Regulating Soil Bacterial Diversity, Enzyme Activities and Community Composition Using Residues from Golden Apple Snails

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Abstract

Golden apple snails (GAS) have become a serious pest for agricultural production in Asia. A sustainable method for managing GAS is urgently needed, including potentially using them to produce commercial products. In this study, we evaluate the effects of GAS residues (shell and meat) on soil pH, bacterial diversity, enzyme activities, and other soil characteristics. Results showed that the amendment of GAS residues significantly elevated soil pH (to near-neutral), total organic carbon (TOC) (by 10-134%), NO_3-N (by 46-912%), NH_4-N (by 18-168%) and total nitrogen (TN) (by 12-132%). Bacterial diversity increased 13% at low levels of amendment and decreased 5% at high levels, because low-levels of GAS residues increased soil pH to near-neutral, while high-levels of amendment substantially increased soil nutrients and subsequently suppressed bacterial diversity. The dominant phyla of bacteria were: Proteobacteria (about 22%), Firmicutes (15-35%), Chloroflexi (12%-22%), Actinobacteria (8%-20%) Acidobacteria, Gemmatimonadetes, Cyanobacteria and Bacterioidetes. The amendment of GAS residues significantly increased the relative abundance of Firmicutes, Gemmatimonadetes, Bacterioidetes and Deinococcus-Thermus, but significantly decreased the relative abundance of Chloroflexi, Actinobacteria, Acidobacteria, Cyanobacteria and Planctomycetes. Our results suggest that GAS residues treatment induces a near-neutral and nutrient-rich soil. In this soil, soil pH may not be the best predictor of bacterial community composition or diversity; rather soil nutrients (*ie*., NH_4-N and NO_3-N) and soil TOC showed stronger correlations with bacterial community composition. Overall, GAS

residues could replace lime for remediation of acidic and degraded soils, not only to remediate physical soil properties, but also microbial communities.

Keywords: bacterial diversity; enzyme activities; golden apple snail; soil nutrients; 16S rRNA

Importance

The wide spreading golden apple snail (GAS) is a harmful pest to crop productions and could result in soil and air pollutions after death. In the previous study, we developed a biocontrol method: adding GAS residues to acidic soil to mitigate the living GAS invasion and spread, improve soil quality, and reduce soil and air pollution. However, the effects of GAS residues amendment on bacterial diversity and community still remain unclear. This study provided insights into bacterial diversity and community compositions to facilitate the evaluation of GAS residues application.

1 Introduction

Invasive golden apple snails (GAS) *Pomacea canaliculata (Lamarck*) have become a serious pest for agricultural production in Asia (1). A series of control methods have been developed (2, 3); the most widely used is chemical control by molluscicides (4), which, could harm environmental and human health (5). A better method for sustainable management is urgently needed, potentially by using them to produce commercial products.

Studies have reported that GAS contains abundant $CaCO_3$ and proteins, and could be used as feed for livestock such as pigs and ducks (6). However, only small GAS can be eaten by ducks or pigs, because the hard shells of large adult GAS make them unpalatable. The $CaCO_3$ in GAS can also neutralize acidic soils, similar to lime. However, lime tends to lead to soil compaction, Si and P deficiency, and reduced soil microbial biomass and diversity.

Anthropogenic N inputs to terrestrial ecosystems have increased three- to five-fold over the past century (7). High levels of N fertilization can drive soil acidification both directly and indirectly (8). J. H. Guo et al. (8) found that anthropogenic acidification driven by N fertilization is at least 10 to 100 times greater than that associated with acid rain. The application and deposition of N is expected to continue to increase (9).

To alleviate soil acidification and control the invasion of the GAS, we have proposed using powered residues from GAS to mitigate soil acidification (unpublished data). The application of GAS residues can significantly increase soil

pH and nitrate nitrogen (NO_3-N) both at a high and low amendment levels, and can increase soil total organic carbon (TOC), total nitrogen (TN), ammonium nitrogen (NH_4- N) and NO_3-N compared with controls and with liming, which has no significant effect on soil nutrients (unpublished data). Although our previous research has indicated that the addition of GAS residues could significantly increase soil microbial biomass and regulate microbial community structure, the mechanisms by which GAS residues regulate microbial community composition, relative abundance and diversity remain unknown.

Previous studies have proposed soil pH and N input as the main predictors of soil microbial diversity in soils (10-12). However, the responses of soil microbes to elevated N inputs and pH are inconsistent. Numerously studies have revealed that N addition led to significant reductions in soil microbial activity (13), diversity (14) and community composition (15) because of increases in C sequestration and/or decreased soil respiration rates (13). Some studies have suggested that neutral soils support greater bacterial diversity than do acidic soils (10, 11). However, some researchers have suggested the opposite—that forest soils with lower pH support greater microbial diversity than agricultural soils with higher pH values (16).

Soil extracellular enzymes play key roles in biological soil processes, specifically in the degradation of soil organic compounds and the mineralization and recycling of nutrients related to C, N, P and S (17). Environmental factors, including water (18), salinity (18), pollution (19), soil nutrients (20), temperature (21) and soil pH can affect the activities of extracellular enzymes directly or indirectly.

Here, we conducted a series of greenhouse experiments amending GAS residues and lime to acidic and degraded soils. We hypothesize that GAS residues and lime—both of which increase soil pH—may differ in their regulation of microbial community structure, diversity and microbial enzyme activities. The objectives of this study were: (1) to explore bacterial community composition and diversity in soils neutralized with powered GAS residues, (2) to determine key factors controlling the composition of bacterial communities, (3) and assess differences in bacterial communities in soils treated with GAS residues and soils treated with lime.

2 Materials and methods

2.1 Testing materials

Golden apple snails (GAS) were collected from the paddy fields at the Xin Tang (Ning Xi) Research and Educational Station of South China Agricultural University, which is located in Zengcheng City, Guangdong Province, China. The snails were washed and frozen at -40℃ in a freezer for 24h. Then the dead snails were dried, grounded into powder and stored in a desiccator. A slightly acidic soil was also collected from the paddy fields with the pH levels ranging from 6.25 to 6.53. The soil predominately consists of medium (38%) and fine (22%) sand, silt (36%) and clay (4%) and has 16.38 g kg^{-1} of TOC, 2.20 g kg^{-1} of TN and 0.58 g kg^{-1} of total phosphorus (TP).

2.2 Experimental design

Experiments were conducted in a greenhouse on the campus of South China

Agricultural University. We implemented three treatments: (1) the control treatment with soil only (CK); (2) soil amended with GAS residues (*i.e.*, shell and meat) (SR); and (3) soil amended with lime (SL). Each treatment had six levels of amendment: 0.5, 1, 2.5, 25, 50, and 100 g kg^{-1}. Amendments were homogenized with soil in each treatment and carefully packaged in a polyvinyl chloride (PVC) column with a diameter of 180 mm and a height of 260 mm. A filter paper was mounted at the bottom of the column to prevent loss of soil or GAS residues. A base plate was also placed at the bottom of the column to position the column. About 400 ml of deionized water (pH = 7.0±0.1) were sprayed to the column each week to prevent the column from drying. Each treatment was triplicated in this study. After 120 days incubation, the soil samples were collected and stored at 4 ^{o}C for about 4h.

2.3 Soil analysis

Soil samples were homogenized by hand and passed through a 2mm sieve to remove rocks, roots and organic residues, and then divided evenly into three subsamples. These three subsamples were treated as follows: the first subsamples were stored at room temperature (about 22 ^{o}C) for about 4h, the second subsamples were stored at 4 ^{o}C, and the third subsamples were stored at -20 ^{o}C for further analysis. The room temperature (first) subsamples were air dried, grounded and analyzed for soil pH, TOC, TN, available phosphorous (AP), ammonium nitrogen (NH_4–N) and nitrate nitrogen (NO_3–N). The second subsamples were analyzed for soil gravimetric moisture and extracellular enzyme activity. The third subsamples were freeze-dried and grounded into power and passed through a 0.25 mm sieve for high-throughput

sequencing analysis.

Soil pH was measured from fresh soil slurries (1 g of soil: 2.5 ml of deionized water) using a handheld multiparameter meter (SX-620, SAN XIN, China). Approximately 20 mg of each powered sample was analyzed for TOC and TN using a C analyzer (TOC-VCSH, Shimadzu Corp., Kyoto, Japan). Concentrations of NH_4–N were analyzed using a UV-vis spectrophotometer at a wavelength of 420 nm (22). Concentrations of NO_3–N were determined using a UV-vis spectrophotometer applying double wavelength of 275 nm and 220 nm (22). Concentrations of AP were analyzed using the molybdenum-antimony anti-spectrophotometric method (23).

2.4 Extracellular enzyme assay

Soil enzyme activities, including β–1,4–glucosidase (BG), acid phosphatase (ACP), β–1,4–N–acetylglucosaminidase (NAG) and β–D–cellobiosidase (cellulose degradation; CB), were measured using fluorometry as described in C. W. Au - Bell et al. (24), with minor modifications. In brief, 1 g of soil was weighted and dissolved in a porcelain evaporating dish with 125 ml of acetate-acetate buffer solution and blended for 30 min on a magnetic stirrer (JB-3, Ronghua, Jianfsu) at 200 rpm. Soil buffer solution (200 µl) was sucked from the dish and injected into a deep-well plate (Labtide, Greystone Biosciences LLC, USA), and then 50 µl each of buffer, substrate and 4–Methylumbelliferyl (MUB) was pipetted and injected into deep-well plates, representing a blank well, sample well and quench well, respectively. We used 200 µl of buffer plus 50 µl of substrate as the negative well and 200 µl buffer plus 50 µl of MUB as the reference standard well. Incubation was conducted in a constant

157 temperature incubator (RXZ, Dongqi, Ningbo, China) at 37 ℃ for 3 h. After
158 incubation, the sample was centrifuged (Eppendorf, USA) at 2900 rpm for 3 min, and
159 the supernate was pipetted and injected into a black flat-bottomed 96-well microplate
160 for fluorescence determination in a microplate reader (SYNERGY H1, BioTek, USA)
161 at the excitation wavelength of 365 nm and emission wavelength of 450 nm.

162 *2.5 Bacterial community and diversity analysis*

163 Bacterial community structure and diversity in soils were determined by 16S
164 rRNA. Soil DNA was extracted with the E.Z.N.A.® soil DNA Kit (Omega Bio-tek,
165 Norcross, GA, USA.) in accordance with manufacturer's instructions. The extracted
166 DNA was quantified by spectrophotometry (Nanodrop 2000, Thermo Scientific, UAS)
167 and stored at -20 °C. Polymerase chain reaction (PCR) was carried out using the
168 universal primer set 338F (5'- ACTCCTACGGGAGGCAGCAG-3') and 806R
169 (5'-GGACTACHVGGGTWTCTAAT-3') by thermocycler PCR system (GeneAmp
170 9700, ABI, USA). PCR was performed to amplify 1 μl of template DNA in a 20-μl
171 reaction system containing 4 μl of 5 × FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.8 μl
172 of each primer (5 μM), 0.4 μl of FastPfu Polymerase and 10 ng of template DNA.
173 Amplification was performed in triplicate as follows: 95 °C for 3 min; 27 cycles of
174 95 °C for 30 s, 30s for annealing at 55 °C, and 45s for elongation at 72 °C; and a final
175 extension at 72 °C for 10 min. PCR reactions were performed in triplicate 20 μl
176 mixture containing 4 μl of 5 × FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.8 μl of each
177 primer (5 μM), 0.4 μl of FastPfu Polymerase and 10 ng of template DNA. The
178 resulting PCR products were extracted from a 2% agarose gel and further purified

using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor TM -ST (Promega, USA) according to the manufacturer's protocol. Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Raw fastq files were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) The reads were truncated at any site receiving an average quality score < 20 over a 50 bp sliding window. (ii) Primers were exactly matched allowing 2 nucleotide mismatching, and reads containing ambiguous bases were removed. (iii) Sequences with overlaps longer than 10 bp were merged according to their overlap sequences. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using confidence threshold of 70%.

2.6 Data analysis

Analysis of Variance (ANOVA) was performed using SPSS. Differences in microbial communities were tested using ANOSIM with 9,999 permutations. A non-metric multidimensional scaling (NMDS) ordination to illustrate the clustering of bacterial community composition variation was conducted using the Vegan software based on the Bray-Curtis distance of genus. We used spearman correlations to identify

significant relationships between soil parameters (involved in six amendment levels of GAS residues and five amendment levels of lime treatments) and the most abundant phyla and genera. To evaluate bacterial diversity in amended soils, Shannon index (25) was applied. To compare microbial communities and identify specialized communities in samples, we used the LEfSe tool (26). Statistical analysis was performed only from the phylum to the genus level to simplify the computation. Non-metric multidimensional scaling (NMDS) of soil bacteria community composition based on Bray-Curtis distances linear regression after amending with lime and GAS residues were conducted to explore the main factors affected soil bacterial community compositions.

3 Results and discussion

3.1 Amendment effects on soil properties

Amendments of GAS residues and lime resulted in increased soil pH (Fig. 1a). The addition of 1.0-2.5 g kg^{-1} GAS residue increased soil pH to neutral (7.0) and additional amendments further increasing pH. Even the smallest amendment of lime (0.5 g kg^{-1}) changed soil pH sharply from acidic to light alkaline (pH > 7.8) (Fig. 1a). The effect of GAS residues on pH may have been mitigated by the decomposition of proteins (from the snail meat) into amino acids and glucose by soil microbes (27).

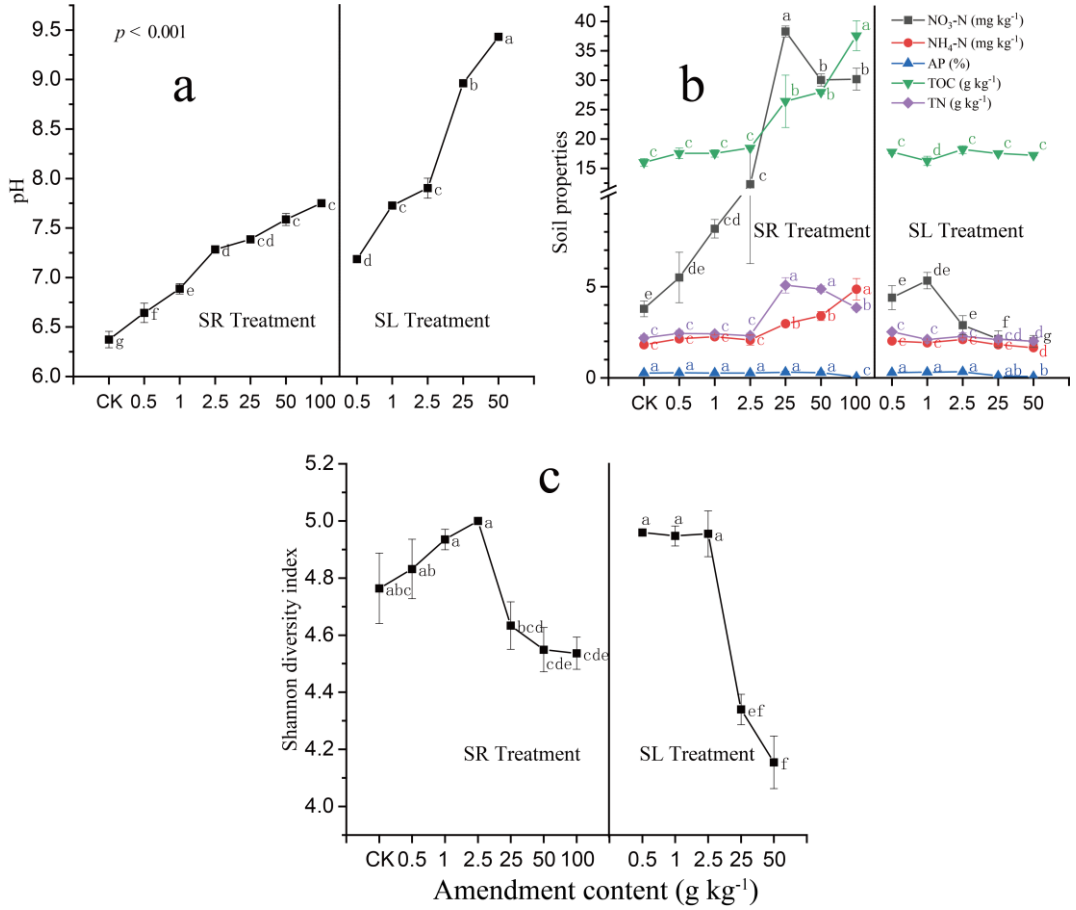


Fig. 1. Variations of Soil pH (a), TOC and nutrients (b), and (c) Shannon diversity index of bacterial induced by the amendment of GAS residues (SR) and lime (SL) in acid soil after incubated for 120 days; where soil properties were indicated by color lines, TOC (Total Organic Carbon, green), NO_3-N (nitrite, black), TN, (Total Nitrogen, purple) NH_4-N (ammonia, red) and AP (Available Phosphorus, blue) (n=3).

Addition of GAS residues also increased soil carbon and soil nutrients—specifically, TOC, TN, NO_3-N and NH_4-N (Fig. 1b). TOC and NH_4-N progressively increased as more GAS residues were added, increasing by 134.28% (TOC) and 167.80 % (NH_4-N) with the amendment of 100 g kg^{-1} GAS residue. Soil nitrogen, TN and NO_3-N showed peak values when 25 g kg^{-1} GAS residues were

232 added, a threshold value prior to which proteins in the GAS residues decomposed or
233 dispersed quickly, but after which anaerobic soils limited the activities of soil
234 microbes and the transfer of proteins into small molecular and inorganic matter so that
235 more NH4-N and NOx were produced and released into the air (27).

236 *3.2 Amendment effects on bacterial diversity*

237 Soil pH and nutrients are the important factors that can affect the soil bacterial
238 community and diversity indicated by Shannon index. Amendment of GAS residues
239 and lime both significantly affected soil bacterial diversity (Fig. 1c). Specifically,
240 amendment of up to 2.5 g kg^{-1} GAS residues increased diversity, as measured by
241 Shannon diversity index, from 4.76 (CK) to 4.99. The addition of more GAS residue
242 decreased bacterial diversity; the addition 75 g kg^{-1} GAS residues resulted in the
243 lowest Shannon diversity index value (4.55) across treatments. Similar to GAS
244 residue, the addition of lime had a positive effect on diversity, but had a negative
245 effect when more than 2.5 g kg^{-1} was added (Fig. 1c).

246 Previous studies have proposed that soil pH affects soil bacterial community
247 structure and diversity (11, 28-30). N. Fierer and R. B. Jackson (10) found that the
248 diversity and richness of soil bacterial communities differed by ecosystem type, and
249 these differences could largely be explained by soil pH (r^2 = 0.70 and r^2 = 0.58,
250 respectively; $P < 0.0001$ in both cases). Bacterial diversity was highest in neutral soils
251 and lower in acidic soils, with soils from the Peruvian Amazon the most acidic and
252 least diverse in their study (10). Our results were consistent with the observations
253 reported in biochar amendment by Q. Li et al. (31), which found that bacterial

254 diversity was relatively low in alkaline soils. We found that soil bacterial diversity
255 increased with the additions of GAS residues and lime amendments between 0.5-2.5 g
256 kg^{-1}, likely because of the shift from acidic to neutral soil pH. Bacterial diversity
257 declined when more GAS residue or lime were added (more than 25 g kg^{-1}), likely
258 due to different factors. For the SL (lime) treatment, high soil pH likely restricted the
259 growth and reproduction of soil bacteria. This is consistent with the results reported
260 by J. Xiong et al. (32), in which soil pH was negatively correlated with soil bacterial
261 diversity in alkaline lake sediments across the Tibetan Plateau. Nevertheless, for the
262 SR (GAS residues) treatment, high soil nutrients, such as NO_3-N and NH_4-N, likely
263 contributed to the simplification of bacterial diversity. These results were contrast to
264 the previous work, in that the addition of N had no significant effect on bacterial
265 diversity while elevated P could increase bacterial diversity marginally (33).

266 However, pH was not likely responsible for the decline of soil bacterial diversity
267 in the SR (GAS residue) treatment, because the peak value of soil pH in the SR
268 treatment was equal or less than 7.8, the pH that yielded maximum bacterial diversity
269 in the SL treatment. Instead elevated soil nutrients likely caused declines in bacterial
270 diversity in the SR treatment. Soil nutrient concentrations were significantly
271 negatively correlated with bacterial diversity index (Table S1). For example, TOC, TN,
272 NH_4-N and NO_3-N were all negatively correlated with Shannon ($p < 0.05$) index. B. J.
273 Campbell et al. (14) and A. Koyama et al. (34) reported similar negative relationships
274 between bacterial diversity and N additions in Arctic tundra soils. Our results contrast,
275 however, with other studies that show positive relationships between soil nutrients

and bacterial diversity (33), which could be caused by differences in the types of soil, and the types and amount of nutrients amended (10, 35, 36).

3.3 Amendment effects on bacterial community composition

Our assessment generated 2016767 high-quality 16S rRNA gene sequences. The bacterial community composition of the soil samples shifted significantly as a result of the amendment of GAS residues and lime, and were clearly distinguished (Fig. 2a, ANalysis Of SIMilarity (ANOSIM), R = 0.823, $P < 0.001$) among the different amendment treatments, and such a pattern was confirmed by hierarchical clustering based on genus (Fig. 2b). Specifically, the analysis identified three groups of bacterial community compositions (*i.e.*, in soils amended with high concentrations of lime, soils amended with high concentration of GAS residues and soils amended with low concentrations of lime or GAS residues). The reason contributed to these changes may attribute to elevated high soil pH and high nutrients availability induced by over amended lime and GAS residues.

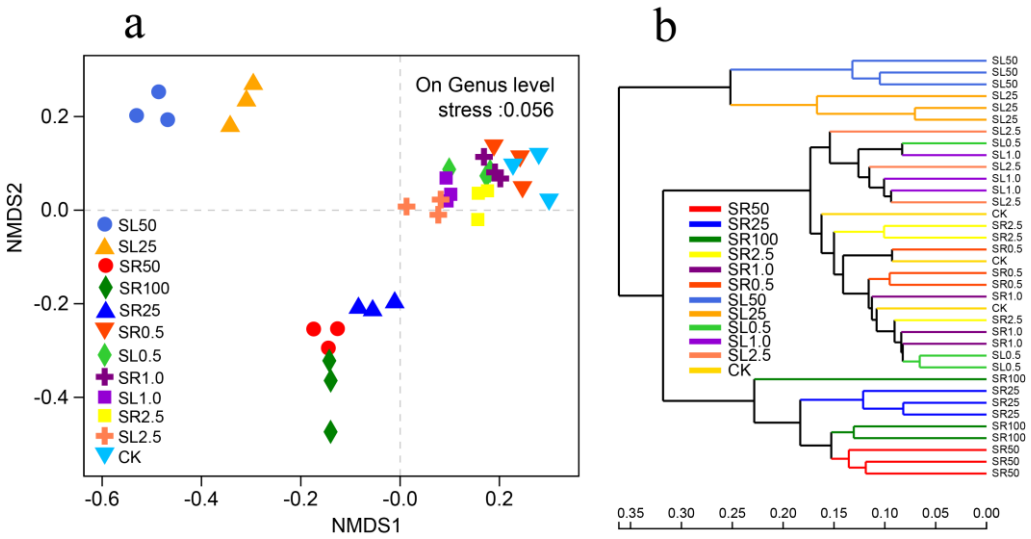


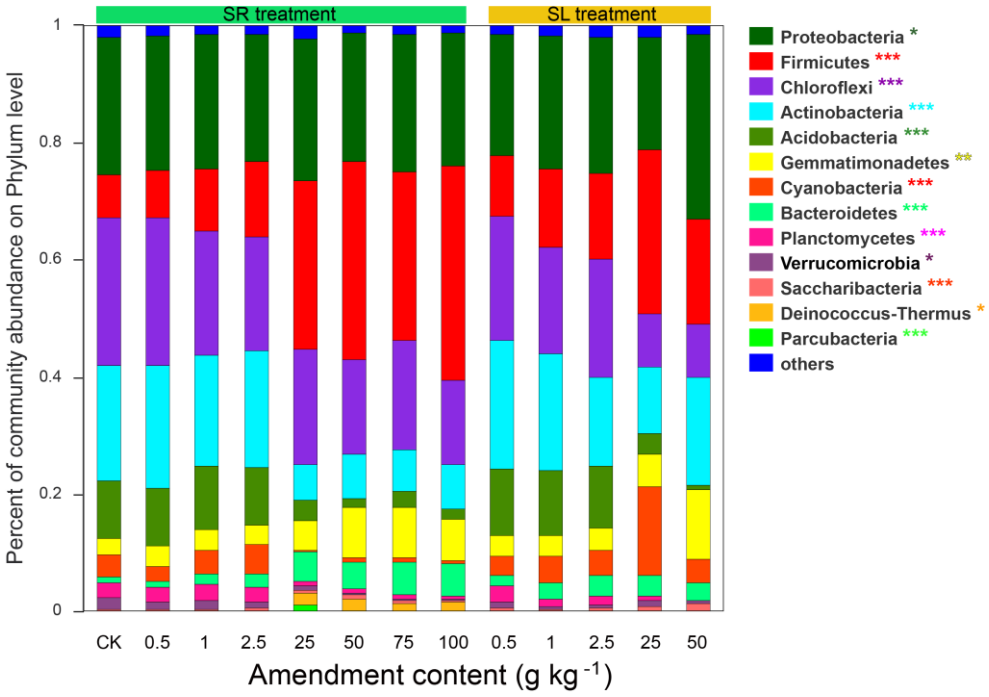
Fig. 2. Non-metric multidimensional scaling (NMDS) of soil bacterial communities

292 based on 16S rRNA within plots as affected amendments (a); and hierarchical
293 clustering of treatments (b). SR represents GAS residues treatments, SL represents
294 lime treatments and the numbers 0.5, 1, 2.5, 25, 50 and 100 represent the amendment
295 levels (g kg^{-1}), CK represents the control (n=3)

296 Amendment of GAS residues and lime induced different changes in bacterial
297 community compositions. The most abundant bacterial phyla detected in soil samples
298 were Proteobacteria (about 22%), Firmicutes (range from 15 -35%), Chloroflexi
299 (range from 12% - 22%), Actinobacteria (range from 8% - 20%), Acidobacteria,
300 Gemmatimonadetes, Cyanobacteria and Bacterioidetes (Fig. 3). The amendment of
301 GAS residues significantly increased the relative abundance of Firmicutes,
302 Gemmatimonadetes, Bacterioidetes and Deinococcus-Thermus, but significantly
303 decreased the relative abundance of Chloroflexi, Actinobacteria, Acidobacteria,
304 Cyanobacteria and Planctomycetes. In SL (lime) treatments, we detected similar
305 decreases in the abundance of Chloroflexi, Acidobacteria and Planctomycetes, but in
306 contrast to SR (GAS residue) treatments, the abundance of Cyanobacteria increased
307 significantly (Fig. 3). Gemmatimonadetes and Bacterioidetes increased in relative
308 abundance in SR treatments but not in SL treatments, possibly because
309 Gemmatimonadetes and Bacterioidetes were responsible for the degradation of soil
310 carbon and the emission of CO_2 (37, 38). Amendment of GAS residues (especially
311 high levels) increased soil TOC, which likely contributed to increases in relative
312 abundance of Gemmatimonadetes and Bacterioidetes. Our results contrast with
313 previous studies that found that high soil pH (with biochar addition) resulted in

314 decreases in Firmicutes abundance (39). That result may reflect the integrated effects
315 of elevated soil pH, C and N (particularly N), because Firmicutes is involved in N
316 cycling (40).

317 Significant changes of soil bacterial communities in SR (GAS residues)
318 treatments can also be explained by copiotroph and oligotroph mechanisms driven by
319 changes in soil nutrients. Copiotrophs preferentially consume labile soil organic C
320 pools, have high nutritional requirements, and can exhibit high growth rates when
321 resources are abundant. In contrast, oligotrophs exhibit slower growth rates and are
322 likely to outcompete copiotrophs in conditions of low nutrient availability due to their
323 higher substrate affinities (41). In our study, the amendments of GAS residues
324 induced oligotrophic soil conditions that may have benefited copiotrophs and
325 disadvantaged oligotrophs. Our results were similar to the previous studies, in that
326 bacteria belonging to the Acidobacteria phylum were most abundant in soils with very
327 low resource availability and their relative abundances declined in soils amended with
328 high concentrations of organic C, N and P (33, 41).



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330 Fig. 3. 16S rRNA sequence-based microbial community composition of GAS residues
331 (SR) and lime (SL) amended soils on the bacterial phylum level. Different colors
332 indicate different phylum as showed in legend; and the symptoms *, * and ***
333 represent significance at 0.05, 0.01 and 0.001 levels (n=3).

334 *3.4 Correlations between soil properties and bacterial community composition*

335 Our results indicated that soil properties were significantly correlated with
336 bacterial phyla (Fig. 4a) and genera (Fig. S1a) in GAS residues amended soils. For
337 example, pH, NH_4-N, NO_3-N and TN had significant positive correlations with the
338 relative abundance of Gemmatimonadetes, Tenericutes, Chlorobi, Firmicutes,
339 Bacteroidetes and Deinococcus-Thermus ($p < 0.01$). In addition, significant negative
340 correlations were detected with Antinobacteria, Cyanobacteria, Nitrospirae,
341 Acidobacteria, Planctomycetes and Verrucomicrobia ($p < 0.01$). As previously
342 discussed, Gemmatimonadetes, Tenericutes, Chlorobi, Firmicutes, Bacteroidetes and
343 Deinococcus-Thermus are all involved in C and N cycling, and amendment of GAS

residues (especially at high concentrations) induced high levels of C and N. The negative effects of SR treatment on Antinobacteria, Cyanobacteria, Nitrospirae, Acidobacteria, Planctomycetes and Verrucomicrobia may reflect sensitivities of some of these groups to higher soil pH; previous studies have found that some of these groups decrease in abundance after fertilizers are applied. For instance, D. R. Nemergut et al. (37) reported that the relative abundance of Verrucomicrobia declined in a fertilized soil. R. T. Jones et al. (42) reported that the abundance of Acidobacteria relative to other bacterial taxa was highly variable across soils, but correlated strongly and negatively with soil pH. B. J. Baker et al. (43) and N. Xu et al. (44) reported that Nitrospirae was active in nitrogen cycling, specifically as nitrite oxidation, and SR treatment may have increased labile nitrogen availability in the soil, possibly restricting growth of Nitrospirae. Although S. N. R. Prasanna (45) reported that Cyanobacteria prefer neutral to slightly alkaline pH for optimum growth, high nutrients may restrict its relative abundance.

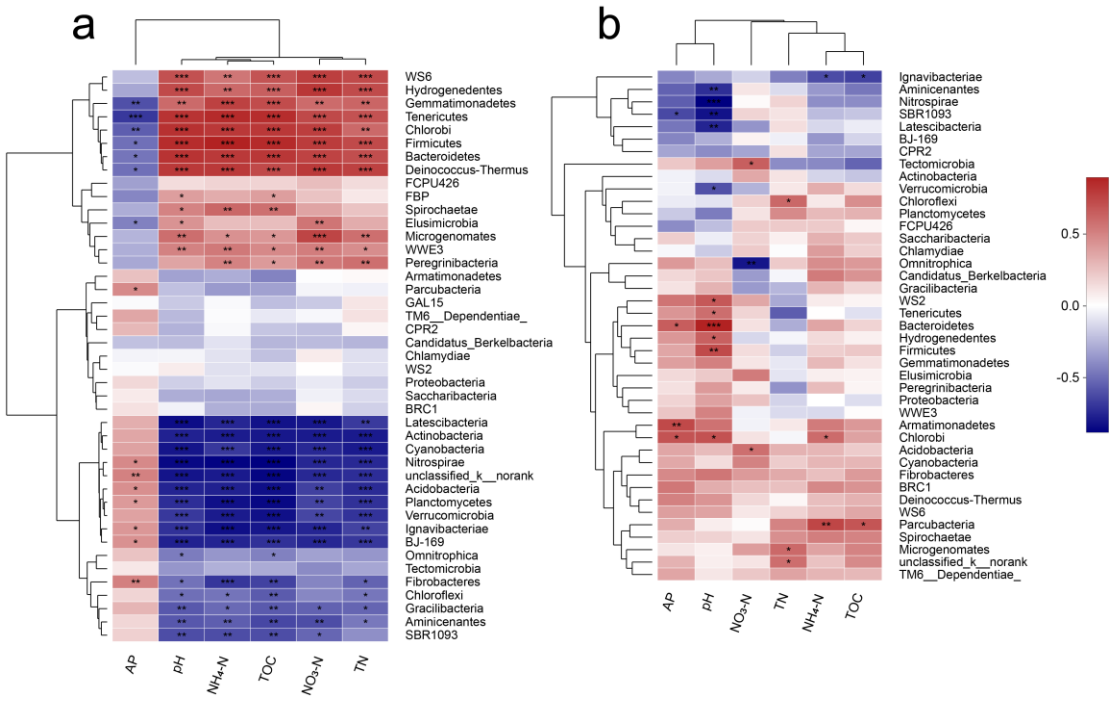
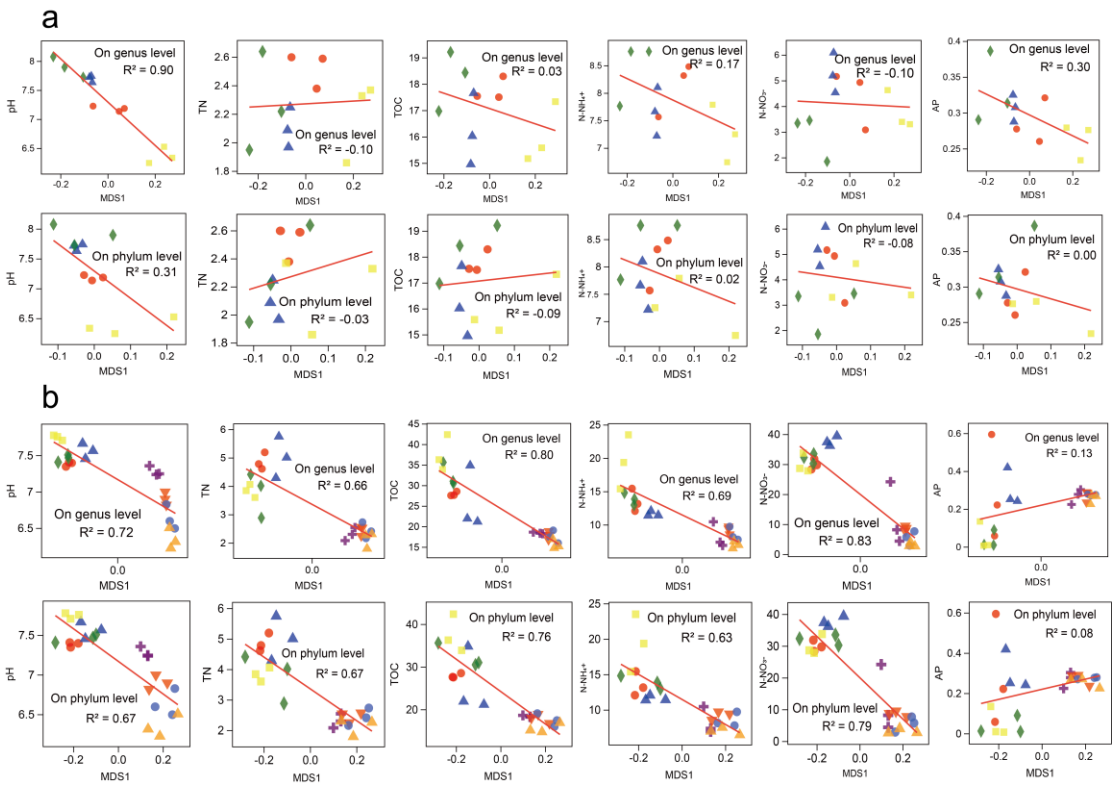


Fig. 4. Spearman correlations between soil properties and bacterial community composition on phylum level after amendment of GAS residues (a) and lime (b); where blue and red colors represent negative and positive correlations,; TOC represents total organic carbon, TN represents total nitrogen, N-NH_4^+ represents ammonia, N-NO_3^- represents nitrite and AP represents available phosphorus, and the symptoms *, ** and *** represent significant levels of 0.05, 0.01 and 0.001(n=3).

For the SR treatments, most significant correlations were found in soil pH; the only groups with relative abundance that was significantly positively related to pH were Bacteroidetes, Firmicutes and Chlorobi with soil pH (Fig. 4a). Fewer phyla showed significant correlations with soil nutrients in the SL treatments compared to the SR treatments. These results suggest that more phyla were affected by C, N (*ie*., TOC, NH_4-N, NO_3-N and TN) in SR treatments. In SR treatments, which increased both soil pH and nutrient contents, soil pH does not appear to be a good predictor of bacterial community composition and diversity.

To evaluate the main factors driving changes in microbial communities in our experimental treatments, we used Non-metric multidimensional scaling (NMDS) of soil bacteria community composition based on Bray-Curtis distances linear regression (Fig. 5). In SL treatments, soil pH was the factor most strongly related to changes in bacterial community composition (R^2 = 0.90) (Fig. 5a). However, in SR treatments soil nutrients tend to shape changes in bacteria composition (Fig. 5b). Our results are consistent with previous research that included a sub-set of the soils included in this survey; they found that the abundances of Bacteroidetes, Betaproteobacteria, and Acidobacteria were most strongly related to estimated carbon availability, not soil pH (41). In addition, soil nutrients (N and P) additions could significantly affect soil bacterial community compositions were also confirmed (33). Nevertheless, our results contrast with other studies that suggest pH as a strong predictor of bacterial community composition and diversity (11, 28, 46).



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392 Fig. 5. Soil properties and Non-metric multidimensional scaling (NMDS) of soil
393 bacteria community composition based on Bray-Curtis distances linear regression
394 after amending with lime (a) and GAS residues (b); TOC represents total organic
395 carbon, TN represents total nitrogen, $N\text{-}NH_4^+$ represents ammonia, $N\text{-}NO_3^-$ represents
396 nitrite and AP represents available phosphorus (n=3).

397 Apart from determining diversity another primary goal of comparing microbial
398 communities is to identify specialized communities in samples. Groups were shown in
399 cladograms, and LDA scores of 2 or greater were confirmed by LEfSe (Fig. S2). In
400 GAS's residues treated soils, 21 bacteria phyla were enriched dominated with
401 Actinobacteria, Bacteroidetes and Firmicutes followed by Acidobacteria,
402 Cyanobacteria and other 16 phyla. Firmicutes were most abundant in the GAS
403 residues 50 g kg^{-1} treatment, which may have been caused by the high soil pH; it was
404 the same pH as in the lime 2.5 g kg^{-1} treatment. To evaluate the effects of amendments

405 on shaping microbial community composition, we compared results from high levels
406 of the GAS residues amendments and low levels of lime amendments with results
407 from low levels of GAS residues amendments, because high levels of GAS residues
408 amendments and low levels of lime amendments showed similar effects on soil pH.
409 The results showed that more specialized bacteria phyla were detected in soils treated
410 with high levels of GAS residues amendments, which suggests that GAS residues can
411 shape bacterial community compositions.

412 *3.5 Correlations between enzyme activities and bacterial community composition*

413 Four enzymes related to C, N and P cycling in soil were determined to uncover
414 the status of soil nutrients and C cycling after the amendment of both GAS residues
415 and lime. We conducted Spearman Correlation analysis to identify the relationships
416 between microbial communities and soil enzyme activities, and significant
417 correlations were found in SR treatment (Fig. 6). For example, we found that β–D–
418 cellobiosidase (CB), β–1,4–N–acetylglucosaminidase (NAG) and β–1,4–glucosidase
419 (BG) activities were significantly correlated with microbial communities both on
420 phylum (e.g., Bacteroidetes, Deinococcus-Thermus, Gemmatimonadetes,
421 Actinobacteria, Acidobacteriae and Nitrospirae) level (Fig. 6a) and genus level (Fig.
422 S3a) in soils treated with GAS residues amendment. Many fewer correlations were
423 found in soils treated with lime amendment (Figs. 6b, S3b). The strong correlations in
424 soils treated with GAS residues may be caused by its influence on substrate such as C
425 and N. M. Waldrop et al. (47) reported that bacterial community composition was
426 correlated with BG, ACP, and sulphatase activity, and concluded that enzyme activity

427 may provide a useful linkage between microbial community composition and carbon
428 processing. As Fig. 6a shows, significant and negative correlations between bacterial
429 phyla such as Actinobacteria, Acidobacteriae and Nitrospirae, and enzyme activities
430 such as CB NAG and BG. Previous studies suggested that N additions resulted in
431 significant reductions in soil microbial activity (13), diversity (14) and community
432 structure composition (15) because of increases in C sequestration and/or decreases in
433 soil respiration rates (13). However, in our study, enzyme activities significantly
434 increased after the addition of GAS residues, which contain abundant N in the form of
435 proteins and amino acids. It is possible that the elevated organic C and N induced by
436 GAS residues treatments promoted hydrolyzation but suppressed the mineralization
437 the C and N (48, 49). Our results are consistent with the results reported by M.
438 Carreiro et al. (50), who found that microbes responded to N by increasing cellulase
439 activity.

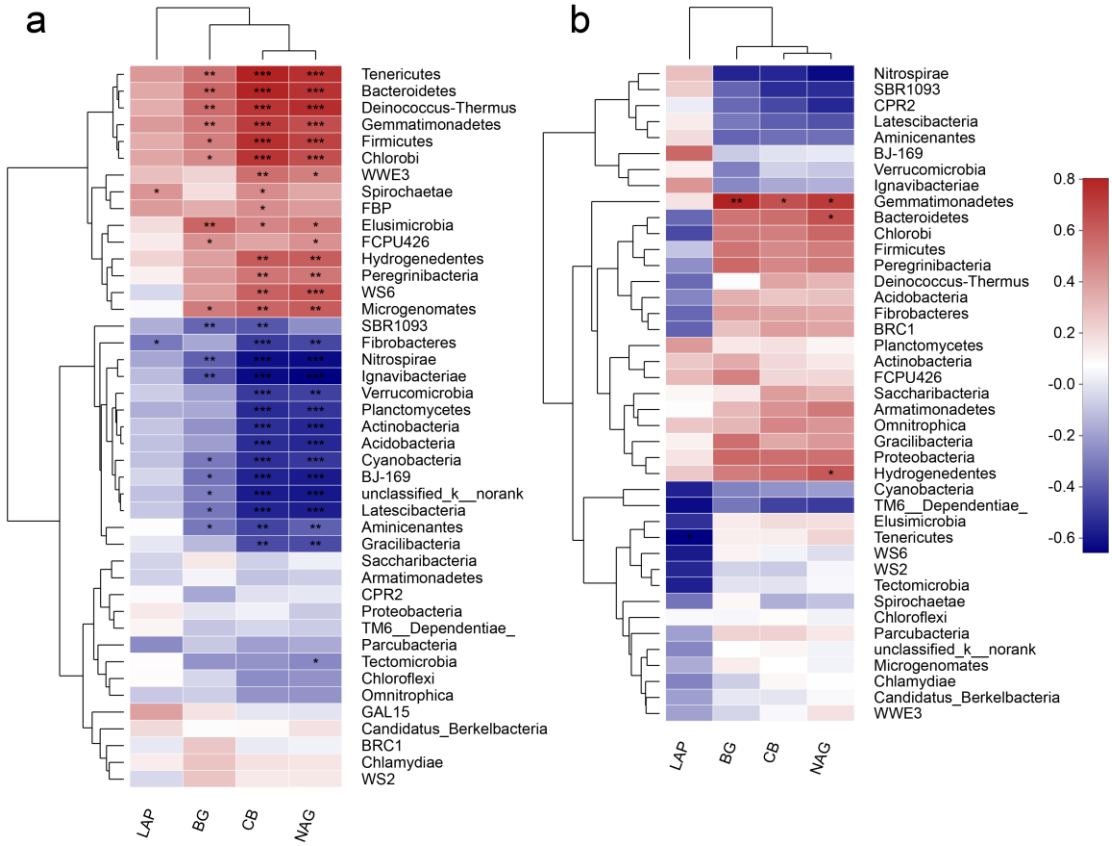

Fig. 6. Spearman correlations between soil enzyme activities and bacterial community composition on phylum level after amendment of GAS residues (SR) (a) and lime (SL) (b); where blue and red colors represent negative and positive correlations; β–1,4–glucosidase (BG), acid phosphatase (ACP), β–1,4–N–acetylglucosaminidase (NAG) and β–D–cellobiosidase (CB); the symptoms *, ** and *** represent significant levels of 0.05, 0.01 and 0.001 (n=3).

Overall, in this study, the amendment of GAS residues significantly increased soil pH due to the $CaCO_3$ component of the GAS's shell. However, GAS residues had weaker effects on soil pH than did lime treatments. For example, at the same amended levels, the pH of soils amended with lime increased sharply, while soils amended with GAS residues rose only to near-neutral pH. In addition, amendment of GAS residues

453 resulted in increased levels of soil nutrients, which could in turn lead to increased
454 bacterial diversity at low amendment levels and decreased bacterial diversity at high
455 amendment levels. That likely attribute to the amendment of GAS residues induced a
456 copiotrophic environment in which, the relative abundance of copiotrophic bacterial
457 communities were increased while oligotrophic bacterial communities were reduced.
458 What's more, soil pH also responsible for the changes of soil bacterial communities.
459 For instance, Gemmatimonadetes, Tenericutes, Chlorobi, Firmicutes, Bacteroidetes
460 and Deinococcus-Thermus were all increased with the addition of GAS residues.
461 Some of them such as Gemmatimonadetes, Tenericutes, Chlorobi and Bacteroidetes
462 were increased due to their roles in C and N cycling, while some of them were
463 decreased because they were suppressed at a higher pH environment. Most
464 researchers suggested that pH was the best predictor for bacterial diversity and
465 community compositions across different types of land-use. Nevertheless, in our study,
466 soil pH may not be the best predictor of bacterial community composition or diversity;
467 rather soil nutrients (*ie*., NH_4-N and NO_3-N) and soil TOC showed stronger
468 correlations with bacterial communities. That likely because the amendment of GAS
469 residues induced elevated soil pH and nutrient content at the same time. Compared
470 with lime treatment, the amendment of GAS residues caused more enriched bacterial
471 phyla, that likely due to the soil nutrients difference between GAS residues treatment
472 and lime treatment. Considering the nutrients cycling, soil enzymes activities related
473 to C, N and P were determined and analyzed, our results suggested that soil enzymes
474 activities showed similar correlations to bacterial communities with soil nutrients.

475 Those indicated that the increases in enzymes activities were attributed to the
476 elevation of soil nutrients induced by the amendment of GAS residues.

477 **4 Conclusion**

478 Our study proposed that GAS residues may be appropriate to remediate acidic
479 soil, improve soil quality and reduce GAS populations in areas subject to GAS
480 invasion. In practice, it may not be practical to dry and crush GAS into powder before
481 applying it to soils. Instead, practitioners could create GAS residues at lower costs by
482 collecting living or dead GAS, spreading them on the soil surface and smashing them
483 using high speed rotary tiller. Also, we suggest applying GAS residues to nonirrigated
484 farmland to reduce the potential water pollution. We suggest amending GAS at 2.5 –
485 25 g kg^{-1}, which appears to be better for soil health and bacterial diversity. These
486 recommendations warrant further testing in the field, but results of our greenhouse
487 experiments suggest they hold promise.

488

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496

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