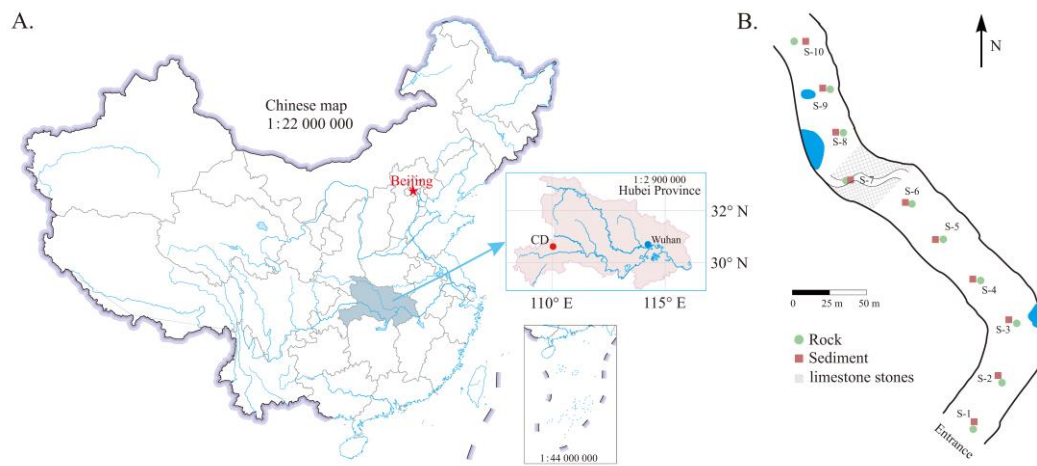


Supplementary Material

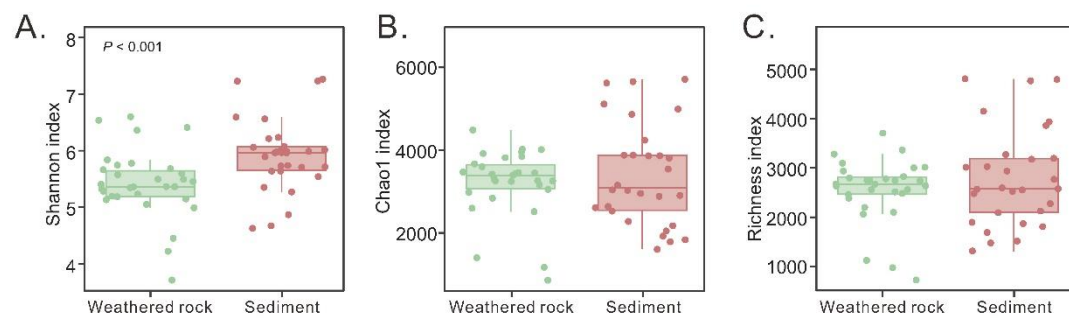
Nitrate determines the bacterial niche specificity and impacts microbial functions in a subsurface karst cave environment

Xiaoyan Liu, Hongmei Wanga*, Weiqi Wang, Xiaoyu Cheng, Yiheng Wang, Qing Li, Lu Li, Liyuan Ma, Xiaolu Lu, Olli H Tuovinen

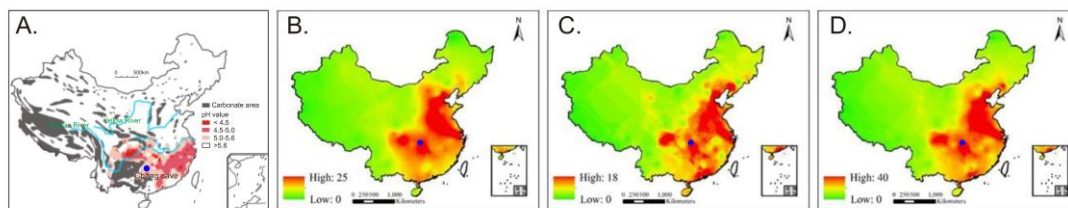
* **Correspondence:** Prof. Hongmei Wang: wanghmei04@163.com or hmwang@cug.edu.cn



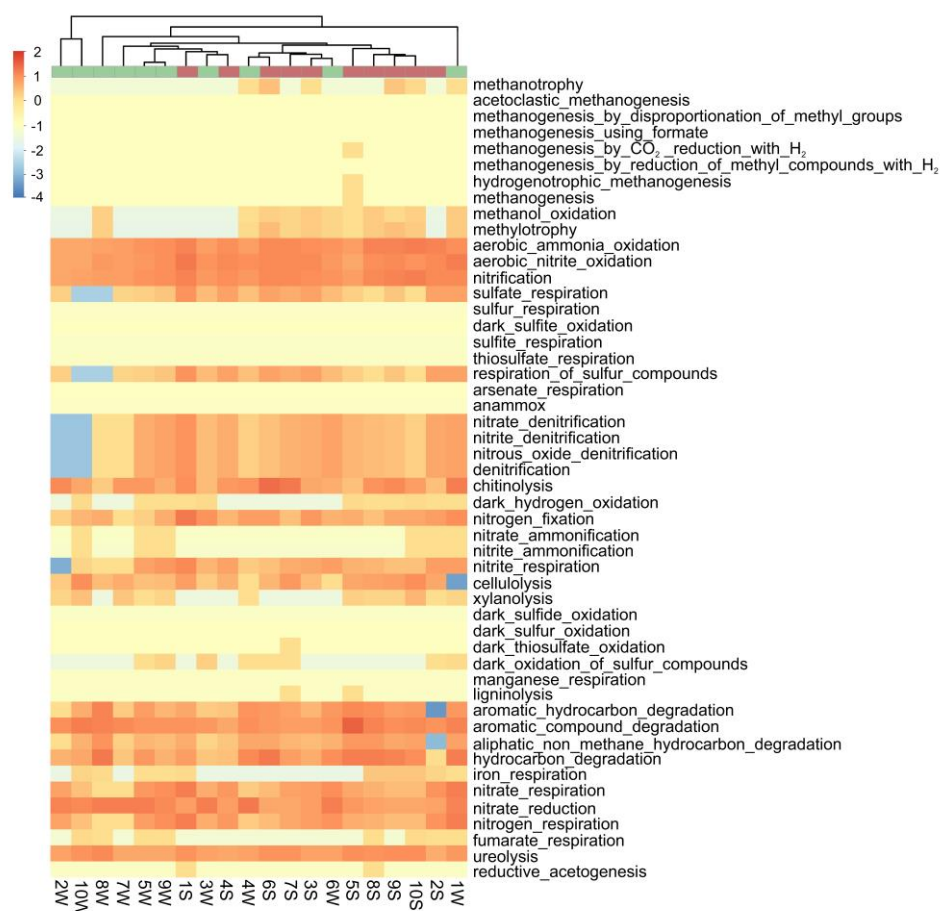
Supplementary Figure 1. The geographic location (30°39'26.01"N, 109°58'27.49" E) (A) and schematic diagram of the sampling site in the Chang Cave (B), Hubei province.



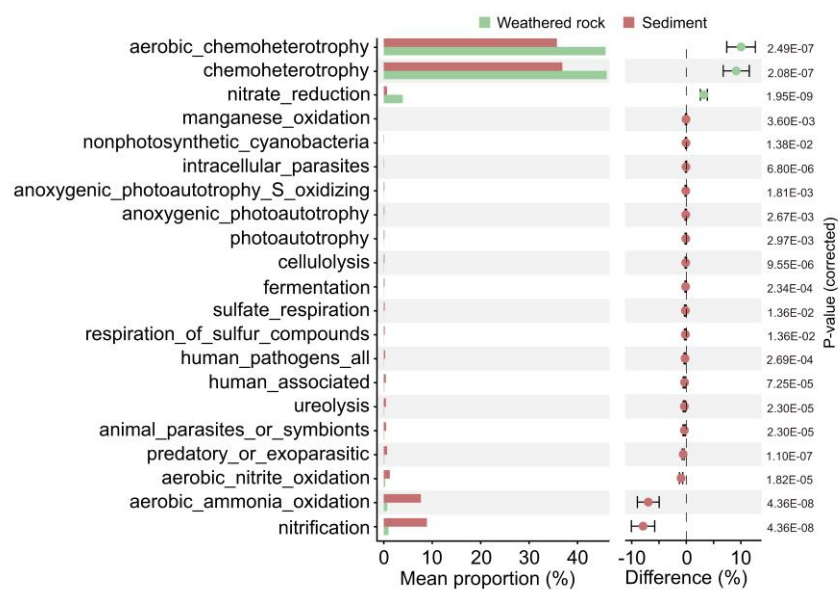
Supplementary Figure 2. The alpha diversity (Shannon index (A), Chao1 index (B) and Richness index (C)) of weathered rocks and sediments in the Chang Cave.



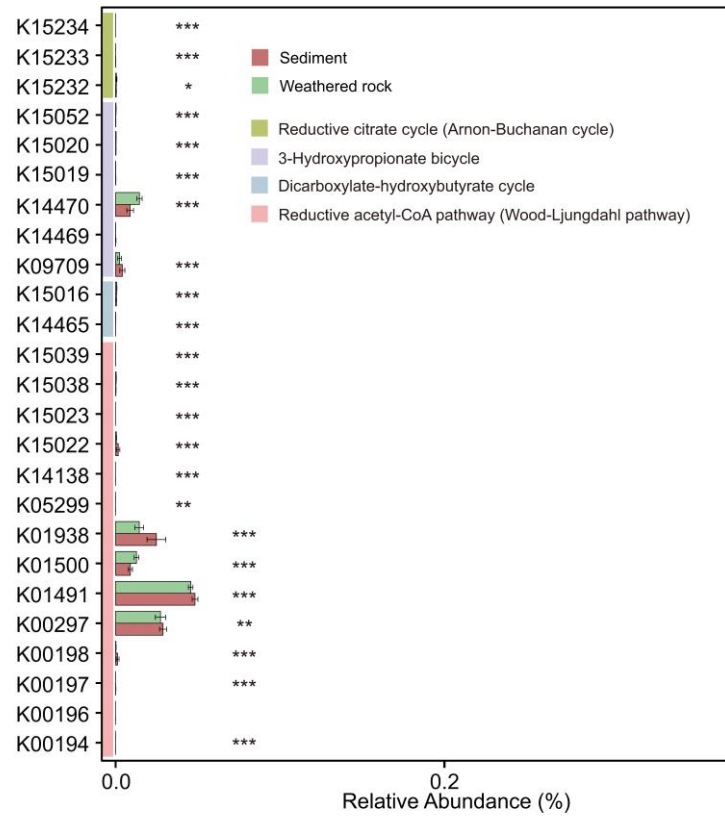
Supplementary Figure 3. (A) Location of the Chang Cave on acid rain distribution map in China, modified after http://english.mep.gov.cn/standards/reports/soe/soe2011/201307/t20130712_255427.htm, Yuan and Cao (2008), and Yun et al. (2016). (B-D) Location of the Chang Cave in the map of spatial distribution characteristics of atmospheric inorganic nitrogen wet deposition in China, 2011-2015, modified after Jia et al. (2019). Data exclude Taiwan and the South China Sea islands of China. (B) $\text{NH}_4^+\text{-N}$; (C) $\text{NO}_3^-\text{-N}$; (D) $\text{NH}_4^+\text{-N} + \text{NO}_3^-\text{-N}$. Unit in $\text{kg N ha}^{-1} \text{ yr}^{-1}$. The blue dot in the map shows the location of the Chang Cave.



Supplementary Figure 4. Heatmap of the top 50 functions predicted by FARPROTAX related to biochemical cycle processes.



Supplementary Figure 5. Predicted functions of biochemical cycle processes in weathered rocks and sediments by FARPROTAX. Only the top 21 functions with significant differences between weathered rocks and sediments, and the relative abundance > 0.3% were selected.



Supplementary Figure 6. Relative abundance of the predicted genes related to prokaryotic carbon fixation in weathered rocks and sediments. Significance level: $P < 0.05$, *, $P < 0.01$, **, $P < 0.001$, ***.

Supplementary Table 1. Microbial classes significantly associated with nitrate with a Spearman rank correlation coefficient < 0.05.

Class	Weathered rock	Sediment
ABY1	-0.49	/
<i>Acidobacteriia</i>	-0.62	/
<i>Actinobacteria</i>	0.48	/
AKAU4049	0.39	/
<i>Anaerolineae</i>	-0.45	0.41
AT-s3-28	/	0.47
BD7-11	-0.38	/
<i>Blastocatellia</i> _(Subgroup_4)	-0.57	/
BRC1	-0.49	/
<i>Campylobacteria</i>	-0.54	/
<i>Chloroflexia</i>	0.45	/
<i>Chthonomonadetes</i>	-0.46	-0.49
<i>Deferribacteres</i>	-0.42	/
<i>Deltaproteobacteria</i>	-0.55	/
FFCH5909	-0.39	/
<i>Gammaproteobacteria</i>	-0.47	/
<i>Gemmatimonadetes</i>	-0.38	-0.53
Gitt-GS-136	0.49	/
<i>Holophagae</i>	-0.65	-0.39
<i>Latescibacteria</i>	-0.4	/
LD1-PA32	/	0.43
Lineage_Ila	-0.53	/
Lineage_Ilb	-0.37	/

<i>Longimicrobia</i>	/	0.47
MB-A2-108	/	-0.42
NC10	-0.42	/
<i>Nitrospira</i>	-0.55	/
OM190	-0.47	/
PAUC43f_marine_benthic_group	0.38	0.37
<i>Planctomycetacia</i>	-0.52	/
<i>Rhodothermia</i>	/	0.56
S0134_terrestrial_group	0.44	/
Subgroup_11	-0.43	/
Subgroup_17	-0.45	/
Subgroup_18	-0.55	/
Subgroup_21	/	0.48
Subgroup_22	-0.55	/
Subgroup_25	-0.45	/
Subgroup_5	-0.39	/
Subgroup_6	-0.68	/
<i>Thermoleophilia</i>	-0.47	/
TK10	-0.6	/
unclassified_ <i>Acidobacteria</i>	-0.55	0.45
unclassified_ <i>Actinobacteria</i>	/	-0.38
unclassified_ <i>Gemmatimonadetes</i>	0.6	/
unculture	-0.39	-0.38
<i>Verrucomicrobiae</i>	-0.5	/
WS6_(Dojkabacteria)	/	0.51
<i>Zixibacteria</i>	-0.57	/

/: no significant correlation.

Supplementary Table 2. Detailed affiliations of the keystone ASVs in the Zi-Pi plots. Bolded species are reported to be involved in nitrogen metabolism previously.

Network	Classification	No. of ASVs	Phylum	Genus
Weathered Rock	Connector	8	<i>Acidobacteria</i>	Subgroup_7 (2), Subgroup_6 (2) , Subgroup_10, RB41 , JGI_0001001-H03, uncultured
		3	<i>Chloroflexi</i>	<i>Thermobaculum</i> , AKYG1722, JG30-KF-CM66
		1	<i>Firmicutes</i>	<i>Bacillus</i>
		8	<i>Gemmatimonadetes</i>	<i>Gemmatimonas</i> , unclassified_ <i>Gemmatimonadaceae</i> , uncultured (6)
		4	<i>Nitrospirae</i>	<i>Nitrospira</i> (4)
		1	<i>Patescibacteria</i>	<i>Saccharimonadales</i>
		14	<i>Proteobacteria</i>	<i>Nitrosospira</i> (3), MND1 (2), <i>Sphingomonas</i> (2) , uncultured (2), <i>Dongia</i> , <i>Woeseia</i> , <i>Escherichia-Shigella</i> , unclassified_ <i>Rhizobiaceae</i> , unclassified_ <i>Hyphomicrobiaceae</i>
		1	<i>Rokubacteria</i>	<i>Rokubacteriales</i>

	89	<i>Actinobacteria</i>	0319-7L14, 67-14 (12), <i>Amycolatopsis</i> , <i>Conexibacter</i> (2), <i>Crossiella</i> (13), <i>Gaiella</i> (4), <i>Haloactinopolyspora</i> , IMCC26256 (2), <i>Kribbella</i> (4), MB-A2-108, <i>Nocardioides</i> , <i>Promicromonospora</i> , <i>Pseudonocardia</i> (12), <i>Rubrobacter</i> (10), <i>Solirubrobacter</i> (3), <i>Sporichthya</i> , <i>Stackebrandtia</i> , <i>Streptomyces</i> , unclassified_ <i>Acidimicrobiia</i> , unclassified_ <i>Actinobacteria</i> (4), unclassified_ <i>Frankiales</i> , unclassified_ <i>Nocardioidaceae</i> , unclassified_ <i>Pseudonocardiaceae</i> , uncultured(10)
Module hub	2	Proteobacteria	wb1-P19, IS-44
	1	Chloroflexi	uncultured
	15	<i>Acidobacteria</i>	<i>Bryobacter</i> , RB41 (2) , Subgroup_10 (3), Subgroup_2 , Subgroup_20, Subgroup_22, Subgroup_6 (4) , Subgroup_9
	58	<i>Actinobacteria</i>	0319-7L14 (2), 67-14 (4), <i>Actinophytocola</i> , <i>Aeromicrobium</i> , <i>Amycolatopsis</i> , Crossiella (10) , <i>Gaiella</i> (3), IMCC26256 (8), MB-A2-108 (3), <i>Nocardioides</i> (2), Pseudonocardia (2) , <i>Quadrisphaera</i> , <i>Streptomyces</i> , unclassified_ <i>Actinobacteria</i> , unclassified_ <i>Propionibacteriaceae</i> , uncultured (17)
	18	<i>Chloroflexi</i>	AKYG1722, Gitt-GS-136 (6), JG30-KF-CM45 (2), JG30-KF-CM66 (2), KD4-96 (5), TK10 (2)
	2	<i>Firmicutes</i>	<i>Bacillus</i> (2)
	1	<i>GAL15</i>	GAL15
	11	<i>Gemmatimonadetes</i>	unclassified_ <i>Gemmatimonadaceae</i> (2), uncultured (9)
	2	<i>Latescibacteria</i>	<i>Latescibacteria</i>

54	<i>Proteobacteria</i>	<i>Acetobacter</i> , <i>bacteriap25</i> , <i>Dongia</i> (2), <i>Dyella</i> , Ellin6055 , Ga0077536 (2) , <i>Haliangium</i> , IS-44 (2) , MND1 (8) , NB1-j , <i>Nordella</i> , <i>Novosphingobium</i> , PLTA13 (2), <i>Sphingomonas</i> , <i>Steroidobacter</i> , SWB02 , TRA3-20, unclassified_ <i>Hyphomicrobiaceae</i> , unclassified_ <i>Methylobacteriaceae</i> , unclassified_ <i>Rhizobiaceae</i> , unclassified_ <i>Sphingomonadaceae</i> , uncultured (11), wb1-P19 (9) , <i>Woeseia</i>
3	<i>Rokubacteria</i>	<i>Rokubacteriales</i> (3)

Supplementary Table 3. The relative abundance of key functional genes in the six microbial carbon fixation pathways.

Microbial carbon fixation pathways	gene	Group	RPM	tax
Calvin cycle	K00314	Weathered rock (+)	1.91E-05	sarcosine dehydrogenase [EC:1.5.8.3]
		Sediment	2.10E-05	
	K00150	Weathered rock	3.70E-06	glyceraldehyde-3-phosphate dehydrogenase (NAD(P)) [EC:1.2.1.59]
		Sediment	2.90E-06	
	K00615	Weathered rock	1.27E-03	transketolase [EC:2.2.1.1]
		Sediment	1.41E-03	
	K00855	Weathered rock	1.83E-05	phosphoribulokinase [EC:2.7.1.19]
		Sediment	5.65E-05	
	K00927	Weathered rock	5.94E-04	phosphoribulokinase [EC:2.7.1.19]
		Sediment	6.64E-04	
	K01601	Weathered rock	4.41E-05	ribulose-bisphosphate carboxylase large chain [EC:4.1.1.39]
		Sediment	1.27E-04	

K01602	Weathered rock	6.84E-06	ribulose-bisphosphate carboxylase small chain [EC:4.1.1.39]
	Sediment	1.85E-05	
K01623	Weathered rock (+)	7.20E-05	fructose-bisphosphate aldolase, class I [EC:4.1.2.13]
	Sediment	8.09E-05	
K01624	Weathered rock (+)	4.89E-04	fructose-bisphosphate aldolase, class II [EC:4.1.2.13]
	Sediment	4.33E-04	
K01807	Weathered rock (-)	3.30E-05	ribose 5-phosphate isomerase A [EC:5.3.1.6]
	Sediment	1.17E-04	
K01808	Weathered rock	3.22E-04	ribose 5-phosphate isomerase B [EC:5.3.1.6]
	Sediment	2.53E-04	
K02446	Weathered rock (+)	4.21E-04	fructose-1,6-bisphosphatase II [EC:3.1.3.11]
	Sediment	2.63E-04	
K03841	Weathered rock (-)	6.75E-05	fructose-1,6-bisphosphatase I [EC:3.1.3.11]
	Sediment	2.31E-04	
K11532	Weathered rock	2.04E-11	fructose-1,6-bisphosphatase II / sedoheptulose-1,7-bisphosphatase

Reductive tricarboxylic acid cycle		Sediment	1.40E-08	[EC:3.1.3.11 3.1.3.37]
	K00169	Weathered rock	5.80E-05	pyruvate ferredoxin oxidoreductase, alpha subunit [EC:1.2.7.1]
		Sediment	7.37E-05	
	K00170	Weathered rock (-)	6.39E-05	pyruvate ferredoxin oxidoreductase, beta subunit [EC:1.2.7.1]
		Sediment	7.83E-05	
	K00171	Weathered rock (-)	6.39E-06	pyruvate ferredoxin oxidoreductase, delta subunit [EC:1.2.7.1]
		Sediment	6.00E-06	
	K00172	Weathered rock (-)	1.72E-05	pyruvate ferredoxin oxidoreductase, gamma subunit [EC:1.2.7.1]
		Sediment	2.71E-05	
	K00174	Weathered rock	7.29E-04	2-oxoglutarate ferredoxin oxidoreductase subunit alpha [EC:1.2.7.3]
		Sediment	6.65E-04	
	K00175	Weathered rock	4.08E-04	2-oxoglutarate ferredoxin oxidoreductase subunit beta [EC:1.2.7.3]
		Sediment	3.87E-04	
	K00176	Weathered rock (-)	3.02E-06	2-oxoglutarate ferredoxin oxidoreductase subunit delta [EC:1.2.7.3]

3-Hydroxypropionate bicycle (key enzyme not detected: Propionyl-CoA synthase)	K00177	Sediment	1.17E-05	2-oxoglutarate ferredoxin oxidoreductase subunit gamma [EC:1.2.7.3]
		Weathered rock (-)	4.37E-06	
	K01637	Sediment	1.76E-05	isocitrate lyase [EC:4.1.3.1]
		Weathered rock (+)	7.09E-04	
	K01643	Sediment	4.74E-04	citrate lyase subunit alpha / citrate CoA-transferase [EC:4.1.3.6 2.8.3.10]
		Weathered rock (-)	7.07E-06	
	K01644	Sediment	2.75E-05	citrate lyase subunit beta / citryl-CoA lyase [EC:4.1.3.6 4.1.3.34]
		Weathered rock (+)	7.33E-04	
	K01646	Sediment	5.20E-04	citrate lyase subunit gamma [EC:4.1.3.6]
		Weathered rock (-)	2.40E-07	
	K14468	Sediment	1.06E-06	malonyl-CoA reductase / 3-hydroxypropionate dehydrogenase (NADP+) [EC:1.2.1.75 1.1.1.298]
		Weathered rock	3.59E-07	
	K08691	Sediment	6.44E-07	malyl-CoA lyase [EC:4.1.3.24]
		Weathered rock (-)	8.39E-05	
		Sediment	6.22E-05	

3-Hydroxypropionate/4-hydroxybutyrate cycle	K14450	Weathered rock	1.83E-07	L-malyl-CoA/beta-methylmalyl-CoA lyase
		Sediment (+)	1.92E-07	
	K08691	Weathered rock (-)	8.39E-05	malyl-CoA lyase [EC:4.1.3.24]
		Sediment	6.22E-05	
	K11263	Weathered rock (+)	1.22E-03	acetyl-/propionyl-CoA carboxylase, biotin carboxylase, biotin carboxyl carrier protein [EC:6.3.4.14]
		Sediment	6.74E-04	
	K15036	Weathered rock	4.09E-06	acetyl-CoA/propionyl-CoA carboxylase [EC:6.4.1.2 6.4.1.3]
		Sediment	4.73E-06	
	K01964	Weathered rock (+)	2.77E-09	acetyl-CoA/propionyl-CoA carboxylase [EC:6.4.1.2 6.4.1.3]
		Sediment (+)	7.99E-09	
	K14534	Weathered rock	9.93E-07	4-hydroxybutyryl-CoA dehydratase / vinylacetyl-CoA-Delta-isomerase [EC:4.2.1.120 5.3.3.3]
		Sediment	2.14E-06	
	K14468	Weathered rock	3.59E-07	malonyl-CoA reductase / 3-hydroxypropionate dehydrogenase (NADP+) [EC:1.2.1.75 1.1.1.298]
		Sediment	6.44E-07	

	K01595	Weathered rock (-)	8.40E-04	phosphoenolpyruvate carboxylase [EC:4.1.1.31]
		Sediment (-)	1.01E-03	
Dicarboxylate/ 4- hydroxybutyrate cycle	K15016	Weathered rock (+)	7.49E-06	3-hydroxybutyryl-CoA dehydratase / 3-hydroxyacyl-CoA dehydrogenase [EC:4.2.1.55 1.1.1.35]
		Sediment (+)	5.01E-06	
	K14465	Weathered rock	9.18E-07	succinate semialdehyde reductase (NADPH) [EC:1.1.1.-]
		Sediment	3.20E-07	
Reductive acetyl-CoA pathway (Wood- Ljungdahl pathway)	K00190	Weathered rock	1.40E-10	carbon monoxide dehydrogenase / acetyl-CoA synthase subunit beta [EC:1.2.7.4 1.2.99.2 2.3.1.169]
		Sediment	6.88E-09	
	K14138	Weathered rock	2.32E-09	carbon monoxide dehydrogenase / acetyl-CoA synthase subunit alpha [EC:1.2.7.4 1.2.99.2 2.3.1.169]
		Sediment	3.45E-08	

The genes with significant differences ($P < 0.05$) between weathered rocks and sediments are shown in bold. +/-: positively/ negatively correlated with nitrate.

Supplementary Table 4. Key enzymes involved in methane metabolism and carbon fixation pathways in prokaryotes with significant correlation with NO₃⁻ in weathered rocks and sediments. Significant Spearman rank correlation coefficients ($P < 0.05$) were shown.

	KO	KO3	KO4	EC	WR	S
Methane oxidation, methanotroph	K14028		mdh1, mxaF; methanol dehydrogenase (cytochrome c) subunit 1	[EC:1.1.2.7]	- 0.46	/
	K14029		mdh2, mxaI; methanol dehydrogenase (cytochrome c) subunit 2	[EC:1.1.2.7]	- 0.55	/
	K16254		mxkJ; mxaJ protein	[EC:1.1.2.7]	- 0.56	/
	K16255	Methan e metabol ism	mxAG; cytochrome c-L	[EC:1.1.2.7]	-0.6	/
	K16256		mxAA; mxaA protein	[EC:1.1.2.7]	- 0.58	/
	K16257		mxAC; mxaC protein	[EC:1.1.2.7]	-0.6	/
	K16258		mxAK; mxaK protein	[EC:1.1.2.7]	-0.6	/
	K16259		mxAL; mxaL protein	[EC:1.1.2.7]	-0.6	/
	K16260		mxAD; mxaD protein	[EC:1.1.2.7]	- 0.62	/

Phosphate acetyltransferase- acetate kinase pathway	K13788		pta; phosphate acetyltransferase	[EC:2.3.1.8]	0.39	/
	K08691		mcl; malyl-CoA/(S)-citramalyl-CoA lyase	[EC:4.1.3.24 4.1.3.25]	- 0.47	/
	K14449		mch, mcd; 2-methylfumaryl-CoA hydratase	[EC:4.2.1.148]	- 0.44	/
	K14472	Carbon fixation pathway s in prokary otes	smtB; succinyl-CoA:(S)-malate CoA-transferase subunit B	[EC:2.8.3.22]	0.44	/
3- Hydroxypropionate bicycle	K15018		3-hydroxypropionyl-coenzyme A synthetase	[EC:6.2.1.36]	0.38	/
	K15019		3-hydroxypropionyl-coenzyme A dehydratase	[EC:4.2.1.116]	0.37	/
	K00240		sdhB, frdB; succinate dehydrogenase / fumarate reductase, iron-sulfur subunit	[EC:1.3.5.1 1.3.5.4]	/	0.37
	K01964		acetyl-CoA/propionyl-CoA carboxylase	[EC:6.4.1.2 6.4.1.3]	/	0.43
	K18594		3-hydroxypropionyl-CoA synthetase (ADP-forming)	[EC:6.2.1.-]	/	0.41
	K18605		biotin carboxyl carrier protein	[EC:6.4.1.2]	/	-0.49
Dicarboxylate- hydroxybutyrate	K14534		abfD; 4-hydroxybutyryl-CoA dehydratase / vinylacetyl- CoA-Delta-isomerase	[EC:4.2.1.120 5.3.3.3]	- 0.57	/

te cycle	K18593	4-hydroxybutyryl-CoA synthetase (ADP-forming)	[EC:6.2.1.-]	/	0.51
	K18605	biotin carboxyl carrier protein	[EC:6.4.1.2]	- 0.42	/
Hydroxypropionate- hydroxybutyrate cycle	K15020	acryloyl-coenzyme A reductase	[EC:1.3.1.84]	/	0.49
	K15036	acetyl-CoA/propionyl-CoA carboxylase	[EC:6.4.1.2 6.4.1.3 2.1.3.15]	/	0.44
	K15052	propionyl-CoA carboxylase	[EC:6.4.1.3 2.1.3.15]	/	0.44
Incomplete reductive citrate cycle	K18209	tfrA; fumarate reductase (CoM/CoB) subunit A	[EC:1.3.4.1]	- 0.38	/
Phosphate acetyltransferase-acetate kinase pathway	K00925	ackA; acetate kinase	[EC:2.7.2.1]	/	-0.47
Reductive acetyl-CoA pathway (Wood- Ljungdahl pathway)	K00196	cooF; anaerobic carbon-monoxide dehydrogenase iron sulfur subunit	[EC:1.2.7.4]	- 0.51	/
	K01938	fhs; formate--tetrahydrofolate ligase	[EC:6.3.4.3]	- 0.51	/
Reductive	K00031	IDH1, IDH2, icd; isocitrate dehydrogenase	[EC:1.1.1.42]	-	/

citrate cycle (Arnon- Buchanan cycle)				0.42	
	K01595	ppc; phosphoenolpyruvate carboxylase	[EC:4.1.1.31]	- 0.37	/
	K01681	ACO, acnA; aconitate hydratase	[EC:4.2.1.3]	- 0.37	/
	K01903	sucC; succinyl-CoA synthetase beta subunit	[EC:6.2.1.5]	- 0.38	/
	K01959	pycA; pyruvate carboxylase subunit A	[EC:6.4.1.1]	- 0.45	/
	K00170	porB; pyruvate ferredoxin oxidoreductase beta subunit	[EC:1.2.7.1]	/	-0.38
	K00239	sdhA, frdA; succinate dehydrogenase / fumarate reductase, flavoprotein subunit	[EC:1.3.5.1 1.3.5.4]	/	0.43
	K01676	E4.2.1.2A, fumA, fumB; fumarate hydratase, class I	[EC:4.2.1.2]	/	-0.39
	K01681	ACO, acnA; aconitate hydratase	[EC:4.2.1.3]	/	-0.37
	K03737	por, nifJ; pyruvate-ferredoxin/flavodoxin oxidoreductase	[EC:1.2.7.1 1.2.7.-]	/	0.43
	K18559	frdD; NADH-dependent fumarate reductase subunit D	[EC:1.3.1.6]	/	-0.45

/: no significant correlation.

Reference

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