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Supplementary Tables

This supplement contains five tables. Table 1 reports the details of the ^{14}C age determinations. Table 2 reports detailed geochemical analyses of the borehole water. Table 3 reports the results of DNA extractions from the multi-layer in-line filter used to filter 15,963 liters of mining water from Beatrix Au Mine. Table 4 reports the morphometrics of *Halicephalobus mephisto* sp. n. Table 5 reports the 16S rRNA gene phylotypes.

Supplementary Table 1. ^{14}C model ages

Borehole	$\Delta^{14}C$ (DIC)	$\delta^{13}C$ (DIC) ‰VPDB	$\Delta^{14}C$ recharge	$\delta^{13}C$ recharge ‰VPDB	Subsurface carbon source	$\delta^{13}C$ ‰VPDB	Age (yr)
Beatrix	-704	-32.0	200	-9 to -12	CH_4	-48 to -53	4,413 to 6,247
Driefontein	-933	-8.0	-405 to -425	-17 to -21	dolomite	0.55	10,104 to 12,084
Tau Tona	-620	-17.7	-405 to -425	-17 to -21	CH_4 or carbon leader	-29 to -38	2,919 to 5,165
Zondereinde	-646	-17.4	-150	-17	CH_4	-19.5	5,798

Supplementary Table 2. Geochemical analyses.

	Driefontein	Beatrix BH2	Zondereinde	Tau Tona
Flow rate (L/m)[1]	6.8	4.5	8	0.33
T°C	24.2	37.2	47.5	47.6
pH	7.5	7.9	8.3	7.7
Eh (mV)	-42.3	-53.7	-64.6	-40.5
DO (μM)	31	13-72	24	28
Conductivity (mS/cm)	4.3	7.2	1.3	28.1
TDS (ppm)	216	3,579	650	14,050
Na^+ (mM)	2.70	60.96	8.35	138.70
Ca^{2+} (mM)	0.60	3.48	1.33	77.00
Mg^{2+} (mM)	0.37	0.09	0.09	0.05
K^+ (mM)	0.05	0.31	0.31	1.02
Cl^- (mM)	1.55	67.35	9.94	316.31
SO_4^{2-} (mM)	0.16	0.17	0.38	0.53
HS^- (mM)	0.01	>10	0.03	>10
NO_3^- (μM)	11.43	<7.3	3.57	<8.1
NO_2^- (μM)	2.2	<2.2	<0.71	2.2
PO_4^{3-} (μM)	<1	<10	<1.05	<1.0
Al^{3+} (μM)	0.26	0.41	1.85	<0.15
Fe_{Total} (μM)	0.52	0.23	1.41	0.18
TOC (mM)	0.18	0.15	0.06	4.03
DOC (mM)	0.17	0.14	0.05	0.49
DIC (mM)	2.39	0.34	0.71	0.18
NH_4 (μM)	35.2	N.A.	25.0	27.0

[1]Except for Tau Tona, water flow was reduced through a valve to prevent too much pressure on the filters, actual flow rate from fully open bore hole is higher.

Samples from Star Diamonds mine and from BH1 at Beatrix Au mine were not analyzed since no nematodes were detected there. Although no nematodes were recovered from Zondereinde Pt mine, the sample was analyzed because of its relevance to the discussion. DO = dissolved oxygen, Eh = oxidation potential, TDS: total dissolved solids. EC = conductivity. TOC = total organic carbon. DOC = dissolved organic carbon. DIC = dissolved inorganic carbon.

Supplementary Table 3. DNA results from Beatrix Au Mine mining water

Filter Component	DNA Concentration (ng/μL)	DNA Yield (ng)	Equivalent Cell Concentration (cells/mL)[1]	DNA fragment size (bp)
Internal filter layer	0.91	90.8	2	N.D.
External filter layer	1.92	192	5	N.D.
Internal net layer	2.04	204	5	300-3500
External net layer	2.26	226	6	200-2500

[1] Equivalent Cell Concentration = (DNA Yield $x10^{-9}$/$2.5x10^{-15}$ g of DNA/cell) /$1.6x10^{7}$ mL. The $2.55x10^{-15}$ g of DNA/cell is based upon analyses of marine planktonic bacteria published by Button and Robertson[85].

Supplementary Table 4. Morphometric data of *Halicephalobus mephisto* sp. n.

	Holotype	Paratypes[1]
L	492	525±17 (490-557)
L'	379	409±11.4 (378-427)
a	27.1	27.1±1.3 (25.5-28.8)
b	5.0	5.8±0.4 (5.5-6.6)
c	4.3	4.5±0.2 (4.3-4.7)
c'	9.2	9.9±0.5 (9.2-10.3)
Max body diam.	18.1	19.6±1.2 (18.0-21.1)
Stoma length	7.6	8.1±1 (7.2-9.6)
Necklength	97.7	91.2±7.4 (78.9-97.6)
Procorpus length	39.8	38.7±2.8 (36.1-42.8)
Isthmuslength	21.0	19.5±1.5 (18.1-21.7)
Procorpus/Isthmus	1.9	2±0.1 (1.9-2)
Taillenght	114	119±7 (111-130)
Anal body diam.	11.4	12±0.7 (11.4-13)
Vulva position	250	262±8.4 (249-273)
V	50.7	49.5±1 (48.3-50.9)
V'	66.0	63.8±1.1 (62.9-65.4)
vulva body diam.	17.6	19.2±1.4 (17.5-21.1)
Gonad (as % L)	30.0	33.7±3.9 (29-37.8)
Vulva-anus distance	129	147±6.3 (128-158)
Vulva-Anus/Tail	1.15	1.3±0.1 (1.1-1.4)
Tail/Vulva-Anus	0.9	0.8±0.1 (0.7-0.9)

[1] Measurements are based upon 9 paratypes and are presented in µm as average ± SD (min-max).

Supplementary Table 5. 16S rRNA Phylotypes

Mine	OTU	# clones	Closest relative/taxonomic position	Acc. Number	% ident.	Source
Beatrix	1, 2	5	*Vibrio* sp. QY102	AY174868	93-99	deep sea water
	3	3	*Klebsiellapneumoniae* AU45	EF032681	98	animal skin
	4	1	*Rheinheimera* sp. 3006	AM110966	93	deep sea sediment
	5, 6	2	*Pseudomonas pachastrellae*PTGa-14	EU603457	93-94	sea sediment
	7	4	*Pseudomonas* sp. E505-11	FJ169986	99	sea sediment
	8	2	*Pseudomonas* sp. LD12	AM913883	98	sea water
	9	1	*Pseudomonas* sp. AMD4	EU600211	97	wastewater
	10	1	*Acinetobacter* sp. PmeaMuc16	EU249988	92	sea water
	11	7	*Thalassolituusoleivorans* SLHC162b	AM279755	95	sea water
	12	1	*Sterolibacteriumdenitrificans*Chol-1S	AJ306683	93	UASB reactor
Zondereinde	13, 15	2	*Bacillus* sp. FE-1	EU271855	93-99	sea water
	14	1	*Bacillus* sp. PK-14	EU685816	96	oil polluted soil
	16	1	*Nitrospira*phylum	AB176701	89	geothermal water
	1	3	*Thiobacillus*-like	AY741698	99	gold mine
	2	7	*Rhodocyclaceae*family	AY770957	99	oilfield
	3	3	*Nitrospira* phylum	EU266868	93	tar contaminated aquifer
	4	4	*Nitrospira* phylum	DQ088762	94	gold mine
	5	8	*Crenarchaeota* phylum	DQ230933	100	platinum mine
Driefontein	1	2	*Thiofabatepidiphila* BDA453	AB304258	98	hot spring
	2	1	*Pseudomonas oleivorans* LCa2	AY623816	99	soil
	3	1	*Lysobacterbrunescens* ATCC29482	AB161360	98	UASB reactor
	4	2	*Thiobacillussajanensis* 4HGT	DQ390445	99	sulfide spring
	5	6	*Hydrogenophaga* sp. EMB33	DQ413146	98	bioreactor
	6	1	*Methyloversatilisuniversalis* EHg5	AY436796	98	lake sediment
	7	3	*Rhodocyclaceae*	AY945901	98	denitrifying reactor
	8	1	*Desulfovibrio* sp. Mlhm	AF193026	96	drinking water
	9	1	*Desulfobacteraceae*	FJ485074	97	phreatic sinkhole
	10	3	*Desulfatiferulaolefinivorans* LM2801	DQ826724	95	wastewater from refinery
	11, 12, 13	17	*Desulfotomaculum* sp. Ox39	AJ577273	90-91	subsurface aquifer
	14	1	*Clostridium straminisolvens* CSK1	AB125279	95	soil
	15	1	*Clostridiales*	DQ513075	84	ridge flank crustal fluid
	16	3	*Chlorobi* phylum	AB354625	94	hot spring
	17	4	*Chlorobi* bacterium Mat9-16	AB478415	88	microbial mat
TauTona	1, 2, 3	34	*Acinetobacter*sp. E11	EF599315	95-99	tannery plant
	4, 5	4	Bacterium OBII5	AF170421	93-94	hydrothermal vent
	6	1	*Stappia*sp. B106	EU726271	98	deep sea

Supplementary Methods

This file contains the details of the sample locations, the sampling methods and the procedures for geochemical, nematological and microbial analyses.

Site Descriptions

The Republic of South Africa hosts the world's deepest mines, some of which exceed 4 km below land surface (kmbls). These deep Au, Pt and diamond mines and their network of tunnels and crosscuts, allow exceptional access to the deep subsurface. During the course of normal mining operations, the advancing tunnels or exploratory boreholes facilitate sampling by intersecting water-bearing fractures. These boreholes typically occur in small cubbies on one side of the tunnel. Sampling sites were chosen at 5 different mines representing different geographic location, geology and depths.

1. Star Diamonds Mine (Petra Diamonds)– This mine is located in Free State Province near Beatrix Au mine in the southern Witwatersrand Basin. The borehole sampled was located on level 14, at 0.5 km below the surface. The water was associated with fractures in a kimberlite dike. The borehole was sealed with a valve. The water from this mine has not been previously studied.
2. Beatrix Au Mine (Gold Fields Ltd.)– Beatrix Au mine is located near the towns of Welkom and Virginia, some 240 km southwest of Johannesburg in the Free State Province of South Africa. Geologically the mine is located along the Southern Rim of the Witwatersrand Basin. It consists of four operating shafts with levels at depths between 600 and 2,155 meters below surface. The fracture water from this mine has been studied previously[13,14,15,30,31]. The two boreholes, BH1 and BH2 are located on level 26 of #3 shaft, 1.3 km below the surface. They are located in the Witwatersrand Supergroup, which at this location is directly overlain by 400-800 m of Carboniferous Karoo sediments[13,14]. Both boreholes were sealed with valves. The flow rate from BH2 was 6,480 L day^{-1} and BH2 was sampled on 6 occasions for a total of ~38,880 L.
3. Driefontein Au Mine (Gold Fields Ltd.)– Driefontein Au mine is situated some 70 km west of Johannesburg near Carletonville in the Gauteng Province of South Africa. Geologically the mine is located on the North Western Rim of the Witwatersrand Basin. It comprises eight producing shafts that mine different contributions from pillars and open ground. Three primary reefs are exploited; the Ventersdorp Contact Reef (VCR) located at the top of the Central Rand Group; the Carbon Leader Reef (CL) near the base and the Middelvlei Reef (MR), which stratigraphically occurs some 50 meters to 75 meters above the CL. It is a large, well-established deep to ultra-deep level Au mine extending to level 50, the lowest working level, at some 3,400 meters below surface. It has been the subject of numerous previous studies[10,13,14,15,17,32,33,34,35,36]. This borehole sampled was located in the intermediate pumping chamber (IPC) at 0.9 km depth of #5 shaft at Driefontein Au Mine (formerly known as East Driefontein Au mine). The IPC is a pump station for lifting water from the 3 km deep mining levels to the surface and is also the location where water from the regional Transvaal dolomite aquifer is used to replenish the mining water lost during recirculation[34]. The borehole that was sampled intersects the dolomite aquifer and was sealed with a valve. The flow rate for the Driefontein IPC borehole was 9,792 L day^{-1} and the borehole was sampled on 3 occasions for a total of 29,376 L.

4. Tau Tona Au Mine (Anglo Gold Ashanti Ltd.) – Tau Tona Au mine is located close to Carletonville not far from Driefontein Au mine and results from this mine have not been previously reported. The sampling site was a nearly horizontal borehole located on level 118 at a depth of 3.6 km in the Witwatersrand Supergroup and intersected the Pretorious fault zone. Borehole was not sealed and flowed intermittently. The flow indicator on the sampling device, however, did not detect a reverse movement of air into the borehole between the intermittent bursts of water indicating that gas was being released from the borehole between the frequent pauses. The flow rate for the Tau Tona 118 borehole was 475.2 L day^{-1} and the borehole was sampled on 4 occasions for a total of 1,901 L.
5. Zondereinde Pt Mine (Northam Platinum Ltd.) – Zondereinde Pt Mine is located in the Transvaal Province within the 2.0 Ga Bushveld Igneous Complex (BIC) and the results on one water sample from this mine have been published previously[15]. The sampled borehole was located on the level 7, at 1.7 km below the surface in the shaft #1. It was located in the Upper Critical Zone of the western lobe of the Rustenburg Layered Suite of the BIC. The borehole was not sealed and flowed continuously. Although the Zondereinde borehole is half the depth of the Tau Tona borehole, its temperature is the same at 48°C. This is because the geothermal gradient of the BIC is 21.9°C km^{-1}, twice that of the Witwatersrand Basin[37,38,39]. This site was chosen because the borehole was fed by a fracture system that runs all the way to the surface and exemplified a possible route for nematodes to migrate down to the deep subsurface. The flow rate for the Zondereinde Pt Mine borehole was 8,640 L day^{-1} and the borehole was sampled only once for a total of 8,640 L.

Sampling and decontamination methods

Samples for nematodes were taken using the same techniques and even the same equipment as used by scientists from the University of the Free State and other institutions, to study the bacterial/archaeal communities present in water emanating from boreholes in the deep South African mines[10]. This was done in order to avoid introducing uncertainty of results due to new sampling techniques. Only the filter apparatus was modified specifically to collect nematodes and had not been used before. Samples were taken by mounting a Margot type expansion plug into existing boreholes. The expansion packer was fitted with a Delran manifold with tygon tubing through which water flowed to a 38 μm pore size membrane filter housed in a cylindrical tube. The plug, manifold and tubing were washed, autoclaved and sealed prior to transport to the mine and quickly assembled and inserted into the boreholes. The borehole water flowed through the plug for several minutes to remove any surface contamination introduced during the insertion of the plug before the manifold and tubing was attached. Water was then allowed to flow through the manifold and tubing for several minutes before the sterile filter apparatus was attached. After the filter was attached, the water flowed through it to a plastic tube equipped with a small flow indicator. To prevent mine air from reaching the filter and to stop reverse contamination by air-borne Metazoa, the bottom of the cylinder holding the filter was fitted with a jagged edge, causing the water, once it passed the filter and reached the bottom of the tube, to swirl, creating a water lock. The small flow indicator slowed the water as well creating a second water lock while maintaining a flow, high enough to prevent nematodes, weighing approximately ~20 nanograms, from entering the filter from the bottom. The setup was equipped with valves at the inlet and outlet, allowing one to isolate the filter apparatus from air before dismounting it from the packer for transport to the laboratory. A typical sampling event involved insertion of the

plug and sampling manifold, filling of a 12 L Cornelius canister for bacterial analysis of the water, over filling of two 1 L amber bottles for ^{3}H and ^{14}C analysis and filling of several smaller volume serum vials and falcon tubes for geochemical analysis following the procedures of Moser et al.[10] and Onstott et al.[13]. After collecting these samples the filter apparatus was attached to the manifold and left on for 24 hours while the borehole water flowed through it. The following day the filter apparatus was disconnected and sealed, the plug and manifold removed and the valve closed. All samples were then transported to the Univ. of Free State laboratory.

To test for possible sources of nematode contamination within the mine, readily available soil was collected with the anticipation that the soils would have far more nematodes/gram than would the mining water, because the latter was treated with disinfectants and for Zondereinde Pt and Tau Tona Au mines the mining water was also chilled.

1) Wet soil within the borehole cubby either from underneath or in the immediate neighborhood of the borehole valve was collected. The reasoning being that if nematodes were present in this soil sample, they may have originated from the borehole water when it flowed out or the mining water when the borehole was first drilled and started living in the soil despite the cooler and drier conditions.
2) Soil samples in the tunnel immediately adjacent to the borehole cubby were collected as a way of checking for the prevalence of nematodes within the mine tunnels open to ventilation and human traffic.
3) Wet soil under or near to the mining water outlets were collected.
4) Two to five liters of mining water were collected directly from a high-pressure tap into plastic bottles and returned to the laboratory for filtration.
5) In the case of Beatrix Au mine access was granted to the mining water system for high volume filtration on two different occasions at two different locations. On first occasion a connection was made at a high-pressure water line located near the top of the Beatrix Au Mine #3 shaft right after the mining water treatment plant using a "Y-split" connection and pressure gauge that allowed the water pressure through the in-line filter never to exceed 50 kPa. Because of the high line pressure, a double open end high-efficiency pleated polypropylene filter with 0.2 μm pore size (Cole Parmer, EW-29830-00) was used. This filter was mounted in a 304 SS housing (Cole-Parmer, EW-01508-40) and autoclaved prior to connecting to the mine water supply system. The mining water sample was filtered for 21 hours at 733 L hr^{-1} for a total volume of 15,963 L. On the second occasion a connection was made to the mining water supply at 25 level of #3 shaft, one level up from the BH2 sample site. The flow of the water was split using two "Y-split" connections with separate valves and pressure gauges to two nematode filters and one double open end high-efficiency pleated polypropylene filter with 0.2 mm pore size. The filtration system was left on the mining water system for ~20 days during which the two nematode filters with an average flow rate of 0.375 L min^{-1} and 0.750 L min^{-1} filtered 10,282 L and 21,300 L, respectively. A total volume of 31,582 L was filtered by the double open end high-efficiency pleated polypropylene filter.

Approximately 100 grams of soil was deposited directly on agar plates in triplicate (9 plates total per sampling site) and the worms were allowed to crawl out. If they did, they were singled out and transferred to a fresh plate and identified. Water samples were concentrated over a 38 μm filter and the filtrate was used to make 1% agar plates. The filters were removed from the filter

housing in a flow through hood, transferred and spread open in 9 cm petri dish filled with water from the sampled borehole. This allowed the nematodes to crawl free from the filter and to be visually inspected using a dissecting microscope. The filters were also placed on the agar plates, including 1% bacteriological agar plates, with the surface where the nematodes should have been, facing the agar. All plates were incubated at room temperature and checked daily for a period of 4 months in the event only an egg or so would be present allowing the nematodes to multiply in sufficient numbers to see them. If, after 4 months, plates were still without nematodes, then it was considered negative result. Those samples yielding nematodes did so within the first 24 hours and they were checked several times daily for signs that they were feeding by observing the pumping motion of the pharyngeal basal bulb.

Nematode Isolation Methods

Since all the samples that yielded nematodes only yielded a single specimen of each species collected, the production of offspring was essential for any further analysis. The nematodes collected were left in the borehole water/petri dish until they produced offspring. This first offspring was used for all analysis of DNA and morphology. When nematodes showed signs of starvation (indicated by the gut becoming transparent) the remaining nematodes were then transferred to a 1% agar dish inoculated with a small amount of *Escherichia coli* OP50 on one side of the dish (if necessary the salinity was adjusted based on borehole water values) and with a microbial pellet from centrifuged borehole water on the other side. Nematode cultures were observed to become unstable once the borehole water bacteria were depleted and only *E. coli* remained indicating the nematodes were clearly adapted to the borehole bacteria as a food source. The inability to get large amounts of well-fed worms prevented us from testing the nematodes extensively, especially with respect to O_2 requirements. One monhysterid female species from the Driefontein Au mine borehole survived but produced no offspring probably because it was a sexual species.

A second monhysterid nematode was identified in the Tau Tona borehole using molecular methods. Borehole water was concentrated and the total DNA was extracted as described for microbial analysis below and PCR-amplified with the eukaryotic primers EukA (5'- AAC CTG GTT GAT CCT GCC AGT -3'), the forward primer for *Saccharomyces cerevisiae* 18S rRNA gene positions 2 to 22, and EukB (5'- TGA TCC TTC TGC AGG TTC ACC TAC -3'), the reverse primer for *S. cerevisiae* 18S rRNA gene positions 1795 to 1772[40] and yielded a 1750 bp fragment. Positive (*Fusarium oxysporium*) and negative controls were used in the PCR reaction. Amplicons were ligated, transformed and positive transformants sequenced as described for the microbial analysis.

Nematode morphological and molecular methods

Nematode specimens for light microscopy observations were collected with the aid of a stereomicroscope, placed in an embryo dish, killed an fixed in hot aqueous 4% formaldehyde + 1% glycerol and processed to anhydrous glycerol following the glycerol-ethanol method[41]. Measurements and illustrations were prepared from camera lucida line drawings, using an Olympus BX 51 DIC microscope (Olympus Optical, Tokyo, Japan) and the drawings were prepared using Illustrator CS software (Adobe Systems, Mountain View, California). Holotype

material is deposited at the Museum voor Dierkunde (collection number UGMD 104141) University of Ghent, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium. Hardcopies of the new species description are available at the libraries of the Royal Belgian Institute for Natural Sciences in Brussels and at the author's institutional addresses.

For SEM analyses, specimens preserved in anhydrous glycerin were transferred to a drop of glycerin in an embryo dish. Two drops of water were added every 10 min. for 2 h. A subsequent ultrasonic treatment for 8 min. of the specimen in a single drop of water removed particles adhering to the body surface. The specimens were progressively dehydrated by exposing them to 20%, 50%, 75%, 95%, and 100% ethanol in 1 h steps, to 100% ethanol overnight and to 100% ethanol for 20 min. the following morning. They were then critical point dried with liquid CO_2, mounted on stubs with carbon discs and coated with Au (25 nm) before observation with a JEOL JSM-840 (Tokyo, Japan, 1985) at 15 kV.

PCR and sequence analysis procedures follow those of Bert et al.[42]. Nearly complete sequences of 18S rRNA (1602-1715 bp) and the D2D3 expansion region of 28S rRNA (536 bp, only for the new species) were obtained. The two 18S rRNA sequences were deposited in NCBI GenBank under the accession numbers GQ892827 and GQ918144 and the D2D3 sequence was deposited in NCBI GenBank under the accession number GU811759. For each analysis, four sequence alignments were obtained, with related sequences from GenBank, using ClustalW[43], MUSCLE[44], MAFFT[45] and by manually deleting the few potentially ambiguous parts of the ClustalW alignment. Bayesian phylogenetic inference was performed with MrBayes v3.1.2[46]. A general time-reversible model with rate variation across sites and a proportion of invariable sites was used as estimated by PAUP/MrModeltest 1.0b (Nylander, 2004: http:// www.ebc.uu.se/systzoo/staff/nylander.html). Two independent analyses were run for $3x10^6$ generations and the trees were generated using the last 1,000,000 generations well beyond the burn-in value and the point of convergence between the two runs was confirmed by the average standard deviation of the split frequencies between the two analyses that approached zero.

Geochemical methods

Temperature, pH, ORP, dissolved O_2, conductivity, resistivity, and TDS were measured on site using a Hanna HI9828 multiprobe. Salinity was measured on site using an ATAGO Pocket refractometer and dissolved O_2 was also measured on site using a Eutech Instruments Cyberscan DO300 in a 1 L flask filled with fracture water. Because a flow cell was not used the O_2 measurements must be considered maximum estimates. Nitrite and sulphide were measured on site using Chemet colorimetric test kits K-7004 and K-9510, respectively. Cation analyses were performed on filtered water samples using a DV ICP-OES (Perkin Elmer Optima 3000). Anion analyses were performed using Ion Chromatography (Dionex DX-120) with an Ionpac AS14 (4x150 mm) analytical column and an Ionpac AG14 (4x50 mm) Guard column. The total organic carbon (TOC) and dissolved organic carbon (DOC) analyses were performed on unfiltered and filtered water samples, respectively, using the persulfate UV oxidation method and a Formacs Low Temperature TOC analyser (Skalar van Holland).The NH_4^+ concentrations were determined using the Nesslerization method. The cation, anion, TOC, DOC and NH_4^+ analyses were conducted at the Institute for Ground Water Studies at University of the Free State. Analyses of the $\delta^{13}C$ and $\Delta^{14}C$ of the dissolved inorganic carbon (DIC) for the water samples were carried out by AMS at the National Isotope Centre, Institute of Geological and Nuclear Sciences Ltd.,

Lower Hut, New Zealand. Samples from Star Diamonds and Beatrix BH1 were not analyzed since they yielded no nematodes. The ^{14}C ages are reported in Table 1 of main text and the detailed model results in Supplementary Table 1 and the Supplementary Discussion.

Microbial methods

Containers, plugs, tubing and manifolds were washed with soapy water and methanol and autoclaved in the lab and sealed until the moment of sampling to avoid any source of external contamination. Water samples for DNA extraction were collected in a Cornelius canister[32]. Once in the lab, water was concentrated through Tangential Flow Filtration (TFF) through a 0.2 μm pore size filter (Amersham Biosciences). Biomass from the concentrate was collected on a polycarbonate filter which was used as starting material for total genomic DNA extraction by using MoBio Soil extraction Kit (MO BIO Laboratories). The 16S rRNA gene was PCR-amplified by using typical domain-specific primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') in combination with 1492R[47] (5'-GGT TAC CTT GTT ACG ACT T-3') and 20F[48] (5'-YTC CSG TTG ATC CYG CSR GA-3') together with 976R[49] (5'-YCC GGC GTT GAM TCC AAT T-3') and utilizing 30 PCR cycles. The size of the PCR product was checked by agarose electrophoresis and was cleaned by DNA Clean and Concentrator (Zymo Research). Cleaned PCR products were ligated into pGEMT-Easy® and transformed into *E. coli* TOPO10 following the manufacturer's guidelines (Promega). Positive transformants were grown, the plasmid was purified and amplified by rolling circle amplification and the insert was sequenced with the flanking SP6 and T7 promoters in MCLab (San Francisco, California).

A total of 145 clones were sequenced. Contigs were obtained and firstly aligned (pairwise) against the Greengenes core data set[50] within the software MOTHUR v.1.15.0[51] by using the default settings (kmer search with 8mers using the Needleman-Wunsch method). Filtering of chimeras was performed with different algorithms provided within MOTHUR. The 142 filtered full-length 16S rRNA gene sequences were imported into ARB Silva SSURef102 database[52] for further manual multiple sequence alignment. The database was updated by adding closely similar 16S rRNA sequences as determined by BLASTn. A distance matrix was obtained and sequences were clustered into OTUs by using MOTHUR[51]. Rarefaction and coverage curves were obtained and community structures compared. The sequences have been deposited in GenBank under accession numbers GQ921326 to GQ921473.

Water samples for microscopy, 45 mL, were fixed on site with 4% formaldehyde and kept refrigerated for 12 hours before being filtered through a 0.22 μm GTTP type filter (Millipore). Cells were stained with DAPI and quantified under a Zeiss Axioskop microscope with a Hg lamp[53]. A Plan-Neofluor 100x/1.30 Oil objective was used to obtain the images. The fluorescent signal was detected through a Zeiss 02 filter set, which is composed of an excitation filter G365, a beam splitter FT395 and a barrier filter LP420. Images were captured with a Nikkon Digital Sight DS-Fi1 camera controlled by the imaging software Nis-Elements D 3.0 on a PC. The image formats were 2560x1920 pixels. Exposure varied between AE 80 to 200 ms, gain between 1.0x and 1.20x, white balance 1.30, 1.00, 2 and enhanced contrast. Noise reduction, saturation, hue, offset and sharpness were null.

The procedure used to extract the DNA from the filter cartridge that filtered the 15,963 L of Beatrix mining water was the same as that employed by Chivian et al.[36] who successfully extracted 46 μg of high molecular weight DNA for metagenomic analyses after having filtered 5,600 L of borehole water. The filter cartridge was removed from its stainless steel housing in a laminar flow-through hood. The end caps were removed by using a sterilized hacksaw and the pleated filter was freed out of the outer plastic cylinder. The pleated filter was separated into its 4 different layers – external net-layer, external filter layer, internal filter layer and internal net-layer. Ten grams of each filter layer (half of the filter) was cut into 1cm x 1 cm pieces and transferred to a 50 mL Falcon tube. DNA extraction buffer [27 mL of 100 mM Tris-Cl, 100 mM EDTA, 1.5M NaCl and 1% CTAB (Cetyltrimethylammonium Bromide) at pH 8], 200 μL of proteinase K (10 mg/ml) and 10 mL of DNAzol® (Invitrogen; Cat no. 10503-027) were added to each sample and incubated at 37ºC for 30 min with gentle end-over-end inversion every 10 min. Three ml of 20% SDS was added to each tube and incubated at 65ºC for 2 hours with gentle end-over-end inversion every 20 min. The aqueous phase was then transferred to a new Falcon tube taking care not to suck up the filter pieces. An equal volume of chloroform-isoamyl alcohol (24:1) was added to the aqueous phase and mixed by end-over-end inversion a few times. Centrifugation was performed at 6,000x g for 10 min at room temperature. The aqueous phase was then transferred to a new 50 mL Falcon tube. Isopropanol (0.7 volume equivalent) was added to the tubes, mixed and precipitated for 1 hour at room temperature. Centrifugation was performed at 10,000x g for 30 min, washed with 5 mL 70% ethanol. The tubes were centrifuged at 10,000x g for 20 min, the tubes were then inverted to drain the ethanol/propanol and the residual ethanol was removed by aspiration. The resulting pellets were air dried and suspended in 1 mL of 10 mM Tris-HCl. The DNA concentration was determined using the Qubit® Quantitation Fluorometer (Invitrogen) and the Quant-iT dsDNA HS assay kit. Twenty μL of isolated DNA was loaded onto a 0.8% gel and electrophoresed at 7V/cm (90V) for 60 min to determine the molecular weight. The BioSpin gel extraction kit (Bioer Technology Co., Ltd.) was used to purify the DNA isolated from the in-line filter according to the manufacturer's protocols.

PCR reactions were performed on isolated DNA using the Eppendorf Mastercycler PCR System. The 18S rRNA gene amplification was performed using the EukA and EukB[40]. Bacterial 16S rDNA PCR was performed using the 27F and 1492R[47]. The two primer sets used for amplification of archaeal 16S rDNA genes were 20F[48] with 976R[49] and 1090R (5'-GGCGATGCACCWCCTCTC-3')[54]. Each 20 μL reaction tube contained ~20 ng of template DNA, 1.5 mM Mg-containing reaction buffer (SuperTherm), 2.5 μM concentration for each of the deoxynucleotides, 1 mg mL^{-1} bovine serum albumin (BSA), 500 nM of the forward and reverse primers and 0.025 U/μL of DNA Taq polymerase (SuperTherm). Thirty-five cycles of PCR were then performed with the Eppendorf Mastercycler and the results were analyzed by a 1% agarose gel electrophoresis. A reaction without DNA template was included in all PCR amplifications as non-template control. Positive controls were also included. Spiked controls were used to rule out PCR inhibitors. PCR reactions were prepared in duplicate and the isolated DNA and purified DNA were added. One set of the duplicates was all spiked with 20 ng of the appropriate positive control. The other set of duplicates contained only the isolated Beatrix samples. If no amplification was observed in reaction tubes spiked with the control, it was interpreted as an indication that the PCR reaction was inhibited.

Supplementary Discussion

This file contains the description of the model used to estimate the ^{14}C age of the DIC, the results from the control samples, the taxonomic description for *Halicephalobus mephisto* followed by a discussion of its phylogenetic relatedness to surface species, and descriptions of the microbial communities followed by a discussion of their relevance.

^{14}C analyses and Modeling

The $\Delta^{14}C$ values for the DIC ranged from -932.8 for the Driefontein borehole to -619.6 for the Tau Tona borehole (Supplementary Table 1) and were higher than previously reported values from other boreholes and mines with the exception of that from the Driefontein borehole[13,55].

Only an unconfined superficial aquifer overlies the BIC at Northam Pt Mine. We would anticipate that the $\delta^{13}C$ of the recharge water would then reflect the isotopic fractionation between soil gas CO_2 and bicarbonate at neutral pH or ~-17‰. This value is very close to that measured for the DIC of the Northam Pt Mine borehole suggesting little geochemical modification. This is consistent with the fact that the Transvaal dolomite occurs beneath the BIC and that carbonate is absent in the ultramafic rock units within the BIC. The only significant carbon source within the ground water is the CH_4, which has a $\delta^{13}C$ value of -19.2‰[15]. In nearby Botswana recharge studies suggest that the average $\Delta^{14}C$ for Karoo groundwater recharge is -150[56]. Using these values the measured $\Delta^{14}C$ of the Northam Pt Mine DIC corresponds to an age of ~5,800 years (Supplementary Table 1).

In the Beatrix Au Mine region of South Africa the $\Delta^{14}C$ of Karoo groundwater DIC ranges from 230 to -530[57]. The $\delta^{13}C$ has not been determined for Karoo groundwater in this region, but further north the $\delta^{13}C$ value for the DIC of Karoo groundwater with the highest $\Delta^{14}C$ concentration yields $\delta^{13}C$ values ranging from -9 to -12‰[58]. Since the $\delta^{13}C$ value of -32‰ for the DIC is much more negative than this, the fracture water DIC may have been altered by organic respiration of dead carbon. The negative $\delta^{13}C$ value for the DIC is consistent with the absence of the Transvaal dolomite in the stratigraphy at Beatrix Au Mine, because if it were present its dissolution would make the $\delta^{13}C$ value for the DIC more positive. The $\delta^{13}C$ value for organic carbon in the Witwatersrand Supergroup averages ~-29‰, which is still too positive. The most likely carbon source, therefore, is the abundant CH_4 for which the $\delta^{13}C$ value ranges from -48 to -53‰[59]. Using these constraints the ^{14}C estimated groundwater age ranges from 4,413 to 6,247 years (Supplementary Table 1).

The $\Delta^{14}C$ values of the groundwater recharging the Transvaal, karstic, dolomitic aquifer in the Driefontein and Tau Tona Au mine region range from -405 to -420. One explanation for these low ^{14}C concentrations is the hydrochemical evolution of groundwater, which partially bypasses the biological soil zone where equilibrium between ^{14}C and bicarbonate is normally established[60]. Instead the slightly acidic recharge water dissolves the Transvaal dolomite host rock with zero ^{14}C concentration resulting in the observed low ^{14}C concentrations. The $\delta^{13}C$ value for the recharge DIC was estimated by using the Mg^{2+}

concentrations of the recharge water and the $\delta^{13}C$ value for the Transvaal dolomite, which is 0.55‰, to determine the amount of dolomite that had dissolved in the recharge groundwater, and these estimates ranged from -17 to -21‰. The $\delta^{13}C$ value for the deeper dolomitic water DIC increased to -8‰ as a result of further dissolution of the Transvaal dolomite. Using these values the ^{14}C age for the dolomite borehole water ranges from 10,104 to 12,084 years (Supplementary Tables 1 and 2).
Given the proximity of Tau Tona Au Mine to Driefontein Au Mine, the same recharge values were assumed for the Tau Tona borehole, which yielded a higher ^{14}C concentration and a more negative $\delta^{13}C$ value even though it is far more saline and located at a much greater depth. If one assumes that the more negative $\delta^{13}C$ value is caused by dead carbon addition from oxidation of the carbonaceous layers within the Witwatersrand with a $\delta^{13}C$ value of -29‰ or from anaerobic oxidation of the abiogenic CH_4 with a $\delta^{13}C$ value of -39‰, then the resulting ^{14}C model age ranges from 2,919 to 5,165 years (Supplementary Table 1).

It is difficult, however, to reconcile the observed values with a flow path for this fracture water that passes through the overlying Transvaal dolomite where the DIC would become enriched in ^{13}C as observed in the Driefontein Au Mine borehole. If one assumes recharge values equivalent to those measured for the Driefontein Au mine borehole then the ^{14}C model age becomes negative. This suggests that the flow path for the paleometeoric water component of the fracture water from this borehole, which intersects the pre-Transvaal Pretorious fault zone, must follow another route to the surface, perhaps northwards ~25 km to surface in the Middelvlei Inlier, a structural dome where the Transvaal dolomite is absent.

Another possible explanation for the high ^{14}C concentration observed in this borehole is mine air CO_2 contamination, if the borehole periodically sucked in air between its intermittent bursts of water. The observed $\delta^{13}C$ value could represent a combination of kinetic fraction of air CO_2 as has been observed in the containment dam water by Takai et al.[61], and the DIC of the saline water which contains little $^{14}C^{13}$. This explanation can be ruled out because the flow meter on the filter array never turned backwards between the water surges (see Supplementary Methods), the dissolved O_2 concentration is lower than that predicted by atmospheric equilibrium and the dissolved gas composition is mostly CH_4 not N_2.

Samples from Star Diamonds mine and from BH1 at Beatrix Au mine were not analyzed since no nematodes were detected there. Although no nematodes were recovered from Northam Pt mine, the sample was analyzed because of its relevance to the discussion.

Results from Nematode Control Samples
Star Diamond Mine

1) The soil sample beneath the borehole valve was very wet and contained a small piece of wood and yielded within days 12 nematodes identified as Dorylaimids, 5 nematodes identified as Mononchids and 3 annelids identified as a single Annelid species.

2) The soil sample from corridor was negative. Sample was dry, but even after wetting on the agar dish, no nematodes or annelids were observed.
3) Four liters of refrigerated mining water were collected on different dates and no nematodes or annelids were observed.
4) The wet soil underneath mining water valve did not yield any nematodes and annelids.

Beatrix Au Mine where *Halicephalobus mephisto* sp. n. was discovered (both boreholes)

1) The soil sample near borehole valve was negative.
2) The soil sample from corridor was very wet, but still negative.
3) Six liters of mining water collected on different dates were all negative.
4) The wet soil underneath mining water valve was negative.
5) The filter through which 15,963 L of mining water passed failed to yield any quality DNA (see discussion below).
6) The two filters through which 31,582 liters of mining water passed failed to yield any nematodes, although some protists were detected. The 1% agar plates were then inoculated with *H. mephisto* to determine whether some procedural error in their preparation had prevented their growth and limited growth was detected on the agar plates ruling out this possibility.

Driefontein Au Mine where *Plectus aquatilis* and a monhysterid nematode were recovered.

1) The wet and chunky, soil sample near the borehole valve yielded the 8 nematodes identified as *Diploscapter coronatus* and 9 nematodes identified as *Rhabditis (Poikilolaimus) regenfussi*.
2) The soil sample from the corridor was very wet, but still negative.
3) Two liters of mining water were negative.
4) Very little soil beneath the mining water tap existed at this site because of the concrete floor. A sample was taken where mining water had made a puddle in the concrete and this sample was negative.

Northam Pt Mine

1) The soil sample near borehole valve was negative.
2) The soil sample from corridor was very wet, but still negative.
3) Two liters of refrigerated mining water were negative.
4) The wet soil underneath the mining water valve was negative.

Tau Tona Au Mine where DNA of monhysterid nematode was recovered.

1) The wet soil near the borehole was highly contaminated with oil or diesel and was negative.
2) The dry soil sample from the corridor outside the borehole cubby was negative and remained negative even upon wetting.
3) Five liters of refrigerated mining water were negative.
4) The chunky wet soil collected near the mining water tap was also negative.

Private companies under contract to the mines perform the treatment and monitoring of the water quality of mining water that is used for drilling or dust control or ventilation underground. In the case of Beatrix Au mine, a Goldfields Ltd. Au mine, Watercare Mining (Pty) Ltd. provides this service. Watercare Mining adds lime to the mining water returning from the drilling platforms to increase its pH from 5 to 8.5. They then add

flocculants to separate mud or clay minerals from the water. They then add BCDMH "Bromochlorodimethylhydantion" and H_2O_2 for disinfection. Once the mining water is pumped to the surface, Na hypochlorite is added for further disinfection and then the pH is adjusted back to 8.5 using Na_2CO_3 before the mining water is pumped back underground. Watercare Mining then assays the number of CFU's of *E. coli* and sulfate reducing bacteria at regular intervals. The first filter sample was collected from this point in the circulation loop, after the addition of the Na hypochlorite and Na_2CO_3 and before it was pumped underground. The pH of the water was 8.2 to 8.3, the Eh was 265 mV, the temperature was 26.4^{o}C and the conductivity was 5.3 mS cm^{-1}. The second filter sample of mining water sample was collected at the 25th level where it is distributed to the drilling teams. Here the pH had decreased to 6.5, the temperature was 23.4^{o}C and the conductivity had increased to 6.3 mS cm^{-1}.

Genomic DNA isolated from all four layers of the filter was only 713 ng with the internal and external net-layers yielding the more DNA relative to the internal and external filter layers (Supplementary Table 3). This amount of DNA is approximately equivalent to 18 bacterial cells mL^{-1}, a value that is two orders of magnitude less than the cellular concentrations in the Beatrix borehole (Table 1). Gel electrophoresis indicated that the fragment size ranged from 200 to 3500 bp suggesting that the genomic DNA is highly degraded. PCR amplification of the 18S rRNA gene (Supplementary Fig. 1B) failed to yield a detectable amplicon for both the isolated DNA and isolated DNA spiked with a positive control (*Yarrowia lipolytica* gDNA) indicating the presence of inhibitors in the isolated DNA. After purification of the isolated DNA from each layer the purified DNA quantity was less than the original isolated DNA (Supplementary Fig. 2A). PCR amplification of the 18S rRNA gene with and without the *Yarrowia lipolytica* gDNA was repeated and the same lack of amplicons and inhibition effects were observed for the PCR runs as with the original isolated DNA (Supplementary Fig. 2B). PCR amplification using bacterial 16S rRNA gene primers with and without an *E. coli* gDNA spike failed to yield detectable amplicons from all four layers (Supplementary Fig. 3). PCR amplification using two sets of archaeal 16S rRNA with and without a *H. salinarum* gDNA spike failed to yield detectable amplicons from all four layers (Supplementary Fig. 4).

The short fragment size of the extracted genomic DNA is consistent with the study of McCarty and Atlas[62] who showed that bleach destroys larger DNA amplicons more rapidly than smaller DNA amplicons. The presence of an inhibitory effect upon the PCR amplification process would also be consistent with the presence of bleach residue in the DNA extracts. The results are also consistent with the lack of CFU's observed by Watercare Mining and also the lack of any nematodes when first 2 liters of this mining water and then 31,582 liters of this mining water were filtered and plated using the procedures described previously.

The absence of nematodes from the mining water and the soil beneath the valves where mining water is frequently released indicates that the disinfectants deter nematode growth in the mining water. This is a good thing since many miners drink the mining water. The absence of nematodes from the tunnel soils that are typically dry suggests that in general

the mining environment is not conducive for the growth and spread of nematodes. Even the wet soils beneath the three nematode-bearing boreholes either didn't contain nematodes or didn't contain nematodes of the same species as that within the borehole. This last observation suggests that the nematodes found in the borehole water have difficulty surviving outside of the borehole in the mine environment.

The Beatrix Au Mine service water (mining water is commonly referred to as service water by all of the South African mines) consists of recycled mining water collected at the dams located at various levels by sump dumps and pumped to the surface where it is diluted with Sedibeng District municipality water (i.e. tap water) to make up for evaporative losses. Mining water contains a small component of fracture water when drilling operations intersect water filled fractures releasing fracture water into the tunnels before the boreholes are closed off by valves and/or the fractures sealed with cement. A relevant question for contamination then is the nematode content of South African tap water. We can assume that tap water has many origins, rain, runoff, rivers, dams, etc. Cohn[63] reported that during the summer the Crocodile River in the Mpumalanga Province of South Africa had a flow of the citrus parasite nematode of $>7x10^9$ larvae hour^{-1}. In this study the authors were only looking for one specific species of nematode. Baujard and Martiny[64] have also reported nematodes in rain. Untreated water that enters the municipal network contains very, very large amounts of nematodes regardless of the origin. In reports of untreated surface water samples of a few liters are routinely taken to obtain workable and relatively accurate population estimates[65]. If we use the little available data on total nematode counts in South African municipality feeds, we can make a very conservative estimate of the nematode count that should be in the water entering the municipality water system prior to treatment. In $6x10^6$ liters of runoff $2.80x10^8$ nematodes or ~50 nematodes L^{-1} have been reported[66,67]. Treatment by the municipality reduces this concentration to < 1 nematode L^{-1}.

Although the tap water meets WHO standards, the recycled mining water may pick up infectious microorganisms from the mining environments as it moves through the tunnels from the mining site to the dams passing by underground toilets, and thus requires further disinfection procedures beyond simple dilution. The company Watercare Mining, Ltd provided the following information about the recycled mining water treatment they provide to Beatrix Au Mine. The company uses a multi-stage technique approach to treating the recycled mining water and has proven ability to eliminate nematodes[68].

1) Water flows from the underground mining site into a dam where its pH is neutralized before moving to settling tanks where a flocculant is added to separate the mud from water. Sedimentation and flocculation are very effective because nematodes do not swim in the water column and thus any nematodes present would settle to the bottom[68] and not be pumped to the surface.
2) BCDMH and H_2O_2 are also added to the water before it is pumped up. Peroxide is a real nematode killer even the sturdy plant parasitic are killed[68].
3) Water is pumped up from the dams to the surface plant where sodium hypochlorite and sodium carbonate are added. Chlorination might kill most nematodes but not all, at least one species of plant parasitic nematode is able to survive such treatment[66], but

no free-living nematode has been reported to survive chlorination. Chlorination is a very efficient fungi and bacteria killer.

4) No biofilms are allowed to form in the surface plant water processor. A potential feeding source for bacteriophagous nematodes like *Halicephalobus mephisto* n. sp. is therefore eliminated preventing the buildup of a nematode population in the surface plant.
5) This water is then pumped back down underground through high-pressure plumbing and is never exposed to the environment until it reaches the drilling rods. In deeper mines this water is chilled to 5-10^{o}C, which would further inhibit any potential nematode growth.

The treatment given to the municipality tap water entering the mine and to the recycled mining water is extremely efficient against nematodes, fungi and bacteria and thus the low concentrations and degraded nature of the DNA leaving the surface water treatment facility and the absence of nematodes in 31,582 liters of treated mining water where it leaves the high-pressure plumbing underground should be no surprise. Furthermore, the rarity of nematodes in the soil samples and the complete absence of nematodes in the Beatrix Au Mine soil samples collected underground further confirms that the recycled mining water is typically too harsh to support nematodes. Given that the mining water is used as a source of drinking water in the mine corridors insufficient treatment for any water-borne organisms that can cause debilitating diseases would have devastating consequences for mine operations. During the course of drilling a ~50m long borehole, ~10^4 liters of mining water is typically used. Upon intersecting a high pressure, water-bearing fracture, the mining water is flushed out by the microbially, chemically and isotopically distinctive fracture water. Any mining water contamination remaining in the borehole becomes highly diluted by this process. In summary the three arguments against *Halicephalobus mephisto* being a mining water contaminant are:

1. No nematodes were present in >10^4 liters of heavily treated mining water, and none are found in the mine tunnels.
2. Even if a nematode were present in 10^5 liters of mining water, as an example, the mining water becomes highly diluted by the high-pressure fracture water that flushes the drill rods and borehole upon intersection of the fracture during the drilling operation and before the borehole is sealed with a valve.
3. Any contaminating nematode that somehow managed not to be flushed out of the borehole with high-pressure fracture water would then have to adapt instantaneously to a hypoxic, high temperature environment that contains very low concentrations of wild-type bacteria once the valve is closed.

Description of *Halicephalobus mephisto*[1]

Body was straight to slightly ventrally arcuate after fixation, 0.52 to 0.56 mm long. The annulation was not visible with light microscopy but is in SEM (Supplementary Fig. 5). The lateral field formed two protruding ridges. The lip region was continuous with neck. Six labial papillae and four cephalic papillae (arrows, Supplementary Fig. 5) were distinct using SEM. Amphidial opening (open arrow) was irregular circular. The stoma

[1] The specific epithet ("he who loves not the light") refers to the devil, Lord of the underworld, in medieval mythology from the Faust legend since the new species is found at -1.3 km into the Earth's crust.

(measured from head end) was 7-10 µm. Lengths of cheilostom, gymnostom and stegostom were in the ratio of 1:3:4. The cheilostom was small, gymnostom clearly cuticularised (about 35% of stoma length); stegostom with indistinct dorsal thickening. Neck length was 79–98 µm, 15 to 18% of body (Supplementary Fig. 5; Supplementary Table 4); cylindrical procorpus, metacarpus swollen, bulb shaped, about 10 µm in length. The isthmus was narrower than the corpus. The basal bulb was ovoid with well-developed valvular apparatus. The nerve ring was at middle of isthmus. The excretory pore was at the middle of isthmus to slightly anterior of the basal bulb. A distinct hemizonid was just anterior excretory pore. The deirid was at the anterior part of basal bulb. The intestine possessed a distinct lumen. The reproductive system was monodelphic, prodelphic, on right side of the intestine, with posterior reflexed ovary extending 99 – 135 µm posterior vulva (Supplementary Fig. 5). Ovary tip is not reflexed back anteriorly. A spermatheca-like structure was not visible. Oviduct with irregular rows of oocytes. Uterus filled with maximum one developing egg, about 30 µm long. Slightly protruding vulva at middle of body. The postvulval sac was short and indistinct, about 5 µm in length. The vagina was perpendicular to the body, about one fourth of body width. The vulva-anus distance was about 1.25 length of tail. The rectum was slightly longer than anal body diameter. The posterior anal lip was slightly protruding. The tail was relatively long and the tail tip filiform, terminus straight to variably curved. Phasmids were not visible. Males were not observed. Using the Andrassy formula[69] and a density of 1.13 g cm^{-3}, the estimated dry weight mass of *H. mephisto* is $2.64x10^{-8}$ g.

Although *Halicephalobus* is a morphologically minimalistic genus, *Halicephalobus mephisto* is a new typological morphospecies (Linnean species concept) as it can be easily differentiated from all other species of *Halicephalobus* by the presence of a long tail (110-130 µm; c': 9-10) with filiform terminus and the absence of reflexed ovary tip. *H. mephisto* sp. n. is most similar to *H. parvus*[70], but it differs from this species by the more anterior vulva (48-51% vs. 53-56%), longer tail (110-130 µm vs. 95 µm) and ovary not reflexed. *Halicephalobus mephisto* n. sp. has a maximally supported sister relationship with *H. gingivalis-Halicephalobus* spp., differing by 168 nucleotides or 10% (Supplementary Fig. 6) from *H. gingivalis* and differs by 8% from *Halicephalobus* species analyzed by Lewis et al.[71]. In comparison, the most closely related different genus, *Procephalobus*, differs 282 nucleotides (17%) from *H. mephisto* n sp. Alternative alignment methods did not have a single effect on the tree topology outcome and resulted in a similar or increased branch support for the *H. gingivalis-Halicephalobus* spp. clade (up to PP 97, MUSCLE alignment). The monophyly of *Halicephalobus* was always maximally supported, independently of the alignment method. Furthermore, 13 and 6 autapomorphic characters were present in the SSU rRNA sequences for the new species and its sister clade, respectively (Supplementary Fig. 6). Additionally, we obtained a D2D3 sequence from the 28S rRNA gene to refine the evolutionary relationships between *H. mephisto* and other *Halicephalobus* species, but phylogenetic analyses based on D2D3 could not resolve relationships within *Halicephalobus*, because the available sequences from GenBank are too short (only 290 comparable homologous positions). Nevertheless in this very short region 5 nucleotide states were clearly different for the *H. mephisto* n. sp. (5 autapomorphic characters). Thus, although based on limited available homologous sequences, multiple autapomorphic characters from two loci (18S and D2D3) and

multiple autapomorphic characters in both sister lineages (18S) indicate lineage exclusivity for *H. mephisto* n. sp. in respect to other *Halicephalobus* species (Supplementary Fig. 6). Hence, the species status of *H. mephisto* fulfills the requirements of an amalgamation of evolutionary and phylogenetic species concepts according to Adams[72] who followed Frost and Kluge[73]. A set of explicit discovery operations minimizing the risk of making systematic errors[72,74] make this approach more stringent than alternative phylogeny–based species concepts[74,75].

Halicephalobus is a cosmopolitan genus that is comprised of primarily free-living and bacteriophagous species, but also opportunistic pathogens. *H. gingivalis* is an opportunistic pathogen found in horses, donkeys and zebras and occasionally in humans and is able to survive at temperatures up to 40 to 42°C. *H. mephisto* is a species of the same genus, which may explain the origin of its tolerance for higher temperatures. Furthermore, *H. gingivalis* can be cultured in axenic medium[76] and is thus not an obligate bacteriophagous species. Consequently, *H. mephisto* may not be an obligate bacteriophagous species either, but given that bacteria are the predominant microorganisms in the subsurface they represent its most likely food source.

The morphologically and morphometrically identified *Plectus aquatilis* from Driefontein Au Mine lies within a completely unresolved clade of other *Plectus* species. The different alignment methods did not have a single effect on the tree topology. *P. aquatilis* Driefontein differs by 6 to 7 18S rRNA nucleotides (0.4%) from two *P. aquatilis* species isolated from the surface, the latter of which differ by three nucleotides (0.2%) from each other (Supplementary Fig. 6B).

Microbial Results

Random colony selection retrieved 31 full-length 16S rRNA sequences from Beatrix, 47 from Driefontein, 25 from Northam and 39 from Tau Tona boreholes. A 97% sequence identity threshold produced 16, 17, 5 and 6 OTUs from each of these sites, respectively. The microbial richness in Northam Pt and Tau Tona Au mines appeared much lower than that of both Beatrix and Driefontein Au mines, which were still distant from reaching saturation (Supplementary Fig. 7). Only the Northam Pt Mine borehole water yielded an archaeal 16S rRNA sequence, and it fell within the *Crenarcheota*, which have been associated with mining water in prior studies[15].

Sequences retrieved from Beatrix Au Mine clustered within the classes of γ and β *Proteobacteria* and the phyla *Firmicutes* and *Nitrospira* (Supplementary Fig. 8). Within the γ *Proteobacteria* class, up to 6 OTUs clustered within the order *Pseudomonadales*, closely related (92 to 98% identity) to several *Pseudomonas* and *Acinetobacter* isolates. Other OTUs within this class were related to the genera *Thalassolituus*, *Rheinheimera*, *Klebsiella* and *Vibrio*. Moreover, these OTUs formed the highest number of clones retrieved from this sample (Supplementary Table 5). Within the β *Proteobacteria* class, one single OTU was related to the family *Rhodocyclaceae*, and its closest (93% identity) bacterial species with a validly published name was the denitrifying *Sterolibacterium denitrificans* Chol-1S. All the OTUs within the phylum *Firmicutes* belonged to the class

Bacillales and were related (93-99% identity) to the genus *Bacillus*. Finally, the only OTU belonging to the phylum *Nitrospira* yielded low identity to any cultured bacterium.

The 16S rRNA clone libraries from the Driefontein borehole revealed some representatives within the order *Pseudomonadales*. Also within the same class, two OTUs were closely related to the sulfur oxidizing bacterium, *Thiofaba,* and the aerobic heterotrophic, *Lysobacter*. Within the β *Proteobacteria*, OTUs clustered within the genus *Hydrogenophaga*, the sulfur-oxidizing *Thiobacillus*, the methylotrophic bacterium, *Methyloversatilis,* and other members from the family *Rhodocyclaceae* (Supplementary Fig. 8). Three OTUs belonging to the δ *Proteobacteria* class were related (95-97% identity) with different anaerobic sulfate reducing bacteria. The sulfate-reducing genus, *Desulfotomaculum,* was also detected in the Driefontein borehole.

Finally, two more OTUs clustered within the phylum *Chlorobi*, normally represented by anaerobic, photoautotrophic bacteria, which would seem at odds with a subsurface environment. Chlorobi-like 16S rRNA sequences have been detected in several other studies of different boreholes in the Witwatersrand Basin[15,31]. Moreover, Chlorobi-like 16S rRNA sequences have also been reported from different subsurface aquifers like an uranium polluted aquifer (GenBank accession number AY532591 retrieved by Gihring et al, unpublished), a granite associated aquifer in Colorado[77] and are ubiquitous in sulfidic cave systems[78]. *Chloroflexi* or *Cyanobacteria*, both phototrophic phyla, are able to grow heterotrophically[79,80] and some of them have lost their pigments to become strict chemoheterotrophic bacteria[79]. Within the *Chlorobi*, a new bacterium, *Ignavibacterium album* gen. nov, sp. nov. strain Mat9-16^T, has been recently isolated from a terrestrial hot spring[81] that is moderately thermophilic, grows fermentatively and is the closest 16S rRNA sequence in the database to the one we report, although still only 90% identical. This new isolate lends credence to the idea that like the *Chloroflexi* or *Cyanobacteria*, members of the Chlorobi are chemoheterotrophic and could inhabit the deep subsurface. This new isolate would also accommodate the new class *Ignavibacteriae* classis nov. and would validate the tentative phylum-taxon level "*Chlorobi*" into *Chlorobi* phyl. nov.

Taxonomic representation was more restricted in the Northam Pt Mine borehole with uncultured members from the β *Proteobacteria* class related to the genus *Thiobacillus* and the phyla *Nitrospira* and *Crenarchaeota*. The sample from the Tau Tona Au Mine borehole also yielded low taxonomic representation. A cluster of OTUs within the class of γ *Proteobacteria* were closely related to the genus *Acinetobacter* (99% identity) and another one (93-94% identity) to the sulphur-oxidizing bacterium OBII5 isolated from a hydrothermal vent (AF170421). The last OTU was related to the genus *Stappia* (98% identity) in the α *Proteobacteria* class (Supplementary Fig. 8).

Cell counting under the fluorescence microscope revealed scarcely 200 cells mL^{-1} in the Northam borehole water and half of that in the Driefontein borehole water. Surprisingly enough, the borehole water from Beatrix BH2 and Tau Tona showed a significantly higher concentration of ~3×10^3 and 3.4×10^3 cells mL^{-1}, respectively. The microbial communities for each borehole appeared different when the morphology was analyzed. Water from Driefontein Au Mine showed few cells, most of them rods and filamentous

bacteria, closely associated. Water from the Northam Pt Mine borehole showed a predominance of cocci-shaped cells. Beatrix and Tau Tona Au Mine boreholes showed a higher density of cells, with more variable morphologies (Supplementary Fig. 9).

In terms of comparative structure, only one OTU was found in more than one borehole when a 97% identity was selected and this between the communities in Beatrix (OTU12) and Driefontein (OTU7). This one belonged to the family *Rhodocyclaceae*. When a 95% identity was chosen, OTU2 from Northam Pt Mine was also shared within the same family. At this same distance, one more OTU related to the genus *Pseudomonas* was shared between the Beatrix (OTU8) and Driefontein (OTU2) boreholes and one more related to *Acinetobacter* between the Beatrix (OTU1) and Tau Tona (OTU1) communities.

Different studies on the deep subsurface have pointed the ubiquity of microbial communities metabolizing exogenous energy-rich substrates (H_2, CH_4, CO, acetate) that are available in these sequestered environments[82,83]. Studies of the Witwatersrand Basin have revealed that radiolytic H_2 sustains sulfate reducing bacteria and/or methanogenic archaea[84]. Molecular signatures obtained in this work pointing towards sulfate reducers are abundant, like the *δ Proteobacteria* (*Desulfovibrio* and *Desulfatibacillus*) and the *Firmicutes* related to the genus *Desulfotomaculum*. Sulfur (or sulfide) oxidizers like *Thiobacillus*, *Thiofaba*, anaerobic photoautotrophic members of the phylum *Chlorobi* or members of the order *Chromatiales* would complete the sulfur cycle. Both the phyla *Nitrospira* and *Crenarchaeota* are also known to possess thermophilic sulfate[85] and sulfur reducers[86], although since most of the sequences belong to clone libraries, any further metabolic inference was not possible. The *β Proteobacteria* represents an important cluster for denitrification[36], and many members of the *Rhodocyclaceae* are known denitrifiers (*Sterolibacterium*, *Thauera*, *Azoarcus*). Some other members reduce (per)chlorates (*Dechlorosomas*, *Dechloromonas*)[87] or are methylotrophic like *Methyloversatilis*, which was also detected in the Driefontein IPC borehole. Whether they are denitrifiers or methylotrophs, the high recurrence of clones within this family shared by different samples needs to be further explored. Nevertheless, regardless of the similarities between the three borehole communities at Beatrix, Tau Tona and Driefontein Au mine, no clear signature pointed towards sulfate as the main electron acceptor in the community inhabiting Beatrix and Tau Tona Au mines. Some strains of *Pseudomonas*, *Rheinheimera*, *Stappia* or *Acinetobacter* are known denitrifiers[88], but the nitrate concentration was below the detection limit (Supplementary Table 2). Many of these genera possess abilities to degrade hydrocarbons[89] and they have been detected and isolated from hydrocarbon-contaminated environments. Abiogenic hydrocarbons in the subsurface of the Witwatersrand Basin and their possible role as energy and carbon sources have been reported before[82]. Whether or not some of the members of this community couple the oxidation of organic matter to nitrate reduction, the community seems well represented by obligate heterotrophic aerobes (*Thalassolituus*, *Lysobacter*, *Klebsiella*), which is in agreement with the geochemical data. The dissolved O_2 concentrations ranged from 31 to 63 μM (Supplementary Table 2), which were higher than previously reported values of <3 to 25 μM from other boreholes and mines[13].

The geochemical and isotopic composition indicate the borehole water represents paleometeoric water inhabited by aerobic and anaerobic bacterial and archaeal populations, which have been systematically detected in recent years in different mines of the Witwatersrand Basin and confirms their indigenous character as a genuine subsurface ecosystem.

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