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13 November 2024

Attn: Jared Leigh
Principal Biodiversity Assessment
BHP WAIO Environment
125 St Georges Tce Perth WA 6000 Australia

Re: Jimblebar Project Stygofauna DNA investigation

Dear Jared,

BHP Western Australian Iron Ore (BHP WAIO) has submitted the Jimblebar Hub Iron Ore Mining Operations Significant Amendment (the Project) to regulators for environmental approvals in December 2023, and requested Biologic undertake some supplementary investigations regarding three stygofauna species of interest to the impact assessment:

1. Phreodrilidae sp. `BOL084`
2. *Atopobathynella* sp. `BSY241` and
3. *Schizopera* sp. 'BHA285'.

These species have not been recorded outside of the groundwater drawdown impact area for the Project to date, based on available sampling and comparisons of DNA sequences undertaken by Bennelongia (2023).

BHP WAIO requested that Biologic undertake a review of available stygofauna and hyporheic fauna specimens collected by Biologic from nearby areas, and DNA sequence any potential targets (i.e. unidentified specimens in the same taxonomic groups as the target species). Comparative DNA sequence data was available for Phreodrilidae sp. `BOL084` (GenBank accession numbers; COI: PP461580, 12S: PP490769) and *Atopobathynella* sp. `BSY241` (GenBank accession numbers; COI: PP461579, 12S: PP490768), both sequenced by Bennelongia.

Blasts of the target sequences against Biologic's BHP sequence collections (aquatic and subterranean) and public DNA sequence libraries revealed no conspecific matches. The closest matches are listed below.

Atopobathynella `sp. BSY241`

- 12S
    - Parabathynellidae `sp. (Pilbara) 21 EM-2019` was the closest result, which was ~20% divergent. This OTU was sampled ~300km north of BSY241.
    - Based on this very high genetic distance, it is a distinct operational taxonomic unit (OTU).
- COI
    - *Atopobathynella* `sp. Biologic-PBAT041` was the closest result, which was 12.8% divergent. This OTU was sampled from Coondiner Ck (~40km from BSY241) during the Western Creeks Aquatic sampling undertaken by our Aquatic team.
    - Pairwise distances this genus for COI above 8% are typically considered distinct OTUs.

Phreodrilidae `sp. BOL084`

- 12S and COI
    - Phreodrilidae `sp. P10 LB-2015` was the closest result, which was 11.5% and 16.2% divergent for 12S and COI, respectively. This OTU was collected 33 km west of BOL084.
    - These genetic distances are much higher than typically observed within OTUs/species of Phreodrillidae, and are therefore considered distinct OTUs.

A review of stygofauna and hyporheic fauna specimens collected by Biologic from nearby BHP sampling areas failed to detect any specimens of *Schizopera* that were available for morphological comparisons. Some Biologic *Schizopera* have been vouchered at the WA Museum, and therefore may be available by arrangement with WAM, but these collections are largely from areas where *Schizopera roberiverensis* occurs (and identified specimens have been resolved as *S. roberiverensis)*, therefore the chances of this material representing the same species as found at Jimblebar are low.

All *Atopobathynella* specimens collected by Biologic are routinely sequenced for identifications, therefore the sequence collection for this genus was considered spatially complete, and no further sequencing was warranted.

This left specimens of Phreodrilidae – of which there was ample material collected via hyporheic sampling - to process through Biologic's DNA sequence laboratory and compare to Phreodrilidae `sp. BOL084`. Only COI was sequenced as this gene is sufficient to detect conspecific matches.

In total, 92 phreodrillid specimens were chosen for DNA sequencing. These were sourced from BHP aquatic monitoring projects, from Western Creek, Minister's North, Weeli Wolli and Skull Springs (See Appendix A). DNA was extracted, amplified and sequenced using standard protocols, targeting the COI region of the mtDNA genome.

Several rounds of amplifications and troubleshooting was required due to the preservation history of these specimens. Aquatic bulk samples are stored at room temperature after sorting, which causes greater DNA degradation over time compared to cold storage. Despite this, after troubleshooting, 82 of the 92 specimens (89%) returned a high quality DNA sequence on target.

These 82 sequences were resolved into 16 OTUs (Table 1). Three OTUs were from the genus *Pristina*, from a different family, Naididae. The remaining 13 OTUs included four previously sequenced OTUs, and 9 new OTUs. No OTUs were conspecific with Phreodrilidae `sp. BOL084`, which was 18.2% divergent from the closest sequence, BMR09838 (Phreodrilidae `sp. Biologic-OLIG168`, sampled from Skull Springs).

Table 1: OTUs derived from 82 DNA sequences

OTU	Number of sequences	New OTU
Antarctodrilus `sp. Biologic-OLIG066`	6	
Phreodrilidae `sp. Biologic-OLIG058`	37	
Phreodrilidae `sp. Biologic-OLIG112`	1	
Phreodrilidae `sp. Biologic-OLIG168`	1	yes
Phreodrilidae `sp. Biologic-OLIG169`	1	yes
Phreodrilidae `sp. Biologic-OLIG170`	3	yes
Phreodrilidae `sp. Biologic-OLIG171`	2	yes
Phreodrilidae `sp. Biologic-OLIG173`	1	yes
Phreodrilidae `sp. Biologic-OLIG174`	1	yes
Phreodrilidae `sp. Biologic-OLIG175`	2	yes
Phreodrilidae `sp. Biologic-OLIG176`	4	yes
Phreodrilidae `sp. Biologic-OLIG177`	1	yes
Phreodrilidae `sp. P19 LB-2015`	19	
Pristina `sp. Biologic-OLIG172`	1	yes
Pristina `sp. WAM-NAIDP001`	1	
Pristina longiseta	1	

Although the target taxa were not recorded on this occasion, the high success rate of the molecular analysis from specimens stored in less-than-ideal conditions was a noteworthy result. These kinds of results, if replicable across different taxonomic groups, suggest a considerable hidden potential in the unidentified, bulk-stored material from hyporheic samples, to expand the available sequence libraries of stygofauna taxa. This may have implications for current and future genetic work, particularly eDNA analyses, the success of which hinges on the breadth of DNA sequence libraries available for comparison.

Thank you again for providing Biologic with this opportunity, and if would like to discuss any details further, please do not hesitate to contact me.

Yours sincerely,

DR. JOEL HUEY

Manager Molecular Systematics | Principal Geneticist

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0423626382

Bennelongia (2023) East Jimblebar Baseline Subterranean Fauna Survey. Draft Report to BHP WA Iron Ore. May 2023.

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Appendix A: Specimen Details

BMR Code	Unique ID	Site	Dec_Lat	Dec_Long	Coll_Date	Collection Method	OTU Name	Reaction State	GenBank Code
BMR09852	30870	WWDD5	-22.8963283	119.2211283	14/05/2022	Hypo	Antarctodrilus `sp. Biologic-OLIG066`	PASS	PQ438875
BMR09854	30871	WWDD5	-22.8963283	119.2211283	14/05/2022	Hypo	Antarctodrilus `sp. Biologic-OLIG066`	PASS	PQ438877
BMR09856	30873	WWDD5	-22.8963283	119.2211283	14/05/2022	Hypo	Antarctodrilus `sp. Biologic-OLIG066`	PASS	PQ438879
BMR09861	30875	WWDD5	-22.8963283	119.2211283	5/11/2022	Hypo	Antarctodrilus `sp. Biologic-OLIG066`	PASS	PQ438883
BMR09862	30876	WWDD5	-22.8963283	119.2211283	5/11/2022	Hypo	Antarctodrilus `sp. Biologic-OLIG066`	PASS	PQ438884
BMR09863	30877	WWDD5	-22.8963283	119.2211283	5/11/2022	Hypo	Antarctodrilus `sp. Biologic-OLIG066`	PASS	PQ438885
BMR12351	30810	MUNJE	-22.5444854	118.7223615	21/03/2021	Hypo	Phreodrilidae `sp. Biologic-OLIG171`	PASS	PQ438892
BMR12352	30811	MUNJE	-22.5444854	118.7223615	21/03/2021	Hypo	Phreodrilidae `sp. Biologic-OLIG171`	PASS	PQ438893
BMR09845	30721	WWUD3/ WWS	-22.9179113	119.1997414	4/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG175`	PASS	PQ438868
BMR09851	30869	WWDD5	-22.8963283	119.2211283	14/05/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG176`	PASS	PQ438874
BMR09853	27807	WWDD5	-22.8963283	119.2211283	14/05/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG176`	PASS	PQ438876
BMR09855	30872	WWDD5	-22.8963283	119.2211283	14/05/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG176`	PASS	PQ438878
BMR09857	30874	WWDD5	-22.8963283	119.2211283	14/05/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG176`	PASS	PQ438880
BMR09850	28134	WWDD4	-22.9043310	119.2158175	14/05/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438873
BMR09957	30862	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438886
BMR12353	30812	WC5	-23.5189013	119.6098178	9/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438894
BMR12354	30813	WC5	-23.5189013	119.6098178	9/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438895
BMR12355	30814	WC5	-23.5189013	119.6098178	9/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438896
BMR12356	30815	WC1	-23.4816983	119.5034461	10/03/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438897
BMR12357	30816	WC1	-23.4816983	119.5034461	10/03/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438898
BMR12358	30817	WC1	-23.4816983	119.5034461	10/03/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438899
BMR12359	30818	WC1	-23.4816983	119.5034461	10/03/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438900
BMR12360	30819	WC1	-23.4816983	119.5034461	10/03/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438901
BMR12361	30820	Fort Riv 1	-23.4451113	119.7742604	11/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438902
BMR12362	30821	Fort Riv 1	-23.4451113	119.7742604	11/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438903
BMR12363	30822	Fort Riv 1	-23.4451113	119.7742604	11/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438904

BMR12364	30823	Fort Riv 1	-23.4451113	119.7742604	11/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438905
BMR12375	30834	WC6	-23.5260023	119.6649611	9/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438916
BMR12376	30835	WC6	-23.5260023	119.6649611	9/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438917
BMR12377	30836	WC6	-23.5260023	119.6649611	9/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438918
BMR12378	30837	WC6	-23.5260023	119.6649611	9/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438919
BMR12379	30838	WC6	-23.5260023	119.6649611	9/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438920
BMR12380	30839	WC6	-23.5260023	119.6649611	9/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438921
BMR12381	30840	WC6	-23.5260023	119.6649611	9/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438922
BMR12382	30841	WC6	-23.5260023	119.6649611	9/05/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438923
BMR12383	30842	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438924
BMR12384	30843	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438925
BMR12385	30844	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438926
BMR12386	30845	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438927
BMR12388	30847	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438929
BMR12390	30849	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438930
BMR12391	30850	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438931
BMR12534	30853	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438934
BMR12535	30854	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438935
BMR12536	30855	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438936
BMR12538	30857	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438937
BMR12539	30858	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438938
BMR12540	30859	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438939
BMR12541	30860	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438940
BMR12542	30861	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG058`	PASS	PQ438941
BMR09860	27714	WWDD4	-22.9043310	119.2158175	5/11/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG112`	PASS	PQ438882
BMR09838	26754	SS	-21.8626821	121.0085309	21/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG168`	PASS	PQ438862
BMR09842	30867	YC3	-22.8245311	119.1636156	8/09/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG169`	PASS	PQ438866
BMR09846	30741	WWUD4	-22.9171760	119.2012274	12/05/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG170`	PASS	PQ438869
BMR09849	27899	WWDD3	-22.9106596	119.2143517	14/05/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG170`	PASS	PQ438872
BMR09858	27810	WWDD5	-22.8963283	119.2211283	14/05/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG170`	PASS	PQ438881
BMR12368	30827	MUNJE	-22.5444854	118.7223615	14/03/2022	Hypo	Pristina `sp. Biologic-OLIG172`	PASS	PQ438909
BMR09841	27738	SS	-21.8626821	121.0085309	11/09/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG173`	PASS	PQ438865
BMR09847	27770	FF	-22.4777007	118.5512263	15/05/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG174`	PASS	PQ438870

BMR09848	27786	WWDD3	-22.9106596	119.2143517	14/05/2022	Hypo	Phreodrilidae `sp. Biologic-OLIG175`	PASS	PQ438871
BMR12387	30846	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Phreodrilidae `sp. Biologic-OLIG177`	PASS	PQ438928
BMR09836	30863	YC4eH	-22.8258155	119.1628052	18/03/2023	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438860
BMR09837	30864	YC4eH	-22.8258155	119.1628052	18/03/2023	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438861
BMR09839	30865	YC4	-22.8256345	119.1629889	18/03/2023	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438863
BMR09840	30866	YC4	-22.8256345	119.1629889	18/03/2023	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438864
BMR09844	30733	YC4	-22.8256345	119.1629889	8/09/2022	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438867
BMR12345	30804	MUNJE	-22.5444854	118.7223615	4/04/2021	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438887
BMR12346	30805	MUNJE	-22.5444854	118.7223615	4/04/2021	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438888
BMR12347	30806	MUNJE	-22.5444854	118.7223615	4/04/2021	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438889
BMR12348	30807	MUNJE	-22.5444854	118.7223615	4/04/2021	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438890
BMR12350	30809	MUNJE	-22.5444854	118.7223615	21/03/2021	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438891
BMR12365	30824	MUNJE	-22.5444854	118.7223615	14/03/2022	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438906
BMR12366	30825	MUNJE	-22.5444854	118.7223615	14/03/2022	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438907
BMR12367	30826	MUNJE	-22.5444854	118.7223615	14/03/2022	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438908
BMR12369	30828	MUNJE	-22.5444854	118.7223615	14/03/2022	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438910
BMR12370	30829	MUNJE	-22.5444854	118.7223615	14/03/2022	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438911
BMR12371	30830	MUNJE	-22.5444854	118.7223615	14/03/2022	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438912
BMR12372	30831	MUNJE	-22.5444854	118.7223615	14/03/2022	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438913
BMR12373	30832	MUNJE	-22.5444854	118.7223615	14/03/2022	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438914
BMR12374	30833	MUNJE	-22.5444854	118.7223615	14/03/2022	Hypo	Phreodrilidae `sp. P19`	PASS	PQ438915
BMR12532	30851	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Pristina longiseta	PASS	PQ438932
BMR12533	30852	WC2	-23.4922154	119.5145079	9/03/2023	Hypo	Pristina `sp. WAM-NAIDP001`	PASS	PQ438933
BMR09859	30445	WWDD3	-22.9106596	119.2143517	4/11/2022	Hypo		FAIL; PCR	n/a
BMR09864	30878	SS	-21.8626821	121.0085309	21/03/2023	Hypo		FAIL; PCR	n/a
BMR09866	30880	SS	-21.8626821	121.0085309	21/03/2023	Hypo		FAIL; PCR	n/a
BMR09867	30881	FF	-22.4777007	118.5512263	6/11/2022	Hypo		FAIL; PCR	n/a
BMR09868	30882	FF	-22.4777007	118.5512263	6/11/2022	Hypo		FAIL; PCR	n/a
BMR12349	30808	MUNJE	-22.5444854	118.7223615	21/03/2021	Hypo		FAIL; PCR	n/a
BMR12389	30848	WC2	-23.4922154	119.5145079	9/03/2023	Hypo		FAIL; PCR	n/a
BMR12537	30856	WC2	-23.4922154	119.5145079	9/03/2023	Hypo		FAIL; PCR	n/a
BMR09843	30868	YC3	-22.8245311	119.1636156	8/09/2022	Hypo		FAIL; contam	n/a
BMR09865	30879	SS	-21.8626821	121.0085309	21/03/2023	Hypo		FAIL; bad sequence	n/a

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