1 *Type of the Paper (Article, Review, Communication, etc.)*

Effects of simulated acid rain on soil respiration rate and soil bacterial diversity in a *Phyllostachyspubescens* forest in subtropical China

5 **NanWang[1,2], XiaochengPan[2,*]**

6 [1] Center for Ecological Research, Northeast Forestry University, Harbin 150040,
7   China;wangnan198110@163.com
8 [2] Zhejiang Provincial Key Laboratory of Carbon Cycling in Forest Ecosystems and Carbon Sequestration,
9   Zhejiang A & F University, LinAn 311300, China.
10 * Correspondence: 419979237@qq.com; Tel.: +86-575-87760102

12 **Abstract:** Acid rain has been regarded as a global environmental concern due to its negative
13 effects on global ecosystems. In this study, we investigated the effects of simulated acid rain (SAR)
14 on soil respiration rate and soil bacterial diversity in a Moso bamboo (*phyllostachyspubescens*) forest
15 in subtropical China. Experimental results showed a similar seasonal pattern of soil respiration
16 rates underdifferent SAR treatments. Seasonal mean soil respiration rates for CK (control, deionized
17 water, pH 6.7), T1 (pH 5.6), T2 (pH 4.0) and T3 (pH 2.5) treatments were 3.44, 4.80, 4.35 and 4.51 μ
18 mol m^{-2} s^{-1}, respectively. One-way analysis of variance indicated that the SAR exposure had no
19 significant effect on soil respiration (p>0.1) and soil microbial biomass (p>0.1).Soil bacterial
20 community diversity was calculated as the Shannon-Wiener diversity index and the results showed
21 that only T3 treatment had significant effects on soil bacterial diversity. The DGGE analysis results
22 revealed that T1 and CK soils had closer association and were related to the T2 soil, while T3 soil
23 was distinctly different from the other treatments. This work highlights that the effects of SAR are
24 important to consider in assessing the soil respiration rate, particularly under the scenario of
25 increasing acid rain pollution.

26 **Keywords:** *phyllostachyspubescens* forest; simulated acid rain (SAR); soil respiration; bacterial
27 community

28

1. Introduction

30   Recently, with the rapid growth of worldwide economy and urban population, more and more
31 sulfur dioxide (SO_2) and nitrogen oxides (NO_x) gases are produced during the combustion of fossil
32 fuels within thermal power plants and automobiles. The emission of these gases into atmosphere
33 could mix with water vapor in the air to form sulfuric and nitric acid, which later falls as acid rain
34 [1, 2]. Acid rain has become a global environmental problem and received worldwide attention due
35 to its environmental damage, including the acidification of soil [3], decrease of microbial
36 community function and enzyme activities [4-6], negative effects on vegetation, in particular forests
37 [7], and the changes of soil species composition [8].

In China, particularly in the southern part, acid rain is also a serious environmental hazard as most of the soil there is acidic. South China, Europe and North America have become the three most severely affected regions by acid rain in the world [9, 10]. As one of the largest CO_2 fluxes in the global carbon (C) cycle, soil respiration contributes to 68-98 Pg-C to the atmosphere annually [11, 12]. Moreover, belowground Caccounts for more than two-thirds of the terrestrial C stock, and roots and microorganisms in the soils play an important role in atmospheric CO_2 respiration. Thus, deep understanding of soil respiration process will help us expand the knowledge of the terrestrial C cycle [13].

During the past decades, researchers mainly focused on how acid rain impacted the soil respiration in forest ecosystems. Acid deposition might directly affect soil respiration by changing microbial activity, enzyme activity, and the composition of the microbial population, as stated above. However, the results obtained in previous study were inconsistent. Chen et al. [9] found little impact on the soil respiration by simulated acid rain (SAR), while Blagodaskaya and Anderson [14] revealed that soil respiration corresponded with SAR loads, which might be associated with the adaptability of bacterial community [15]. Likewise, acid deposition might indirectly influence soil respiration due to the decrease of pH. Spain [16] revealed that organic C content in soil increased when pH in soil decreased, while Baath and Anderson [17] observed an increase of soil respiration rates along with the decrease of pH in soil.

Phyllostachyspubescens, a typical and economic bamboo species, is widely grown in South China, due to its rapid growth rate and forest formation, high revenue, widespread use, and high regeneration capacity. In China, the bamboo-growing area is increasing year by year and *phyllostachyspubescens* (referred as bamboo forest afterwards) forest has become an important ecosystem type. Unfortunately, few investigations have focused on the impacts of acid rain on soil respiration in bamboo forest, and to our knowledge, the long-term *in situ* experiment was missing. Also, the information about the effect of acid rain on bacteria microbial community in bamboo forest is rare. Thus, studying soil respiration and microbial community in bamboo forest (subtropical forest) subject to SAR treatments is important for understanding their functions in C cycling/ecosystem C flux.

In this study, we measured the soil respiration, soil microbial biomass (characterized as soil microbial biomass C (C_{mic}) and soil microbial biomass nitrogen (N_{mic})) and microbial community change in a bamboo forest, a subtropical soil environment. The selected forest was subject to 10 months of artificial acid rain to evaluate if soil respiration and the microbial community is altered by different SAR levels.

2. Materials and Methods

2.1 Site description

In 2015, experiments were conducted at Tianmu Mountain (30.30°N, 119.45°E) near Hangzhou city, in Zhejiang province, China. The detailed information about Tianmu Mountain could be seen in Wang et al. [18]. The bamboo forest is at 500 m elevation and adjoins evergreen broad-leaf forest and commonly mixed grow with *Castanopsissclerophylla*, *Castanopsismysinaefolia*, *Zelkovaschneideriana*, and *Liquidambar formosana*. The area of bamboo forest occupies 875,000m², and understory species are rare, including *Camellia sinensis*, *Euryahebeclados*, *Cyclobalanopsisgracilis*,

79   *Cyclobalanopsismyrsinifolia*, *Rhododendron ovatum* and *Lithocarpusbrevicaudatus*. The properties of soil

80   at the end of the experiment are shown in Table 1.

81   *2.2 SAR treatment*

82   The experiment was conducted since September, 2016. Twelve sample plots (10 m×10 m) were

83   selected and divided into four groups, and the twelve sample plots were almost at the same

84   elevation to avoid the effect of mountain slope gradient on soil respiration as much as possible.

85   According to the acid rain characteristic and acid deposition levels in Lin'An, Jiangsu province,

86   four groups of experiments were designed as follows: control experiment (termed as CK), only

87   deionized water was applied to the experimental sites and the pH was approximately 6.7; T1-T3

88   experiment, prepared acid rain was applied to the corresponding sites and the pH was 5.6, 4.0 and

89   2.5 respectively. The simulated acid rain was prepared from deionized water and contained H_2SO_4

90   and HNO_3 with a mole ratio of 4.5:1 [19]. Throughout the duration of the experiment, 10 L of the

91   simulated acid rain were applied to each site twice a week (or postponed in case of rain or high soil

92   humidity). In order to ensure the acid rain permeated into the soil evenly, we used a simulation

93   apparatus capable of delivering droplet sizes in the range of 1.0 to 1.2 mm diameter. The

94   experimental sites were subjected to simulated acid rain treatments for 10 months.

95

96   *2.3 Soil respiration rate measurement*

97   Soil respiration rate was measured according to Chen et al. [1]. The PVC soil collar (20 cm in

98   diameter) was permanently installed (5 cm) into the soil in each site and soil packing by PVC collar

99   was minimized. In order to minimize the impact of aboveground respiration by living plants

100   during soil respiration rate measurement, we removed the living plants within the soil collar

101   completely prior to measurements. Measurements were generally implemented once a month from

102   September 2016 to July 2017. The detailed information about soil temperature and moisture

103   measurement can be found in Chen et al. [1]. Each measurement started at 09:00 am and the whole

104   process lasted for about 2-3 hour, including 30 min for preheat, and transport [20]. In order to

105   guarantee the accuracy, a preheat measurement was performed to exclude the residual gas inside

106   the instrument prior to formal measurement. Furthermore, during each measurement, the distance

107   between surveyors and gas analyzer should be more than 2 m to avoid the disturbance. The moist

108   soil samples at each site were collected and pretreated for C_{mic} and N_{mic} analysis [21]. In brief, The

109   C_{mic} and N_{mic} values were determined on the <2-mm mesh field-moist samples. Soil C_{mic} was

110   estimated on a 7.3-g oven-dry equivalent of field-moist soil sample by the

111   chloroform-fumigation-extraction method and soil N_{mic} was determined by

112   chloroform-fumigation-incubation method using a 7.3-g oven-dry equivalent of field-moist soil

113   sample, after adjusting the moisture content to 55%. The correction factors applied to C_{mic} and N_{mic}

114   calculation were 0.45 and 0.57. Soil temperature (ºC) and moisture (g water/kg soil) at the depth of 5

115   cm were monitored adjacent to each PVC collar using a probe connected to the Li-8100 during the

116   soil respiration rate measurements.

117

118   *2.4 Statistical analysis*

119   Soil respiration rate in each treatment was calculated as the mean of the measurements from 3

120   collars. The significant level of the soil respiration rate among the SAR treatments (including CK)

121   was tested using a *t*-test proposed by Luo et al. [42]. One-way ANOVA was used to test the SAR

122   effects on bacterial diversity and microbial biomass. All the statistical analyses were performed

123   using Excel 2007 (Microsoft Inc. Seattle, WA, USA) and the SPSS software version 11.0.

124

125   *2.5Bacterial community*

126   At the end of the experiments, soils in twelve sample plots were collected to analyze bacterial

127   microbial community and ten cores (5 cm diameter × 20 cm length) were taken from each sampling

128   plot and mixed. The twelve samples were shipped to lab as soon as possible and subsequently

129   sieved through a 2 mm mesh to avoid the interference of plant debris and soil fauna. Total DNA

130   were firstly extracted according to Zhou et al. [22] and then purified as per Cahyani et al. [23]. The

131   DNA yield, quality and purity were assessed as described by Chang et al. [24]. The universal

132   bacterial primers, PRBA338f and PRUN518r, located at the V3 region of the 16S rRNA genes of

133   bacterioplankton, were used to amplify the variable V3 region of 16S rDNA. The detailed procedure

134   for PCR amplication and DGGE analysis was implemented as described previously by Chang et al.

135   [24]. Bacterial community diversity was calculated as the Shannon-Wiener diversity index [22].

136

137   **3. Results and Discussion**

138   *3.1 Effect of SAR on soil respiration rates*

139   Soil temperature was measured periodically during the whole experiment and the measured

140   results showed that the temperature in soil seasonally changed accompanying with the change of

141   air temperature in experimental site (**Table 2**). Soil temperature and moisture are generally

142   considered two basic factors in controlling soil respiration process. In our study, when the entire

143   experimental period was considered, seasonal variability of soil respiration rate was mainly

144   controlled by soil temperature, as the absolute value of difference in moisture content among all the

145   sampled soil was small. The moisture content probably mediated the responses of soil respiration

146   rate to temperature [43].

147   **Figure 1** showed the seasonal variation of soil respiration rates subject to SAR exposure. For

148   each group experiment, the soil respiration rates varied seasonally following a similar trend with

149   soil temperature: from September 2016 to January 2017, soil respiration rate decreased during

150   winter, followed by a sharp increase during spring and summer from March 2017 to July 2017. This

151   observation was in accordance with that reported by Chen et al. [1]. Seasonal mean soil respiration

152   rate for the CK, T1, T2 and T3 treatments were 3.44, 4.80, 4.35 and 4.51 $\mu mol\ m^{-2}\ s^{-1}$, respectively

153   (**Figure 2**). However, the soil respiration rates among the four group experiments were not regular.

154   For example, the soil respiration rate of T2 was lowest at the beginning of the experiment while it

155   reached to highest value at the end of the experiment. At each sampling point, no typical trend with

156   the strength of SAR was observed, which suggested that it's not easy to clearly state how the

157   different SAR treatments impacted on the soil respiration rates herein.

158

159   Compared with CK (control, deionized water, pH 6.7), all the SAR treatments in this study

160   (including T1 (pH 5.6), T2 (pH 4.0), T3 (pH 2.5)) induced a positive effect on the soil respiration rate.

161   Soil respiration rates under T1, T2 and T3 treatments were enhanced by 39.5%, 26.5% and 31.1%,

162 respectively, relative to that of CK treatment. However, ANOVA resultsindicated that different
163 SAR exposure of T1, T2 and T3 had no significant effects on soil respiration (p>0.1), although SAR
164 are generally considered to have inhibition effect on soil respiration rate [40]. Although effect of
165 SAR treatments on soil respiration has been widely studied, results from these studies were
166 inconsistent. For example, Will et al. [25] revealed that acid rain impacted little on the soil CO_2 flux
167 while Zelles et al. [26] observed a decreased CO_2 emission when an artificial acidic soil subject to
168 SAR exposure and this negative effects might be contributed to the inhibited activity of soil
169 microbes by low pH in the soil. What's more, an enhanced soil CO_2 flux was also reported when a
170 low concentration of simulated acid rain was applied to the soil [27], which was in consistent with
171 our results in this study. Organisms in soil may mediate themselves to the changing acid
172 environment and meanwhile soil could buff the SAR effects on soil to a certain degree.The
173 enhanced effect may result from nutrients, such as nitrate nitrogen (NO_3^--N), that are used to
174 acidify the simulated acid rain [41].The inconsistent results with regard to the effect of SAR on soil
175 respiration could be justified using several lines of reasoning, including the length of experimental
176 period [1], and the applied simulated acid rain treatment[28]. The soil respiration rate almost
177 maintained constant at pH 4 and pH 6, while decreased 20% at pH 3. Also, the effect of SAR on soil
178 respiration rate was reported to be relied on geographic locations [28]. Compared with microbes in
179 temperate soil, SAR treatment impacted the microbes in subarctic soil more greatly. And soil
180 respiration rates are more easily to be affected by SAR in dry nutrient-poor forests than in medium
181 and mesic forests.

182   Soil enzyme activity is the direct expression of the soil microbial community to the metabolic
183 requirements and available [9]. The soil enzymatic activities, including phosphatase, urease and
184 sucrasewere shown in **Table 1**. SAR treatments (T1 and T2) resulted in a decrease in urease and
185 sucrase activity, although the differences between CK and SAR treatments were not significant.
186 This finding might be attributed to a potential trade-off between the organisms showing positive
187 responses to SAR and others showing negative responses.

188

189 *3.2 Effect of SAR on soil microbial biomass*

190   **Figure 3** showed the variations of soil microbial biomass subject to SAR exposure and the
191 average values were plotted in the figure. The soil microbial biomass of CK treatment in September,
192 2014 was not included in the figure as the soil samples were lost during transportation.

193   In winter (November/December, 2016), the concentrations of Cmic and Nmic were generally
194 lower than that in the other sample dates. Possible explanation for the observation would be the
195 lower microbial activity of the soil in the winter [46]. It was observed a more pronounced temporal
196 fluctuation of Nmic values compared with those of Cmic, in agreement with those reported by
197 Moore et al. [21]. Although C and N are the main compositions of microorganisms, their
198 concentrations in microbes vary greatly, especially the N content, and notably depend on the
199 growth stage of microbes. The soil pH held a clearly effect on the microbial biomass and low soil
200 pH would lead to a low microbial biomass content [29]. However, ANOVA analysis results
201 indicated that the difference in soil microbial biomass subject to SAR exposure was not significant
202 (p>0.1) in this study. Furthermore, the concentrations of Cmic and Nmic at all the SAR treatments
203 were comparable or even stimulated compared with CK treatment, which was in consistent with

204　the trend of soil respiration rate. The microbial community might have adapted to the simulated
205　acid rain conditions during the long-term experiment. Neither Cmic nor Nmicwas significantly
206　correlated with soil pH. Possible explanations for this observation were that the pH of soils used in
207　this study was acidic, and a small change of pH caused by acid rain had a minimum effect on the
208　soil microbial biomass. Carter and Rennie[30] revealed that higher pH would enhance microbial
209　biomass content and microbial biomass content would conversely get lost with a lower pH applied.
210　Besides the soil pH, the microbial biomass content would also be affected by other environmental
211　conditions. Wolters [31] found that the decreased soil pH did not impact microbial biomass content
212　until the soil pH was lower than 2 or 3. Besides, the type of acid rain (sulfuric acid or nitric acid)
213　would influence the microbial biomass content. Nitric acid has proven to be capable of positively
214　and negatively impacting microbial biomass, and the variable effects mainly depend on the soil's N
215　threshold value. For example, Bewley and Stotzky[32] found an inhibitory effect of nitric acid on
216　microbial biomass and activity; while Killham et al. [33] reported that nitric acid showed a
217　stimulated effect on microbial biomass and activity exposure to nitric acid.

218

219　*3.3 Effect of SAR on soil bacterial community diversity*

220　The bacterial community diversity was presented as Shannon index. As shown in **Table 3**, only
221　soil bacterial diversity of T3 was significantly different from that of CK based on the obtained value
222　of LSD0.05. Cluster analysis showed that T1 and CK soils had closer association and were related to
223　the T2 soil, while T3 soil was distinctly different from the other treatments (**Figure 4**). The result
224　clearly demonstrated that DGGE profiles revealed marked differences in the response of soil
225　bacterial communities under different SAR treatments.

226　Soil bacterial microbial diversity may be influenced by simulated acid rainexposure [44, 45].
227　However, the obtained findings with regard to the effect of SAR on soil bacterial microbial diversity
228　seemed controversial. For example, Pennanen et al. [34] reported that the amount of bacterial
229　measured decreased with increasing pH and bacteria were more affected than fungi by the
230　acidification. Anderson and Domsch[35] also found that the total microbial biomass was more
231　sensitive to acidic pH than to a neutral pH. Besides, McColl and Firestone [36] and Stemmer et al.
232　[37] observed that acid rain hardly impacted bacterial community in soil. In contrast, Pennanen et
233　al. [38] showed that the bacteria in soil microbial community would increase when humus pH was
234　decreased and the bacteria in soil could adapt to the new acidic environment step by step. Similarly,
235　Wang et al. [39] found a stimulated effect on soil microbial diversity after being subjected to
236　simulated acid rain (pH 4.5/5.5). In our study, a high acid not only stimulated soil respiration
237　(compared with CK experiment) but also increased soil bacterial community diversity. Up to now
238　there was no consensus in this argument and many factors could contribute to this contradiction,
239　like the treatment period, species, and the soil sampling procedure used, which deserves further
240　research in future. In present study, bacterial community was analyzed by DGGE method with
241　universal bacterial primers, which could reflect the genetic diversity of a microbial community and
242　has the advantages of being reliable, reproducible, rapid, and allows screening of multiple samples.
243　However, it also has several limits, such as effect of variable DNA extraction efficiency on DGGE
244　profiles, similarities in the mobility characteristics of the polyacrylamide gel of DNA fragments
245　with different sequences, and dependence of DGGE profiles on soil type being tested and choice of

primers [24]. Therefore, different approaches such as PLFA analysis, Biolog and molecular technology should be used to better investigate the effect of acid rain on soil microbial communities.

4. Conclusions

It can be concluded that the different SAR treatments showed similar seasonal pattern of soil respiration rate in *phyllostachyspubescens* forest in subtropical China. SAR had no significant effects on both soil respiration rate and soil microbial biomass. The soil bacterial diversity analysis indicated that only T3 treatment (pH 2.5) showed a significant effect on soil bacterial diversity relative to that of CK, and the higher acid load (T2 and T3) increased the soil bacterial diversity in terms of Shannon index.Overall, the impacts of acid rain in bamboo forest ecosystem was highlighted in this study and studying soil respiration and microbial community in bamboo forest (subtropical forest) subject to SAR treatments could contribute for understanding their functions in C cycling/ecosystem C flux.

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Conflicts of Interest: The authors declare no conflict of interest.

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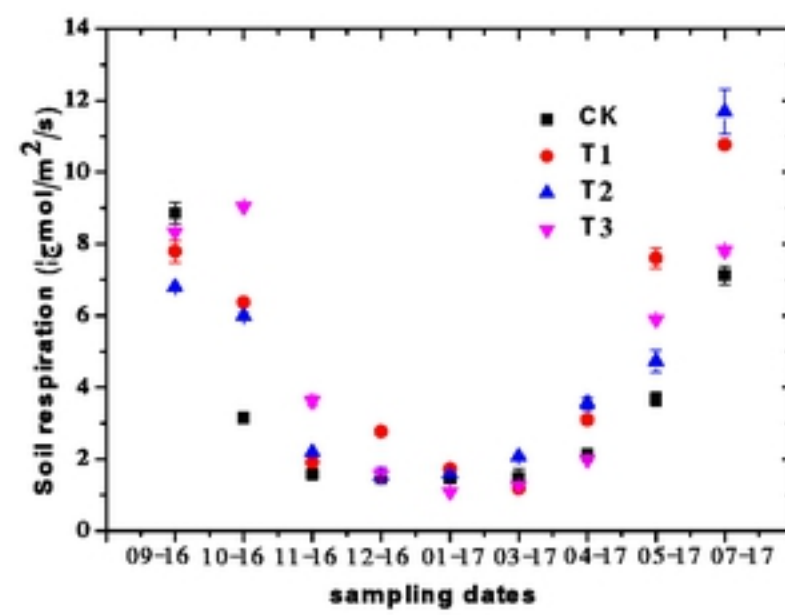
Figure 1. Variation of soil respiration under different SAR treatments
(sampling date: month-year)
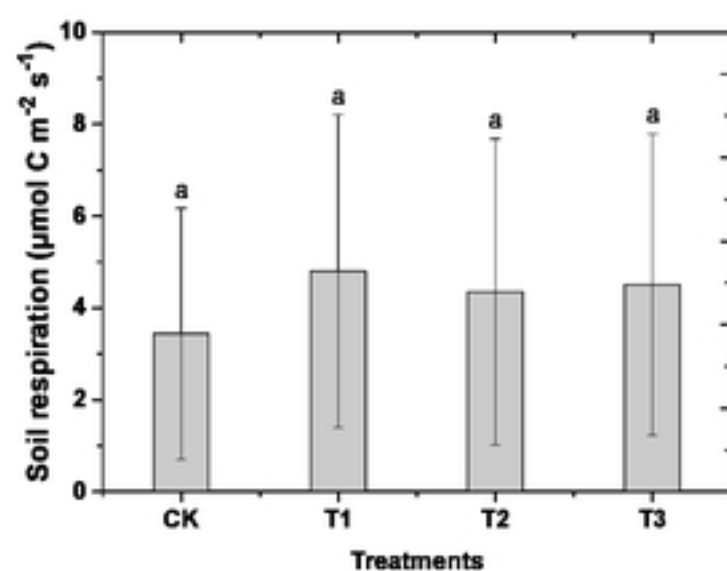
Figure 2 Mean soil respiration rates for different SAR treatments (Error bars are standard error of the mean ... above the column are not significantly different between different treatments)
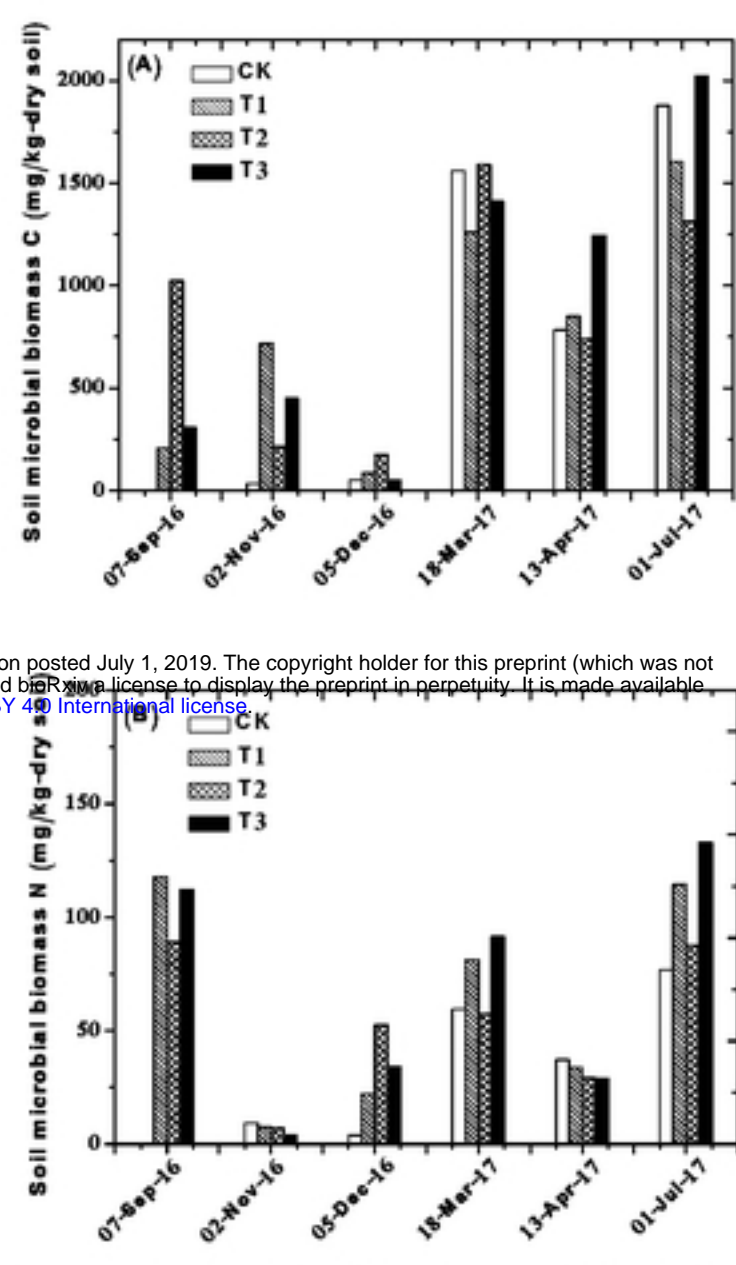
Figure3 Seasonal variations of soil microbial biomass C (A) and N (B) under different SAR treatments
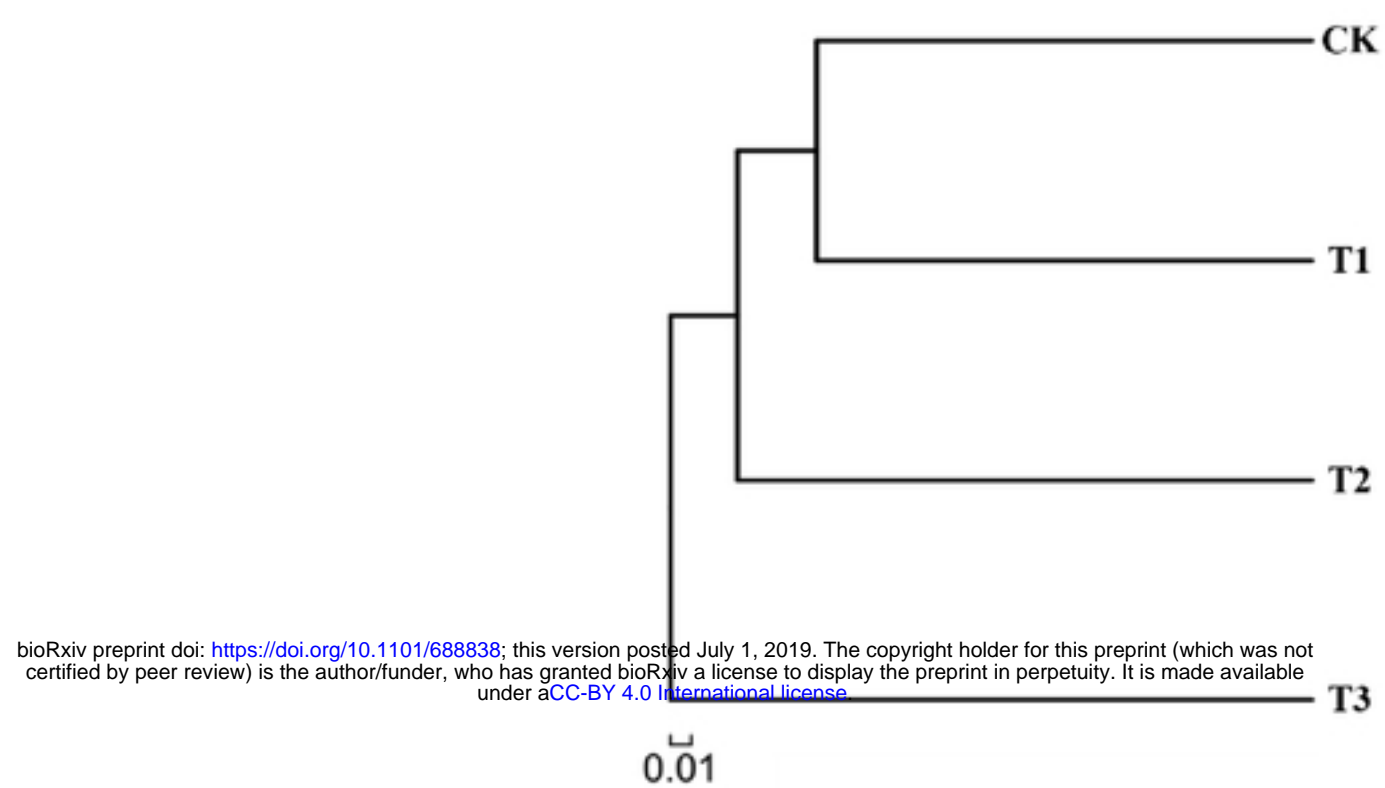
Figure 4 Cluster analysis of the DGGE profiles of 16S rDNA amplified from the tested soils