*								
	Diplopoda	Polydesmida							*							
		Polyxenida									*					
	Insecta					*	*	*	*		*	*	*	*		*

Phylum	Class	Order	Family	Genus	Species	HEOP0425	EOP0253R	HEOP0574M	HEOP504M	W152	OB23REG1	HEV0008M	HEO0022M	W029	T399	EOP0334R
		Blattodea				*	*	*	*			*				
			Rhinotermitidae	*Heterotermes*	*Heterotermes sp. Hpa.2*									*		
			Termitidae											*		
				Amitermes	*Amitermes sp. ANIC 0041*			*								
				Drepanotermes	*Drepanotermes sp. ANIC 0081*			*								
		Coleoptera	Coccinellidae										*			
				Coccinella	*Coccinella transversalis*			*								
			Dermestidae	*Anthrenus*	*Anthrenus verbasci*			*			*					
			Scarabaeidae	*Mimeoma*	*Mimeoma maculata*			*			*					
		Diptera	Calliphoridae	*Calliphora*			*				*			*		
				Lucilia				*								
			Ceratopogonidae	-	*Ceratopogonidae sp. SAEVG Morph0114*			*								
			Chironomidae				*		*		*	*	*			*
				Chironomus	*Chironomus sp. 'februarius'*											*
					Chironomus tepperi		*	*	*				*	*		*
				Larsia	*Larsia sp. WA17.2.3*							*				
				Microchironomus	*Microchironomus sp. B1*						*		*	*		
				Polypedilum	*Polypedilum nubifer*		*				*					*
				Smittia			*									
				Tanytarsus		*	*				*		*	*		*
			Chloropidae								*			*		
				-	*Chloropidae sp.*				*							
				Olcella									*			
			Culicidae	*Culex*									*			
			Drosophilidae	-	*Drosophilidae sp. sc_02914*								*			
			Muscidae	*Musca*				*	*	*		*	*			
			Psychodidae	*Psychoda*	*Psychoda sp. BOLD:AAP4716*		*									
			Sarcophagidae	*Protomiltogramma*	*Protomiltogramma nigriseta*				*					*		
			Sciaridae								*	*	*			
			Tachinidae	*Exorista*	*Exorista sorbillans*				*							
		Hemiptera				*			*	*	*	*	*	*		*
			Alydidae	*Melanacanthus*	*Melanacanthus marginatus*		*									
			Aphididae	*Hyalopterus*	*Hyalopterus pruni*	*			*							
			Cicadellidae	*Exitianus*	*Exitianus nanus*								*			
				Orosius	*Orosius orientalis*						*					
			Delphacidae	*Toya*	*Toya sp. BOLD:AAF8686*						*		*			
			-	-	*Hemiptera sp. BOLD:AAG8997*									*		
			Miridae	*Campylomma*		*										

Phylum	Class	Order	Family	Genus	Species	HEOP0425	EOP0253R	HEOP0574M	HEOP504M	W152	OB23REG1	HEV0008M	HEO0022M	W029	T399	EOP0334R
			Nabidae	*Nabis*					*							
			Pachygronthidae	*Stenophyella*							*		*	*		
			Reduviidae	*Coranus*	*Coranus sp. 00001510*								*			
		Hymenoptera	Formicidae	*Iridomyrmex*	*Iridomyrmex anceps group sp. BOLD:ADF6290*						*	*	*			*
			Scelionidae	*Telenomus*	*Telenomus sp. M432*								*			
		Lepidoptera	Cossidae	*Endoxyla*	*Endoxyla duponchelii*	*	*		*							
			Erebidae	*Dasypodia*	*Dasypodia selenophora*										*	*
				Eudesmeola	*Eudesmeola lawsoni*								*			
				Pandesma	*Pandesma submurina*		*				*	*				
			Lycaenidae	*Nacaduba*	*Nacaduba biocellata*		*		*				*	*		*
				Theclinesthes								*				
			Noctuidae	*Athetis*	*Athetis tenuis*							*				
				Chrysodeixis	*Chrysodeixis argentifera*											*
				Heliocheilus								*				*
					Heliocheilus canusina	*										
			Nymphalidae	*Danaus*		*	*		*				*			
			Oecophoridae	*Mixodetis*	*Mixodetis sp. ANIC4*								*			
				Pachybela	*Pachybela sp. ANIC15*								*			
			Papilionidae	*Papilio*							*					
			Pterophoridae	*Trichoptilus*	*Trichoptilus adelphodes*		*									
			Pyralidae	*Meyrickiella*	*Meyrickiella homosema*	*	*		*	*	*		*	*		*
			Tortricidae	*Strepsicrates*			*									
		Neuroptera	Chrysopidae	-	*Chrysopidae sp. sc_00077*				*				*			
				Mallada					*		*		*	*		
		Odonata	Aeshnidae	*Anax*	*Anax papuensis*				*					*		
		Orthoptera	Acrididae										*			
				Acrida	*Acrida conica*								*			
		Psocoptera	Liposcelididae	*Liposcelis*	*Liposcelis bostrychophila*			*			*		*			*
					Liposcelis brunnea					*						
		Thysanoptera	Phlaeothripidae	*Haplothrips*	*Haplothrips froggatti*		*									*
		Trichoptera											*			
	Malacostraca	Amphipoda	Paramelitidae	*Chydaekata*	*Chydaekata acuminata*			*	*	*	*	*	*			
				-	*Paramelitidae sp. 3 TLF-2008*			*				*	*			
		Bathynellacea	Bathynellidae	*Pilbaranella*	*Pilbaranella ethelensis*									*		
	Pauropoda	-	-	-	*Pauropoda sp. WAM-PAUD002*								*			
		Tetramerocerata	Pauropodidae	*Decapauropus*	*Decapauropus sp. WAM PAUD002*								*			
					Decapauropus sp. WAM PAUD005				*							*
Ascomycota									*							*

Phylum	Class	Order	Family	Genus	Species	HEOP0425	EOP0253R	HEOP0574M	HEOP504M	W152	OB23REG1	HEV0008M	HEO0022M	W029	T399	EOP0334R
	Dothideomycetes	Cladosporiales	Cladosporiaceae	*Cladosporium*			*	*			*	*	*	*		*
					Cladosporium herbarum								*			
					Cladosporium sphaerospermum	*	*					*				
		-	-	-	*Dothideomycetes sp. NU30*			*		*			*	*		*
		Pleosporales				*		*	*		*	*	*			
			Didymellaceae				*						*			
				Phoma	*Phoma sp. 1 OB-2014*	*	*	*	*	*	*	*	*	*		*
			Pleosporaceae				*	*	*			*				
				Bipolaris	*Bipolaris sorokiniana*	*			*		*		*			
	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Cladophialophora*	*Cladophialophora bantiana*		*				*				*	*
			Trichomeriaceae	*Arthrocladium*	*Arthrocladium fulminans*			*								*
		Eurotiales	Aspergillaceae	*Aspergillus*		*	*	*	*	*	*	*	*	*		*
					Aspergillus affinis	*							*			
					Aspergillus puulaauensis	*	*	*	*	*	*	*	*	*		*
					Aspergillus terreus	*	*	*	*	*	*	*	*	*		*
				Penicillium			*	*	*		*	*	*	*		*
					Penicillium canescens						*					
			Thermoascaceae	*Paecilomyces*	*Paecilomyces variotii*									*		
			Trichocomaceae	*Talaromyces*	*Talaromyces marneffei*						*		*		*	*
					Talaromyces pinophilus		*					*				
	Leotiomycetes								*		*		*			
		Erysiphales	Erysiphaceae	*Blumeria*	*Blumeria graminis f. sp. tritici*		*	*	*							
	Sordariomycetes							*			*	*	*	*		*
		Hypocreales	Bionectriaceae	*Gliomastix*	*Gliomastix murorum var. felina*			*	*					*		
			Cordycipitaceae	*Cordyceps*	*Cordyceps pruinosa*				*							
				Parengyodontium	*Parengyodontium album*	*	*		*	*	*	*	*			
			Hypocreaceae	*Trichoderma*			*									
					Trichoderma pseudokoningii									*		
			Nectriaceae	*Fusarium*		*	*	*	*	*		*	*	*		*
					Fusarium solani									*		
		Xylariales	Sporocadaceae	*Pestalotiopsis*	*Pestalotiopsis fici*			*					*			
Basidiomycota	Agaricomycetes	Agaricales	Agaricaceae	*Macrolepiota*	*Macrolepiota fuliginosa*				*	*						*
		Cantharellales	Ceratobasidiaceae	*Rhizoctonia*									*			
	Cystobasidiomycetes	Cystobasidiales	Cystobasidiaceae	*Cystobasidium*	*Cystobasidium sp.*								*			
	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Rhodotorula*	*Rhodotorula mucilaginosa*		*		*							*
	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Filobasidium*	*Filobasidium floriforme*								*			
	Ustilaginomycetes	Ustilaginales	Ustilaginaceae											*		
				Anthracocystis	*Anthracocystis panici-leucophaei*	*	*	*	*		*					

Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**HEOP0425**	**EOP0253R**	**HEOP0574M**	**HEOP504M**	**W152**	**OB23REG1**	**HEV0008M**	**HEO0022M**	**W029**	**T399**	**EOP0334R**
Cryptophyta	Cryptophyceae	Pyrenomonadales	Geminigeraceae	*Guillardia*	*Guillardia theta*			*								
Evosea	Eumycetozoa	Stemonitida	Stemonitidae	*Stemonitis*	*Stemonitis flavogenita*	*		*	*		*		*			
Loukozoa	Malawimonadea	Malawimonadida	Malawimonadidae	*Malawimonas*	*Malawimonas jakobiformis*			*					*			
Mucoromycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Entomortierella*	*Entomortierella parvispora*						*		*			
	Mucoromycetes	Mucorales				*		*	*			*				
			Lichtheimiaceae	*Lichtheimia*	*Lichtheimia ramosa*	*	*	*	*			*	*			
Nematoda	Chromadorea	Rhabditida	Rhabdiasidae	*Rhabdias*	*Rhabdias collaris*						*					
			Rhabditidae	*Diploscapter*	*Diploscapter sp.*						*	*				
	Enoplea	Dorylaimida	Longidoridae	*Xiphinema*	*Xiphinema brevicolle*				*		*					
Ochrophyta	Chrysophyceae	Chromulinales									*					
			Paraphysomonadaceae	*Paraphysomonas*	*Paraphysomonas sp.*							*	*	*		
Oomycota											*		*			
	-	Albuginales	Albuginaceae	*Wilsoniana*	*Wilsoniana amaranthi*	*			*	*			*			
	Peronosporea	Peronosporales	Peronosporaceae	*Peronospora*							*		*			
			Pythiaceae			*	*	*	*	*	*	*	*	*		*
				Globisporangium	*Globisporangium lacustre*								*			
Rotifera	Eurotatoria	Ploima	Brachionidae	*Euchlanis*	*Euchlanis dilatata*											*
Sulcozoa	Diphyllatea	Diphylleida	Collodictyonidae	*Diphylleia*	*Diphylleia rotans*	*	*	*								
Tubulinea	Echinamoebida	-	-	*Vermamoeba*	*Vermamoeba vermiformis*		*				*		*			
Zoopagomycota	Entomophthoromycetes	Entomophthorales	Ancylistaceae	*Conidiobolus*	*Conidiobolus sp.*				*		*					

5.0 SUMMARY

Across the two assays, several stygofauna families/species were detected. While a broader range was detected with the 18S assay, these are not able to be resolved to the species-level due to the nature of the gene region targeted. While not the target of this study, a number of detections were made in non-stygofauna phyla, potentially providing context for the detections made as well as an indication of co-occurring biota.

ARCHIVING OF STUDY DATA

The DNA extracts derived from this study will be stored within eDNA Frontiers' premises for a period of 12 months. If samples are required to be stored longer a sample archiving service can be provided.

All electronic data relating to the study is stored in an offsite secure server. This includes; all laboratory raw data; personnel records; and the study report. Hard copy documents are archived by study number into a locked area of the test facility located in eDNA Frontiers, Curtin University administration area.

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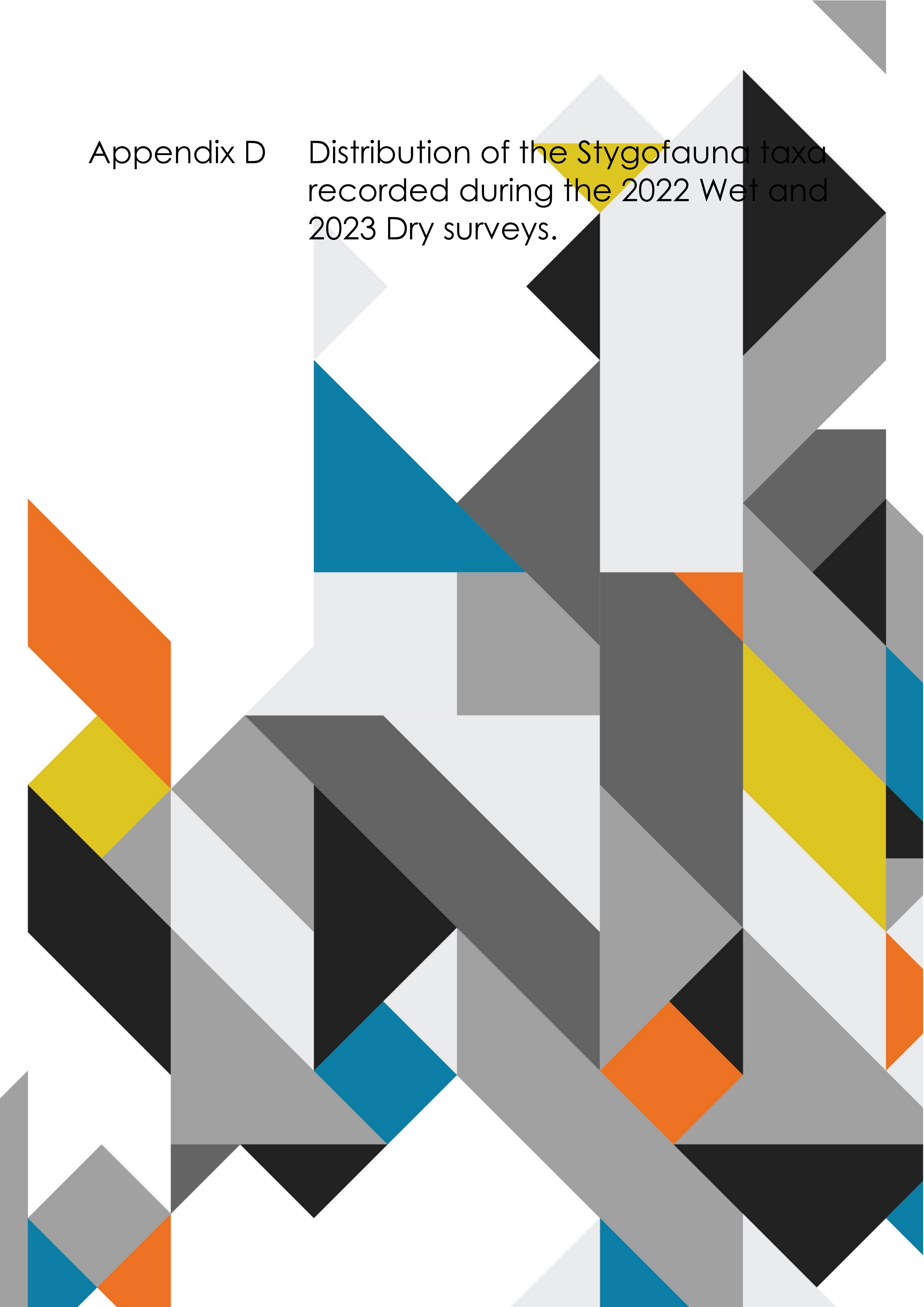
APPENDIX ONE

Table A1. Diversity detected from water samples using a COI assay (Ar5) targeting invertebrates. Presence of the species at each site is indicated by the symbol *. Taxonomy was assigned as per NCBI and classifications were standardised according to the Global Biodiversity Information Facility (accessed April 2023). Blank cells indicate where taxa could not be resolved to a lower taxonomic level; species-level taxonomy is only shown for matches ≥97%.

Phylum	Class	Order	Family	Genus	Species	W152	T399	HEOP0504M	W029	EOP0334R	HEOP0425	EOP0253R	HEOP0574M	OB23REG1
Bacteria														
Pseudomonadota	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas*								*		
					Brevundimonas mediterranea					*				
				Caulobacter				*					*	*
		Hyphomicrobiales	Boseaceae	*Bosea*										*
					Bosea sp. AS-1								*	*
			Brucellaceae											*
				Brucella										*
			Devosiaceae											*
			Hyphomicrobiaceae	*Hyphomicrobium*				*			*			
					Hyphomicrobium sp. DMF-1	*		*		*	*		*	
			Methylobacteriaceae							*		*		
				Methylorubrum									*	
			Methylocystaceae	*Methylosinus*				*						
			Nitrobacteraceae	*Bradyrhizobium*		*		*					*	*
			Phyllobacteriaceae	*Mesorhizobium*	*Mesorhizobium sp. 8*								*	
					Mesorhizobium sp. L-8-10									*
			Rhizobiaceae	*Rhizobium*	*Rhizobium jaguaris*					*	*			
					Rhizobium sp. AB2/73									*
			Xanthobacteraceae	*Labrys*										*
		Rhodospirillales	Azospirillaceae	*Niveispirillum*									*	
			Geminicoccaceae	*Tistrella*	*Tistrella mobilis KA081020-065*					*			*	
			Rhodospirillaceae	*Hypericibacter*	*Hypericibacter adhaerens*			*	*					
			Stellaceae	*Stella*							*			
		Sphingomonadales	Sphingomonadaceae	*Sphingopyxis*				*						
			Sphingosinicellaceae	*Sphingosinicella*	*Sphingosinicella microcystinivorans*								*	
Eukaryota														
Amoebozoa	Lobosa	Amoebida	Acanthamoebidae	*Acanthamoeba*	*Acanthamoeba byersi*								*	
					Acanthamoeba comandoni					*	*		*	
					Acanthamoeba sp.						*			
Annelida	Clitellata	Tubificida	Phreodrilidae	-	*Phreodrilidae sp. P11*						*			
				-	*Phreodrilidae sp. P15*						*			
Arthropoda	Arachnida							*						

Phylum	Class	Order	Family	Genus	Species	W152	T399	HEOP0504M	W029	EOP0334R	HEOP0425	EOP0253R	HEOP0574M	OB23REG1
	Branchiopoda	Diplostraca	Macrotrichidae	*Macrothrix*	*Macrothrix sp. HE-364*				*					
	Insecta	Blattodea	Rhinotermitidae						*					
			Termitidae	*Drepanotermes*	*Drepanotermes sp. ANIC 0081*								*	
		Diptera	Chironomidae	*Tanytarsus*	*Tanytarsus bispinosus*						*		*	
			Sciaridae											*
			Sphaeroceridae	*Pullimosina*	*Pullimosina heteroneura*					*				
	Protura	Protura	Fujientomidae	*Fujientomon*	*Fujientomon dicestum*					*				
Loukozoa	Malawimonadea	Malawimonadida	Malawimonadidae	*Malawimonas*	*Malawimonas jakobiformis*								*	
Oomycota	Peronosporea	Peronosporales	Pythiaceae	*Pythium*				*		*			*	*
Tracheophyta	Liliopsida	Poales	Poaceae								*			*
			Typhaceae								*			
	Magnoliopsida					*	*						*	
		Brassicales	Brassicaceae									*		
		Fabales	Fabaceae						*	*	*	*	*	*

Appendix D Distribution of the Stygofauna taxa recorded during the 2022 Wet and 2023 Dry surveys.



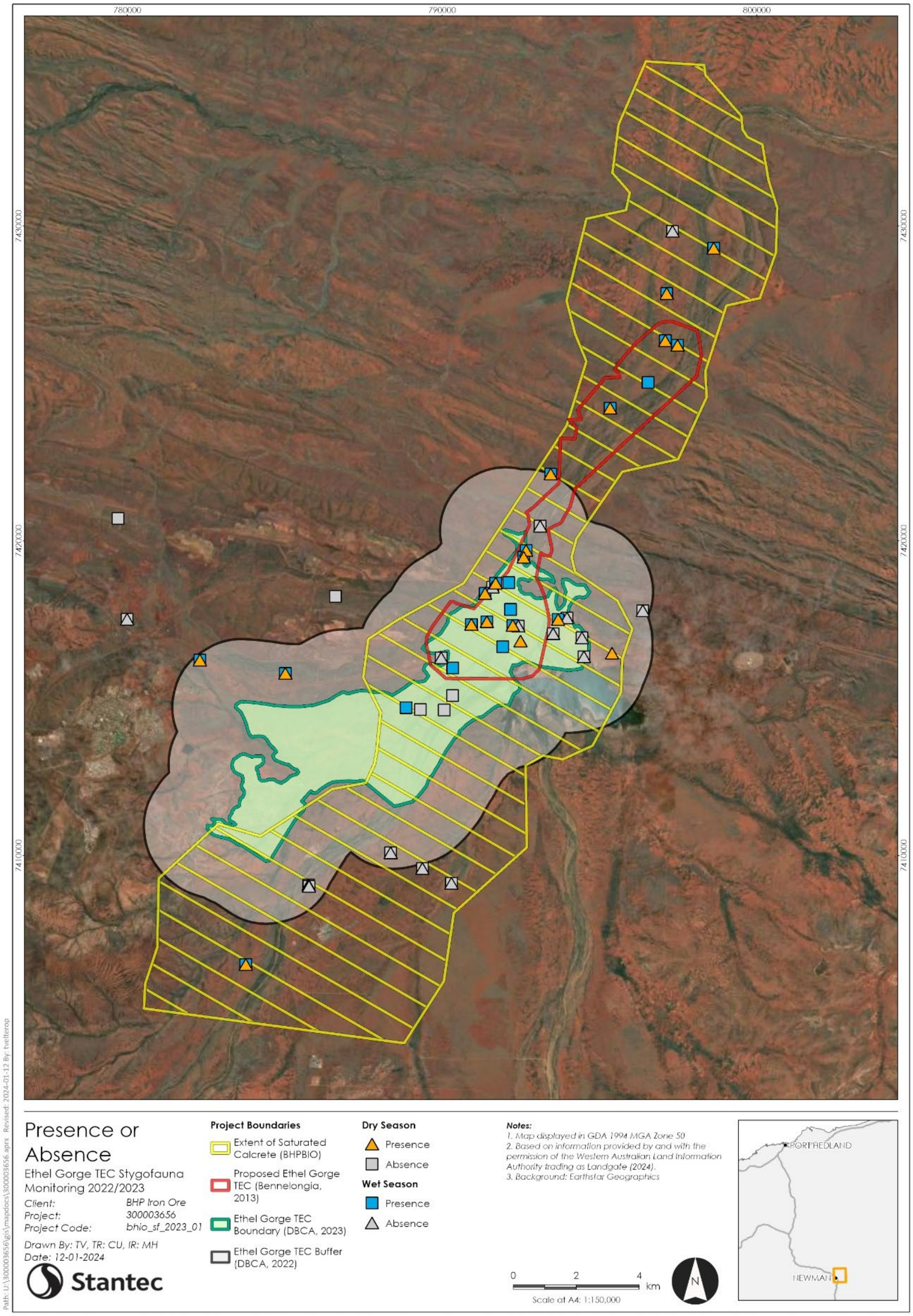
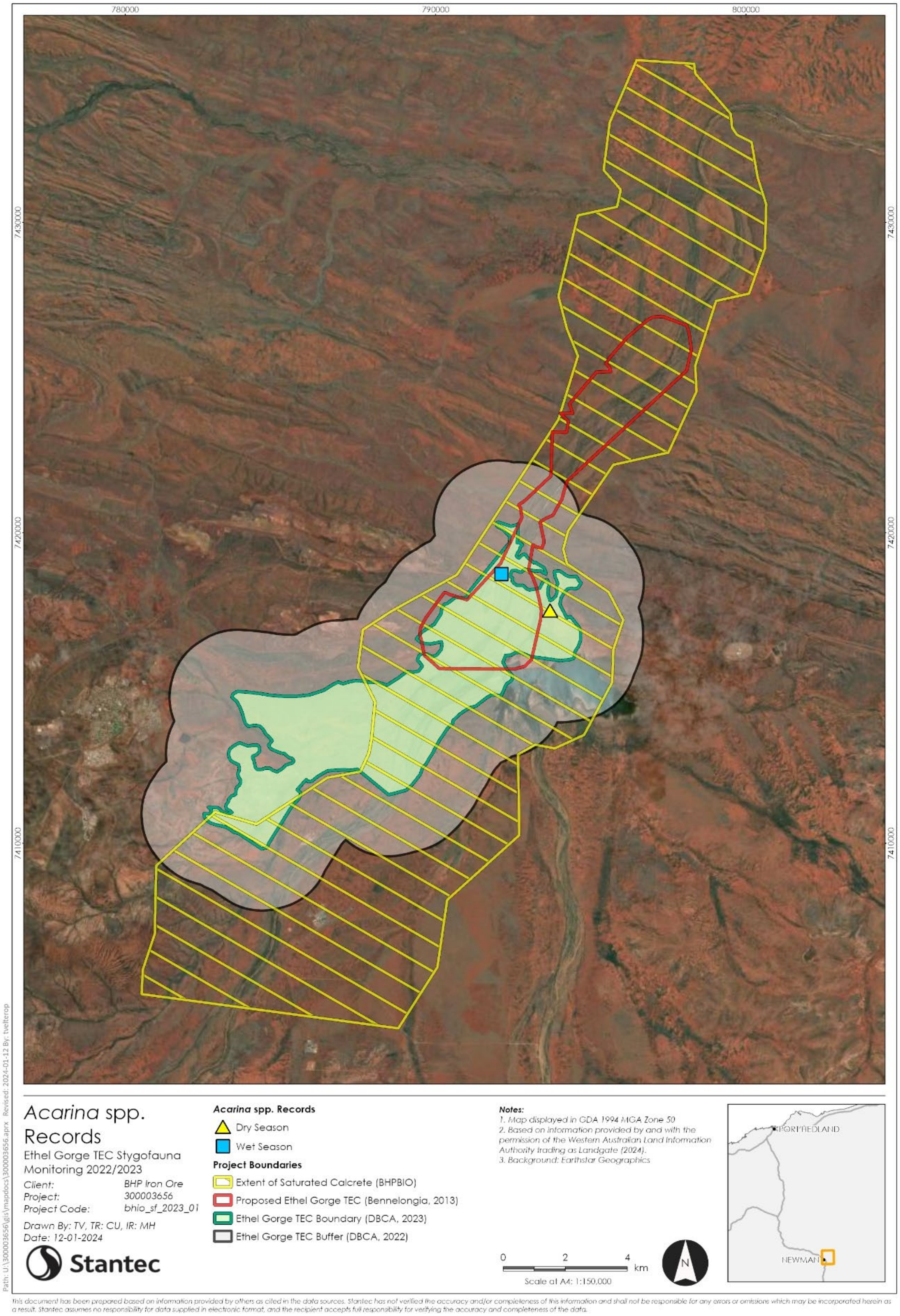


Figure D-1 Presence/Absence of Stygofauna within the Project area in 2022 Wet and 2023 Dry.



Notes:
1. Map displayed in GDA 1994 MGA Zone 50
2. Based on information provided by and with the permission of the Western Australian Land Information Authority trading as Landgate (2024).
3. Background: Earthstar Geographics

This document has been prepared based on information provided by others as cited in the data sources. Stantec has not verified the accuracy and/or completeness of this information and shall not be responsible for any errors or omissions which may be incorporated herein as a result. Stantec assumes no responsibility for data supplied in electronic format, and the recipient accepts full responsibility for verifying the accuracy and completeness of the data.

Figure D-2 Presence of Acarina species within the Project area in 2022 Wet and 2023 Dry.

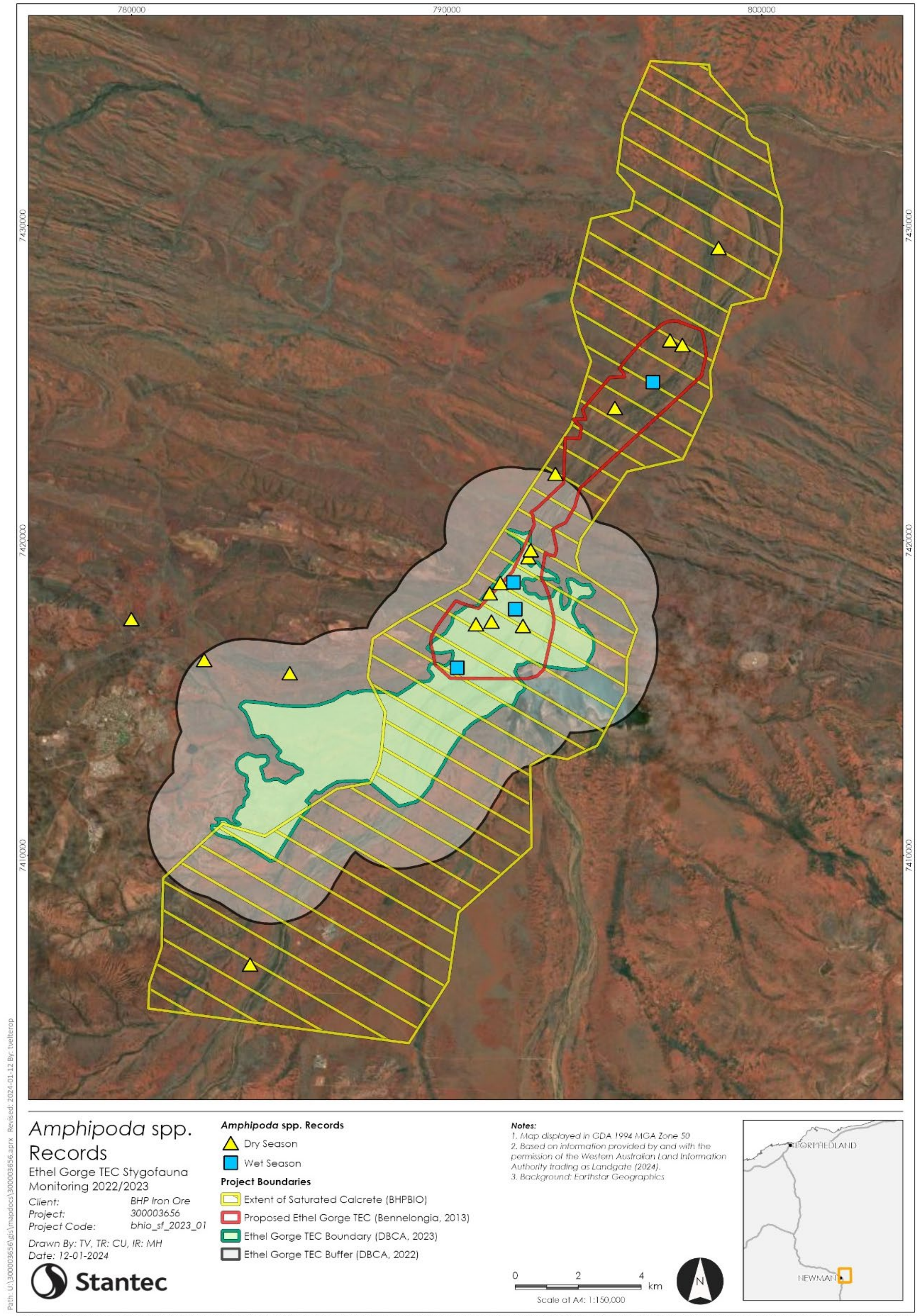


Figure D-3 Presence of Amphipoda species within the Project area in 2022 Wet and 2023 Dry.

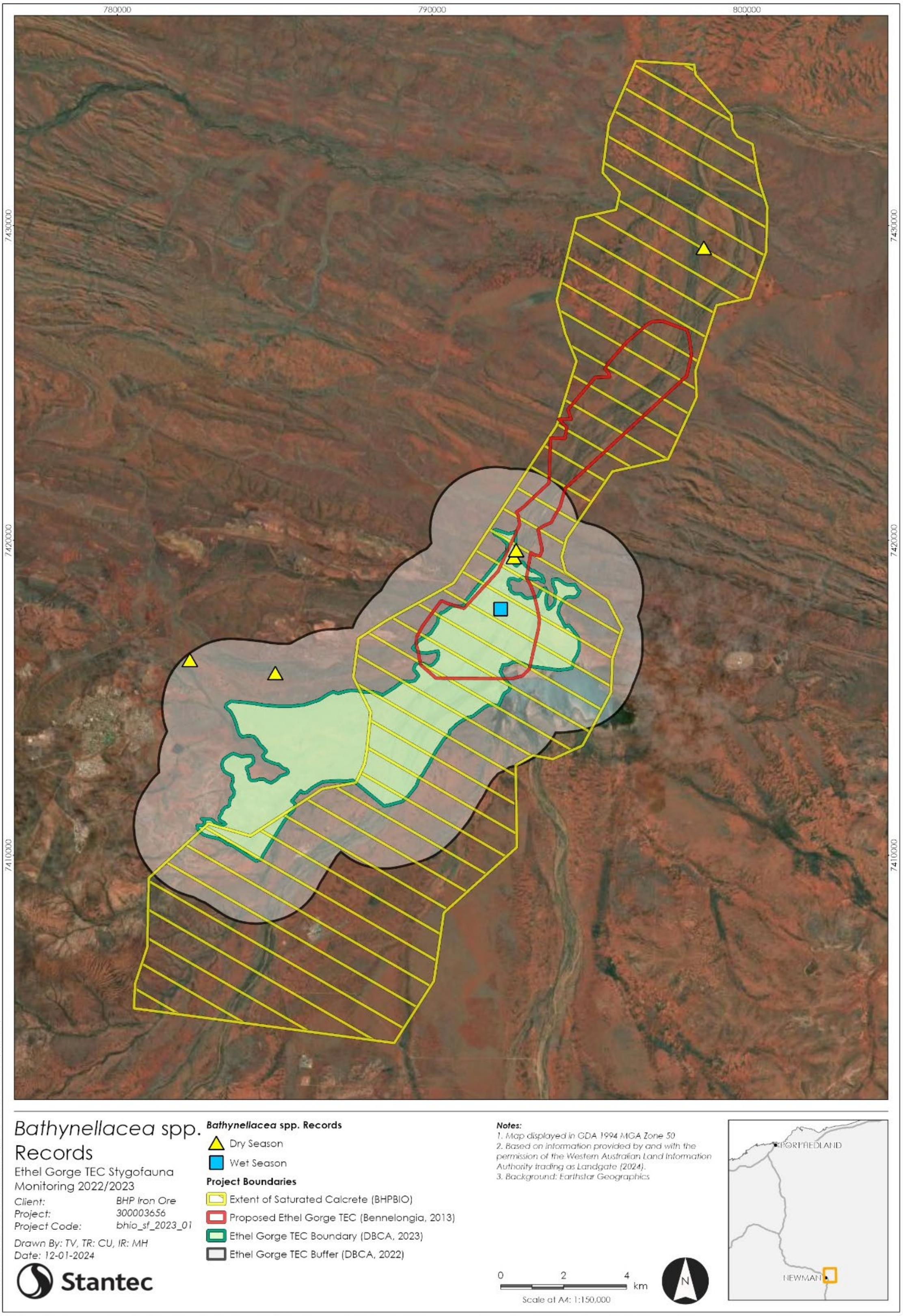


Figure D-4 Presence of Bathynellacea species within the Project area in 2022 Wet and 2023 Dry.

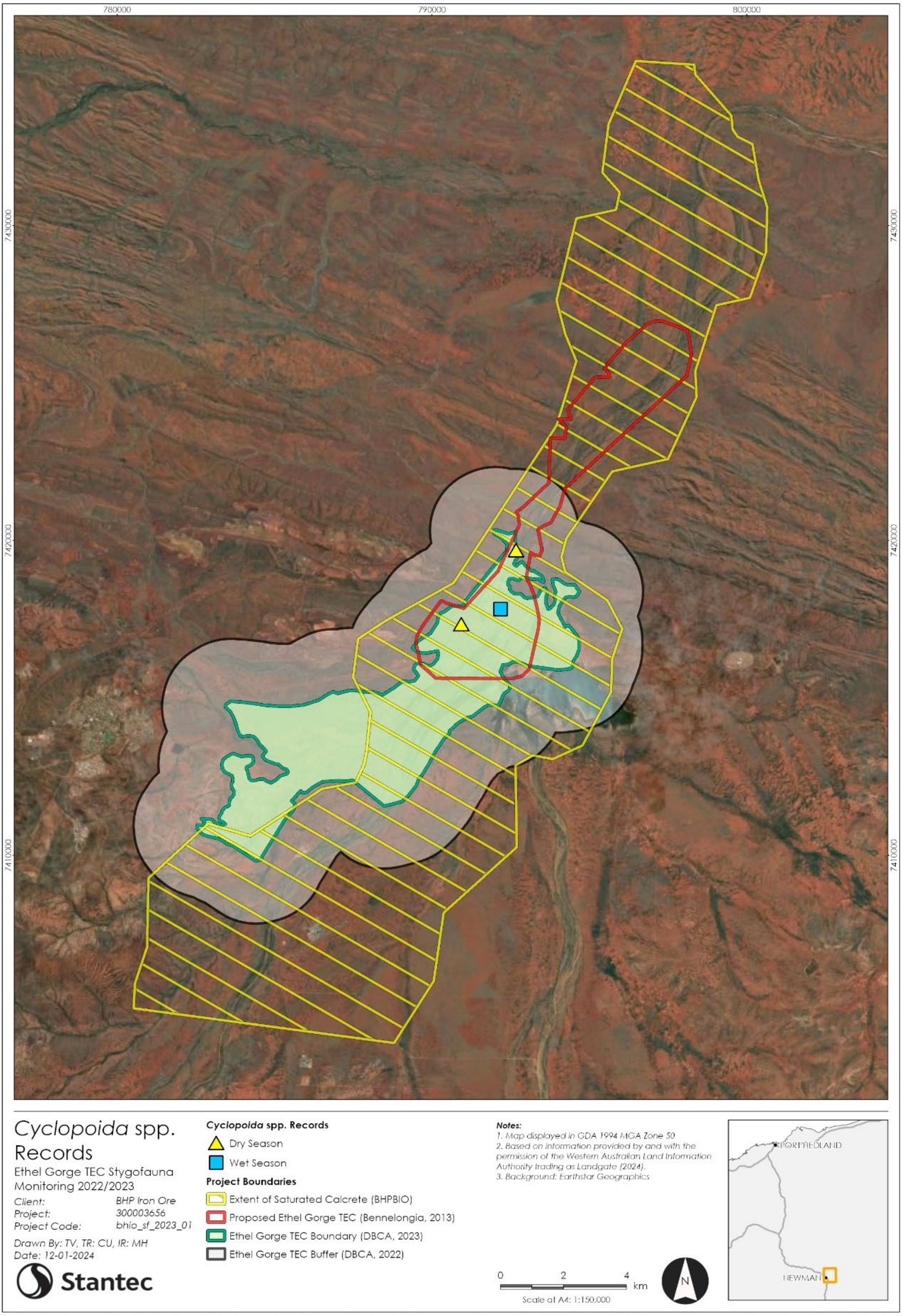


Figure D-5 Presence of Cyclopoid copepod species (other than Diacyclops spp.) within the Project area in 2022 Wet and 2023 Dry.

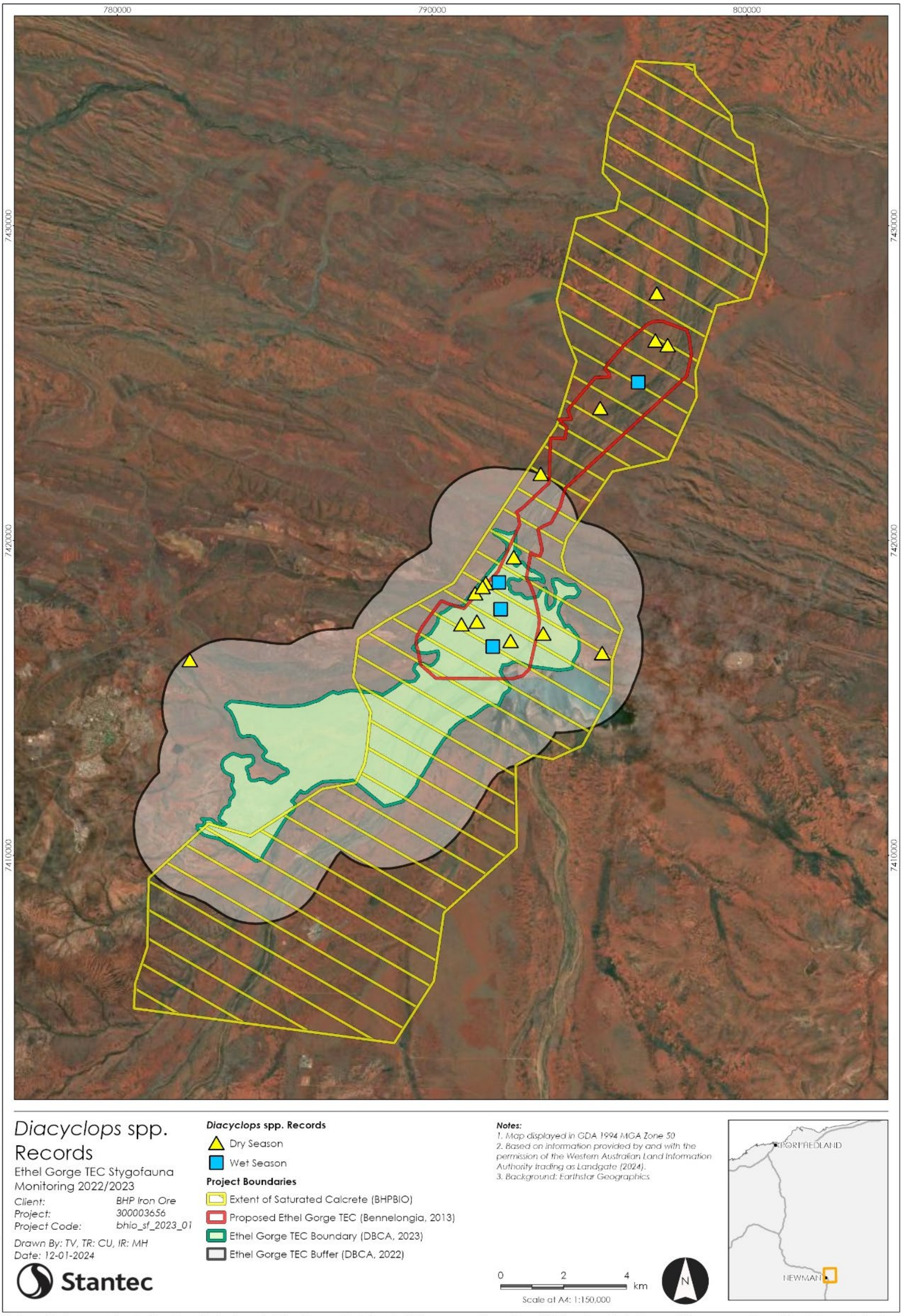


Figure D-6 Presence of Diacyclops spp. (Copepoda) species within the Project area in 2022 Wet and 2023 Dry.

Figure D-7 Presence of *Archinitocrella newmanensis* (Copepoda: Harpacticoida)within the Project area in 2022 Wet and 2023 Dry.

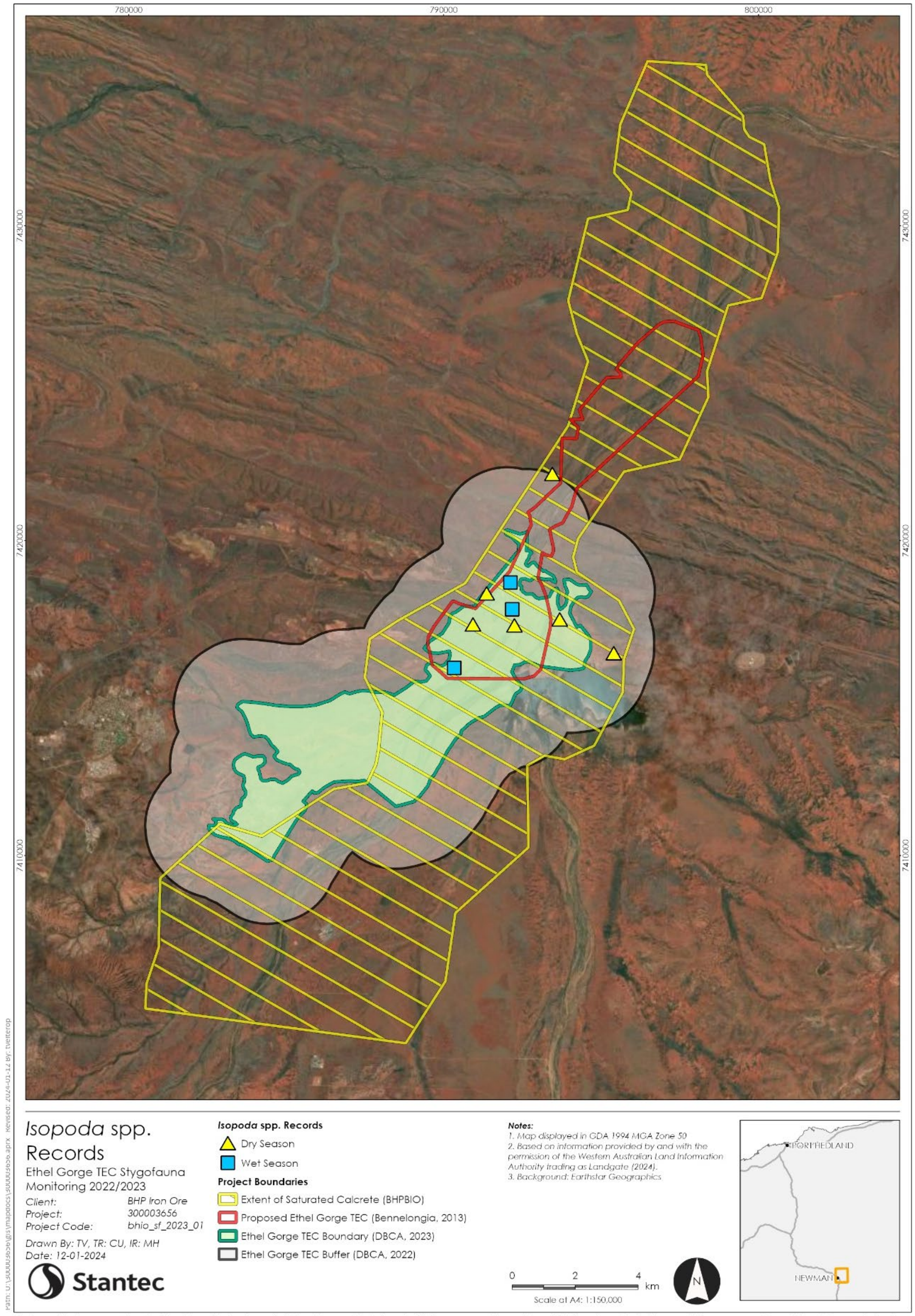


Figure D-8 Presence of Isopoda species within the Project area in 2022 Wet and 2023 Dry.

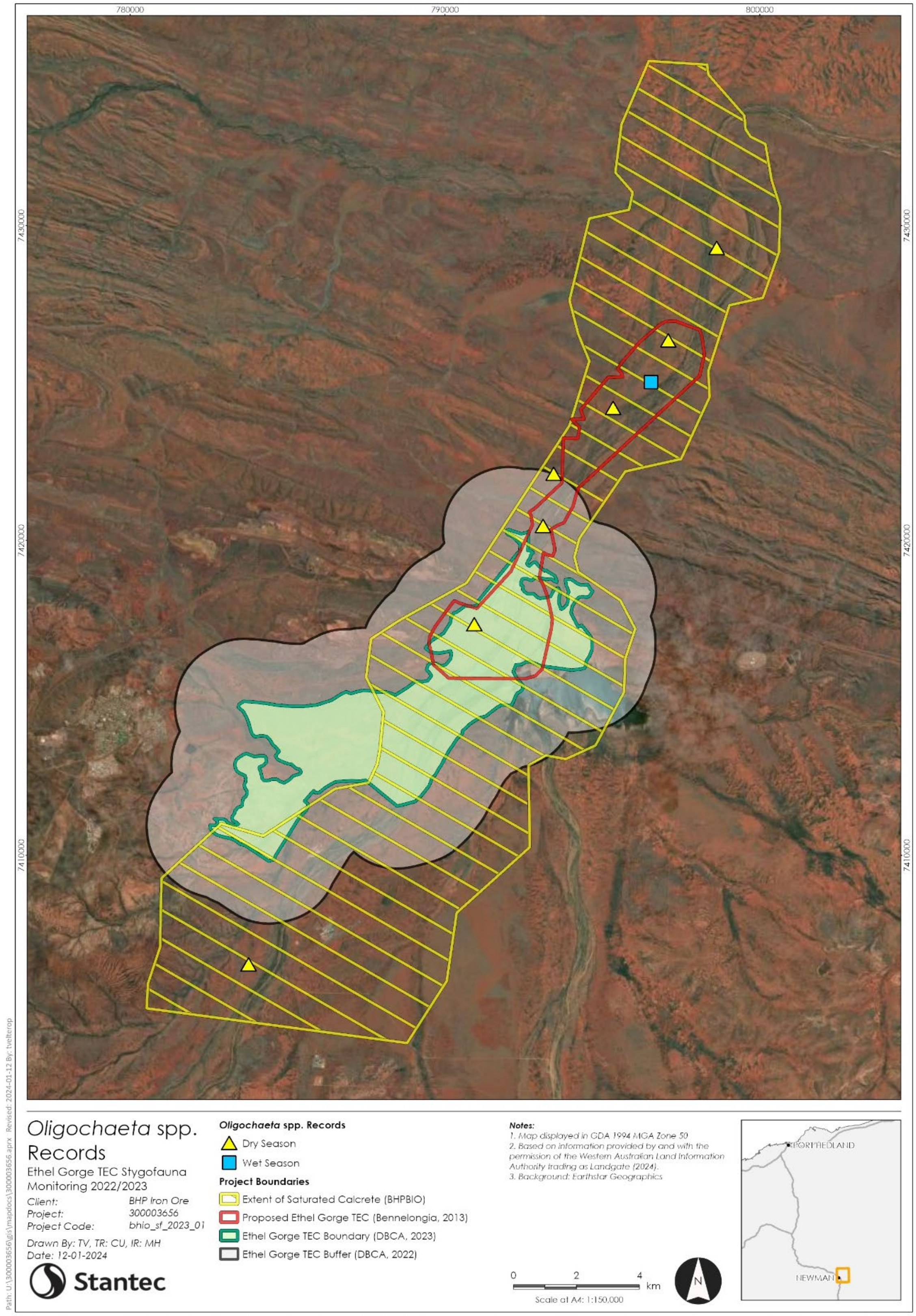


Figure D-9 Presence of Oligochaete species within the Project area in 2022 Wet and 2023 Dry.

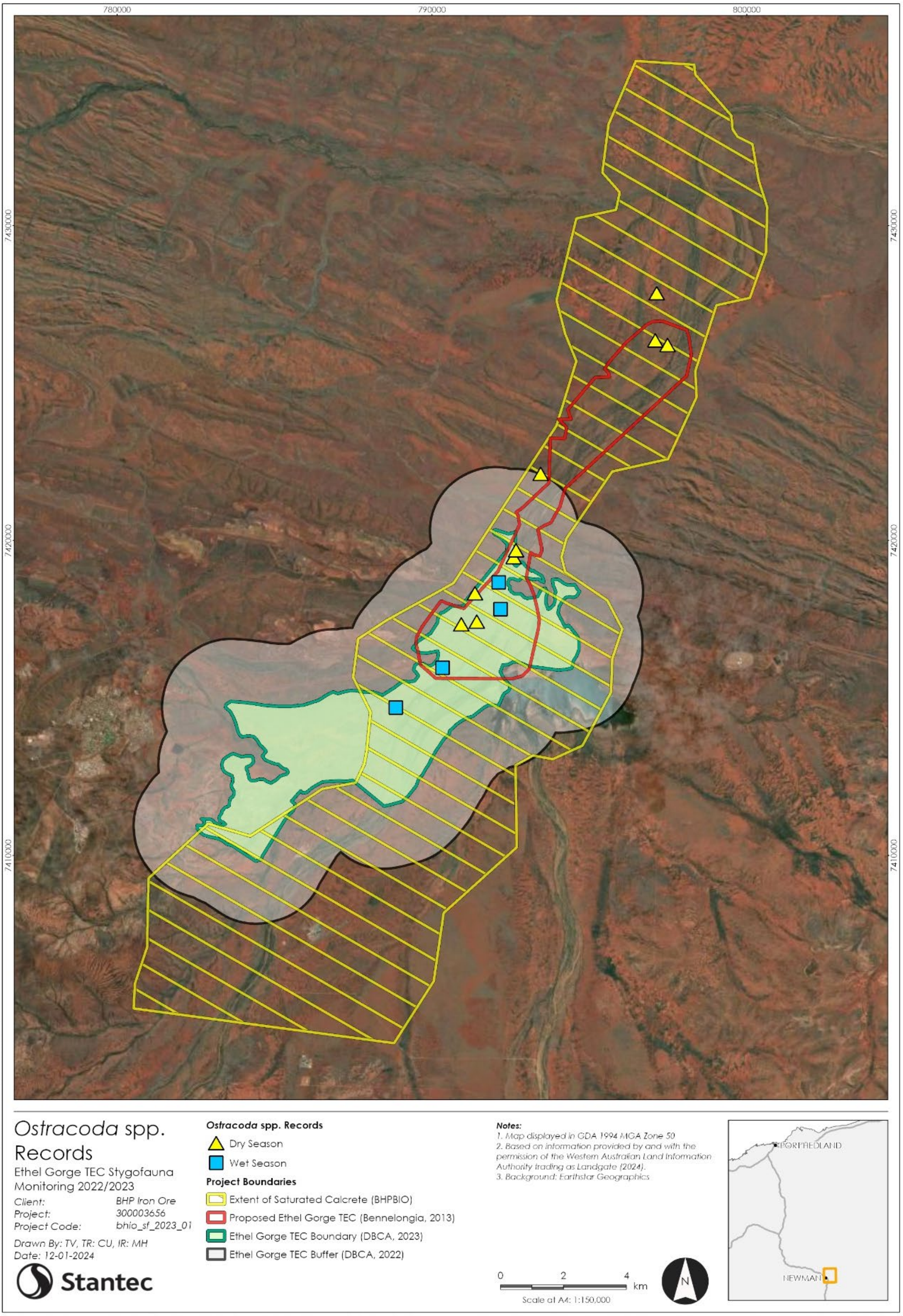


Figure D-10 Presence of Ostracoda species within the Project area in 2022 Wet and 2023 Dry.

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