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1 Response of Soil Bacterial Communities to
2 Sulfadiazine Present in Manure: Protection and
3 Adaptation Mechanisms of Extracellular
4 Polymeric Substances

5

6 *Linlin Qiu,^a Jingjing Wu,^a Wenchao Du,^b Muhammad Nafees,^a Ying Yin,^a Rong Ji,^a*
7 *Steven A. Banwart,^{c,d} Hongyan Guo^{a,*}*

8

9 ^a State Key Laboratory of Pollution Control and Resource Reuse, School of the
10 Environment, Nanjing University, Nanjing, Jiangsu 210023, China

11 ^b School of Environment, Nanjing Normal University, Nanjing 210023, China

12 ^c School of Earth and Environment, University of Leeds, Leeds, LS2 9JT, UK

13 ^d Global Food and Environment Institute, University of Leeds, Leeds, LS2 9JT, UK

14

15 * Corresponding author. Tel.: +86-25-89680263; Fax: +86-25-89680263.

16 E-mail address: hyguo@nju.edu.cn (H. Guo).

17

18 **Abstract**

19 Extracellular polymeric substances (EPS) play a dominant role in protective biofilms.
20 However, studies exploring the underlying protective mechanism of EPS have mainly
21 focused on activated sludge, whereas their positive roles in protecting soil microbes
22 from environmental stress have not been elucidated. In this study, we revealed the
23 response of soil bacterial communities to various dosages of sulfadiazine (SDZ) present
24 in manure, with a special emphasis on the role of EPS. Sequencing analysis showed
25 that the bacterial community demonstrated stronger symbiotic relationships and weaker
26 competitive interaction patterns to cope with disturbances induced by SDZ. EPS was
27 mainly composed of tyrosine-like and tryptophan-like substances, and moreover,
28 carboxyl, hydroxyl and ether groups were the main functional groups. An adaptation
29 mechanism, namely the enhanced secretion of tryptophan-like substances, could help
30 alleviate the SDZ stress effectively in the biofilms occurring in soil that experienced
31 long-term manure application. Furthermore, the existence of EPS weakened the
32 accumulation of antibiotic resistance genes (ARGs) in soil. Our results for the first time
33 systematically uncover the joint action of biofilm tolerance and ARGs in resisting SDZ
34 stress, which enhances understanding of the protective role of EPS and the underlying
35 mechanisms governing biofilm functions in soil environments.

36 **Keywords:** EPS, antibiotic resistance, sulfonamides

37

38 **1. Introduction**

39 Sulfonamides with low price and high efficiency of restraining the growth of
40 micro-organisms have seen extensive usage since the 1940s in worldwide animal
41 husbandry for therapeutic and prophylactic use to enable intensification of food animal
42 production (Baran et al., 2011). The poor assimilation and incomplete metabolism of
43 sulfonamides that occurs in the gut of animals has led to a large proportion excreted
44 unchanged or modified to other bioactive metabolites in faeces and urine (Schauss et
45 al., 2009; Jechalke et al., 2014). Manure application could accelerate the development
46 and epidemic spread of antibiotic resistance genes (ARGs) not only because the co-
47 excretion of parent substance and metabolites can provide selective pressure, but
48 because excreta is an important reservoir of antibiotic resistance bacteria (ARB) and
49 ARGs (Jechalke et al., 2014; Udikovic-Kolic et al., 2014). The increasing prevalence
50 of ARGs will pose global threats to public health, and even result in the emergence of
51 multidrug-resistant superbugs and is regarded as one of the worst-case scenarios for
52 emerging global risks to public health and medical treatments (Udikovic-Kolic et al.,
53 2014; Zhu et al., 2013).

54 The bloom of ARGs in the natural environment results from three principle
55 mechanisms which exist in combination, namely the proliferation of ARB due to
56 selective pressures, genetic mutation and recombination, along with the horizontal gene
57 transfer of ARGs (Berendonk et al., 2015). Mobile genetic elements (MGEs) loaded
58 with ARGs shuttles between bacterial hosts resulting in the dissemination of antibiotic
59 resistance (Gootz, 2010, Bellanger et al., 2014). Molecular mechanisms of antibiotic

60 resistance mainly fall into two groups: those that impede antibiotic access to target
61 receptors through reduction of cell wall permeability to slow uptake and increasing
62 efflux of active compounds out of the cell; those that hinder antibiotic binding to target
63 receptors through inactivation of the antibiotic and modification of target receptors
64 (Blair et al., 2015). To date, research on antibiotic resistance in soil environments
65 mainly focuses on the role of ARGs (Liang et al., 2017; Hall et al., 2020; Wang et al.,
66 2018a; Muurinen et al., 2017; Xie et al., 2016). In fact, microbes within a biofilm state
67 could become less susceptible to antibiotic compounds even if lacking ARGs and
68 associated MGEs (Anderl et al., 2000) and noting that numerous soil microorganisms
69 exist within biofilm communities (Redmile-Gordon et al., 2014).

70 Biofilms are consortia of microbes connected through extracellular polymeric
71 substances (EPS), which take up 80% dry mass of soil biofilms (Chenu, 1993). EPS
72 secreted by microorganisms are mainly present as a complicated matrix predominantly
73 composed of proteins, polysaccharides and extracellular DNA (Redmile-Gordon et al.,
74 2014; Costa et al., 2018). There are a series of mechanisms behind biofilm tolerance
75 and resistance, including transport limitation, sacrificial reaction and nutrient gradient
76 formation (Stewart, 2002). As the principal member in EPS, proteins contain many
77 functional groups and hydrophobic regions that can adsorb a wide range of organic
78 micropollutants including antibiotics (Xu et al., 2013; Wang et al., 2018b; Pi et al.,
79 2019; Wang et al., 2018c; Zhang et al., 2018). Compared to work on the soil
80 environment, research on EPS is more advanced in wastewater treatment plant systems.
81 Studies have reported that EPS in activated sludge served as important reservoirs of

82 sulfonamides and could alleviate sulfonamides stress effectively (Xu et al., 2013; Wang
83 et al., 2018b; Xu and Sheng, 2020). Proteins played a dominate role in the interaction
84 between EPS and sulfonamides, and moreover, the binding between them was achieved
85 by hydrophobic interactions and functional groups of EPS (Xu et al., 2013; Wang et al.,
86 2018b). However, studies on soil EPS lag behind due to methodological challenges. In
87 fact, only limited research considers the critical roles played by soil EPS in improving
88 water retention, soil aggregate stability and microbial metabolic activity (Adessi et al.,
89 2018; Sher et al., 2020; Wu et al., 2019). Until now, the key role of EPS in antibiotic
90 resistance has not been reported in soil environments. In the light of this important
91 knowledge gap, we hypothesize that soil EPS also play an important part in antibiotic
92 resistance of soil microbes and the generation of EPS can occur independently from the
93 presence of ARGs in soil microorganisms. Hence, there is an urgent need to gain
94 knowledge on the joint action of biofilm tolerance and ARGs in response to antibiotic
95 stress in soil environments.

96 To gain insight into the role of EPS in biofilm resistance mechanisms in the soil
97 environment, cation exchange resin extraction methods were employed to obtain EPS
98 from soil. Fluorescence excitation–emission matrix (EEM) spectroscopy combined
99 with parallel factor analysis (PARAFAC) were adopted to gain detailed information
100 about EPS composition. Additionally, Fourier transform infrared (FTIR) spectra
101 analysis was carried out to understand the functional groups of EPS. The objectives of
102 this studies were to explore (i) the influence of applying manure spiked with
103 sulfadiazine (SDZ) on soil bacterial community composition and co-occurrence

104 network; (ii) the protective role of EPS and ARGs in response to SDZ stress; and (iii)
105 the adaptation mechanism exhibited by biofilms resulting from long-term exposure to
106 antibiotics.

107 **2. Materials and methods**

108 *2.1 Experimental design and growth condition*

109 Two soil samples with the same mineral parentage were collected separately using
110 spade within 10 cm of surface soil from a bamboo forest and a farmland in Ningbo city,
111 Zhejiang province, China. These two soils are characterized as Regosol according to
112 the classification of the world reference base of soils (WRB). The bamboo forest soil
113 with no history of manure addition while the farmland soil experienced decades of
114 stable manure application. The physicochemical characteristics of the two soil samples
115 are displayed in Table S1 in the Supporting Information (SI). Soil samples were left
116 standing in a ventilated space at room temperature for at least a fortnight to remove
117 moisture and then passed through 2 mm sieves. Bench-scale pot experiments were
118 designed using manure spiked with different doses of SDZ to mimic a gradient of
119 contamination, namely clean (0 mg kg⁻¹), low (5 mg kg⁻¹), moderate (10 mg kg⁻¹) and
120 high (100 mg kg⁻¹) contaminated manure. Low dosing in our study corresponded to
121 about 10 fold of an average environmentally relevant concentration (Deng et al., 2018).
122 These four mixtures of manure and SDZ were incorporated individually with the two
123 soil samples to form 8 treatments with four replicates each and 32 pots in total. Each
124 pot was filled with 200 g soil, 8 g manure and some water to maintain at 60% of the
125 soil's water-holding capacity. Ten seeds of pak choi (*Brassica rapa* subsp. *chinensis*)

126 were scattered in the surface soil and thinned out during germination. All of the pots
127 were placed randomly side by side in the greenhouse (temperature of 25 °C, relative
128 humidity of 60-70% and 14-h photoperiod). During the growth of pak choi, equal
129 amount of tap water was added to every pot approximately every two days. The pak
130 choi plants were harvested on the 40th day after planting. Simultaneously, soil samples
131 were collected after harvesting the pakchoi plants for further analysis.

132 *2.2 Extraction protocol and characterization of EPS*

133 The method adopted to extract EPS was cation exchange resin extraction as described
134 by Redmile-Gordon et al. (2014), which is described in detail in Text S1. The
135 concentration of dissolved organic carbon (DOC) was used as an indicator of EPS
136 quantity. The EPS extract was passed through 0.45 µm polytetrafluoroethylene
137 membranes and then determined using a TOC analyzer (vario TOC, Elementar,
138 Germany). The composition of EPS was characterized by Fluorescence
139 excitation–emission matrix (EEM) spectroscopy and Fourier transform infrared (FTIR)
140 spectra respectively. To avoid the inner filtering effects, EPS samples were diluted with
141 ultra-pure water to ensure that the DOC concentration was lower than 10 mg L⁻¹. The
142 fluorescence EEM spectra was recorded with excitation ranges between 220-490 nm
143 and emission ranges between 250-550 nm using a F-7000 fluorescence spectrometer
144 (Hitachi High Technologies, Japan). The EEMs were determined by subtracting the
145 ultra-pure water signal first and then removing the Rayleigh and Raman scatter signals.
146 Subsequently PARAFAC was conducted using MATLAB 2019a with a DOMFluor
147 toolbox as described by Stedmon and Rasmus (2008). Split half analysis, residual

148 analysis and sum of squared error analysis were carried out during the process of
149 determining the number of components.

150 The lyophilized EPS extract and infrared grade KBr were mixed with a ratio of 1:100
151 and homogenized in an agate grinder. About 100 mg of the mixture was analyzed by a
152 FTIR spectrometer (NEXUS870, NICOLET, USA) with a spectral range of 4000-400
153 cm^{-1} , 32 scans and a spectral resolution of 4 cm^{-1} .

154 *2.3 Two-Dimensional (2D) FTIR Correlation Spectroscopy*

155 To get the structural variation information of EPS, 2D correlation spectra were
156 conducted following the method of Noda and Ozaki (2004). In this study, SDZ
157 concentration was considered as an external perturbation. Then synchronous and
158 asynchronous correlation spectroscopy was generated using the 2Dshige software
159 (Kwansei-Gakuin University, Japan). The region from 1400 to 800 cm^{-1} was focused
160 and analyzed in detail here, because the major bands were included in this zone.
161 Spectral coordinates, intensities and signs of correlation peaks appearing on 2D spectra
162 could be interpreted according to well-established principles (Noda and Ozaki, 2004;
163 Noda, 2012).

164 *2.4 Sequencing and bioinformatics analysis*

165 Microbial DNA was extracted from lyophilized soil for qPCR and sequencing
166 following the protocol of MoBio DNeasy Powersoil Kit (MoBio, Carlsbad, CA, USA).
167 The modified primer pair 341F/518R was used to amplify the V3 region of 16S rRNA
168 (Klindworth et al., 2012). Unique 12-nt barcode oligonucleotides were connected to the

169 5'-ends of the forward primer to distinguish different soil microbial DNA in the mixed
170 pool. The reaction mixtures and the amplification protocol followed a previous study
171 (Li et al., 2018). The size and quality of PCR products were checked through visualizing
172 on a 2% agarose gel. The checked products were purified using the E-Z 96 Cycle Pure
173 Kit (Omega, U.S.A.). Qubit dsDNA HS Assay Kits (Invitrogen, U.S.A.) were used to
174 determine the concentration of clean PCR products. Then, products were pooled in
175 equimolar concentrations and were subjected to library preparation followed the
176 procedure of Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific, U.S.A.).
177 Afterwards, all of the products were diluted to 100 pM. The diluted products were
178 subjected to cluster generation and sequenced in the Ion Proton sequencer (Life
179 Technologies, U.S.A.). QIIME2 (v.2019.7) was employed to analyze raw sequence data
180 through the following process: demultiplex, quality control, and taxonomy
181 classification (Klindworth et al., 2012). Sequence quality control and the generation of
182 amplicon sequence variants (ASVs) were performed using the DADA2 method
183 (Callahan et al., 2016). After that, a feature table was produced. Silva database was
184 choose to conduct the taxonomy annotation for each sequence (Quast et al., 2012)

185 *2.5 ARGs abundance analysis*

186 The qPCR was conducted using an ABI QuantStudio 12K Real-Time PCR System
187 (Applied Biosystems, USA). The primer sequences are demonstrated in Table S2. Four
188 standard plasmids including *sul1*, *sul2*, *intI1* and 16S rRNA were employed to generate
189 calibration curves. The protocol of making standard plasmids has been describe
190 elsewhere (Chen et al., 2018). A 20 μ L qPCR mixture was made up of 2 \times SYBR Green

191 Premix Ex Taq (Takara, 5 μ L), forward/reverse primers (10 μ M, 0.8 μ L), DNA template
192 (2 μ L), ddH₂O (3 μ L) and ROX (0.2 μ L). The amplification condition was as follows:
193 95 °C for 5 min followed by 35 cycles of 95 °C for 5 s, annealing at 55 °C (16S rRNA,
194 *sul1* and *sul2*) or 63 °C (*int11*) for 30 s and 72 °C for 30 s. Triplicate reactions were
195 carried out for each sample to check the reproducibility and weaken the potential bias
196 of PCR.

197 2.6 Statistical analysis

198 Statistical comparisons of EPS quantity, the abundance of ARGs and Shannon index
199 were examined by one-way analysis of variance (ANOVA) with Fisher's least
200 significant difference (LSD) tests in SPSS 26.0 software (SPSS, Chicago, IL, USA).
201 Principal coordinate analysis (PCoA) was conducted based on the Bray-Curtis distances
202 to evaluate the influence of SDZ on bacterial community composition (Anderson and
203 Willis, 2003). Moreover, Permutational multivariate analysis of variance
204 (PERMANOVA) was employed to determine effect size and significances on beta-
205 diversity using "Adonis" function in "Vegan" package of R project (Anderson, 2001).
206 An on-line linear discriminant analysis of effect size (LefSe) analysis was carried out
207 at <http://huttenhower.sph.harvard.edu/galaxy> to detect markedly different species for
208 different groups (Segata et al., 2011). The alpha value for the factorial Kruskal-Wallis
209 test among treatments was 0.05 and the threshold on the logarithmic LDA score for
210 discriminative features was 2.0. Samples from bamboo forest soil and farmland soil
211 under SDZ exposure (Low, Moderate and High) were used to perform network analysis
212 to investigate variations in interactions between microbes caused by SDZ exposure.

213 The co-occurrence network was constructed by calculating a similarity matrix based on
214 the Spearman correlation coefficient. The correlation threshold was above 0.8 and the
215 p value was below 0.01. To identify highly associated nodes, Molecular Complex
216 Detection (MCODE) was employed on the Cytoscape platform (Bader and Hogue,
217 2003). Finally, Gephi software was used to achieve network visualization (Bastian and
218 Jacomy, 2009).

219 **3. Results and discussion**

220 *3.1 Shift in bacterial community and co-occurrence networks in the presence of SDZ*

221 Natural soil from bamboo forest was compared with farmland soil receiving long-
222 term manure application to explore the acute and chronic response of the soil bacterial
223 communities with the emergence of SDZ as a pollutant of concern. A total of 1,816,492
224 high-quality bacterial sequences were detected through the Ion Torrent sequencing
225 platform. For both soils, the alpha-diversity of the bacterial community measured by
226 the Shannon index was significantly increased with high concentrations SDZ ($p < 0.05$,
227 Figure 1a). To detect if there existed phylogenetic relations between the soil bacterial
228 communities, a PCoA was conducted based on the Bray-Curtis metric distance. PCoA
229 demonstrated a clear separation between the bamboo forest and farmland soils (Figure
230 1b). Furthermore, a PERMANOVA test indicated that the bacterial community was
231 noticeable influenced by the soil type (11.4%, $p = 0.001$), which also demonstrated the
232 pronounced differences in bacterial community between two soils.

233 To extend the understanding of bacterial community changes, we next sought to

234 examine the difference in the soil microbiome composition at taxonomic level (Figure
235 1c). The general landscape of bacterial taxa showed that all samples exhibited similar
236 taxonomic composition and that the dominant phyla were *Proteobacteria*,
237 *Actinobacteria*, *Acidobacteria*, *Gemmatimonadetes*, *Planctomycetes*, *Bacteroidetes*,
238 *Chloroflexi* and *Firmicutes*. *Proteobacteria* and *Actinobacteria* were the most abundant
239 phyla, accounting for 28.62–32.33% and 21.90–34.57% in bamboo forest and farmland
240 soils respectively. For the bacterial community of the bamboo forest soil,
241 *Actinobacteria* was enriched at low concentrations of SDZ, while *Acidobacteria* and
242 *Gemmatimonadetes* were overrepresented at moderate SDZ concentrations, and
243 moreover *Chloroflexi* and *Nitrospirae* were enriched at high SDZ concentrations. With
244 regard to farmland soil, the low concentrations shared an extremely similar bacteria
245 community composition with the control soil, while the bacterial communities exposed
246 to moderate SDZ concentrations demonstrated a community composition that was
247 similar to that for high SDZ exposure. In general, the relative abundance of
248 *Proteobacteria*, *Acidobacteria*, *Gemmatimonadetes* and *Nitrospirae* increased in the
249 presence of moderate and high concentrations of SDZ, while there was a noticeable
250 decline in *Actinobacteria* and *Firmicutes* abundance. In fact, *Actinobacteria* is
251 composed of various crucial microbes in soil playing important ecological roles
252 including recycling of substances, degradation of complex organic matter and
253 bioremediation of xenobiotics (Alvarez et al., 2017). Moreover, *Actinobacteria* is a
254 critical component of antibiotic-producing bacteria and some self-resistance
255 mechanisms has been developed by them to survive (D'Costa et al., 2006). In our study,

256 *Actinobacteria* with a significant decline in the relative abundance might lead to a
257 significant change in the overall soil microbial metabolic pattern and self-resistance
258 ability.

259 To further probe the observed differences, LEfSe was carried out to detect marked
260 different species between treatments (Segata et al., 2011). In general, the bamboo forest
261 soil held a greater diversity of marked different species than the farmland soil (Figure
262 S1), indicating that bacteria in farmland soil had stronger tolerance ability to SDZ. For
263 bamboo forest soil, the low concentrations treatment showed a predominance of
264 *Actinobacteria*, *Thermoleophilia* and *Bacilli*, the moderate concentrations treatment
265 exhibited a dominance of *Acidobacteria* and *Gemmatimonadetes*, and the high
266 concentrations treatment was dominated by *Chloroflexia* at the class level (Figure 1d).
267 In contrast, for the bacterial community in the farmland soil, the low concentrations
268 treatment demonstrated an ascendance of *Actinobacteria*, whereas at the moderate SDZ
269 concentrations *Bacteroidetes* (Phylum) and *Gemmatimonadetes* were dominant and
270 with high concentrations conditions showed a predominance of *Acidobacteria* and
271 *Dehalococcoidia* at the class level (Figure 1e).

272 Besides changes in community composition, microbial interactions might also
273 change when responding to external disturbances. To this end, for both soils it was
274 critical to acquire information on co-occurrence networks of soil bacterial communities
275 with SDZ exposure. Results showed that the network of the bacterial community with
276 the addition of SDZ composed of 88 nodes (214 edges) in the bamboo forest soil and
277 96 nodes (185 edges) in the farmland soil (Table S4). The comparable number of nodes

278 but more edges number in bamboo forest soil indicated that more interactions existed
279 between microbes in bamboo forest soil under SDZ exposure. Through the analysis of
280 the taxonomic feature, we found that the taxa of the bamboo forest soil bacterial
281 network mainly belonged to Bacteroidetes (13.64%), while *Proteobacteria* (15.62%)
282 was the dominant taxa in the farmland soil. Additionally, there were great positive
283 degrees of correlation between bacterial taxa in two soils with the exposure of SDZ
284 (Table S4), which suggested that there was strong symbiotic relationship between
285 microbes with the presence of SDZ. More specifically, the SDZ-treated network of
286 bacterial communities from farmland soil showed the largest positive correlations, over
287 92%. The bacterial networks were clustered into modules in which the species were
288 assigned functional interdependences and harbored similar ecological niches
289 (Layeghifard et al., 2017). One more modules were identified in the network of
290 bacterial community of bamboo forest soil than that of farmland soil in the presence of
291 SDZ (Figure 2c and 2d), which also suggested that bacteria in bamboo forest showed
292 closer interdependence when coping with SDZ stress.

293 Overall, changes in the microbiome communities and co-occurrence networks were
294 related to the external SDZ disturbance in both soils. The bacterial community
295 composition of the farmland soil with the long-term manure application exhibited the
296 capability to tolerate low concentrations of SDZ. Moreover, bacterial community of
297 two soils both demonstrated patterns of stronger symbiotic relationships and weaker
298 competitive interactions to survive with the existence of SDZ disturbance.

299 3.2 Extracellular Interaction shielding by EPS

300 Many microbes have the capacity to excrete EPS and form protective biofilms to
301 tolerate stresses that emerge in the environment (Stewart, 2002). Variations of the
302 amount of EPS were examined between two soils with the application of manure in the
303 absence and presence of various levels of SDZ (Figure 3a). Compared to the soil from
304 the bamboo forest ($208.65 \pm 6.15 \text{ mg kg}^{-1}$), the soil from the farmland contained more
305 EPS ($300.05 \pm 18.51 \text{ mg kg}^{-1}$) with the absence of SDZ in the manure. This result might
306 be related to the fact that manure usually contains some pollutants, such as heavy metals,
307 antibiotics and estrogens (Jechalke et al., 2014; Hanselman et al., 2004), leading to
308 selection of soil microorganisms that can produce more EPS as a survival mechanism.
309 In addition, the farmland soil holds more organic matter mainly due to the long-term
310 application of manure (Table S1), which was conducive to abundant carbon and energy
311 resources for microbial growth and metabolism. When the added manure contaminated
312 high concentrations of SDZ, EPS production increased immensely in both soils. This
313 may indicate the production of microbial EPS as a selection response that confers
314 greater tolerance to SDZ.

315 Aside from being a physical barrier in the extracellular space, EPS could also provide
316 active sites containing functional groups for interaction with organic contaminants to
317 decrease exposure and environmental stress (Zhang et al., 2018; Sheng et al., 2010).
318 FTIR was carried out to identify the main chemical functional groups present in the
319 EPS. Five main absorbance peaks were observed in the FTIR spectra (Figure 3b), which
320 supplied crucial information about the composition and function of the EPS. The band
321 at 1360 cm^{-1} confirmed the presence of carboxyl (Do et al., 2020). The band around

322 1160 cm^{-1} corresponds to the stretching vibration of C-O (Yin et al., 2015; Jia et al.,
323 2017; Zhu et al., 2012). The band at about 1075 cm^{-1} was assigned to the ring vibration
324 of C-O-C (Jia et al., 2017; Zhang et al., 2020). The vibration around 950 cm^{-1} is
325 potentially related to the presence of nucleic acids (Jia et al., 2017). The band at
326 approximately 860 cm^{-1} was attributed to the vibration of C-C and C-OH (Jia et al.,
327 2017). Generally, the functional groups composition of the EPS from the bamboo forest
328 soil was similar to that of the EPS from the farmland soil that experienced long-term
329 manure application.

330 In order to gain more precise information and facilitate the deconvolution of
331 overlapping peaks, 2D-COS analysis was conducted based on the FTIR spectrum. In
332 general, six auto-peaks were observed near 860, 950, 1020, 1110, 1250 and 1360 cm^{-1}
333 in the synchronous map of the EPS from the bamboo forest soil (Figure 3c). Among
334 these peaks, the band at 950 cm^{-1} had the strongest intensity, showing that the O-P-O
335 structure was more susceptible to SDZ concentrations than other groups. All of the
336 cross-peaks were positive, implying that the intensities of these groups proceeded in
337 the same direction with the increase in SDZ concentration. Based on Noda's rule, the
338 intensity variation followed the sequential order C-OH > C-O-C > O-P-O > COOH >
339 C-C (C-OH). In the case of EPS derived from farmland soil, six auto-peaks around 1360,
340 1200, 1130, 1020, 950 and 860 cm^{-1} appeared in the synchronous map obtained from
341 IR spectrum (Figure 3d). Moreover, the peak centered around 1020 cm^{-1} had the
342 strongest intensity, which implied that C-OH was the most susceptible groups to SDZ
343 addition (Oliveira et al., 2017). The cross-peaks were all positive, also showing that

344 changes in the intensities of these groups varied in the same direction with increasing
345 SDZ concentration. The same rule could be applied to the synchronous and
346 asynchronous map and it could be concluded that the sequential order of intensity
347 variation was C-O-C > C-OH > COOH > O-P-O > C-C (C-OH).

348 To eliminate the overlapped absorbance of EEM fluorescence spectra, a PAFAFAC
349 model was employed to provide more reliable and detailed information about EPS
350 composition. Through split half analysis, sum of squared error and residual analysis
351 (Figure S2), all the EEMs (64 samples) could be divided into two protein-like
352 components successfully (Figure 4a). Component 1 representing a tyrosine-like
353 substance with a characteristic peak at Ex/Em (230/295 nm) (Table S5) (Wu et al., 2018;
354 Maqbool et al., 2016; Zhang et al., 2019; Zhang et al., 2016). Component 2 exhibited a
355 fluorescence peak at Ex/Em (270/370 nm) that corresponded well with a tryptophan-
356 like substance observed in previous studies (Table S5) (Wu et al., 2018; Maqbool et al.,
357 2016; Zhang et al., 2019; Zhang et al., 2016; Zhu et al., 2015). In general, the content
358 of C1 in the EPS was approximately double that of C2 (Figure 4b). EPS derived from
359 all the bamboo forest soil shared extremely similar patterns no matter the content of
360 SDZ present in the manure (Figure 4b). Regarding the EPS from the farmland soil,
361 changes in the relative content of the two components became stronger. More
362 specifically, a steady and significant decline could be seen in the proportion of C1 along
363 with the rise of SDZ contained in the manure, decreasing from $65.21 \pm 3.00\%$ in the
364 EPS of control soil to $59.64 \pm 2.31\%$ in that of soil with high SDZ addition (Figure 4b).
365 In contrast, the content of C2 grew steady from $34.78 \pm 3.00\%$ to $40.36 \pm 2.31\%$ with

366 increasing concentration of SDZ (Figure 4b).

367 In summary, it appeared that microbes in two soils adopted different strategies to
368 deal with the increasing concentration of SDZ present in the environment. Microbes in
369 the bamboo forest soil seemed to produce more EPS (Figure 3a), and then they could
370 provide more functional groups to adsorb external SDZ to relief stress. Besides
371 increased EPS production and the adsorption of functional groups, microbes in the
372 farmland soil tended to enhance the secretion of tryptophan-like component existed in
373 the EPS to cope with SDZ stress, and this may be related to a mechanism for increased
374 tolerance to SDZ exposure.

375 *3.3 Intercellular Response dominated by ARGs*

376 As SDZ accumulates in the environment, EPS might not hinder antibiotic compound
377 access to the intracellular environment. The transport of SDZ into microbial cells might
378 lead to the excessive production of ARGs. The absolute copy number and relative
379 abundance of class 1 integrons (*intI1*) and two sulfonamide resistance genes (*sul1* and
380 *sul2*) were estimated using the qPCR technology (Figure 5). In general, *intI1* constituted
381 the largest abundance of all targeted ARGs, whereas *sul1* was the least abundant in all
382 samples. Microbes from the farmland soil with the absence of SDZ held higher ARGs
383 abundance than the bamboo forest soil, a result which agreed with previous studies
384 (Muurinen et al., 2017; Tang et al., 2015; Heuer et al., 2011). In terms of the bamboo
385 forest soil, the absolute abundance of these three genes increased immensely with the
386 introduction of moderate to high SDZ concentration in the manure ($p < 0.05$, Figure

387 5a). The moderate concentration of SDZ may cause the significant increase of *sul2*
388 absolute abundance in the farmland soil ($p < 0.05$), while there was only tiny difference
389 in the abundance of *sul1* and *intI1*. It can be concluded that SDZ first enhanced the
390 action of the plasmid in which the *sul2* gene is located (Sköld, 2000). Subsequently,
391 microbes might activate the excessive expression of *sul1* located in the Tn21 type
392 integron to cope with growing SDZ in the environment (Sköld, 2000). For both soils,
393 only the occurrence of high concentrations of SDZ coincided with a significant increase
394 of the relative abundance of *sul1* and *sul2* ($p < 0.05$, Figure 5b). However, there was no
395 noticeable change in the relative abundance of *intI1* between the two soils.

396 *3.4 Protective Mechanisms*

397 The above-mentioned results are consistent with the role of EPS as a microbial first
398 line of defense against external SDZ exposure. Microbes may be shielded by EPS
399 through functional groups that could absorb organic micropollutants. With regard to
400 bamboo forest soil, the acute response of microbes is most likely binding SDZ through
401 the functional group in the EPS, such as carboxyl, hydroxyl, phosphoric and ether group
402 when coping with the low concentration of SDZ (Figure 6a). With the increase in
403 concentration of SDZ, the active sites would be consumed and the EPS would gradually
404 lose its protective action. This may cause antibiotic access to cell and occurrence of
405 ARGs. Even though increased SDZ concentrations may induce microbes to produce
406 more EPS in the extracellular space to reduce the stress of antibiotic exposure, it was
407 still inevitable to induce the bloom of ARGs in cell (Figure 6b). Because of the long-
408 term application of manure, microbes from the farmland soil possessed more EPS and

409 ARGs in the original soil state (Figure 3a and 5). For this reason, the soil microbes
410 appear to have greater tolerance to SDZ (Figure 6c). When confronted with high
411 concentration of SDZ, the enhanced secretion of tryptophan-like substance occurred
412 and may be part of a mechanism for microbes to ease the chemical stress of SDZ (Figure
413 6d). Owing to such a possible protective pathway, the bloom of ARGs did not occur,
414 even when exposed to high concentrations of SDZ. On the other hand, a previous study
415 showed that Ca^{2+} could electrostatically bind with the function groups of EPS,
416 consequently, the bridge between plasmids and EPS was established (Hu et al., 2019).
417 This bond could impede the horizontal transfer of plasmid-borne ARGs into recipient
418 cells (Hu et al., 2019). Microbes in farmland soil had a stronger capability of producing
419 EPS, thus, the inhibition of lateral transfer may also be stronger. There are also other
420 biofilm-specific mechanism of resistance and tolerance identified to deal with
421 exogenous disturbance, such as biofilm-specific expression of efflux pumps and
422 protection of oxidative stress (Van et al., 2014). More detailed works about molecular
423 mechanisms of biofilms tolerance and resistance in soil environment are needed in the
424 future.

425 **4. Conclusions**

426 In summary, our study for the first time investigated the joint action of biofilm
427 tolerance and ARGs in resisting SDZ stress in the soil environment. We found that
428 tyrosine-like and tryptophan-like substances were the main components of soil EPS and
429 the main functional groups included carboxyl, hydroxyl and ether groups. EPS likely
430 played a protective role through their functional groups that provided active sites to

431 adsorb SDZ in the extracellular space, thereby reducing bioavailability. Soil microbial
432 communities that experienced long-term manure applications developed adaptation
433 mechanisms to ease the external SDZ stress. The enhanced secretion of tryptophan-like
434 substances might help avoid the bloom of ARGs in soil environment.

435 **CRedit authorship contribution statement**

436 Linlin Qiu: Writing - Original Draft, Writing - Review & Editing. Jingjing Wu:
437 Investigation, Resources. Wenchao Du: Writing - Review & Editing. Muhammad
438 Nafees: Writing - Review & Editing. Ying Yin: Conceptualization, Writing - Review
439 & Editing. Rong Ji: Conceptualization, Writing - Review & Editing. Steven A. Banwart:
440 Conceptualization, Writing - Review & Editing. Hongyan Guo: Conceptualization,
441 Project administration, Funding acquisition.

442 **Declaration of Competing Interest**

443 The authors declare that they have no known competing financial interests or personal
444 relationships that could have appeared to influence the work reported in this paper.

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678 **Figure Captions**

679 Figure 1. Comparison of bacterial communities between bamboo forest soil and
680 farmland soil with a gradient of SDZ addition in the manure. (a) alpha diversity
681 estimated by Shannon index (b) PCoA plot of bacterial community composition based
682 on Bray-Curtis metric distance (c) taxonomic compositions of bacterial communities
683 and cladogram generated by LEfSe indicating difference of taxa induced by the addition
684 of SDZ in bacterial community of bamboo forest soil (d) and farmland soil (e).

685 Figure 2. The bacterial co-occurrence networks under SDZ presence (Low, Moderate
686 and High) in the manure based on correlation analysis. A node stands for an ASV and a
687 connection represents a strong Spearman's correlation with $p > 0.8$ and significant at p
688 value < 0.01 . The bacterial modules (Score > 3) in the networks were clusters of closely
689 interconnected nodes. The nodes of the SDZ presence networks are colored by phyla
690 (a, c) and modules (b, d) respectively for two soils and the size of each is proportional
691 to the number of connections (degree). The red edges represent positive interactions
692 between two ASVs, while the blue stand for negative interactions.

693 Figure 3. Characterization of EPS expressed using general spectroscopic observations.
694 (a) The amount of EPS represented by DOC concentration (b) FTIR spectra of EPS
695 extracted from two soils and synchronous and asynchronous 2D correlation maps
696 generated from FTIR spectrum of EPS obtained from (c) bamboo forest soil and (d)
697 farmland soil.

698 Figure 4. Fluorescence components information identified by PARAFAC analysis (a)

699 the spectral shapes and (b) relative amount of two components obtained using
700 fluorescence maximum and the number of replicates for each treatment was eight.

701 Figure 5. The (a) absolute abundance (copies g⁻¹) and (b) relative abundance (gene
702 copies 16S rRNA⁻¹) of ARGs in soil. Error bars indicated the standard deviation and
703 different lowercase letters indicated significant difference ($p < 0.05$).

704 Figure 6. Schematic diagram of protective mechanisms potentially occurred in the
705 extracellular and intercellular space of bamboo forest soil (a, b) and farmland soil (c, d)
706 in the presence of low and high concentration.

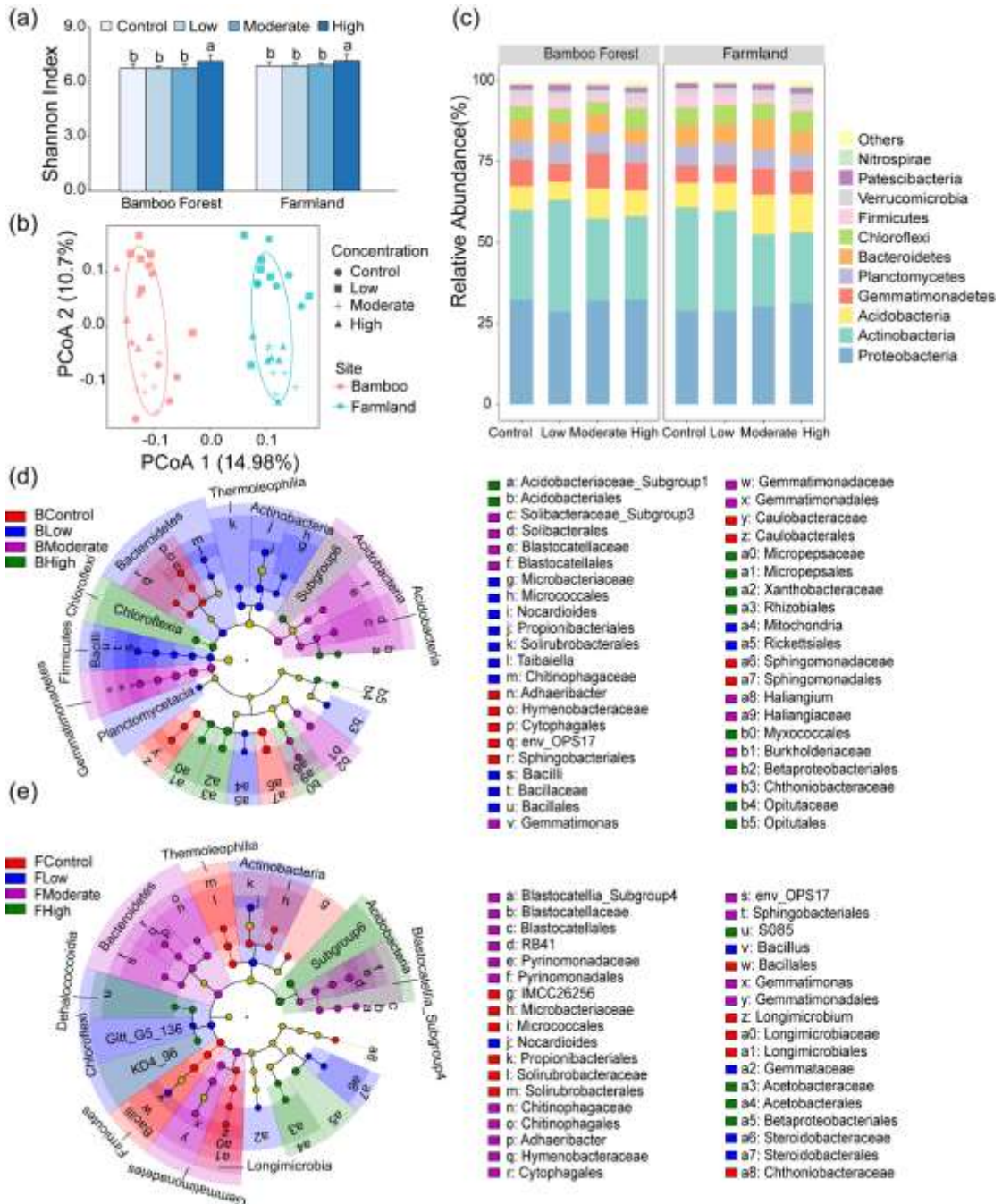


Figure 1.

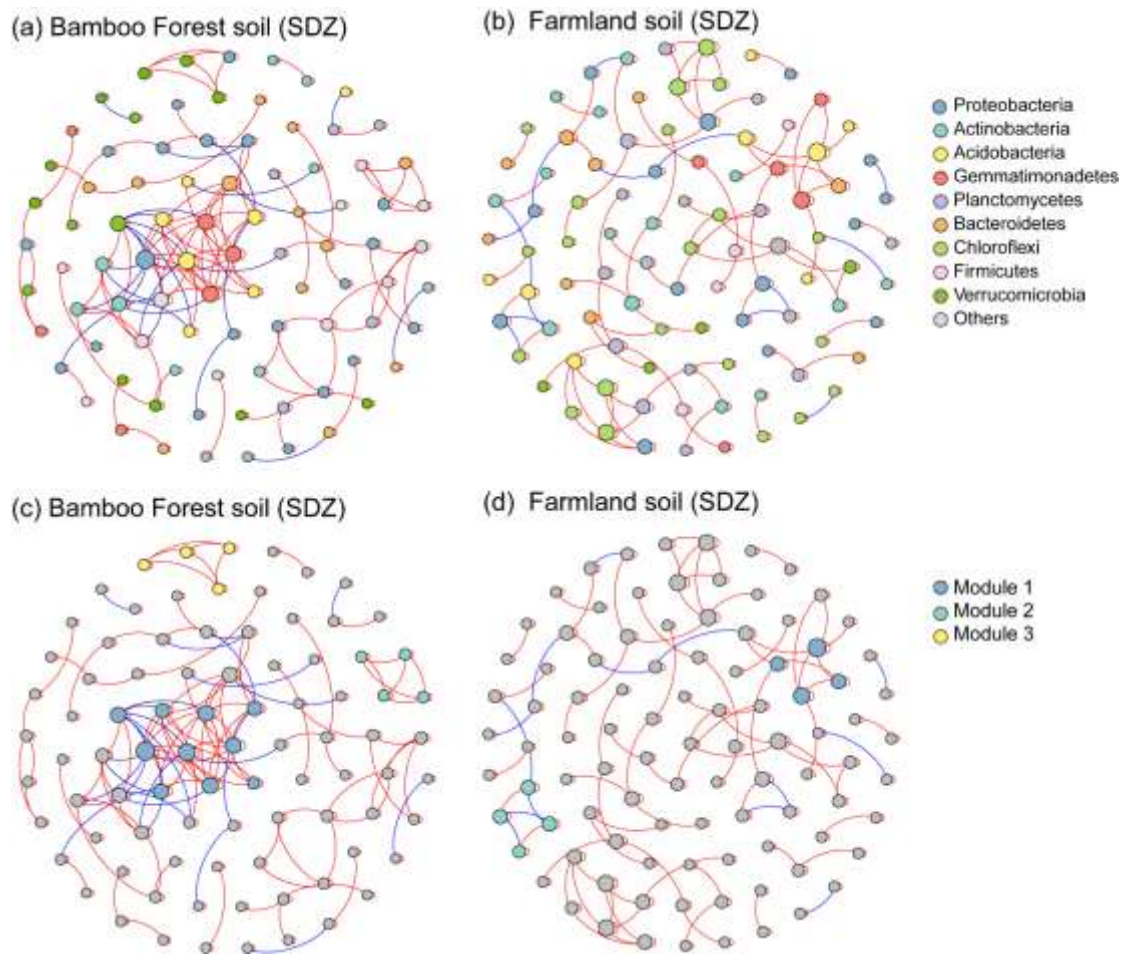


Figure 2.

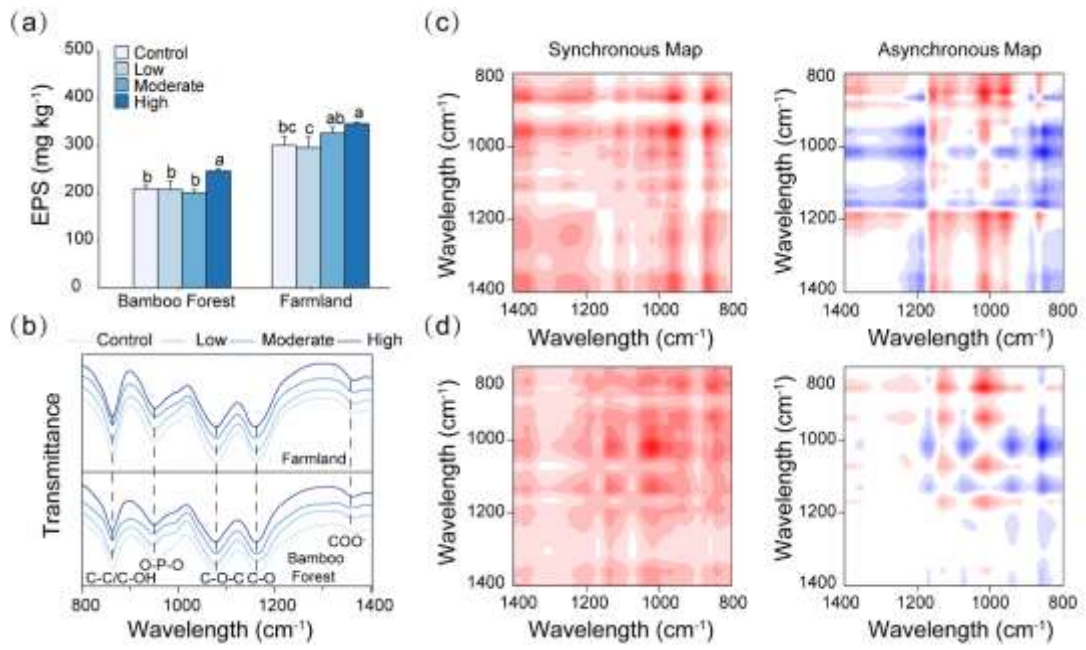


Figure 3.

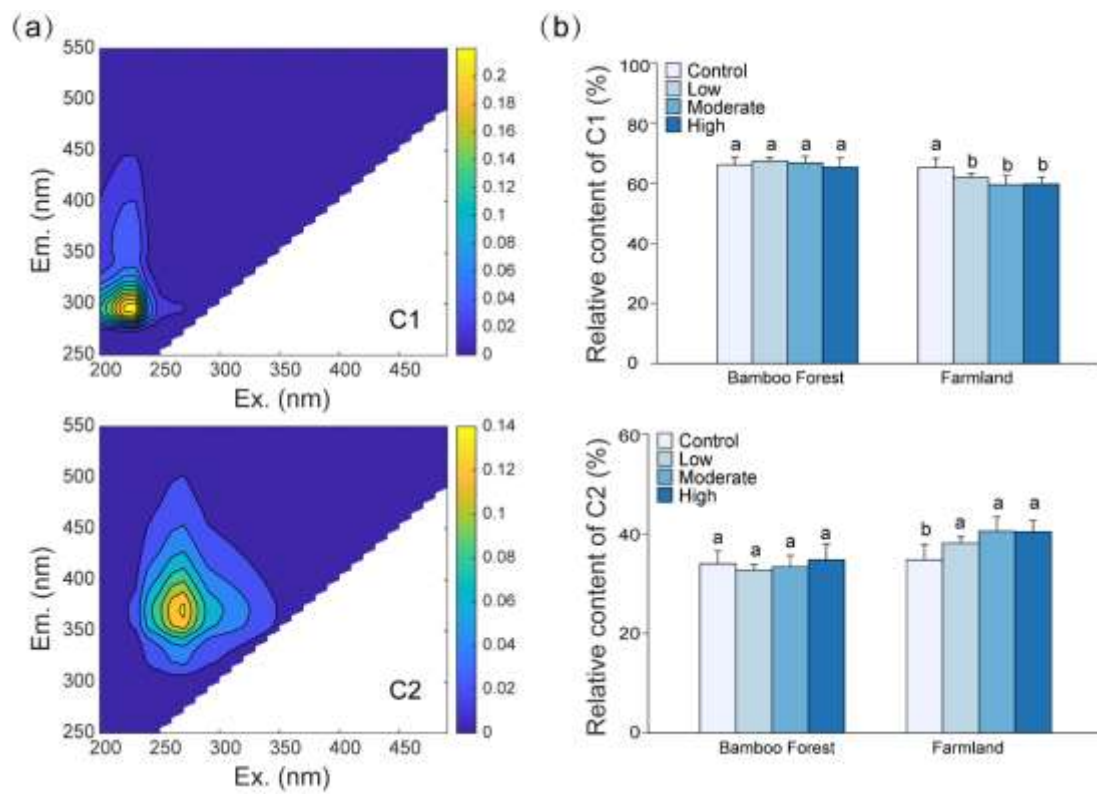


Figure 4.

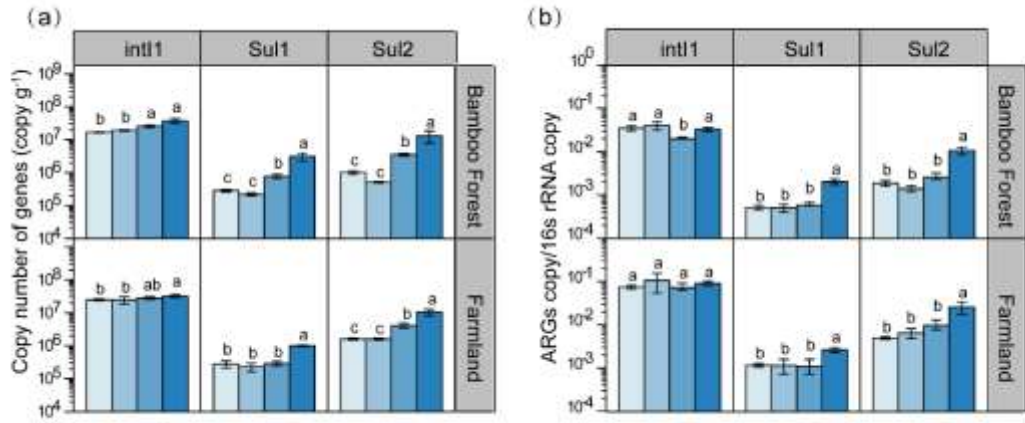


Figure 5.

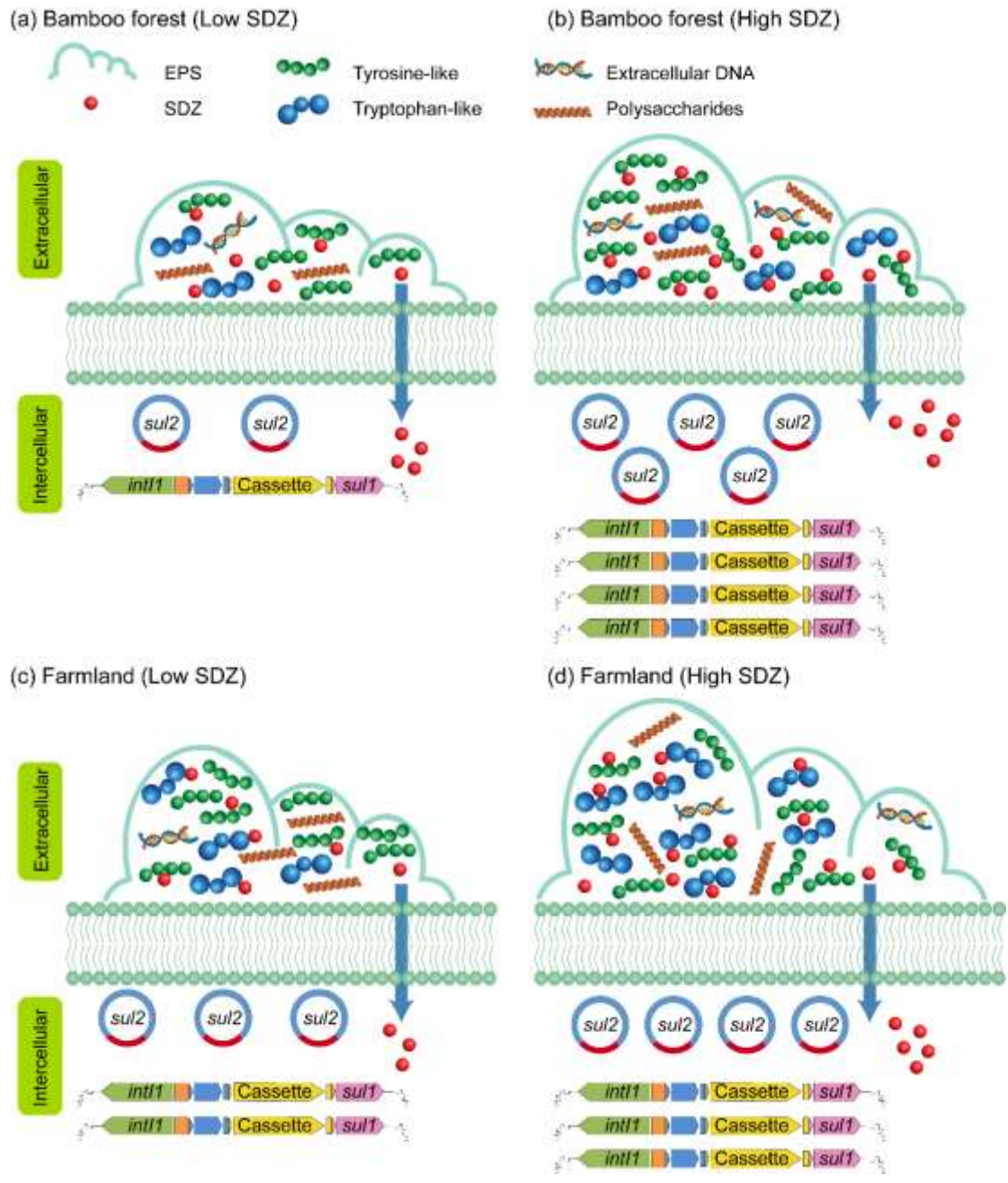


Figure 6.