



(51) International Patent Classification:

C02F 3/34 (2006.01)

C02F 101/30 (2006.01)

C02F 3/30 (2006.01)

(21) International Application Number:

PCT/US2018/052710

(22) International Filing Date:

25 September 2018 (25.09.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/565,480

29 September 2017 (29.09.2017) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,

HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

— with sequence listing part of description (Rule 5.2(a))

(54) Title: OXIDANT ENHANCED FEAMMOX ACTIVITY

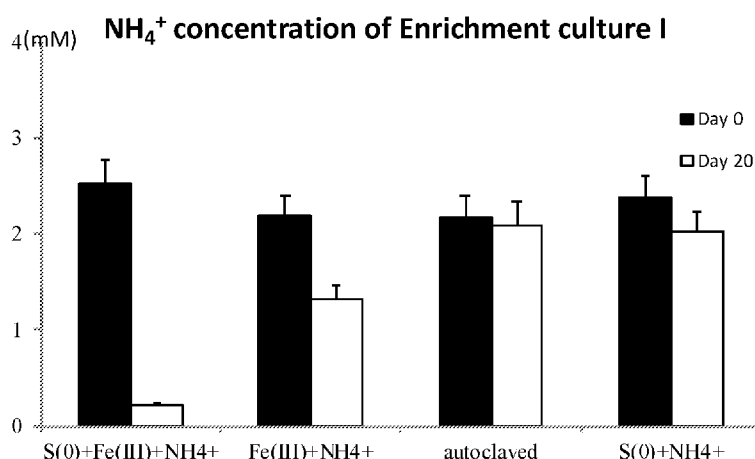


FIG. 32

(57) Abstract: In one aspect, oxidant is employed for enhancing Feammox activity. Briefly, a medium comprises an ammonium containing contaminant, an iron component, an oxidant, and a Feammox bacterium and/or enzyme thereof capable of oxidizing ammonium with reduction of Fe(II) to Fe(III), wherein the oxidant regenerates Fe(III) via Fe(II) oxidation. In some embodiments, the medium is soil and/or water.

OXIDANT ENHANCED FEAMMOX ACTIVITY

GOVERNMENT SUPPORT

This invention was made with government support under Grant No. DE-SC0006847 awarded by the Department of Energy and Grant No. CBET-1433101 awarded by the National Science Foundation. The government has certain rights in this invention.

RELATED APPLICATION DATA

The present invention claims priority pursuant to 35 U.S.C. § 119(e) to United States Provisional Patent Application Serial Number 62/565,480 filed September 29, 2017 which is hereby incorporated by reference in its entirety. The Sequence Listing titled "Sequence Listing," having a file size of 2,867,945 bytes, created on September 29, 2017 and filed herewith is incorporated herein by reference as if fully set forth.

FIELD

The present application relates compositions and methods for environmental remediation and, in particular, to compositions and methods employing Feammox bacteria and an oxidant.

BACKGROUND

The removal of ammonium and other contaminants from soil and water is an important environmental task. Wastewater treatment plants in the developed world and in many developing countries oxidize NH_4^+ to NO_3^- before discharging the treated wastewater. This is done to decrease oxygen demand in the receiving waters. Additionally, nitrogen excess in near shore environments has been identified as a major environmental problem leading to eutrophication and anoxia. Legislations are being implemented requiring the conversion of NO_3^- to N_2 in conventional waste water treatment plants. The nitrogen compounds that are present in contaminated water, such as ammonium NH_4^+ , nitrite (NO_2^-) and nitrate (NO_3^-) will have to be converted to

elemental nitrogen N_2 , which can be released in the gaseous state into an open environment.

Nitrogen removal via nitrification and denitrification can be performed by microorganisms. Numerous studies have focused on the aerobic oxidation of ammonia or NH_4^+ to first nitrite (NO_2^-) and then nitrate (NO_3^-). Oxidation of NH_4^+ to NO_2^- is the first and rate limiting biological step in the nitrification, and also a required step prior to anaerobic NH_4^+ oxidation (anammox), which is the biological conversion of NH_4^+ and NO_2^- to nitrogen gas in the absence of oxygen. Anammox is the most common anaerobic NH_4^+ oxidation pathway in soils environments known so far, using NO_2^- as the electron acceptor. Anaerobic NH_4^+ oxidation is performed by anammox bacteria. Aerobic NH_4^+ oxidation is performed by two groups of organisms, ammonia-oxidizing bacteria (AOB), and ammonia-oxidizing archaea (AOA).

In the wastewater treatment plants, biological ammonium oxidation is conducted by aerobic nitrifying bacteria and requires aeration in the step with the highest energy input. An alternative is the partial nitrification (nitritation) Anammox system, which has been implemented in some treatment plants, with the goal of saving energy costs, since only half of the NH_4^+ is converted to NO_2^- aerobically. However, these Anammox based wastewater treatment systems need to operate between 28°C and 35°C.

An NH_4^+ oxidation process coupled to iron (Fe) reduction called Feammox was identified (Clement JC et al. 2005 Soil Biol Biochem 37:2323–2328; Sawayama S. 2006; J Biosci Bioeng 101:70–72; Shrestha J et al. 2009 Soil Sci 174:156–164; Yang WH et al. 2012 Nat Geosc 5: 538–541, all of which are incorporated herein by reference as if fully set forth). Feammox is a process that can be described as the oxidation of NH_4^+ in the absence of molecular oxygen, with iron oxides [Ferric iron, Fe(III)] as the electron acceptor. In this reaction Fe(III) is reduced to Ferrous iron Fe(II), while NH_4^+ is transformed to NO_2^- , nitrogen gas (N_2), or other nitrogen forms. Feammox may provide

benefits for development of an improved system that does not require aeration or heating of the wastewater in temperate climates.

SUMMARY

In one aspect, media are described herein comprising components for removal of ammonium containing contaminants. Briefly, a medium comprises an ammonium containing contaminant, an iron component, an oxidant, and a Feammox bacterium and/or enzyme thereof capable of oxidizing ammonium with reduction of Fe(III) to Fe(II), wherein the oxidant regenerates Fe(III) via Fe(II) oxidation. In some embodiments, the medium is soil or water. Moreover, the medium can further comprise at least one additional contaminant for removal.

In another aspect, systems for environmental remediation are provided. A system, for example, comprises a reactor including a medium comprising an ammonium containing contaminant, an iron component, an oxidant, and a Feammox bacterium and/or enzyme thereof capable of oxidizing ammonium with reduction of Fe(III) to Fe(II), wherein the oxidant regenerates Fe(III) via Fe(II) oxidation. In some embodiments, the reactor comprises an inlet and outlet for the medium, such as water and/or soil. The reactor, for example, can be operated continuously or in a batch mode. The medium, in some embodiments, can further comprise at least one additional contaminant for removal.

In another aspect, methods of environmental remediation are described herein. A method comprises providing a medium including one or more ammonium containing contaminants and disposing a Feammox bacterium and/or enzyme thereof in the medium. The Feammox bacterium and/or enzyme thereof participates in the oxidation of ammonium coupled with reduction of Fe(III) to Fe(II). An oxidant in the medium subsequently regenerates Fe(III) via Fe(II) oxidation. In some embodiments, oxidation of Fe(II) by the oxidant generates hydrogen ions that are consumed in the ammonium

oxidation. Additionally, in some embodiments, the oxidant forms a compound with Fe(II) in the medium.

In another aspect, various compositions are described herein. In some embodiments, a composition comprises an *Acidimicrobiaceae* Feammox bacterium A6 having Accession Deposit Number PTA-122488, at least one of soil, wastewater, and a carrier, and elemental sulfur. The bacterium is capable of oxidizing ammonium coupled with reduction of Fe(III) to Fe(II). In other embodiments, a composition comprises at least one enzyme capable of oxidizing ammonium into nitrite, at least one of soil, wastewater, and a carrier, and elemental sulfur. The soil or wastewater comprises an ammonium containing contaminant, and the at least one enzyme comprises a sequence with at least 70% identity to the sequence selected from the group consisting of SEQ ID NOS: 29 - 49.

In another aspect, systems employing the various compositions are provided. In some embodiments, a system comprises a reactor and a composition that includes an *Acidimicrobiaceae* Feammox bacterium A6 having Accession Deposit Number PTA-122488, at least one of soil, wastewater and a carrier, and elemental sulfur. The bacterium is capable of oxidizing ammonium coupled with reduction of Fe(III) to Fe(II).

In another aspect, processes for reducing a nitrogen contaminant in soil and/or water are described herein. A process, in some embodiments, comprises mixing the wastewater with an *Acidimicrobiaceae* Feammox bacterium A6 having Accession Deposit Number PTA-122488 to form a mixture. The process also comprises adding elemental sulfur to the mixture. In some embodiments, the water can be wastewater or water from the ground water system.

In a further aspect, various hosts are provided including nucleic acids comprising a sequence with at least 70% identity to a reference sequence selected from the group

consisting of SEQ ID NOS: 8 - 28. Additionally, compositions comprising such hosts are also contemplated. A composition, for example, can comprise one or more hosts, at least one or soil, wastewater, and a carrier and elemental sulfur. The soil or wastewater can comprise an ammonium containing contaminant.

These and other embodiments are described in further detail in the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A - 1B illustrate concentration of Fe(II) (FIG. 1A) and NH_4^+ (FIG. 1B) in incubation with three different Fe(III) sources: ferrihydrite (open circle), ferric chloride (closed square), and ferric citrate (closed triangle).

FIGS. 2A - 2B illustrate concentration of Fe(II) (FIG. 2A) and NH_4^+ (FIG. 2B) in 25-day incubation with NH_4Cl and ferrihydrite (open circle), NH_4Cl and goethite (closed circle).

FIGS. 3A - 3B illustrate concentration of Fe(II) (FIG. 3A) and NH_4^+ (FIG. 3B) during the 180 day incubation.

FIGS. 4A - 4B illustrate concentration of Fe(II) (FIG. 4A) and NH_4^+ (FIG. 4B) in 25-day incubations in samples with NH_4Cl (open square), ferrihydrite (open circle), sterilized soil with NH_4Cl and ferrihydrite (closed triangle).

FIGS. 5A - 5B illustrate concentration of NO_3^- (open circle) and NO_2^- (closed square) (FIG. 5A) and DOC (open triangle) and TN (open square) (FIG. 5B) during the 180 day incubation.

FIG. 6 illustrates comparison of DGGE analysis profiles of soil communities during anaerobic incubations.

FIG. 7 illustrates abundance of total bacteria (closed rhombus) during 180 days of anaerobic incubation, 16S rRNA gene (black bars) and RNA copy numbers (gray bars) of *Acidimicrobiaceae* bacterium A6 in soil samples with 1.20 mmol L⁻¹ NaHCO₃ addition, 16S rRNA gene (white bars) and RNA copy numbers (dotted bars) of bacterium A6 with 0.20 mmol L⁻¹ NaHCO₃ addition.

FIGS. 8A - 8D illustrate concentration of NH₄⁺ (FIG. 8A), NO₂⁻ (FIG. 8B), NO₃⁻ (FIG. 8C), and N₂O (FIG. 8D) in the samples incubated with (open circle) or without (closed square) C₂H₂.

FIG. 9 illustrates stoichiometry between the Fe(II) production and NH₄⁺ consumption during 180 day incubation.

FIG. 10 illustrates a graph of ΔG vs. pH.

FIG. 11 illustrates a schematic representation of a membrane reactor for NH₄⁺ oxidation via Feammox.

FIGS. 12A-12B illustrate ordination plots of the canonical correspondence analysis (CCA) for the first two dimensions of CCA of the relationship between the environmental factors and Feammox bacteria vs. environment factor of soil analysis and relation of Feammox to other bacterial communities affecting the nitrogen cycle.

FIG. 12A illustrates the total variance in the bacterial abundance and the cumulative variance of the bacteria-environment relationship. FIG. 12B illustrates the total

variance in the microbial composition and the cumulative variance of the bacteria-sampling location relationship.

FIG. 13 illustrates NH_4^+ oxidized over 6 days by *Acidimicrobiaceae* bacteria A6.

FIG. 14 illustrates uranium U(VI) removed from solution over 6 days.

FIG. 15 illustrates reduction of Fe in nontronite over 12 days.

FIG. 16 illustrates total U and U(VI) concentrations over 11 days.

FIGS. 17A - 17B illustrate oxidation of NH_4^+ (FIG. 17A) and bioreduction of Cu(II) (FIG. 17B) by Feammox. Filled bars are for results with A6, and open bars are controls.

FIGS. 18A - 18D illustrate an effect of biodegradation of trichloroethylene (TCE) (FIG. 18A), tetrachloroethylene (PCE) (FIG. 18B), benzene (FIG. 18C) and phenanthrene (FIG. 18D) by Feammox. Filled bars are for results without FMO inhibitor, open bars are for results with FMO inhibitor and crosshatched bars are no NH_4^+ controls.

FIGS. 19A - 19D illustrate A6 bacterial cultures and cells. FIG. 19A illustrates growth of the A6 bacterium in the liquid medium. FIG. 19B illustrates A6 growth on the solid inorganic NH_4^+ -ferric iron medium. FIGS. 19C - 19D show rod-shaped A6 cells.

FIG. 20 illustrates anaerobic oxidation of ammonium by the A6 isolate. Filled bars are for results with NH_4^+ and stripped bars are results with NO_2^- .

FIG. 21 illustrates an effect of NH_4^+ concentration on its consumption by A6. 10 mM NH_4^+ results are reported in the left filled bar of each of days 0, 5, 10 and 20 panels. 5 mM NH_4^+ are reported in the middle dotted bar of each of days 0, 5, 10 and 20 panels. 1 mM NH_4^+ is reported in the right open bar of each of days 0, 5, 10 and 20 panels.

FIG. 22 illustrates iron reduction rates. Fe (II) results are reported in the left bar of each of the day 0, 10, and 20 panels. Fe(III) results are reported in the right bar of each of the days 0, 10, and 20 panels.

FIG. 23 illustrates an effect of iron concentration on Feammox. 20 mM NH_4^+ results are reported in the left filled bar of each of days 0, 5, 10 and 20 panels. 10 mM NH_4^+ results are reported in the middle dotted bar of each of days 0, 5, 10 and 20 panels. 2 mM NH_4^+ results are reported in the right open bar of each of days 0, 5, 10 and 20 panels.

FIG. 24 illustrates the relative ammonium removal rate constants (k) by *Acidimicrobiaceae*-bacteria A6 at 15°C (top line), 20°C (middle line) and 35°C (lower line).

FIGS. 25A - 25B illustrate determination of the effect of pH on NH_4^+ consumption (FIG. 25A) and bacterial growth (FIG. 25B). pH =2 results are reported in the far left bar of each of days 0, 5, 10, 15 and 20 panels. pH=4.5 results are reported in the left middle bar of each of days 0, 5, 10, 15 and 20 panels. pH=6.5 results are reported in the right middle bar of each of days 0, 5, 10, 15 and 20 panels. pH=8 results are reported in the far right bar of each of days 0, 5, 10, 15 and 20 panels.

FIG. 26 illustrates a schematic representation of the genome of the *Acidimicrobiaceae* Feammox bacterium A6.

FIG. 27 illustrates the FMO activity.

FIG. 28 illustrates oxidation of NH_4^+ by *Acidimicrobiaceae* bacterium A6 when the FMO activity in the presence (left bars for each of 0, 12, 22, 30 and 37 day panels) and

absence (right bars for each of 0, 12, 22, 30 and 37 day panels) of a broad enzyme inhibitor (NaS).

FIG. 29 illustrates a scheme of the electrogenic microbial reactor.

FIG. 30 illustrates the concentration of NH_4^+ after 20 days of incubation with *Acidimicrobiaceae* Feammox bacterium A6 in the presence of elemental sulfur and different controls according to some embodiments.

FIG. 31 illustrates concentration of Fe(III) after 20 days of incubation with *Acidimicrobiaceae* Feammox bacterium A6 in the presence of elemental sulfur and different controls according to some embodiments.

FIG. 32 illustrates the concentration of NH_4^+ after 20 days of incubation with an enrichment culture of *Acidimicrobiaceae* Feammox bacterium A6 in the presence of elemental sulfur and different controls according to some embodiments.

FIG. 33 illustrates the concentration of Fe(III) after 20 days of incubation with an enrichment culture of *Acidimicrobiaceae* Feammox bacterium A6 in the presence of elemental sulfur and different controls according to some embodiments.

FIG. 34 illustrates the concentration of NH_4^+ after 20 days of incubation with an enrichment culture of *Acidimicrobiaceae* Feammox bacterium A6 in the presence of elemental sulfur and different controls according to some embodiments.

FIG. 35 illustrates the concentration of Fe(III) after 20 days of incubation with an enrichment culture of *Acidimicrobiaceae* Feammox bacterium A6 in the presence of elemental sulfur and different controls according to some embodiments.

FIG. 36 illustrates copy number of 16S rRNA gene of *Acidimicrobiaceae* Feammox bacterium A6 during 20 days of incubation in the presence of elemental sulfur and different controls according to some embodiments.

FIG. 37 illustrates the effects of elemental sulfur additions in a continuous flow membrane Feammox reactor according to some embodiments.

DETAILED DESCRIPTION

Certain terminology is used in the following description for convenience only and is not limiting. The words “right,” “left,” “top,” and “bottom” designate directions in the drawings to which reference is made. The words “a” and “one,” as used in the claims and in the corresponding portions of the specification, are defined as including one or more of the referenced item unless specifically stated otherwise. This terminology includes the words above specifically mentioned, derivatives thereof, and words of similar import. The phrase “at least one” followed by a list of two or more items, such as “A, B, or C,” means any individual one of A, B or C as well as any combination thereof.

“Synthetic nucleic acid sequence,” “synthetic polynucleotide,” “synthetic oligonucleotide,” “synthetic DNA,” or “synthetic RNA” as used herein refers to a nucleic acid, polynucleotide, oligonucleotide, DNA, or RNA that differs from one found in nature by having a different sequence than one found in nature or a chemical modification not found in nature. The definition of synthetic nucleic acid includes but is not limited to a DNA sequence created using biotechnology tools. Such tools include but are not limited to recombinant DNA technology, chemical synthesis, or directed use of nucleases (so called “genome editing” or “gene optimizing” technologies).

Embodiments described herein can be understood more readily by reference to the following detailed description and examples and their previous and following

descriptions. Elements and apparatus described herein, however, are not limited to the specific embodiments presented in the detailed description. It should be recognized that these embodiments are merely illustrative of the principles of the present invention. Numerous modifications and adaptations will be readily apparent to those of skill in the art without departing from the spirit and scope of the invention.

In one aspect, media are described herein comprising components for removal of ammonium containing contaminants. A medium comprises an ammonium containing contaminant, an iron component, an oxidant, and a Feammox bacterium and/or enzyme thereof capable of oxidizing ammonium with reduction of Fe(III) to Fe(II), wherein the oxidant regenerates Fe(III) via Fe(II) oxidation. In some embodiments, the medium is soil or water. The medium, for example, is a ground water system or wastewater. Moreover, the medium can further comprise at least one additional contaminant for removal.

Turning now to specific components, the medium comprises one or more ammonium containing contaminants. An ammonium containing contaminant may be, but is not limited to, an industrial, agricultural or human municipal waste. Ammonium containing contaminants, for example, can comprise fertilizers, domestic sewage, or industrial effluents. The ammonium containing contaminant may comprise ammonium chloride and/or any other ammonium salt. The ammonium containing contaminant may also be a nitrogen containing organic compound, wherein nitrogen may be hydrolyzed to ammonium.

The medium also comprises a Feammox bacterium and/or enzyme thereof capable of oxidizing ammonium with reduction of Fe(III) to Fe(II). The Feammox bacterium may be an *Acidimicrobiaceae* bacterium or a bacterium with a similar genetic composition. The Feammox bacterium may be a bacterial strain that was isolated from wetland soils

collected in New Jersey after a series of enrichment incubations. The soil samples were collected at the location identified as 40°15' N–74°30' W or within 100 m of the identified location. The Feammox bacterium may be the bacterial strain designated the *Acidimicrobiaceae* Feammox bacterium A6 and described herein. The *Acidimicrobiaceae* Feammox bacterium A6 was submitted for deposit with the American Type Culture Collection (ATCC; 10801 University Blvd. Manassas, Virginia 20110-2209, USA) on April 27, 2015, the submission was supplemented on May 7, 2015, and was assigned Accession Deposit Number PTA-122488 on September 17, 2015. The *Acidimicrobiaceae* Feammox bacterium may have a genome comprising, consisting essentially of, or consisting of a nucleic acid sequence with at least 70, 72, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identity to SEQ ID NO: 1. The Feammox bacterium may have the genome size of 3.3 mega base pairs (Mb) and guanine-cytosine content 52%. The bacterial genome may further include a gene encoding a Feammox Ammonium Monooxygenase. As used herein, the term “Feammox Ammonium Monooxygenase” (*FMO*) refers to an enzyme that plays a key role in oxidizing ammonium coupled with ferric iron reduction. The *FMO* also refers to genes encoding clones or different variants of the Feammox Ammonium Monooxygenase. The gene may include a nucleic acid comprising, consisting essentially of, or consisting of a nucleic acid sequence with at least 70, 72, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identity to a sequence selected from the group consisting of: SEQ ID NOS: 8 - 28. The Feammox Ammonium Monooxygenase may include an amino acid comprising, consisting essentially of, or consisting of a nucleic acid sequence with at least 70, 72, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identity to a sequence selected from the group consisting of: SEQ ID NOS: 29 - 49. The Feammox bacterium may be live or lyophilized.

Determining percent identity of two nucleic acid sequences may include aligning and comparing the nucleotides at corresponding positions in the two sequences. If all positions in two sequences are occupied by identical nucleotides then the sequences are

said to be 100% identical. Percent identity may be measured by the Smith Waterman algorithm (Smith TF, Waterman MS 1981 "Identification of Common Molecular Subsequences," J Mol Biol 147: 195 -197, which is incorporated herein by reference as if fully set forth).

As described herein, the medium may comprise one or more enzymes, such as FMO, in addition to the Feammox bacterium for the oxidation of ammonium coupled with reduction of Fe(III) to Fe(II). Alternatively, one or more enzymes, such as FMO, can be present in the medium in the absence of the Feammox bacterium. In such embodiments, the one or more enzymes can be responsible for the ammonium oxidation and/or reduction of Fe(III) to Fe(II). In some embodiments, for example, FMO is isolated from Feammox bacterium and employed in the media. In other embodiments, the one or more enzymes may be fabricated via synthetic chemical techniques.

The medium also comprises an iron component. In some embodiments, the iron component is a natural component of the medium. The iron component can naturally occur in soil, for example. In other embodiments, the iron component is added to the medium. The iron component can be the source of ferric ion, Fe(III), reduced to Fe(II) in conjunction with ammonium oxidation. Any iron component or source consistent with the environmental objectives of the present invention can be employed in the medium. In some embodiments, the iron component comprises iron oxide, a goethite, elemental iron, a nontronite, iron-rich clay or various mixtures thereof. Iron oxide can include hydrated forms, such as ferrihydrite. Ferrihydrite includes a dark brown or yellow brown mineral composed of about 20% (FeO₄) and 80% (FeO₆) polyhedral. The term "goethite" refers to an iron oxyhydroxide containing ferric iron. The term "nontronite" refers to the Fe(III) rich clay mineral having a typical structural formula Ca_{0.5}(Si₇Al₈Fe₂)(Fe_{3.5}Al₄Mg₁)O₂₀(OH)₄. The iron component may be scrap metal, or any other source of ferric iron. The iron component can also serve as culture media for the

Feammox bacterium in the medium, in some embodiments. For example, the iron component can comprise NH_4^+ –ferric iron media in liquid or solid form.

The iron component can be present in the medium in any amount to support the oxidation of ammonium, as described herein. In some embodiments, ferric ion is present in the medium at a concentration of 1 mM to 200 mM. The concentration of ferric iron may also be from 1 mM to 2 mM, from 2 mM to 3 mM, from 3 mM to 4 mM, from 4 mM to 5 mM, from 5 mM to 6 mM, from 6 mM to 7 mM, from 7 mM to 8 mM, from 8 mM to 9 mM, from 9 mM to 10 mM, from 10 mM to 20 mM, from 20 mM to 30 mM, from 30 mM to 40 mM, from 40 mM to 50 mM, from 50 mM to 60 mM, from 60 mM to 70 mM, from 70 mM to 80 mM, from 80 mM to 90 mM, from 90 mM to 100 mM, from 100 mM to 150 mM, or from 150 mM to 200 mM.

An oxidant is also present in the medium, wherein the oxidant regenerates Fe(III) via oxidation of Fe(II). As discussed further below, regeneration of Fe(III) by the oxidant can conserve the Fe(III) used in the ammonium oxidation reaction and enhance or accelerate ammonium decomposition in the medium. In this way, iron is recycled in the ammonium decomposition process, and less iron is required for environmental remediation according to processes described herein. Additionally, in some embodiments, oxidation of Fe(II) by the oxidant can produce hydrogen ions. Hydrogen ions produced in the oxidation can be consumed in the ammonium oxidation reaction detailed in Equation 1 below. Accordingly, generation of hydrogen ions in the oxidation of Fe(II) can assist in maintaining a stable pH of the medium during ammonium oxidation. In some embodiments, pH of the medium is 4 to 8 or 5 to 7.

Subsequent to Fe(II) oxidation, the reduced oxidant may react with Fe(II) to provide one or more additional chemical species to the medium. Reaction of the reduced oxidant with Fe(II) can inhibit the accumulation of Fe(II) in the medium. In some embodiments, the reduced oxidant forms a precipitate with Fe(II). Alternatively, the

chemical species formed between the reduced oxidant and Fe(II) is fully or partially soluble in aqueous-based media or water.

Any oxidant having the functionality of Fe(II) oxidation without interfering with ammonium oxidation processes can be employed. Suitable oxidant may be in elemental or molecular form. In some embodiments, the oxidant is elemental sulfur, S(0). Elemental sulfur can be present in the medium in any amount consistent with the environmental remediation objectives described herein. For example, elemental sulfur may be present in the medium in an amount in excess of the solubility limit of elemental sulfur in water. The amount of elemental sulfur may be in a range from 1 mg/L to 2 kg/L of the composition. The amount of sulfur may be any amount in a subrange within the range of 1 mg/L to 2 kg/L, where the subrange includes (1) a lower limit selected from 1 mg/L increments from 1 mg/L to 1.999 kg/L, and (2) a higher limit that is higher than the lower limit and selected from 1 mg/L increments from 1 mg/L to 2 Kg/L. For example, the lower limit may be 1 mg/L, 2 mg/L, 3 mg/L ... 1.999 Kg/L, and the higher limit may be 2 mg/L, 3 mg/L, 4 mg/L ... 2 Kg/L. The amount of elemental sulfur may be selected from any value within the range or subrange, or an endpoint of the range or subrange.

As described herein, the medium can comprise water, soil or mixtures thereof. The water can be any source of water, including ground water, lakes, streams and/or reservoirs. In some embodiments, the water is wastewater. As used herein, the term "wastewater" refers to any water that has been adversely affected in quality by anthropogenic influence. Wastewater may be municipal wastewater, industrial wastewater, agricultural wastewater, surface runoff, stormwater, or wastewater combining wastewater from multiple sources. Wastewater may be treated in a wastewater treatment plant. Wastewater may include nitrogen contaminants. As used herein, the term "contaminant" refers to compounds that are not occurring in water naturally, pose health or ecological risks or are subject to state and federal regulation.

Nitrogen contaminants may be an ammonium containing contaminant. The ammonium containing contaminant can comprise an industrial, agricultural, or human municipal waste. Ammonium containing contaminants, for example, may include fertilizers, domestic sewage, or industrial effluents. The ammonium containing contaminant may be ammonium chloride or any other ammonium salt. The ammonium containing contaminant may be a nitrogen containing organic compound, wherein nitrogen may be hydrolyzed to ammonium.

Similarly, soil may be any soil that has been adversely affected in quality by anthropogenic influence. The soil may include any nitrogen contaminants described herein. The soil may include groundwater. The groundwater may comprise wastewater described herein. The groundwater may contain any nitrogen contaminant described herein.

Water or soil medium may include at least one additional contaminant. The at least one additional contaminant may be inorganic contaminants. Inorganic contaminants may be but are not limited to heavy metals, radionuclides or trace metals. Inorganic contaminants may be copper, uranium, ferric iron, lead, zinc, arsenic, chromium, mercury or silver. The at least one additional contaminant may be organic contaminant(s). The organic contaminants can comprise chlorinated volatile organic compounds, perchloroethylene (PCE), trichloroethylene (TCE), trichloroethane, dichloroethane, vinyl chloride, polychlorinated biphenyls, fuel constituents, benzene, ethylbenzene, toluene, xylene, phenanthrene, methyl tert butyl ether, tertiary butyl alcohol, polyaromatic hydrocarbons, or ethylene dibromide.

In an embodiment, the ammonium containing contaminant may be added to a composition that would otherwise be low or deficient in ammonium when reactions in addition to or in place of ammonium oxidation are of interest.

In some embodiments, the medium may further comprise a carrier. The carrier may support growth of the Feammox bacterium. The carrier may comprise a filter, beads, agarized medium, or any surface that allows bacterial attachment. The carrier may include media for culturing the Feammox bacterium. The media may be inorganic NH_4^+ -ferric iron media. The inorganic NH_4^+ -ferric iron media may be solid media or liquid media. The liquid media may include but not limited to the following components: NH_4Cl , $(\text{NH}_4)_2\text{SO}_4$, NaHCO_3 , KHCO_3 , KH_2PO_4 , 100 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The liquid media may further include ferrihydrite, AQDS, trace element solution or vitamins. Vitamins may be but are not limited to ATCC® vitamins. The liquid media may have a pH in a range from 4.0 to 5.0. The media may include traces of dissolved oxygen. The solid medium may have the same composition as the liquid media but include elements to solidify the mixture. The solid media may be solidified with 0.8 % agar. The solid media may include ferrihydrite that is spread on the surface of the medium.

In another aspect, systems for environmental remediation are provided. A system, for example, comprises a reactor including a medium comprising an ammonium containing contaminant, an iron component, an oxidant, and a Feammox bacterium and/or enzyme thereof capable of oxidizing ammonium with reduction of Fe(III) to Fe(II), wherein the oxidant regenerates Fe(III) via Fe(II) oxidation. In some embodiments, the reactor comprises an inlet and outlet for the medium, such as water and/or soil. The reactor, for example, can be operated continuously or in a batch mode. Components of the medium, including the ammonium containing contaminant, iron component, oxidant, and Feammox bacterium and/or enzyme thereof can have any properties and/or compositions described hereinabove. In some embodiments, the medium can comprise at least one additional contaminant. Additional contaminants can have any composition described hereinabove. Moreover, the medium can comprise water and/or soil. Water of the system can be any source of water, including wastewater, ground water, lakes, streams and/or reservoirs.

The reactor may be a continuous membrane reactor or a sequential batch reactor. In a non-limiting example the continuous membrane reactor may be a reactor illustrated in FIG. 11. FIG. 11 illustrates a scheme of a membrane reactor for NH_4^+ oxidation via Feammox. Referring to FIG. 11, 1 refers to the feed solution, 2 refers to the feed pump, 3 refers to the membrane module, 4 refers to the floater connected to an electrical on/off switch, 5 refers to the ceramic diffuser, 6 refers to the N_2 supplier, 7 refers to the water bath, 8 refers to outflow, and 9 refers to the outflow pump. The reactor may be an electrogenic microbial reactor. The electrogenic microbial reactor may be any bioelectrochemical system that extracts energy from a substrate (Call and Logan 2011 *Biosen. Bioelectron.* 26(11): 4526-4531, which is incorporated herein by reference as if fully set forth). The electrogenic microbial reactor may be any bioelectrochemical system that extracts energy from a substrate (Call and Logan 2011 *Biosen. Bioelectron.* 26(11): 4526-4531, which is incorporated herein by reference as if fully set forth). In the bioelectrochemical system, electrons may be harvested biologically, and then transferred to the anode that functions as the terminal electron acceptor for the microorganisms in the system, and H_2 is produced at the anode. The electrogenic microbial reactor may be a Microbial Fuel Cell (MFC) or Microbial Electrolysis cell (MEC). A non-limiting example of the electrogenic microbial reactor is illustrated in FIG. 29. Referring to FIG. 29, the reactor includes a tank, a graphite anode connected via titanium wire to the positive terminal of the power supply, a stainless steel cathode connected via stainless steel wire to the negative terminal of the power supply. MECs require a small potential applied from an external power source ($E_{\text{AP}} > 0.25 \text{ V}$), resulting in H_2 production at the anode (Logan, Hamelers et al. 2006 *Environ. Sci. Technol.* 40(17):5181-5192; Call and Logan 2008 *Environ. Sci. Technol.* 42(9):3401-3406; Call and Logan 2011, all of which are incorporated by reference herein as if fully set forth).

In an embodiment, a reactor may be an industrial-type reactor. The reactor may operate within a water treatment plant. The reactor may be a treatment pond or a reservoir. The reactor may be a tank for wastewater storage.

Reactor conditions may include a temperature in a range from 4°C to 35°C. The temperature may be in a range between any two integer value temperatures selected from 4°C to 35°C. The temperature may be in a range between and including 4°C to 10°C, 10°C to 15°C, 15°C to 20°C, 20°C and 25°C, 25°C and 30°C, 30°C and 35°C. The temperature may be any one integer value temperature selected from those including and between 4°C and 35°C or 15°C to 35°C. Temperatures between room temperature and 35°C may be used. The temperature may be any one temperature including and between room temperature and 35°C. Temperatures between 20°C and 35°C may be used. The temperature may be any temperature including and between 20°C and 25°C.

The reactor may be operated for a period of time ranging from 2 hours to 45 days. The time period may be 5 hours, 10 hours, 15 hours, 20 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 10 days, 15 days, 20 days, 25 days, 30 days, 35 days, 40 days or 45 days. The time period may be any one integer value selected from those including and between value points, endpoints inclusive. The time period may be greater than 45 days. The time period may be less than 1 day. In continuous flow reactors or in batches, the process may last from several hours to several months. For continuous flow reactors, the time period may depend on the bacterial concentration in the inoculum. Higher bacterial concentration in the inoculum may result in a shorter remediation time. The time period may depend on hydraulic retention capacity of a continuous flow reactor. Lower retention capacity of the continuous flow reactor may result in a shorter remediation time. Hydraulic residence time for the continuous flow reactors may be from 3 hours to 4 hours, from 3 hours to 5 hours, from 3 hours to 6 hours, from 3 hours to 7 hours, from 3 hours to 8 hours, from 3 hours to 10 hours, from 3 hours to 15 hours, from 3 hours to 20 hours, from 3 hours to 1 day, from 3 hours to 2 days, from 3 hours to

3 days. Hydraulic residence time may be any integer value selected from those including and between value points, endpoints inclusive.

pH of the medium in the reaction can range from 2.0 to 8.0. The pH of the medium may be in a range between and including 2.0 and 3.0, 3.0 and 4.0, 4.0 and 5.0, 5.0 and 6.0, 6.0 to 7.0, 7.0 to 7.5. The pH may be any one integer value pH selected from those including and between 2.0 and 7.5. The pH may be any pH including and between 4.0 and 7.0.

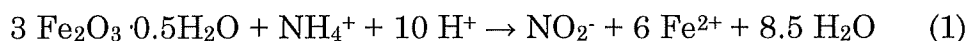
In some embodiments, the reactor and associated processes may have nitrification and denitrification phases. The process may include a nitrification phase followed by a denitrification phase. The nitrification and denitrification phases may be performed in parallel. The parallel performance of nitrification and denitrification phases may occur at low levels of oxygen. The nitrification and denitrification phases may be performed in the same reactor. The reactor utilized in the process may be configured to allow a nitrification phase and a denitrification phase. There may be two separate reactors for each of the nitrification and denitrification phases. The process may further include adding Anammox bacteria to the reactor. The nitrification phase may be performed by the Feammox bacteria. In the nitrification phase, ammonium may be converted to nitrite. The denitrification phase may be performed by denitrifying bacteria. In the denitrification phase, nitrite may be converted to gaseous nitrogen (N_2). The denitrification phase may be performed by the Anammox bacteria.

In another aspect, methods of environmental remediation are described herein. A method comprises providing a medium including one or more ammonium containing contaminants and disposing a Feammox bacterium and/or enzyme thereof in the medium. The Feammox bacterium and/or enzyme thereof participates in the oxidation of the ammonium coupled with reduction of Fe(III) to Fe(II). An oxidant in the medium subsequently regenerates Fe(III) via Fe(II) oxidation. In some embodiments,

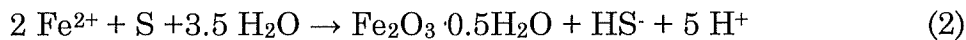
oxidation of Fe(II) by the oxidant generates hydrogen ions that are consumed in the ammonium oxidation. Additionally, in some embodiments, the oxidant forms a compound with Fe(II) in the medium, as described hereinabove. The compound may be a precipitate, in some embodiments. Components of the medium, including the ammonium containing contaminant, iron component, oxidant, and Feammox bacterium and/or enzyme thereof can have any properties and/or compositions described hereinabove. In some embodiments, the medium can comprise at least one additional contaminant. Additional contaminants can have any composition described hereinabove. Moreover, the medium can comprise water and/or soil. Water can be of any source, including wastewater, ground water, lakes, streams and/or reservoirs.

In a non-limiting example, the oxidant can be element sulfur, S(0). Elemental sulfur oxidizes Fe(II) to regenerate Fe(III). The regenerated Fe(III) can subsequently participate in Feammox reactions occurring in the medium. The oxidation of Fe(II) by elemental sulfur can also produce hydrogen ions. The hydrogen ions may be consumed in Feammox reactions occurring in the medium as provided in Equation 1 below. The production of hydrogen ions can assist in maintaining a stable pH of the medium. Additionally, the regeneration of Fe(III) can reduce the iron content required for sufficient ammonium oxidation and/or removal of other contaminants from the medium. In reduced form, the elemental sulfur can be present as sulfide. The sulfide can react with Fe(II) to precipitate iron sulfide (FeS). Reaction of the reduced oxidant with Fe(II) inhibits accumulation of Fe(II) in the media. As shown in the examples below, presence of the oxidant can substantially enhance ammonium decomposition rates. While not wishing to be bound by any theory, the overall reaction scheme involving elemental sulfur oxidant, S(0), is presented in equations (1-4):

Oxidation of ammonium by Feammox bacterium and/or enzyme(s) thereof:



Oxidation of Fe²⁺ by S(0):



Precipitation of FeS:



Overall reaction:



Accordingly, with proper oxidant, a redox cycle can be established for enhancing ammonium oxidation and/or decomposition and removal of other contaminants from the media, while conserving Fe(III).

Methods of environmental remediation described herein may occur in low levels of oxygen or in the absence of oxygen. In some embodiments, methods may include removing oxygen. As used herein, “absence of oxygen” may include a complete lack of oxygen, low levels of oxygen or oxygen levels below detection. Absence of oxygen may include low oxygen concentrations that allow for iron reduction. Absence of oxygen for example, may be 2 mg/l of dissolved oxygen or less. Absence of oxygen may be 0.5 mg/l of dissolved oxygen or less. Low levels of oxygen may be created by organisms degrading organic carbon when oxygen is consumed by organisms degrading organic carbon, and no or insufficient aeration is provided to compensate for low levels of oxygen. The process may include maintaining low levels of oxygen.

In another aspect, various compositions are described herein. In some embodiments, a composition comprises an *Acidimicrobiaceae* Feammox bacterium A6 having Accession Deposit Number PTA-122488, at least one of soil, wastewater, and a carrier, and elemental sulfur. The bacterium is capable of oxidizing ammonium coupled with reduction of Fe(III) to Fe(II). In other embodiments, a composition comprises at least one enzyme capable of oxidizing ammonium into nitrite, at least one of soil, wastewater, and a carrier, and elemental sulfur. The soil or wastewater comprises an ammonium containing contaminant, and the at least one enzyme comprises a sequence

with at least 70% identity to the sequence selected from the group consisting of SEQ ID NOS: 29 - 49.

In another aspect, systems employing the various compositions are provided. In some embodiments, a system comprises a reactor and a composition that includes an *Acidimicrobiaceae* Feammox bacterium A6 having Accession Deposit Number PTA-122488, at least one of soil, wastewater and a carrier, and elemental sulfur. The bacterium is capable of oxidizing ammonium coupled with reduction of Fe(III) to Fe(II).

In another aspect, processes for reducing a nitrogen contaminant from soil and/or water are described herein. A process, in some embodiments, comprises mixing the wastewater with an *Acidimicrobiaceae* Feammox bacterium A6 having Accession Deposit Number PTA-122488 to form a mixture. The process also comprises adding elemental sulfur to the mixture. In some embodiments, the water can be wastewater or water from a ground water system.

In a further aspect, various hosts are provided including nucleic acids comprising a sequence with at least 70% identity to a reference sequence selected from the group consisting of SEQ ID NOS: 8 - 28. Additionally, compositions comprising such hosts are also contemplated. A composition, for example, can comprise one or more hosts, at least one of soil, wastewater, and a carrier and elemental sulfur. The soil or wastewater can comprise an ammonium containing contaminant.

In some embodiments, a synthetic nucleic acid encodes an enzyme capable of oxidizing ammonium coupled with iron reduction. The enzyme may be Feammox Ammonium Monooxygenase (*FMO*). The synthetic nucleic acid may comprise consists essentially of, or consists of a sequence with at least 70, 72, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identity to a reference sequence selected from the group consisting of: SEQ ID NOS: 8 - 28.

Additionally, expression cassettes are provided that include any synthetic nucleic acid described herein. An expression cassette, for example, may include the synthetic nucleic acid encoding the FMO. The expression cassette may be introduced into a vector. Suitable vectors may be cloning vectors, transformation vectors, expression vectors, or virus-based vectors. The expression cassette portion of a vector may further include a regulatory element operably linked to a synthetic nucleic acid encoding the FMO. In this context, operably linked means that the regulatory element imparts its function on the synthetic nucleic acid. For example, a regulatory element may be a promoter, and the operably linked promoter would control expression of the synthetic nucleic acid.

Expression of a synthetic nucleic acid encoding the FMO from the expression cassette may be under the control of a promoter, which provides for transcription of the synthetic nucleic acid in a host. The promoter may be a constitutive promoter or, tissue specific, or an inducible promoter. A constitutive promoter may provide transcription of the nucleic acid throughout most cells and tissues of the host and during many stages of development but not necessarily all stages. An inducible promoter may initiate transcription of the synthetic nucleic acid sequence only when exposed to a particular chemical or environmental stimulus. A tissue specific promoter may be capable of initiating transcription in a particular host tissue. The promoter may provide transcription of a synthetic nucleic acid having a sequence with at least 70, 72, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identity to a reference sequence selected from the group consisting of SEQ ID NOs: 8 - 28 and expression of the FMO that is capable of oxidizing ammonium coupled with iron reduction.

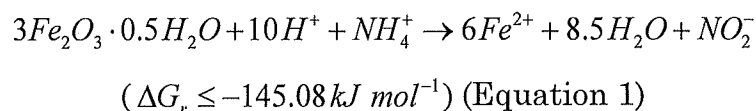
In some embodiments, any one of the vectors or expression cassettes described herein may be introduced to a host. The host can comprise a bacterium, plant, algae, or yeast.

An embodiment includes a host herein comprising any one of the vectors or expression cassettes described herein.

The following non-limiting examples are provided to illustrate particular embodiments. The embodiments throughout may be supplemented with one or more detail from one or more example below, and/or one or more element from an embodiment may be substituted with one or more detail from one or more example below.

Example 1. Development of an enrichment culture capable of ammonium oxidation under iron reducing conditions

An anaerobic NH_4^+ oxidation process coupled to iron reduction was first noted in a forested riparian wetland in New Jersey. In this reaction, NH_4^+ is the electron donor, which is oxidized to nitrite (NO_2^-), and ferric iron [Fe(III)] is the electron acceptor, which is reduced to ferrous iron [Fe(II)]. The stoichiometry and change in free energy when ferrihydrite is the Fe(III) source is:



These pathways have been reported to oxidize NH_4^+ to NO_2^- , to NO_3^- , or directly to N_2 , using Fe(III) as electron acceptor (Clement *et al.*, 2005; Shrestha *et al.*, 2009; Sawayama, 2006; Yang *et al.*, 2012, all of which are incorporated by reference as if fully set forth).

Soil samples were collected from the same location and used for laboratory incubation experiments as well as to set up an enrichment system for Feammox in a continuous flow membrane reactor. Various incubation conditions [Fe(III) sources, inorganic carbon content, NH_4^+ concentration, $^{15}\text{NH}_4^+$, and acetylene gas (C_2H_2) as a selected inhibitor] were used to study the Feammox mechanism. Molecular biology methods, such as denaturing gradient gel electrophoresis (DGGE), 454 pyrosequencing, and real-

time quantitative PCR (qPCR) analysis were used to investigate the bacterial community change during incubations. Production of both nitrite (NO₂⁻) and ferrous iron were measured repeatedly during incubations when soil slurries were supplied with iron oxide (ferrihydrite or goethite) and ammonium chloride.

The Feammox process provided denitrifiers and anammox bacteria with the necessary NO₂⁻ under this anaerobic incubation, and achieved total nitrogen loss via denitrification and anammox pathways. Therefore, Feammox is an important process in the nitrogen cycle in soil environments under oxygen limited conditions, and reveals a new linkage between these two significant biogeochemical cycles (iron and nitrogen cycle).

Example 2. Sample collection and processing

Soils for all the experiments described in examples herein were taken from a temperate forested riparian wetland at the Assunpink Wildlife Management Area, New Jersey. Ten soil cores were collected from 10 cm below the surface with polyethylene column containers (8 cm diameter and 30 cm long) and transported to the laboratory within 2 hours. The soil pH was between 3.5 and 4.5, and no manganese oxides were detected. The detailed physicochemical characteristic of these wetland soils have been described elsewhere (Clement *et al.*, 2005 Soil Biol Biochem 37: 2323-2328, which is incorporated by reference as if fully set forth). Prior to all incubation experiments, soil slurry from the field site was aerated for a month to degrade much of the labile organic carbon. After a 30 days of aeration, the dissolved organic carbon (DOC) content was stable at 2.06 ± 0.20 mg g⁻¹. Following the aeration treatment, the soil was divided into 400×10 g (air-dry equivalent) subsamples, and added into 50mL serum vials, with 30 mL deionized water. The soil slurries were purged thoroughly with a CO₂:N₂ (80:20) mixture, resulting in a final pH of ~ 4 to 4.5. The vials were sealed tightly with rubber stoppers and were stored in an anaerobic glove box for 30 days at ambient temperature

to allow for stabilization before starting the incubations.

Example 3. Batch incubation experiments

All incubations, addition of reagents, and sampling were conducted in an anaerobic glove box with a solution of resazurin as the redox indicator. Soil samples were first incubated with different Fe(III) sources to determine which source would yield a more active Feammox process: 6-line ferrihydrite ($\text{Fe}_2\text{O}_3 \cdot 0.5\text{H}_2\text{O}$) or goethite $[\text{FeO}(\text{OH})]$ + NH_4^+ addition; ferric chloride + NH_4^+ addition; ferric citrate + NH_4^+ addition; either only ferrihydrite or NH_4^+ addition; and autoclaved soil with ferrihydrite + NH_4^+ addition ($n = 30$ per treatment). 6-line ferrihydrite ($\text{Fe}_2\text{O}_3 \cdot 0.5\text{H}_2\text{O}$) or goethite $[\text{FeO}(\text{OH})]$ was prepared according to Cornell and Schwertmann, 2003 *The Iron Oxides: Structure, Properties, Reactions, Occurrences, and Uses*. John Wiley and Sons Ltd., which is incorporated herein by reference as if fully set forth. pH was adjusted to 4.5 in the ferrihydrite/goethite augmented samples, and to between 3.5 ~ 4.0 in the ferric chloride/citrate augmented samples. Soil-slurry samples, which were prepared to have an initial concentration of 12.0 mmol L^{-1} Fe(III) and/or 2.00 mmol L^{-1} NH_4^+ , were incubated in a series of 50 ml vials with an oxygen-free headspace, created by purging with a $\text{CO}_2:\text{N}_2$ (80:20) mixture. Triplicate samples were collected destructively every two days to analyze iron and nitrogen species.

The second incubation was conducted to extend the anoxic incubation with ferrihydrite to 180 days, with repeated NH_4Cl additions after the NH_4^+ in solution was exhausted. The initial concentration of Fe(III) was 25.0 mmol L^{-1} and 1.00 mmol L^{-1} NH_4^+ was added on days 4, 24, and 60, furthermore, 0.20 mmol L^{-1} NaHCO_3 was added on day 50 and day 90 of the incubation. On day 125, incubation vials were divided into two sets to study the effect of different inorganic carbon contents on Feammox. Either 1.20 mmol L^{-1} or 0.20 mmol L^{-1} of NaHCO_3 plus 2 mmol L^{-1} of NH_4Cl were added to each set. NaHCO_3 was then added every 10 days, which increased the soil pH to ~5 in the samples amended with 1.20 mmol L^{-1} of NaHCO_3 . For this incubation, samples were

collected every four days. Finally, soil samples collected on day 180 of the incubations were used to enrich the Feammox bacteria in a membrane reactor. To study how the organic carbon content affects the Feammox bacteria, 1.00 mmol L⁻¹ sodium citrate was also supplied on day 125 to four of the 1.20 mmol L⁻¹ NaHCO₃ amended samples.

In the third experiment, inorganic nitrogen species were quantified through incubations in the presence of C₂H₂. Soil slurries were first incubated for 90 days in eighty 50 mL vials. The Fe(III) concentration at the beginning of the incubations was 25 mmol L⁻¹. One mmol L⁻¹ NH₄Cl and 0.20 mmol L⁻¹ NaHCO₃ was added on days 24, 60, and 90. After this incubation, 5 mL of pure C₂H₂ gas were added to 40 vials, which resulted in a final C₂H₂ concentration of 100 L⁻¹. Samples with and without C₂H₂ were then incubated anaerobically for 20 days. The headspace gas was sampled every 24 hours for N₂O analysis, and soil samples were analyzed every two days for Fe and N species.

Example 4. Continuous flow membrane Feammox reactor

Soil samples collected on day 180 from the incubation with ferrihydrite, NH₄Cl, and 1.20 mmol L⁻¹ NaHCO₃ additions were inoculated into a continuous flow membrane reactor, which was operated under anaerobic conditions by constantly purging N₂ through the reactor's headspace at a room temperature (25°C), and with a 48 hour hydraulic retention time (Abbassi *et al.*, 2014 *J. Environ. Management* 142: 53-59, which is incorporated herein by reference as if fully set forth).

The enrichment medium contained the following components per liter: 177 mg NH₄Cl, 77.9 mg (NH₄)₂SO₄, 19.8 mg NaHCO₃, 71.0 mg KHCO₃, 9.00 mg KH₂PO₄, 100 mg MgSO₄·7H₂O, and 60.0 mg CaCl₂·2H₂O. After autoclaving, 1 mL trace element solution was added to the medium (Van de Graaf *et al.*, 1996 *J. Microbiol.*, 142:2187–2196, which is incorporated herein by reference as if fully set forth). 50.0 mmol L⁻¹ ferrihydrite were added once every two weeks directly into the reactor. To aid in

maintaining anaerobic conditions, 0.10 - 0.20 mmolL⁻¹ sodium citrate was fed to the reactor about twice per month. pH was controlled at around 4~5, and dissolved oxygen was < 0.10 mg/L. Samples from the outflow were collected every two days, and sludge samples from reactor were collected and kept at -20 °C for molecular biology analysis.

Finally, ¹⁵N isotope tracer incubations were conducted using slurries collected from the stable Feammox membrane reactor. Five treatments (n = 3 per treatment) were conducted: (1) control with only anoxic deionized (DI) water; (2) ¹⁵NH₄Cl addition; (3) ¹⁵NH₄Cl + Fe(III) addition; (4) ¹⁵NH₄Cl and C₂H₂ addition; (5) ¹⁵NH₄Cl, C₂H₂, and Fe(III) addition. The headspace gas of each 50mL incubation vial was sampled every 24 hours for ¹⁵N₂O analysis.

Example 5. Chemical analyses

For each sample collection during the incubations, a set of vials was destructively sampled in a glove box under oxygen-free conditions and the pH was measured immediately using a pH electrode. An extraction with 0.5N HCl was conducted for 24 hours at room temperature to determine acid-extractable Fe(II) and NH₄⁺ concentrations in the soils. Fe(II) was analyzed using the ferrozine assay method (Stookey, 1970 Anal Chem 42: 779–781; Komlos *et al.*, 2007 Water Res 41: 2996–3004, which are incorporated herein by reference as if fully set forth). Extraction efficiency of Fe(II) was affected by the HCl concentration and the extraction time. About 5-10 % more Fe(II) could be extracted with either 1N HCl extraction over 24 hours or with 0.5 N HCl over 36 hours as opposed to 0.5 N HCl over 24 hours. Furthermore, after more Fe(II) was produced in the system with increasing incubation time, the Fe(II) extraction efficiency improved. Only a 1-2% difference was observed in the Fe(II) extracted over 24 hours using 0.5N vs. 1N HCl towards the end of the incubation period. Clays, present in the soil incubations, typically sorb Fe(II) more efficiently when the total Fe(II) is low, furthermore ferrihydrite is slowly converted to magnetite, resulting in relatively different associations to different phases of the Fe(II) over the

duration of the incubation. All of which leads to incomplete Fe(II) extractions, especially when the Fe(II) is low. Fe(II) data obtained herein via 0.5N HCl extractions over 24 hours to ensure that the methods and hence data are comparable to those reported by other researchers focusing on iron reduction and iron bioavailability.

NH₄⁺ was analyzed using a Dionex™ Ion Chromatograph (LC3000) with a CS-16 Colum and a CS-16 guard column (flow rate = 1.00 mL min⁻¹, detection limit = 0.012 ppm). NO₃⁻ and NO₂⁻ were extracted with DI water for 1 hour anaerobically, and measured via Ion Chromatography, using an AS-22 Column along with an AG-22 guard column (flow rate = 1.20 mL min⁻¹, detection limit = 0.016 ppm). For the total organic carbon (TOC) and total nitrogen (TN) analyses a Shimadzu TOC-5000(A) was used. N₂O concentrations were determined on a gas chromatograph Shimadzu 2014 equipped with an electron capture detector.

Example 6. DNA and RNA isolation

DNA and RNA samples were extracted from soils collected at the wetland prior to any laboratory incubation, from the samples taken at different time points during the incubation experiments and from the reactor. DNA was extracted from 500 mg soil or sludge samples using the FastDNA® spin kit for soil (MP Biomedicals, USA) as described by the manufacturer, and RNA using the FastRNA® pro Soil Direct Kit. The concentrations were measured using a Nano-drop 2000 spectrophotometer (Thermo Scientific, USA).

Example 7. PCR-DGGE and 454 pyrosequencing analysis

Bacterial universal 16S rRNA gene primer sets V3-2/V3-3 were used for PCR amplification (Jensen *et al.*, 1998 FEMS Microbiol Ecol 26: 17–26, which is incorporated herein by reference as if fully set forth). DGGE was performed with an 8% polyacrylamide gel containing a gradient from 40% to 80% denaturant using the gradient gel electrophoresis system (C.B.S. SCIENTIFIC, USA). The electrophoresis

was carried out at 60 V for 15 hours. After that the gel was stained with 0.1 $\mu\text{L mL}^{-1}$ SYBR Green I and visualized with an UV transilluminator. All visible bands were excised from the gel and used as templates for re-amplification, using the primer set V3-1/ V3-2 and followed by cloning (Jensen *et al.*, 1998, which is incorporated herein by reference as if fully set forth). PCR products were purified via agarose gel extraction and cloned into a pGEM-T vector (Promega). A total of 10 to 30 positive recombinant clones for each band were identified by colony PCR, and were sent for sequencing to avoid erroneous interpretations. DNA sequencing was then conducted by Genewiz, Inc. Bacteria were classified and the phylogenetic tree of *Acidimicrobiaceae*-related sequences was constructed using the Bayesian inference (Huelsenbeck *et al.*, 2001 Science, 294:2310–2314, which is incorporated herein by reference as if fully set forth). Sequences obtained in this study were submitted to the GenBank database on February 4, 2013, and are available therein under accession numbers KC581755 - KC581779. See also Huang S and Jaffe PR, *Characterization of incubation experiments and development of an enrichment culture capable of ammonium oxidation under iron reducing conditions*, 2014, Biogosciences Discuss, 11, 12295-12321, which is incorporated herein by reference as if fully set forth. To further confirm the changes in the bacterial community, 454 pyrosequencing was performed with samples collected from the incubation on days 0, 30, 90, 160 and from the membrane reactor after 150 days of reactor operation. Domain-specific primers Bact-338F1/909R, targeting the V3-V5 region of the 16S rDNA of bacteria were amplified and sequenced according to Pinto *et al.* (2012, PLoS One 7: 43093, which is incorporated herein by reference as if fully set forth).

Example 8. Quantitative PCR (qPCR) assay

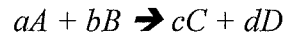
qPCR experiments were carried using a StepOnePlus™ Real-Time PCR System (Life Technologies, USA), represented by 16S rRNA genes, using primer sets 1055f/1392r for total bacteria (Harms *et al.*, 2003), Amx368f/Amx820r for anammox bacteria (Schmid *et al.*, 2000; Schmid *et al.*, 2003), acd320f - 432r which were developed for

Acidobacteriaceae bacteria, and acm342f - 439r which were developed for *Acidimicrobiaceae* bacteria. See Harms *et al.* 2003 *Environ Sci Technol* 37: 343–351, for total bacteria; a Schmid *et al.*, 2000 *Syst Appl Microbiol* 23:93–106, and Schmid *et al.*, 2003 *Syst Appl Microbiol* 26:529–538 for anammox bacteria. For the detection of denitrifiers, AOB and AOA, denitrifying functional genes (*nirS* and *nirK*), ammonia monooxygenase structural gene (*amoA*) were quantified with primer sets NirS3F/NirS5R, NirK1F/NirK5R, amoA-1F/ amoA-2R for AOB-*amoA* and Arch amoA-F/ amoA-R for AOA-*amoA* (Braker *et al.*, 1998 *Appl Environ Microbiol* 64:3769–3775; Rotthauwe *et al.*, 1997 *Environ Microbiol* 9:1761–1771; Francis *et al.*, 2005 *Proc. Natl. Acad. Sci.* 102:14683–14688, all of which are incorporated by reference herein as if fully set forth). Primer pairs CrenamoA23f /616r, was also used to quantify the thaumarchaeal *amoA* genes which represented acidophilic ammonia oxidizers (Tourna *et al.*, 2008 *Environ Microbiol* 10: 1357–1364, which is incorporated herein by reference as if fully set forth). For DNA quantification, each qPCR mixture (20 μ L) was composed of 10 μ L of SYBR Premix Ex Taq® II (Takara, Japan), 0.8 μ L 10 μ M of each primer, and ~ 10 ng DNA template. RNA quantification was conducted through a real-time quantitative reverse transcription-PCR (RT-qPCR) analysis, by using the One Step SYBR® PrimeScript® RT-PCR Kit (Takara, Japan) according to the manufacturer's recommendations. Thermal cycling conditions for total 16S rDNA, *nirS* and AOB-*amoA* gene numbers was initiated for 30 s at 94 °C, followed by 40 cycles of 5 s at 94°C, 30 s at 57°C, and 30 s at 70°C. 16S rDNA numbers of anammox, *Acidobacteriaceae* and *Acidimicrobiaceae* bacteria were performed at 56°C, 55°C, and 58°C as annealing temperature respectively, with the same program. For AOB-*amoA* gene and thaumarchaeal *amoA* genes, annealing temperature was 53°C and 55°C, and the annealing time was adjusted to 45 s. For RNA quantification, the cycling conditions were identical to those described for measuring gene numbers, with the exception that an initial incubation was conducted for 5 min at 42°C to facilitate reverse transcriptase activity. Each assay contained a standard using a serial dilution of plasmids containing specific target genes, independent triplicate templates for each soil sample,

and triplicate no template controls (NTC).

Example 9. 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 ° Kelvin (297.15 K). Free energies of formation were obtained from Stumm and Morgan (1996): $\Delta G_f^0(\text{NH}_4^+) = -79.37 \text{ kJ mol}^{-1}$, $\Delta G_f^0(\text{NO}_2) = -37.2 \text{ kJ mol}^{-1}$, $\Delta G_f^0(\text{H}_2\text{O}) = -237.18 \text{ kJ mol}^{-1}$, $\Delta G_f^0(\text{Fe}^{2+}) = -78.87 \text{ kJ mol}^{-1}$. $\Delta G_f^0(\text{Fe}_2\text{O}_3 \cdot 0.5\text{H}_2\text{O}) = -711 \text{ kJ mol}^{-1}$ (Stumm and Morgan 1996 Aquatic Chemistry: Chemical Equilibria and Rates in Natural Waters (*John Wiley, New York*); Majzlan *et al.*, 2004 Geochim. Cosmochim. Acta. 68, 1049–1059, which are incorporated herein by reference as if fully set forth). 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 *Brock Biology of Microorganisms*, 10th ed. Appendix1: energy calculations in microbial bioenergetics, which is incorporated by reference herein as if fully set forth). The chemical activity values used in the calculation are based on our incubation experiments: $C_{\text{NH}_4^+} = 2 \text{ mmol L}^{-1}$, $C_{\text{NO}_2} = 10 \text{ } \mu\text{mol L}^{-1}$, $C_{\text{Fe}^{2+}} \leq 0.01 \text{ } \mu\text{mol L}$ (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) oxides. 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.

$$\begin{aligned}
 & 3Fe_2O_3 \cdot 0.5H_2O + 10H^+ + NH_4^+ \rightarrow 6Fe^{2+} + 8.5H_2O + NO_2^- \text{ (Equation 1)} \\
 & \Delta G_r \leq [6\Delta G_{fFe^{2+}}^0 + 8.5\Delta G_{fH_2O}^0 + 1\Delta G_{fNO_2^-}^0 - 3\Delta G_{fFe_2O_3 \cdot 0.5H_2O}^0 - 10\Delta G_{fH^+}^0 - 1\Delta G_{fNH_4^+}^0] \\
 & \quad + (0.008314 \text{ kJ mol}^{-1})(297.15 \text{ K}) \ln \frac{(C_{Fe^{2+}})^6 (C_{H_2O})^{8.5} (C_{NO_2^-})^1}{(C_{Fe_2O_3 \cdot 0.5H_2O})^3 (C_{H^+})^{10} (C_{NH_4^+})^1} \\
 & \Delta G_r \leq [6(-78.87) + 8.5(-237.18) + 1(37.2) - 3(-711) - 10(4 \times -5.69) - 1(-79.37)] \\
 & \quad + (0.008314 \text{ kJ mol}^{-1})(297.15 \text{ K}) \ln \frac{(10^{-8})^6 (1)^{8.5} (10^{-5})^1}{(1)^3 (10^{-4})^{10} (2 \times 10^{-3})^1} \\
 & \Delta G_r \leq -145.08 \text{ kJ mol}^{-1}
 \end{aligned}$$

The $\leq$ sign is because an upper limit (detection limit) was used for the Fe(II) concentration.

FIG. 10 illustrates a graph of ΔG vs. pH. Referring to this figure, pH shows that when maintaining all species concentrations constant except H^+ , Feammox should not be feasible when the pH is above 6.5. Hence, Feammox is expected to occur in acidic environments.

Example 10. PCR amplification, DGGE analysis and pyrosequencing

Bacterial universal 16S rRNA gene primer sets V3-2/V3-3 and 27f/519r were used for PCR amplification (Jensen *et al.*, 1998; Lane, 1991, both of which are incorporated herein by reference as if fully set forth). Each 25 μ L reaction mixture contained 2.5 μ L 10 $\times$ PCR Buffer (500 mM KCl, 25 mM MgCl₂, 200 mM Tris-HCl [pH 8.4], 0.1% Triton X-100), 2.0 μ L 2.5mM DNTP mixture (Takara, Japan), 0.3 μ L of 10 μ M V3-2 and V3-3, 0.13 μ L 5U Taq polymerase, 1 μ L of template DNA, and 18.77 μ L 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 $\mu\text{L mL}^{-1}$ 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, which is incorporated herein by reference as if fully set forth). 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 *Appl. Environ. Microbl.* 71, 1501–1506, which is incorporated herein by reference as if fully set forth). Six groups of bacteria were classified via a phylogenetic analysis using the Bayesian inference (BI), implemented with MrBayes version 3.1.2 (Huelsenbeck *et al.*, 2001; Ronquist *et al.*, 2003 *Bioinformatics.* 19, 1572–1574, which are incorporated herein by reference as if fully set forth). A best fit model of nucleotide substitution was identified using the Akaike information criterion (AIC) (Akaike, 1973) as implemented in MrModelTest 2.3 (Nylander, 2004). Bayesian analysis was carried out using GTR+I+G model selected by MrModelTest 2.3, in which model parameters were treated as unknown and estimated through the BI. The

following settings were applied: implementing two Markov chain Monte Carlo (MCMC) runs, running four simultaneous Markov chains for 19 million generations, and sampling the Markov chains every 100 generations. Tracer V1.5 (Rambaut and Drummond, 2009) was used to judge convergence of the Bayesian Markov chain Monte Carlo runs. The first 10,000 sampled trees were discarded as burn-in. A consensus tree was constructed from the remaining sampled trees. Sequences obtained in this study were submitted to the GenBank database on February 4, 2013, and are available therein under accession numbers KC581755 - KC581779. See also Huang S and Jaffe PR, *Characterization of incubation experiments and development of an enrichment culture capable of ammonium oxidation under iron reducing conditions*, 2014, Biogeosciences Discuss, 11, 12295-12321, which is incorporated herein by reference as if fully set forth. Approximately 2.5 ng of each DNA extract from samples collected from the incubation on days 0, 30, 90, 160 and from the membrane reactor after 150 days of reactor operation were used for 454 pyrosequencing analysis. To amplify a 16S rRNA gene fragment of the appropriate size and sequence variability for the 454 pyrosequencing, specific primers Bact-338F1(CCTACG GGRGGCAGCAG) (SEQ ID NO: 2) /909R(CCGTCAATTYHTTTTRAGT) (SEQ ID NO: 3), targeting the V3-V5 region of the 16S rRNA gene of bacteria were chosen (Pinto and Raskin 2012, which is incorporated herein by reference as if fully set forth). The PCR conditions used were 94°C for 2 min, 20 cycles of 94°C, 45 s denaturation; 55°C, 45 s annealing and 72°C, 1 min extension; followed by 72°C, 6 min. After 20 rounds of amplification, another 3 rounds of amplification were done to add the A and B adapters required for 454 pyrosequencing to specific ends of the amplified 16S rRNA fragment for library construction (Margulies *et al.*, 2005 Nature, 437, 376–380, which is incorporated herein by reference as if fully set forth). Approximately 4 ng/μl of 16S rRNA gene fragment from each soil samples was required to construct the five libraries for 454 sequencing. Polymerase chain reaction products were cleaned using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions, quantified using a Qubit Fluorometer (Invitrogen), and then sent for 454-pyrosequencing using a Roche/454 GS

FLX sequencer.

A total of 19,021 partial 16S rRNA sequences were obtained from the five soil samples. The sequences were then passed through the DOTUR program to further reduce errors as outlined previously (Schloss and Handelsman, 2005, which is incorporated herein by reference as if fully set forth). Briefly, after trimming, pre-clustering, removal of chloroplast sequences and alignments, a total of 10172 sequences remained. These were clustered with the average neighbor algorithm with a 3% dissimilarity cutoff, which resulted in 1015 OTUs (Pinto and Raskin 2012, which is incorporated herein by reference as if fully set forth).

Example 11. Primer design for real-time PCR assay

Two sets of primers, acd320f (5'-CGG TCC AGA CTC CTA CGG GA -3') (SEQ ID NO: 4) - 432r (5'-GAC AGG GTT TTA CAG TCC GAA GA -3') (SEQ ID NO: 5) and acm342f (5'-GCA ATG GGG GAA ACC CTG AC-3') (SEQ ID NO: 6) - 439r (5'-ACC GTC AAT TTC GTC CCT GC -3') (SEQ ID NO: 7) 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.

Example 12. Isotope tracer incubations

After 270 days of operation, Feammox enrichment slurries collected from the Feammox membrane reactor were used for isotope tracer incubations. Slurries were first incubated for 20 days in 50 mL vials under anaerobic conditions. Five treatments (n =

3 per treatment) were conducted as follows: (1) control treatment with only anoxic DI water; (2) $^{15}\text{NH}_4\text{Cl}$ addition; (3) $^{15}\text{NH}_4\text{Cl} + \text{Fe(III)}$; (4) $^{15}\text{NH}_4\text{Cl}$ and C_2H_2 addition; (5) $^{15}\text{NH}_4\text{Cl}$, C_2H_2 and Fe(III) addition. The final concentration of $^{15}\text{NH}_4\text{Cl}$ was 1 mmol L^{-1} , and 5 mmol L^{-1} of ferrihydrite was added as the Fe(III) source. 5 mL of pure C_2H_2 gas was added to the vials, which resulted in a final C_2H_2 concentration of $100 \text{ } \mu\text{mol L}^{-1}$. Samples were then incubated anaerobically for 7 days. The headspace gas was sampled every 24 hours for $^{15}\text{N}_2\text{O}$ analysis. All these processes were conducted in an anaerobic glove box. N_2O was determined by isotope ratio mass spectrometry (IRMS, Thermo Finnigan Delta V Advantage, Bremen, Germany). $^{15}\text{N}_2\text{O}$ concentration was also calculated as $^{15}\text{N}_2\text{O}$ atom% excess above its natural abundance, following methods described by Ding *et al.* 2014 *Environ Sci Technol.* 48(18):10641-7, which is incorporated herein by reference as if fully set forth. $^{15}\text{N}_2\text{O}$ production rates were calculated from the linear change in $^{15}\text{N}_2$ concentrations in the vial headspace between two given time points.

Example 13. Change in Fe and N species under different operational conditions

Batch incubation experiments were conducted. Batches included soil slurry included with three different Fe(III) sources. FIGS. 1A - 1B illustrate concentration of Fe(II) (FIG. 1A) and NH_4^+ (FIG. 1B) in incubation with three different Fe(III) sources: ferrihydrite (open circle), ferric chloride (closed square), and ferric citrate (closed triangle). Referring to these figures, the values represent the mean and standard error ($n=3$). After incubating the pre-treated soil slurry with the three different Fe(III) sources for 30 days, only samples to which either ferrihydrite or goethite had been added showed measurable NH_4^+ oxidation. Referring to FIGS. 1A - 1B, in samples incubated with ferric citrate and NH_4Cl , Fe(III) reduction was much faster than in those supplied with Fe(III) oxides, but the NH_4^+ concentration remained fairly constant. FIGS. 2A - 2B illustrate concentration of Fe(II) (FIG. 2A) and NH_4^+ (FIG. 2B) in 25-day incubation with NH_4Cl and ferrihydrite (open circle), NH_4Cl and goethite (closed circle). The values represent the mean and standard error ($n=3$). FIGS. 3A - 3B

illustrate concentration of Fe(II) (FIG. 3A) and NH_4^+ (FIG. 3B) 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. 1.2 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). FIGS. 4A - 4B illustrate concentration of Fe(II) (FIG. 4A) and NH_4^+ (FIG. 4B) in 25-day incubations in samples with NH_4Cl (open square), ferrihydrite (open circle), sterilized soil with NH_4Cl and ferrihydrite (closed triangle). Referring to these figures, the values represent the mean and standard error (n=3). Still referring to FIGS. 4A - 4B, no detectable Fe(II) reduction or NH_4^+ oxidation was found in the sterilized soils amended with ferrihydrite and NH_4Cl . Referring to FIGS. 2A - 2B, faster iron reduction and NH_4^+ removal was observed in ferrihydrite than in goethite-amended sediments.

Since samples incubated with ferrihydrite and NH_4Cl resulted in the fastest NH_4^+ oxidation, the anaerobic incubation with ferrihydrite was extended to 180 days. Ferrihydrite as the Fe(III) source results in a larger negative ΔG value than goethite. The NH_4^+ oxidation rate increased as NH_4Cl was supplied repeatedly, especially after 125 days of incubation when the NaHCO_3 additions were increased from 0.20 to 1.20 mmol L^{-1} in addition to the 2.00 mmol L^{-1} NH_4^+ added. Referring to FIGS. 4A - 4B, the increased NaHCO_3 dosing also increased the generation of Fe(II). FIG. 9 illustrates stoichiometry between the Fe(II) production and NH_4^+ consumption during 180 day incubation. Referring to FIG. 9, 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 ratio (n=3). During the 180-day incubation, the ratio of Fe(II) produced to NH_4^+ removed gradually increased until it reached 5.3:1 by day 160 after which it remained stable.

FIGS. 5A - 5B illustrate concentration of NO_3^- (open circle) and NO_2^- (closed square)

(FIG. 5A) and DOC (open triangle) and TN (open square) (FIG. 5B) during the 180 day incubation. Referring to these figures, 25 mmol L⁻¹ Fe(III) was added on day 0. 1.0 mmol L⁻¹ NH₄⁺ was added on days 4, 24, and 60. 0.2 mmol L⁻¹ NaHCO₃ was added on day 50 and day 90 of the incubation; 1.20 mmol L⁻¹ + 2 mmol L⁻¹ of NH₄Cl were added on day 125. The values represent the mean and standard error (n=3).

Referring to FIGS, 5A - 5B, NO₂⁻ appeared within a few days after the addition of NH₄⁺, with a maximum concentration 0.44 ± 0.17 mmol L⁻¹ in the second NH₄⁺ oxidation cycle. Referring to FIG. 5A, NO₂⁻ did not accumulate in the system and was immediately consumed after generation. Referring to FIGS. 5A - 5B, NO₃⁻ production showed a similar pattern to that of NO₂⁻ (FIG. 5A), and TN loss similar to the decrease in NH₄⁺ (FIG. 5B). During 180 days of incubation, the system experienced a loss of TN of 57.2 ± 3.13 mg L⁻¹. Referring to FIG. 5B, the DOC content fluctuated slightly in the early stage of incubation, but overall, the DOC concentration was relatively stable at around 45~50 mg L⁻¹.

A 64.5% NH₄⁺ removal, between inflow and outflow was achieved in the membrane reactor after 150-days of operation.

Example 14. Phylogenetic analysis of the microbial community based on 16S rRNA gene

FIG. 6 illustrates comparison of denaturing gradient gel electrophoresis (DGGE) analysis profiles of soil communities during anaerobic incubations. Samples from 0, 30, 90 and 160 days of incubation with ferrihydrite + NH₄Cl + NaHCO₃ (lane 1-4); 160 days of incubation with only ferrihydrite (lane 5); ferric chloride + NH₄Cl (lane 6); ferric citrate + NH₄Cl (lane 7); 120 days incubation with ferrihydrite + NH₄Cl + NaHCO₃ + organic carbon (band 8); ferrihydrite + NH₄Cl + organic carbon (lane 9).

Still referring to FIG. 6, samples from 6 and 120 days of incubation without any addition (lanes 10 and 11) were used as controls.

Still referring to FIG. 6, all visible bands observed in the DGGE analysis (significant bands were marked) were excised from the gel and sequenced after cloning. Clone libraries from 12 samples resulted in 721 sequences of partial 16S rRNA gene fragments, and six groups of bacteria were classified via a phylogenetic analysis. During this 180-day anaerobic incubation with ferrihydrite and NH_4Cl , the microbial communities shifted dramatically and the microbial diversity decreased with time. See FIG. 6, lanes 1-4. Some DGGE bands disappeared gradually with time, such as band A5 and band A7. Band A5, represents a dissimilatory iron-reducing bacteria, *Geobacter* sp., which existed in this Fe(III)-rich wetland soil and reappeared for a short time during the initial anaerobic incubation. Band A7, represents an ammonia-oxidizing bacterium, *Nitrosomonas* sp., which showed a strong presence in the samples at 30 days of incubation and was attenuated after longer incubation times. In contrast, DGGE bands A6, A8 and A9 became more significant as the incubation time increased, showing that there were three groups of bacteria dominating in the system after 160 days of incubation. Band A6 represents a group of bacteria belonging to the *Acidimicrobiaceae* family. Bacteria from the *Acidobacteriaceae* family are represented by band A8. Some species in this family have been described as iron reducers and obligate heterotrophs (Kishimoto *et al*, 1991 Water Res 41: 2996–3004; Rowe *et al.*, 2007 Environ Microbiol 9:1761–1771; Coupland and Johnson, 2008 FEMS Microbiol Lett 279:30–35). DGGE band A9 represents bacteria of the *Rhodocyclaceae* family. This family contains mainly denitrifying bacteria, which exhibit very versatile metabolic capabilities (Smith *et al*, 2005 Water Res 39: 2014–2023; Huang *et al.*, 2011 Biogeosciences 8: 5251–5280).

Microbial communities also differed between samples incubated with various Fe(III) sources, and between samples with or without the addition of inorganic carbon.

Samples supplied with either ferric chloride or ferric citrate as the Fe(III) source plus NH_4^+ , and samples supplied with just ferrihydrite and no NH_4^+ , had a decreased diversity in their bacterial communities. See FIG. 6, lanes 5-7. Samples supplied with both organic carbon (1.00 mmol L⁻¹ sodium citrate) and inorganic carbon (1.20 mmol L⁻¹ NaHCO_3) had a higher bacterial diversity. See FIG. 6, lane 8.

Changes in the microbial community after 180 days of incubation were also confirmed via 454-pyrosequencing, and the obvious growth of *Actinobacteria*, *Acidobacteria* and β -*Proteobacteria* groups (which band A6, A8 and A9 belong to) was consistent with the DGGE results, where the *Actinobacteria* cell number increased the most. *Planctomycetes* phylum, with which anammox bacteria are affiliated, was detected in the first 90 days of incubation, but disappeared or was below detection on day 160. *Actinobacteria* were also the dominant species in the Feammox enrichment reactor based on the results of the 16S rDNA library obtained via pyrosequencing.

The *Acidimicrobiaceae* bacterium, represented by band A6, which belongs to the *Actinobacteria* phylum, was the dominant species in the incubation experiments after 180 days of incubation (14.8% in total 16S rRNA gene sequences) as well as in the membrane reactor after 150 days of operation (40.2% in total 16S rRNA gene sequences). Its similarity to other *Acidimicrobiaceae*-related sequences was shown using a phylogenetic tree. The tree was constructed using the Bayesian inference (BI) method with 16S rRNA gene sequences from DGGE band A6 and bacteria from the *Acidimicrobiaceae* family from other studies. Unlike the bacteria represented by band A8 and A9, which were also found in samples that did not show Feammox transformations, this *Acidimicrobiaceae* bacterium was only detected in incubations (or reactor) that were augmented simultaneously with ferrihydrite, NH_4Cl , and NaHCO_3 and have shown Feammox activity.

Example 15. Changes of bacterial abundance and activity during incubations and in the

reactor

The total bacterial abundance determined via the 16S rRNA gene copy number, decreased during the 180-day incubation. FIG. 7 illustrates abundance of total bacteria (closed rhombus) during 180 days of anaerobic incubation. 16S rRNA gene (black bars) and RNA copy numbers (gray bars) of *Acidimicrobiaceae* bacterium A6 in soil samples with 1.20 mmol L⁻¹ NaHCO₃ addition. 16S rRNA gene (white bars) and RNA copy numbers (dotted bars) of bacterium A6 with 0.20 mmol L⁻¹ NaHCO₃ addition. Referring to FIG. 7, both, 16S rRNA gene and rRNA fragment copies of *Acidimicrobiaceae* bacteria (DGGE band A6) increased during the incubation, particularly after 90 days. Still referring to FIG. 7, the rRNA numbers increased slowly during the first 3 months and doubled between day 130 and day 140 of the incubation period. rRNA as a biomarker for changes of protein level, even though not as specific as mRNA, is a good indicator for bacterial activity (Poulsen *et al.*, 1993 Appl Environ Microbiol 59: 1354–1360; Park *et al.*, 2010 Environ Sci Technol 44: 6110–6116), both of which are incorporated by reference herein as if fully set forth. Increase in the denitrifier activity was most likely stimulated by the NO₂⁻ generated via Feammox. The number of the *amoA* gene, representing the abundance of ammonia-oxidizing bacteria, decreased sharply with time and was hardly detected after 90 days of incubation. Through quantification of thaumarchaeal *amoA* genes, none of the acidophilic ammonia oxidizers were detected in the system.

In the Feammox reactor, the copy number of *Acidimicrobiaceae* bacterium A6, Anammox bacteria and *nirS* gene were 0.37×10^7 , 0.13×10^6 , and 0.92×10^6 copies g⁻¹ dw, respectively, while the *amoA* gene was not detected.

Example 16. Changes of bacterial abundance and activities with NaHCO₃ amendment

Abundance and activity of *Acidimicrobiaceae* bacteria, represented by band A6, were compared between samples incubated under the same conditions except the amounts of NaHCO₃ added (0.20 mmol L⁻¹ vs. 1.20 mmol L⁻¹). From day 125 to day 180 of the

incubation, both 16S rRNA gene and rRNA fragment numbers of *Acidimicrobiaceae* bacteria were higher in the soils with the higher inorganic carbon content. Referring to FIG. 7, the 16S rRNA gene copies of samples augmented with 1.20 mmol L⁻¹ NaHCO₃ were four times higher than those in samples that had been augmented with only 0.20 mmol L⁻¹ NaHCO₃. The rRNA copies of the *Acidimicrobiaceae* bacteria, showed even larger differences in response to the amounts of NaHCO₃ added. In the samples augmented with 1.20 mmol L⁻¹ NaHCO₃, the rRNA copy number increased from $0.04 \pm 0.06 \times 10^6$ to $0.19 \pm 0.09 \times 10^6$ copies g⁻¹ dw over 50 days of incubation (day 130 to day 180). Still referring to FIG. 7, however, in the samples to which only 0.2 mmol L⁻¹ NaHCO₃ were added on day 125, the rRNA number gradually decreased from $0.29 \pm 0.10 \times 10^5$ to $0.19 \pm 0.05 \times 10^5$ copies g⁻¹ dw during the same 50 days incubation.

Example 17. Nitrogen species changes in samples incubated in the presence of C₂H₂

C₂H₂ can inhibit the oxidation of NH₄⁺ to NO₂⁻ under aerobic conditions, and the reduction of N₂O to N₂ as well as the anammox pathway under anaerobic conditions (Yoshinari *et al.*, 1977 Soil Biol Biochem 9: 177–183; Jensen *et al.*, 2007 FEMS Microbiol Ecol 26: 17–26; Kartal *et al.*, 2011 Nature 479:127–130, all of which are incorporated by reference herein as if fully set forth). To gain further insights into the nitrogen removal process observed, incubations with C₂H₂ were conducted. FIGS. 8A–8D illustrate concentration of NH₄⁺ (FIG. 8A), NO₂⁻ (FIG. 8B), NO₃⁻ (FIG. 8C), and N₂O (FIG. 8D) in the samples incubated with (open circle) or without (closed square) C₂H₂. The values represent the mean and standard error (n=3). After 20 days of incubation, less NH₄⁺ was oxidized in the samples amended with C₂H₂, compared to those incubated without C₂H₂. Referring to FIG. 8A, NO₂⁻, which is postulated to be the direct product of the NH₄⁺ oxidation, accumulated slowly in samples incubated with C₂H₂. Referring to FIG. 8B, NO₃⁻ reached a higher concentration in samples without C₂H₂ than in samples incubated with C₂H₂. Referring to FIG. 8C, N₂O, a product of NO₂⁻ reduction, accumulated in samples incubated with C₂H₂, which inhibits the reduction of N₂O to N₂. Fe(II) production was not much affected by the presence of

C₂H₂, and after 20 days incubation was 4.36 ± 0.72 and 5.71 ± 0.67 mmol L⁻¹ in sample incubated with and without C₂H₂, respectively.

In ¹⁵N isotope tracer incubations, detectable ¹⁵N-N₂O was only found in samples amended with both, ¹⁵NH₄Cl and Fe(III), with ¹⁵N-N₂O production rates 2.14 ± 0.059 or 0.072 ± 0.023 µg g⁻¹ d⁻¹ in samples incubated with or without C₂H₂ treatment.

Referring to FIG. 6, DGGE band A5 represents dissimilatory iron-reducing bacteria, which appeared for a short time at the beginning of the anaerobic incubation. For longer incubation times these heterotrophic bacteria decreased rapidly. Referring to FIG. 2A and FIG. 4A, over a 25 day incubation period, more than three times the mass of Fe(II) was produced in samples amended with ferrihydrite and NH₄⁺ as compared to the samples amended only with ferrihydrite, indicating that most of the Fe(III) reduction came from the Feammox reaction, and not from dissimilatory Fe(III) reduction.

Referring to FIG. 6, AOB represented by DGGE band A7, as well as the *amoA* gene, decreased after 30 days of incubation. Also, no AOA or acidophilic ammonia oxidizers were detected although the pH condition seems suitable for them in these incubations.

Oxygen deficiency was the most likely reason for the decline in AOB in this system over time (Laanbroek *et al.*, 1994 *Arch Microbiol* 161:156–162). Referring to FIGS. 4A - 4B, even though a small amount of AOB would be enough for NH₄⁺ oxidation, in control samples to which no Fe(III) was added, and which AOB do not require, no NH₄⁺ consumption was detected. Moreover, the decrease in *amoA* gene at a time of increasing NH₄⁺ oxidation also indicates that neither AOB nor acidophilic ammonia oxidizers were the drivers of the NH₄⁺ oxidation in the later incubation times.

An uncultured *Acidimicrobiaceae* bacterium became the dominant species during the 180-day anaerobic incubation period, increasing from 0.92% on day 0 in terms of cell

numbers to 14.8% on day 160. Referring to FIGS. 1A - 1B, FIGS. 2A - 2B, and FIG. 6, in the incubation experiments conducted (which included controls with only NH_4^+ , only iron, autoclaved, and various Fe(III) sources), this *Acidimicrobiaceae* bacterium was only detected and growing in samples to which NH_4^+ was supplied as an electron donor, ferrihydrite was supplied as electron acceptor, and NaHCO_3 was supplied as a carbon source. The abundance and activity of this *Acidimicrobiaceae* bacterium increased along with the Feammox activity during the incubations. Referring to FIG. 7, during the incubation period its rRNA changed from $(0.22 \pm 0.01) \times 10^5$ copies g^{-1} dw to $(0.28 \pm 0.07) \times 10^6$ copies g^{-1} dw, indicating a substantial increase in its activity. In the continuous flow membrane reactor, which had a high NH_4^+ removal and Fe(III) reduction rate, this *Acidimicrobiaceae* bacterium was enriched from an initial 14.8% to 40.2% after 150 days operation, and no other known NH_4^+ oxidizers (AOB or anammox) were detected. These results indicated that this *Acidimicrobiaceae* bacterium might play an important role in the Feammox reactions described in this study. According to a phylogenetic analysis, this bacterium has a 92% identity with *Ferrimicrobium acidiphilum* sp. *F. acidiphilum*, which belongs to the *Acidimicrobiaceae* family, and was first isolated from mine environments, and *F. acidiphilum* strain T23 is the only pure strain with a comprehensive characterization. Uncultured *Ferrimicrobium* sp. has been detected in mine water, but so far not in wetland soils. *Ferrimicrobium* sp. is an acidophilic heterotrophic ferrous iron oxidizing bacterium, which can also reduce Fe(III) under anoxic conditions (Johnson *et al.*, 2009). The uncultured *Acidimicrobiaceae* bacterium, also has a 90% identity with *Acidimicrobium ferrooxidans*, a facultative autotroph in the same family that can reduce Fe(III) in anaerobic environments while oxidizing sulfide to sulfur and exists widely in soil environments.

According to a phylogenetic comparison with similar clones from studies reported in the GenBank, and taking into account its special growth characteristics (stimulated by inorganic carbon, oxidizing NH_4^+ coupled to Fe(III) reduction), also its gradual activity

increase with increased Feammox activity, as well as a strong link with a Feammox enrichment reactor, this uncultured *Acidimicrobiaceae* bacterium A6 is probably a previously unreported species in the *Acidimicrobiaceae* family that might be either responsible or play a key role in the Feammox process described here. Referring to FIGS. 3A - 3B and FIG. 7, *Acidimicrobiaceae* bacterium A6 was more active and the Feammox pathway was faster in samples with higher NaHCO_3 amendments, which, in addition to the fact that ΔG in Equation 1 is negative, indicates that if this *Acidimicrobiaceae* bacterium is actually responsible for conducting the Feammox reaction as depicted in equation 1, it may be an autotroph. Growth of *nirS* gene suggested that denitrification pathways were also active in the incubations described here. NO_2^- that was being produced during the anaerobic NH_4^+ oxidation was reduced to N_2 by denitrifiers, and NO_2^- did not accumulate in the system.

Referring to FIGS. 1A - 1B and FIGS. 2A - 2B, the Feammox reaction studied here proceeded only when iron oxides (ferrihydrite or goethite) were supplied as electron acceptor, whereas samples incubated with ferric chloride or ferric citrate as the Fe(III) source showed no measurable NH_4^+ oxidation. In the incubations to which ferric citrate was added as the Fe(III) source, Fe(III) was reduced rapidly by dissimilatory iron reducers, using organic carbon as electron donor. Referring to FIG. 6, the DGGE results for incubations with ferric citrate (lane 7) show that the most dominant species was an *Actinobacterium*, known to reduce iron under anaerobic conditions. *Acidimicrobiaceae* bacterium A6 was not detected in these incubations. Since acidic conditions as well as minimal dissolved Fe(II) and NO_2^- concentrations are required to make the Feammox reaction energetically favorable as shown in Equation 1, the presence of iron oxides as the main Fe(III) source may have helped to maintain the concentrations of Fe(II) in solution below the detection limit through the incubation since iron oxides can sorb Fe(II) and/or incorporate it into their structure.

Various NH_4^+ oxidation products, i.e. NO_3^- , NO_2^- and N_2 , generated through the

Feammox process are thermodynamically feasible, and were reported in different Feammox studies (Sawayama, 2006; Shrestha *et al.*, 2009; Yang *et al.*, 2012, which are incorporated by reference herein as if fully set forth). Because there was no initial nitrate or nitrite in the system, because all experiments were conducted under strict oxygen free conditions, and because of the rapid decrease of *amoA* genes, neither NO_3^- reduction nor aerobic NH_4^+ oxidation could be the reason for the formation of NO_2^- during the incubations. In all incubations where NH_4^+ was removed, the production of NO_2^- was observed, although NO_2^- did not accumulate. Most of the NO_2^- produced from the NH_4^+ oxidation was reduced rapidly by denitrifiers, which were present in the incubations.

When C_2H_2 was used to stop the reduction of N_2O to N_2 , the total N_2O (0.72 ± 0.23 mmol L^{-1}) plus NO_2^- produced (0.13 ± 0.07 mmol L^{-1}) was equal to the NH_4^+ consumed, showing that NH_4^+ was not oxidized directly to N_2 in the samples. ^{15}N - NH_4^+ incubations, as an extension of C_2H_2 treatment, showed that ^{15}N - N_2O built up when $^{15}\text{NH}_4\text{Cl}$ was added as the NH_4^+ source, demonstrating that NH_4^+ was oxidized during the Feammox process rather than be adsorbed or taken uptake by microorganisms in the system.

Although nitrification might happen in suboxic environments (oxygen $< 5\mu\text{M}$, Lam *et al.*, 2007), NH_4^+ oxidation in the presence of C_2H_2 has never been reported. C_2H_2 is an inhibitor of ammonia monooxygenase (AMO), and can restrain aerobic NH_4^+ oxidizers from using oxygen by binding covalently to AMO (Hynes and Knowles, 1982; Hyman and Wood, 1985; Gilch *et al.*, 2009). C_2H_2 can also inhibit the NH_4^+ -activation step of anammox cells, which use NO_2^- as the oxidant (Kartal *et al.*, 2011). Therefore these Feammox bacteria might differ from common NH_4^+ oxidizers, by using an alternative NH_4^+ oxidation pathway that is not inhibited by C_2H_2 , and AMO might not play a role in Feammox. The fact that NH_4^+ oxidation was not affected by the presence of acetylene is a further indication that AOB are not responsible for this process since

they would be affected by acetylene. Furthermore, in the isotope tracer incubations, $^{15}\text{N}\text{-N}_2\text{O}$ was below the detection limit in samples to which Fe(III) was not supplied, showing again that NH_4^+ oxidation proceeded only when iron was being reduced.

Referring to FIG. 9, the role of anammox during the incubations was also evaluated. During the incubations the ratio of NH_4^+ oxidized to Fe(III) reduced increased gradually from 1:1.9 to 1:5.3, which is close to the stoichiometry of 1:6, shown in Equation 1. Although the discrepancies in the Feammox stoichiometry between iron and NH_4^+ are attributed in part to incompletely Fe(II) extraction, the influence of anammox activity in the earlier incubations would have contributed to a lower Fe(II) produced to NH_4^+ removed ratio than the theoretical value of 1:6.

The Feammox reaction became more dominant in terms of NH_4^+ oxidation after 125 days of incubation due to a relative increase in the activity of the Feammox bacteria. A parallel pathway to Feammox, such as anammox, could as mentioned above, explain the lower stoichiometric ratio, especially at earlier incubation times. In the samples taken before the incubation, $0.17 \pm 0.05 \times 10^6$ copies g^{-1} dw of anammox rRNA gene were found, which decreased to $0.09 \pm 0.06 \times 10^5$ on day 130. It was postulated that anammox was responsible for some initial NH_4^+ and NO_2^- removal, and denitrification became more dominant for NO_2^- removal later during the incubation period. NH_4^+ removal via Anammox in the early incubations may also explain why the observed NH_4^+ oxidation rates and the abundance of *Acidimicrobiaceae A6* did not change proportionally over the full incubation period.

Example 18. Influence of environmental factors on Feammox pathway in soil environments

Sample collection. Soils for all the experiments described in this study were taken from a series of local wetland-, upland-, as well as storm-water detention pond-sediment at

New Jersey and labeled according to the samples location as L, A, P, C, B, V and W. Three soil cores were collected from 0 cm to 20 cm soil from each sample location, and transported to the laboratory within 2 hours for further analysis.

Analysis of chemical properties. Physicochemical characteristic of these soils such as soil pH; Fe content [Fe (III) and Fe(II)]; nitrogen species (NO_3^- , NO_2^- and NH_4^+); sulfate (SO_4^{2-}); phosphate (PO_4^{3-}); dissolve organic carbon (DOC) were determined. pH was measured immediately using a pH electrode. A 0.5N HCl extraction was conducted for 24 hour at room temperature to determine acid-extractable Fe(II) and NH_4^+ concentrations in the soils. Fe(II) was analyzed using the ferrozine assay method (39), and NH_4^+ was analyzed using a Dionex™ Ion Chromatograph (LC3000) with a CS-16 Colum and a CS-16 guard column (flow rate = 1.0 mL min^{-1} , detection limit = 0.012 ppm). NO_3^- , NO_2^- , SO_4^{2-} and PO_4^{3-} concentrations were measured from anaerobic DI water extraction for 1 hour, via Ion Chromatography, using an AS-22 Colum along with an AG-22 guard column (flow rate = 1.2 mL min^{-1} , detection limit = 0.016 ppm). For the DOC analyses a Shimadzu TOC-5000(A) was used.

Batch incubation. A series of incubation experiments were conducted. Soil samples from each location were divided into $12 \times 10 \text{ g}$ (air-dry equivalent) subsamples, and added into 50mL serum vials, with 30 mL deionized water. The soil slurries were purged thoroughly with a $\text{CO}_2:\text{N}_2$ (80:20) mixture, and then incubated under anaerobic condition in glove box.

In the first incubation experiment, Feammox activities of each sample were checked. In day 0 to 15, no NH_4^+ or Fe(III) was added to the incubations. On day 16, samples were amended with $5 \text{ mmol L}^{-1} \text{ NH}_4\text{Cl}$ and 30 mmol L^{-1} ferrihydrite, and continue incubated under anaerobic condition. On day 45, $2 \text{ mmol L}^{-1} \text{ NH}_4\text{Cl}$ and 5 mmol L^{-1} ferrihydrite were added again after NH_4^+ was exhausted. Autoclaved sterilized soils were incubated as controls. Triplicate samples were collected from two vials every four

days to analyze iron and nitrogen species.

The second incubation was conducted to check how pH and NO_2^- concentration control Feammox reaction. For pH incubation, enrichment culture with *Acidimicrobiaceae* bacterium A6 were exposed to different pH gradients, pH=2, 4.5, 6.5, 8 (adjusted with HCl and NaOH). For NO_2^- incubation, enrichment cultures were added with different concentration of NO_2^- (0.02, 0.2, 1.0, 2.0 $\text{mmol} \cdot \text{L}^{-1}$ as final concentrations). The initial concentrations of Ferrihydrite were 25 $\text{mmol} \cdot \text{L}^{-1}$ and 2 $\text{mmol} \cdot \text{L}^{-1}$ NH_4^+ was added. Triplicate samples were collected every two days to analyze iron, nitrogen species, and also the copy number of *Acidimicrobiaceae* bacterium A6.

DNA was isolated as described in Example 6.

QPCR was carried out for total bacteria, anammox bacteria and *Acidimicrobiaceae* bacteria, represented by 16S rRNA genes, using primer sets 1055f/1392r, Amx368f/Amx820r and acm342f/439r, respectively. (Harms et al. 2003; Schmid et al, 200, 2003 and Huang and Jaffe, 2013, all of which are incorporated by reference as if fully set forth) For the detection of denitrifiers, denitrifying functional genes (*nirS*) were quantified with primer sets NirS3/NirS5 (Braker et al., 1998, which is incorporated by reference herein as if fully set forth). Thermal cycling conditions for total 16S rDNA and *nirS* gene numbers were essentially as described in Example 7.

Statistical analysis. Correlations between the bacterial distributions vs. the environmental factors were analyzed with the canonical correspondence analysis (CCA) using R (22). Analyses of Spearman rank, multivariate and stepwise linear regressions of environmental and bacterial abundance variables were carried out using SPSS v12 on both raw and log-transformed data. All data were analyzed using one-way analysis of variance (ANOVA) with the significant level $P < 0.05$.

It has been shown that anammox activity is optimal at a pH of 7.8 - 8.0 and temperatures between 28 °C - 35 °C. In anammox reactors, the DO concentration is usually maintained between 0.2 mg/L - 1mg/L to allow for partial nitrification, which is required to achieve satisfactory NH_4^+ removal. In soils environments, anammox bacteria are usually present under slightly oxic conditions, which again, ensures that AOB are able to provide adequate NO_2^- levels. Both AOB and anammox bacteria are autotrophic, therefore the organic carbon content in soils does not affect their NH_4^+ oxidation rate, except for *Nitrosopumilis maritimus* (an AOB species) which was reported to be inhibited by organic substrates even at very low concentrations.

AOB and anammox bacteria usually coexist in hypoxic environments, since AOB provide the NO_2^- for the anammox reaction. Hence, Feammox bacteria, which also produce NO_2^- while oxidizing NH_4^+ , may enhance anammox in environments where AOB are not active. Batch incubations of soil samples at sites where Feammox activity was observed have shown that after NH_4^+ was oxidized to NO_2^- by the Feammox process, nitrogen loss proceeded via denitrification and/or anammox. Since, as shown by equation 1, removal of NO_2^- makes the reaction more favorable, the coexistence of Feammox with denitrifiers and/or anammox may be key for anaerobic ammonium oxidation in soil environments.

Therefore, influence of the different environment factors on the Feammox presence/activity in soil environments was investigated, the relationships between Feammox bacteria and other microorganisms responsible for nitrogen transformations in soils were determined.

Chemical properties of samples collected from different locations Soil and sediment samples collected for this study cover a wide range of chemical properties: pH from 4.26 to 7.21; Fe(III) from 120 to 1243 mg kg⁻¹; Fe(II) from 7.92 to 356 mg kg⁻¹; NH_4^+ from 6.02 to 86.0 mg kg⁻¹; NO_3^- from 6.71 to 274.8 mg kg⁻¹; NO_2^- from 4.74 to 152.8 mg kg⁻¹;

SO₄²⁻ from 4.89 to 128.6 mg kg⁻¹; PO₄³⁻ from 2.97 to 28.5 mg kg⁻¹; DOC from 9.37 to 28.09 g kg⁻¹.

Most of the soil and sediment samples selected for this study were acidic. pH value of surface soil samples (top 5 cm of soil,) was higher than that of soil below 10 cm. Concentrations of NO₃⁻ and DOC were also higher in the surface soils. NO₃⁻ were only showed in samples from 0 to 5 cm of soil, but hardly detected in soils collected from 10 to 20 cm in L7. On the contrary, NO₂⁻ was found more rich below 10 cm than the upper soil samples in most of the locations.

All of these soil samples were iron rich, compared to the total iron levels usually reported for wetland soils (100 mg kg⁻¹ to 620 mg kg⁻¹) and sediment environments (30 to 120 mg kg⁻¹). Fe(III) concentrations in vegetation-covered soil samples were obvious higher than the other samples. In additional, Fe(III) concentrations in soil collected from rhizosphere, were much higher than that from non-rooting zone at the same location. Fe (II) abundance shown a different trend, and was found negatively correlated ($r = -0.61$ $p = 0.001$) with Fe(III) in the same sample locations.

Among these samples, samples originated from a forested riparian wetland area that was 0.5 km near some agricultural lands, have higher NH₄⁺ and PO₄³⁻ contents. The distribution of SO₄²⁻ in samples of this study was similar to that of NO₃⁻ between different sample locations but shown no obvious discrepancy with sample depth.

Feammox activity and abundance of Acidimicrobiaceae bacterium A6 Fe (II) production and NH₄⁺ consumption were checked after two weeks anaerobic incubation to monitor the Feammox activity in different samples. After 15 days of anaerobic incubation without the addition of extra NH₄⁺ and Fe(III), all samples showed clearly iron reduction, form 2.10 to 8.86 mmol L⁻¹.

The existence and abundance of the Feammox *Acidimicrobiaceae* bacterium A6 in soil and sediment samples were determined via qPCR analysis. Abundance of *Acidimicrobiaceae* bacterium A6 in the field samples shown consistent with the anaerobic NH_4^+ consumption in incubations, and a positively correlation ($r = 0.58$ $p = 0.012$) were revealed between them.

Feammox activity in incubation with different pH and NO_2^- concentrations. The Feammox reaction was most active in samples with pH 4.5. When pH increased or decreased, NH_4^+ oxidation and Fe reduction slow down, and Feammox reaction seem to stop at a pH 2.0 or 8.0. Abundance of Feammox *Acidimicrobiaceae* bacterium A6 increased after 14 days anaerobic incubation in samples with pH 2, 4.5, or 6.5 and slightly decreased in alkaline conditions. There was no significant difference between number of *Acidimicrobiaceae* bacterium A6 in samples incubated with pH 4.5 and 6.5, which is 2.37×10^4 and 2.41×10^4 copies mL^{-1} , respectively.

Feammox activity was sensitive to NO_2^- concentration in the samples. Feammox reaction processed when NO_2^- concentrations were 0.02 and 0.2 $\text{mmol} \cdot \text{L}^{-1}$, but it was inhibited when NO_2^- reached to 1.0 $\text{mmol} \cdot \text{L}^{-1}$. Changes of *Acidimicrobiaceae* bacterium A6 abundance were consistent with Feammox activities with different concentration of NO_2^- were added. In samples with 1.0 and 2.0 $\text{mmol} \cdot \text{L}^{-1}$ NO_2^- were added, the numbers of *Acidimicrobiaceae* bacterium A6 were decreased after incubation.

CCA analysis of Feammox distributions in soil samples.

Soils for all the experiments described in this study were taken from three areas: New Jersey and South Carolina in Unities States, Guangdong province in China. A total of 52 locations were selected and cover 4 different types of soil samples, which are wetland soils (W), river sediments (R), forest soils (F), paddy soils (P). FIGS. 12A-12B illustrate ordination plots of the canonical correspondence analysis (CCA) for the first

two dimensions of CCA of the relationship between the environmental factors and Feammox bacteria vs. environment factor of soil analysis and relation of Feammox to other bacterial communities affecting the nitrogen cycle. FIG. 12A illustrates the total variance in the bacterial abundance and the cumulative variance of the bacteria-environment relationship. FIG. 12B illustrates the total variance in the microbial composition and the cumulative variance of the bacteria-sampling location relationship.

Referring to FIG. 12A, the CCA analysis of Feammox bacteria and nitrogen microorganisms in response to environmental variables confirmed the influence shown in this figure. The first two CCA axes (CCA1 and CCA2) explained 73.2% of the total variance in the bacterial abundance and 80.6% of the cumulative variance of the bacteria-environment relationship. pH value ($p = 0.030$) and Fe(III) content ($p = 0.021$) had significant effects on the distributions of *Acidimicrobiaceae* bacterium A6, and NO_2^- seemed to inhibit this bacteria by showing a negatively correlation. Concentrations of NO_2^- were supporting anammox bacterial existence ($p = 0.025$), and the combined effects of SO_4^{2-} and NO_2^- affects the distribution of *nirS genes*. NH_4^+ content was not limited for either Feammox or aerobic/anaerobic NH_4^+ oxidation bacteria. Referring to FIG. 12B, the first two CCA axes (CCA1 and CCA2) explained 71 % of the total variance in the microbial composition and 81 % of the cumulative variance of the bacteria-sampling location relationship. The CCA analysis showed that CCA1 represented the pH level of the soils. Referring to FIG. 12B, sample with Feammox activity assembled in a low pH area, while other soil samples distributed in the opposite pH area showed no Feammox reaction. CCA2 represents the vertical distributions of soil samples, soils collected from similar depth gathered together. More aerobic or anaerobic NH_4^+ oxidation bacteria were found in surface soil samples. It was observed that Feammox bacteria and denitrifiers tended to be active at depths below 10 cm of the soil environments.

Example 19. Reduction of Uranium by Acidimicrobiaceae bacteria A6 with ferrihydrite as the iron source

Acidimicrobiaceae bacteria A6 from a pure culture were prepared for uranium (U) bioreduction experiments. Twelve 15-mL vials of bacteria in their normal growth medium were centrifuged after which the bacteria were resuspended in a modified anaerobic growth medium. Of the twelve vials, three contained bacteria that had been autoclaved to establish a control condition with dead bacteria. The centrifugation and washing was repeated twice. The modified anaerobic growth medium consisted of NaHCO_3 , KHCO_3 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, CaCl_2 , and AQDS. It differed from the normal growth medium in that it did not contain KH_2PO_4 , which was removed due to its high sorption of U, or NH_4Cl , which was added separately. Uranyl acetate was added to all vials such that the initial concentration of U was approximately 64 μM .

The twelve vials were divided into four groups: three different control conditions and the experimental condition as shown in Table 1. Ammonium (NH_4^+) in the form of NH_4Cl was added to the three experimental vials as an electron source for the bacteria. In the first control condition, NH_4Cl was also added, but the microbial monooxygenase enzyme was inhibited by the addition of Na_2S . The second control condition, mentioned earlier, contained bacteria that had been killed in an autoclave. NH_4Cl was added to this condition, as well. No NH_4Cl was added to the third control condition to establish an electron donor-limited control. No iron (Fe) was added to any vials, but some ferrihydrite, a common ferric oxyhydroxide, which acted as the Fe source in the original pure culture medium remained with the bacteria through the washing process.

Table 1. Experimental and control conditions in U bioreduction experiment with ferrihydrite as Fe source

Experimental	Control 1: Enzyme	Control 2:	Control 3:
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		Inhibited	Autoclaved Bacteria	No NH_4^+
NH_4^+ , mM	3.98 ± 0.01	3.96 ± 0.01	4.01 ± 0.02	0.03 ± 0.01
Initial total U, μM	64.8 ± 15.6	65.5 ± 6.3	65.4 ± 6.1	59.3 ± 17.0
Na_2S , μM	0	20	0	0
<i>Acidimicrobiaceae</i> bacteria A6	Live	Live	Dead	Live

In Table 1, values given for NH_4^+ and U concentrations are the averages of three vials $\pm$ one standard deviation

The extent of *Acidimicrobiaceae* bacteria A6 activity in each condition was monitored by measuring concentrations of U and NH_4^+ on the day the experiment started and again six days later. Samples for NH_4^+ analysis were filtered using 0.2 μm nylon filters and stored at 4°C until they were analyzed using a Dionex™ Ion Chromatograph ICS3000 with a CS-16 column, a CS-16 guard column, and a CERS 500 (4 mm) suppressor. Samples for U(VI) analysis were also filtered at 0.2 μm to remove reduced U(IV) precipitates and were preserved in 2% HNO_3 . Unfiltered samples for total U analysis were mixed in a 1:1 ratio with 0.2 M NaHCO_3 to dissolve any U(IV) that had been reduced by the bacteria. After a 24-hour extraction at room temperature, these samples were also preserved in 2% HNO_3 . U measurement in acidified samples was completed on a Thermo Scientific Element 2 inductively-coupled plasma mass spectrometer (ICP-MS).

Reduction of Uranium by Acidimicrobiaceae bacteria A6 with Ferrihydrite as the Iron Source. NH_4^+ analyses indicate that *Acidimicrobiaceae* bacteria A6 were only active in the experimental condition which contained live bacteria with NH_4^+ and U. FIG. 13 illustrates NH_4^+ oxidized over 6 days by *Acidimicrobiaceae* bacteria A6. Uranium (U)

was present in all conditions. Referring to FIG. 13, percentages shown are an average of three replicates. Error bars indicate one standard deviation. Referring to FIG. 13, over 6 days, bacteria in this condition oxidized an average of 7.0 mg L^{-1} of NH_4^+ which is equivalent to the removal of 9.8% of the initial NH_4^+ . NH_4^+ oxidation was not seen in any of the control conditions which suggests that *Acidimicrobiaceae* bacteria A6 were not active in these conditions. In the control conditions in which the relevant monooxygenase enzyme was inhibited, the average NH_4^+ concentration increased by 0.31 mg L^{-1} indicating no NH_4^+ oxidation. Similarly, in the control condition containing autoclaved bacteria, the average NH_4^+ concentration decreased by 0.20 mg L^{-1} indicating no significant NH_4^+ oxidation. In the control in which no NH_4^+ was added, the concentration of NH_4^+ actually increased from an average concentration of 0.52 mg L^{-1} to 1.83 mg L^{-1} . This increase was seen in all three replicates and could indicate nitrogen release through the death and decay of *Acidimicrobiaceae* bacteria A6 that were deprived of their requisite electron donor NH_4^+ .

Measurement of U concentrations indicates that biological reduction is one of multiple pathways by which U was removed from solution in these experiments. Across all experimental and control conditions, between 74% and 91% of the added U disappeared from solution within 2 hours of uranyl acetate addition. It is likely that this U(VI) sorbed to ferrihydrite which remained with the bacterial cells through the centrifugation and washing procedure. As a result, the effective initial aqueous U(VI) concentrations varied from $7.2 \text{ }\mu\text{M}$ to $13.2 \text{ }\mu\text{M}$ U(VI). FIG. 14 illustrates uranium U(VI) removed from solution over 6 days. Referring to FIG. 14, the difference between the “Live and active A6” condition and both the “Autoclaved A6” and the “Limitation of electron donor” conditions is attributable to U reduction by *Acidimicrobiaceae* bacteria A6. Percentages shown are an average of three replicates. Error bars indicate one standard deviation.

Referring to FIG. 14, significant U(VI) removal in all conditions continued after the

initial removal by sorption. In the three control cases, the lack of NH_4^+ oxidation indicates that *Acidimicrobiaceae* bacteria A6 were inactive and thus the U(VI) removal in those cases must have been abiotic. It is likely that U diffusion into the matrix of ferrihydrite caused the removal of 51.4 % and 65.6% of effective initial aqueous U(VI) over 6 days in the NH_4^+ -limited and autoclaved controls, respectively. This two-step uranyl sorption to ferric oxyhydroxides is an established phenomenon (Hsi CKD and Langmuir D 1985 *Geochim Cosmochim Acta* 49, 1931-1941; Morrison, SJ et al., 1995 *J Contam Hydrol* 17, 333-346, both of which are incorporated herein by reference as if fully set forth). In the enzyme inhibited control, even more U(VI) was abiotically removed over 6 days than in the autoclaved or NH_4^+ -limited control. The additional U(VI) removal in this case is likely due to abiotic U(VI) reduction by the added enzyme inhibiting compound, Na_2S . The sulfide in Na_2S is able to act as an electron donor for U(VI) reduction (Hua B et al. 2006 *Environmental Science & Technology* 40, 4666-4671, which is incorporated herein by reference as if fully set forth).

Referring to FIG. 14, comparison of U(VI) removal in the experimental condition and the controls with autoclaved bacteria and with NH_4^+ limitation suggests that biotic reduction of U(VI) by *Acidimicrobiaceae* bacteria A6 is significant. Without the help of reductants like Na_2S , the U(VI) removal in vials with live and active bacteria exceeds the highest removal in vials with dead bacteria by 25%. Since these conditions are identical except for the state of the bacteria, it is reasonable to conclude that the additional U(VI) reduction is performed by *Acidimicrobiaceae* bacteria A6.

Example 20. Reduction of uranium by *Acidimicrobiaceae* bacteria A6 with nontronite as the iron source

Cell suspension experiments with nontronite, an Fe-rich clay, were performed to study U bioreduction by *Acidimicrobiaceae* bacteria A6 in an environment rich in electron accepting Fe(III) to promote *Acidimicrobiaceae* bacteria A6 activity. This experiment

was similar to the cell suspension experiments without the addition of an Fe source as described above. Pure culture of *Acidimicrobiaceae* bacteria A6 in twelve 15-mL vials was washed by centrifugation and resuspension in anaerobic growth medium consisting of NaHCO_3 , KHCO_3 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, CaCl_2 , and AQDS.

Nontronite (NAu-2) from the Source Clay Repository of the Clay Minerals Society was selected as an electron acceptor for this experiment due to its inability to sorb significant amounts of U at slightly acidic pH. The structural formula of this clay is $\text{M}^{+0.72}[\text{Si}_{7.55}\text{Al}_{0.45}][\text{Fe}_{3.83}\text{Mg}_{0.05}]\text{O}_{20}(\text{OH}_4)$ where M^+ represents monovalent interlayer cations (Keeling JL et al. 2000 *Clay Clay Miner* 48, 537-548, which is incorporated herein by reference as if fully set forth). This clay is 23.4% Fe by weight, of which 99.4% is Fe(III) (Liu D et al. 2011 *Geochim Cosmochim Ac* 75, 1057-1071, which is incorporated herein by reference as if fully set forth). A stock solution of 20 g L^{-1} nontronite was prepared using a previously established protocol (Liu et al. 2011, which is incorporated herein by reference as if fully set forth). Briefly, the clay was ground by hand with mortar and pestle before it was soaked in 0.5 mM NaCl overnight. The solution was centrifuged to eliminate all but the 0.02-0.5 μm size fraction. These remaining particles were washed with deionized water and dried in an oven at 100 °C. The clay was diluted to 20 g L^{-1} in deionized water and autoclaved to create a sterile stock solution. This stock solution was added to all 12 vials so that the final concentration of nontronite in each vial was 5 g L^{-1} .

Again, the twelve vials were divided into four groups: one experimental group and three control groups as described in Table 2. To establish the experimental condition with live and active *Acidimicrobiaceae* bacteria A6, NH_4Cl and uranyl acetate were added so that the initial concentrations of NH_4^+ and U were 3.726 mM and 12.5 μM , respectively. The first control condition contained bacteria that had been autoclaved, but NH_4Cl and uranyl acetate were added to these control vials so that they were otherwise identical to the experimental condition. The second control condition

contained live bacteria and U, but no NH_4^+ was added to these vials to distinguish the effects of live and active bacteria from live and inactive bacteria. The third control condition contained live bacteria and added NH_4^+ , but did not contain any U in order to determine whether the presence of U altered the activity of *Acidimicrobiaceae* bacteria A6.

Fe(II) was measured at intervals of 24 to 48 hours throughout the experiment to gauge Fe-reducing activity of *Acidimicrobiaceae* bacteria A6. 20 μL of unfiltered samples were deposited in 1 mL of 0.5 N HCl. After 15 minutes, Fe(II) was measured using the well-established ferrozine method (Komlos J and Jaffe PR, 2004 *Biodegradation* 15, 315-325, which is incorporated herein by reference as if fully set forth). Briefly, Fe(II) was measured by adding 30 μL of HCl-extracted sample to 1.5 mL of ferrozine and measuring the absorbance after 30 minutes at a wavelength of 562 nm in a Spectronic Genesys 2 spectrophotometer. Absorbances were converted to molar concentrations of Fe(II) using a standard curve.

Table 2. Experimental and control conditions in U bioreduction experiment with nontronite as Fe source

	Experimental	Control 1: Autoclaved Bacteria	Control 2: No NH_4^+	Control 3: No U
NH_4^+ , mM	3.726 mM	3.726 mM	0 mM	3.726 mM
Initial total U, μM	12.5 ± 2.3	11.8 ± 0.3	12.4 ± 0.5	0
Nontronite, g L^{-1}	5	5	5	5
<i>Acidimicrobiaceae</i> bacteria A6	Live	Dead	Live	Live

Concentrations of Fe(II) measured by the ferrozine method include aqueous Fe(II) and approximately 74% of the Fe(II) sorbed to nontronite surfaces (Zhang GX *et al.* 2009 *Geochim Cosmochim Acta* 73, 3523-3538, which is incorporated herein by reference as if fully set forth). Therefore, these measurements were used only in relation to each other to track bacterial Fe(II) production indicating *Acidimicrobiaceae* bacteria A6 activity. U, NH_4^+ , nitrate (NO_3^-), and nitrite (NO_2^-) concentrations were measured after 0, 7, and 11 days to determine activity of *Acidimicrobiaceae* bacteria A6. Total U and U(VI) concentrations were measured by ICP-MS as described above. NH_4^+ , NO_3^- , and NO_2^- concentrations were measured on a Dionex™ Ion Chromatograph ICS3000. Columns used for NH_4^+ were identical to those described above. NO_3^- and NO_2^- analysis was carried out with an AS-22 column, an AG-22 guard column, and an ASRS 300 (4 mm) suppressor. It had previously been determined that NH_4^+ could sorb to nontronite, so total NH_4^+ was also measured. Sorbed NH_4^+ was extracted from nontronite by adding 20 μL samples from each vial to 1 mL of 0.5 N HCl. After 20 hours, the acid extractions were filtered on 0.2 μm nylon filters and preserved at 4°C until the NH_4^+ concentrations were measured on a Dionex™ Ion Chromatograph.

Purity of bacterial cultures was confirmed after the experiment using DNA isolation and quantitative polymerase chain reaction (qPCR) assay. 1 mL samples of the slurry containing bacteria, growth medium, and nontronite were centrifuged at 1000 rpm for 10 minutes before DNA was extracted from the pellets using the FastDNA® spin kit for soil (MP Biomedicals, USA) as described by the manufacturer. Total bacterial abundance was represented by the number of copies of 16S rRNA genes quantified by qPCR with primers BACT1369F-PROK1492R and the TaqMan probe1389F (Suzuki MT *et al.* 2000 *Appl Environ Microb* 66, 4605-4614, which is incorporated herein as if fully set forth). *Acidimicrobiaceae* bacteria A6 bacteria were enumerated with the primer set acm342f-439r (Huang S and Jaffe PR 2015 *Biogeosciences* 12, 769-779, which is incorporated herein by reference as if fully set forth). Several other U-reducing species were also targeted to check the samples for contamination. To

investigate the presence of sulfate and Fe reducing bacteria, *δ-Proteobacteria* was enumerated by qPCR using primer set 361F-685R and the TaqMan probe 1839F (Stults JR et al. 2001 *Appl Environ Microb* 67, 2781-2789, which is incorporated herein by reference as if fully set forth). *Geobacter*, the dominant Fe-reducing microorganisms in sediment environments, was enumerated by qPCR primer set 561F-825R and TaqMan probe Gbc2 (Stults et al. 2001, which is incorporated herein by reference as if fully set forth). *Anaeromyxobacter* spp., a metal-reducing group of bacteria, was enumerated with the 60F-461R primer pair (Petrie L et al. 2003 *Appl Environ Microb* 69, 7467-7479, which is incorporated herein by reference as if fully set forth). All qPCR experiments were carried out using a StepOnePlus™ Real-Time PCR System (Life Technologies, USA). For DNA quantification, each qPCR mixture (20 µL) was composed of 10 µL of SYBR Premix Ex Taq® II (Takara, Japan), 0.8 µL of 10 µM of each primer, and ~ 10 ng DNA template. Each assay contained a set of standards produced by serial dilution of plasmids containing specific target genes, independent triplicate templates for each slurry sample, and triplicate no template controls (NTC).

Reduction of Uranium by Acidimicrobiaceae bacteria A6 with Nontronite as the Iron Source. Measurements of Fe(II) confirm that *Acidimicrobiaceae* bacteria A6 remain active in the presence of U and nontronite. FIG. 15 illustrates reduction of Fe in nontronite over 12 days. Values shown are averages of three replicates. Error bars indicate one standard deviation. Referring to FIG. 15, Fe(II) production was similar in rate and extent in conditions with and without U, indicating that the addition of 10 µM uranyl acetate is not toxic to *Acidimicrobiaceae* bacteria A6. In cases with and without added U, *Acidimicrobiaceae* bacteria A6 were able to use the Fe(III) in nontronite as an electron acceptor for NH₄⁺ oxidation. Still referring to FIG. 15, in the condition with autoclaved *Acidimicrobiaceae* bacteria A6, the Fe(II) remained constant throughout the 12-day experiment. This result indicates that there was no abiotic Fe cycling occurring in the vials.

Results indicate that *Acidimicrobiaceae* bacteria A6 can reduce U in addition to Fe. FIG. 16 illustrates total U and U(VI) concentrations over 11 days. Values shown are averages of three replicates. Error bars indicate one standard deviation. Referring to FIG. 16, essentially all of the U(VI) disappeared from solution in 7 days in the condition with live *Acidimicrobiaceae* bacteria A6, NH_4^+ , and U. This change cannot be attributed to activity of other bacteria, as qPCR results indicated that only *Acidimicrobiaceae* bacteria A6 were present in all vials with live bacteria. Referring to FIG. 16, this change in U(VI) also cannot be attributed to sorption of U to nontronite or ingredients of the growth medium because the aqueous U(VI) concentrations remained essentially equivalent to total U concentrations in vials in which *Acidimicrobiaceae* bacteria A6 had been killed. The similarity between U(VI) and total U in the condition with autoclaved bacteria also demonstrates that no abiotic U reduction occurred. The difference between vials with dead and live *Acidimicrobiaceae* bacteria A6 indicate that *Acidimicrobiaceae* bacteria A6 can reduce aqueous U(VI) to U(IV) solids. It has previously been determined that *Shewanella oneidensis* MR-1 are also able to completely reduce U in the presence of nontronite (Zhang GX et al 2009, which is incorporated herein by reference as if fully set forth). *Shewanella oneidensis* MR-1 are only able to reduce U in the presence of AQDS; though this present study did not examine the effect of AQDS on U reduction by *Acidimicrobiaceae* bacteria A6, it is likely that they similarly require AQDS (Zhang GX et al 2009, which is incorporated).

In all vials except one, the total U measured by bicarbonate extraction decreased over 11 days. This change likely resulted from the slow incorporation of U into the structure of nontronite where it would be unaffected by the extraction.

Unexpectedly, Fe and U reduction occurred in vials without NH_4^+ for the live *Acidimicrobiaceae* bacteria A6 to oxidize. In these vials, the Fe(II) increased and U(VI) decreased more slowly and to a lesser extent over 12 days than they did in vials with NH_4^+ . These results could indicate that the live *Acidimicrobiaceae* bacteria A6 area

able to use a structural component of the nontronite as an electron donor rather than NH_4^+ . Alternatively, biomass turnover and decomposition could provide the remaining live *Acidimicrobiaceae* bacteria A6 with NH_4^+ . In this case, the lower proportion of live bacteria and the lower concentrations of NH_4^+ would explain the slower rates and smaller extent of Fe and U reduction.

Example 21. Bioreduction of Cu(II) by Feammox

FIGS. 17A - 17B illustrate oxidation of NH_4^+ (FIG. 17A) and reduction of Cu (II) (FIG. 17B) by Feammox. Around 10^4 cell per ml of pure A6 were incubated in inorganic NH_4^+ -Ferric iron liquid medium with 20 ppm Copper (II) chloride (CuCl_2) under anaerobic conditions for 40 days. Referring to FIG. 17B, 64.2 % of Cu(II) was reduced and the total dissolved Cu [including Cu(II) and Cu(I)] decreased by 23.1% over the incubation period, while the concentrations of Cu(II) and Cu(I) remained constant for the autoclaved treatment.

After the incubation, the samples were aerated to reoxidize the reduced copper. This resulted in full recovery of the initial Cu(II).

Example 22. Investigation of selected organic contaminants biodegradation by Feammox

Around 10^4 cell per ml of pure A6 were incubated in inorganic NH_4^+ -Ferric iron liquid medium with 1000ppb Trichloroethylene (TCE) /1000ppb tetrachloroethylene (PCE) / 100ppb benzene/ 100ppb phenanthrene under anaerobic conditions for 40 days. Six 15-mL vials of incubations were conducted for each organic contaminant. The six vials were divided into three groups: two different control conditions and the experimental condition as shown in Table 3.

Table 3. Experimental and control conditions in selected organic contaminants

biodegradation by Feammox

	Experimental	Control 1: Enzyme Inhibited	Control 3: No NH_4^+
NH_4^+ , mM	2.96 ± 0.12	2.97 ± 0.05	0.02 ± 0.01
Initial total TCE/PCE/benzene/ phenanthrene, ppb	1000/1000/100/ 100/	1000/1000/100/ 100/	1000/1000/ 100/100/
Na_2S , μM	0	50	0
<i>Acidimicrobiaceae</i> bacteria A6	Live	Live	Live

Subsamples were taken on day 0, 12, 22, 30 and 37 under anoxic conditions. Samples for NH_4^+ analysis were filtered using 0.2 μm nylon filters and stored at 4°C until they were analyzed using a DionexTM Ion Chromatograph ICS3000 with a CS-16 column, a CS-16 guard column, and a CERS 500 (4 mm) suppressor. TCE, PCE, benzene or phenanthrene were measured with a Thermo Scientific Vanquish UHPLC system. FIGS. 18A - 18D illustrate an effect of biodegradation of trichloroethylene (TCE) (FIG. 18A), tetrachloroethylene (PCE) (FIG. 18B), benzene (FIG. 18C) and phenanthrene (FIG. 18D) by Feammox. Around 10^4 cell per ml of pure A6 were incubated in inorganic NH_4^+ -Ferric iron liquid medium with 1000 ppb Trichloroethylene (TCE) (FIG. 18A), 1000 ppb tetrachloroethylene (PCE) (FIG. 18B), 100 ppb benzene (FIG. 18C), 100 ppb phenanthrene (FIG. 18D) under anaerobic conditions for 40 days.

Referring to FIGS, 18A -18B, TCE, PCE, benzene and phenanthrene degradation were found in all Feammox active samples, but not in the samples that included Feammox enzyme inhibitor (sodium bisulfide) and samples without NH_4^+ . It was observed that 36 % of TCE, 28% of PCE, 20% of benzene and 8.8 % of phenanthrene was degraded over an incubation period of approximately one month. NH_4^+ oxidation was not seen in any of the control conditions, which suggests that *Acidimicrobiaceae bacteria A6* were not active in these conditions that also did not result in the degradation of the organics.

It should be noticed that all of the above are extremely recalcitrant compounds, especially in anaerobic environments. Higher degradation rates can be achieved by having larger bacterial numbers. The bacterial numbers for higher degradation may be 10^6 , 10^8 or 10^{10} cells per ml of pure A6.

Example 23. Characteristics of Feammox Acidimicrobiaceae bacterium A6 and enzyme identification

Feammox bacteria were isolated in inorganic NH_4^+ -Ferric iron solid medium from wetland soils collected in New Jersey as described in Examples 2 - 17. The bacteria were cultured in liquid and solid media.

Inorganic NH_4^+ -Ferric iron liquid medium contained the following components per liter: 177 mg NH_4Cl , 77.9 mg $(\text{NH}_4)_2\text{SO}_4$, 19.8 mg NaHCO_3 , 71.0 mg KHCO_3 , 9.00 mg KH_2PO_4 , 100 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 60.0 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. 3.37g ferrihydrite, 18.42 mg antraquinone-2,6-disulfonate (AQDS) and 1 mL trace element solution and vitamin (ATCC®). Inorganic NH_4^+ -Ferric iron solid medium was prepared with liquid medium and 0.8 % agar, 0.2 mL 3.37g L^{-1} ferrihydrite was spread on the surface.

FIGS. 19A - 19D illustrate A6 bacterial cultures and cells. FIG. 19A illustrates growth of the A6 bacterium in the liquid medium. Cultures of A6 became increasingly turbid and black-colored (due to accumulation of ferrous iron) during incubation. Culture doubling times of the A6 isolate were 10.2 days. FIG. 19B illustrates A6 growth on the solid inorganic NH_4^+ -ferric iron medium. Colonies of A6 are displayed with black spot (iron-encrusted) on the solid medium. FIGS. 19C - 19D show rod-shaped A6 cells. The cells were 1.5 - 3 mm long by 0.5 mm wide. The A6 bacteria are gram-positive bacteria. On the solid medium, colonies with black spot (accumulation of ferrous iron) were collected and transferred to inorganic NH_4^+ -Ferric iron liquid medium. Their Feammox

activity was verified by the NH_4^+ oxidation and iron reduction rates. A strain of *Actinobacteria* was isolated and identified as Feammox bacteria, and named *Feammox Acidimicrobiaceae* bacterium A6. The pH optimum for growth of A6 was 4.5, but reasonable activity was detected from pH 2 to 7. Its temperature optimum was 25°C, and a doubling time of 10.2 days. A ^{13}C labeled CO_2 amendment was conducted, and the ^{13}C in cells of A6 increased from 1.80% to 10.3% after 14 days incubation. In a separate incubation, $^{15}\text{NH}_4\text{Cl}$ was added with a final concentration of 0.5 mmol L^{-1} , and 0.133 mmol L^{-1} of $^{15}\text{NO}_2^-$ was detected, while no $^{15}\text{NO}_3^-$ was produced.

The genome size and guanine-cytosine content of A6 are 3.3 mega base pairs (Mb) and 52 %. The Feammox ammonium Monooxygenase (*FMO*) gene (SEQ ID NO: 8), was identified. *FMO* is considered to be the functional gene coding ammonium oxidation coupled to ferric iron reduction in A6.

Isolation, purification and cultivation of bacteria: Continuous flow membrane Feammox enrichment reactor Soil samples from wetland soils collected in New Jersey were inoculated into a continuous flow membrane reactor, which was operated under anaerobic conditions by constantly purging N_2 through the reactor's headspace at a room temperature (25°C), and with a 48 hour hydraulic retention time. FIG. 11 illustrates a scheme of a membrane reactor for NH_4^+ oxidation via Feammox. Referring to this figure, 1 refers to the feed solution, 2 refers to the feed pump, 3 refers to the membrane module, 4 refers to the floater connected to an electrical on/off switch, 5 refers to the ceramic diffuser, 6 refers to the N_2 supplier, 7 refers to the water bath, 8 refers to outflow, and 9 refers to the outflow pump. Inorganic NH_4^+ -Ferric iron liquid medium contained the following components per liter: 177 mg NH_4Cl , 77.9 mg $(\text{NH}_4)_2\text{SO}_4$, 19.8 mg NaHCO_3 , 71.0 mg KHCO_3 , 9.00 mg KH_2PO_4 , 100 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 60.0 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. 3.37g ferrihydrite, 18.42 mg AQDS and 1 mL trace element solution and vitamin (ATCC®). pH was controlled at around 4~5, and dissolve oxygen was < 0.10

mg/L. Samples from the outflow were collected every two days, and sludge samples from reactor were collected and kept at -20 °C for molecular biology analysis.

454 pyrosequencing was performed with samples collected from the membrane reactor after 150 days of reactor operation with Domain-specific primers, targeting the V3-V5 region of the 16S rDNA of bacteria were amplified following methods suggested by Pinto *et al.* 2012, PLoS One 7: 43093, which is incorporated herein by reference as if fully set forth. And the results were compared with samples from previous incubation on days 0, 30, 90, 160. *Acidimicrobiaceae* bacterium A6, which belongs to the *Actinobacteria* phylum, was the dominant species in the incubation experiments after 180 days of incubation (14.8% in terms of cell numbers) and increased in the membrane reactor after 150 days of operation (40.2% in terms of cell numbers).

Growth on solid media: Inorganic NH_4^+ -Ferric iron solid media was prepared with liquid media and 0.8 % agar, 0.2 mL 3.37g L^{-1} ferrihydrite was spread on the surface.

On the solid medium, colonies with black spot (accumulation of ferrous iron) were collected and transferred to inorganic NH_4^+ -Ferric iron liquid media. FIG. 20 illustrates anaerobic oxidation of ammonium by the A6 isolate. Referring to FIG. 20, bacterial cells were incubated for 20 days on the inorganic, NH_4^+ -ferric iron media, pH 4.5. The A6 concentration was 10^5 copies per mL. 0.738 mM of NH_4^+ was oxidized and 0.710 mM of NO_2^- was produced. Still referring to FIG. 20, the Feammox activity of the bacterial cells was verified by the NH_4^+ oxidation and iron reduction rates. A strain of *Actinobacteria* was isolated and identified as Feammox bacteria, and named *Feammox Acidimicrobiaceae* bacterium A6. Referring to FIGS, 19C - 19D, microscopic examination showed that cells were rod-shaped, 1.5–3 mm long by 0.5 mm wide, gram-positive.

Growth on liquid media: In ferric iron-containing liquid media, cultures of A6 became

increasingly turbid and black-coloured (due to the accumulation of ferrous iron) during incubation. Microscopic examination showed that endospores were produced. The pH optimum for growth of isolate A6 were 4.5, and its temperature optimum was 25°C. Under optimum conditions of pH and temperature, culture doubling times of isolate A6 were 10.2 days.

Anaerobic oxidation of ammonium - ammonium oxidation rates

The A6 isolate was incubated for 20 days on the inorganic, NH_4^+ -Ferric iron media, at a pH 4.5. The A6 bacterial concentration was 10^5 copies per mL. FIG. 20 illustrates concentrations of NH_4^+ and NO_2^- during anaerobic oxidation of ammonium. After incubation, 0.738 mM of NH_4^+ was oxidized and 0.710 mM of NO_2^- was produced.

Effect of NH_4^+ concentration on Feammox. FIG. 21 illustrates an effect of NH_4^+ concentration on its consumption by A6. Referring to this figure, the isolate A6 was incubated for 20 days in the inorganic NH_4^+ -Ferric iron media, pH4.5. A6 included 10^5 copies per mL. 10 mM ferrihydrite and three different amounts (10 mM, 5 mM, 1mM) of NH_4^+ were added initially. Higher NH_4^+ consumption rates by Feammox were found for higher NH_4^+ concentration incubation.

^{15}N isotope incubation $^{15}\text{NH}_4\text{Cl}$ was added with a final concentration of 0.5 mmol L^{-1} and 0.133 mmol L^{-1} of $^{15}\text{NO}_2^-$ was detected after 7 days incubation, while no $^{15}\text{NO}_3^-$ was produced. The data was summarized in Table 4.

Table 4. Summary of ^{15}N isotope incubation

	¹⁴ N	¹⁵ N
D1-NH ₄ ⁺	0.892	0.108
D1-NO ₂ ⁻	0.995	0.005
D1-NO ₃ ⁻	0.995	0.005
D7-NH ₄ ⁺	0.847	0.153
D7-NO ₂ ⁻	0.757	0.243
D7-NO ₃ ⁻	0.992	0.008
	D1	D7
15N-NH ₄ ⁺	0.5192	0.3862
15N-NO ₂ ⁻	0.0001	0.1330
15N-NO ₃ ⁻	0.0001	0.0001

The data confirmed that NO₂⁻ is the main product of NH₄⁺ oxidation in Feammox reaction.

Reduction of ferric iron reduction rates: FIG. 22 illustrates iron reduction rates. The A6 cells were incubated for 20 days on the inorganic, NH₄⁺-ferric iron medium, pH 4.5. The A6 concentration was 10⁵ copies per mL. After incubation, 4.330 mM of NH₄⁺ was oxidized, and 4.317 mM of Fe(II) was produced.

Effect of iron concentration on Feammox: FIG. 23 illustrates an effect of iron concentration on Feammox. Referring to FIG. 23, the isolate A6 was incubated for 20 days in the inorganic NH₄⁺-Ferric iron media, at pH4.5. A6 included 10⁵ copies per mL. 2 mM NH₄⁺ and three different amounts (20 mM, 10mM, 2mM) of ferrihydrite were added initially. No significant different of NH₄⁺ consumption by Feammox with different ferrihydrite amended during incubation was found. As long as there was sufficient iron included in the media, no effect of iron concentration on Feammox was observed.

Determination of pH and temperature optima: Isolate A6 incubation for 20 Days under different pH gradient in inorganic, NH_4^+ -Ferric iron media. A6 is 105 copies per mL. pH adjust to 2, 4.5, 6.5, 8.0 by HCl and NaOH. FIGS. 25A - 25B illustrate determination of the effect of pH on NH_4^+ consumption (FIG. 25A) and bacterial growth (FIG. 25B). pH=2 results are reported in the far left bar of each of days 0, 5, 10, 15 and 20 panels. pH=4.5 results are reported in the left middle bar of each of days 0, 5, 10, 15 and 20 panels. pH=6.5 results are reported in the right middle bar of each of days 0, 5, 10, 15 and 20 panels. pH=8 results are reported in the far right bar of each of days 0, 5, 10, 15 and 20 panels. The pH optimum for growth of A6 was 4.5, but reasonable activity was detected from pH 2 to 7.

Temperature gradient: FIG. 24 illustrates the relative ammonium removal rate constants (k) by *Acidimicrobiaceae*-bacteria A6 at 15°C, 20°C and 35°C. Referring to this figure, the highest rate was measured at 20°C, $k=0.03\text{hr}^{-1}$, followed by 35°C, $k=0.019\text{hr}^{-1}$, and 15°C, $k=0.01\text{hr}^{-1}$.

Utilization of organic substrates: Growth yields of isolate A6 have no growth difference in media containing glycerol, citric acid and glutamic acid with in control cultures NaHCO_3 added. The data shows that A6 is an autotroph utilizing inorganic carbon but the presence of different organic carbons does not affect its growth.

It was observed that culturing of the isolate A6 in yeast extract media required one or more additional growth factors.

Heavy metal tolerance: This gram-positive isolate was able to grow in media containing elevated concentrations of the heavy metals tested. The A6 isolate was tested for the ability to grow in the media supplemented with 100 μM -100 mM of copper, 10 -200 mM of zinc, 20 -100 μM of uranium, 10 - 200 mM of ferric iron or 10 - 200 mM of ferrous

iron. It was observed that the isolate A6 grew in 260 mM on ferric iron. However, addition of ferrous iron did not inhibit the bacterial growth. Growth also occurred in the presence of 100 mM, but not 200 mM, copper. It was also observed that 50 mM zinc inhibited growth. The A6 bacteria was growing in the presence of the tested concentrations of uranium but it was found that uranium was toxic to the bacterial cells at 200 μ M.

Autotrophic growth and CO₂ fixation - assimilation of CO₂ in cultures of isolate A6: A6 was able to grow in organic carbon- free liquid media. A ¹³C labeled CO₂ amendment was conducted with a Thermo MAT 253™ stable isotope ratio mass spectrometer, and the ¹³C in cells of A6 increased from 1.80% to 10.3% after 14 days incubation.

Table 5. Summary of assimilation of CO₂

	Cell (with methanol wash)		DNA	
	¹² C	¹³ C	¹² C	¹³ C
Day 0	98.2%	1.80%	98.9%	1.10%
Day 14	89.7%	10.3%	82.0%	18.0%

Screening of RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) genes:

RuBisCO genes were amplified from cell lysates of A6. Both *cbbL* and *cbbM* genes were successfully amplified.

Full sequencing and functional gene-single-copy gene analysis: FIG. 26 illustrates a schematic representation of the genome of the *Acidimicrobiaceae* Feammox bacterium A6. The genome size and guanine-cytosine content of A6 are 3.3 mega base pairs (Mb) and 52 %. Genotypic traits are summarized in Table 6.

Table 6. Genotypic traits

Attribute	Value
Genome size (bp)	3,106,468
Plasmid size(bp)	106,011
DNA Coding region (bp)	2,991,461
DNA G+C content (bp)	1,709,039
RNA genes	42
rRNA operons	2
Protein-coding genes	2032
Pseudo genes	58
Genes with function prediction	1579
Genes assigned to COGs	1392
Genes assigned Pfam domains	1497
Genes with transmembrane helices	538

Functional gene: The gene encoding Feammox ammonium Monooxygenase (FMO) having a sequence of SEQ ID NO: 12 was identified. The gene was located in the bacterial plasmid. The FMO is considered to be the functional gene coding ammonium oxidation coupling to ferric iron reduction in A6.

FMO activity. FIG. 27 illustrates the FMO activity. Referring to FIG. 27, isolate A6 was incubated for 20 days under pH 4.5, in inorganic NH_4^+ -Ferric iron media. Copy number of Feammox gene, FMO-a, was analyzed during the incubation which showed active Feammox reaction. It was observed that that FMO activity increased as total microbial as well as *Acidimicrobiaceae* Feammox bacterium A6 activity increased.

FIG. 28 illustrates oxidation of NH_4^+ by *Acidimicrobiaceae* bacterium A6 and the FMO activity in the presence (without FMO-a) and absence (with FMO-a) of a broad enzyme

inhibitor (NaS). Referring to FIG. 28, it was observed that the NH_4^+ oxidation rate decreased in the samples without FMO-a.

Phylogenetic analysis: According to the phylogenetic analysis, Acidimicrobiaceae bacterium A6), belonging to the Acidimicrobiaceae family, whose closest cultivated relative is *Ferrimicrobium acidiphilum* (with 92% identity) and *Acidimicrobium ferrooxidans* (with 90% identity).

Example 24. Feammox applications

Feammox process could be applied for waste water treatment for ammonium removal without the need to supply oxygen. Oxygen supply is the highest energy cost in waste treatment plants. The process may also be used in the design of engineered wetlands for nitrogen removal. That would be important in the agricultural and livestock industry. There might be many other applications.

Ammonium oxidation requires oxygen. Anammox process works with nitrite, but it requires high temperatures (around 82°F). Since the bacterium was isolated at a New Jersey wetland, it is reasonable that it will function at lower temperatures. This is important for waste treatment, since in the winter waste water temperatures are around 40°F. Feammox may be applied for developing more temperature robust treatment processes that do not need aeration. Means to recycle iron and/or use scrap grounded up iron may be developed to be supplied for Feammox. The process similar to the one tested in incubation experiments will be utilized in a simple prototype reactor. Table 6 describes NH_4^+ removal using the reactor illustrated in FIG. 11 contrast Anammox and Feammox.

Table 7. Comparison of Feammox and Anammox reactors

	Feammox reactor	Anammox reactor	Conditions
--	-----------------	-----------------	------------

Temperature	20-25°C	28-35°C	Feammox can work if T~15°C
pH	4.5-6	circumneutral	Feammox can work if pH~2
Aeration time	0h/day	Non or 1.5h/day	Feammox require Fe(III)
Doubling time	8 - 12 days	10 -12 days	Both are slow growing bacteria
NH ₄ ⁺ removal	85%	41% (no aeration) 72% (with aeration)	NH ₄ ⁺ inflow concentration 5mM; Retention Time 48h

It was shown that the Feammox reactor is as efficient if not more than the anammox reactor. Finally, sludge was mixed from the Feammox and Anammox reactors, showing that a combined Feammox/Anammox mode is feasible and does not require partial aeration, which is required by Anammox to convert some NH₄⁺ to NO₂⁻, which in the combined process is done by Feammox. Operating conditions were different for the Feammox/Anammox reactor.

A combined Feammox/Anammox process results in less iron demand than a pure Feammox process. Anammox with 20% Feammox sludge may result in 70% NH₄⁺ removal without aeration.

Example 25. Feammox in electrogenic microbial reactors

Electrogenic microbial reactors such as Microbial Fuel Cells (MFCs) and Microbial Electrolysis cells (MECs) are bioelectrochemical systems that extract energy from a substrate (Call and Logan 2011 *Biosen. Bioelectron.* 26(11): 4526-4531, which is incorporated herein by reference as if fully set forth). This process harvests electrons biologically, which are then transferred to the anode that functions as the terminal electron acceptor for the microorganisms in the system, and H₂ is produced at the anode. FIG. 29 illustrates a scheme of the electrogenic microbial reactor. Referring to FIG. 29, the reactor includes a vial, a graphite anode connected via titanium wire to

the positive terminal of the power supply, a stainless steel cathode connected via stainless steel wire to the negative terminal of the power supply. MECs require a small potential applied from an external power source ($E_{AP} > 0.25$ V), resulting in H_2 production at the anode (Logan, Hamelers et al. 2006 *Environ. Sci. Technol.* 40(17):5181-5192; Call and Logan 2008 *Environ. Sci. Technol.* 42(9):3401-3406; Call and Logan 2011, all of which are incorporated by reference as if fully set forth). When the anode in a MFC or MEC works in place of a finite electron acceptor (*i.e.*, Fe(III)), the bacterial growth is no longer limited by the consumption of that electron acceptor.

Some electrogenic iron reducing microorganisms; *e.g.*, *Geobacter* sp., are capable of transferring the electrons to an anode instead of Fe(III) and grow in MECs (Logan 2009, which is incorporated herein by reference as if fully set forth). Thus, we have tested A6's ability to grow in small MECs has been tested. It was found that under constant mixing, and with a voltage input of 0.7V, MECs seeded with a pure culture of A6 to which 5 mM ammonium was added produced a current density (I) that increased from 0.02 A/m³ to up to ~36 A/m³, while the autoclaved control increased to only 1.3 A/m³ (Call and Logan 2011, which is incorporated herein by reference as if fully set forth). The A6 biomass increased from 10⁴ copies of DNA/ml to 9x10⁵ over 14 days of operation. An electron shuttling compound, AQDS (Anthraquinone-2,6-disulfonate) had to be added to the solution, since unlike *Geobacter*, A6 does not seem to colonize the anode. When an electron shuttling compound like AQDS is added, the electron will be transferred from NH_4^+ to AQDS in solution and then to the anode. Hence, MECs and MFCs may be the ideal Feammox reactor configuration for NH_4^+ removal from wastewater via Feammox that solves the Fe(III) supply problem. Other configurations and materials can also be used to construct MECs as well as MFCs.

Although the equation in FIG. 29 shows that the solution pH should decrease while NH_4^+ is being oxidized, it was observed that pH increases. Our hypothesis is that this is due to the CO_2 consumption by A6, which is an autotroph. Hence, CO_2 control may be

for the growth of a pure A6 culture. CO₂ addition should not be needed when organics are degraded simultaneously since CO₂ is produced during the biodegradation of organics.

Example 26. Addition of elemental sulfur stimulates Feammox activity

Standard Feammox incubations were conducted with the addition of S(0). It was shown that by adding S(0) to the medium the growth rate of *Acidimicrobiaceae* bacterium A6 was increased, as well as the removal of ammonium, and the production of Feammox related enzymes (Accession numbers AF5136601-AF5136621). SEQ ID NOS of amino acid and nucleic acid sequences Feammox related enzymes ERCFMO_0001-FERCFMO_0021 (correspond to AF5136601-AF5136621) are listed in Table 8 below.

Table 8. SEQ ID NOS of FMO Related Enzymes and Genes

FMO clone*	SEQ ID NO (Gene)	SEQ ID NO (Enzyme)
ERCFMO_0001	8	29
ERCFMO_0002	9	30
ERCFMO_0003	10	31
ERCFMO_0004	11	32
ERCFMO_0005	12	33
ERCFMO_0006	13	34
ERCFMO_0007	14	35
ERCFMO_0008	15	36
ERCFMO_0009	16	37
ERCFMO_0010	17	38
ERCFMO_0011	18	39
ERCFMO_0012	19	40
ERCFMO_0013	20	41
ERCFMO_0014	21	42
ERCFMO_0015	22	43
ERCFMO_0016	23	44
ERCFMO_0017	24	45
ERCFMO_0018	25	46
ERCFMO_0019	26	47
ERCFMO_0020	27	48
ERCFMO_0021	28	49

* Clone refers to the clone of uncultured bacterium particulate methane monooxygenase/ammonia monooxygenase subunit A, partial cds, also referred to as the FMO.

Based on the findings that Feammox can degrade TCE/PCE and PAHs, it was investigated whether one or a combination of these enzymes are involved in these reactions.

Table 9 describes an increase in copy numbers of the genes encoding clones of the FMO enzyme in response to the addition of S(0).

Table 9. Increase in Copy Numbers of the Genes Encoding Clones of the FMO Enzyme in Response to Addition of S(0)

		Number of A6 cells (x10 ⁴) /mL of culture				Gene copy number (x10 ⁴)/mL of culture		
Gene Encoding Clone of the FMO Enzyme	Origin of the Clone	initial	after 20 days incubation with S(0)	after 20 days incubation without S(0)	with S(0)/ without S(0)	after 20 days incubation with S(0)	after 20 days incubation without S(0)	with S(0)/with- out S(0)
ERCFMO_0001	Pure*	23.00	121.77	48.88	2.49	258.00	38.91	6.631
ERCFMO_0002	Pure*	41.79	253.49	210.48	1.20	138.00	72.59	1.901
ERCFMO_0003	Enrichment **	294.98	113.65	75.53	1.50	162.00	73.54	2.203
ERCFMO_0004	Enrichment **	22.81	215.65	183.08	1.18	212.00	31.71	6.686
ERCFMO_0005	Pure*	59.30	75.09	21.85	3.44	78.00	25.13	3.104
ERCFMO_0006	Enrichment **	226.56	275.09	236.90	1.16	261.00	20.52	12.718
ERCFMO_0007	Pure*	65.38	146.68	29.33	5.00	137.00	16.84	8.135
ERCFMO_0008	Enrichment **	199.70	196.86	155.78	1.26	308.00	133.40	2.309
ERCFMO_0009	Enrichment **	93.68	312.54	236.33	1.32	237.00	215.13	1.102
ERCFMO_0010	Enrichment **	80.59	217.89	120.70	1.81	259.00	129.07	2.007
ERCFMO_0011	Pure*	51.70	70.82	77.63	0.91	70.00	25.65	2.729
ERCFMO_0012	Enrichment **	24.33	76.92	98.05	0.78	308.00	111.97	2.751
ERCFMO_0013	Enrichment **	439.43	504.46	388.55	1.30	329.00	225.75	1.457
ERCFMO_0014	Enrichment **	15.21	80.67	38.05	2.12	234.00	125.65	1.862
ERCFMO_0015	Pure*	15.21	133.99	62.73	2.14	132.00	32.71	4.036

ERCFMO_0016	Enrichment **	316.27	372.08	413.23	0.90	541.00	407.03	1.329
ERCFMO_0017	Pure*	28.09	141.41	74.44	1.90	159.00	82.74	1.922
ERCFMO_0018	Enrichment **	482.01	574.22	423.33	1.36	560.00	180	3.111
ERCFMO_0019	Enrichment **	206.79	226.83	250.00	0.91	410.00	120	3.417
ERCFMO_0020	Enrichment **	428.79	537.18	341.00	1.58	323.23	134.00	2.412
ERCFMO_0021	Pure*	13.68	102.00	26.67	3.83	120.00	23.00	5.217

* Pure bacterial culture (10^5 /mL *Acidimicrobiaceae* bacterium A6 only, in series of 20mL vials)

** Enriched bacterial culture (10^{6-7} /mL *Acidimicrobiaceae* bacterium A6; include~ 35%-45% of the total bacteria number)

It was observed that incubation of the bacterium A6 with S(0) resulted in increase in the number of A6 cells and the number of the copies of the genes encoding clones ERCFMO_0001, ERCFMO_0002, ERCFMO_0005, ERCFMO_0007, ERCFMO_0011, ERCFMO_0015, ERCFMO_0017, and ERCFMO_0021 of the FMO enzyme. Elemental sulfur S(0) was added to the cultures in the amount between 0.5 mM to 20 mM. However, no difference was observed between the amount of sulfur and the reaction rate in the cultures. It was observed that the small amount of sulfur added at the beginning resulted in short time of the reaction. However, more sulfur added over time prolonged the reaction time. The solubility of sulfur is $1.9(\pm 0.6) \times 10^{-8}$ mole $S_8 \cdot kg^{-1}$. Therefore, the cumulative amount of sulfur added needs to be in excess of 0.05 mM/L to affect the reaction if added once. The maximum sulfur that can be added is believed to depend on the density of sulfur (≈ 2 kg/L). Sulfur can be added in the amount between 1 mg/L and 2 kg/L.

The references cited throughout this application are incorporated for all purposes apparent herein and in the references themselves as if each reference was fully set forth. For the sake of presentation, specific ones of these references are cited at particular locations herein. A citation of a reference at a particular location indicates a manner(s) in which the teachings of the reference are incorporated. However, a citation of a reference at a particular location does not limit the manner in which all of the teachings of the cited reference are incorporated for all purposes.

Example 27 – Oxidant enhanced Feammox activity

Batch incubations with elemental sulfur, S(0).

To set up the incubations, 30ml of inorganic Fe(III)-NH₄⁺ enrichment medium (iFeN) was added into 50 mL sealed serum vials. The composition of the iFeN medium (pH 4.5, which was adjusted by adding dropwise HCl) consisted of 69.55 mg/l NH₄Cl, 77.9 mg/l (NH₄)₂SO₄, 19.8 mg/l NaHCO₃, 71.0 mg/l KHCO₃, 9.00 mg/l KH₂PO₄, 100 mg/l

MgSO₄•7H₂O, and 60.0 mg/l CaCl₂•2H₂O, in addition to 1 mg/l of a trace element solution and 1 mg/l of a vitamin solution (ATCC® MD-VS™). The cultures were also supplemented with 6-line ferrihydrite (Fe₂O₃•0.5H₂O; prepared according to Cornell and Schwertmann, 1990), to obtain a final amount of 10 mmol/l of Fe(III). 200 mg elemental sulfur [S(0)] was then added to these 30ml iFeN media. After inoculating the iFeN media with about 10⁵ /ml of *Acidimicrobiaceae* sp. A6, or with enrichment Feammox cultures containing similar numbers of *Acidimicrobiaceae* sp. A6, the headspace of each incubation vial was vacuumed and then flushed with a N₂/CO₂ (80:20) mixture, which was repeated three times. The enrichment cultures were then kept in dark at 25°C and incubated under anaerobic conditions. Three sets of control samples were prepared, 1) autoclaved control, 2) no S(0) added control, 3) no Fe(III) added control. Extra 3.0 mM of NH₄Cl was added to the second incubation experiments of enrichment cultures. Anaerobic incubations were performed for 20 days, and concentrations of Fe(III) and NH₄⁺ were analyzed daily.

Results

Results of the Feammox batch incubations showing the effect of S(0) addition on ammonium removal and iron reduction with the pure culture of *Acidimicrobiaceae* sp. A6 are shown in Figures 30 and 31, and with the *Acidimicrobiaceae* sp. A6 enrichment culture (two separate experiments) in Figures 32 through 35. As illustrated in these figures, rates of ammonium removal and iron reduction in the presence of S(0) were significantly enhanced relative to the control compositions. The effect of adding S(0) on the abundance of *Acidimicrobiaceae* sp. A6 during the incubation with the pure culture are shown in Figure 36. As evidenced in Figure 36, the abundance of *Acidimicrobiaceae* sp. A6 was substantially enhanced by the presence of elemental sulfur relative to the various control compositions. Increases in the *Acidimicrobiaceae* sp. A6 population can also increase removal rates of ammonium and/or other contaminants.

Example 28 – Continuous flow membrane Feammox reactor employing elemental sulfur, S(0)

A 2L continuous flow membrane Feammox reactor (Huang and Jaffe, 2015), was operated under anaerobic conditions by constantly purging N₂ through the reactor's headspace at a room temperature (25°C), and with a 48-hour hydraulic retention time. A 35.5~40.2% NH₄⁺ removal, between inflow and outflow was achieved in the membrane reactor during a one-year operation. The medium in the inflow contained the following components per liter: 177 mg NH₄Cl, 77.9 mg (NH₄)₂SO₄, 19.8 mg NaHCO₃, 71.0 mg KHCO₃, 9.00 mg KH₂PO₄, 100 mg MgSO₄·7H₂O, and 60.0 mg CaCl₂·2H₂O, and 1 mL trace element solution (Van de Graaf *et al.*, 1996). 10.0 mmol/l of ferrihydrite were added once every week directly to the reactor. To aid in maintaining anaerobic conditions, 0.10 - 0.20 mmolL⁻¹ sodium citrate was feed to the reactor about twice per month. pH was controlled at around 4~5, and the dissolve oxygen was maintained at 0.10-0.30 mg/l.

After the reactor was running under stable conditions, 200 mg of S(0) was added to the reactor and set up day 0, twice 64 mg of S(0) were added on day 14 and 32. Samples from the inflow and outflow were collected and analyzed every day, and sludge samples from reactor were collected and kept at -20 °C for molecular biology analysis.

As illustrated in Figure 37, after adding S(0), the ammonium removal rate between inflow and outflow increased by greater than 10 percent.

Various embodiments of the invention have been described in fulfillment of the various objects of the invention. It should be recognized that these embodiments are merely illustrative of the principles of the present invention. Numerous

modifications and adaptations thereof will be readily apparent to those skilled in the art without departing from the spirit and scope of the invention.

CLAIMS

1. A medium comprising:
an ammonium containing contaminant, an iron component, an oxidant, and a Feammox bacterium and/or enzyme thereof capable of oxidizing ammonium coupled with reduction of Fe(III) to Fe(II), wherein the oxidant regenerates Fe(III) via Fe(II) oxidation.
2. The medium of claim 1, wherein the Fe(II) oxidation generates hydrogen ions.
3. The medium of claim 2, wherein the hydrogen ions are consumed in the ammonium oxidation.
4. The medium of claim 1, wherein the iron component comprises Fe(III).
5. The medium of claim 4, wherein the iron component comprises ferrihydrite, iron oxide, elemental iron, a goethite, a nontronite, an iron-rich clay or mixtures thereof.
6. The medium of claim 1, wherein the medium is aqueous-based.
7. The medium of claim 1, wherein the medium is wastewater.
8. The medium of claim 6 having a pH of 2 to 8.
9. The medium of claim 1, wherein the medium is soil.
10. The medium of claim 1, wherein the reduced oxidant forms a compound with

Fe(II) in the medium.

11. The medium as in any one of claims 1-9, wherein the oxidant is elemental sulfur.

11. The medium as in any one of claims 1-10 further comprising at least one additional contaminant.

12. The medium of claim 11, wherein the at least one additional contaminant is selected from the group consisting of halogenated organic contaminants, chlorinated volatile organic compounds, perchloroethylene (PCE), trichloroethylene (TCE), trichloroethane, dichloroethane, vinyl chloride, polychlorinated biphenyls, fuel constituents, benzene, ethylbenzene, toluene, xylene, phenanthrene, methyl tert butyl ether, tertiary butyl alcohol, polyaromatic hydrocarbons, and ethylene dibromide.

13. The medium as in any one of claims 1-12, wherein the Feammox bacterium is an *Acidimicrobiaceae* bacterium.

14. A system comprising:

a reactor, the reactor comprising a medium including an ammonium containing contaminant, an iron component, an oxidant, and a Feammox bacterium and/or enzyme thereof capable of oxidizing ammonium coupled with reduction of Fe(III) to Fe(II), wherein the oxidant regenerates Fe(III) via Fe(II) oxidation.

15. The system of claim 14, wherein the Fe(II) oxidation generates hydrogen ions.

16. The system of claim 15, wherein the hydrogen ions are consumed in the ammonium oxidation.
17. The system of claim 14, wherein the iron component comprises Fe(III).
18. The system of claim 17, wherein the iron component comprises ferrihydrite, iron oxide, elemental iron, a goethite, a nontronite, an iron-rich clay or mixtures thereof.
19. The system of claim 14, wherein the medium is aqueous-based.
20. The system of claim 14, wherein the medium is wastewater.
21. The system of claim 20, wherein the aqueous-based medium has a pH of 2 to 8.
22. The system of claim 14, wherein the medium is soil.
23. The system of claim 14, wherein the reactor provides anaerobic conditions.
24. The system of claim 14, wherein the reduced oxidant forms a compound with Fe(II) in the medium.
25. The system as in any one of claims 14-24, wherein the oxidant is elemental sulfur.
26. The system as in any one of claims 14-25 further comprising at least one additional contaminant.

27. The system of claim 26, wherein the at least one additional contaminant is selected from the group consisting of halogenated organic contaminants, chlorinated volatile organic compounds, perchloroethylene (PCE), trichloroethylene (TCE), trichloroethane, dichloroethane, vinyl chloride, polychlorinated biphenyls, fuel constituents, benzene, ethylbenzene, toluene, xylene, phenanthrene, methyl tert butyl ether, tertiary butyl alcohol, polyaromatic hydrocarbons, and ethylene dibromide.
28. The medium as in any one of claims 14-27, wherein the Feammox bacterium is an *Acidimicrobiaceae* bacterium.
29. A method of environmental remediation comprising:
providing a medium including one or more ammonium containing contaminants;
disposing an oxidant and a Feammox bacterium and/or enzyme thereof in the medium; and
oxidizing the ammonium with the Feammox bacterium and/or enzyme thereof coupled with reduction of Fe(III) to Fe(II); and
regenerating Fe(III) with the oxidant via Fe(II) oxidation.
30. The method of claim 29 further comprising adding an iron component to the medium.
31. The method claim 29, wherein hydrogen ions are generated by the Fe(II) oxidation.
32. The method of claim 31, wherein the hydrogen ions are consumed in the

ammonium oxidation.

33. The method of claim 30, wherein the iron component comprises Fe(III).

34. The method of claim 33, wherein the iron component comprises ferrihydrite, iron oxide, elemental iron, a goethite, a nontronite, an iron rich silicate or mixtures thereof.

35. The method of claim 29, wherein the medium is aqueous-based.

36. The method of claim 29, wherein the medium is wastewater.

37. The method of claim 35, wherein the medium has a pH of 2 to 8.

38. The method of claim 29, wherein the medium is soil.

39. The method of claim 29 further comprising forming a compound with the reduced oxidant and Fe(II).

40. The method of claim 29, wherein the oxidant is present in the medium in excess of the oxidant's solubility limit in water.

41. The method as in any of claims 29 to 40, wherein the oxidant is elemental sulfur.

42. The method of claim 29, wherein the oxidant increases the rate of ammonium oxidation.

43. The method as in any of claims 29 to 42, wherein the medium further comprises at least one additional contaminant.
44. The method of claim 43 further comprising removing the at least one additional contaminant from the medium.
45. The method as in any one of claims 29 to 44, wherein the Feammox bacterium is an *Acidimicrobiaceae* bacterium.

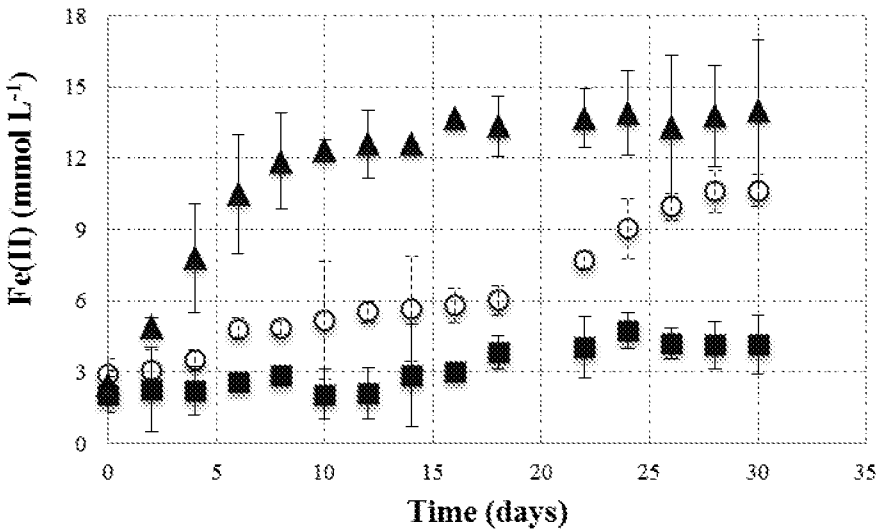


FIG. 1A

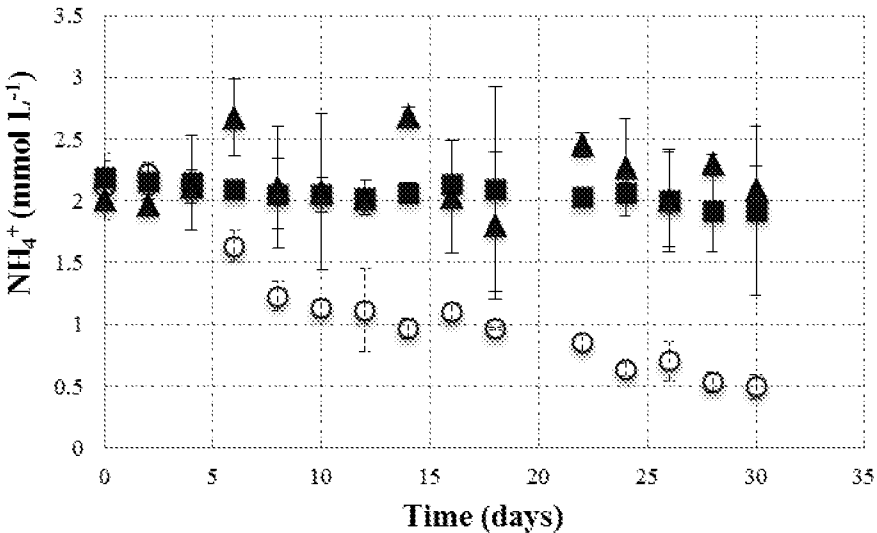


FIG. 1B

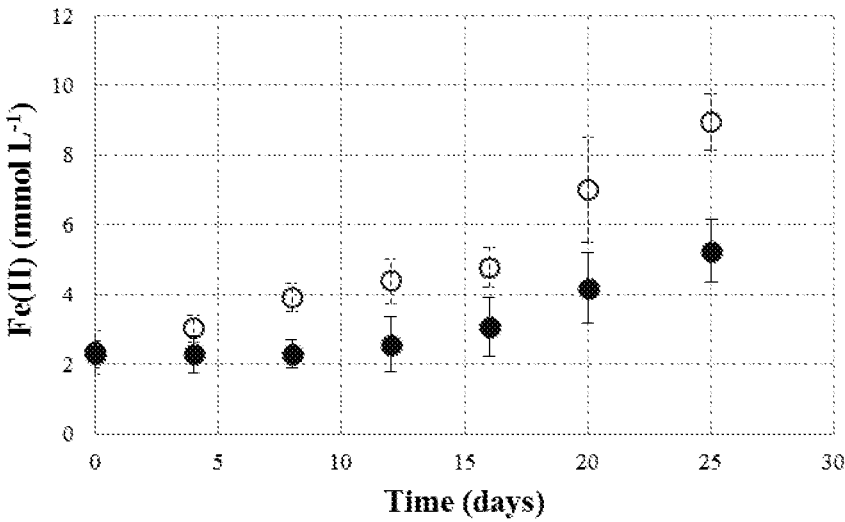


FIG. 2A

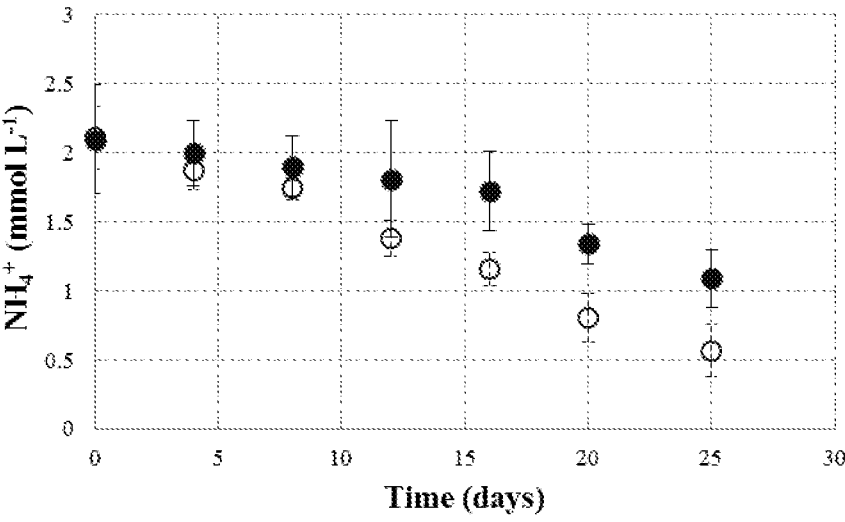


FIG. 2B

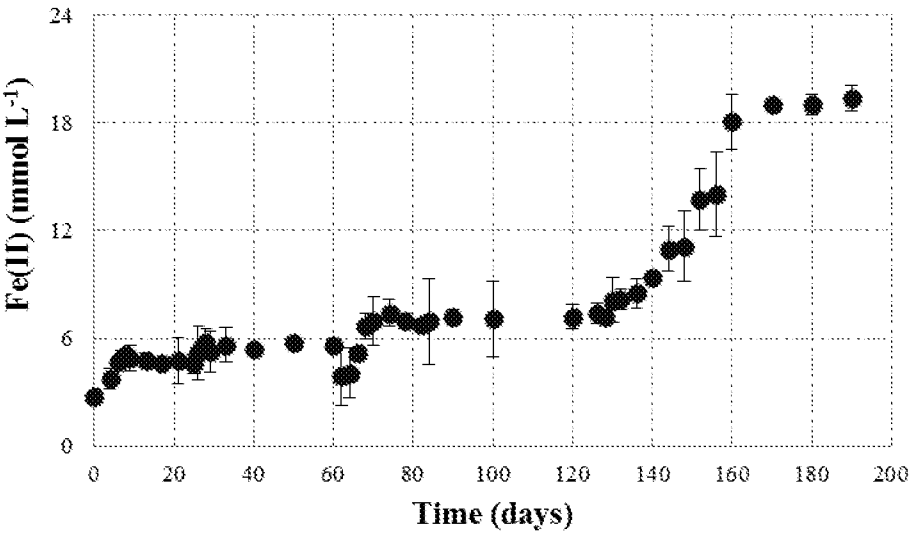


FIG. 3A

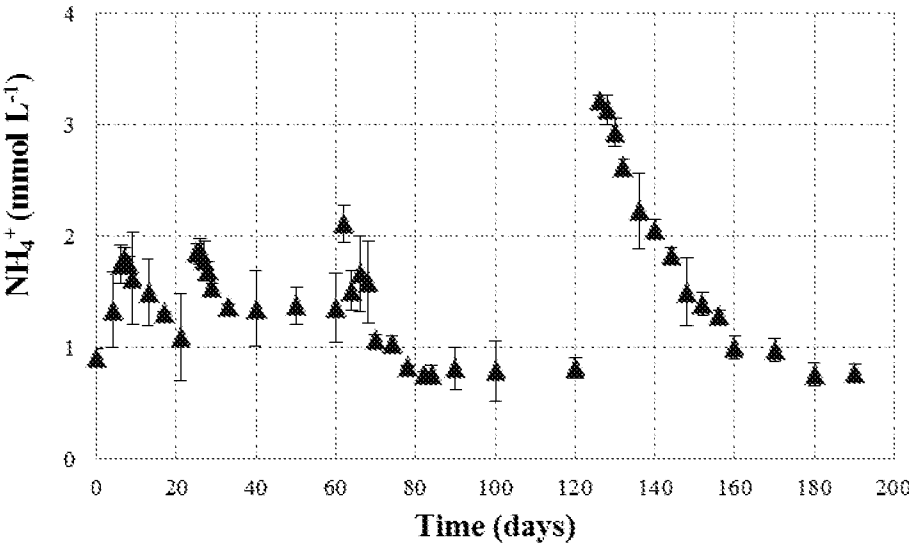


FIG. 3B

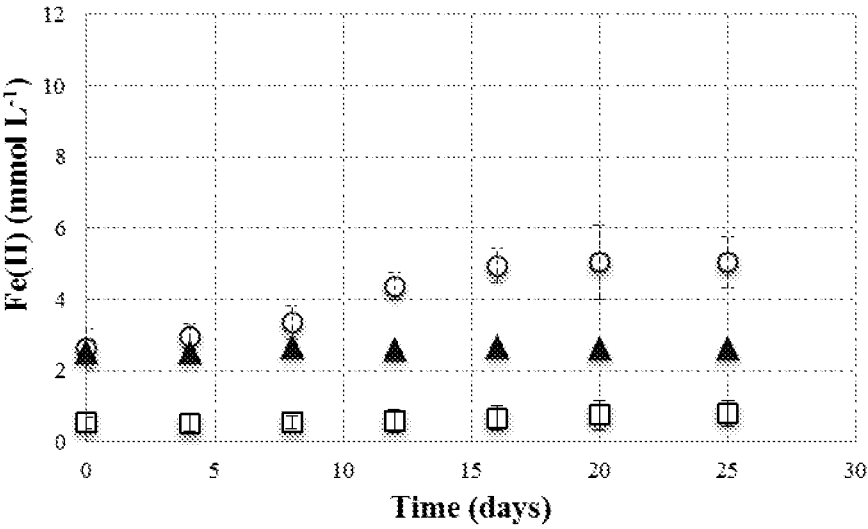


FIG. 4A

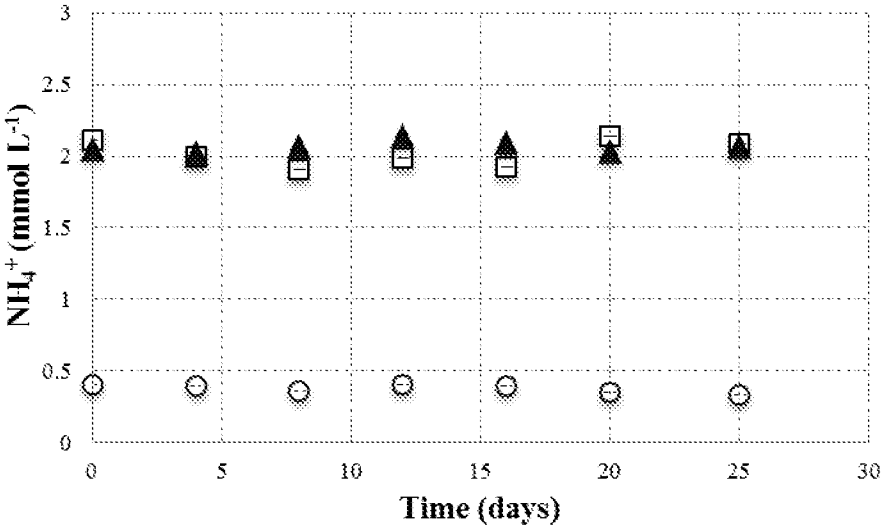


FIG. 4B

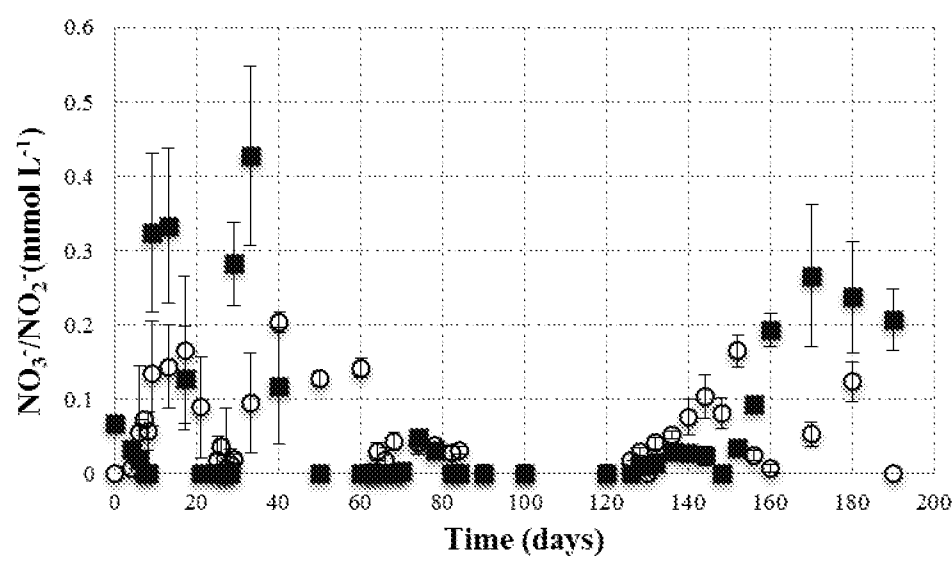


FIG. 5A

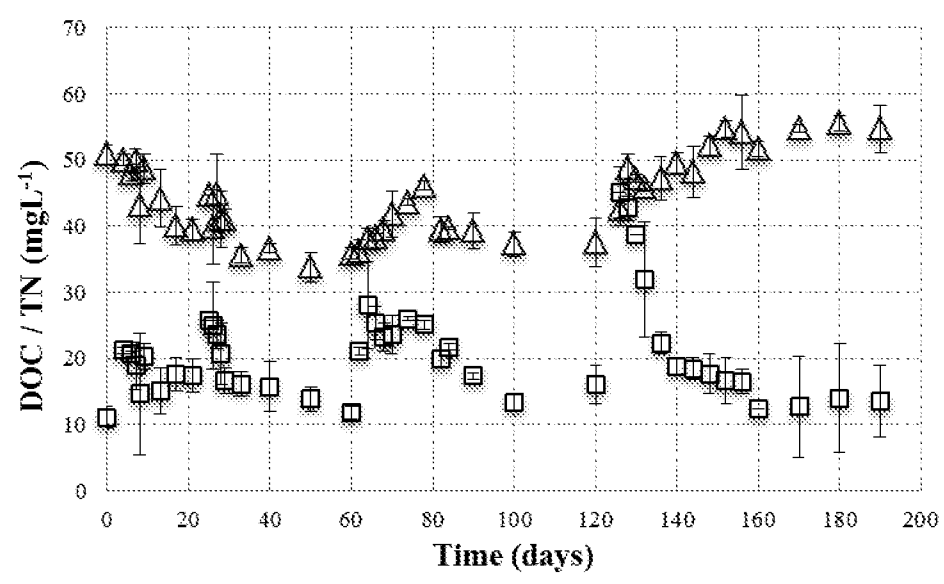


FIG. 5B

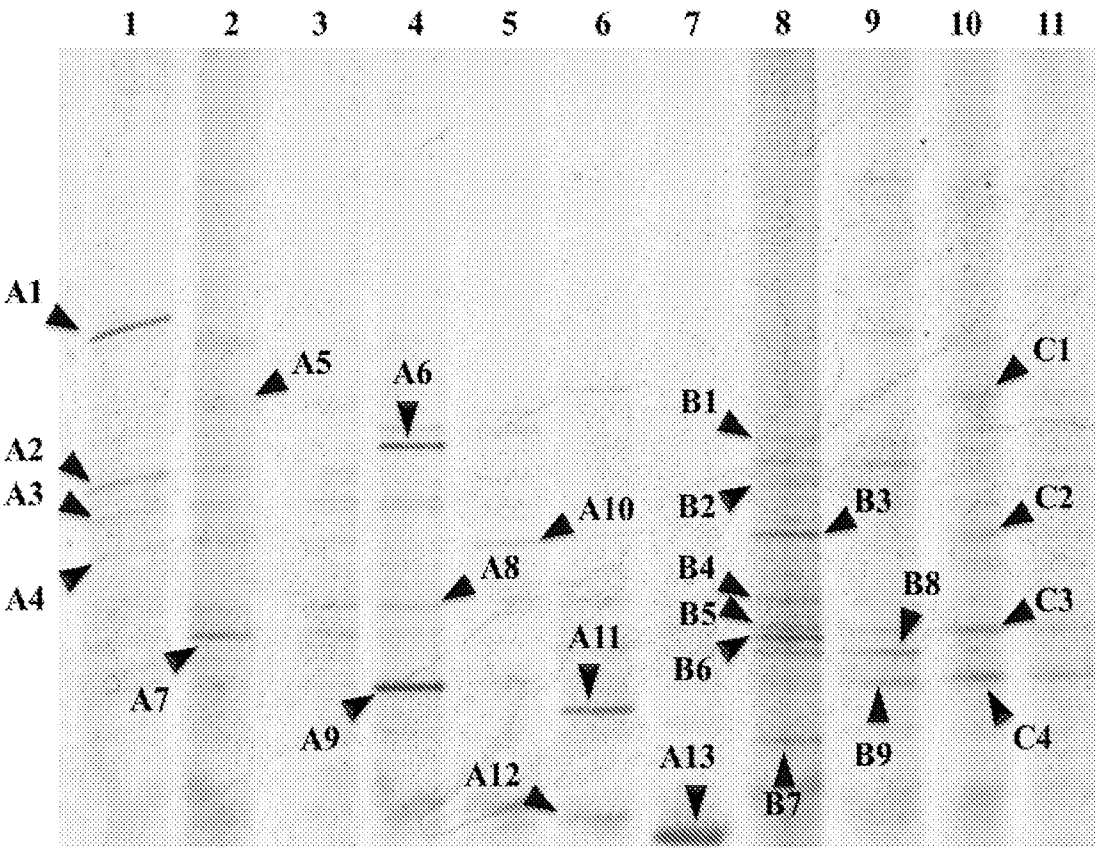


FIG. 6

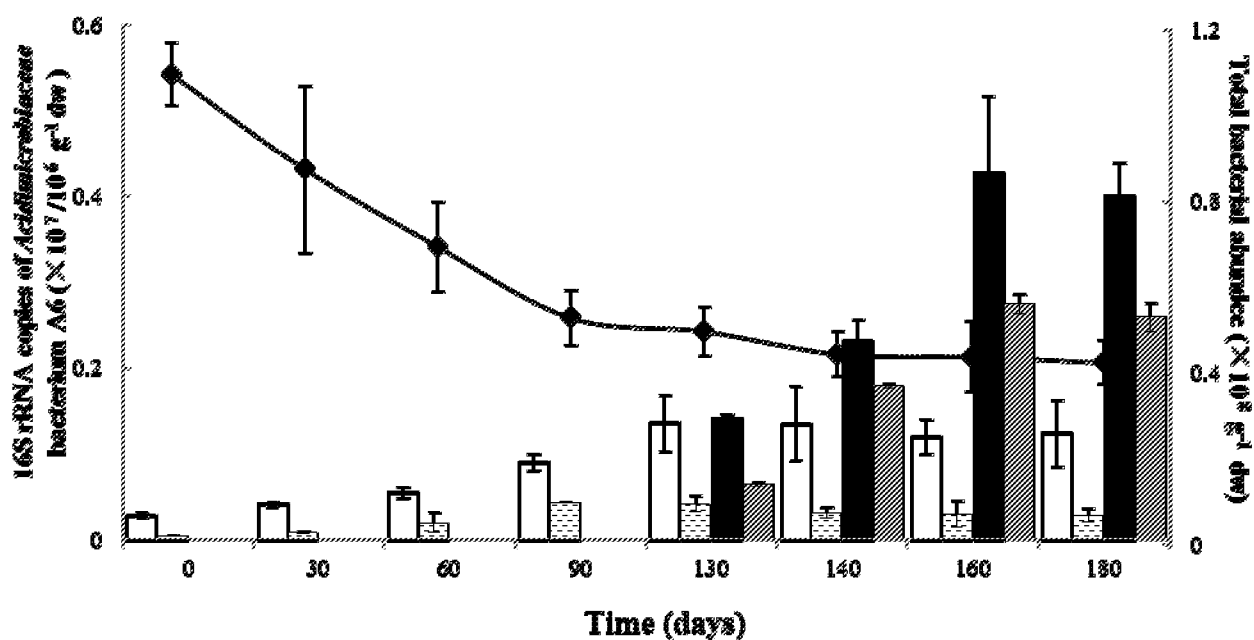


FIG. 7

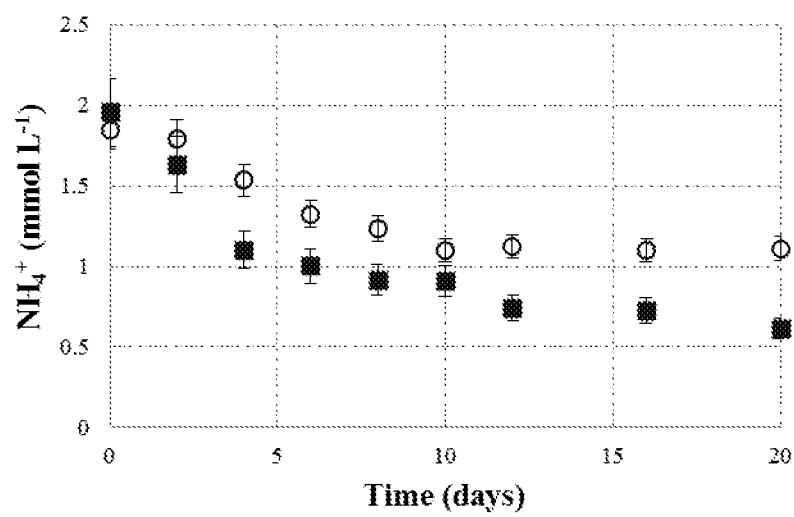


FIG. 8A

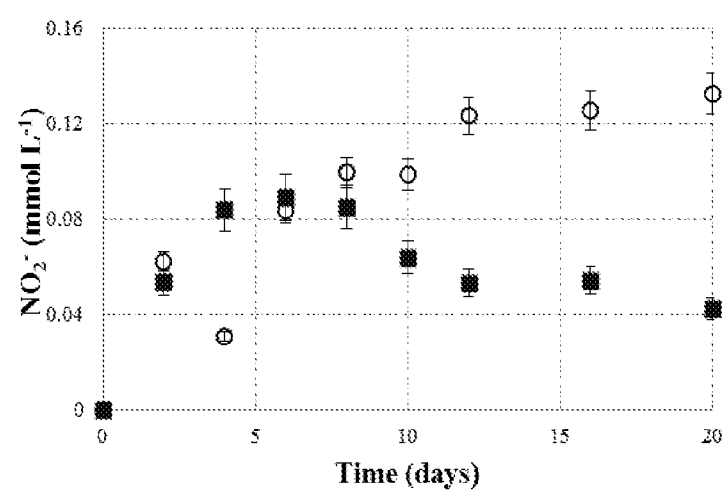


FIG. 8B

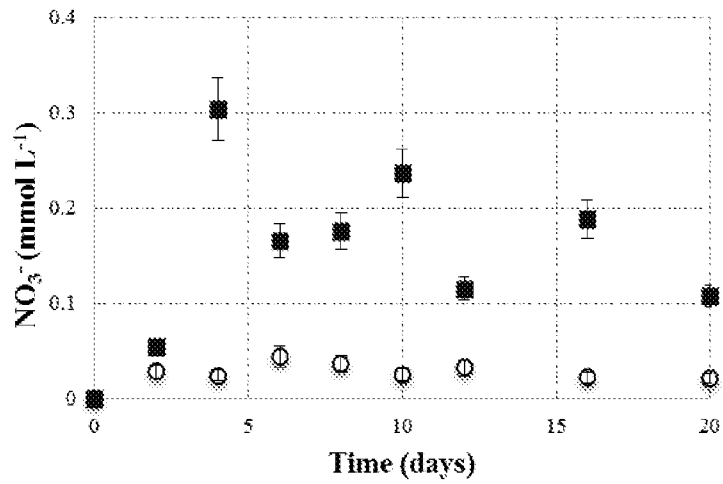


FIG. 8C

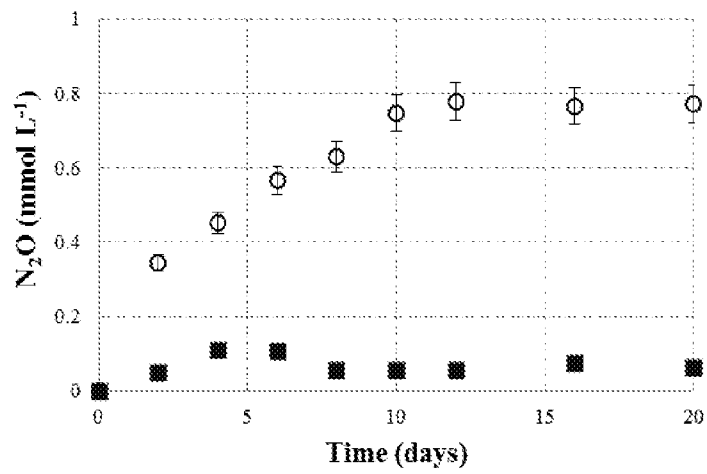


FIG. 8D

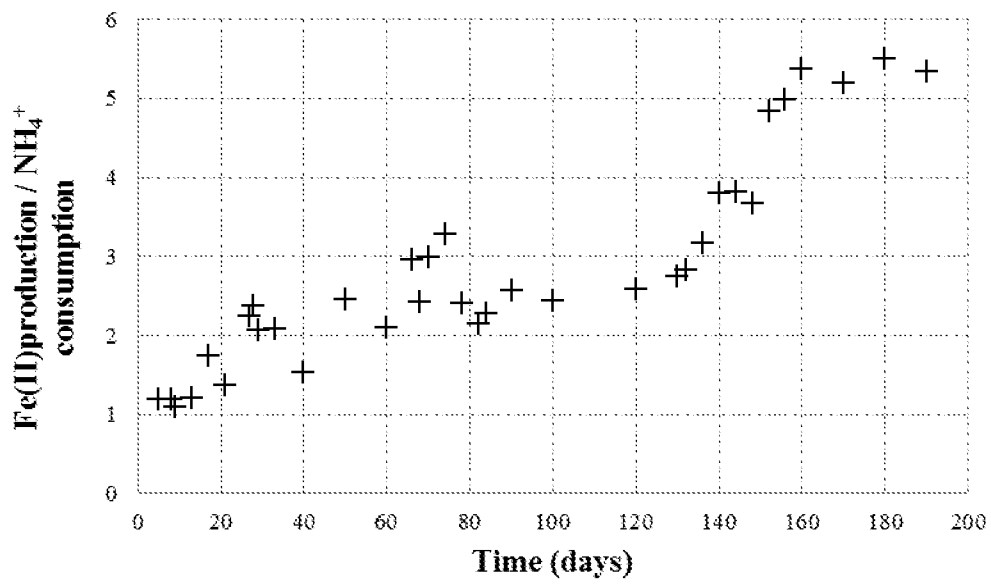


FIG. 9

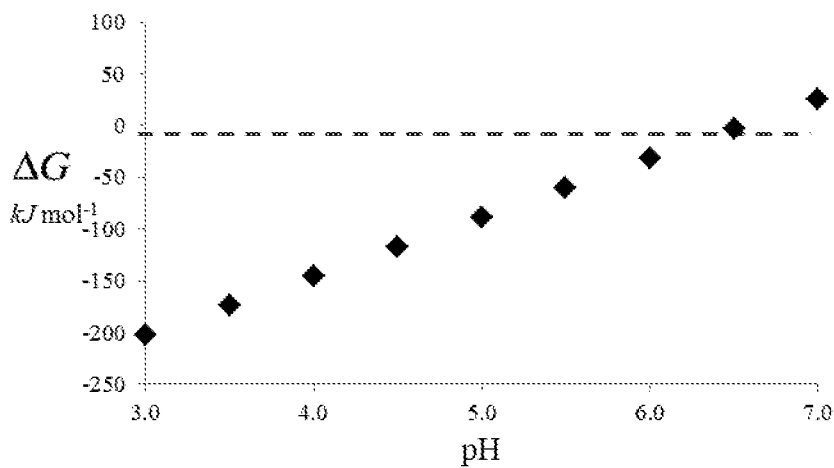


FIG. 10

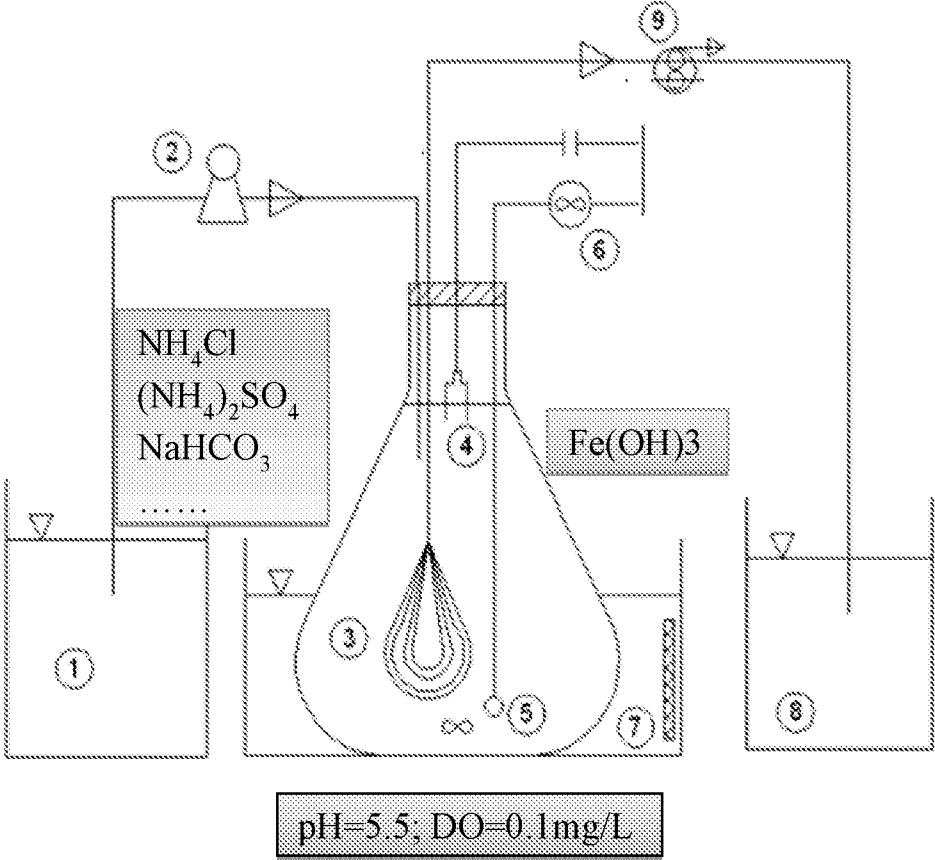


FIG. 11

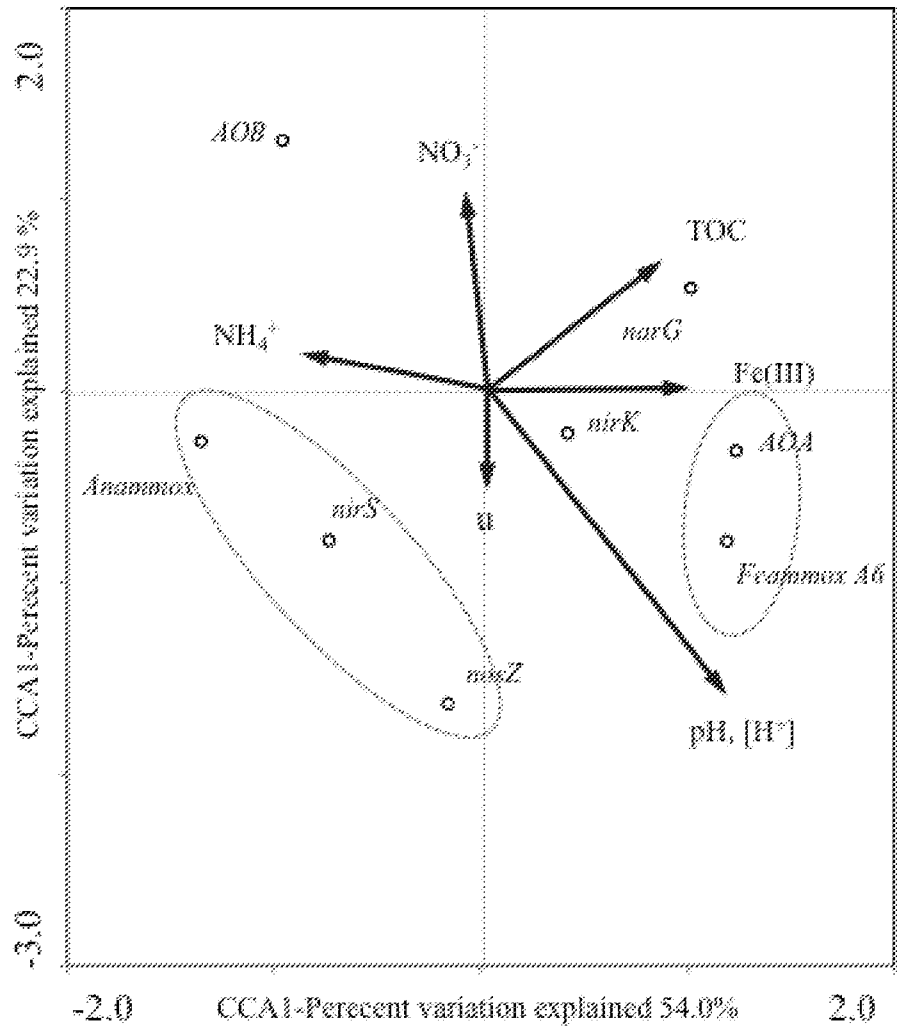


FIG. 12B

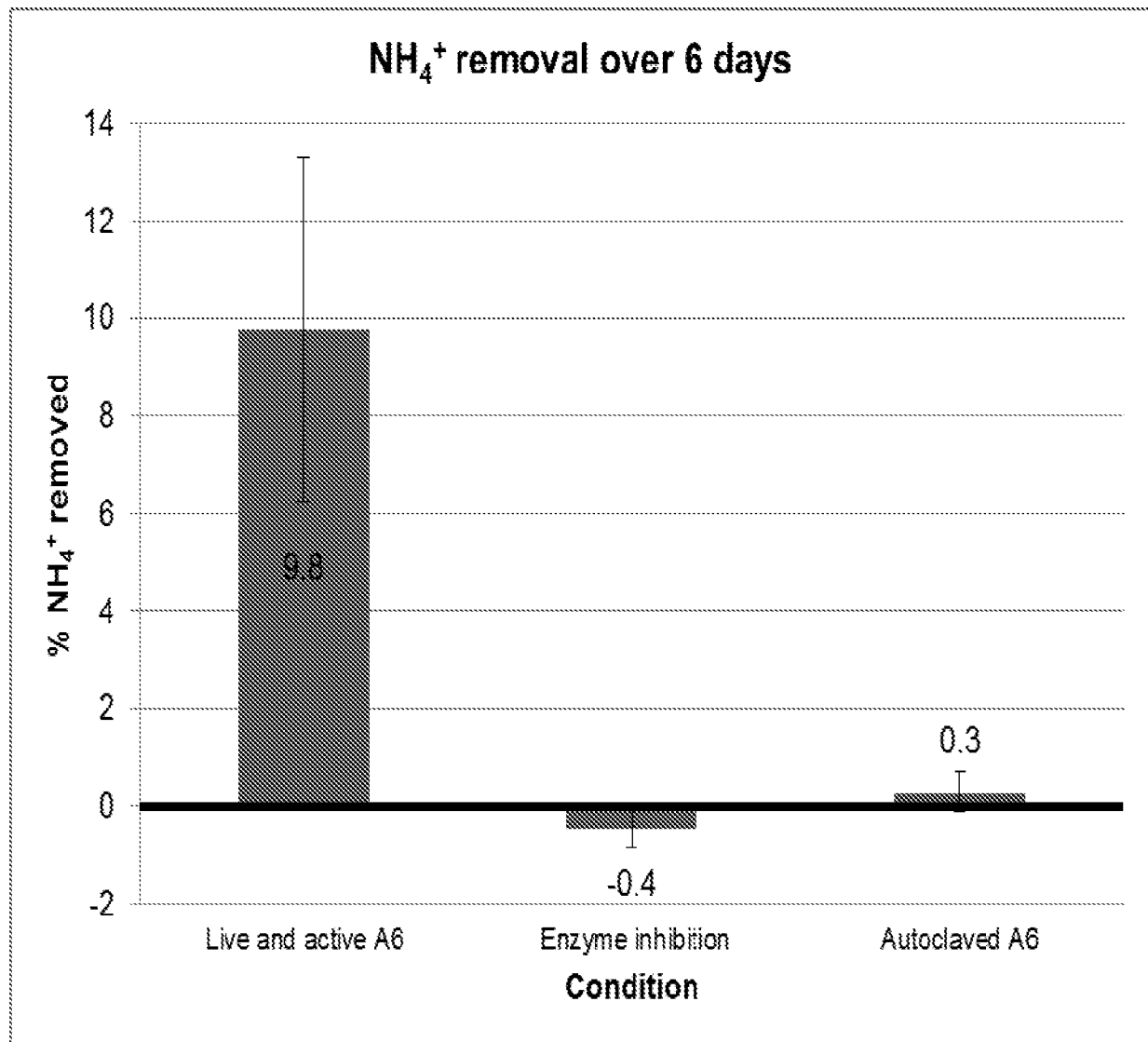


FIG. 13

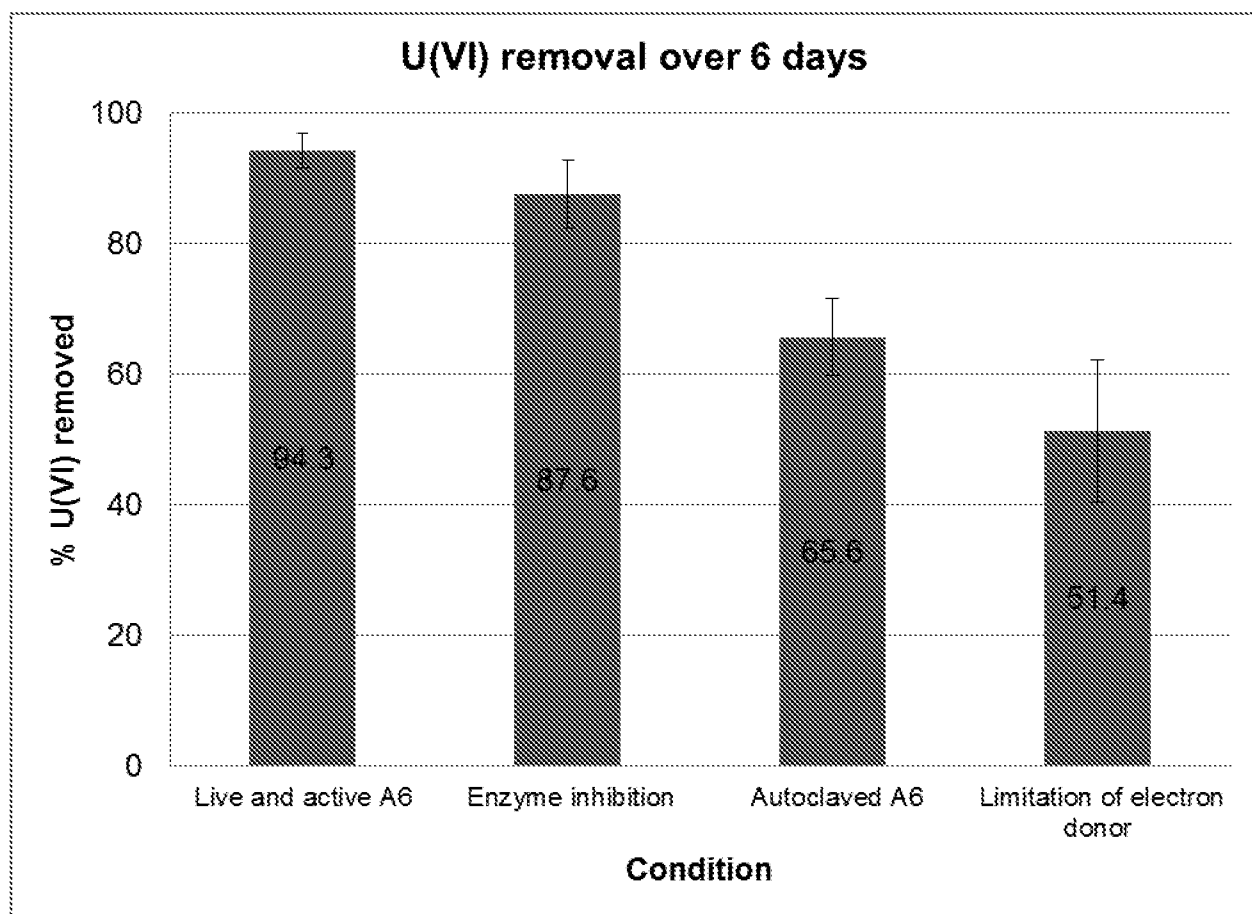


FIG. 14

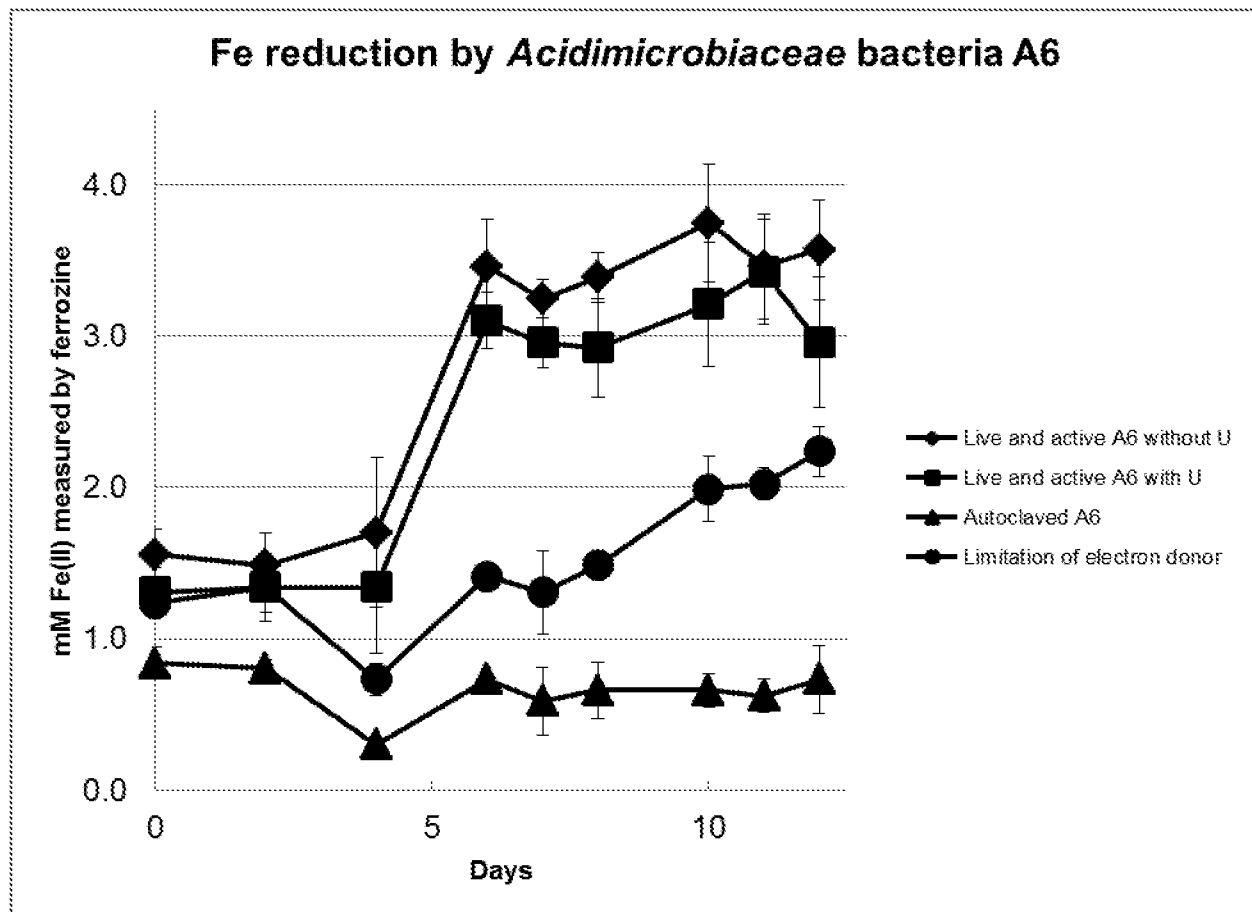


FIG. 15

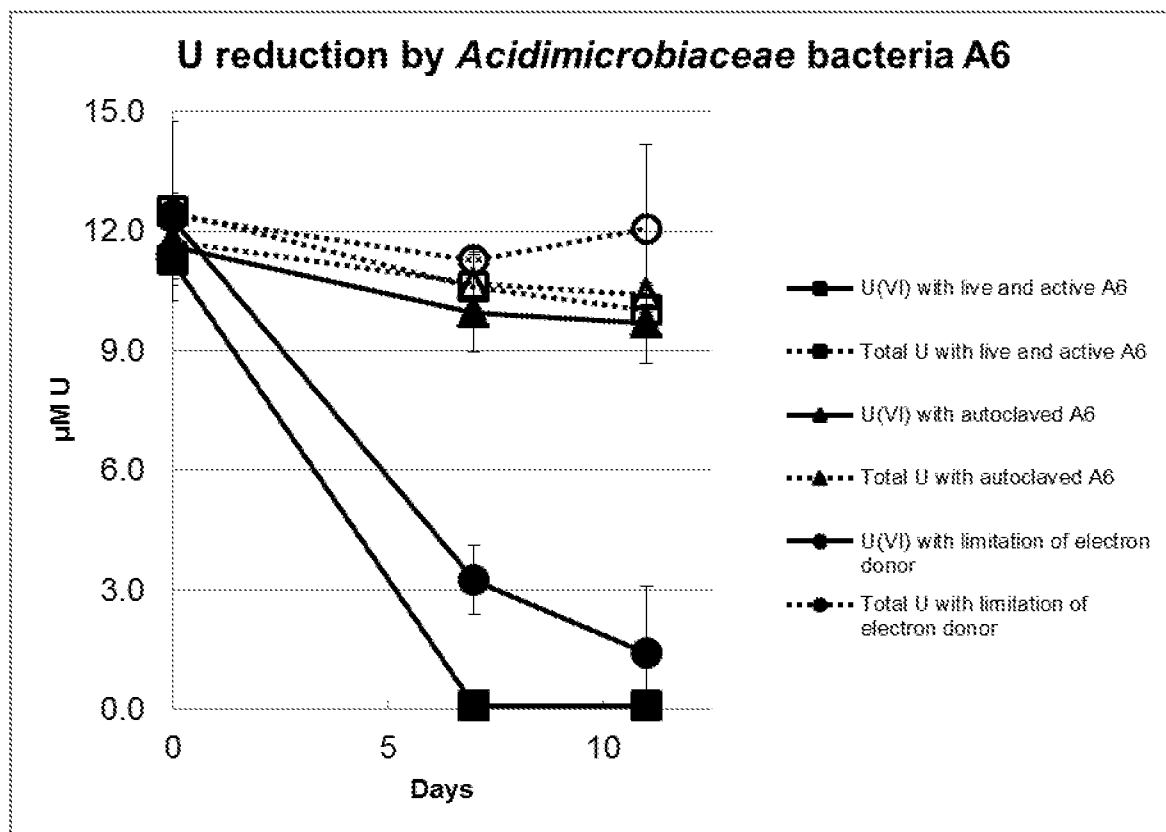


FIG. 16

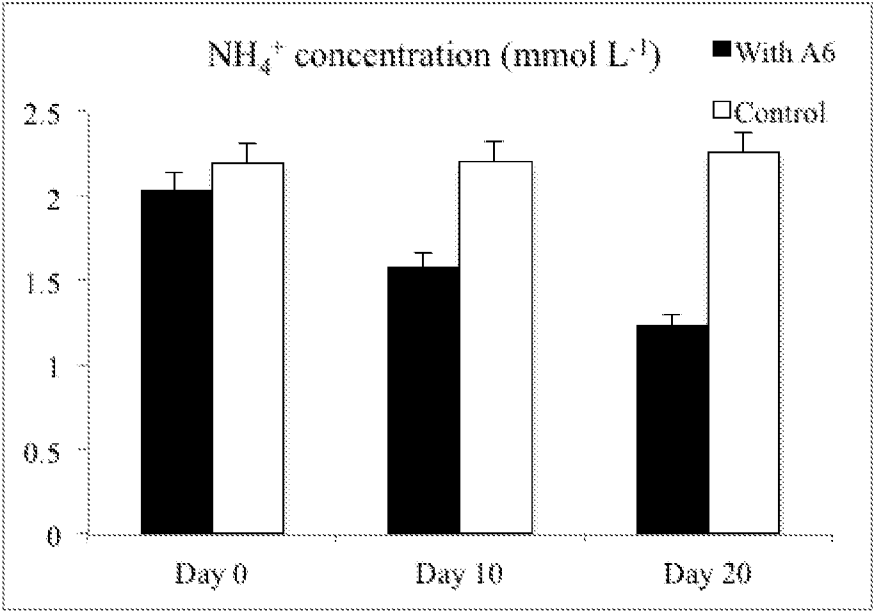


FIG. 17A

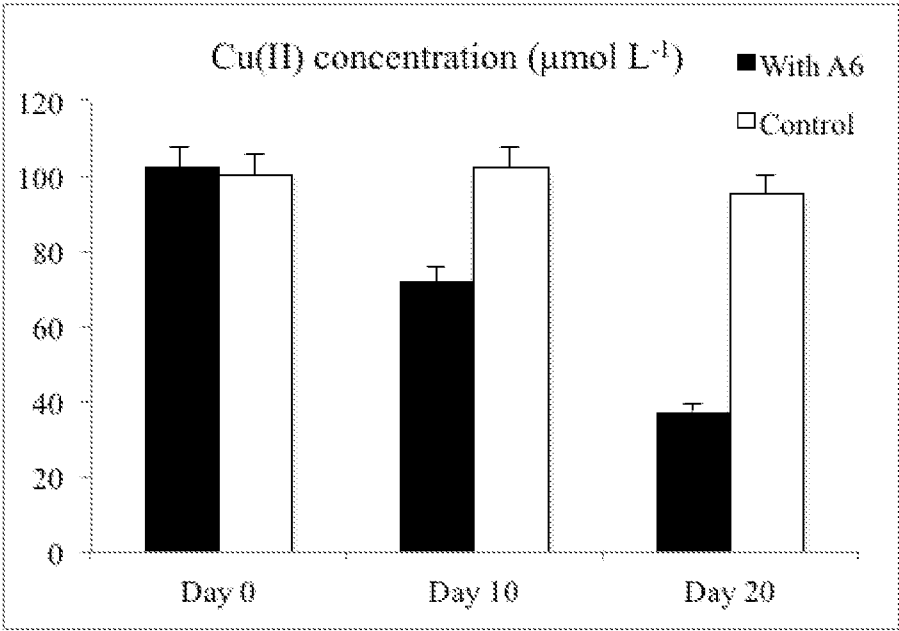


FIG. 17B

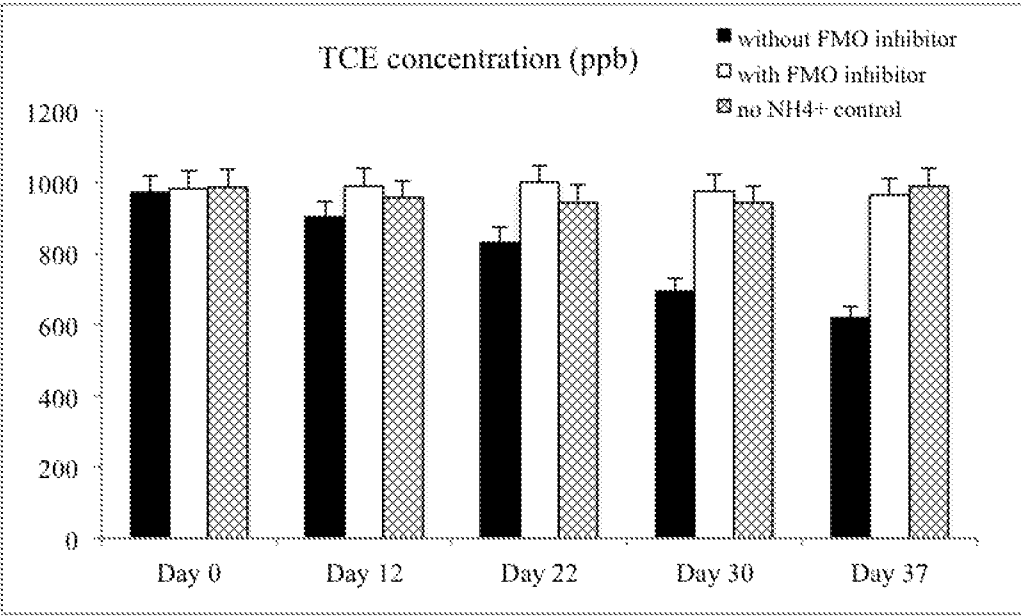


FIG. 18A

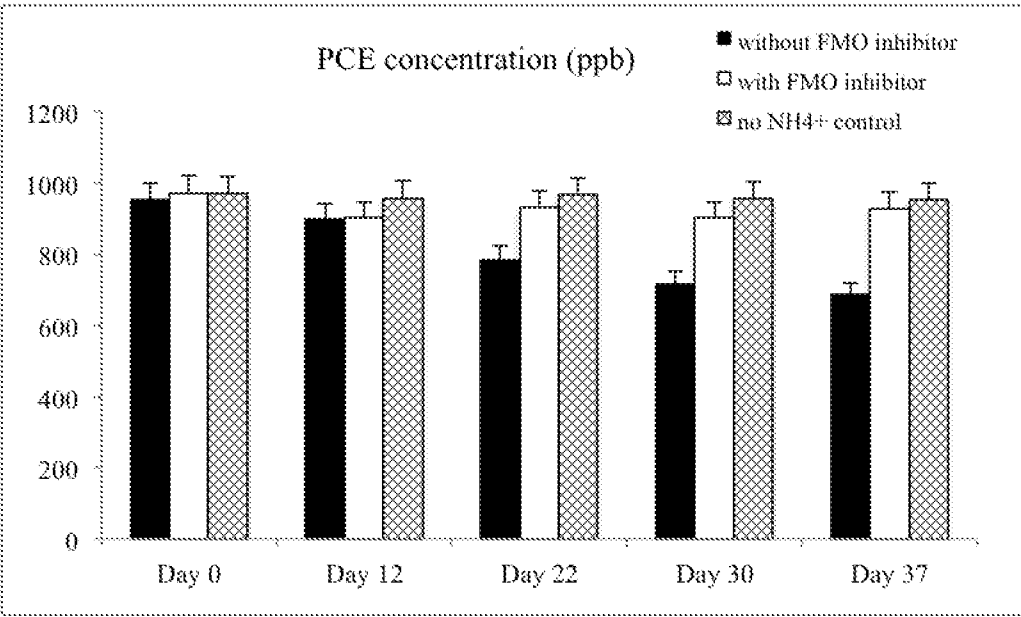


FIG. 18B

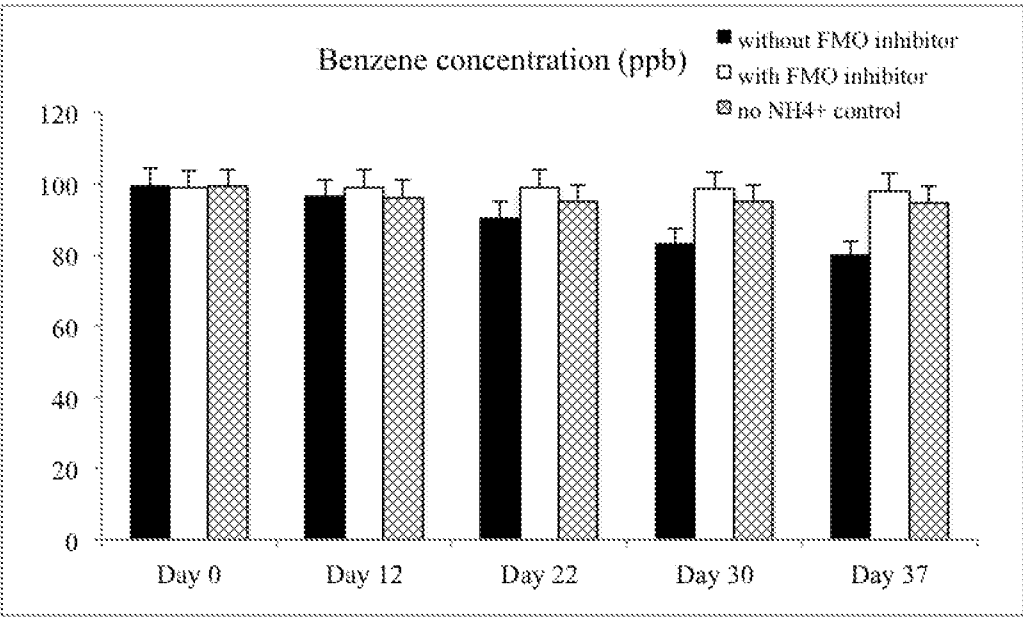


FIG. 18C

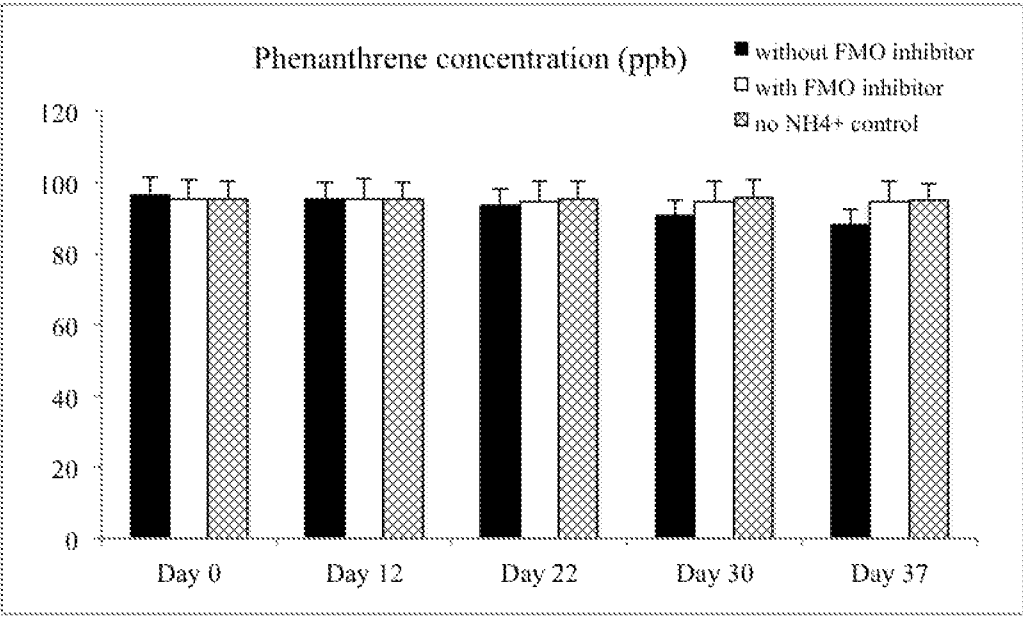


FIG. 18D



FIG. 19A

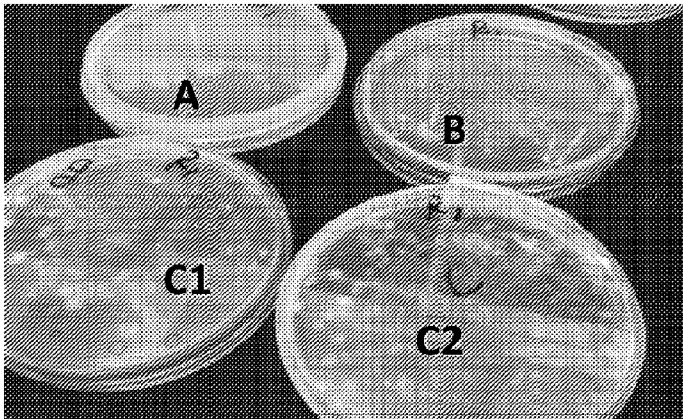


FIG. 19B

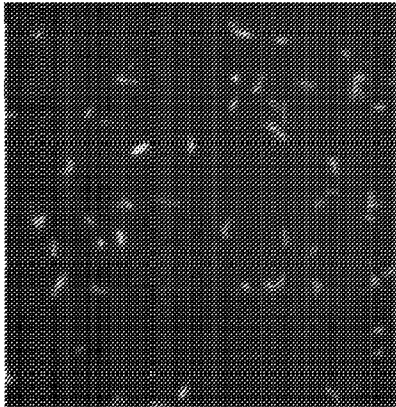


FIG. 19C

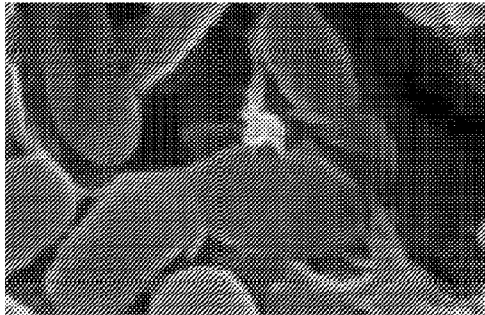


FIG. 19D

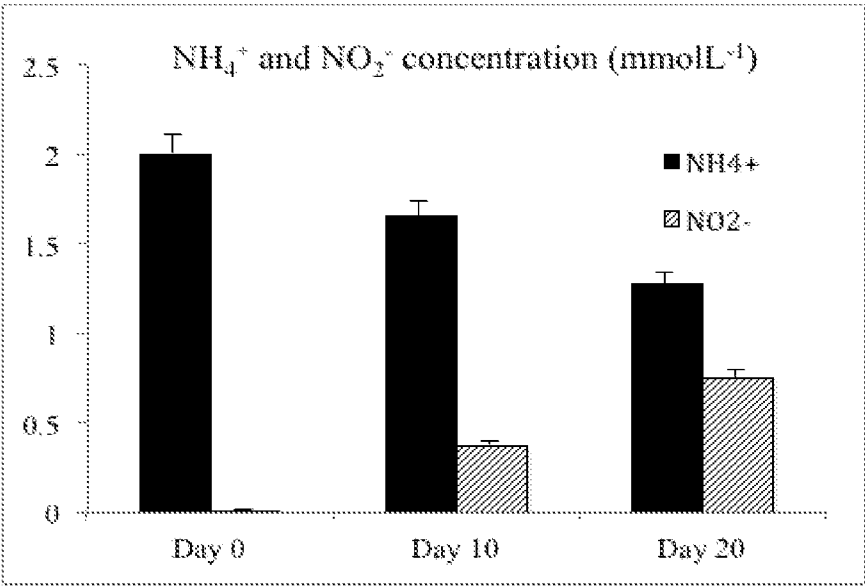


FIG. 20

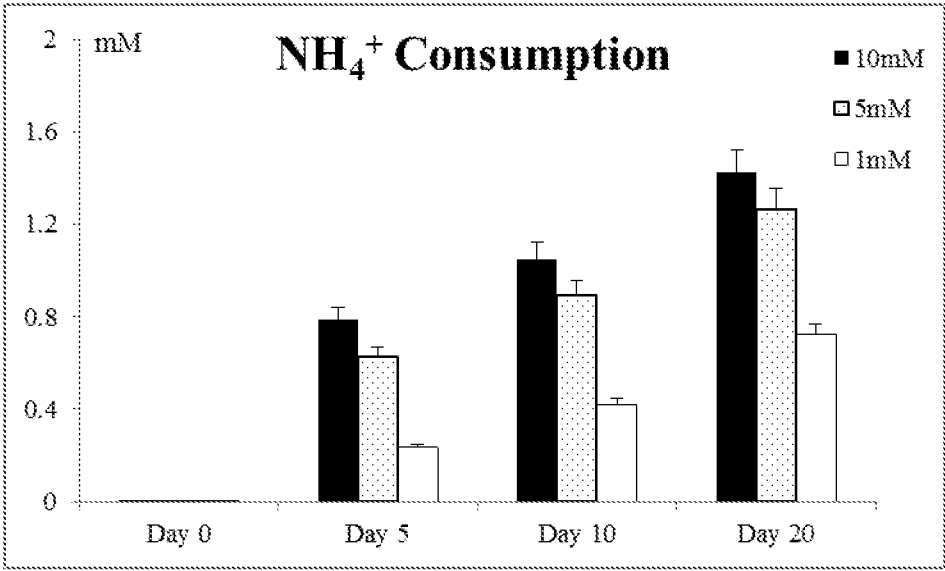


FIG. 21

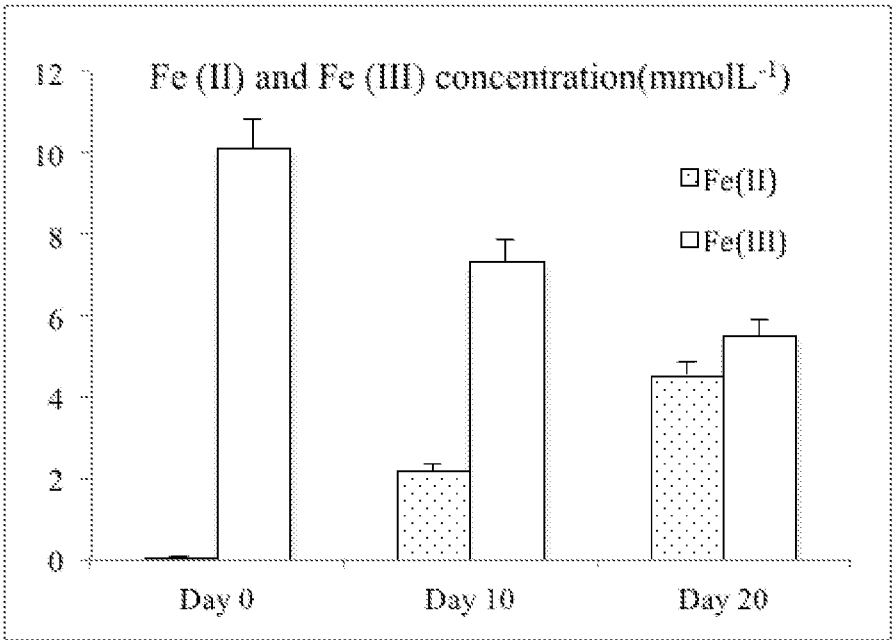


FIG. 22

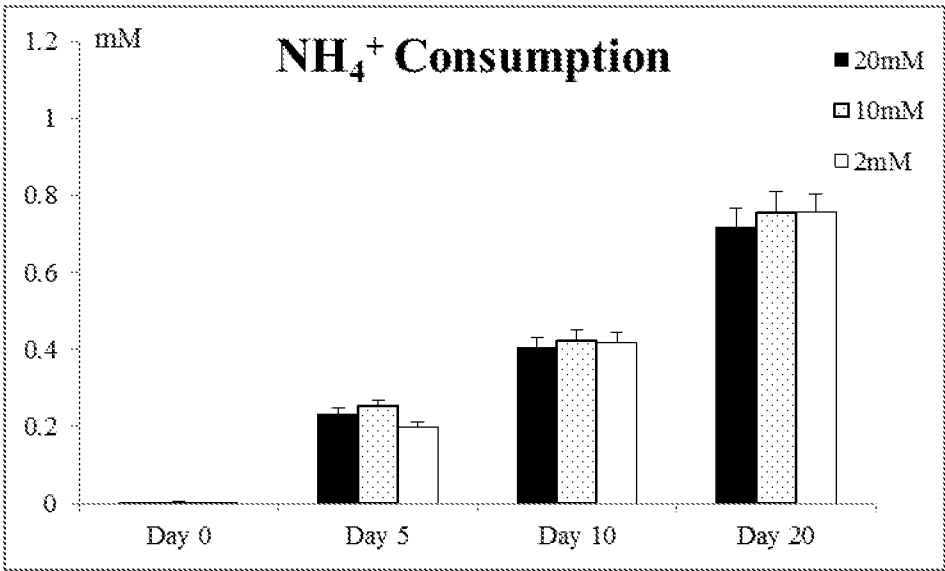


FIG. 23

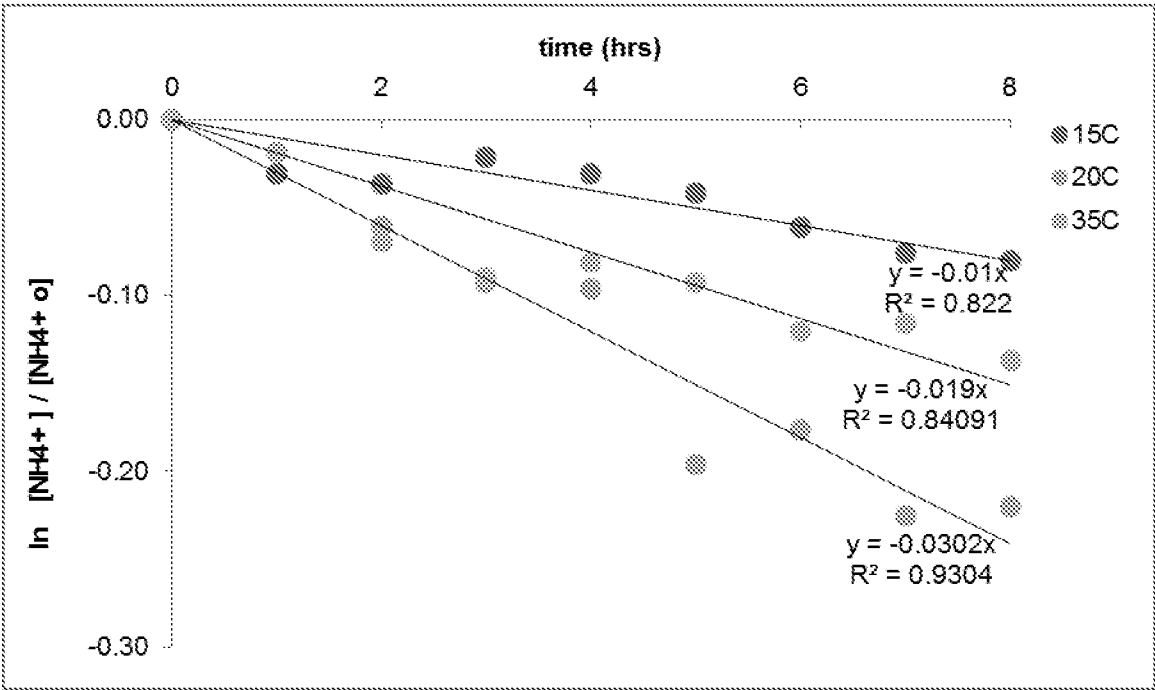


FIG. 24

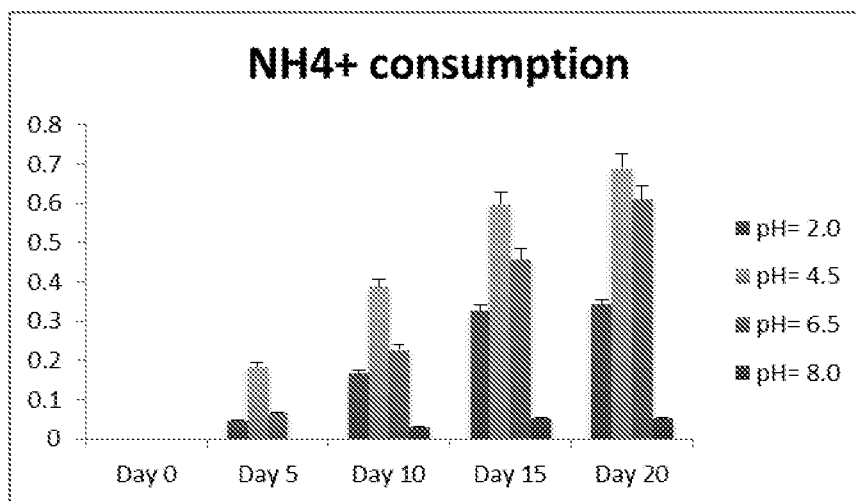


FIG. 25A

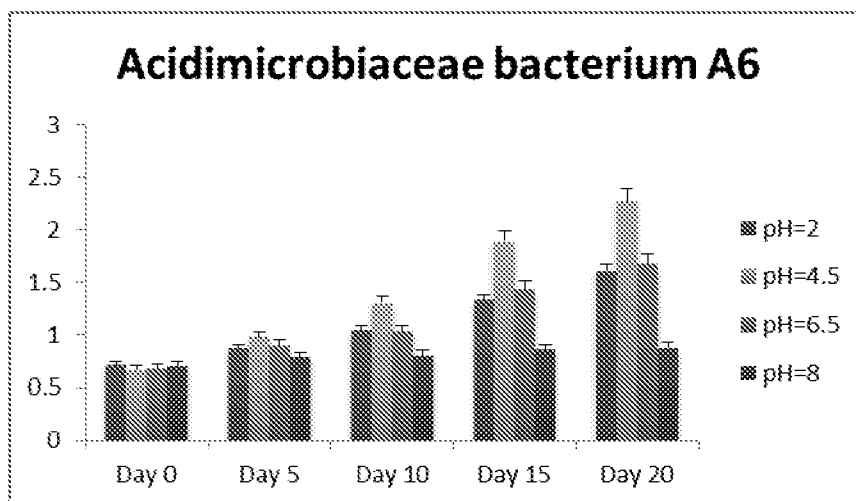


FIG. 25B

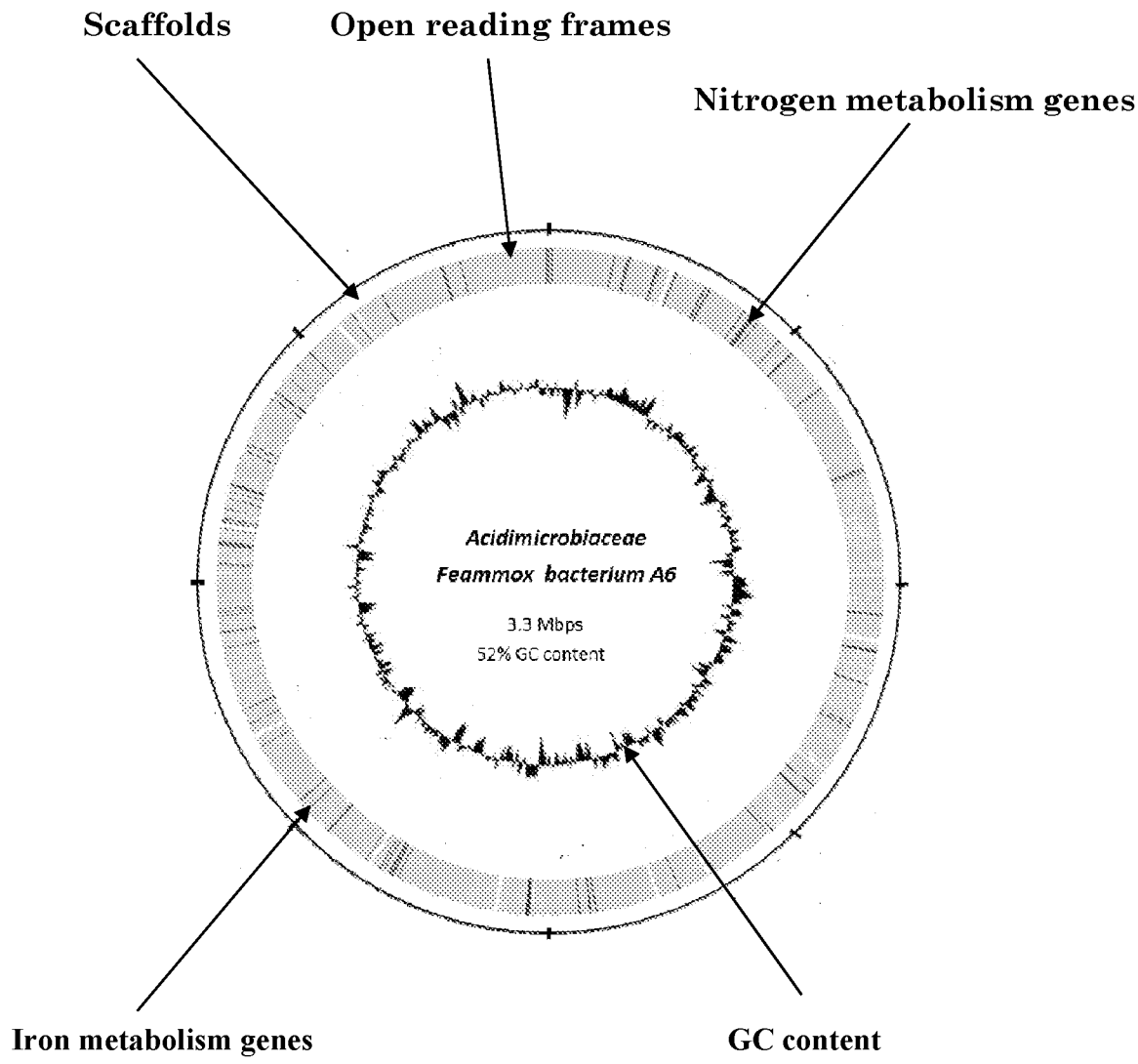


FIG. 26

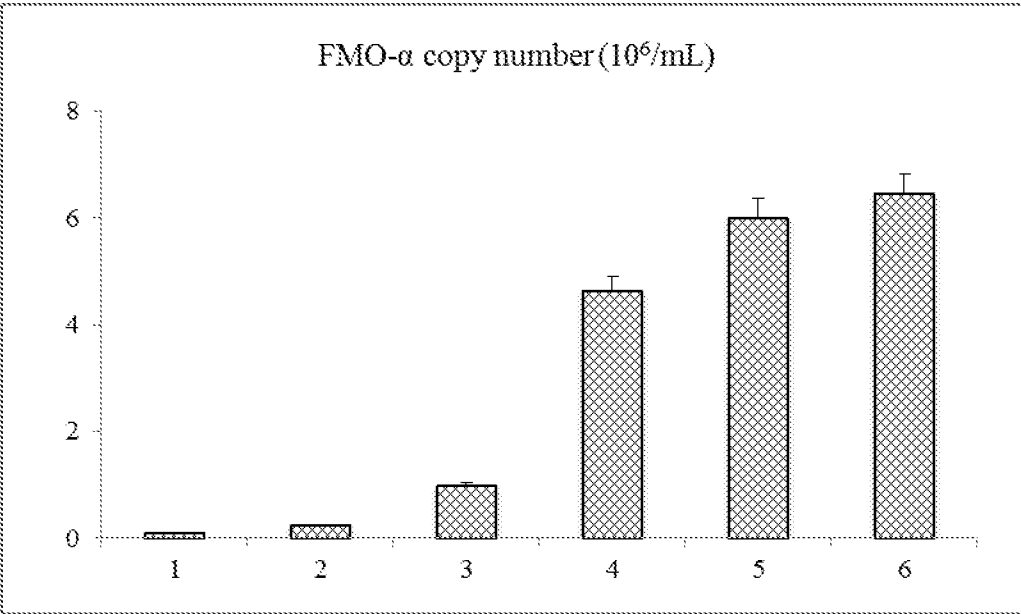


FIG. 27

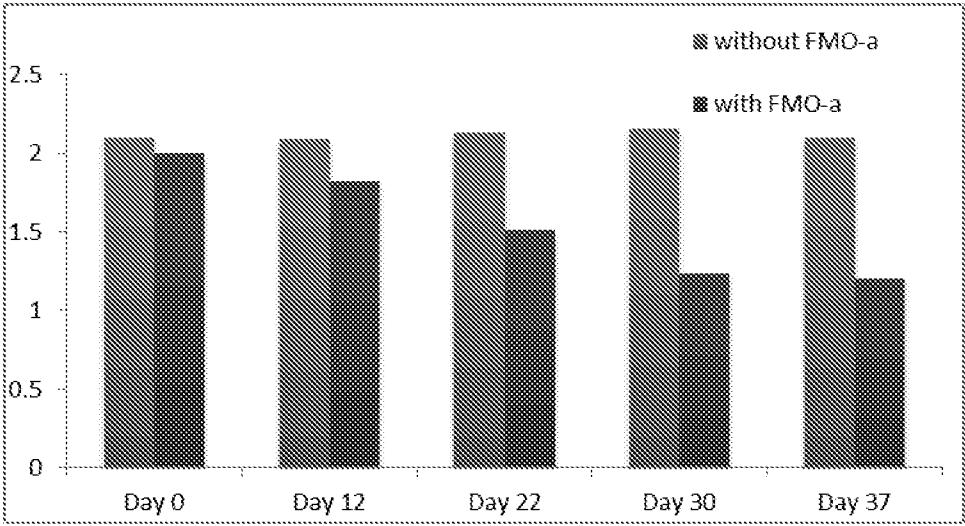


FIG. 28

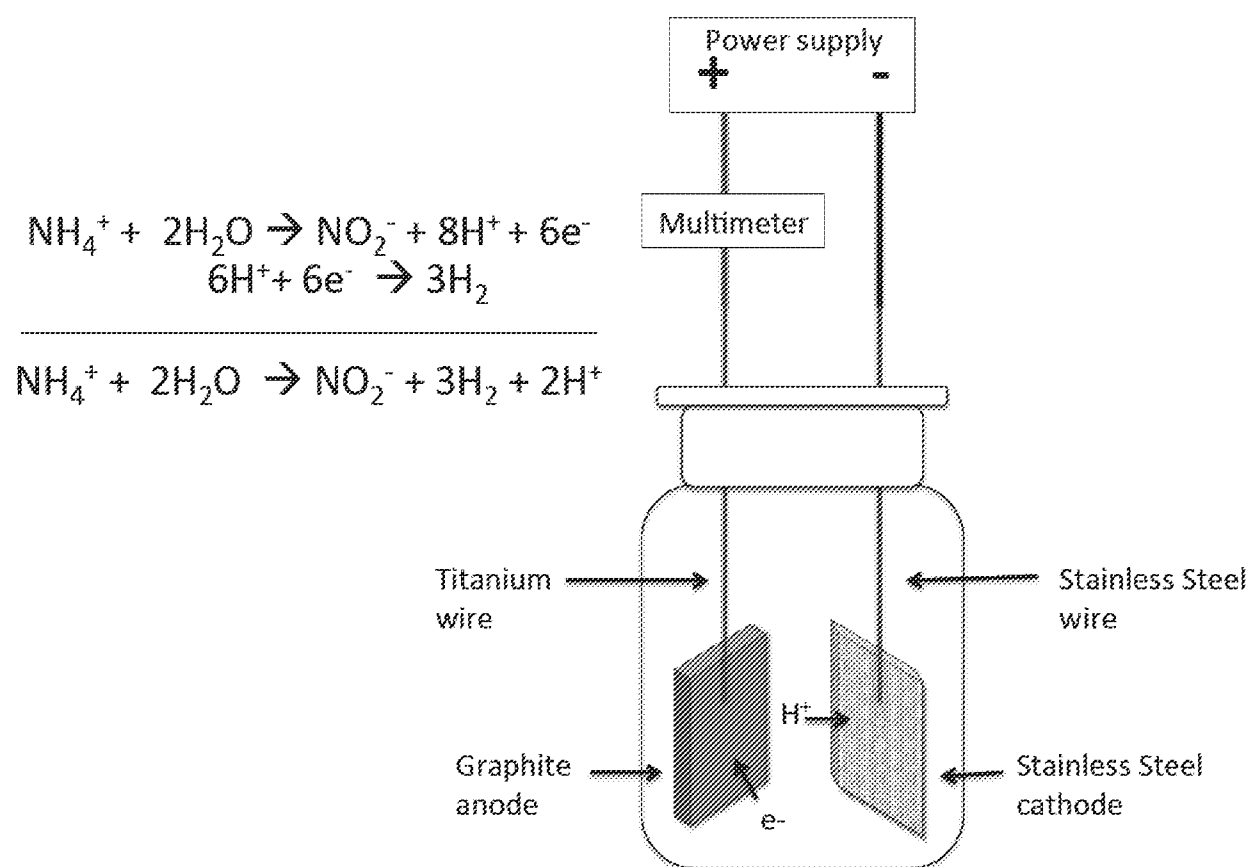


FIG. 29

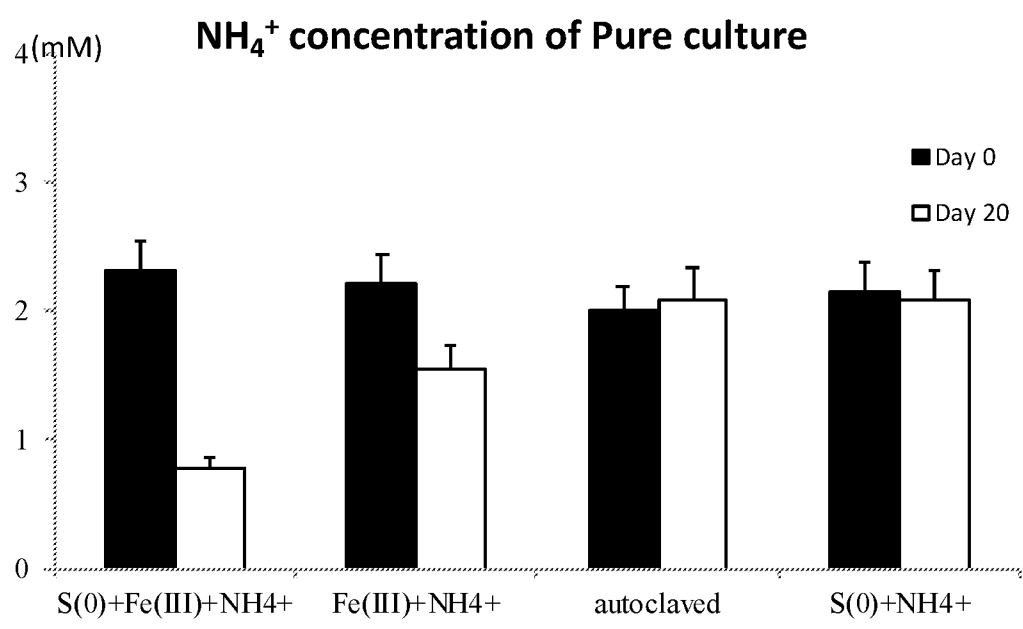


FIG. 30

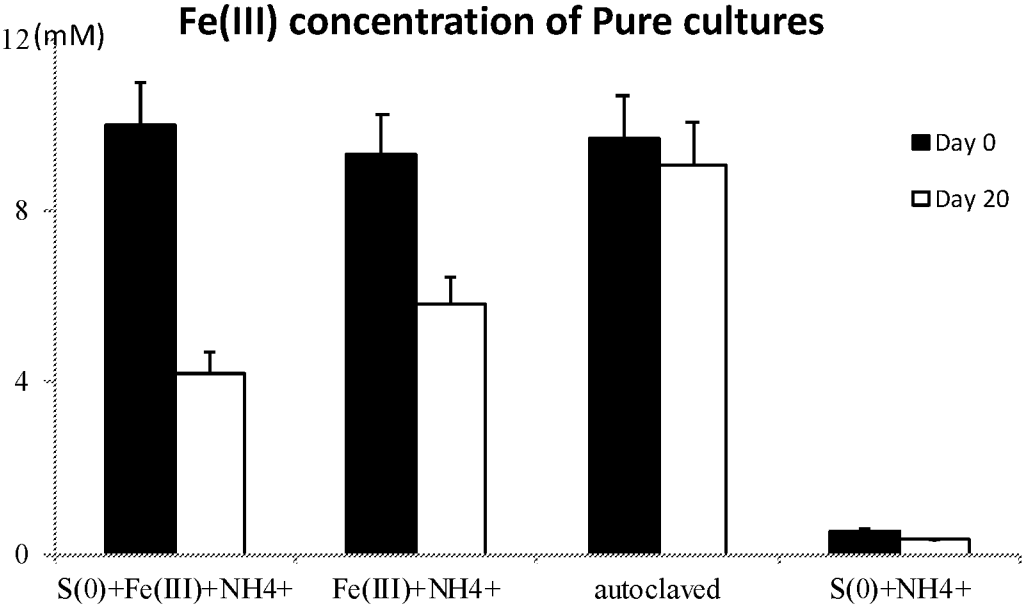


FIG. 31

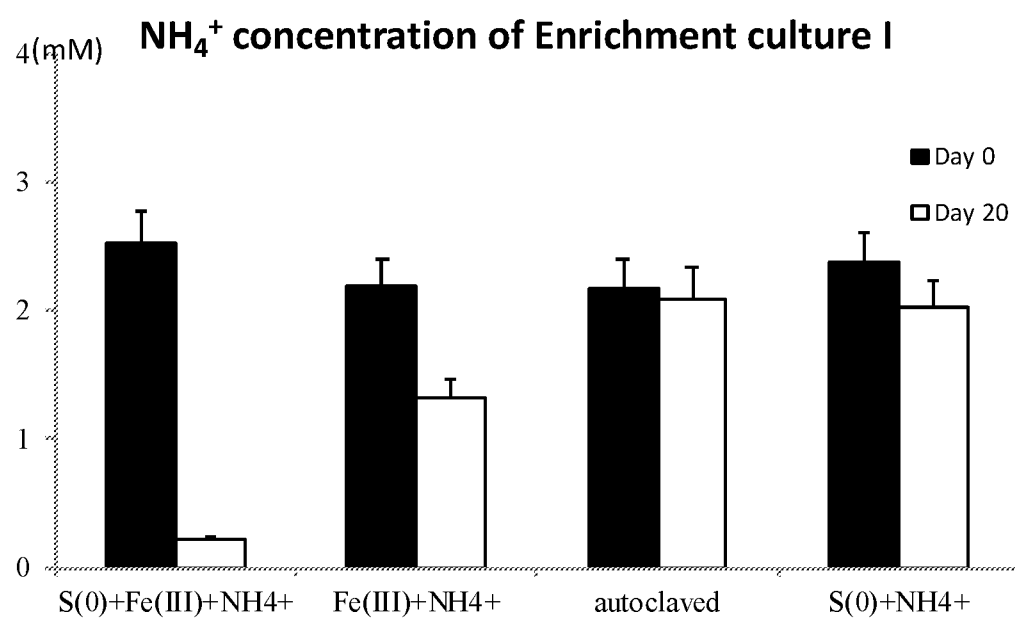


FIG. 32

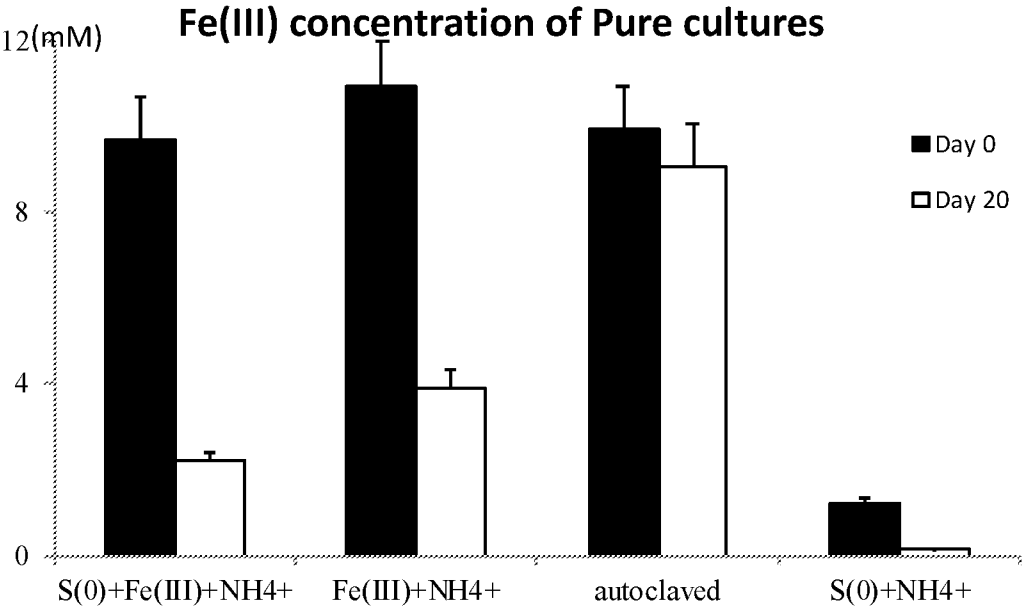


FIG. 33

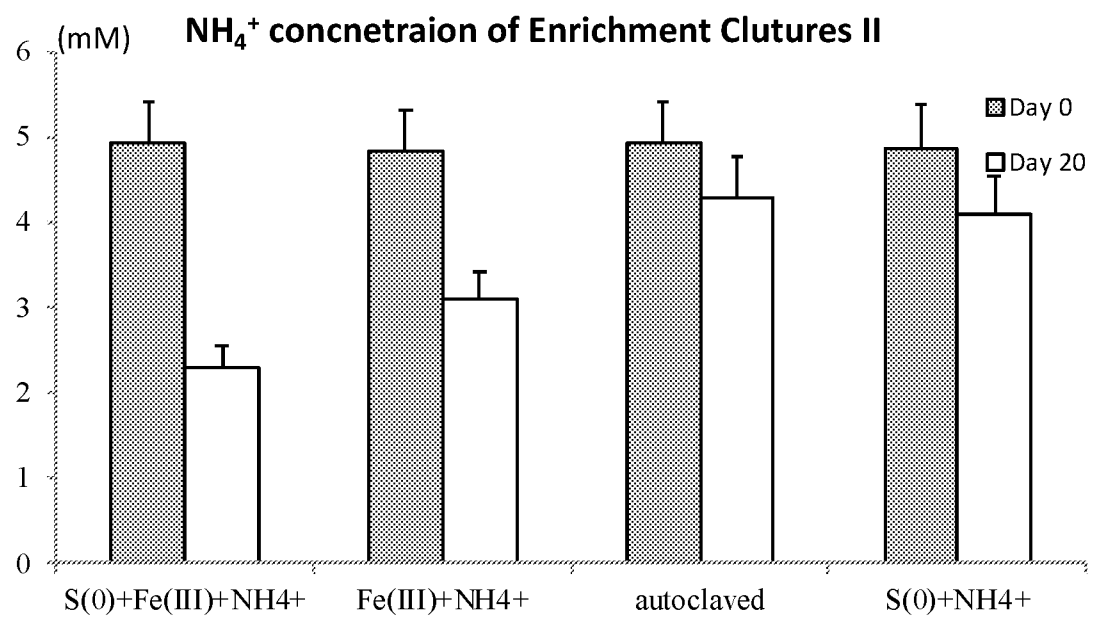


FIG. 34

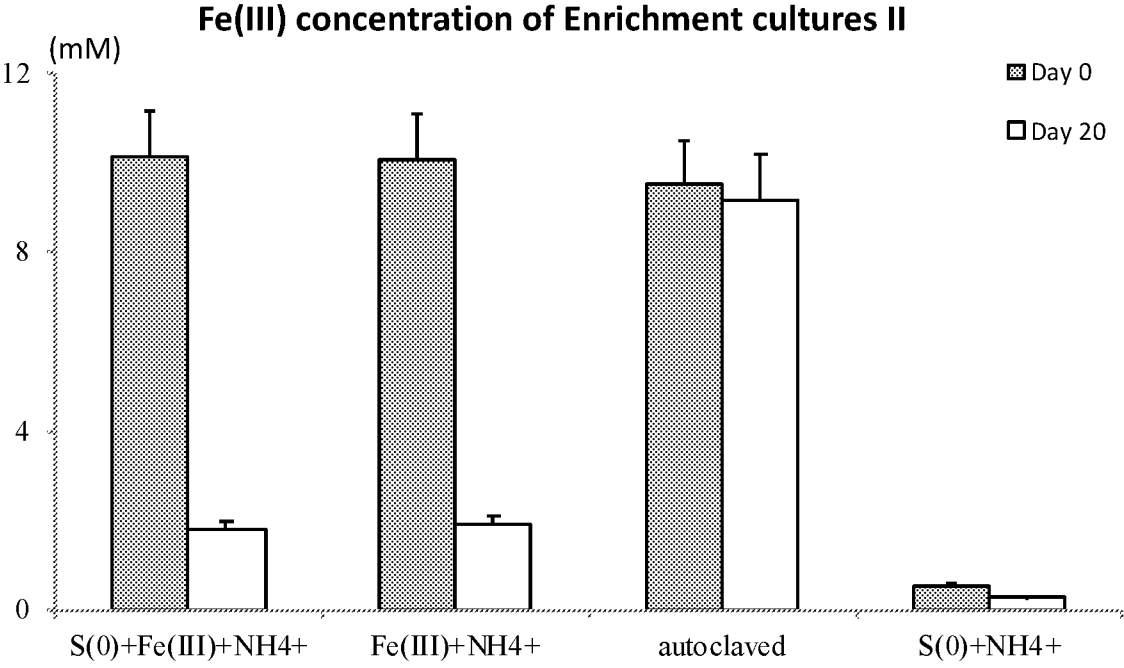


FIG. 35

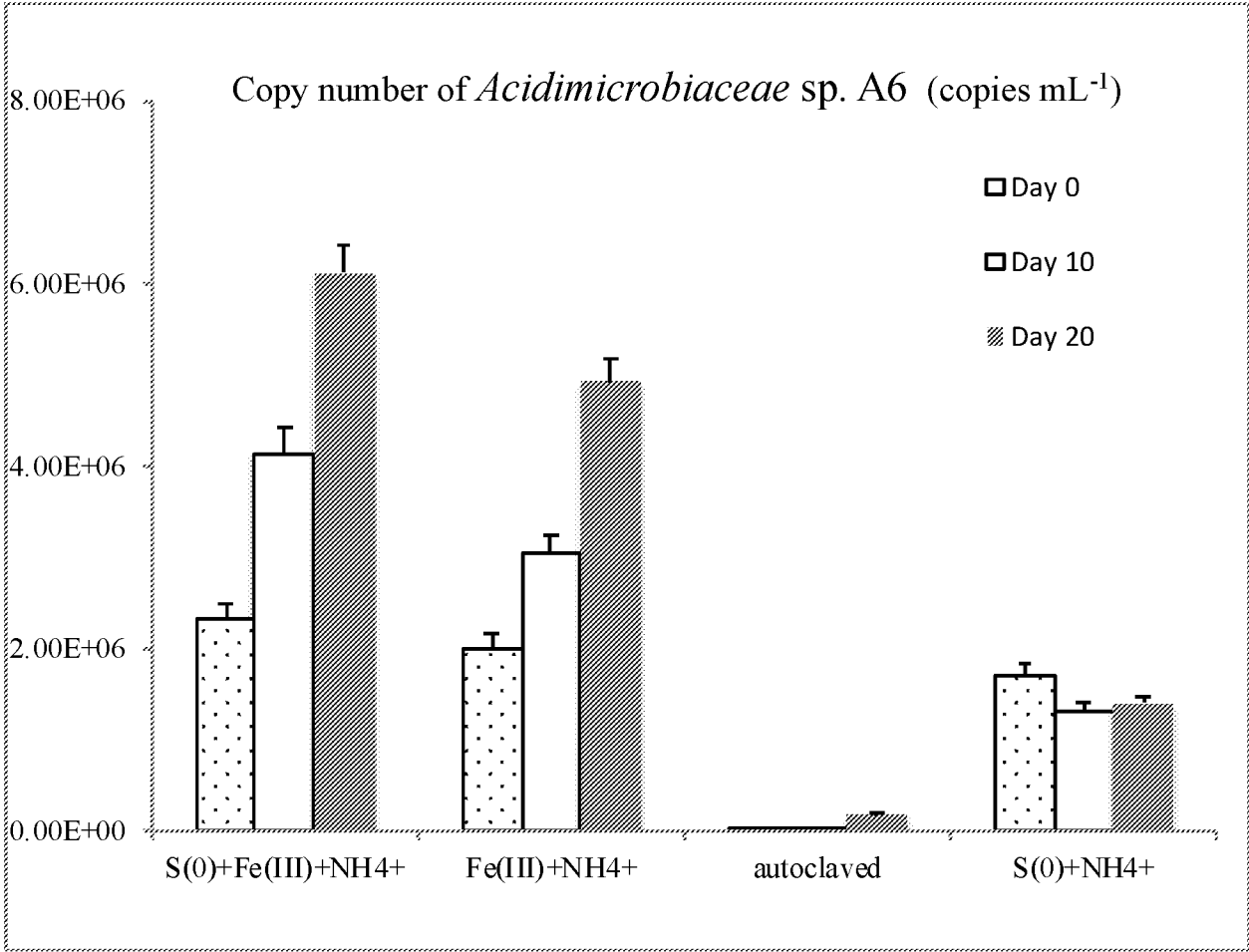


FIG. 36

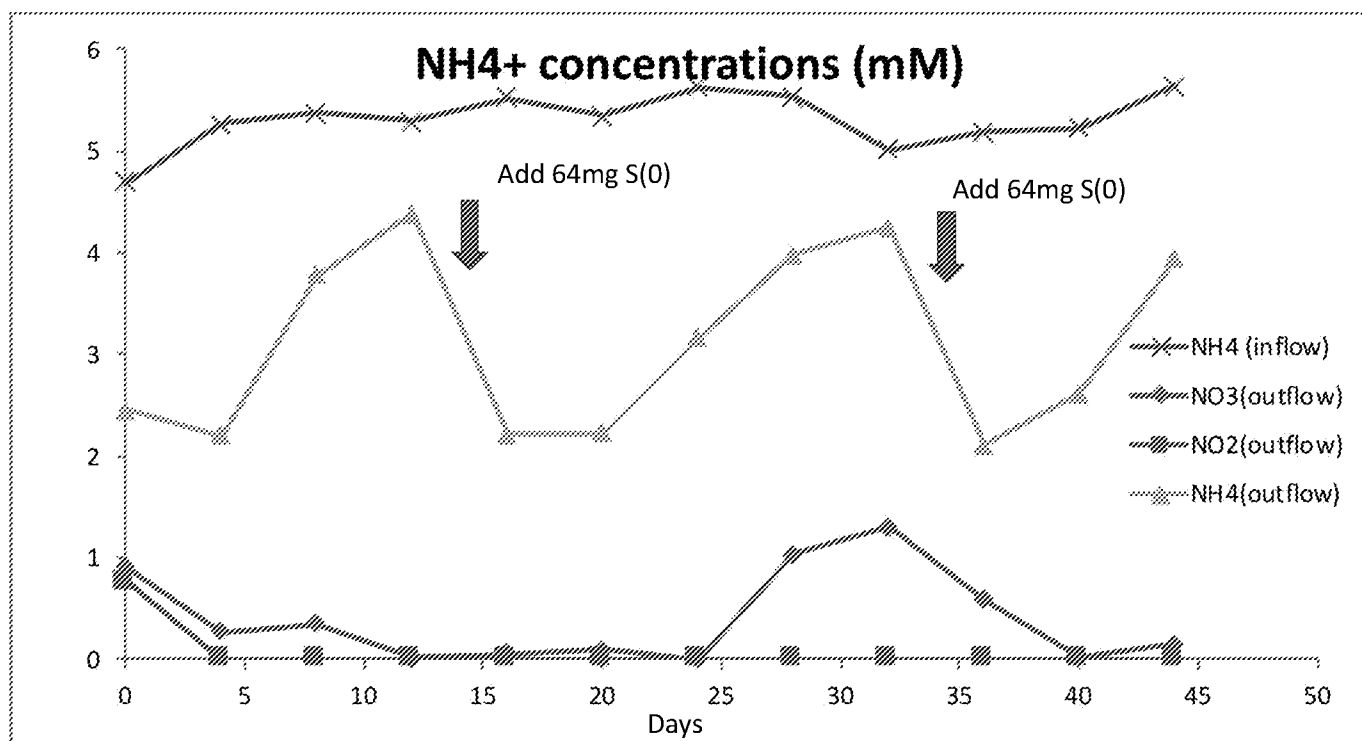


FIG. 37

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US18/52710

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C02F 3/34, 101/16, 3/30, 101/30 (2018.01)

CPC - C02F 3/345, 3/341, 3/301, 3/302

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2015/0321933 A1 (THE TRUSTEES OF PRINCETON UNIVERSITY) 12 November, 2015; paragraphs [0010]-[0011], [0052], [0080], [0092], [0117], [0199]; claims 1-9, 25, 32	1-10, 11a/1-9, 14-24, 25/14-24, 29-40, 41/29-40, 42
Y	(MULOPO, J et al.) Biological Regeneration of Ferric (Fe ³⁺) Solution During Desulphurisation of Gaseous Streams: Effect of Nutrients and Support Material. Water Science and Technology. 2015; Vol. 71, No. 11; pages 1672-1678; abstract; page 1674, column 1, paragraph 2; DOI: 10.2166/wst.2015.144	1-10, 11a/1-9, 14-24, 25/14-24, 29-40, 41/29-40, 42
Y	(RICKARD, D) Kinetics of Pyrite Formation by the H ₂ S Oxidation of Iron (II) Monosulfide in Aqueous Solutions Between 25 and 125°C: The Rate Equation. Geochimica et Cosmochimica Acta. 01 January 1997; Vol. 61, No. 1; pages 115-34.; abstract; page 121, column 2, paragraph 1	10, 24, 25/24, 39, 41/39
Y	(HENSHAW, PF et al.) Extraction of Elemental Sulfur from an Aqueous Suspension for Analysis by High-Performance Liquid Chromatography. Analytical Chemistry. 01 August, 1997; Vol. 69, No. 16; pages 3119-3120; page 3119, column 1, paragraph 2	40, 41/40
P, X	US 2018/0029909 A1 (THE TRUSTEES OF PRINCETON UNIVERSITY) 01 February, 2018; whole document	1-10, 11a/1-9, 14-24, 25/14-24, 29-40, 41/29-40, 42

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 November 2018 (13.11.2018)

Date of mailing of the international search report

12 DEC 2018

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US18/52710

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 11b, 12-13, 26-28, 43-45
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.