Published in final edited form as:
Soil Biol Biochem. 2009 August ; 41(8): 1612–1621.

Humic acids buffer the effects of urea on soil ammonia oxidizers and potential nitrification

Lianhua Dong[a,b], **Ana Lucía Córdova-Kreylos**[b,1], **Jinshui Yang**[a], **Hongli Yuan**[a,*], and **Kate M. Scow**[b]

[a] State Key Lab for Agrobiotechnology, College of Biological Sciences and Center of Biomass Engineering, China Agricultural University, No. 2 Yuanmingyuan West Road, Beijing 100193, PR China

[b] Department of Land, Air and Water Resources, University of California, Davis, CA 95616-5200, USA

Abstract

Humic acids (HAs) play an important role in the global nitrogen cycle by influencing the distribution, bioavailability, and ultimate fate of organic nitrogen. Ammonium oxidation by autotrophic ammonia-oxidizing bacteria (AOB) is a key process in ecosystems and is limited, in part, by the availability of NH_4^+. We evaluated the impact of HAs on soil AOB in microcosms by applying urea (1.0%, equal to 10 mg urea/g soil) with 0.1% bHA (biodegraded lignite humic acids, equal to 1 mg/g soil), 0.1% cHA (crude lignite humic acids) or no amendment. AOB population size, ammonium and nitrate concentrations were monitored for 12 weeks after urea and HA application. AOB densities (quantified by real-time PCR targeting the *amoA*) in the Urea treatments increased about ten-fold (the final abundance: 5.02×10^7 copies (g of dry soil)$^{-1}$) after one week of incubation and decreased to the initial density after 12 weeks incubation; the population size of total bacteria (quantified by real-time PCR with a universal bacterial probe) decreased from 1.12×10^{10} to 2.59×10^9 copies (g of dry soil)$^{-1}$ at week one and fluctuated back to the initial copy number at week 12. In the Urea + bHA and Urea + cHA treatments, the AOB densities were 4 and 6 times higher, respectively, than the initial density of approximately 5.07×10^6 copies (g of dry soil)$^{-1}$ at week 1 and did not change much up to week 4; the total bacteria density changed little over time. The AOB and total bacteria density of the controls changed little during the 12 weeks of incubation. The microbial community composition of the Urea treatment, based on T-RFLP using CCA (canonical correspondence analysis) and pCCA (partial CCA) analysis, was clearly different from those of other treatments, and suggested that lignite HAs buffered the change in diversity and quantity of total bacteria caused by the application of urea to the soil. We hypothesize that HAs can inhibit the change in microbial community composition and numbers, as well as AOB population size by reducing the hydrolysis rate from urea to ammonium in soils amended with urea.

Keywords

Humic acids; Ammonia oxidizer; *amoA*; SYBR green real-time PCR; *Taq*Man real-time PCR; T-RFLP; Potential nitrification

* Corresponding author. Tel.: +86 10 62733464; fax: +86 10 62734008. hlyuan@cau.edu.cn (H. Yuan)..
[1] Present address: Marrone Organic Innovations, 2121 Second St., Suite 107B, Davis, CA 95618, USA.

1. Introduction

Large amounts of N fertilizer are applied annually to agricultural soils with serious consequences for climate change and water quality (Clinton et al.,1995). Any ammonia not absorbed by plants is rapidly oxidized by autotrophic ammonia-oxidizing archaea (AOA) and bacteria (AOB). Ammonia oxidation, the chemolithoautotrophic oxidation of NH_3 to NO_2^- via hydroxylamine (Wood, 1987), is limited in many ecosystems by the availability of NH_3 (Belser, 1979; Laanbroek and Woldendorp, 1995). In soils where ammonia oxidizers compete with plants and heterotrophic bacteria for available NH_3 (Bodelier et al., 1998), humic acids (HAs) may play a role in regulating the soil availability of NH_3, due to their adsorption properties (Mackowiak et al., 2001).

HAs are polyelectrolytic macromolecular compounds originating from chemical and biological degradation of plant and animal residues, and microbial cells (Hayes and Wilson, 1997). HAs play an important role in the global nitrogen cycle through their influence on the distribution, bioavailability, and ultimate fate of sedimentary organic nitrogen (Davies and Ghabbour, 1998; Lichtfouse et al., 1998; Stankiewicz and Van-Bergen, 1998; Christl et al., 2000). Humic substances can incorporate nitrogen into their structure either directly through chemical reactions or indirectly through microbial activities and subsequent decomposition of microbial biomass (Clinton et al., 1995). Several reports indicate that ammonia–N may be abiotically fixed to soil organic matter, lignin, peat or coal (Nommik and Vathras, 1982; Lapierre et al., 1994; Bosatta and Agren, 1995) when the C/N ratio of plant residue during humification is higher than 10 (Knicker et al., 1997). Thorn and Mikita (1992), using ^{15}N and ^{13}C NMR techniques, detected that ^{15}N-labeled ammonia was incorporated into HAs in the laboratory incubation and that the average N content of HAs increased from 0.88 to 3.17%.

It is important to note that HAs can vary in the chemical characteristics and properties based on their origin. Weathered lignite contains 40–85% HAs, while soils on average contains only 1–5% HAs. Lignite HAs contain more carbonyl carbon (about 16%) and less aliphatic carbon (27%) than soil HAs with about 11% carbonyl carbon and 31% aliphatic carbon (Zheng, 1991). HAs made from low grade coal, such as lignite, has a long history of use as a fertilizer in combination with urea. In China alone, 350,000 tons HAs are used in agriculture every year (Zheng, 1991; Jiang and Zhang, 2002; Liang et al., 2007). It has been shown in previous studies that lignite HAs can increase crop yields relative to urea-only treatments (Zheng, 1991), indicating a synergistic effect between the two compounds, However, little is known regarding the mechanism by which lignite HAs increase the benefits of urea application. Based on earlier reports (Thorn and Mikita, 1992; Clinton et al., 1995) and our previous study (Dong et al., 2006), two possible mechanisms are suggested: 1) part of the ammonium generated from urea mineralization is incorporated into lignite HAs, this reducing the net loss due to volatilization, 2) lignite HAs inhibit the activity of urease, which decomposes urea to NH_3, resulting in a lower rate of urea hydrolysis. This reduced rate of hydrolysis reduces the loss of NH_3, increasing urea availability for plants. The increased availability of NH_3 could in turn affect the structure or population size of AOB communities in the soil (Bollmann and Laanbroek, 2001).

Other potential effects of HAs on microbial communities are structure stabilization: buffering the changes in size or abundance of some microbial groups by chelating unavailable nutrients (thus making them available) and buffering pH (Mackowiak et al., 2001; Pertusatti and Prado, 2007). Additionally, HAs may reduce negative effects of direct application of urea and other chemical fertilizers on soil bacteria or fungi. The buffering of pH is an important determinant of AOB and total bacteria community structure (Frostegård et al., 1993; Pennanen et al., 1998; Kelly et al., 1999; Enwall et al., 2007). HAs have been

shown to buffer pH between 5.5 and 8.0 (Pertusatti and Prado, 2007). So we hypotheses that HAs can buffer the community change caused by increasing or decreasing pH.

With the present study, we aimed to clarify the mechanisms by which lignite HAs amplify the effects of urea on crop yields. To test this effect, we measured the effects of lignite HAs on microbial community structure and population size, and more specifically on AOB and total bacteria in urea-amended soil. We assumed that lignite HAs could either decrease the AOB population size or change the AOB community composition, and stabilize the diversity of soil total bacteria after the application of urea. Compared to the original formulation extracted from crude lignite (cHA), biodegraded lignite HA (bHA) has a relatively higher nitrogen content and lower molecular mass, with greater potential to stimulate biological activity in soil (Dong et al., 2006; Yuan et al., 2006). Soils were treated with two different kinds of HAs (cHA: crude lignite humic acids, and bHA: biodegraded lignite humic acids) after urea application in microcosms. Changes in the microbial community structure were monitored by Terminal Restriction Fragment Length Polymorphism (T-RFLP) and the population sizes of total bacteria and AOB were measured by real-time PCR. Other parameters measured during the incubation included pH, ammonium and nitrate concentration, potential nitrification and urease activity.

2. Material and methods

2.1. Lignite sample and HA extraction

Lignite was collected from the Huolingele Minerals Administration coal mine, Inner Mongolian Autonomous Region, Northwest China. Air-dried crude lignite or biodegraded lignite was pulverized and passed through a 70-mesh sieve. cHA and bHA were extracted according to a previously described protocol (Dong et al., 2006). Briefly, lignite or biodegraded lignite was suspended in a NaOH solution and stirred at 20 °C for 24 h, then centrifuged at 6000 × g for 15 min. The supernatant was filtered and the pH was adjusted to 2.0 with HCl. The solution was centrifuged to precipitate HA which was washed with distilled water three times and dried at 60 °C.

2.2. Experimental design

2.2.1. Soil sampling—In April 2006, soil cores from depths of 0 to 10 cm were collected at the China Agriculture University farmlands in, Beijing, China. A corn crop had been harvested in October 2005, and no vegetation was present at the time of sampling. Physical and chemical characterization of the soil yielded the following results: pH, 7.47; organic matter, 10.83%; total N, 0.53%; inorganic N, 69 mg/kg; soluble P, 9.83 mg/kg; bulk density, 1.32 g cm $^{-3}$. Based on its chemical and physical properties the soil at this site is classified as a Cinnamon soil (Institute of Soil Science, CAS). The soil was air dried, passed through a 1-mm sieve and stored at 4 °C.

2.2.2. Microcosm preparation—Four soil treatments were added as solutions of (1) Urea (1.0% urea, equal to 4.7 mgN/g dry soil), (2) Urea + bHA (1.0% urea + 0.1% bHA), (3) Urea + cHA (1.0% urea + 0.1% cHA), and (4) untreated control Urea and HAs were added to the soil as described by Venterea and Rolston (2000) to minimize spatial stratification. The influent solutions were prepared as follows: 1 g HA dissolved in 1 L 0.01 M NaOH, and pH was adjusted to 7.0 with 0.1 M HCl. After that, 10 g urea was added to the HA solution. Soils were flushed slowly with their respective HA + urea solutions until effluent urea–N concentrations (measured by spectroscopic photometry (Peiqi and Mile, 1991) were equal to the influent level. The flushed solution volumes used were 2000, 2015 and 2011 ml for the Urea, Urea + bHA, Urea + cHA treatment respectively. Soils were then

drained with a pressure plate to equilibrate to 0.05 MPa. The control was flushed with 2000 ml dH_2O instead of the Urea + HAs solution.

Soil microcosms were constructed in 5 L plastic pots. A total of 12 pots (four treatments in triplicate) with 2 kg soil each were incubated at 28 °C. Samples were collected from each pot after 0, 2, 3, 5, 7, 9, 12, 16, 28 and 84 days of incubation and analyzed for pH, NH_4^+–N, NO_3^-–N, urease activity and potential nitrification. Additionally, five-gram soil from each pot was collected at 1, 4 and 12 weeks of incubation. These samples were stored at –80 °C for subsequent DNA extraction.

2.3. Chemical measurements

Soil pH was determined using a combined glass electrode in 1:1 (w:v) ratios of soil with distilled 1 M KCl. To measure ammonium and nitrate concentrations, 100 ml of 0.01 M $CaCl_2$ was added to 10 g of soil, shaken for 1 h on a reciprocating shaker and centrifuged at 4100 × *g* for 5 min at 5 °C. The supernatant was collected and stored at –20 °C until analysis. NH_4^+ and NO_3^- concentrations in the extracts were determined by a continuous flow analytical system (TRAACS 2000, Bran & Lubbe, Norderstedt, Germany).

2.4. Potential nitrification

Potential nitrifications were estimated in triplicate samples after 0, 1, 4 and 12 weeks of incubation. Ten-gram soil samples were placed in 100-ml beakers amended with 2 ml 2.5 mgN/ml $(NH_4)_2SO_4$; simultaneously, 2 ml sterile water was added to another beaker with 10 g soil as the control. All beakers with soil were incubated in the dark at 28 °C while continually shaken. At 1, 2, 3, 5 and 7 d, six subsamples from each beaker were used to measure the concentration of NH_4^+–N and NO_3^-–N as described above. As nitrite from ammonium oxidation is quickly oxidized to nitrate, its concentration was negligible in our incubations. Potential nitrification was calculated by linear regression of nitrate concentration against incubation time. The increase in nitrate concentration was linear from day 0 until the time when nitrate concentration peaked.

2.5. Urease activity assay

Urease activity was measured in triplicate using a modified indophenol colorimetric method which comprised incubation of soil with buffered urea solution, extraction of ammonium with 0.01 M $CaCl_2$ and colorimetric NH_4^+ determination by an indophenol reaction (Kandeler and Gerber, 1988). A 5 g soil sample was incubated with 0.1 M urea in citric acid buffer (pH 6.7) for 4 h at 37 °C. Meanwhile, the same amount of soil was incubated in citric acid without urea as a control. A soil-free incubation of the reagents was used as a blank. The urease activity was expressed as the transformed amount (mg) of ammonium-N per 100 g dry soil per day.

2.6. DNA extraction from soil

A humic acid removal step was performed prior to DNA extraction. Two-gram soil was washed with humic extraction buffer (Cavagnaro et al., 2007) and DNA was extracted from 0.5 g of the washed soil using a Bio 101 Fast DNA SPIN kit (for soil) as described by the manufacturer (Bio 101, Inc., CA, USA). The quantity (relative to DNA size marker) and quality of the extracted DNA were analyzed by electrophoresis on a 1.0% agarose gel and by the Qubit fluorometer (Invitrogen, USA).

2.7. Oligonucleotide primers and PCR amplification

Differences in bacterial community composition were determined through terminal restriction fragment length polymorphism (T-RFLP) analysis of amplified bacterial 16S rRNA gene for total bacteria and *amoA* (encoding a subunit of ammonia mono-oxygenase) for AOB. The 16S rRNA gene was amplified using eubacterial 16S rRNA gene primers: P0/P6 (Cello et al., 1997). Because of the low abundance of AOB, amplification of *amoA* was performed using a nested PCR approach, with aliquots of the first-round PCR products used as templates in the second round of PCR. The first-round PCR primers used were amoA-2F/5R (Webster et al., 2002), and amoA-1F/2R (Rotthauwe et al., 1997) in the second round. The P0 and amoA-1F primers were labeled with carboxyfluorescein (6-FAM) for detection of the terminal restriction fragments (TRF). Amplification was carried out in 25 μl reactions containing 2.5 μl 10× PCR buffer, 0.5 μl 10 mM dNTP, 0.5 μl of each primer (10 pmol μl^{-1}), 19 μl sterile water, 0.3 μl of Taq DNA polymerase (5 U μl^{-1}) (Promega, USA), and 1 μl template. Amplification was performed with a PTC-200 Thermocycler (Biorad, Hercules, CA, USA.). The cycling program for 16S rRNA gene consisted of a 5 min initial denaturation step at 95 °C followed by 5 cycles of 95 °C/30 s, 60 °C/30 s, and 72 °C/2 min; and 5 cycles of 95 °C/30 s, 55 °C/30 s, and 72 °C/2 min; and then 25 cycles of 95 °C/30 s, 50 °C/30 s, and 72 °C/2 min, and a 10 min final extension step at 72 °C.

For *amoA* amplification we used touchdown PCR (Webster et al., 2002) for the first round, and the conditions for the second-round PCR were as follows: 95 °C/5 min followed by 40 cycles of 95 °C/30 s, 60 °C/30 s, 72 °C/40 s and a 10 min final extension step at 72 °C. Each PCR product (4 μl) was visualized after electrophoresis on 1.0% agarose TAE gels and subsequent staining with ethidium bromide (1 mg l^{-1}) for 15 min. All reactions were performed in triplicate under the conditions described above. Triplicates were pooled (150 μl) and purified using QIA quick columns (Qiagen, USA) to remove unincorporated nucleotides and labeled primers. DNA was eluted in a final volume of 50 μl.

2.8. T-RFLP analysis

Purified PCR products were digested for 3 h in a water bath using Hae III (GG'CC), Hha I (GCG'C) and Msp I (C'CGG) for 16S rRNA gene at 37 °C, and TaqI (T'CGA) for *amoA* at 65 °C (Horz et al., 2000). The restriction–digestion mixture contained 6 μl of purified PCR product, 2 μl of enzyme buffer, 11 μl sterile water and 1 μl of 10 U of restriction endonuclease. Aliquots (20 μl) of the digest were purified by ethanol precipitation. The precipitate was mixed with 0.3 μl of GeneScan ROX 500 size standard (Applied Biosystems, NJ, USA) and 10 μl of deionized formamide. The fluorescently labeled terminal fragments were separated by capillary electrophoresis using an ABI PRISM 3100-Avant Genetic Analyser automated sequence analyzer (Applied Biosystems, NJ, USA). The T-RFLP profiles, plotted as peak areas (relative abundances) against fragment sizes, were analyzed using GENEMAKER software (version 1.6, Applied Biosystems, NJ, USA). Only TRFs between 60 and 600 bp with peaks higher than 80 fluorescence units were included in the analysis (Blackwood et al., 2003). The same procedure was used for *amoA*-TRFs, but only those with length sizes 45–500 bp were included since there could be a 48 bp TRF belonging to AOB when digesting the 491 bp PCR product amplified with amoA-1F/2R with TaqI restriction enzyme (Park and Noguera, 2004).

2.9. Total bacteria population size determination: TaqMan real-time quantitative PCR

Population sizes of total bacteria were determined using the real-time quantitative *Taq*Man PCR method. Real-time PCR was performed in a 20 μl reaction mixture that consisted of 5 μl of template DNA (1 ng μl^{-1}), 10 μl of *Taq*Man Universal PCR Master Mix (Applied Biosystems, NJ, USA), 3 μl of H_2O, 0.8 μl of each primer and 0.4 μl of probe. The primers Bact1369F, Prok1492R and probe TM1389 (Suzuki et al., 2001) were used at concentrations

of 800 nM, 800 nM and 200 nM, respectively. The PCR protocol for 16S rRNA gene quantification has been described by Okano et al. (2004). The fluorescence signal was used to calculate C_T (threshold cycle) values by the thermocycler's internal software (7300 System SDS software, V1.2.2, Applied Biosystems, CA, USA).

2.10. AOB population size determination: SYBR green real-time quantitative PCR

Population sizes of AOB were determined using an SYBR green real-time quantitative PCR (qPCR) chemistry. Primers A189 (Holmes et al., 1995) and amoA-2R′ (Okano et al., 2004) were used to amplify the *amoA* gene. The PCR conditions and protocol for *amoA* quantification using the 7300 real-time PCR system (Applied Biosystems, CA, USA) have been described by Cavagnaro et al. (2007). The *amoA* copy numbers in environmental samples were determined using an external standard curve generated by cloning the gene in a plasmid (Okano et al., 2004). *Nitrosomonas europaea* ATCC 19718 purchased from the American Type Culture Collection (ATCC) was used as an *amoA* standard cloned into a plasmid for the qPCR and cultured in ATCC liquid medium 221 at 28 °C in the dark with periodic pH adjustment.

2.11. Standard curve and quantification of amoA and 16S rRNA gene in soil samples

The procedures for preparing the external standard curve and quantification of *amoA* and bacterial 16S rRNA gene in soil samples were as reported by Okano et al. (2004). The AOB and total bacterial population sizes reported here were corrected for efficiency of extraction from soil by dividing the copies per μl from qPCR results by the DNA concentration (ng/μl) used in the qPCR to obtain copies/ng DNA. Then, this value was multiplied by the total amount of DNA (ng DNA/extraction) extracted from soil to get the copies/extraction. Lastly, dividing copies/extraction by the dry weight of soil per extraction yields the normalized copies/g dry soil.

2.12. Statistical analysis

All reported values are given as mean ± SD. To determine the statistical significance of differences in potential nitrification, pH, richness and diversity, *amoA* and 16S rRNA gene copy numbers between treatments, the averages were compared using one-way analysis of variance (ANOVA) and the Student–Newman–Keuls test. Microbial community fingerprints and correlations with environmental variables were analyzed by canonical correspondence analysis (CCA) and partial CCA (pCCA) (Lepš and Šmilauer, 2003; Córdova-Kreylos et al., 2006) carried out with the CANOCO software (Microcomputer Power, Ithaca, N.Y.). TRFs were expressed as relative abundance and only those TRFs present in 10% or more of the samples were used in CCA and pCCA to reduce environmental noise and account for the analytical method detection limit. The Monte Carlo permutation test was used to test the significance of the correlations between microbial community composition and measured environmental variables.

3. Results

3.1. Urease activity, ammonium and nitrate concentrations

Urease activity of the four treatments is shown in Fig. 1A. The urease activity in soils treated with urea and Urea + HAs was similar during the first 3 days and increased significantly compared to day 0 ($P < 0.05$). However, in the Urea treatment, urease activity increased sharply and peaked at day 5, while those of Urea + HAs changed little ($P > 0.05$), which indicated the urease activity of Urea + HAs was suppressed by the addition of bHA and cHA. At day 5, urease activity was significantly higher in urea-only treatments compared to all other treatments. After 5 days, the urease activity of urea-only treatment

began decreasing rapidly ($P < 0.05$) and was much lower than those of the Urea + HAs treatments after day 7, and remained lower until day 28. Urease activity in the control changed little during the period of incubation ($P > 0.05$).

NH_4^+ and NO_3^- concentration changes were measured after adding urea and HAs (Fig. 1B and C). Ammonium concentrations increased significantly during the first 7 days in urea-only treatments and during the first 16 days in Urea HAs treatment ($P < 0.05$). In the control soil, the NH_4^+ concentrations remained low and changed little over time ($P > 0.05$, Fig. 1B). The Urea treatment showed an immediate NH_4^+ release and the highest NH_4^+ concentration was detected at day 7. The same NH_4^+ pulse was observed in the Urea + HAs treatments, lasting until day 7, but then they showed a short period of stability that lasted until day 9, after which NH_4^+ concentrations peaked at day 16. At day 84, NH_4^+ concentrations of all treatments were similar to that of the control (about 0.03 mgN/dry soil, data not shown).

In the control soil, NO_3^- concentration did not fluctuate much over time and remained at around 0.2 mgN (g dry soil)$^{-1}$ after day 3. However, in all the other treatments NO_3^- concentrations increased substantially after day 3 ($P < 0.05$). For the Urea treatment, the highest concentration was detected at day 16 and then remained at approximately 3 mgN (g dry soil)$^{-1}$. For the Urea + HAs treatments, the NO_3^- concentration increased with a delay from day 9 to day 16 during the one month incubation time ($P < 0.05$), but there was no difference between Urea + bHA and Urea + cHA. Differences in nitrate concentration between treatments were minimal on day 84 (data not shown) compared to day 28. The final NO_3^- concentration (3 mgN (g dry soil)$^{-1}$) was lower than the urea–N (4.7 mgN (g dry soil)$^{-1}$), which indicated partial loss of ammonium through volatilization. However, comparing Fig. 1B and C, it is noticeable that the final nitrate concentration exceeded the total ammonium concentration, which may be due to part of ammonium was not detected because of quick transformation by nitrification.

3.2. Potential nitrification and pH

The changes of soil pH are shown in Fig. 2B. To determine the effects of lignite HAs on AOB activity and biomass we measured the potential nitrification at weeks 1, 4 and 12 (Fig. 2). At the beginning of the incubation (day 0), the potential nitrification and pH values did not differ among the treatments (Fig. 2B). After one week of incubation, the potential nitrification in the Urea treatment had increased by 8 times and the pH decreased from 7.51 to 6.93. For Urea + bHA and Urea + cHA, the pH did not change but the potential nitrification was 3.5 and 2.1 times higher than the initial rate, respectively. At week 4, the pH of the Urea treatment changed little and the potential nitrification greatly decreased compared to week 1; for the Urea + HAs treatments, the pH decreased while the potential nitrification increased. At week 12, the potential nitrification in all treatments returned to their initial levels. The pH values of the Urea and Urea + HAs treatments were lower than that of the control ($P < 0.05$), While the pH of the Urea + HAs treatments was higher than those of the Urea treatments ($P < 0.05$).

3.3. Impact of HA on AOB and total bacteria population size

A standard curve was generated by plotting serial dilutions ranging from 10^2 to 10^7 copies of plasmid containing the amoA against their corresponding C_T values. The parameter C_T (threshold cycle) is the cycle number at which the fluorescence emission crosses a threshold within the logarithmic increase phase. The threshold was defined as 10 times the standard deviation around the average intensity of background fluorescence from non-template controls. The standard curve ($C_T = -3.495 \times \log 10$ (*amoA*) + 39.628) had an $R^2 = 0.993$, indicating a strong linear relationship between C_T value and the log 10 number of *amoA*

copies over 6 orders of magnitude, and the reaction efficiency was calculated to be 93.1% (based on the formula $E = 10^{(-1/\text{slope})}-1$). The PCR products had melting profiles similar to *amoA* standard, indicating that the peaks measured using the SYBR green approach were the desired product.

A standard curve for quantification of bacterial 16S rRNA gene was similarly constructed. Standard solutions ranged from 10^3 to 10^8 genome copies of *Escherichia coli*. Quantification was based on the following equation: $C_T = -3.470 \times \log 10$ (16S rRNA gene) + 41.749; $R^2 = 0.999$, efficiency = 94.1%.

AOB population sizes were not significantly different among treatments on day 0 ($P = 0.15$). The AOB population density in the control treatment did not change much over time and remained between 5.10×10^6 and 7.62×10^6 copies (g of dry soil)$^{-1}$ (Fig. 3A). In the Urea treatment, the AOB numbers increased rapidly and significantly ($P < 0.05$) up to ten-fold higher after one week of incubation. In the Urea + bHA and Urea + cHA treatments, the AOB population density increased 3 and 5 times after 1 week, respectively. After 12 weeks, AOB population size of the Urea and Urea + HAs treatments decreased by about 6.25×10^6 copies (g of dry soil)$^{-1}$ and there was no significant difference among the treatments ($P = 0.18$).

Total bacterial population sizes were not significantly different among treatments on day 0 ($P = 0.15$). In the control, total bacteria density changed little over time ($P = 0.08$). However, in the Urea treatment, the total bacterial density decreased significantly at week 1 and week 4 ($P = 0.01$) (Fig. 3B). In the Urea + HAs treatments, the numbers of total bacteria differed little at week 1 ($P = 0.15$) and increased at week 4 ($P = 0.05$). After 12 weeks, there was no difference the total bacterial numbers among the 4 treatments ($P = 0.15$).

The average ratio of AOB to total bacterial populations in all treatments was 1.31×10^{-3} at the beginning of incubation (Fig. 3C). After one week, the ratio significantly changed in Urea and Urea + bHA treatments ($P = 0.01$ and $P = 0.04$ respectively), and did not change in Urea + cHA treatment ($P = 0.21$). However, the ratio in the control changed little (0.1–0.3%) and there was no significant difference after 12 weeks among all treatments ($P = 0.15$).

3.4. Effects of HAs on total bacteria and AOB community composition and abundance

During the processing of PCR, one of the replicate samples was lost, and so results about T-RFLP presented here are only duplicates per treatment. The T-RFLP patterns obtained with 16S rRNA gene digested with all three restriction enzymes (HhaI, MspI and Hae III) were essentially the same (data not shown). Only the results obtained with HhaI are shown. Across all samples, there were 104 unique TRFs (Fig. 4A), ranging from 16 to 53. T-RFLP profiles of the communities were explored using CCA and pCCA (Table 1). The first CCA ordination axis explained 39.7% of the total variation ($r = 0.994$) and separated the samples to two groups (P0.002): one group included samples at time 4; another one included samples at time 1, time 2 and time 3 (Fig. 4B). The second axis explained 18.8% of total variation ($r = 0.954$). Most TRFs congregated around the Urea + HAs treatments, and fewer TRFs were associated with the Urea treatment (Fig. 4A and 4B). The projection of environmental variables revealed a strong correlation with time 4 ($P = 0.002$, $r = 0.965$, Fig. 4C).

Because the CCA established the importance of time as an overarching controlling variable, time was treated as covariable in pCCA to extract the effects of other variables on the microbial community T-RFLP profiles. Using pCCA, T-RFLP analysis discriminated communities into three main groups (Fig. 5A). One group consisted of the Urea treatment, the second group of the Urea + HAs and the third group of control samples. The pCCA (Fig.

5) explained 36.0% and 24.8% of the variation on the first two axes. In the pCCA diagram (Fig. 5B), the most important variables are represented by longer arrows. The copy number of *amoA* was positively correlated with the Urea treatment and negatively correlated with Urea + HAs and NH_4^+ concentration. The quantity of total bacteria (16S) was negatively correlated with the Urea treatments and positively with Urea + HAs and NH_4^+ concentration. The NO_3^- concentration was negatively correlated with pH.

The TRFs found in our treated soils belong to previously reported AOB lineages in the β-Proteobacteria. TRF 219/491 bp belonged to *N. europaea/Nitosomonas eutropha* lineage, TRF 48/354/441//491 bp attributed to other *Nitrosomona* lineage including Nitrosomonas oligotropha/Nitrosomonas cryotolerans/Nitrosomonas marina/Nitrosomonas communis lineage, and TRF 283 bp belonged to *Nitrosospira* lineage, respectively (Horz et al., 2000; Park and Noguera, 2004; Siripong and Rittmann, 2007). At week 1 (Fig. 6A), the detected TRFs in the control were: 48 bp, 219 bp, 283 bp and 491 bp. These same TRFs were detected in Urea and Urea + HAs treatments, but their relative abundances varied by treatment: TRF 48 bp decreased while 283 and 491 bp increased compared to the control ($P < 0.05$). In addition, a new low-abundance TRF (441 bp) was detected in the Urea treatment. At week 4 (Fig. 6B), the TRFs in the controls changed little ($P = 0.16$), whereas the *Nitrosospira* lineage (283 bp) continued to increase in the Urea treatment and abundances of other TRFs decreased. In the Urea + HAs treatments, the 283 bp increased slightly whereas other TRFs changed little ($P > 0.05$). At week 12 (Fig. 6C), the *Nitrosospira* lineage TRFs represented the dominant group in all the treatments, especially in the Urea treatment whereas all other TRFs could barely be detected.

4. Discussion

Advances in molecular techniques enable assessment of presence and abundance of specific microbial groups such as AOB. T-RFLP, a culture-independent technique, is a reproducible tool widely used for rapid characterization of microbial community structure and dynamics (Osborn et al., 2000; Kitts, 2001; Lueders and Friedrich, 2003). Limitations of PCR-based methods, such as TRFLP, include bias associated with selective amplification of target genes, differences in terms of base-length of the real and observed TRF and appearance of pseudo TRFs (Wang and Wang, 1996; Polz and Cavanaugh, 1998; Egert and Friedrich, 2003). To reduce the potential bias of one kind of restriction enzyme, we generated multiple T-RFLP profiles using three different endonucleases for each sample and demonstrated a high degree of reproducibility with different enzymes. In addition, results of statistical assessments of community differences were the same whether we employed T-RFLP data based on presence/absence or relative abundance of TRFs and this suggested that bias due to PCR differential amplification was minimal in this study (data not presented).

4.1. Effect of HAs on total bacteria

The CCA analysis based on T-RFLP showed relationships between community composition and different HA treatments and incubation time. To discern HAs or urea effects from other environmental effects on communities, we employed pCCA (Córdova-Kreylos et al., 2006) to statistically extract the variability associated with time from other treatments effects. This enabled identification of several key variables with which microbial community composition shifts appeared significantly related. The microbial communities in the Urea treatment were clearly different from those in the other treatments, which may be attributable to the low pH or fast ammonium release and nitrate accumulation in the Urea treatment. Several studies have shown that pH change can affect soil microbial community structure (Frostegård et al., 1993;

Pennanen et al., 1998; Kelly et al., 1999; Fierer and Jackson, 2006). The pH and numbers of total bacteria indicated by 16S rRNA gene copies based on real-time *Taq*Man probe PCR quantification were negatively correlated in the Urea treatment. Low pH could be caused by released acidity after application of urea when ammonium is converted to nitrate by soil bacteria; this was suggested by the negative correlation between pH and nitrate concentration. However, the change in pH may be not big enough to lead to a major difference in total bacterial community. On the other hand, sudden increase of NH_4^+ and quick accumulation of NO_3^- could enhance nitrifier and other active group growth which might change the total bacterial community. All of the analytical results showed that there was no significant difference between cHA and bHA during the entire period of the incubation, supporting the lack of differentiation observed between these groups in pCCA plots.

While total bacterial quantities in the Urea + HAs treatments did not change much compared to the control during the incubation, they were much higher than that in the Urea treatment. Moreover, several TRFs detected in the control and Urea + HAs treatments were not detected in Urea treated soils, perhaps because their density decreased at week 1 and 4. In addition, a new TRF attributed to AOB was detected in the Urea treatment. This new TRF may be a result of an increased population of AOBs associated with fast increase of ammonium concentrations.

4.2. AOB population densities

The AOB TRFs detected in this study all belonged to the *Nitrosomonas* and *Nitrosospira* lineages. Strains from the *Nitrosospira* lineage were dominant in all treatments, which are consistent with previous findings that soils are dominated by strains belonging to the genus *Nitrosospira*, rather than *Nitrosomonas* (Hastings et al., 1998; Mendum et al., 1999). The number of *Nitrosospira* appeared to increase significantly, especially after the application of urea. Although PCR-based measurements are not strictly quantitative, differences among *amoA*-based T-RFLP patterns have been demonstrated to correlate with differences in the input ratios of *amoA* sequence types in the PCR (Horz et al., 2000). In addition, we found other TRF (441 bp) related to *N. cryotolerans* lineage in the Urea treatment, potentially due to the increased abundance of AOB after the application of urea. The AOB numbers in Urea treatment increased promptly while those in the Urea + HAs treatment changed gradually because of the slow release of ammonium, which caused the difference in the AOB community. On the other hand, it has been reported that pH appears to be a major factor regulating the soil AOB community, both by direct impact on the AOB and indirect effects on a range of soil processes (Enwall et al., 2007). However, in the present study, the change from 7.56 to 6.83 in pH might not be great enough to lead the change in AOB community.

Quantification for *amoA* was performed using SYBR green. Generally, compared to *Taq*Man, SYBR green is considered to have slightly lower resolution, but it is advantageous in applications where it is impossible to perform the sequencing and phylogenetic analysis of AOB diversity needed to ensure that the TaqMan probe can detect all AOB present in the sample (Cavagnaro et al., 2007). Thus, most AOB sequences detected during T-RFLP were included in the real-time PCR quantification using this method. The total AOB numbers ranged between 5.0×10^6 and 5.0×10^7 copies (g of dry soil)$^{-1}$ in all treatments and the largest population size occurred in the Urea treatment. These numbers are comparable to observations from an earlier study using *Taq*Man PCR in a different agricultural soil: 1.0×10^7 copies (g of dry soil)$^{-1}$ with the largest population size in the ammonium treated soil (Okano et al., 2004). In addition, the proportion of AOB making up the total bacterial populations increased (from 0.1 to 5.4%) during the first 4 weeks of incubation in the Urea

treatment, whereas in the control it remained about the same (0.1–0.3%), which corresponded to the decrease of diversity after urea application.

However, it has been reported that ammonia-oxidizing archaea (AOA), found capable of oxidizing ammonia in agricultural and grassland soils, are more abundant in soils than their well-known bacterial counterparts, so future investigations must include also the evaluation of AOA and their contribution to potential nitrification s (Leininger et al., 2006; Nicol and Schleper, 2006).

4.3. Effect on potential nitrification

Strong relationships between NH_4^+ disappearance and NO_3^- accumulation in urea and Urea + HAs treatments over time (Fig. 1B) suggested that rates of net nitrogen mineralization and immobilization were similar, and denitrification was minimal in this well-aerated soil (Okano et al., 2004). Additionally, the linear regression analysis showed a significant inverse correlation between NH_4^+ and NO_3^- concentrations in Urea treatment from day 7 to day 28 ($r = -0.98$, $P = 0.0003$, $n = 12$) and in Urea + HAs from day 16 to day 28 ($r = -0.98$, $P = 0.03$, $n = 12$). It has reported that NH_4^+ concentration can regulate potential nitrification (Stehr et al., 1995), stimulating nitrifying activity within a restricted range (Magalhaes et al., 2005) as a function of the environmental conditions and AOB species. The *Ks* (half-saturation constants) values are considered to be important characteristics of organisms living in nitrogen-limited environments. Generally, for activity and growth of AOB in pure culture, the *Ks* for $NH_4^+ + NH_3$ ($NH_4^+ + NH_3$ concentration at which the half-maximum oxidation rate is achieved) ranged between 0.051 and 14 mM (Prosser, 1989). Additionally Stehr et al. (1995) reported the *Ks* for $NH_4^+ + NH_3$ of the sediment AOB isolates ranged between 0.03 and 3.3 mM. In this study, our highest ammonium concentration was only 0.113 mM [1.4 mgN (g dry soil)$^{-1}$], and it is still within the range of concentrations known to stimulate nitrifying activity. Therefore the higher concentration of NH_4^+ might explain the increase of activities of AOB in the Urea treatment at week 1 and exhaustion of NH_4^+ may partially explain the substantial decrease at week 4. Similarly, the reason why the potential nitrification in the Urea + HAs treatments was much lower than in the Urea treatment at week 1, but much higher at week 4 could be due to NH_4^+ availability. HAs slowed down the release of ammonium in soil by inhibiting urease activity which caused the delay of nitrification in Urea + HAs treatments. Another possible explanation is that ammonia–N is abiotically incorporated into soil organic matter, peat, coal or lignin (Nommik and Vathras, 1982; Thorn and Mikita, 1992; Lapierre et al., 1994; Bosatta and Agren, 1995), but this possibility can be excluded because final NO_3^- levels were the same in all three Urea treatments.

Potential nitrification s can be an indirect measure of AOB abundance, though rates may also be sensitive to a phenotypic or physiological change in AOB community (Henriksen, 1980; Mendum et al., 1999). The positive correlation between potential nitrification and AOB population size in Urea treatment ($r = 0.66$, $P = 0.34$), in Urea + bHA ($r = 0.99$, $P = 0.006$) and Urea + cHA ($r = 0.84$, $P = 0.016$) also support this conclusion. Indeed, we detected high activity and high densities of AOB after a short time of incubation (one week), and this is inconsistent with an earlier report of high nitrifying activity but no change in AOB population quantified by DGGE after 4 weeks (Avrahami et al., 2002). The incubation temperature differed between the two studies, and 4 °C in the Avrahami et al. (2002) study was not the optimal growth temperature for most AOB. However, a greater abundance but not activity of AOB was observed at day 28 in the Urea treatment. This lack of correspondence between numbers and activity might be due to the persistence of DNA from AOB inactive cells, which may be why the positive correlation between potential

nitrification and AOB population size is not significant in the Urea treatment ($r = 0.66$, $P = 0.34$). Additionally, the potential nitrification s in the Urea + HAs treatments continued to increase over the 4 weeks of incubation, possibly responding to a continual slow release of NH_4^+. This slow supply of NH_4^+ could, in turn, support higher AOB densities in the Urea + HAs treatments.

4.4. Possible mechanisms

The ammonium concentration profile in all treatments showed that HAs delayed the release of ammonium during the urea hydro-lysis. We hypothesize that HAs indirectly affect the availability of NH_4^+ through stabilization of extracellular enzymes (Nannipieri et al., 1996), such as urease (Dong et al., 2006, 2008). Since urease is the key enzyme which decomposes urea to ammonia, application of urea along with HAs slows down the transformation of urea to ammonium. Indeed, we observed that the activity of urease in the Urea + HAs treatments was much lower than that in the Urea treatment during the first 5 days and then the activity was maintained at 50–60% for the following 16 days. Thus HAs can alter the release of ammonia by impacting urease activity, while the substrate availability (ammonia concentration in soil) is crucial to the development of population size and activity of nitrifiers.

On the other hand, the potential nitrification was significantly different after addition of bHA and cHA, which indicated a difference of nitrifier activity and biomass. Since the nitrification was regulated by ammonium concentration, so HAs can impact potential nitrification indirectly through affecting the ammonia availability. Soil HAs may also have an effect on soil urease, but the behavior of the urea-only treatment and the difference between Urea and Urea + HAs indicated that all the major effects we discussed above are caused by lignite HAs.

In conclusion, in soil amended with urea, addition of lignite HAs, regardless of their degree of biodegradation, can buffer the change in microbial community composition and numbers, as well as AOB population size and potential nitrification, by reduction of the urea to ammonium hydrolysis rate through the inhibition of urease activity.

Acknowledgments

This research was part of project No.2006AA10A213 supported by the Ministry of Science and Technology of China (the 863 program) and No.30670071 supported by the National Science Foundation of China. Additional funding was provided by National Institutes of Environmental Health Science Superfund Basic Research Program (5P42 ES004699).

References

Avrahami S, Conrad R, Braker G. Effect of soil ammonium concentration on N_2O release and on the community structure of ammonia oxidizers and denitrifiers. Applied and Environmental Microbiology. 2002; 68:5685–5692. [PubMed: 12406765]

Belser LW. Population ecology of nitrifying bacteria. Annual Review of Microbiology. 1979; 33:309–333.

Blackwood CB, Marsh TL, Kim S-H, Paul EA. Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. Applied and Environmental Microbiology. 2003; 69:926–932. [PubMed: 12571013]

Bodelier PLE, Duyts H, Blom CWPM, Laanbroek HJ. Interactions between nitrifying and denitrifying bacteria in gnotobiotic microcosms planted with the emergent macrophyte *Glyceria maxima*. FEMS Microbiology Ecology. 1998; 25:63–78.

Bollmann A, Laanbroek HJ. Continuous culture enrichments of ammonia-oxidizing bacteria at low ammonium concentrations. FEMS Microbiology Ecology. 2001; 37:211–221.

Bosatta E, Agren GI. Theoretical analyses of interaction between inorganic nitrogen and soil organic matter. European Journal of Soil Science. 1995; 46:109–114.

Cavagnaro TR, Jackson LE, Scow KM, Hristova KR. Effects of arbuscular mycorrhizas on ammonia oxidizing bacteria in an organic farm soil. Microbial Ecology. 2007; 54:618–626. [PubMed: 17955326]

Cello FD, Bevivino A, Chiarini L, Fani R, Paffetti D, Tabacchioni S, Dalmastri C. Biodiversity of a *Burkholderia cepacia* population isolated from the maize rhizosphere at different plant growth stages. Applied and Environmental Microbiology. 1997; 63:4485–4493. [PubMed: 9361434]

Christl I, Knicker H, Kogel IK, Kretzschmar R. Chemical heterogeneity of humic substances: characterization of size fractions obtained by hollow-fibre ultrafiltration. European Journal of Soil Science. 2000; 51:617–625.

Clinton PW, Newman RH, Allen RB. Immobilization of ^{15}N in forest litter studied by ^{15}N CPMAS NMR spectroscopy. European Journal of Soil Science. 1995; 46:551–556.

Córdova-Kreylos AL, Cao Y, Green GP, Hwang H-M, Kuivila KM, LaMontagne MG, Werfhorst LCVD, Holden PA, Scow KM. Diversity, composition, and geographical distribution of microbial communities in California salt marsh sediments. Applied and Environmental Microbiology. 2006; 72:3357–3366. [PubMed: 16672478]

Davies, G.; Ghabbour, EA. Humic Substances: Structures, Properties and User. Royal Society of Chemistry; Cambridge: 1998.

Dong LH, Yuan Q, Yuan HL. Changes of chemical properties of humic acids from crude and fungal transformed lignite. Fuel. 2006; 85:2402–2407.

Dong LH, Yang JS, Wang ET, Yuan HL, Chen WX. Chemical characteristic and influences of two fractions of Chinese lignite humic acids on urease. European Journal of Soil Biology. 2008; 44:166–171.

Egert M, Friedrich MW. Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. Applied and Environmental Microbiology. 2003; 69:2555–2562. [PubMed: 12732521]

Enwall K, Nyberga K, Bertilssonb S, Cederlunda H, Stenstroma J, Hallin S. Long-term impact of fertilization on activity and composition of bacterial communities and metabolic guilds in agricultural soil. Soil Biology and Biochemistry. 2007; 39:106–115.

Fierer N, Jackson RB. The diversity and biogeography of soil bacterial communities. Proceedings of the National Academy of Sciences. 2006; 103:626–631.

Frostegård A, Bååth E, Tunlid A. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biology and Biochemistry. 1993; 25:723–730.

Hastings RC, Saunders JR, Hall GH, Pickup RW, McCarthy AJ. Application of molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake. Applied and Environmental Microbiology. 1998; 64:3674–3682. [PubMed: 9758784]

Hayes, MHB.; Wilson, WR. Humic Substances, Peats and Sludges. Royal Society of Chemistry; Cambridge: 1997.

Henriksen K. Measurement of in situ rates of nitrification in sediment. Microbial Ecology. 1980; 6:329–337.

Holmes AJ, Costello A, Lidstrom ME, Murrell JC. Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiology Letters. 1995; 132:203–208. [PubMed: 7590173]

Horz H-P, Rotthauwe J-H, Lukow T, Liesack W. Identification of major subgroups of ammonia-oxidizing bacteria in environmental samples by T-RFLP analysis of *amoA* PCR products. Journal of Microbiological Methods. 2000; 39:197–204. [PubMed: 10670766]

Jiang JP, Zhang XJ. Effect of urea coated with humic acid on increase yield of cotton. Soils Fertility Science. 2002; 3:33–34. (in Chinese).

Kandeler E, Gerber H. Short-term assay of soil urease activity using color-imetric determination of ammonium. Biology and Fertility of Soils. 1988; 6:68–72.

Kelly JJ, Häggblom M, Tate RL III. Changes in soil microbial communities over time resulting from one time application of zinc: a laboratory microcosm study. Soil Biology and Biochemistry. 1999; 31:1455–1465.

Kitts CL. Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. Current Issues in Intestinal Microbiology. 2001; 2:17–25. [PubMed: 11709853]

Knicker H, Ludemann HD, Haider K. Incorporation studies of NH4+ during incubation of organic residues by ^{15}N CPMAS-NMR spectroscopy. European Journal of Soil Science. 1997; 48:431–441.

Laanbroek, HJ.; Woldendorp, JW. Activity of chemolithotrophic nitrifying bacteria under stress in natural soils.. In: Jones, JG., editor. Advances in Microbial Ecology. Plenum Press; New York: 1995. p. 275-304.

Lapierre C, Monties B, Meier D, Faix O. Structural investigation of kraft lignins transformed via oxo-ammoniation to potential nitrogenous fertilizer. Holzforschung. 1994; 48:63–68.

Leininger S, Urich T, Schloter M, Schwark L, Qi J, Nicol GW. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature. 2006; 442:806–809. [PubMed: 16915287]

Lepš, J.; Šmilauer, P. Multivariate Analysis of Ecological Data Using CANOCO. University Press; Cambridge: 2003.

Liang TB, Wang ZL, Liu LL, Wang RJ, Chen XG, Zhang XD, Shi CY. Effects of humic acid urea on yield and nitrogen absorption, assimilation and quality of ginger. Plant Nutrition and Fertilizer Science. 2007; 13:903–909.

Lichtfouse E, Chenu C, Baudin F, Leblond C, Da-Silva M, Behar F. A novel pathway of soil organic matter formation by selective preservation of resistant straight-chain biopolymers: chemical and isotope evidence. Organic Geochemistry. 1998; 28:411–415.

Lueders T, Friedrich MW. Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and mcrA genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. Applied and Environmental Microbiology. 2003; 69:320–326. [PubMed: 12514011]

Mackowiak CL, Grossl PR, Bugbee BG. Beneficial effects of humic acid on micronutrient availability to wheat. Soil Science Society of American Journal. 2001; 64:1744–1750.

Magalhaes CM, Joye SB, Moreira RM, Weibe WJ, Bordalo AA. Effect of salinity and inorganic nitrogen concentrations on nitrification and depotential nitrification s in intertribal sediments and rocky biofilms of the Douro River Estuary, Portugal. Water Research. 2005; 39:1783–1794. [PubMed: 15899276]

Mendum TA, Sockett RE, Hirsch PR. Use of molecular and isotopic techniques to monitor the response of autotrophic ammonia-oxidizing populations of the beta subdivision of the class Proteobacteria in arable soils to nitrogen fertilizer. Applied and Environmental Microbiology. 1999; 65:4155–4162. [PubMed: 10473429]

Nannipieri, P.; Sequi, P.; Fusi, P. Humus and enzyme activity.. In: Piccolo, A., editor. Humic Substances in Terrestrial Ecosystems. Elsevier; Amsterdam: 1996. p. 293-298.

Nicol GW, Schleper C. Ammonia-oxidising Crenarchaeota: important players in the nitrogen cycle? Trends in Microbiology. 2006; 14:207–212. [PubMed: 16603359]

Nommik, H.; Vathras, K. Retention and fixation of ammonia and ammonium in soil.. In: Stevenson, FJ., editor. Nitrogen in Agricultural Soils. American Society Of Agronomy; Madison: 1982. p. 123-171.

Okano Y, Hristova KR, Leutenegger CM, Jackson LE, Denison RF, Gebreyesus B, Lebauer D, Scow KM. Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. Applied and Environmental Microbiology. 2004; 70:1008–1016. [PubMed: 14766583]

Osborn AM, Moore ERB, Timmis KN. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environmental Microbiology. 2000; 2:39–50. [PubMed: 11243261]

Park HD, Noguera DR. Evaluating the effect of dissolved oxygen on ammonia-oxidizing bacterial communities in activated sludge. Water Research. 2004; 38:3275–3286. [PubMed: 15276744]

Peiqi, AL.; Mile, RH. Soil Analysis Methods. Chinese Agricultural Science and Technology Press; Beijing: 1991.

Pennanen T, Perkiomaki J, Kiikkila O, Vanhala P, Neuvonen S, Fritze H. Prolonged, simulated acid rain and heavy metal deposition: separated and combined effects on forest soil microbial community structure. FEMS Microbiology Ecology. 1998; 27:291–300.

Pertusatti J, Prado AGS. Buffer capacity of humic acid: thermodynamic approach. Journal of Colloid and Interface Science. 2007; 314:484–489. [PubMed: 17603069]

Polz MF, Cavanaugh CM. Bias in template-to-product ratios in multi-template PCR. Applied and Environmental Microbiology. 1998; 64:3724–3730. [PubMed: 9758791]

Prosser JI. Autotrophic nitrification in bacteria. Advance in Microbial Physiology. 1989; 30:125–181.

Rotthauwe JH, Witzel KP, Liesack W. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Applied and Environmental Microbiology. 1997; 63:704–712.

Siripong S, Rittmann BE. Rittmann diversity study of nitrifying bacteria in full-scale municipal wastewater treatment plants. Water Research. 2007; 41:1110–1120. [PubMed: 17254627]

Stankiewicz BA, Van-Bergen PF. Nitrogen and N-containing macromolecules in the bio- and geosphere: an introduction. American Chemistry Society Symposium Series. 1998; 707:1–12.

Stehr G, Bottcher B, Dittberner P, Rath G, Koops HP. The ammonia-oxidizing nitrifying population of the River Elbe Estuary. FEMS Microbiology Ecology. 1995; 17:177–186.

Suzuki MT, Beja, Taylor LT, Delong EF. Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton. Environmental Microbiology. 2001; 3:323–331. [PubMed: 11422319]

Thorn KA, Mikita MA. Ammonia fixation by humic substances: a nitrogen-15 and carbon-13 NMR study. Science of the Total Environment. 1992; 113:67–87.

Venterea RT, Rolston DE. Mechanisms and kinetics of nitric and nitrous oxide production during nitrification in agricultural soil. Global Change Biology. 2000; 6:303–316.

Wang GC, Wang Y. The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. Microbiology. 1996; 142:1107–1114. [PubMed: 8704952]

Webster G, Embley TM, Prosser JI. Grassland management regimens reduce small-scale heterogeneity and species diversity of (beta)-proteobacterial ammonia oxidizer populations. Applied and Environmental Microbiology. 2002; 68:20–30. [PubMed: 11772604]

Wood, PM. Monooxygenase and free radical mechanisms for biological ammonia oxidation.. In: Cole, JA.; Ferguson, SJ., editors. The Nitrogen and Sulfur Cycles. Cambridge University Press; Cambridge: 1987. p. 219-243.

Yuan HL, Yang JS, Wang FQ, Chen WX. Degradation and solubilization of Chinese lignite by Penicillium sp P6. Applied Biochemistry and Microbiology. 2006; 42:52–55.

Zheng, P. The Application and Production of Peat Humic Acids. Chemical Industry Press; Beijing: 1991.

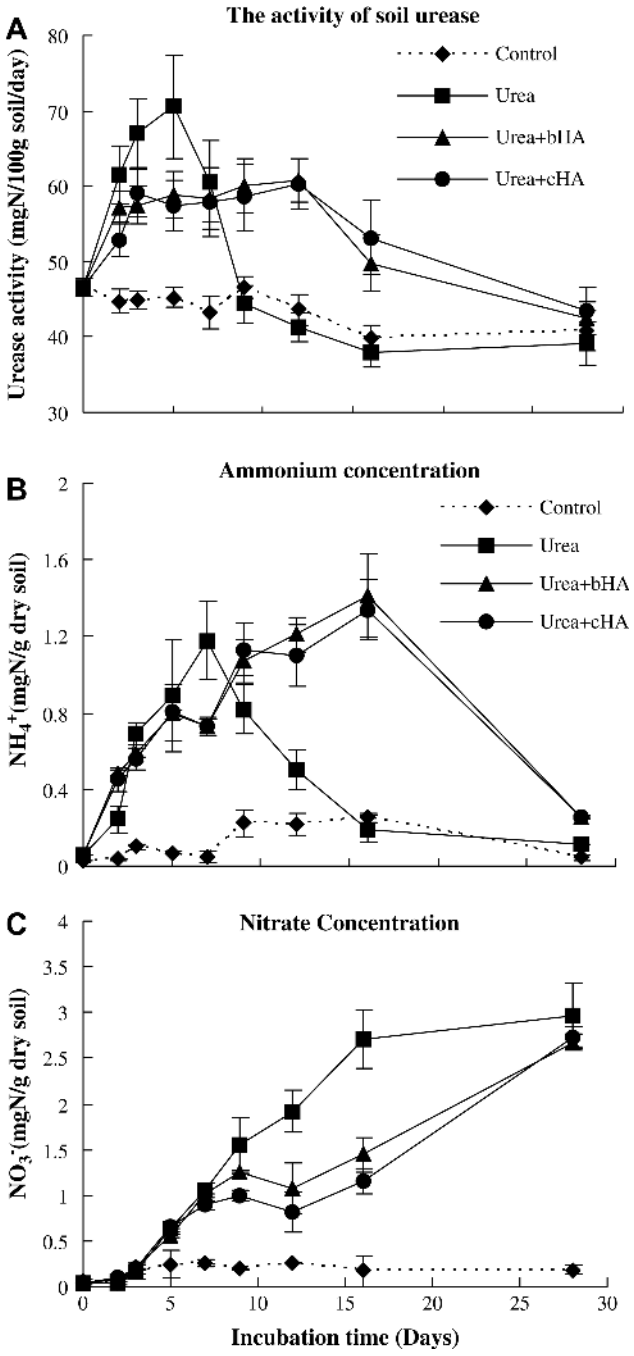

Fig. 1.

Changes in urease activity (A) ammonium (B) and nitrate concentration (C) after application of urea. Sampling points were at day 0, 2, 3, 5, 7, 9, 12, 16 and 28. Diamond with dash line, square, triangle and sphere with solid line are Control (no urea and HAs), Urea only, Urea + bHA and Urea + cHA treatments, respectively.

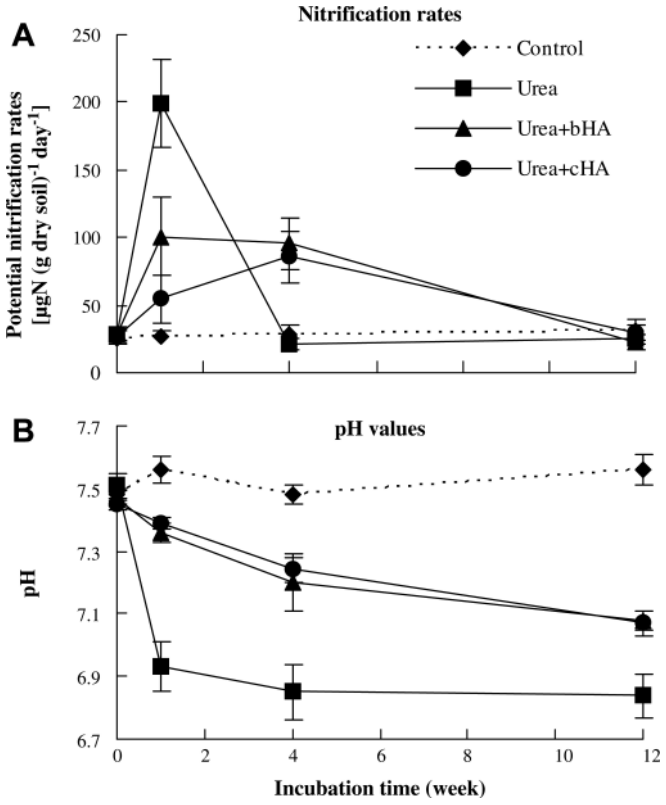

Fig. 2.

Potential nitrification s (A) and pH values (B) in different treatments at different sample points. Sampling points were at week 0, 1, 4 and 12. Diamond with dash line, square, triangle and sphere with solid line are Control (no urea and HAs), Urea only, Urea + bHA and Urea + cHA treatments, respectively.

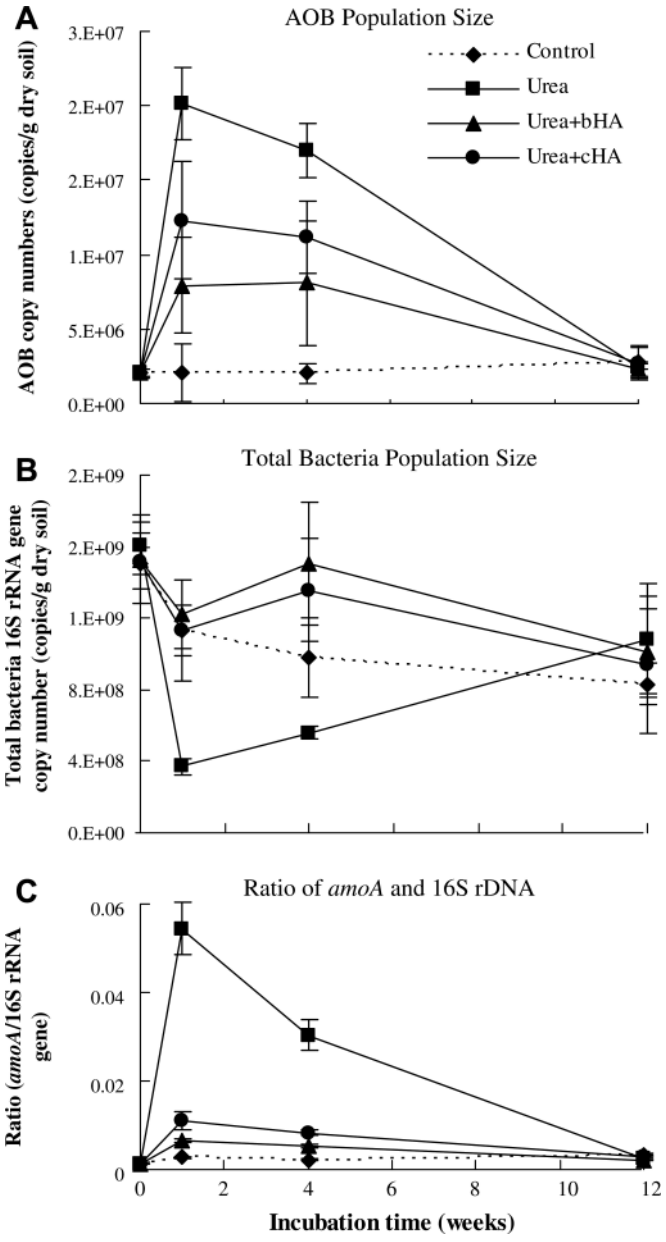

Fig. 3.

Changes in AOB population size (A) and total bacterial population size (B), and the ratio of AOB and total bacterial population size (C) after application urea. Sampling points were at week 0, 1, 4 and 12. Diamond with dash line, square, triangle and sphere with solid line are Control (no urea and HAs), Urea only, Urea + bHA and Urea + cHA treatments, respectively.

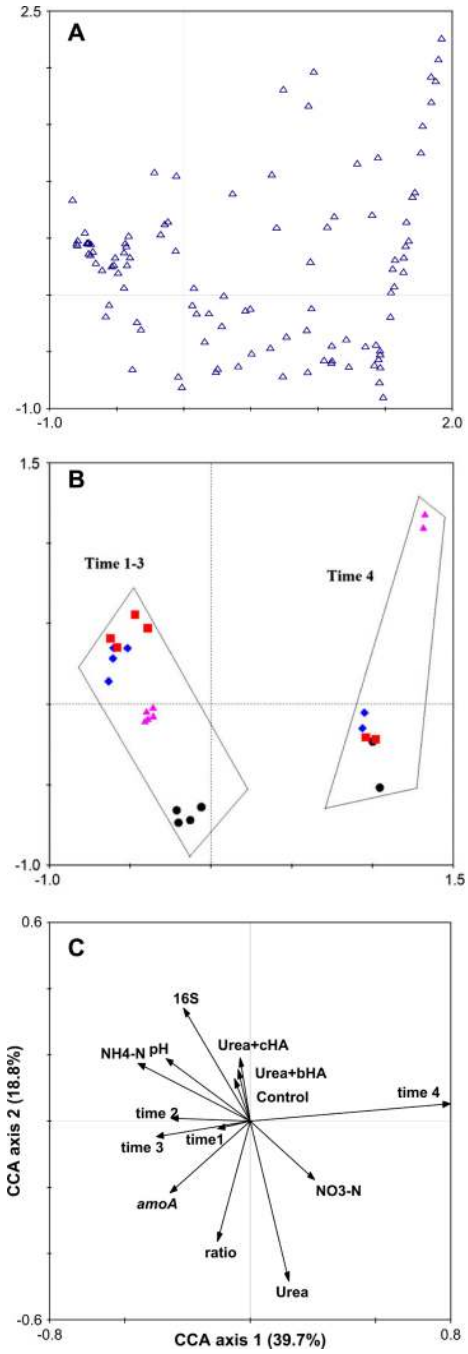

Fig. 4.

Species (A, open triangles), samples (B, solid symbols) and environmental variables (C, arrows) ordination plots of CCA results for T-RFLP fingerprint. The analysis included all variables before pCCA. The soil sample before treating with urea was analyzed as zero (time 1). Four treatments (triangle, Control; sphere, Urea only; square, Urea + cHA; diamond, Urea + bHA) with replicates were sampled at week 1 (time 2), 4 (time 3) and 12 (time 4) during the incubation. 16S: total bacterial 16S rRNA gene copies; *amoA*: AOB *amoA* gene copies; ratio: the ratio of *amoA* to 16S rRNA gene.

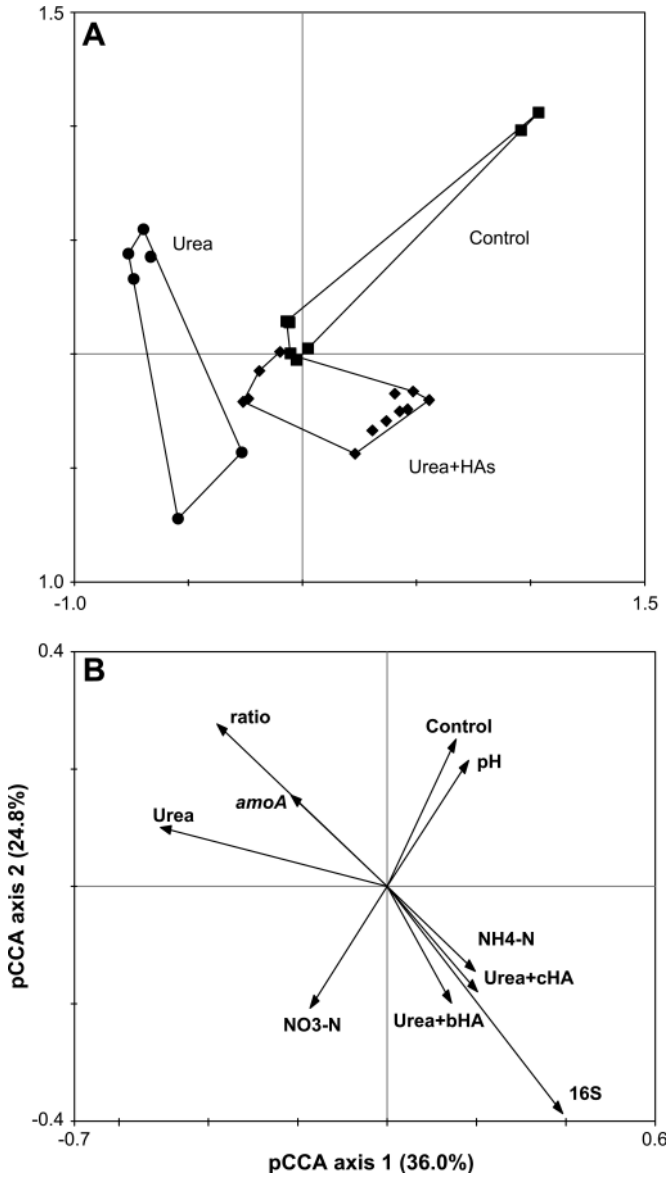

Fig. 5.

Sample (A) and environmental variables (B) ordination plots of pCCA results for T-RFLP fingerprint. Time was a covariable. Polygons indicate sample groups. Sampling points were at week 0, 1, 4 and 12. Sphere: Urea-only treatment; Diamond: Urea + HAs; Square: Control. 16S: total bacterial 16S rRNA gene copies; *amoA*: AOB *amoA* gene copies; ratio: the ratio of *amoA* to 16S rRNA gene.

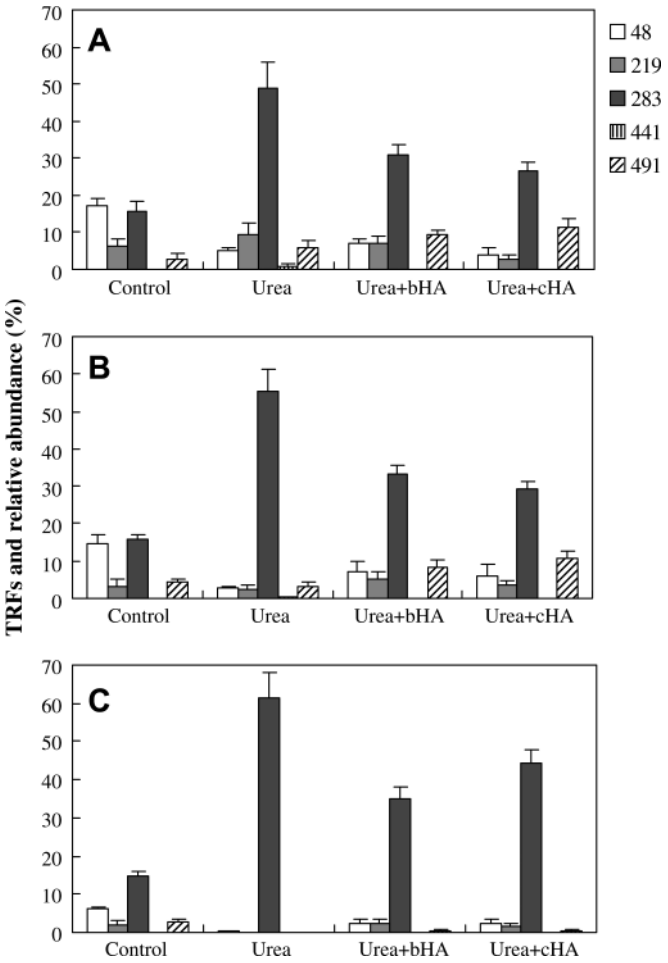

Fig. 6.

Relative abundances of specific AOB in T-RFLP profiles obtained from soil amended with HA or urea. Relative abundances determined at week 1 (A), week 4 (B) and week 12 (C). Assignations were obtained from T-RFLP profiles of *amoA* digested with TaqI.

Table 1

Summary statistics from all ordination analyses.

Microbial community analysis	Ordination technique	Environmental variables (no. of variables)	Covariables (no. of covariables)	% inertia explained by first two canonical axes[a]	% inertia explained by all canonical axes	*P* value for the first canonical axis[b]	*P* value for all canonical axes
T-RFLP	CCA	Time (4)	–	58.5	81.3	0.002	0.002
	pCCA	Treatment (4)	Time (4)	68.8	87.7	0.004	0.002
	pCCA	Treatment, pH, NH_4^+, NO_3^-(7)	Time (4)	60.8	78.2	0.04	0.01

[a]Refers to % of total inertia, which is the variance in ordination scores, or as in pCCA, refers to % of residual inertia after accounting for the time effects using time variables as covariables.

[b]*P* values based on Monte Carlo permutation test, as described in Materials and methods.