

Effect of swine wastewater irrigation on soil microbial diversity and structure

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Abstract: Soil acidification will promote the mobility of some toxic elements and decrease crop yield, resulting in economic loss and public health issues at the same time. Hence it has always been a problem to agriculturists. To solve it people have come up with all kinds of treatments such as lime, boron slurry, and some manure. Recently, the swine industry in China is facing massive closures due to the swine wastewater disposal crisis. Untreated swine wastewater would pollute the water body for it is high in bicarbonate and nutrient concentration that will cause algae bloom. Therein lies the reason why in pre-study we believe swine wastewater has the potential to remediate acid soil. Wastewater's capabilities of lowering soil acidity and exchangeable aluminum, increasing soil fertility, and affecting deeper layer soil have been verified in pre-study. However, our understanding of whether wastewater affects microbial diversity in soils is very limited. Therefore, we use 16S rDNA analysis to do metagenomic analysis and compare microbial diversity and microbial community structure in soil samples with or without wastewater treatment. In the result we found that the abundance of acidophilic bacteria in soil has declined. The result also showed that wastewater has introduced digestive tract bacteria to the soil environment. In general, wastewater irrigation had no significant effect on soil microbial diversity but had some effect on microbial community structure. Further research is needed to explore the potential effects on soil environment, plants, or humans caused by this change.

1. Introduction

Soil acidification is a worldwide problem that has bothering farmers for years. Acid soil, meaning soil pH lower than 5.5, occupied 30% of the world's ice free land [1]. In China, 18% of arable land has a pH below 5.5[2]. Similarly in the UK, most arable land and grassland has their soil below optimum pH [3]. As pH gets lower, some harmful metals, like aluminum (Al), will become mobilized. Al ion is known as an inhibitor of plant growth and crop yield [4]. This is urgent in the context of global food shortages. Al ion also threatened human health for there is evidence proving that it has neurotoxicity [5]. If this soil acidification is left alone with no intervention, there is strong evidence supporting that pH decreasing would become faster and extend to deeper layers of soil, increasing the time and economic cost of harness[1]. Nevertheless, soil acidification would negatively alter soil microbial population and release toxic metals such as chromium, manganese, and cadmium, pollute soil, waterbody and eventually harm human health through the food chain [2]. Therefore, soil acidity correction is important and urgent.

There are many factors, both spontaneous and factitious, causing soil acidification such as acidic precipitation, acidifying fertilizing and growth of legumes, base cation removal, and mineral libation of organic matter. Among all these, the growing use of nitrogen fertilizer makes the most significant contribution [1] [2]. In China 2007, the consumption of N fertilizer has reached 1.91 times larger than in early 1980s. The pH of some arable areas has dropped 0.8 units from the 1980s to the 2000s [1].

Traditionally people use lime to neutralize acid soil. Some bicarbonate-rich waste products would also accomplish the same purpose. The previous study of this study has tested swine wastewater in acid soil correction [6]. This waste water cannot be discharged directly. This is because excessive nitrogen and phosphorus emission will lead to serious water pollution, and swine wastewater happens

to contain high concentrations of nitrogen and phosphorus compounds and bicarbonate. In order to meet discharge standards, pig farmers have to pay a high cost to treat it. Hence in pre-study we propose to use swine wastewater to irrigate acid soil. In this way we can not only correct acidic soil with high concentration of bicarbonate, but also significantly reduce the large cost of purifying and discharging sewage. Nitrogen and phosphorus compounds can increase the fertility of soil, too. In this case using swine wastewater in acid soil treatment becomes a win-win solution for both soil correction and wastewater treatment. The pre-study showed a very positive result in soil pH rising and exchangeable Al ion fixing. Wastewater treatment is statistically significantly more efficient than lime treatment in both tests. Wastewater also has a better mobility than lime. It is effective in all layer soil, that is total 30cm depth, while lime application is only effective in top 2 layers, that is 0 to 6cm depth. From all points of view, swine wastewater seems like a perfect, efficient and environmentally friendly solution.

However, microbial communities are an important component of the soil micro environment. Bacteria can nourish plants by metabolizing organic matter in the soil or by fixing nitrogen. Change in microbial diversity or abundance of any kind may lead into change in soil chemical environment, and affect plant growth. Microbial diversity is usually highest in neutral soil. As the pH value of soil gets lower microbial diversity would also decrease [7]. Many studies have shown that different microbes have different tolerance to acid [8]. Whether a change in pH increases the abundance of good or bad bacteria is of interest. In addition, whether the application of swine wastewater will lead to the emergence of foreign microorganisms in the soil is also the focus of our research. Hence in this article we compared the soil microorganism diversity before and after applying swine wastewater, to see what changes it brings to the soil microenvironment.

2. Methods

2.1 Soil incubation

The soil consisting of Inceptisol was sampled from a tea farm in Hangzhou, China. Then air dried, grounded, and passed through a 2 mm sieve. The swine wastewater was obtained from the outlets of anaerobically digested effluent in a swine farm in Fuzhou, China. Place 1.2 kg soil sample in two polyethylene pots respectively and incubate it at 25°C, then assign the two to the control group and experimental group. There were 3 replications in each group.

Control group: Irrigating with deionized water at a rate of 1.2 liter per kilogram of soil.

Experimental group: Irrigating with swine wastewater at a rate of 1.2 liter per kilogram of soil.

2.2 Bulk genomic DNA extraction, amplification and sequencing

2.2.1 Bulk genomic DNA extraction

Extract bulk genomic DNA from original soil samples before irrigation and incubation (S1), from control group after 14 days of incubation (S2), from experimental group after 14 days of incubation (S3), and from the wastewater samples before irrigation (F). Use a FastDNA SPIN Kit for Soil (MP Biomedicals, CA, USA) following the manufacturer's instructions.

2.2.2 Amplification

Run a PCR program to amplify 16S rDNA genes with primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3').

2.2.3 Sequencing

Amplified PCR products were sequenced with an Illumina HiSeq PE250 sequencing platform (Illumina, San Diego, CA, USA). Sequences were clustered into operational taxonomic units (OTUs) with USEARCH11 via a sequence similarity threshold of 0.97 [9].

2.3. 16S data analysis

Sample analysis was completed by Novogene <https://www.novogene.com>. There are 4 sets of data. S1.1-1.3 indicates data from acid soil before any irrigation; S2.1-2.3 indicates data from acid soil after pure water irrigation; S3.1-S3.3 indicates data from acid soil after wastewater irrigation; and F1.1-F1.3 indicates data from swine wastewater itself.

3. Result and discussion

3.1 Clustering analysis

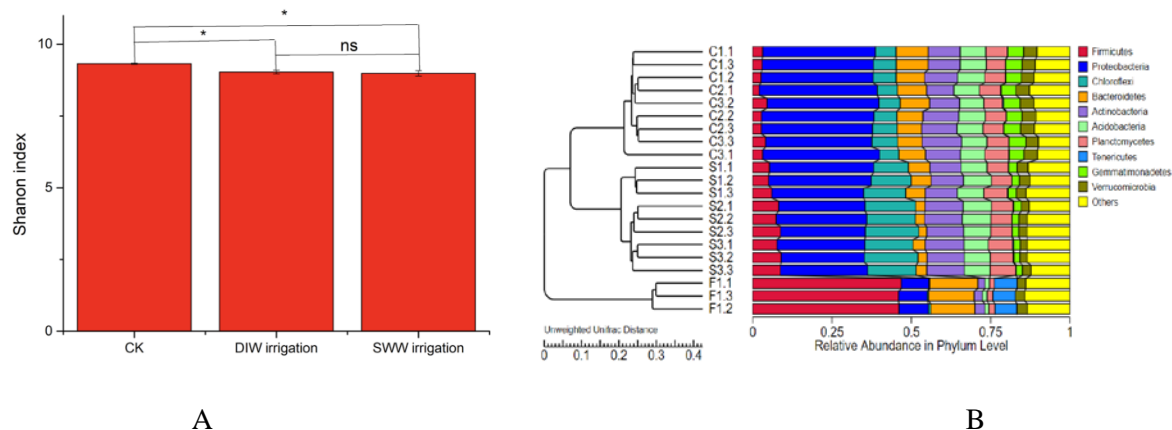


Fig. 1 Shannon index (A) and UPGMA cluster tree (B)

*Sample C1, C2, C3 are not related to this study

Unweighted pair group method with arithmetic mean (UPGMA) is a tool that shows the dissimilarity between the considered samples [10]. The larger the similarity the closer they are on the tree. As shown in UPGMA cluster tree (Fig. 1B), there was no mutual contamination between the samples. Because as the graph shows sample groups C, S, and F are separated from each other very early. Samples in sample group S1, S2, S3, and F1 all came together in the end. The relative abundance in Phylum level similarity between samples S2 and S3 is relatively large. Shannon index (Fig. 1A) also gives the same result because there is little difference between DIW (deionized water) irrigation bar and SWW (swine wastewater) irrigation bar. Meaning that wastewater irrigation may not affect microbial diversity in soil.

3.2 Dominant microorganisms in each sample in Class level

In order to analyze whether the structure of dominant bacteria in soil was changed, the change of the abundance of bacteria in Class classification was firstly concerned. Top 10 ranking Class (Fig.2) in relative abundance for sample S1, S2, and S3 is slightly different from each other. As shown in Fig.1, compare with S2 and S3, S1 has a higher relative abundance of Sphingobacteriia, Acidobacteria, Betaproteobacteria, and Alphaproteobacteria; and a lower relative abundance of Ktedonobacteria and Clostridia. By comparing S2 and S3, the relative abundance of Gammaproteobacteria, Ktedonobacteria, and Clostridia is higher in S3; the relative abundance of Acidobacteria is lower in S3. Which means that after irrigation (with deionized water or wastewater), the abundance of Sphingobacteriia, Acidobacteria, Betaproteobacteria, and Alphaproteobacteria decreased while the abundance of Ktedonobacteria and Clostridia increased. Bacteria in Acidobacteria are mostly acidophilic species. Water dilutes hydrogen ions in the soil and raises the pH of it, thus resulting in an abundance decrease. On this basis bicarbonate in wastewater can neutralize hydrogen ions, causing the pH of the soil to increase even more, which has been verified in pre-study. Therefore, compared with deionized water irrigation, the abundance of Acidobacteria is lower after wastewater treatment.

Alphaproteobacteria and Betaproteobacteria abundance both dropped after both irrigation. But there is little difference between the effect of deionized water or wastewater on their abundance. The

abundance of Gammaproteobacteria and Betaproteobacteria are positively correlated with pH value and dissolved organic carbon concentration in soil[11]. Hence we predict that their abundance will increase after wastewater irrigation. But only Gammaproteobacteria has a significant increase in abundance after wastewater treatment. The reason for this needs further study but it is speculated that other bacteria in soil may inhibit these two kinds of bacteria. Or the experimental conditions are not suitable for the growth of these two kinds of bacteria.

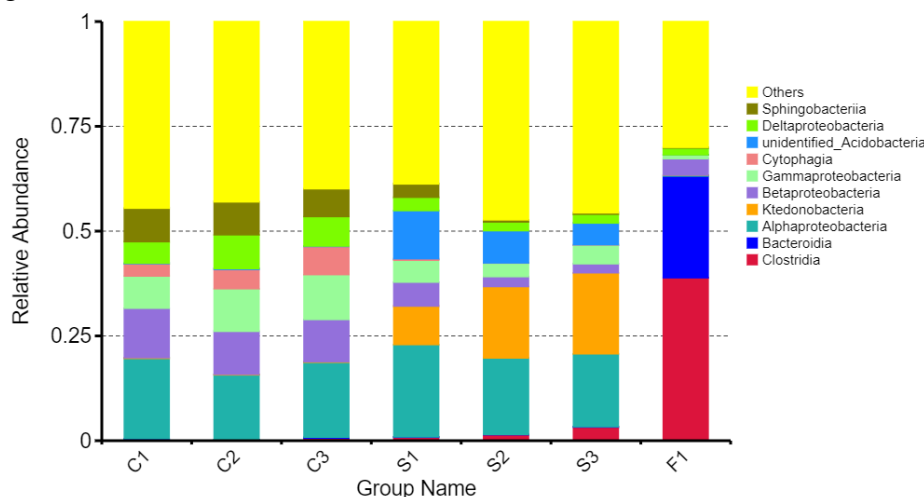


Fig.2 Top 10 ranking Class in relative abundance in each sample group

*Sample C1, C2, C3 are not related to this study

In wastewater, Bacteroidales has the most abundance. But this didn't increase the abundance of Bacteroidales in S3. Bacteroidales are obligate anaerobes parasitic mostly in human or animal intestines, oral cavity, upper respiratory tract and reproductive tract. Hence although they have certain pathogenicity, they do not affect humans through agricultural irrigation. Clostridia are widely distributed in soil. It also has the second most abundance in wastewater. Compared with S2, the abundance of it has doubled in S3.

3.3 Dominant microorganisms in each sample in Genus level

The function of bacteria is usually analyzed at the taxonomic level of the genus. In Genus level the abundance of Sphingomonas, Rhizomicrobium, Gemmatimonas, Haliangium, and Burkholderia-Paraburkholderia has decreased after any irrigation; The abundance of unidentified_Nitrospiraceae, Variibacter, unidentified_Chloroplast, and Bradyrhizobium has increased after any irrigation. Compare S2 with S3, the abundance of unidentified_Nitrospiraceae, unidentified_Chloroplast, and Clostridium_sensu_stricto_1 has increased; the abundance of Candidatus_Solibacter, Actinospica, Rhizomicrobium, Acidothermus, and Sphingomonas has decreased. In wastewater samples (F1), vadinBC27_wastewater-sludge_group, Christensenellaceae_R-7_group, Proteiniphilum, Methanosaeta, and unidentified_Synergistaceae have high abundance but these organisms do not appear in sample S3.

Gram-positive and -negative bacteria have different tolerance to acid. Usually, Gram-negative bacteria are more sensitive to the change of pH and their abundance is positively related to it[8]. Therefore theoretically speaking, after irrigating wastewater Gram-negative bacteria abundance would increase. But the results do not meet with this hypothesis. All kinds of bacteria, except Actinospica, whose abundance decreased after wastewater irrigation were gram-negative. Actinospica is Gram-positive acidophilic bacteria, its change in abundance meets the hypothesis. Other bacteria, however, need further study to find the reason for this result. The assumption we can make now is Gram-negative bacteria abundance is positively related with pH value, their optimum pH range may be more narrow and acidic, and the wastewater pushes the soil pH beyond that range.

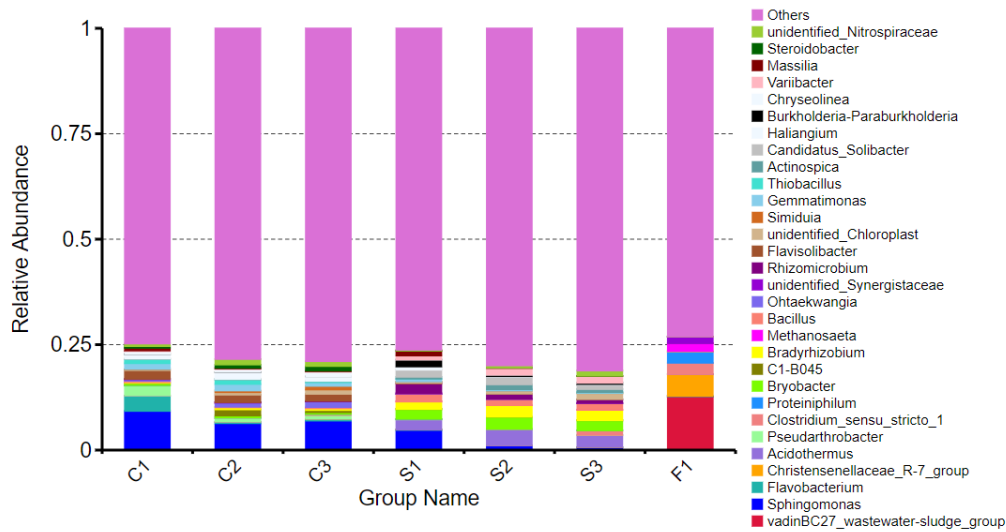


Fig.3 Top 10 ranking Genus in relative abundance in each sample group

*Sample C1, C2, C3 are not related to this study

The abundance of Chloroplast has increased after wastewater irrigation. This indicates that there is a large increase in the abundance of photoautotrophic microorganisms. This may be related to the nutrients in wastewater. This fact supports the ability of wastewater to improve soil fertility. Nitrospiraceae also has a significant increase in S3. It is capable of partial nitrification and complete nitrification. Its increase in abundance also indicates that the soil Nitrogen concentration has increased.

3.4 T-test analysis

T-test bar graph gives an intuitive view of which genera differ most from sample to sample. Compared with S1, there are fewer genera with significant differences between S2 and S3. As shown in Fig.4, after applying wastewater the abundance of *Clostridium_sensu_stricto_1*, *Terrisporobacter*, *Romboutsia*, *Turcibacter*, *Singulisphaera*, and *Clostridium_sensu_stricto_5* is significantly higher than applying deionized water. *Candidatus_Solibacter*, *Terracidiphilus*, and *Telmatobacter* are lower in abundance after applying wastewater. *Clostridium_sensu_stricto_1*, *Clostridium_sensu_stricto_5*, *Terrisporobacter*, and *Romboutsia* are all digestive tract bacteria. Among them, *Terrisporobacter* has the potential to cause disease. There has been a case report of a patient undergoing postoperative infection with a kind of *Terrisporobacter* (*Terrisporobacterglycolicus*) at the site of an open fracture, resulting in death [12]. But that seems to be rare. *Terrisporobacter* is not present in both S1 and S2 samples, hence this genus is introduced into the soil through wastewater irrigation. There is no evidence that these bacteria have any effect on plants. *Candidatus_Solibacter*, *Telmatobacter*, and *Terracidiphilus* are all Acidobacteria and commonly found in soil. They are mildly acidophilic and all participate in the carbon cycle in the soil. The decrease in their numbers after irrigation was expected. *Singulisphaera* is also commonly found in soil. It is a weak acidophilic bacterium [13]. In the previous study we found that wastewater irrigation raised the soil pH from 3.85 to 5.5, hence the slight increase of this kind of bacteria is expected. In the study of Zhou C et. al., *Singulisphaera* can increase the amount of available phosphorus in soil that plants can uptake [14]. Because it is a phosphate-solubilizing bacteria. The proliferation of such bacteria is certainly good for plants, and it may also mean that repairing acidic soil may optimize its microbes network.

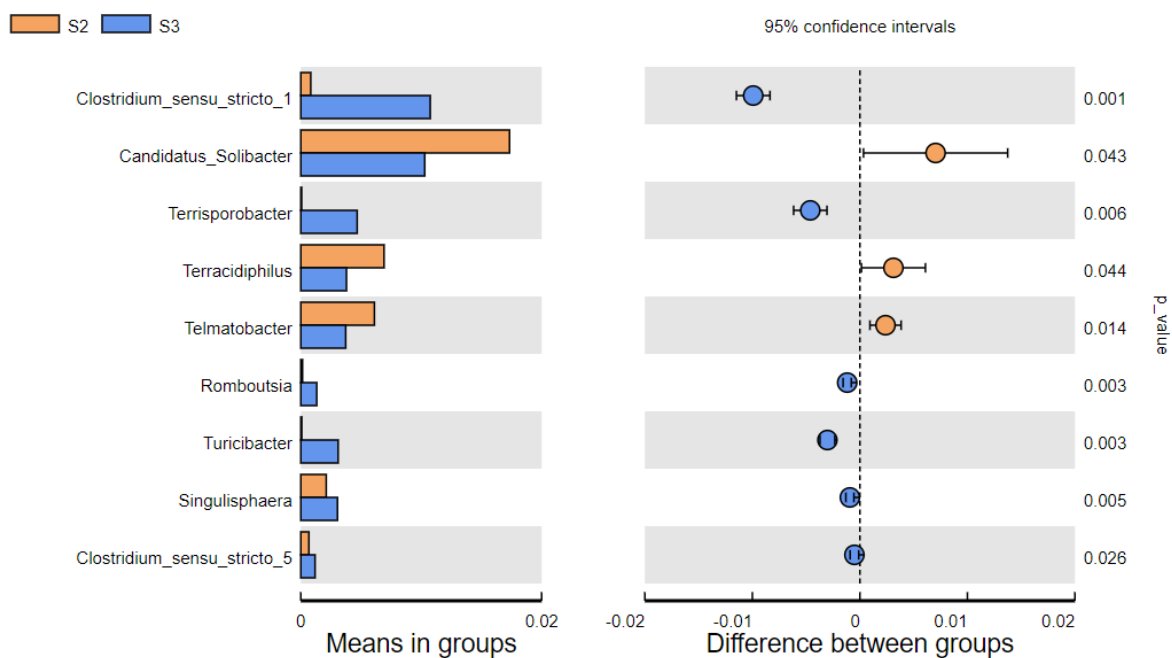


Fig.4 T-test bar graph of sample group S2 and S3

The symbiotic or competitive relationship among soil microorganisms forms a complex network [15][16]. Swine wastewater irrigation has no significant effect on the diversity of soil microbes but it does alter the microbial community structure. In Rong, L et. al. 's research, adding LDPE micro plastics to soil lowered the complexity of soil bacterial community network and also changed the structure of nitrogen-cycling bacterial community[17]. In this study, swine wastewater introduced new bacteria to soil and also altered the abundance of the original bacteria. Hence the bacterial community network has great potential of being changed. More research is needed to explore whether it will affect the nitrogen and carbon cycles in soil.

4. Conclusions

Increasing crop yields has always been one of the most important tasks for agricultural workers, but acidification of the soil caused by increased fertilizer application becomes another problem that needs to be solved. Traditionally people use lime and plant ashes for soil remediation. Instead of applying these, we explored a new method to neutralize soil in this study, that is applying swine wastewater. This treatment is more efficient than the traditional lime treatment, but its effect on the soil micro environment is unknown. The purpose of this study is to find out what effect this wastewater will do to soil micro environment. After comparative analysis, the diversity of soil microorganisms did not change significantly, but the microbial community structure slightly changed.

This study also has some shortcomings and limitations. The first is the time span. The data for this study was collected over a period of only one week. The possible consequences of some interactions between microbes may not be noticeable in such a short time. Different source of wastewater may also be a variable. Different pig farm management methods, geographical locations, and feeding formulas can change the chemical composition and microbial structure of the wastewater produced. Further studies are needed to explore whether such changes will affect the original symbiotic or competitive relationship of the soil microbial community. Subsequent studies should extend the time span to see if long term irrigation of wastewater will have any different effect on soil microbes. Then study whether wastewater from different pig farms differs from one another and whether such differences have different effects on soil microbes or soil remediation. Whether wastewater from other animal farms can also be used for acid soil remediation is also worth studying.

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