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Characterization of incubation experiments and development of an enrichment culture capable of ammonium oxidation under iron reducing conditions

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1. Supplemental Methods

1.1 Thermodynamic Consideration of Feammox

The change in Gibbs free energy of Equation 1 was calculated to determine the thermodynamic feasibility of the Feammox reactions using the following equation:

$$\Delta G_r = \Delta G_r^0 + RT \ln \frac{(C)^c (D)^d}{(A)^a (B)^b}$$

and: $$\Delta G_r^0 = c\Delta G_{fC}^0 + d\Delta G_{fD}^0 - a\Delta G_{fA}^0 - b\Delta G_{fB}^0$$

R is the gas constant, which equals 0.008314 kJ mol^{-1}. K, and T is the absolute temperature in ^{o} Kelvin (297.15 K). Free energies of formation were obtained from Stumm and Morgan (1996): $\Delta G_f^0(NH_4^+) = -79.37$ kJ mol^{-1}, $\Delta G_f^0(NO_2^-) = -37.2$ kJ mol^{-1}, $\Delta G_f^0(H_2O) = -237.18$ kJ mol^{-1}, $\Delta G_f^0(Fe^{2+}) = -78.87$ kJ mol^{-1}. $\Delta G_f^0(Fe_2O_3 \cdot 0.5H_2O) = -711$ kJ mol^{-1} (Majzlan *et al*., 2004). For biogeochemical reactions involving H^+, requires converting from standard condition (pH=0) to biochemical conditions: $\Delta G^{0'} = \Delta G^0 + m\Delta G_f^{'}(H^+)$, where m is the net number of H^+ in the reaction and $\Delta G_f^{'}(H^+)$ is calculated as -5.69 kJ mol^{-1} per pH unit (Madigan *et al*., 2002). The chemical activity values used in the calculation are based on our incubation experiments:

$C_{NH_4^+}$ = 2 mmol L^{-1}, $C_{NO_2^-}$ = 10μmol L^{-1}, $C_{Fe^{2+}} \leq$ 0.01μmol L^{-1} (detection limit), respectively, and pH = 4.0. The dissolved Fe(II) was below the ferrozine method detection limit in the solution due to its sorption onto the Fe(III) oxide. Measurable dissolved Fe was only present in the samples extracted with 0.5M HCl. An activity of 1 was used for the solid-phase Fe(III) oxide minerals, and water.

$$3Fe_2O_3 \cdot 0.5H_2O + 10H^+ + NH_4^+ \rightarrow 6Fe^{2+} + 8.5H_2O + NO_2^- \quad \text{(Equation 1)}$$

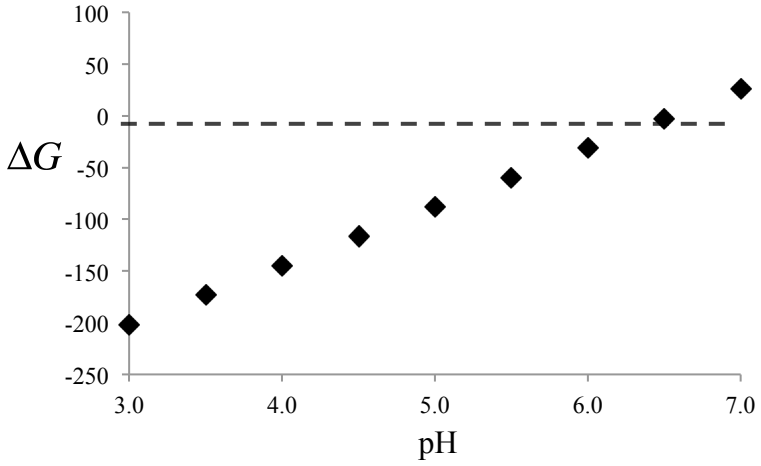
$$\Delta G_r \leq [6\Delta G^0_{fFe^{2+}} + 8.5\Delta G^0_{fH_2O} + 1\Delta G^0_{fNO_2^-} - 3\Delta G^0_{fFe_2O_3 \cdot 0.5H_2O} - 10\Delta G^0_{fH^+} - 1\Delta G^0_{fNH_4^+}]$$
$$+(0.008314 kJ\ mol^{-1})(297.15K)\ln\frac{(C_{Fe^{2+}})^6(C_{H_2O})^{8.5}(C_{NO_2^-})^1}{(C_{Fe_2O_3 \cdot 0.5H_2O})^3(C_{H^+})^{10}(C_{NH_4^+})^1}$$

$$\Delta G_r \leq [6(-78.87) + 8.5(-237.18) + 1(37.2) - 3(-711) - 10(4 \times -5.69) - 1(-79.37)]$$
$$+(0.008314 kJ\ mol^{-1})(297.15K)\ln\frac{(10^{-8})^6(1)^{8.5}(10^{-5})^1}{(1)^3(10^{-4})^{10}(2 \times 10^{-3})^1}$$

$$\Delta G_r \leq -145.08 kJ\ mol^{-1}$$

The ≤ sign is because we used an upper limit (detection limit) for the Fe(II) concentration.

A graph of ΔG vs. pH shows that when maintaining all species concentrations constant except H^+, Feammox should not be feasible when the pH is above 6.5. Hence, we should expect Feammox to occur in acidic environments.



Note: Ferrihydrite is unstable, and with a few exceptions (Majzlan *et al*., 2004), not many values for its ΔG_f^0 have been reported. Hence, many authors use $Fe(OH)_3$ as a stand in for ΔG_r calculations involving ferrihydrite. Using $Fe(OH)_3$ will also result in a negative ΔG_r., when NH_4^+ is oxidized to NO_2^- and $Fe(OH)_3$ reduced to Fe(II) (Yang *et al*., 2012). The same is true for goethite as the Fe(III) source (Clement *et al*., 2005).

1.2 PCR amplification and DGGE analysis

Bacterial universal 16S rRNA gene primer sets V3-2/ V3-3 (Jensen *et al*., 1998) and 27f /519r (Lane, 1991) were used for PCR amplification. Each 25 μL reaction mixture contained 2.5 μL 10×PCR Buffer (500 mM KCl, 25 mM MgCl2, 200 mM Tris-HCl [pH 8.4]. 0.1% Triton X-100), 2.0 μL 2.5mM DNTP mixture (Takara, Japan), 0.3 μL of 10 μM V3-2 and V3-3, 0.13 μL 5U Taq polymerase,1 μL of template DNA, and 18.77 μL sterilized ddH2O. The PCR protocol was as follows: 30 s initial denaturation at 94 °C; 10 cycles with each cycle consisting of 30 s of denaturation at 94 °C, 30 s of annealing at

61°C (the temperature of anneal decreased 0.5 °C after each cycle), and 40 s extension at 72 °C; 25 cycle with each cycle included 30 s denaturation at 94°C, 30 s annealing at 55 °C, and 40 s extension at 72 °C; followed by a final 5 min extension at 72 °C. PCR products stained with 0.02 μL mL^{-1} Genefinder were visualized on 1% (w/v) agarose gel at 120 V for 20 min, and visualized under SYNGENE Genesnap. A much higher degree of diversity was observed with primer sets V3-2/ V3-3, hence its DGGE products were used for the following analysis.

After the DGGE was performed, all visible bands were excised from the gel and used as templates for re-amplification, using primer set V3-1/V3-2 (Jensen *et al*., 1998). The PCR program was initiated with 30 s at 94 °C, followed by 40 cycles of 5 s at 94 °C, 30 s at annealing at 56°C, and 30 s at 70 °C. The PCR products were purified using Qiaquick PCR preps (Qiagen, Valencia, CA) and cloned into a pGEM-T vector (Promega, USA). Positive recombinant clones were identified by PCR, and the PCR products were cleaned with ExoSap treatment and sequences were conducted by Genewiz, Inc., USA. Clone libraries from 12 samples resulted in 721 sequences of partial 16S rRNA gene fragments and the sequences were grouped into operational taxonomic units (OTUs) based on a 5% sequence distance cutoff calculated using the DOTUR program (Schloss and Handelsman, 2005). Six groups of bacteria were classified via a phylogenetic analysis using the Bayesian inference (BI) (Huelsenbeck *et al*., 2001), implemented with MrBayes version 3.1.2 (Ronquist *et al*., 2003). Sequences reported in this study were deposited in GenBank database under accession numbers KC581755 -KC581779.

1.3 Primer design for real-time PCR assay

Two sets of primers, acd320f (5'-CGG TCC AGA CTC CTA CGG GA -3') - 432r (5'-GAC AGG GTT TTA CAG TCC GAA GA -3') and acm342f (5'- GCA ATG GGG GAA ACC CTG AC-3') - 439r (5'-ACC GTC AAT TTC GTC CCT GC -3') were designed for *Acidobacteriaceae* bacteria A8 and *Acidimicrobiaceae* bacterium A6 respectively from clone libraries in this study, using an NCBI Primer-Blast program (http://www. ncbi.nlm.nih.gov/tools/primer-blast). This program did not show any putative sequences deposited in the GenBank, that amplified with the selected primers, could interfere with the experiment. The sequences of *Acidobacteriaceae* bacteria and *Acidimicrobiaceae* bacterium A6 acquired from this study did not exhibit any mismatches with the above primer sequences. Primers were then used for real-time PCR amplification in the soil samples from the incubation experiments.

2. Supplemental Results

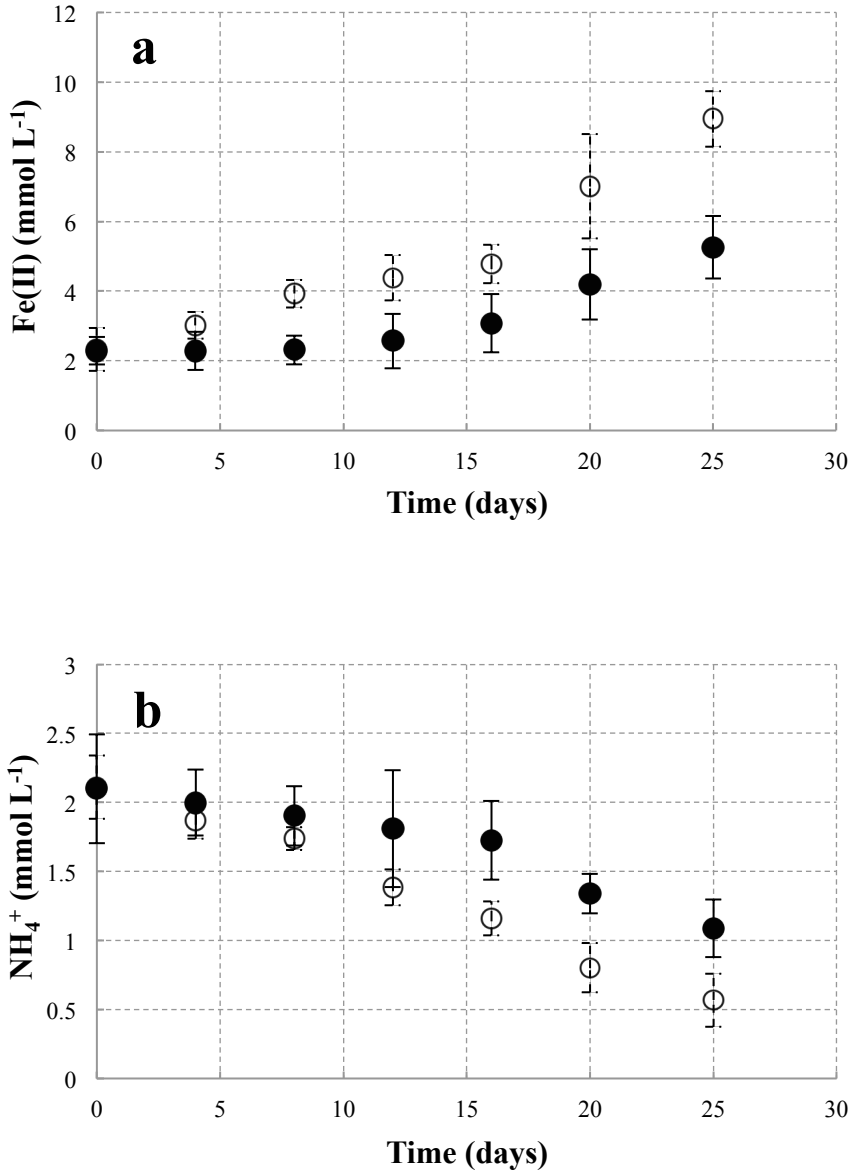


Figure S1. Concentration of Fe(II) and NH_4^+ in 25-day incubation with NH_4Cl and ferrihydrite (○), NH_4Cl and goethite (●). The values represent the mean and standard error (n=3).

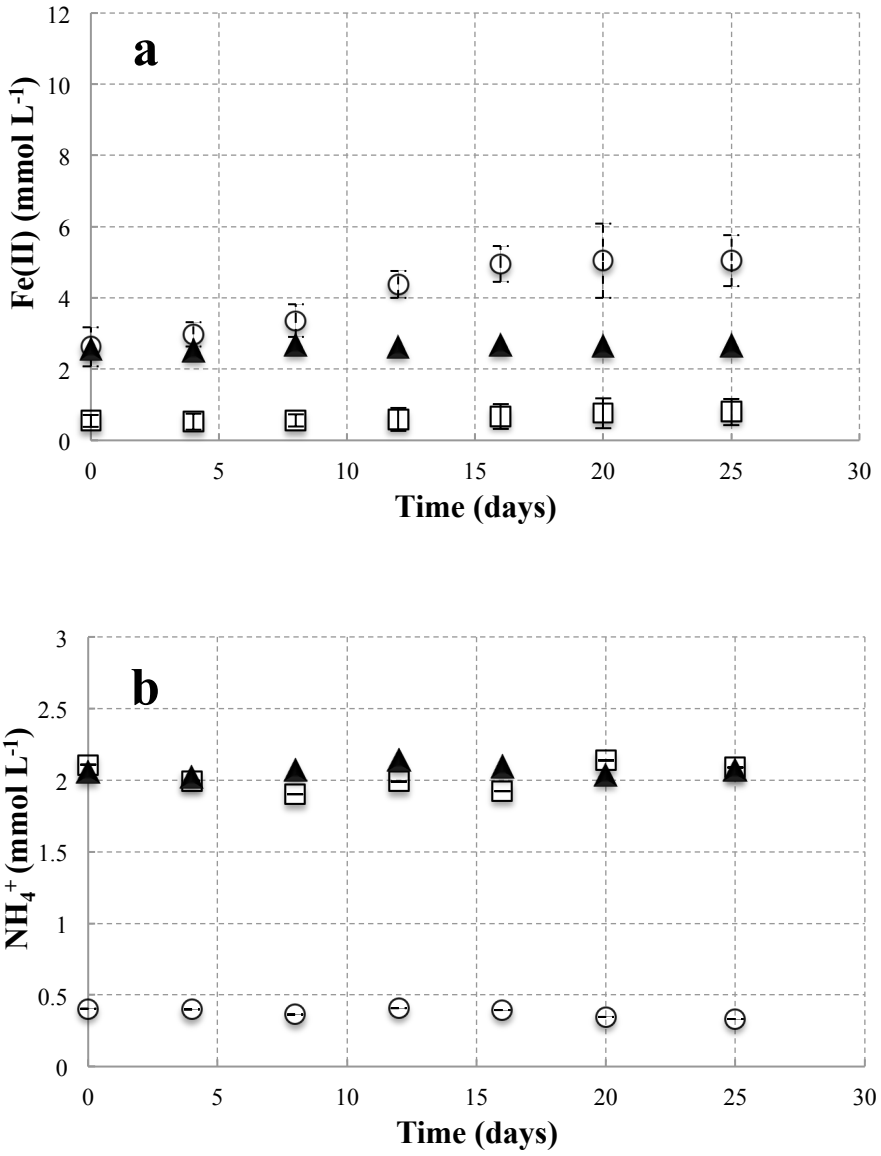


Figure S2. Concentration of Fe(II) and NH_4^+ in 25-day incubations in samples with NH_4Cl (□), ferrihydrite (○), sterilized soil with NH_4Cl and ferrihydrite (▲). The values represent the mean and standard error (n=3).

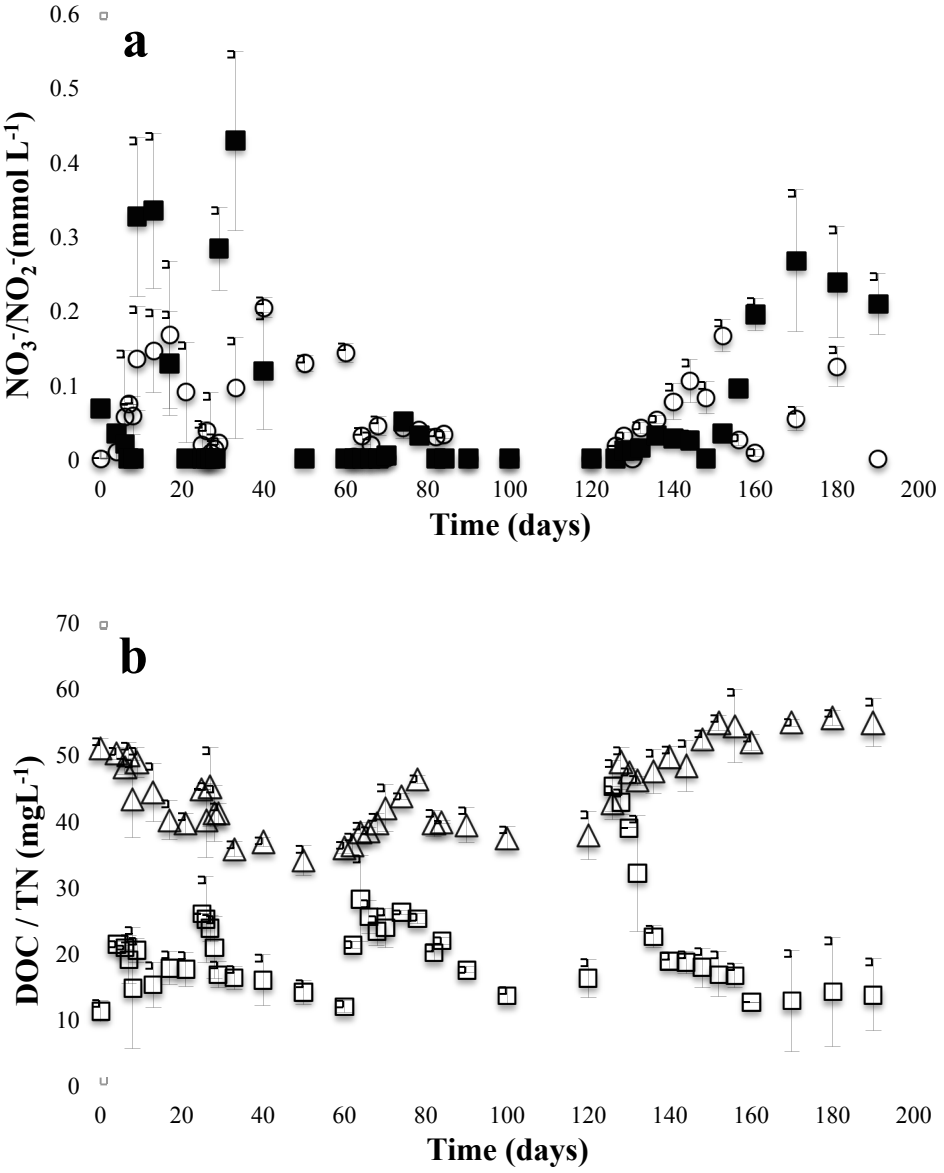
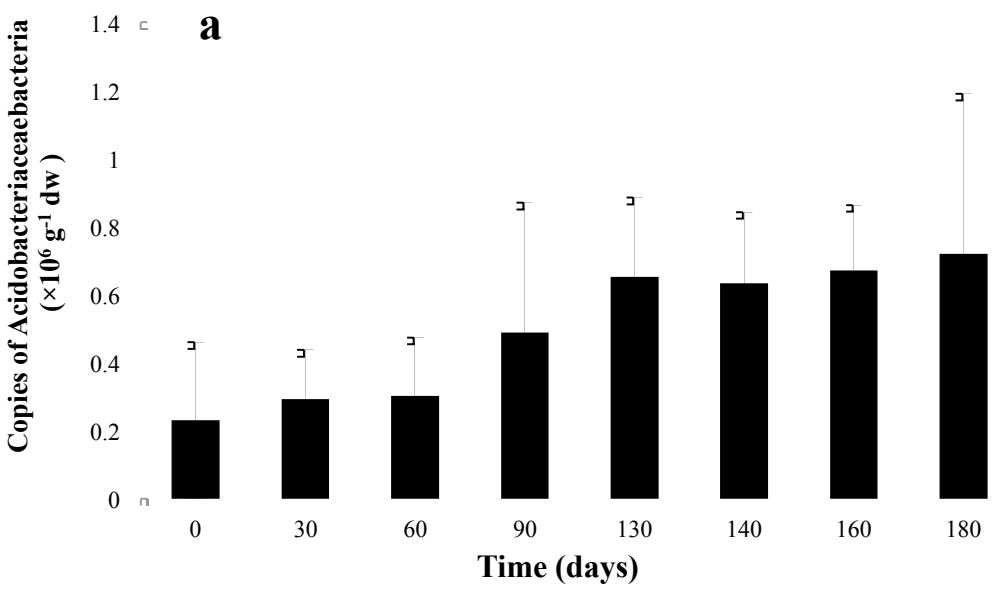
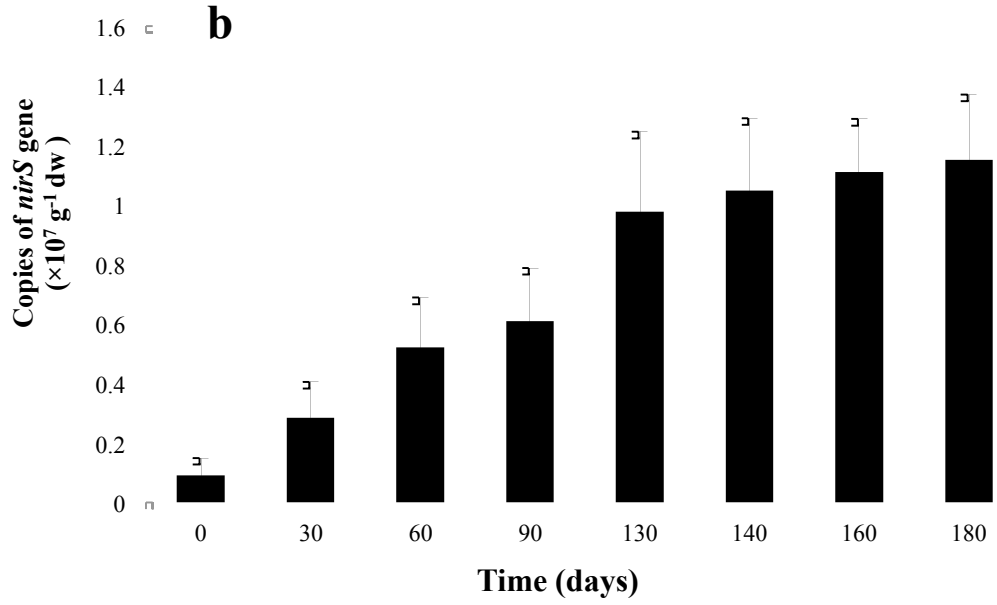


Figure S3. Concentration of (a) NO_3^-(○) and NO_2^- (■) and (b) DOC (Δ) and TN (□) during the 180 day incubation. 25 mmol L^{-1} Fe(III) was added on day 0. 1.0 mmol L^{-1} NH_4^+ was added on days 4, 24, and 60. 0.2 mmol L^{-1} $NaHCO_3$ was added on day 50 and day 90 of the incubation. 1.20 mmol L^{-1} + 2 mmol L^{-1} of NH_4Cl were added on day 125. The values represent the mean and standard error (n=3).


a
Copies of Acidobacteriaceaebacteria (×10^6 g^-1 dw)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
30
60
90
130
140
160
180
Time (days)


b
Copies of nirS gene (×10^7 g^-1 dw)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
30
60
90
130
140
160
180
Time (days)

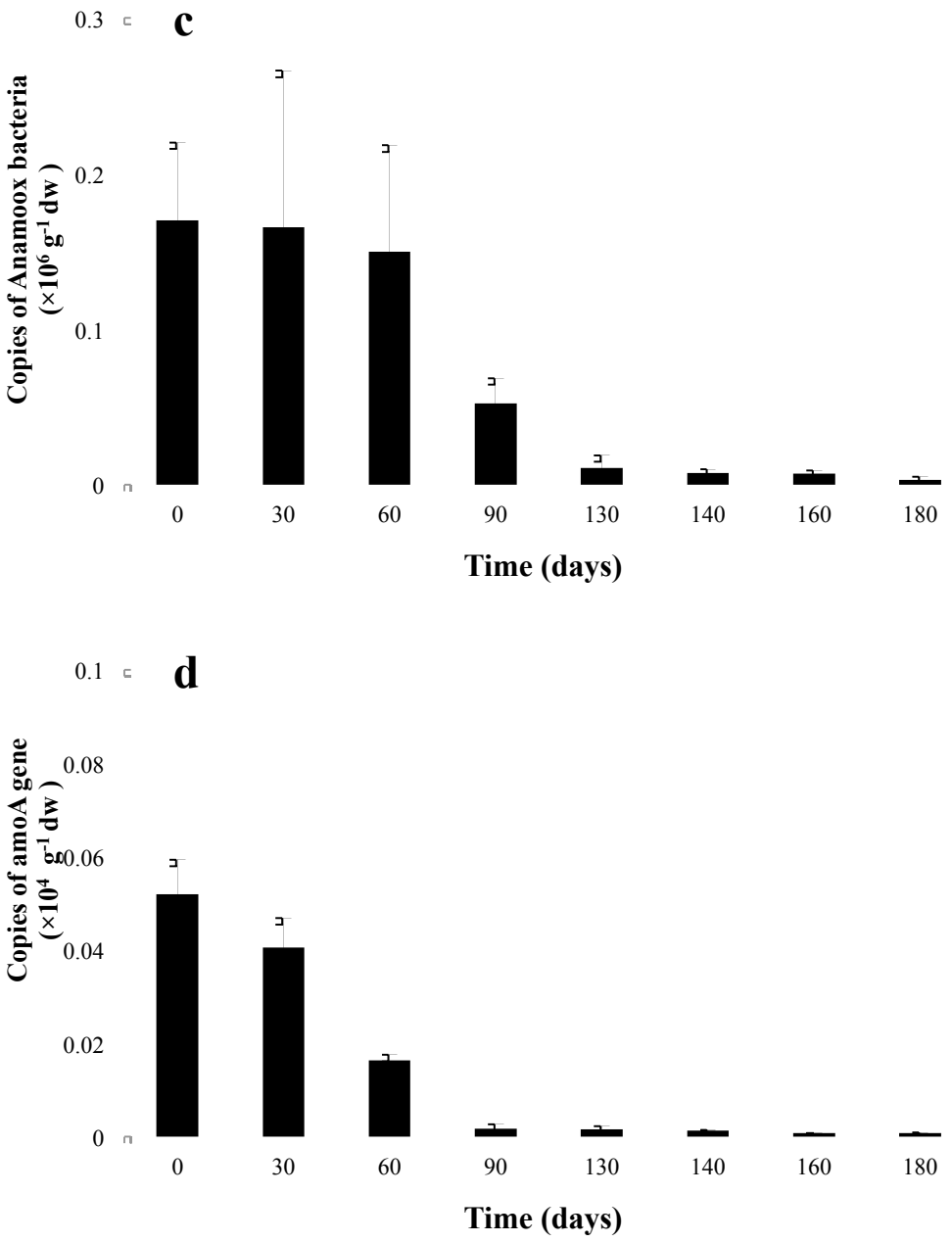
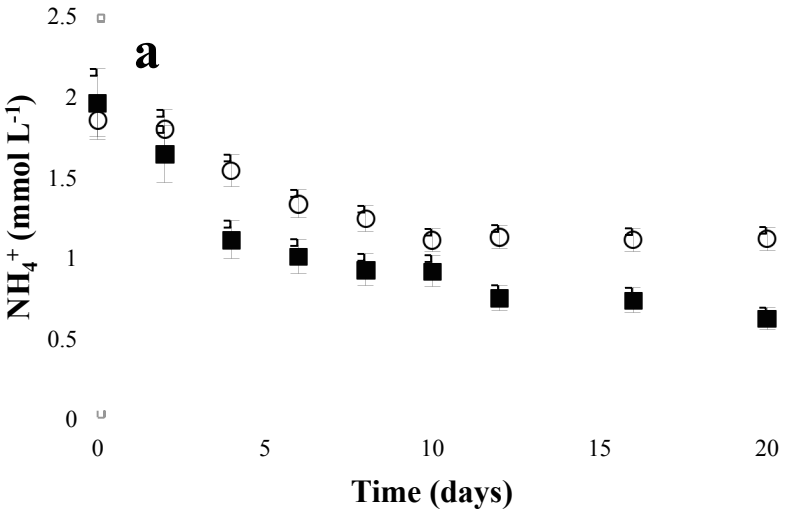
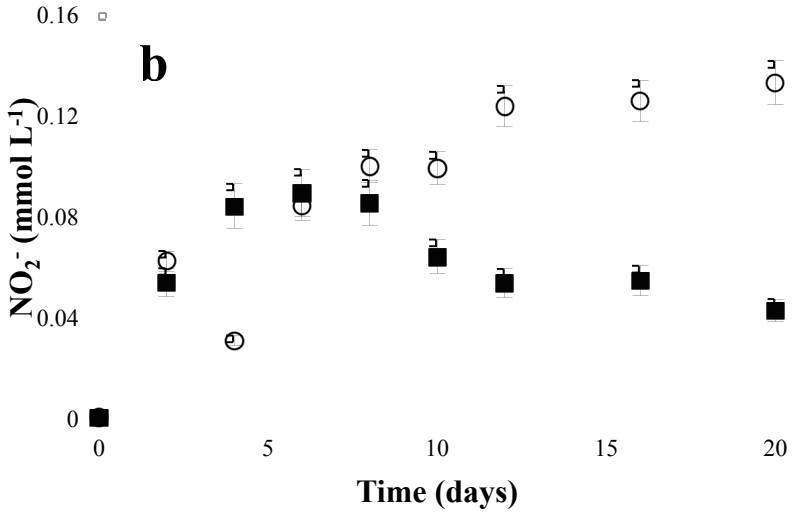


Figure S4. Copy numbers of (a) Acidobacteriaceae bacteria (DGGE band A8), (b) *nirS* gene, (c) anammox bacteria and (d) *amoA* gene during 180 days of anaerobic incubation.


a
NH_4^+ (mmol L^{-1})
2.5
2
1.5
1
0.5
0
0
5
10
15
20
Time (days)


b
NO_2^- (mmol L^{-1})
0.16
0.12
0.08
0.04
0
0
5
10
15
20
Time (days)

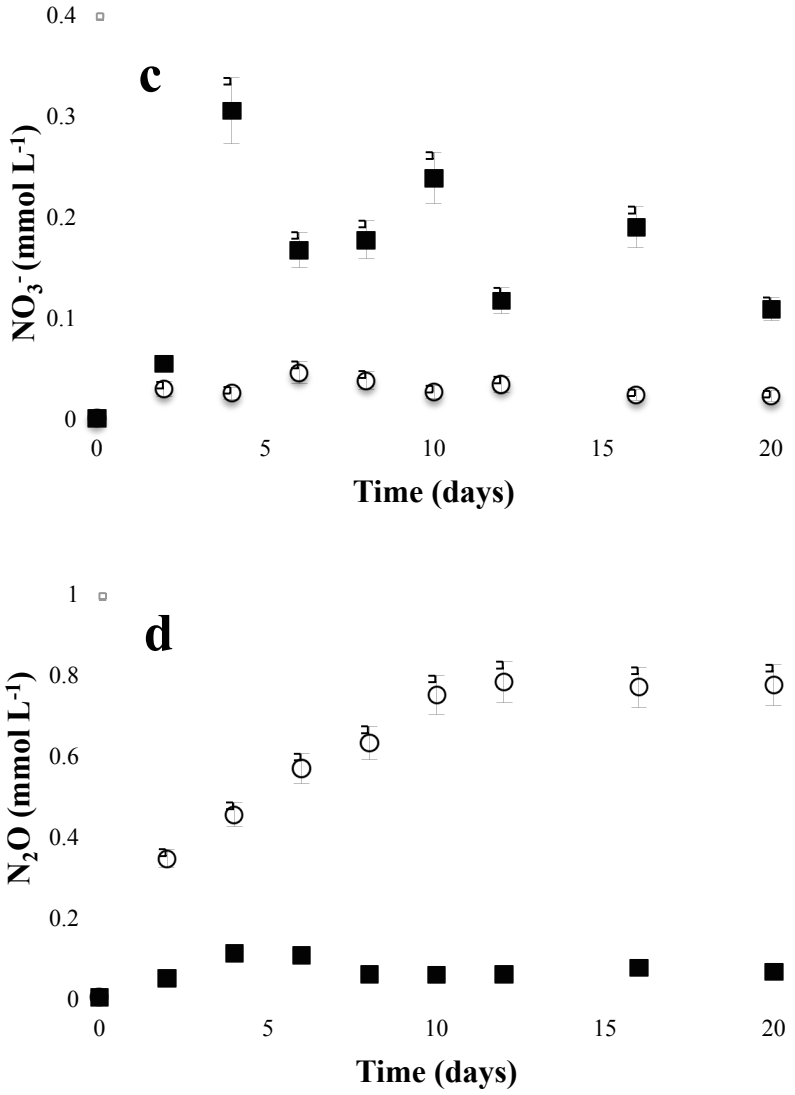


Figure S5. Concentration of NH_4^+, NO_2^-, NO_3^-, and N_2O in the samples incubated with (○) or without (■) C_2H_2. The values represent the mean and standard error (n=3).

Table S1. Sequence analysis of bands excised from DGGE gels

Phylogenetic group	Band	Related sequence	Identity (%)
Bacteroidetes	A1	Uncultured Sphingobacteria bacterium clone ADK-BTh02-48 16S ribosomal RNA gene (EF520590)	93
		Flavobacterium sp. GNNN5_III 16S ribosomal RNA gene (JQ072049)	95
Chloroflexi	A3	Uncultured Bellilinea sp. clone 058 16S ribosomal RNA gene (GU556275)	99
		Ktedonobacter racemifer gene for 16S rRNA, partial sequence (AB510917)	93
	A12	Uncultured Chlorobi bacterium partial 16S rRNA gene, clone JML-1 (FN423885)	96
Firmicutes	A4	Eubacterium hadrum partial 16S rRNA gene, type strain DSM 3319T, clone 2 (FR749933)	99
	A11	Bacillus pocheonensis strain BJC15-D23 16S ribosomal RNA gene (JX483732)	100
	A10	Uncultured Paenibacillus sp. clone T1A4B 16S ribosomal RNA gene (HQ916801)	94
Actinobacteria	A6, B1, D6	Ferrimicrobium acidiphilum strain T23 16S ribosomal RNA gene (AF251436)	92
		Acidimicrobium ferrooxidans strain TH3 16S ribosomal RNA gene (EF621760)	90
	A6	Uncultured Ferrimicrobium sp. clone D.an-41 16S ribosomal RNA gene (JX505108)	95
	A13	Uncultured Actinobacteria Kmlps6-6 16S ribosomal RNA gene (AF289904)	98
Acidobacteria	B4	Uncultured Acidobacteria bacterium clone GYs1-54 16S ribosomal RNA gene (JX493091)	93
	A8, D11	Uncultured Acidobacteria bacterium clone 3OL11 16S ribosomal RNA gene(GQ342349)	97
		Geothrix sp. enrichment culture clone AP-FeEnrich1 16S ribosomal RNA gene (JX828409)	94

Proteobacteria

Alphaproteobacteria	B8	Uncultured Sphingomonas sp. clone B119 16S ribosomal RNA gene (HM452498)	100
Deltaproteobacteria	A5, C1,	Uncultured Geobacter sp. clone HZ-1d-7 16S ribosomal RNA gene (HQ875514)	99
	C2, B3	Uncultured Cystobacteraceae bacterium clone H3-27 16S ribosomal RNA gene, partial sequence (JF703480)	97
Gammaproteobacteria	A2	Uncultured Pseudomonas sp. isolate ODP1176A6H 26 B 16S ribosomal RNA gene (AY191355)	99
	A9	Acinetobacter sp. ACA7 16S ribosomal RNA gene (JN703731)	98
BetaProteobacteria	B2,B7	Azoarcus denitrificans Td-15 16S ribosomal RNA gene (L33688)	96
	B6	Uncultured Ferribacterium sp. Clone GS40to44-70 16S ribosomal RNA gene (JQ288478)	99
		Uncultured Nitrosospira sp. isolate DGGE gel band 6 16S ribosomal RNA gene (JX901178)	96
	A7,B5, C3	Uncultured Nitrosomonadaceae bacterium clone PM5_-0.3-14 16S ribosomal RNA gene (JQ177857)	98
	A9,B9, C4, D14	Uncultured Rhodocyclus sp. clone W4S68 16S ribosomal RNA gene (AY691423)	97
	A9	Comamonas sp. 'ARUP UnID 223' 16S ribosomal RNA gene (JQ259419)	97

3. Supplemental References

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