This is the preprint version of the contribution published as:

Sun, Z., **Zheng, T.**, Xin, J., Zheng, X., Hu, R., Subhan, F., **Shao, H.** (2018):
Effects of alkali-treated agricultural residues on nitrate removal and N_2O reduction of denitrification in unsaturated soil
J. Environ. Manage. **214** , 276 – 282

The publisher's version is available at:

http://dx.doi.org/10.1016/j.jenvman.2018.02.078

Effects of alkali-treated agricultural residues on nitrate removal and N_2O reduction of denitrification in unsaturated soil

Zhaoyue Sun[a,b], Tianyuan Zheng[c,d*], Jia Xin[a,b], Xilai Zheng[a,b], Rongting Hu[a,b], Fazle Subhan[e], Haibing Shao[c,d]

a. Key Lab of Marine Environmental Science and Ecology, Ministry of Education, Qingdao, China

b. Shandong Provincial Key Laboratory of Marine Environment and Geological Engineering, Qingdao, China

c. Helmholtz Centre for Environmental Research – UFZ, Permoserstraße 15, 04318 Leipzig, Germany

d. Applied Environmental Systems Analysis, Dresden University of Technology, Germany

e. Department of Chemistry, Abdul Wali Khan University Mardan, Pakistan

Abstract： Lignocellulosic agricultural residues were utilized as denitrification carbon substrates to improve the purification capacity of unsaturated soil and alleviate nitrate pollution of groundwater. In this study, corncob and wheat straw were treated by calcium hydroxide to improve biodegradability and enhance denitrification potential. Calcium hydroxide treatment decreased the contents of lignin (i.e., from 16.7 wt% to 15.2 wt% in corncob and from 21.9 wt% to 20.6 wt% in wheat straw), increased potential biodegradable carbon by 4.4-5.3 times, reached complete nitrate removal 7-14 days earlier and decreased $N_2O/(N_2O+N_2)$ ratios by 85-99%. The results provide an insight into the application of alkali-treated agricultural residues as denitrification carbon sources to alleviate nitrate transport to groundwater and reduce potential greenhouse effect.

Keywords： Denitrification; Alkaline treatment; Nitrate removal; $N_2O/(N_2+N_2O)$ ratio; Quantitative PCR

1. Introduction

Improper disposal of residues from agricultural activities poses a threat to

environment and health. As an important source of nutrients, agricultural residues can be reused and applied in soil or groundwater remediation (Medina et al., 2015). Contamination of groundwater with nitrate is caused by excessive use of nitrate-bearing fertilizers, crop irrigation with wastewater, and fertilization with manure (Gibert et al., 2008; Liu et al., 2009; Rivett et al., 2008; Rocca et al., 2007). Groundwater with a high nitrate concentration induces health problems such as methemoglobinemia in infants and cancer (Ashok and Hait, 2015; Vosoughifar et al., 2005; Wang and Wang, 2012). The European Union and World Health Organization have set the threshold concentration of nitrate to be 50 mg L^{-1} in potable water (Ashok and Hait, 2015).

Denitrification is the main process of nitrate reduction which happens in vadose zone and groundwater (Ashok and Hait, 2015), but the limited amount of organic carbon in vadose zone is regarded as the limitation of natural denitrification process (Gibert et al., 2008; Pu et al., 2014). It indicated that an organic layer under topsoil at point sources (e.g., septic tank drainage fields and composting sites) increased nitrate removal (Robertson et al., 2000; Schipper and McGill, 2008). Agricultural residues (e.g., pine bark (Huang et al., 2015), distillers' grains (Wan et al., 2015), wood chips (Damaraju et al., 2015) and corncob (Wang et al., 2013)) have been employed as carbon sources. Agricultural residues have several advantages, such as high C/N ratio, low cost, high permeability, long persistence (Chang et al., 2001; Warneke et al., 2011a) and high denitrification performance. However, labile cellulose and hemicellulose are often tightly bounded to lignin to form a recalcitrant structure, which is very difficult to be composed by bacteria (Kallioinen et al., 2013). Warneke et al. (2011b) reported that high removal rates of carbon substrates were not sustainable due to the decrease of organic carbon release in the later phase of the column experiment. Alkaline treatment

can reduce the steric hindrance of hydrolytic enzymes and enhance carbohydrate digestibility (Renu et al., 2014), which has been widely used in wetlands (Wen et al., 2010) and water purification (Feng et al., 2017). For example, Wen et al. (2010) investigated effects of plant biomass with and without NaOH pretreatment on nitrate removal and transformation of carbon sources in subsurface-flow constructed wetlands. Nitrate removal rates were greatly affected by the content of carbohydrates (e.g., cellulose and hemicellulose) and the C/N ratio in biomass. Feng et al. (2017) reported that solid-phase denitrification systems with alkali pretreated corncob, rice straw and rice hulls exhibited good performances in nitrogen removal. It can be concluded that alkali pretreatment was favorable to increase lignin removal and the exposure of biodegradable compositions to bacteria. However, alkaline treatment is rarely used in denitrification layer in soil system.

Denitrification involves the reduction of via a series of microbial reduction reactions which lead to the production of gaseous products (i.e., NO, N_2O and N_2). N_2O is an active gas leading to ozone depletion (Ciarlo et al., 2007) and global warming (Wang et al., 2011). Warneke et al. (2011b) found that the removal of denitrification bed with corncobs was higher, but dissolved N_2O emission was also observed. The emission of N_2O is affected by the availability of carbon (Senbayram et al., 2012), lignin content (Millar and Baggs, 2004; Zhu et al., 2013), C:N ratios of crop residues (Huang et al., 2004; Li et al., 2013), and denitrifiers possessing N_2O reductase function gene (*nosZ*).

The unique contribution of this work is to get alkaline treated lignocellulose carbon materials to accelerate removal and reduce the N_2O emission simultaneously. Besides, the relationship between bacterial activities (the abundance of total bacteria and denitrifiers possessing *nosZ* gene) and denitrification potential in different bioavailable

carbon amended soils is built. The results are important to guide in-situ remediation of nitrate contamination in groundwater.

2. Materials and methods

2.1. Soil and crop residues

The soil was collected from a farm land in Dong Zhuangtou village (120°20′E, 36°43′N, Qingdao, China) in November 2015. In view of the high denitrification activity of soil at 20-50 cm depth, the samples were collected at this depth. After air-dried to water holding capacity of 45%, the soil was sieved (< 2 mm) to homogenize. After removal of stones, plant residues and small insects, it was stored at 4 °C. The chemical composition of soil was determined to have a total element carbon of 3.3 g kg^{-1} and element nitrogen of 0.5 g kg^{-1}. Its bulk density was measured to be 1.6 g cm^{-1}, with 44.7 wt% silt, 40.3 wt% sand and 15.0 wt% clay along with a water holding capacity (WHC) of 15.0 wt%. The soil texture belonged to medium textural class according to the FAO's soil classification. The raw agricultural residues were corncob and wheat straw, which were collected from crops in local field.

2.2. Preparation of denitrification carbon materials

Untreated corncob (UC) and wheat straw (UWS) were washed 3 times with fresh tap water and dried at 60 °C in oven till constant weight. Then, the materials were grinded to 24-80 mesh, followed by washing and drying. The carbon materials were pretreated with $Ca(OH)_2$ according to previous studies of Hu et al. (2017) and Chang et al. (1998). The mixture A was prepared with 5 g corncob, 50 mL distilled water and 0.5 g $Ca(OH)_2$ (Sinopharm Chemical Reagent Co. Ltd, Analytical grade). Compared to corncob, the volume of grinded wheat straw (5 g) was larger and 25 mL more distilled water was used to make sure a high dispersion of wheat straw in the mixture B.

Therefore, mixture B contained 5 g wheat straw, 75 mL distilled water and 0.5 g $Ca(OH)_2$. Mixture A and B were separately put in 150 mL Erlenmeyer flask and treated at 150 rotate-per-minute (rpm) for 6 h at 70 ºC and 24 h at 95 ºC, respectively. The treated corncob (TC) and wheat straw (TWS) were washed with distilled water to a pH value of 7.0-7.5 and dried at 75 ºC for 24 h. The properties of carbon materials are shown in Table 1.

Table 1 Properties of carbon materials (UC=untreated corncob, TC=treated corncob, UWS=untreated wheat straw, TWS=treated wheat straw).

	Lignin [a] wt %	Hemicellulose [b] wt %	Cellulose [c] wt %	C [d] wt %	N [d] wt %	C/N	Reducing sugar yield [e] (mg glucose g^{-1} dry mass)
UC	16.7±0.22	43.4±0.40	38.5±0.60	46.73	0.44	107.2	120.9±4.21
TC	15.2±0.50	34.1±0.34	47.0±0.99	43.72	0.26	167.3	657.1±7.50
UWS	21.9±0.41	40.9±0.07	34.3±0.47	46.48	0.33	139.6	58.3±3.24
TWS	20.6±0.15	23.5±0.11	50.6±0.06	44.53	0.21	215.6	368.1±6.74

[a] was determined based on Huang et al. (2004).

[b, c] were determined based on Lin et al.(2010).

[d] were analyzed on a Thermo EA2000 Elemental Analyzer.

[e] were measured based on Chang et al. (2001).

2.3. Denitrification performance

Prior to incubation experiment, the initial concentration of -N was adjusted to 399 mg kg^{-1} dry soil by adding KNO_3 solution of 0.5M. The pH value of soil was adjusted to 6.0 by adding NaOH solution of 0.1M. Finally, the soil moisture was adjusted to 15.0 % by distilled water and named as CK. Then, a mixture of CK (50.0 g) and carbon materials (1.0 g) were in an anaerobic bottle with N_2 atmosphere. To calculate the production of N_2, 10% of N_2 was replaced by C_2H_4 (Gregorich and Carter, 2007). The anaerobic bottles were shaken every day to facilitate gas diffusion. The anaerobic

bottles were at 25 ºC for 28 days.

The products involved in denitrification (e.g. , N_2O, N_2 and CO_2) were determined as follows. The soil sample without C_2H_4 addition was extracted with a 0.5 M K_2SO_4 solution (1:5 w/v) by shaking at 20 ºC for 1 h (Miller et al., 2008). Then the extract was filtered through 0.45 μm cellulose acetate membrane and stored at -20 ºC before analysis. Nitrate and nitrite were assayed according to Chinese SEPA standard methods (China, 2002). Dissolved organic carbon (DOC) was monitored as an indicator of the carbon available for the bacterial activity to take place (Gibert et al., 2008). DOC concentration in extract was measured with a total organic carbon analyser (TOC-VCPN, Shimadzu). Concentrations of N_2O were determined via gas chromatography (GC-14CP, Shimadzu) equipped with an electron capture detector (ECD) using Ar (95%) and CH_4 (5%) as carrier gas. N_2O was separated in the Porapak Q chromatography column (Agilent Technologies; column length, 3.0 m; inner diameter, 1 mm). Operating conditions for the GC were as follows: injector temperature 85 ºC, column temperature 60 ºC and detector temperature 300 ºC. The limits of detection and quantification for N_2O analysis are 0.03 and 0.10 ppm, respectively. N_2 was determined by the subtraction between N_2O with and without C_2H_2 (Zhu et al., 2013). CO_2 was analyzed by N_2 flushing for 15 min and trapped in barium hydroxide solution absorbent without C_2H_2 (China, 2000).

2.4. DNA extraction and quantitative PCR

The governing molecular-biological mechanism of denitrification was measured by quantitative PCR. 2 g subsamples were sampled from each aerobic bottle without acetylene on day 7, 14, 21 and 28. The Power Soil DNA Isolation kit (MoBio, USA) was used to extract 0.25 g thoroughly mixed fresh soil sample. The extracted DNA was

assessed using Picodrop microliter spectrophotometry (Picodrop, Ltd, UK). Bacterial abundance was measured using quantitative PCR.

The 16S rRNA and *nosZ* functional genes were targeted for the total bacterial communities and denitrifier communities, respectively. All qPCR assays were carried out in triplicate, and were performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) including thermal cycling, fluorescent data and data analysis using SYBR-green based detection. The primers and thermal cycling conditions used for the total bacterial 16S rRNA PCR were previously described by He et al. (2015). The 20 μL qPCR mixture contained the following reagents: 10 μL Fast Start Universal SYBR Green Master (Rox) (Roche Diagnostics, Germany), 0.3 μM each primer, 0.2 μg μL^{-1} bovine serum albumin (BSA) and 2.0 μL template DNA. *nosZ* gene fragments were amplified by using *nosZ*1F (5'-WCSYTGTTCMTCGACAGCCAG-3') and *nosZ*1R (5'-ATGTCGATCARCTGVKCRTTYTC-3') primer pair (Henry et al., 2006). Real time PCR for *nosZ* was performed in a volume of 25 μL, and the assay mixture contained 10 μL Rox, 0.6 μM of each *nosZ* primer, 2 μg μL^{-1} BSA, and 2μL of template DNA. The thermal cycling conditions for the *nosZ* primers were as follows: an initial cycle of 95 °C for 15 min; 40 cycles of 95 °C for 45 s, 58 °C for 60 s. For each primer set, the C_T and logarithmic values of plasmid copy numbers were determined by using a standard curve generated with three replicate 10-fold serial dilutions of a known quantity of plasmids carrying a fragment of the 16S rRNA and *nos*Z gene. The plasmids carrying the target gene fragments were extracted from *E. coli* hosts using a Fast Plasmid Mini kit (CWBIO, Beijing, China). Plasmid DNA concentrations were measured with Picodrop microliter spectrophotometry (Picodrop, Saffron Walden, Essex, UK). The

data were analyzed with ABI PRISM 7500 SDS software (version 1.3.1; Applied Biosystems).

2.5. Data analysis

Statistical analyses were performed using SPSS version 19.0 (IBM Corporation, USA), with $p<0.05$ used as the criterion for statistical significance. Significant differences among treatments were evaluated by one-way ANOVA using Duncan test.

3. Results and discussion

3.1. DOC emission and removal

The reducing sugar yields of materials to investigate the potential hydrolysis performance of agricultural residues (Chang et al., 1998; Chang et al., 2001) are listed in Table 1. The reducing sugar yields were in the order of TC > TWS > UC > UWS. Apparent increases of sugar yields were in treated carbon materials, which indicated that the agricultural residues could have a higher biodegradability potential to favor more electron donors emission after alkaline treatment.

To further explore the carbon availability of different carbon materials in denitrification, DOC emissions over the incubation were compared (Fig. 1a). The DOC concentrations in UC and UWS ranged between 50-170 mg C kg^{-1} dry soil. Compared to UC and UWS, DOC concentrations in TC and TWS increased from 56.6 to 823.1 mg C kg^{-1} dry soil and 75.9 to 604.9 mg C kg^{-1} dry soil on day 28, respectively. It indicated more degradable and available carbon for microorganisms in TC and TWS than UC and UWS. Lignin has been proved to retard enzyme access to glucan chains by its protective sheathing and reduce cellulase effectiveness by unproductive binding and steric hindrance (Lin et al., 2010; Mosier et al., 2005). This is consistent with our results in Table 1, which showed that the treated carbon materials consist of a smaller proportion

of lignin and a greater proportion of cellulose than untreated carbon materials. Thus, when the hydrolysis barriers of substrates were broken by alkaline treatment, microbial enzymes easily decompose cellulose into small carbon fraction to release much more DOC.

In anaerobic environments, the possible fates of include assimilatory nitrate reduction (immobilization), DNRA (dissimilatory nitrate reduction to ammonia), and denitrification (Tiedje, 1988). It has been reported that anaerobic environments with high C/N ratios tends to facilitate DNRA (Gibert et al., 2008). DNRA converts nitrate to ammonium rather than N_2 in anaerobic reduction reaction. In our study, the concentration of each treatment was below 3 mg N kg^{-1} dry soil during the whole experiment, thus, the conversion of nitrate to ammonium through DNRA was considerably low. Moreover, removal of immobilization was relatively smaller than that of denitrification, therefore, its contribution was assumed to be negligible (Gibert et al., 2008; Greenan et al., 2006). Compared with the -N concentration in CK (3.1%, Fig. 1b), was entirely removed after 28 days with carbon substrates. The maximum removal efficiencies of -N removal in soils were 28, 21, 28 and 14 days for UC, TC, UWS and TWS, respectively. Treated carbon groups exhibited higher -N removal rates than untreated carbon groups due to a high level of DOC emission. DOC emission patterns could be attributed to the dynamic equilibrium of carbon consumptions by microbes and carbon emissions by microbial hydrolysis. Thus, when the amount of released DOC exceeds the need of microbes for both growth and denitrification (i.e., TC and TWS), DOC increased (Shen and Wang, 2011).

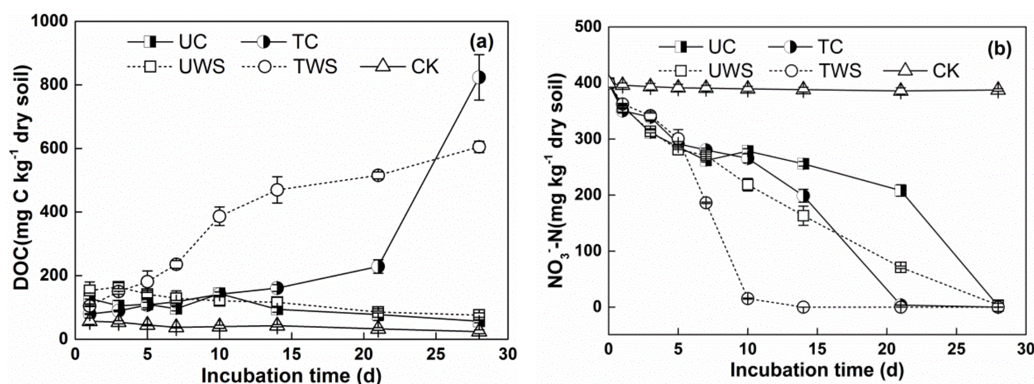

Fig. 1. Time courses of (a) DOC (mg C kg^{-1} dry soil) concentrations and (b) -N (mg kg^{-1} dry soil) concentrations in soil with UC, TC, UWS, TWS and CK. Error bars denote SD (n=3).

3.2. CO_2 emissions

Fig. 2a shows the microbial respiration as estimated by CO_2 production. Compared with CK (p<0.05), organic carbon increased the respiration of microbes. Cumulative CO_2 emission of CK was 2.0-17.7 mg C kg^{-1} dry soil during the 0-28th days. After adding carbon substrates, cumulative CO_2 emissions had a slow increasing initial stage (0-7 day), and rapidly increased during the 7-28th day. The carbon mineralization percentages in the total carbon of substrates of UC, TC, UWS and TWS were 4.3%, 8.1%, 5.5% and 14.3%, respectively. The higher amount of carbon mineralization with treated materials could be attributed to a lower recalcitrant lignin contents.

In addition, the kinetic curve of the amount of nitrate removal versus CO_2 emission can be divided into 2 stages (Fig. 2b). In the first stage, CO_2 emission correlated highly with -N losses (R^2=0.96), which indicated that the mineralization of organic carbon had a positive correlation with -N depletion. However, when more than 99% of nitrate has been consumed (stage 2), the amount of CO_2 emissions still increased. Thus, this portion of CO_2 might be mainly released by non-denitrifiers (e.g., manganese and iron oxides, then sulphate, and then hydrogen and carbon dioxide) (Rivett et al., 2008).

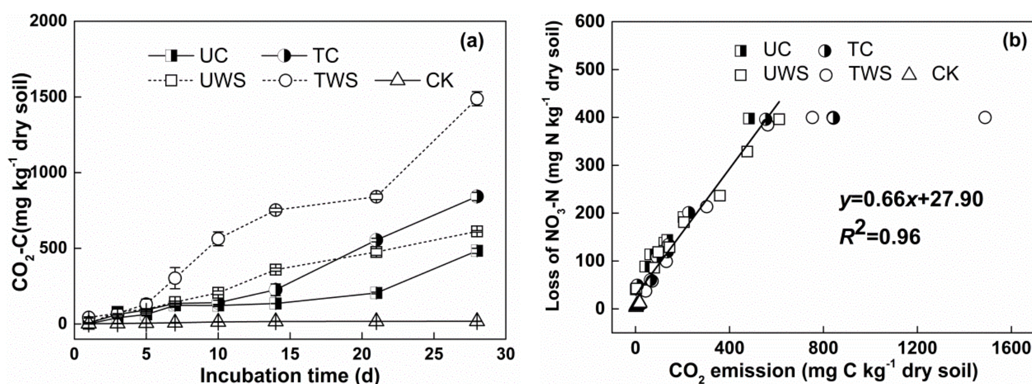

Fig. 2. (a) Time courses of CO_2 (mg C kg^{-1} dry soil) emissions over 28 day incubation time and (b) -N losses (mg kg^{-1} dry soil) versus cumulative CO_2 emissions (mg C kg^{-1} dry soil) of UC, TC, UWS, TWS and CK. Error bars denote SD (n=3).

3.3. Cumulative denitrification and production of -N, N_2O and N_2O/(N_2O+N_2)

The cumulative denitrification was calculated by the sum of N_2 and N_2O emissions similar to that reported by Miller et al. (2008) (Fig. 3a). The denitrification rates were calculated by fitting a zero-order model to the estimated cumulative denitrification at each sampling point (Fig. 3b). Denitrification rates were accelerated in treated carbon groups. According to difference in denitrification rate, the denitrification process could be divided into as three periods i.e., the lag adaption period (0-7 days), the rapid denitrification period (7-21 days) and the final slowness period (21-28 days). In the first period, the denitrification rates were in the range of 0.1 to 18.2 mg N kg^{-1} dry soil with treated carbon groups, which were lower than that in the second period. It was caused by the substantial production of new enzymes and growth of microorganisms (Gregorich and Carter, 2007). In the second period, intense denitrifying activities occurred and the maximum denitrification rates were observed in TC, UWS and TWS. In the last period, the denitrification rates declined due to consumed nitrate substrates. However, the nitrate substrate was not completely consumed in UC (Fig. 1b) which reached the highest denitrification rates. This resulted in the relatively stable and

constant denitrification rates. The time of maximum denitrification rate of UC was delayed due to the limitation of DOC concentration.

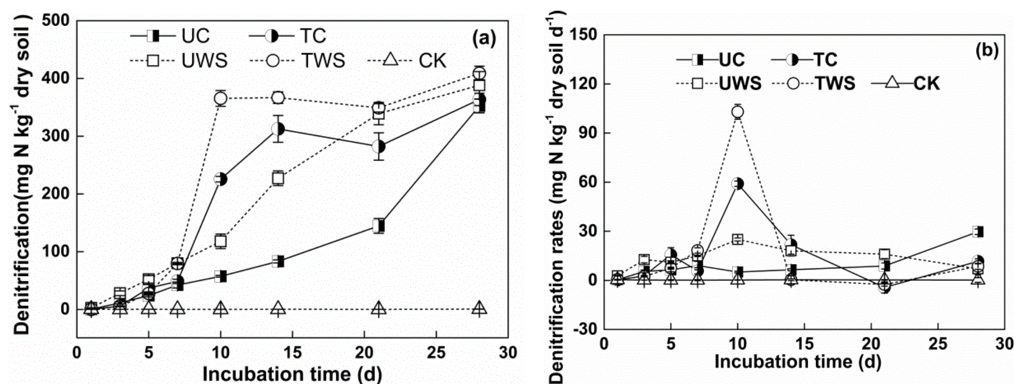

Fig. 3. Time courses of (a) cumulative denitrifications (N_2O+N_2) and (b) denitrification rates over 28 day incubation time of UC, TC, UWS, TWS and CK. Error bars denote SD (n=3).

Nitrite, the intermediate product of nitrate reduction, was also observed (Fig. 4). Cumulative -N concentrations in soil increased sharply to the peak value during the lag period. It is followed by a decrease to a level of < 3 mg N kg^{-1} dry soil. When -N production rate was greater than the consumption rate, the nitrite quickly accumulated. In view of extremely low values of final nitrite in all groups, nitrite did not affect the distribution of nitrogen at the end of incubation.

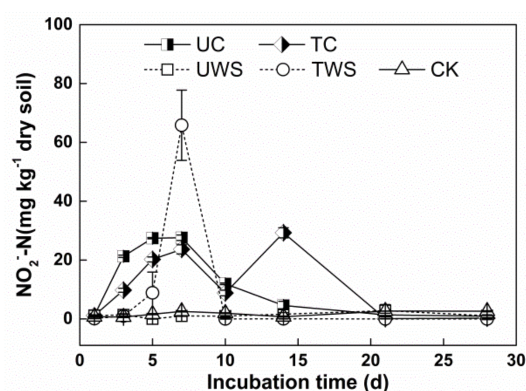

Fig. 4. Time courses of -N (mg kg^{-1} dry soil) concentrations in soil of UC, TC, UWS, TWS and CK. Error bars denote SD (n=3).

As a by-product in denitrification, N_2O is an important issue to address due to greenhouse effect. The cumulative N_2O emission patterns are shown in Fig. 5a.

Cumulative N_2O emissions of UC and UWS increased with time throughout the incubation and reached 336.5 and 130.3 mg N kg^{-1} dry soil at the end of incubation, respectively. In contrast, cumulative N_2O emissions of TC and TWS reached the highest point of 122.9 mg N kg^{-1} dry soil and 79.5 mg N kg^{-1} dry soil on day 14 and 7 respectively, and followed by a decrease to < 50 mg N kg^{-1} dry soil on the 28th day. Cumulative N_2O emissions of untreated carbon groups were 74 times greater than that of the treated carbon groups in the end. In addition, given that N_2O has a global warming potential 296 times than that of CO_2 (Henderson et al., 2010). Although it was observed that the accumulated emissions of CO_2 by respiration of TC and TWS were 1.3 and 1.7 times of UC and UWS, respectively (Fig. 2a), the alkali-treated carbon materials still played a crucial role in mitigating greenhouse effects.

In Fig. 5b, N_2O molar ratios changed with time from 0.96 to 0.004 with carbon materials in soils and N_2O ratios were lower compared with that of untreated carbon materials. $N_2O/(N_2+N_2O)$ ratio was affected by the concentrations (Senbayram et al., 2012; Zhu et al., 2013), organic carbon availability (Drenovsky et al., 2004; Huang et al., 2004; Jahangir et al., 2012; Li et al., 2016) and enzyme activities (Zhu et al., 2013) etc. Previous studies suggested that was a prior electron acceptor than N_2O in denitrification (Miller et al., 2008). Therefore, increased level could inhibit N_2O reductase activity (Rivett et al., 2008; Senbayram et al., 2012). As a result, when the lack of electron donors or supply exceeded the denitrifying microbes reducing demand, N_2O may accumulate (Miller et al., 2008; Swerts et al., 1996). As shown in section 3.1, untreated carbon groups contained much more recalcitrant carbon compounds (e.g. lignin) to induce insufficient electron donors. As expected, soils with treated residues contributed to the majority of gaseous release in the form of N_2.

In addition, the type and amount of carbon species were investigated. As shown in Table 1, the percentage of carbon in treated materials (TC and TWS) only decreased by 4.2-6.5% compared with untreated materials (UC and UWS). Meanwhile, the potential biodegradable carbon (i.e. reducing sugar yield) in treated materials increased by 4.4-5.3 times compared with untreated materials. The responds of $N_2O/(N_2O+N_2)$ ratios in treated carbon groups decreased by 85-99% compared with untreated carbon groups. This suggested that the impact of alkaline treatment played a positive role in the increasing of bioavailable carbon, which reduced $N_2O/(N_2O+N_2)$ ratio as well.

Moreover, the C/N ratio in agricultural residue increased as follows: UC < UWS < TC < TWS. Agricultural residues with high C/N ratios caused limitation for soil denitrifiers, thereby promoting to reduce to N_2 completely (Li et al., 2013). In addition, this observation is consistent with addition of plant materials with high C/N ratio leading to lower N_2O production as reported by Li et al. (2013) and Millar and Baggs (2004). However, Miller et al. (2008) investigated that during the 144h incubation period, barley straw with higher C/N ratio (C/N = 45:1) exhibited a higher N_2O molar ratio than red clover with lower C/N ratio (C/N =13:1). It was attributed to less labile carbon in barley straw compared with red clover. In view of the above two issues, it can be concluded that when the labile carbon is the major part of total carbon in crop residues, higher C/N ratio could indicate lower N_2O emission. Otherwise, the C/N ratio showed to be a poor predictor of nitrogen release as these residues contained high amounts of lignin and polyphenols as reported by Millar and Baggs (2004). It is clear that treated carbon materials not only had more available carbon but also had higher C/N ratio than untreated materials, which could favor more N_2O transformation to N_2.

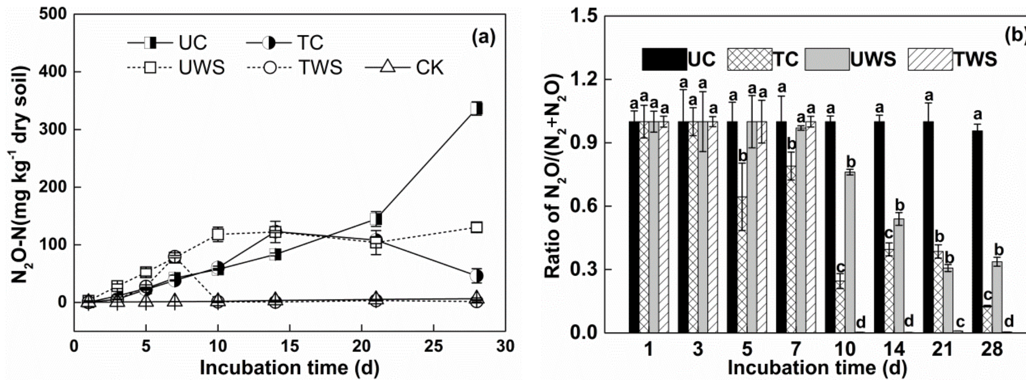

Fig. 5. Time courses of (a) cumulative byproducts N_2O of UC, TC, UWS, TWS and CK. (b) N_2O molar ratios ($N_2O/(N_2+N_2O)$) over 28 day incubation time. Error bars denote SD (n=3).The same letters suggested that the values were not significantly different in the same incubation time (p>0.05) based on a Duncan test.

3.4. Response of 16S rRNA and *nosZ* gene with different carbon substrates

To obtain a deeper insight into the abundance of the total bacteria with different carbon, 16S rRNA gene copy numbers of community were measured (Fig. 6a). Total bacterial abundances of a short-term incubation apparently increased with carbon substrates in soils. The total bacteria abundances increased for all groups over the 7 to 14th day except for CK, while the total bacterial abundances of TC and TWS declined from 4.9×10^{10} to 3.2×10^{10} copy numbers g^{-1} soil and from 4.1×10^{10} to 3.1×10^{10} copy numbers g^{-1} soil over the 21 to 28th day, respectively. The highest 16S rRNA copy numbers at the end of the incubation were measured in UC and UWS, and were both 5.4×10^{10} copy numbers g^{-1} dry soil. In comparison, the untreated carbon groups had greater total bacterial abundances but lower denitrification performance at the end of incubation, which suggested that non-denitrifiers were major part of total bacteria in untreated carbon substrates amended soils. This demonstrated that the available carbon substrates in untreated carbon were mainly consumed by non-denitrifiers (e.g., fermentative anaerobic bacteria).

To assess the diversity of denitrifying bacteria responsible for N_2O consumption,

the investigators have focused on components of denitrifier community using the N_2O reductase (*nosZ*) as molecular marker. Although some denitrifiers lack *nosZ*, the level of *nosZ* expression in the soil relative to N_2O production determines whether is reduced to N_2 or N_2O (Ma et al., 2008). Different carbon materials affected the ratios of denitrifiers bearing *nosZ* relative to total bacteria numbers (Fig. 6b). During the first week, the denitrifier gradually acclimatized and the nitrous oxide reductase (*nosZ*) was just activated. The low relative densities of *nosZ* were coincided with the accumulation of N_2O. When the relative densities of *nosZ* in TWS reached a high level (0.13%) on the day 14, N_2 production reached a plateau with 366 mg N kg^{-1} soil (Fig. 3). The maximum value of *nosZ* relative abundance in TC peaked at 0.12% on the day 21, which corresponded with the decrease of N_2O as shown in Fig. 5a. For untreated carbon groups, the relative densities of *nosZ* in UWS remained 0.026%-0.050% throughout the incubation, and the *nosZ* relative densities remained at a low level (< 0.027%) in UC till the 21th day. Overall, the relative densities of *nosZ* fragments in treated carbon groups were greater than untreated carbon groups, which contributed to more N_2O reduction.

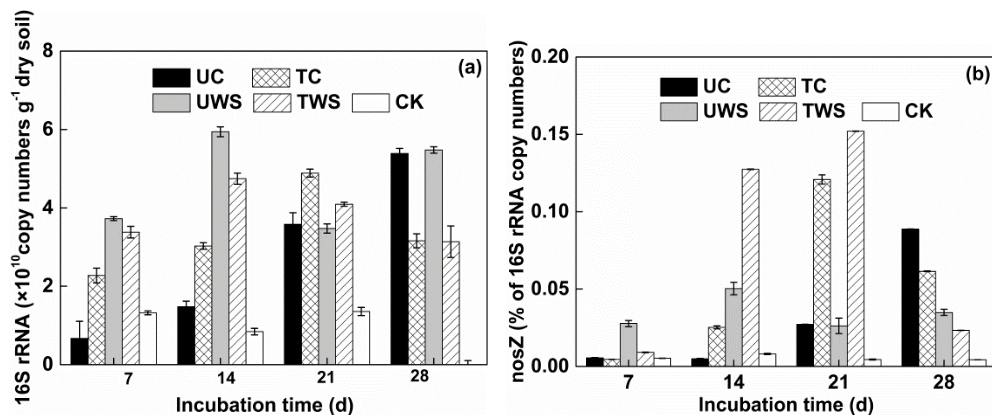

Fig. 6. (a) Quantification of 16S rRNA copy numbers and (b) relative densities of *nosZ* gene fragments in soil over 28d in response to different carbon addition treatments (i.e., UC, TC, UWS and TWS). Error bars denote SD (n=3). Standard curve descriptors for *nosZ* and 16S rRNA gene numbers are as follows: y=-3.21x+35.59, R^2=0.998 (*nosZ*); y=-2.83x+38.550, R^2=0.994.

4. Conclusions

In this study, effects of alkali-treated agricultural residues on denitrification in unsaturated soil were investigated by batch experiments in anaerobic condition. Compared with untreated agricultural residues, $Ca(OH)_2$ treatment increased biodegradability potential of residues by 4.4-5.3 times. Increased total bacterial and community abundances possessing *nosZ* contributed to a higher nitrate removal rates and facilitated N_2O-to-N_2 transformation. Alkali-treated agricultural residue, as a potential carbon material for in situ denitrification layer, could efficiently retard nitrate plume transport into groundwater and minimize the risk of greenhouse gas emission. Considering the complexity of the in situ denitrification layer, the lifetime of alkali-treated agricultural residue and the long-term performance need a further investigation.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 41731280) and the National Key Research Project (Grant No.2016YFC0402810).

References

Ashok, V., Hait, S., 2015. Remediation of nitrate-contaminated water by solid-phase denitrification process-a review. Environ. Sci. Pollut. Res. 22, 8075-8093.

Chang, V.S., Nagwani, M., Holtzapple, M.T., 1998. Lime pretreatment of crop residues bagasse and wheat straw. Applied Appl. Biochem. Biotechnol. 74, 135-159.

Chang, V.S., Nagwani, M., Kim, C., Holtzapple, M.T., 2001. Oxidative lime pretreatment of high-lignin biomass. Appl. Biochem. Biotechnol. 94, 1-28.

China MOHO., 2000. Methods for determination of carbon dioxide in air of public places. GB/T 18204.24-2000 pp. 92-98.

China SEPA., 2002. Monitoring and analysis methods of water and wastewater fourth ed. China Environmental Science Press, Beijing, China.

Ciarlo, E., Conti, M., Bartoloni, N., Rubio, G., 2007. The effect of moisture on nitrous oxide emissions from soil and the $N_2O/(N_2O+N_2)$ ratio under laboratory conditions. Biol. Fertil. Soils 43, 675-681.

Damaraju, S., Singh, U.K., Sreekanth, D., Bhandari, A., 2015. Denitrification in biofilm configured horizontal flow woodchip bioreactor: Effect of hydraulic retention time and biomass growth. Ecohydrology & Hydrobiology 15, 39-48.

Drenovsky, R.E., Vo, D., Graham, K.J., Scow, K.M., 2004. Soil water content and organic carbon availability are major determinants of soil microbial community composition. Microb. Ecol. 48, 424-430.

Feng, L.J., Chen, K., Han, D.D., Zhao, J., Lu, Y., Yang, G.F., Mu, J., Zhao, X.J., 2017. Comparison of nitrogen removal and microbial properties in solid-phase denitrification systems for water purification with various pretreated lignocellulosic carriers. Bioresour. Technol. 224, 236-245.

Gibert, O., Pomierny, S., Rowe, I., Kalin, R.M., 2008. Selection of organic substrates as potential reactive materials for use in a denitrification permeable reactive barrier (PRB). Bioresour. Technol. 99, 7587-7596.

Greenan, C.M., Moorman, T.B., Kaspar, T.C., Parkin, T.B., Jaynes, D.B., 2006. Comparing carbon substrates for denitrification of subsurface drainage water. J. Environ. Qual. 35, 824-829.

Gregorich, E.G., Carter, M.R., 2007. Soil Sampling and Methods of Analysis, Second

ed. CRC Press, Canada.

He, H., Zhen, Y., Mi, T.Z., Xu, B.C., Wang, G.S., Zhang, Y., Yu, Z.G., 2015. Community composition and distribution of sulfate- and sulfite-reducing prokaryotes in sediments from the Changjiang estuary and adjacent East China Sea. Estuar. Coast. Shelf. Sci. 165, 75-85.

Henderson, S.L., Dandie, C.E., Patten, C.L., Zebarth, B.J., Burton, D.L., Trevors, J.T., Goyer, C., 2010. Changes in denitrifier abundance, denitrification gene mRNA levels, nitrous oxide emissions, and denitrification in anoxic soil microcosms amended with glucose and plant residues. Appl. Environ. Microbiol. 76, 2155-2164.

Henry, S., Bru, D., Stres, B., Hallet, S., Philippot, L., 2006. Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. Appl. Environ. Microbiol. 72, 5181-5189.

Hu, R.T., Zheng, X.L., Xin, J., Sun, Z.Y., Zheng, T.Y., 2017. Selective enhancement and verification of woody biomass digestibility as a denitrification carbon source. Bioresour. Technol. 244, 313-319.

Huang, G.X., Huang, Y.Y., Hu, H.Y., Liu, F., Zhang, Y., Deng, R.W., 2015. Remediation of nitrate-nitrogen contaminated groundwater using a pilot-scale two-layer heterotrophic-autotrophic denitrification permeable reactive barrier with spongy iron/pine bark. Chemosphere 130, 8-16.

Huang, Y., Zou, J.W., Zheng, X.H., Wang, Y.S., Xu, X.K., 2004. Nitrous oxide emissions as influenced by amendment of plant residues with different C:N ratios. Soil. Biol. Biochem. 36, 973-981.

Jahangir, M.M.R., Khalil, M.I., Johnston, P., Cardenas, L.M., Hatch, D.J., Butler, M., Barrett, M., O Flaherty, V., Richards, K.G., 2012. Denitrification potential in subsoils: A mechanism to reduce nitrate leaching to groundwater. Agr. Ecosyst. Environ. 147, 13-23.

Kallioinen, A., Hakola, M., Repo, T., Leskelä, M., von Weymarn, N., Siika-aho, M., 2013. A novel alkaline oxidation pretreatment for spruce, birch and sugar cane bagasse. Bioresour. Technol. 140, 414–420.

Li, X.C., Hu, F., Shi, W., 2013. Plant material addition affects soil nitrous oxide production differently between aerobic and oxygen-limited conditions. Appl. Soil Ecol. 64, 91-98.

Li, X.X., Sørensen, P., Olesen, J.E., Petersen, S.O., 2016. Evidence for denitrification as main source of N_2O emission from residue-amended soil. Soil Biol. Biochem. 92, 153-160.

Lin, L.L., Yan, R., Liu, Y.Q., Jiang, W.J., 2010. In-depth investigation of enzymatic hydrolysis of biomass wastes based on three major components: Cellulose, hemicellulose and lignin. Bioresour. Technol. 101, 8217-8223.

Liu, H.J., Jiang, W., Wan, D.J., Qu, J.H., 2009. Study of a combined heterotrophic and sulfur autotrophic denitrification technology for removal of nitrate in water. J. Hazard. Mater. 169, 23-28.

Ma, W.K., Bedard-Haughn, A., Siciliano, S.D., Farrell, R.E., 2008. Relationship between nitrifier and denitrifier community composition and abundance in predicting nitrous oxide emissions from ephemeral wetland soils. Soil Biol. Biochem. 40, 1114-1123.

Medina, J., Monreal, C., Barea, J.M., Arriagada, C., Borie, F., Cornejo, P., 2015. Crop

residue stabilization and application to agricultural and degraded soils: A review. Waste Management, 42, 41-54.

Millar, N., Baggs, E.M., 2004. Chemical composition, or quality, of agroforestry residues influences N_2O emissions after their addition to soil. Soil Biol. Biochem. 36, 935-943.

Miller, M.N., Zebarth, B.J., Dandie, C.E., Burton, D.L., Goyer, C., Trevors, J.T., 2008. Crop residue influence on denitrification, N_2O emissions and denitrifier community abundance in soil. Soil Biol. Biochem. 40, 2553-2562.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol. 96, 673-686.

Pu, J.Y., Feng, C.P., Liu, Y., Li, R., Kong, Z., Chen, N., Tong, S., Hao, C.B., Liu, Y., 2014. Pyrite-based autotrophic denitrification for remediation of nitrate contaminated groundwater. Bioresour. Technol. 173, 117-123.

Renu, S., Shukla, A., Tiwari, S., Srivastava, M., 2014. A review on delignification of lignocellulosic biomass for enhancement of ethanol production potential. Renew. Sust. Energ. Rev. 713-728.

Rivett, M.O., Buss, S.R., Morgan, P., Smith, J.W.N., Bemment, C.D., 2008. Nitrate attenuation in groundwater: A review of biogeochemical controlling processes. Water Res. 42, 4215-4232.

Robertson, W.D., Blowes, D.W., Ptacek, C.J., Cherry, J.A., 2000. Long-term performance of in situ reactive barriers for nitrate remediation. Ground Water 38, 689-695.

Rocca, C.D., Belgiorno, V., Meriç, S., 2007. Overview of in-situ applicable nitrate

removal processes. Desalination 204, 46-62.

Schipper, L.A., McGill, A., 2008. Nitrogen transformation in a denitrification layer irrigated with dairy factory effluent. Water Res. 42, 2457-2464.

Senbayram, M., Chen, R., Budai, A., Bakken, L., Dittert, K., 2012. N_2O emission and the $N_2O/(N_2O+N_2)$ product ratio of denitrification as controlled by available carbon substrates and nitrate concentrations. Agr. Ecosyst. Environ. 147, 4-12.

Shen, Z.Q., Wang, J.L., 2011. Biological denitrification using cross-linked starch/PCL blends as solid carbon source and biofilm carrier. Bioresour. Technol. 102, 8835-8838.

Swerts, M., Merckx, R., Vlassak, K., 1996. Denitrification, N_2-fixation and fermentation during anaerobic incubation of soils amended with glucose and nitrate. Biol. Fert. Soils. 23, 229-235.

Tiedje, J.M., 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. Environmental Microbiology of Anaerobes. 179-244.

Vosoughifar, H.R., Shamsai, A., Ebadi, T., 2005. Discretization of flow contain nitrate in porous media by finite volume technique. Int. J. Environ. Sci. Technol. 1, 317-323.

Wan, R., Zheng, X., Chen, Y.G., Wang, H.C., 2015. Using cassava distiller's dried grains as carbon and microbe sources to enhance denitrification of nitrate-contaminated groundwater. Appl. Microbiol. Biot. 99, 2839-2847.

Wang, R., Willibald, G., Feng, Q., Zheng, X., Liao, T., Brüggemann, N., Butterbach-Bahl, K., 2011. Measurement of N_2, N_2O, NO, and CO_2 emissions from soil with the Gas-Flow-Soil-Core technique. Environ. Sci. Technol. 45, 6066-6072.

Wang, X., Xing, L., Qiu, T., Han, M., 2013. Simultaneous removal of nitrate and pentachlorophenol from simulated groundwater using a biodenitrification reactor

packed with corncob. Environ. Sci. Pollut. Res. 20, 2236-2243.

Wang, X.M., Wang, J.L., 2012. Denitrification of nitrate-contaminated groundwater using biodegradable snack ware as carbon source under low-temperature condition. Int. J. Environ. Sci. Technol. 9, 113-118.

Warneke, S., Schipper, L.A., Bruesewitz, D.A., McDonald, I., Cameron, S., 2011a. Rates, controls and potential adverse effects of nitrate removal in a denitrification bed. Ecol. Eng. 37, 511-522.

Warneke, S., Schipper, L.A., Matiasek, M.G., Scow, K.M., Cameron, S., Bruesewitz, D.A., McDonald, I.R., 2011b. Nitrate removal, communities of denitrifiers and adverse effects in different carbon substrates for use in denitrification beds. Water Res. 45, 5463-5475.

Wen, Y., Chen, Y., Zheng, N., Yang, D.H., Zhou, Q., 2010. Effects of plant biomass on nitrate removal and transformation of carbon sources in subsurface-flow constructed wetlands. Bioresour. Technol. 101, 7286-7292.

Zhu, T.B., Zhang, J.B., Yang, W.Y., Cai, Z.C., 2013. Effects of organic material amendment and water content on NO, N_2O, and N_2 emissions in a nitrate-rich vegetable soil. Biol. Fertil. Soils. 49, 153-163.