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Diverse microbial species survive high ammonia concentrations

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Abstract: Planetary protection regulations are in place to control the contamination of planets and moons with terrestrial micro-organisms in order to avoid jeopardizing future scientific investigations relating to the search for life. One environmental chemical factor of relevance in extraterrestrial environments, specifically in the moons of the outer solar system, is ammonia (NH_3). Ammonia is known to be highly toxic to micro-organisms and may disrupt proton motive force, interfere with cellular redox reactions or cause an increase of cell pH. To test the survival potential of terrestrial micro-organisms exposed to such cold, ammonia-rich environments, and to judge whether current planetary protection regulations are sufficient, soil samples were exposed to concentrations of NH_3 from 5 to 35% (v/v) at −80 °C and room temperature for periods up to 11 months. Following exposure to 35% NH_3, diverse spore-forming taxa survived, including representatives of the *Firmicutes* (*Bacillus, Sporosarcina, Viridibacillus, Paenibacillus, Staphylococcus* and *Brevibacillus*) and *Actinobacteria* (*Streptomyces*). Non-spore forming organisms also survived, including *Proteobacteria* (*Pseudomonas*) and *Actinobacteria* (*Arthrobacter*) that are known to have environmentally resistant resting states. *Clostridium* spp. were isolated from the exposed soil under anaerobic culture. High NH_3 was shown to cause a reduction in viability of spores over time, but spore morphology was not visibly altered. In addition to its implications for planetary protection, these data show that a large number of bacteria, potentially including spore-forming pathogens, but also environmentally resistant non-spore-formers, can survive high ammonia concentrations.

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Introduction

High ammonia (NH_3) concentrations are postulated to exist in the subsurface of Titan, a moon of Saturn. Current modelling suggests that a primordial subsurface Titan ocean may have had concentrations of ammonia up to 15 wt%. However, today, because of the shrinkage of the ocean, Titan might host a subsurface water body with concentrations as high as 33 wt% (Fortes 2000). Although the connection between the surface and subsurface of Titan has not been demonstrated, the possibility of ammonia–water cryovolcanism has been suggested (Mitri *et al*. 2008), raising the question of the fate of any organics or terrestrial micro-organisms on spacecraft that come into contact with high ammonia solutions. Can terrestrial micro-organisms survive or even proliferate in these extraterrestrial environments of the outer solar system? The answer to this question will be informative for persons responsible for planetary protection regulations for missions to these cold worlds.

Ammonia is known to be toxic to micro-organisms (Deal *et al.* 1975). There are three major mechanisms by which it can exert a toxic effect. First, un-ionized ammonium (NH_3) is easily transported across the cell membrane and accumulates inside the cell as ammonium ions, where it interferes with the proton gradient across the cell wall by removing free H^+. This causes a collapse of the proton motive force driving energy acquisition. Secondly, the reaction with water ($NH_3 + H_2O > NH_4^+ + OH^-$) is a redox reaction, potentially interfering with cellular redox reactions. Ammonia, a weak base, also causes an increase in pH. Deleterious effects on cell survival could potentially be caused by an increase in pH.

Free NH_3 at concentrations of 6–12 mM (0.011–0.022%) have been suggested as the highest concentrations to be tolerable by a range of micro-organisms, the highest of these levels reported in methanogens (Mc Carty & McKinney 1961; Braun *et al.* 1981; De Baere *et al.* 1984; Koster & Lettinga 1984; Angelidaki & Ahring 1993). Much of the existing work has been conducted in sludges and digestion reactors where high concentrations of free NH_3 accumulate. Siegel *et al.* reported evidence for the capability of a mutant strain of *Penicillium notatum* to take up a radiolabelled precursor of DNA, RNA and protein in a glycerol–NH_3 medium at −40 °C and apparent localization of labelled DNA precursor in the nucleoid region (Siegel *et al.* 1968), although these results have not been corroborated. In other studies survival of micro-organisms including a strain of *Penicillium brevicompactum*

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was reported on plants exposed to atmospheres with a high NH_3 concentration up to 95% (Siegel & Giumarro 1965).

Although actively growing cells are affected by low concentrations of ammonia, there is little data on its effects on inactive organisms or those possessing resting states. The effects of ammonium ions on spore germination have been studied previously and were suggested to enhance *Bacillus* spore germination at sub-molar concentrations (Wolf & Thorley 1957; Preston & Douthit 1984). Although ammonium is widely reported to be cytotoxic, experiments investigating the effects of ammonium on bacteria (*Corynebacterium glutamicum, Escherichia coli* and *Bacillus subtilis*) showed that sodium ions could elicit the same retardation of growth as ammonium when present at concentrations of 750 mM (1.5% v/v) or more, suggesting that ionic or osmotic effects caused growth inhibition, not the presence of ammonium *per se* (Müller *et al.* 2006).

The purpose of this study was to ascertain whether organisms on spacecraft would remain intact after exposure to high liquid-ammonia concentrations (up to 35% v/v) in concert with low temperatures, and whether transient exposure to ammonia would compromise their ability to grow when re-exposed to favourable growth conditions.

Experimental procedures

Culturability of soil microbes exposed to NH_3

Most micro-organisms within spacecraft assembly clean rooms are either human-associated or of environmental origin. Spore-forming micro-organisms such as *Bacillus* spp. are common contaminants, as well as other organisms that are resistant to desiccation or have environmentally robust resting states (La Duc *et al.* 2009).

To sample a large diversity of potential micro-organisms of interest, subsurface soil (circa 2–6 cm depth) was collected from an unvegetated site adjacent to a lawn at The Open University in Milton Keynes, UK. It was mixed before dividing into two portions. One portion was subjected to 'activation' by heating in a water bath at 70 °C for 22 minutes, before being left at room temperature (RT) for 26 hours. The 'unactivated' soil was likewise incubated at RT. To 10 g soil in 50 ml tubes, 40 ml of a solution containing 5, 17.5 or 35% (v/v) NH_3 was added. Ammonia solutions were prepared from a 35% (v/v) stock solution (0.88 S.G., Fisher Scientific, Leicestershire, UK) and diluted as required in distilled water. Controls contained soil and 40 ml distilled water only. Tubes were shaken thoroughly by hand after NH_3 addition and were incubated immediately at −80 °C or RT. Each treatment had four replicates. In addition to NH_3 treatments, additional treatments without NH_3 but with pHs amended to correspond as closely as possible to those of NH_3 solutions of 5% (pH 11.1), 17.5% (pH 12.1) and 35% (13.1) were prepared by amending distilled water with NaOH prior to addition to soil.

Tubes were sampled (following mixing by hand) after incubation for 1, 12 and 48 weeks, except for RT treatments, which were not sampled at 48 weeks. At each sampling −80 °C treatments were thawed at RT prior to sample removal and returned to −80 °C as quickly as possible. Samples were serially diluted in distilled water and plated in triplicate on nutrient agar (Oxoid, Basingstoke, UK). Plates were incubated aerobically or anaerobically at RT for 9 days before recording colony forming units (cfu). Regression analyses and analysis of variance (ANOVA) were used to determine whether activation treatment, exposure time and NH_3 significantly influenced culturability.

Determination of NH_3 by distillation

To determine whether the concentration of NH_3 had decreased during the soil-exposure experiment, the concentration in the liquid phase of treatments was determined at 48 weeks. Samples were diluted in dH_2O and 0.5 ml added to a solution containing 5 ml boric acid (1% w/v) and a mixed indicator, before distillation in a Vapodest 30. The distilled sample was titrated in duplicate against 0.2 N H_2SO_4 using a Titronic 96 (Schott). Blanks were also titrated, in addition to the NH_3 stock solution. Total N per ml of sample was calculated as

$$N(\%) = (T - B) \times N \times 14 \times (1/Vp) \times (1/W) \times (100/1000)\%$$

where T = ml of standard acid to titrate sample, B = ml of standard acid to titrate blank, N = normality of standard acid, 14 = equivalent weight, Vp = volume distilled, W = weight of sample.

Identification of bacteria isolated following NH_3 exposure

Selected bacterial isolates (labels preceded with LKS) were identified by partial sequencing of the amplified 16S rRNA genes (Mclab, USA). Amplicons were generated by colony PCR with primers pA and pH (Bruce *et al.* 1992) where each 50 µl reaction mixture contained 0.2 µM each primer, 200 µM each dNTP (New England Biolabs), 2.5 U *Taq* DNA polymerase, 2 mM $MgCl_2$ and 1 × PCR buffer (200 mM Tris/HCl (pH 8.4) and 500 mM KCl) (Invitrogen Corporation, Paisley, UK). Amplifications were performed in a G-Storm GS1 thermal cycler (GRI Ltd, Essex, UK), with an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 1 minute, 55 °C for 40 seconds and 72 °C for 40 seconds, with a final extension for 10 minutes at 72 °C. Amplicons were sequenced using primer pH and/or pA. Isolate sequences were searched against the RDP (Ribosomal Database Project) (Cole *et al.* 2009) to determine their phylogenetic affiliations. A neighbor-joining phylogenetic tree was constructed from partial 16S rRNA gene sequences of isolates and their closest database relatives, aligned in BioEdit (Hall 1999) over *E. coli* nucleotide positions 112–673. Trees were constructed in MEGA4 (Tamura *et al.* 2007) using the Jukes–Cantor (Jukes *et al.* 1969) algorithm. Isolate sequences have been deposited in GenBank under accession numbers JF502702–JF502828.

Exposure of spores to NH_3 at −80 °C

B. subtilis DSM10, which was used as a defined control, and soil isolate LKS-9 (JF502810) were grown on nutrient sporulation medium (NSM), consisting of nutrient agar with 1 mM $MgCl_2 \cdot 6H_2O$. Biomass from 5-month-old plate cultures,

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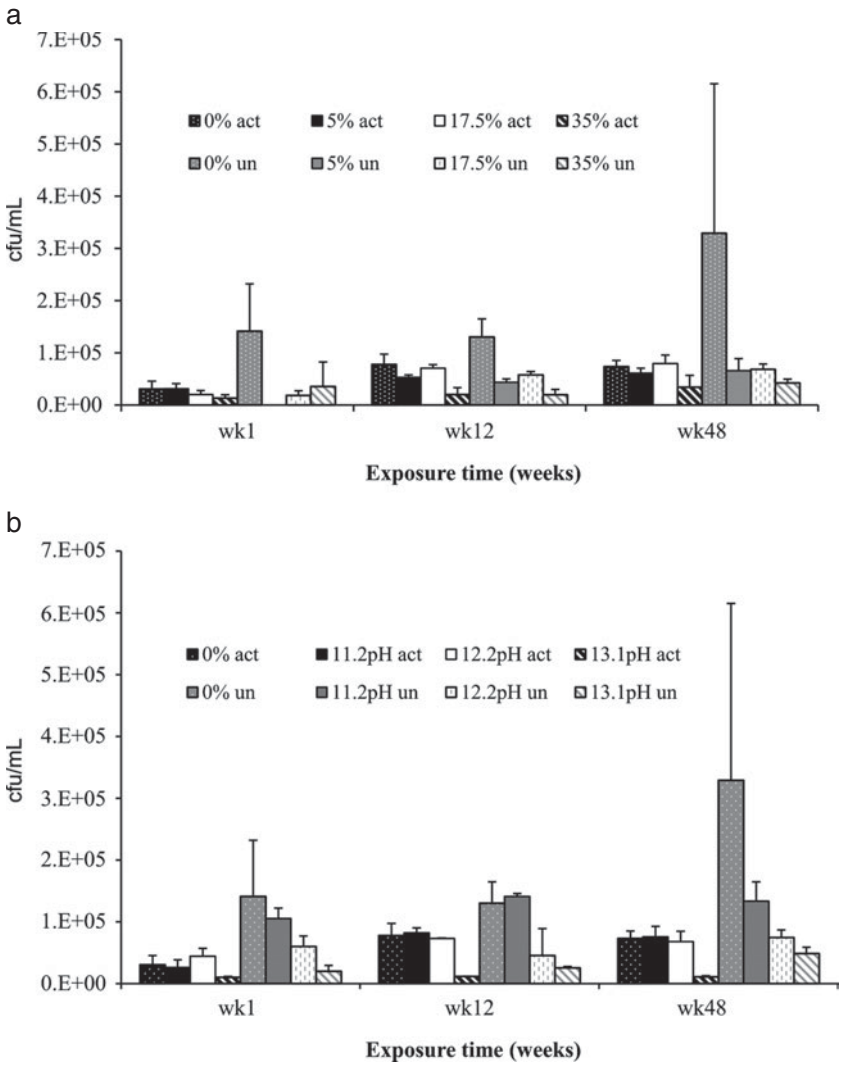


Fig. 1. Colony forming units (cfu) obtained on nutrient agar following exposure of activated (act) and unactivated (un) soil at −80 °C, to (a) NH_3 and (b) alkaline pH conditions over time.

grown and maintained at RT, was incubated at −80 °C in water containing NH_3 at concentrations of 0, 5 and 35% (v/v). All treatments were performed in triplicate. Prior to the addition of biomass, tubes (15 ml) containing NH_3 and/or water were cooled on ice. Inocula were prepared in chilled sterile distilled water and added rapidly to prevent spore germination prior to inoculation. Immediately after inoculation, tubes were mixed vigorously to ensure homogeneous suspensions and contents aliquoted into smaller (1.7 ml) tubes to prevent repeated freeze–thaw cycles during future samplings. These tubes were incubated immediately at −80 °C. The initial number of cfu was determined by plating immediately on nutrient agar, in triplicate, following serial dilution. Plates were incubated at RT for 6 days prior to counting. Subsequent samplings were performed 44 and 77 days.

Scanning electron microscopy (SEM)

In addition to determinations of cfu, spores of *B. subtilis* DSM10 and LKS-9 exposed to NH_3 in water (as above) or nutrient broth at −80 °C, were observed at intervals by SEM. Samples of 1 ml volume were filtered onto 0.2 µm pore size black polycarbonate filters (Fisher Scientific, Leicestershire, UK). Filters were gold coated prior to observation in secondary electron mode with a field emission gun scanning electron microscope (FEGSEM) (Zeiss Supra VP), using an accelerating voltage of 8 kV and a 14 mm working distance.

Results

Culturability of soil microbes exposed to NH_3

The effects of NH_3 on the culturability of soil microbes was assessed by incubating soil, both at RT and −80 °C, in the presence of up to 35% (v/v) NH_3. Microbial culturability, represented by cfu at 1, 12 and 48 weeks, is shown in Fig. 1. On some plates accurate determination of cfu was hampered by the presence of spreading growth forms with obscured the ability to count colonies, accounting for high standard deviations.

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Table 1. P-*values resulting from a one-way ANOVA analysis to determine the effects of exposure time at −80 °C on cfu abundance for activated and unactivated soil at each NH_3 concentration and pH. Results in bold indicate those relationships for which regression analysis returned a non-significant* P-*value (⩾ 0.05)*

[NH_3]/pH	Activated	Unactivated
0% NH_3	0.000	0.020
5% NH_3	0.000	0.010
17.5% NH_3	0.000	0.000
35% NH_3	0.003	**0.113**
pH 11.2	0.000	0.010
pH 12.2	0.001	0.024
pH 13.1	**0.574**	0.000

Table 2. P-*values resulting from the one-way ANOVA analysis to determine the effect of activation treatment on soil cfu abundance following exposure at −80 °C for 1, 12 and 48 weeks. Results in bold indicate those relationships for which regression analysis returned a non-significant* P-*value (⩾ 0.05)*

[NH_3]/pH	Week 1	Week 12	Week 48
0% NH_3	0.003	0.000	0.011
5% NH_3	No data	0.041	**0.492**
17.5% NH_3[a]	**0.913**	0.007	**0.061**
35% NH_3	**0.118**	**0.914**	**0.377**
pH 11.2	0.000	0.000	0.000
pH 12.2	**0.070**	**0.052**	**0.462**
pH 13.1	0.020	0.000	0.000

[a]A high number of fungi on plates in this treatment obscured bacterial counts and may account for this value.

Exposure time significantly affected cfu abundance for both activated and unactivated soils at −80 °C (Table 1), as revealed by a one-way ANOVA analysis and confirmed by regression analysis. The activation state of soil was shown to be non-significant in the presence of NH_3 at −80 °C, with the exception of 17.5% after 12 weeks of exposure (Table 2). Two-way ANOVA analyses revealed a significant interaction of NH_3 with time at −80 °C (not shown). Significant effects of activation treatment were observed for 11.2 and 13.1 pH treatments, however, significant effects at pH 12.2 were not observed. With some exceptions, differences in culturability between NH_3 and the corresponding pH treatment were not significant (not shown).

Determination of NH_3 by distillation

The % concentration of N in soil treatments did not decrease substantially during the course of the experiment. The average N% in 35% treatments was 0.7752, compared with 0.7952 in the stock solution. The N% of the control tubes was 0.0224.

Identification of bacteria isolated following NH_3 exposure

DNA sequencing of bacteria isolated on nutrient agar following soil exposure to NH_3 revealed the dominance of *Firmicutes*, particularly the order *Bacillales* (Fig. 2). Within this order, bacteria belonging to the genera *Bacillus, Sporosarcina, Viridibacillus, Paenibacillus, Staphylococcus* and *Brevibacillus* were identified following exposure to 35% NH_3. Also identified, however, were isolates belonging to the phyla *Actinobacteria* (*Arthrobacter* and *Streptomyces*) and *Proteobacteria* (*Pseudomonas*), albeit at a much lower frequency. The phylogenetic placement of these isolates relative to their closest matches within the RDP is shown in Fig. 2.

Exposure of spores to NH_3 at −80 °C

The survival of spores of *B. subtilis* DSM10 and soil *Bacillus* isolate LKS-9 (JF502810) at −80 °C, in the presence of up to 35% (v/v) NH_3 was observed by culturing on nutrient agar following 44 and 77 days incubation. Counts, relative to those at the time of inoculation are shown in Fig. 3.

SEM

Spores were observed in suspensions prepared in water and stored at −80 °C. Spores were also observed in 35% (v/v) NH_3 after storage at −80 °C (Fig. 4). Preparations containing NH_3 and media had mineral rosette crystal structures associated with them (Fig. 4(e)). The composition of a typical crystal, as determined by Energy Dispersive Spectroscopy (EDS) was 57.6% C, 22.7% O, 0.3% Na, 6.9% Mg, 11.5% P and 0.9% K.

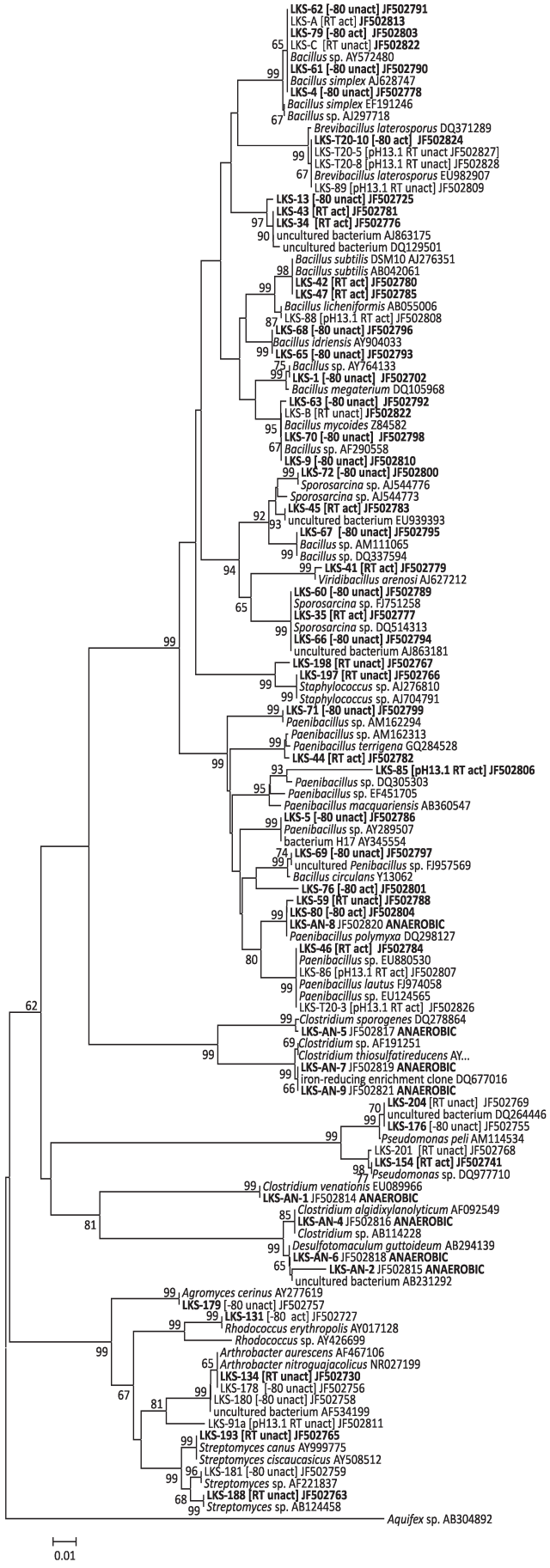
Discussion

To test the hypothesis that a diversity of micro-organisms of interest to planetary protection could survive solutions of high ammonia concentrations, samples of soil were exposed to varying concentrations of ammonia at RT and −80 °C.

After 3 months of exposure, a large number of organisms were isolated. Most culturable organisms that were identified were from spore-forming genera, suggesting that these resting states conferred resistance to ammonia. The species isolated, however, may also reflect the specific cultivation methods. Spore-formers are also the organisms most likely to survive on spacecraft surfaces. The majority of isolates were from the phylum *Firmicutes*, particularly the order *Bacillales*. Isolates of another spore-forming genus, *Streptomyces* (phylum Actinobacteria) were also obtained. Following exposure to high ammonia concentrations, soil incubated under anaerobic growth conditions yielded a prevalence of organisms phylogenetically affiliated to the genus *Clostridium*.

However, non-spore-forming bacteria were also obtained. The survival of the organisms closely affiliated with the non-spore-forming genus *Arthrobacter*, is consistent with the known environmental tolerances of this genus. They are known to be capable of inhabiting dry, oligotrophic environments (Cacciari & Lippi 1986; Reddy *et al.* 2002). They have been isolated from the surface of rocks (Gounot, 1967; Heyrman *et al.* 2005) and are known to sequester metals from rocks (Brantley *et al.* 2001). They are especially known for their capacity to inhabit cold polar environments (Nelson &

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Parkinson 1978a, b; Vishnivetskaya *et al.* 2006; Cockell *et al.* 2010), which is relevant to the context here (i.e. the planetary protection of cold outer solar system bodies). However, this is the first time that tolerance to high ammonia concentrations has been reported. Species affiliated with *Pseudomonas*, a non-spore-forming *Proteobacteria*, were recovered. Some representatives of pseudomonads are well characterized with respect to organic solvent tolerance (Huertas *et al.* 1998), although tolerance to high ammonia concentrations is not reported. Identification of organisms was not exhaustive and although every effort was made to sample multiple representatives of the dominant morphologies, other genera may have been overlooked.

The death of vegetative organisms and organisms without robust resting states might be attributed to the factors discussed earlier involving ammonia toxicity. At the extremely high ammonia concentrations examined in this study the solutions are very alkaline. Before the addition of soil, the NH_3 solutions at 5, 17.5 and 35% (v/v) had pHs of approximately 11.2, 12.2 and 13.1. The alkaline pH alone was capable of causing significant reductions in the viable population of organisms, suggesting that this factor alone is an important determinant in ammonia toxicity at the concentrations studied here. Although direct cytotoxic effects of ammonia cannot be ruled out, there was no statistically significant difference between effects of NH_3 and basic conditions on cfu. The time of exposure to NH_3 and basic conditions did have a statistically significant effect on the number of cfu, showing that these conditions were deleterious over time.

The purpose of heat treating the soil prior to use was to germinate at least some spores present within the soil, thus leaving a greater proportion of spores in the 'unactivated' soil (Ghosh *et al.* 2009). We hypothesized that spore-formers would be more resistant to NH_3 treatment and therefore the unactivated soil would yield higher counts of culturable micro-organisms. Our results, however, suggest that this prior heat treatment had no significant effect on cfu abundances in NH_3 treatments −80 °C. As the original soil community was of unknown composition, we cannot be sure that the heat treatment had the desired effect, due to different requirements among micro-organisms for germination and sporulation.

Fig. 2. Neighbor-joining phylogenetic tree of partial 16S rRNA gene sequences from soil bacteria isolated after exposure to NH_3, and their closest database representatives. Trees were constructed as described in methods, using 1000 bootstrap replications (values shown at nodes). Scale bar represents 1% sequence divergence. Isolates from soil exposed to 17.5 or 35% (v/v) NH_3 are indicted in bold. Temperature and soil activation treatment are indicated in parenthesis. Sequences from isolates cultured at RT under anaerobic conditions are also indicated. Additional sequenced isolates belonging to the genera *Bacillus, Paenibacillus* and *Sporosarcina*, and identical or almost identical to those shown have been excluded to avoid repetition.

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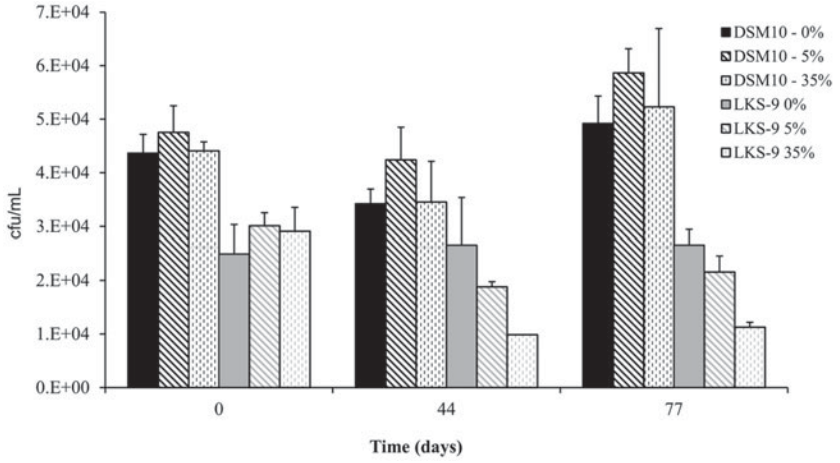


Fig. 3. Culturability (cfu) of spores of *B. subtilis* DSM10 and soil isolate LKS-9 (JF502810, a *Bacillus* sp.) both prior to and following exposure at −80 °C to NH_3 at concentrations of 0, 5 and 35%.

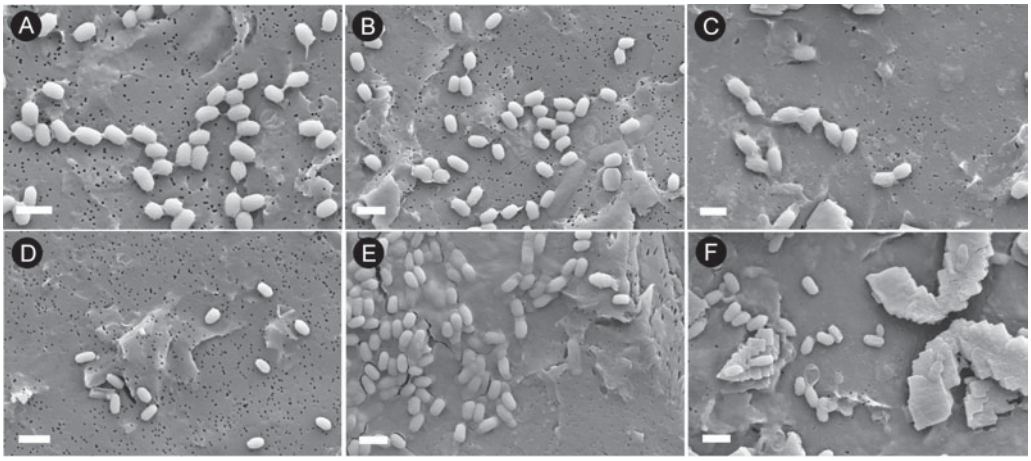


Fig. 4. SEM of spores of isolate LKS-9 (JF502810) (a–c) and DSM-10 (d–f) exposed to 0 or 35% (v/v) NH_3. (a, d) Spores in water (0% NH_3) at RT. (b, e) Spores at −80 °C in water with 0% NH_3. (c, f) Spores at −80 °C in media with 35% NH_3.

Although spores did survive high ammonia concentrations, the use of spores of an isolate from this experiment (LKS-9 (JF502810)) showed that over time the viability of spores was reduced as ammonia concentrations were increased. However, the percentage loss of viability of spores of this species at 77 days was similar to 44 days, suggesting that an early loss of viability results in a population in which subsequent loss of viability might be reduced.

An intriguing observation was a greater germination rate in DSM10 after 77 days compared with 44 days. This data may be consistent with the observation that two of the unactivated treatments (5 and 17.5%) and the 17.5% activated, had higher relative cfu after storage at −80 °C at 12 (84 days) weeks compared with week 1, suggesting that long-term exposure to low temperatures improved spore germination.

Our results show that many spore-forming and non-spore forming organisms with environmentally tolerant vegetative states (the very organisms found in spacecraft assembly facilities) can survive after exposure to many months of high ammonia concentrations in combination with low (−80 °C) temperatures and subsequently grow. Concomitantly, these results show the potential survival of organisms and their component organics under high ammonia concentrations. Thus, exposure to high extraterrestrial ammonia concentrations, although generally cytotoxic, could not be reliably expected to kill all organisms on a spacecraft or destroy its organic components.

Finally, our data yield new insights into organisms potentially capable of surviving high ammonia concentrations in bioreactors and sludge processing plants, where ammonia concentrations are generally very high (Angelidaki & Ahring 1993). We note that many of the organisms we cultured (*Bacillus* and *Clostridium*) belong to genera with pathogenic taxa and thus very high ammonia concentrations generated in many anaerobic facilities, although considered highly cytotoxic, will not kill these organisms.

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References

Angelidaki, I. & Ahring, B.K. (1993). Thermophilic anaerobic digestion of livestock waste the effect of ammonia. *Appl. Microbiol. Biotechnol*. **38**, 560–564.

Braun, R., Huber, P. & Meyrath, J. (1981). Ammonia toxicity in liquid piggery manure digestion. *Biotechnol. Lett*. **3**, 159–164.

Brantley, S.L., Liermann, L., Bau, M. & Wu, S. (2001). Uptake of trace metals and rare earth elements from hornblende by a soil bacterium. *Geomicrobiol. J*. **18**, 37–61.

Bruce, K.D., Hiorns, W.D., Hobman, J.L., Osborn, A.M., Strike, P. & Ritchie, D.A. (1992). Amplification of DNA from native populations of soil bacteria by using the polymerase chain reaction. *Appl. Environ. Microbiol*. **58**, 3413–3416.

Cacciari, I. & Lippi, D. (1986). Arthrobacters, successful arid soil bacteria. A review. *Arid Soil Res. Rehab*. **1**, 1–30.

Cockell, C.S., Osinski, G.R., Banerjee, N.R., Howard, K.T., Gilmour, I. & Watson, J. (2010). The microbe–mineral environment and gypsum neogenesis in a weathered polar evaporite. *Geobiology* **8**, 293–308.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M. *et al.* (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucl. Acids Res*. **37**, D141–D145.

Deal, P.H., Souza, K.A. & Mack, H.M. (1975). High pH, ammonia toxicity, and the search for life on the Jovian planets. *Origins of Life* **6**, 561–573.

De Baere, L.A., Devocht, M., van Assche, P. & Verstrate, W. (1984). Influence of high NaCl and NH_4Cl salt levels on methanogenic associations. *Water Res*. **18**, 543–548.

Fortes, A.D. (2000). Exobiological implications of a possible ammonia–water ocean inside Titan. *Icarus* **146**, 444–452.

Ghosh, S., Zhang, P., Li, Y.-Q. & Setlow, P. (2009). Superdormant spores of *Bacillus* species have elevated wet-heat resistance and temperature requirements for heat activation. *J. Bacteriol*. **191**, 5584–5591.

Gounot, A.M. (1967). Biologic role of Arthrobacter in subterranean soils. *Ann Inst Pasteur Paris*. **113**, 923–945.

Hall, T.A. (1999). BioEdit; a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser*. **41**, 95–98.

Heyrman, J., Verbeeeren, J., Schumann, P., Swings, J. & De Vos, P. (2005). Six novel *Arthrobacter* species isolated from deteriorated mural paintings. *Int. J. Syst. Evol. Microbiol*. **55**, 1457–1464.

Huertas, M.J., Duque, E., Marqués, S. & Ramos, J.L. (1998). Survival in soil of different toluene-degrading *Pseudomonas* strains after solvent shock. *Appl. Environ. Microbiol*. **64**, 38–42.

Jukes, T.H., Cantor, C.R. & Munro, H.N. (1969). *Mammalian Protein Metabolism*. Academic Press, New York, pp. 21–132.

Koster, I.W. & Lettinga, G. (1984). The influence of ammonia–nitrogen on the specific activity of pellitized methanogenic sludge. *Agric. Wastes* **9**, 205–216.

La Duc, M.T., Osman, S., Vaishampayan, P., Piceno, Y., Andersen, G., Spry, J.A. & Venkateswaran, K. (2009). Comprehensive census of bacteria in clean rooms by using DNA microarray and cloning methods. *Appl. Environ. Microbiol*. **75**, 6559–6567.

Mc Carty, P.L. & McKinney, R.E. (1961). Salt toxicity in anaerobic digestion. *J. Water Pollut. Control Fed*. **33**, 399–415.

Mitri, G., Showman, A.P., Lunine, J.I. & Lopes, R.M.C. (2008). Resurfacing of Titan by ammonia–water cryomagma. *Icarus* **196**, 216–224.

Müller, T., Walter, B., Wirtz, A. & Burkovski, A. (2006). Ammonium toxicity in bacteria. *Curr. Microbiol*. **52**, 400–406.

Nelson, L.M. & Parkinson, D. (1978a). Growth characteristics of three bacterial isolates from an arctic soil. *Can. J. Microbiol*. **24**, 909–914.

Nelson, L.M. & Parkinson, D. (1978b). Effect of starvation on survival of three bacterial isolates from an arctic soil. *Can. J. Microbiol*. **24**, 1460–1467.

Preston, R.A. & Douthit, H.A. (1984). Stimulation of germination of unactivated *Bacillus cereus* spores by ammonia. *J. Gen. Microbiol*. **130**, 1041–1050.

Reddy, G.S.N., Prakash, J.S.S., Matsumoto, G.I., Stackebrandt, E. & Shivaji, S. (2002). *Arthrobacter roseus* sp. nov., a psychrophilic bacterium isolated from an Antarctic cyanobacterial mat sample. *Int. J. Syst. Evol. Microbiol*. **52**, 1017–1021.

Siegel, S.M. & Giumarro, C. (1965). Survival and growth of terrestrial microorganisms in ammonia-rich atmospheres. *Icarus* **40**, 37–40.

Siegel, S.M., Nathan, H.C. & Roberts, K. (1968). Experimental biology of ammonia-rich environments: optical and isotopic evidence for vital activity in *Penicillium* in liquid ammonia–glycerol at −40 °C. *Proc. Natl. Acad. Sci. USA* **60**, 505–508.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol*. **24**, 1596–1599.

Vishnivetskaya, T.A., Petrova, M.A., Urbance, J., Ponder, M., Moyer, C.L., Gilichinsky, D.A. & Tiedje, J.M. (2006). Bacterial community in ancient Siberian permafrost as characterised by culture and culture-independent methods. *Astrobiology* **6**, 400–414.

Wolf, J. & Thorley, C.M. (1957). The effects of various germination agents on the spores of some strains of *B. subtilis*. *J. Appl. Bacteriol*. **20**, 384–389.

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