Supplementary information

Effects of norfloxacin, copper, and their interactions on microbial communities in estuarine sediment

Xiaohan Chen[a,1], Jinjin Chen[a,1], Xiaoxuan Yu[a], Edmond Sanganyado[a,b,c]*, Lin Wang[a], Ping Li[a,b], Wenhua Liu[b,a]*

[a]*Provincial Key Laboratory of Marine Biotechnology, Institute of Marine Science, Shantou University, Shantou, Guangdong 515063, China;* [b]*Southern Marine Science and Engineering Guangdong Laboratory, Guangzhou 511458, China;* [c]*Department of Applied Sciences, Northumbria University, Newcastle upon Tyne, NE1 8ST, UK*

* corresponding author: esang001@ucr.edu (E.S.) and whliu@stu.edu.cn (W.L.)

[1] XC and JC contributed equally.

S1 Methods

S1.1 Determination of norfloxacin in the sediment sample

Sediment sample was freeze-dried, and 2 g dw was added in a 30-mL glass tube. Afterwards, 100 μL 1.0 mg/L standard solutions of norfloxacin was added into the tube, mixed and stored at 4 °C overnight. After addition of 10 mL acetonitrile and 10 mL citric acid buffer (pH 3), the tube was vortex mixed for 1 min, and ultrasonicated for 15 min, followed by 10 min centrifugation at 1370 g. The supernatant was transferred to a round-bottom flask. After the extraction procedure repeated twice, the supernatants were combined. After rotary evaporation of the extract at 55 °C for removal of organic solvent, the concentrate was diluted by Milli-Q water to 200 mL. Cleanup was conducted using SAX cartridges (6 mL, 500 mg) and HLB cartridges (6 mL, 200 mg) as described in (Zhou et al., 2011). Finally, the background concentration of NFX was determined using a liquid chromatography mass spectrometer (Thermo TSQ-Endura).

S1.2 Determination of Cu in the sediment sample:

Briefly, the freeze-dried sediment sample was sieved and grounded. Concentrated hydrofluoric acid and nitric acid were used in microwave digestion. Afterwards, 0.3 g digested sample was weighted and the Cu concentration by dry weight was analyzed by a Jena PQ 9000 ICP-OES (Analytik Jena AG, Germany) (Zhuang et al., 2019). The experiments were conducted in duplicates. Method blanks were also conducted for quality assurance and quality control.

Table S1 Treatments of sediment samples.

Notations	NFX (μg g^{-1})	Cu (μg g^{-1})	Notes
Blank 1	AO	AO	The original sediment, as the control group on Day 1
Blank 2	AO	AO	The control group on Day 28
NFX1	1.0	AO	
NFX10	10	AO	
NFX20	20	AO	
Cu	AO	40	
NFX1Cu	1	40	
NFX10Cu	10	40	
NFX20Cu	20	40	

AO: as original.

Table S2 Normality test for bacterial taxa at genus a level for genera with >0.1% in relative abundance

	Kolmogorov-Smirnov test[a]			Shapiro-Wilk test		
	Statistics	Freedom	Significance	Statistics	Freedom	Significance
Blank 1	0.348	140	0.000	0.307	140	0.000
Blank 2	0.369	140	0.000	0.249	140	0.000
NFX1	0.374	140	0.000	0.233	140	0.000
NFX10	0.378	140	0.000	0.220	140	0.000
NFX20	0.384	140	0.000	0.213	140	0.000
Cu	0.377	140	0.000	0.233	140	0.000
NFX1Cu	0.376	140	0.000	0.231	140	0.000
NFX10Cu	0.368	140	0.000	0.253	140	0.000
NFX20Cu	0.371	140	0.000	0.259	140	0.000

a. Lilliefors Significance Correction

Table S3 Normality test for bacterial taxa at genus level for the top 30 genera in relative abundance

	Kolmogorov-Smirnov test[a]			Shapiro-Wilk test		
	Statistics	Freedom	Significance	Statistics	Freedom	Significance
Blank 1	0.398	31	0.000	0.360	31	0.000
Blank 2	0.417	31	0.000	0.361	31	0.000
NFX1	0.443	31	0.000	0.367	31	0.000
NFX10	0.444	31	0.000	0.362	31	0.000
NFX20	0.401	31	0.000	0.370	31	0.000
Cu	0.391	31	0.000	0.383	31	0.000
NFX1Cu	0.419	31	0.000	0.375	31	0.000
NFX10Cu	0.386	31	0.000	0.375	31	0.000
NFX20Cu	0.424	31	0.000	0.370	31	0.000

a. Lilliefors Significance Correction

Table S4 Levene's test for homogeneity of variance of bacterial population.

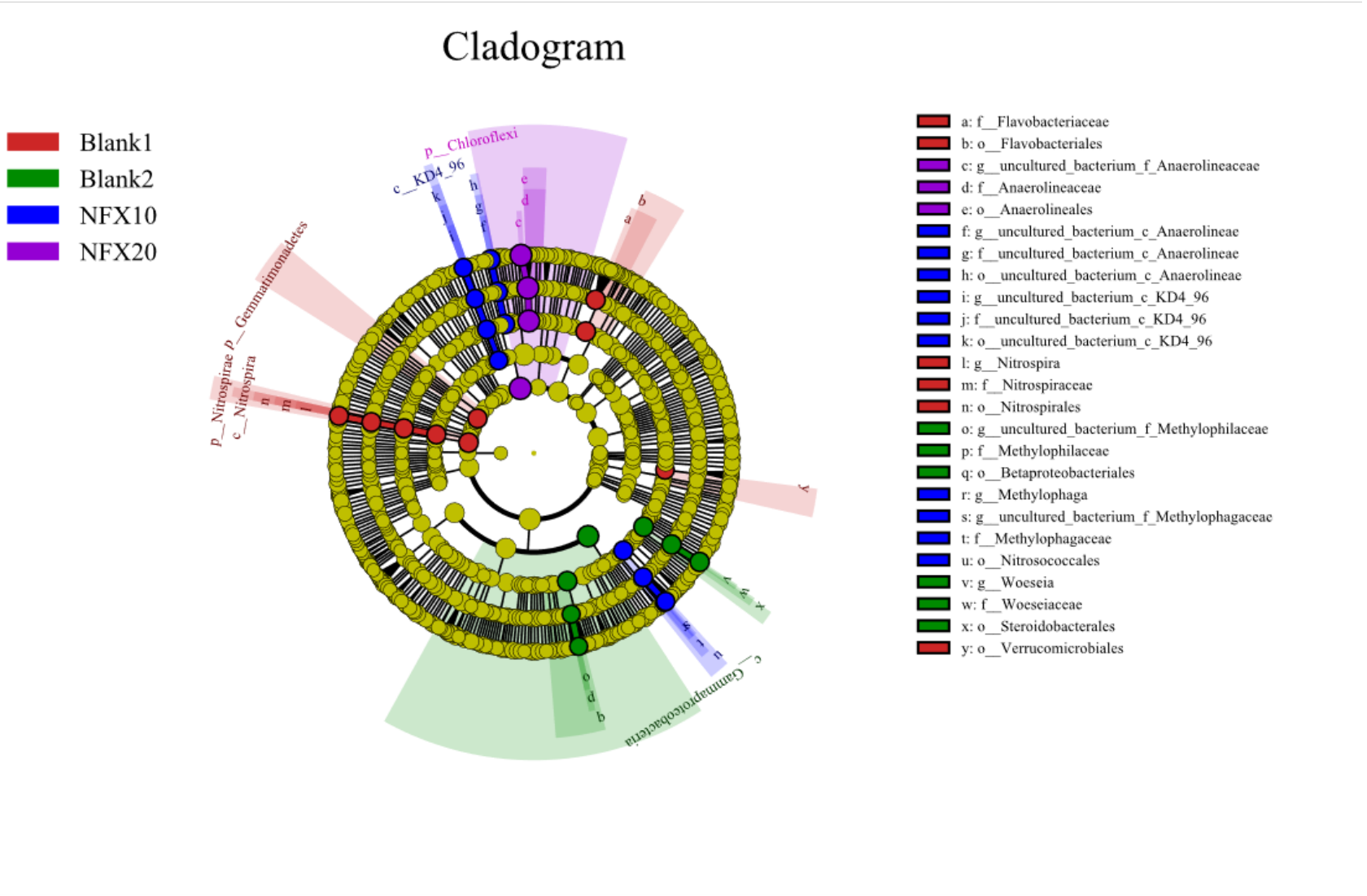
Day	Significance		
	NFX[a]	Cu[c]	NFX+Cu[f]
7	0.808	0.062[d]	0.202
14	0.025[b]	0.020[e]	0.106
21	0.927	0.031[e]	0.139
28	0.775	0.183[d]	0.072

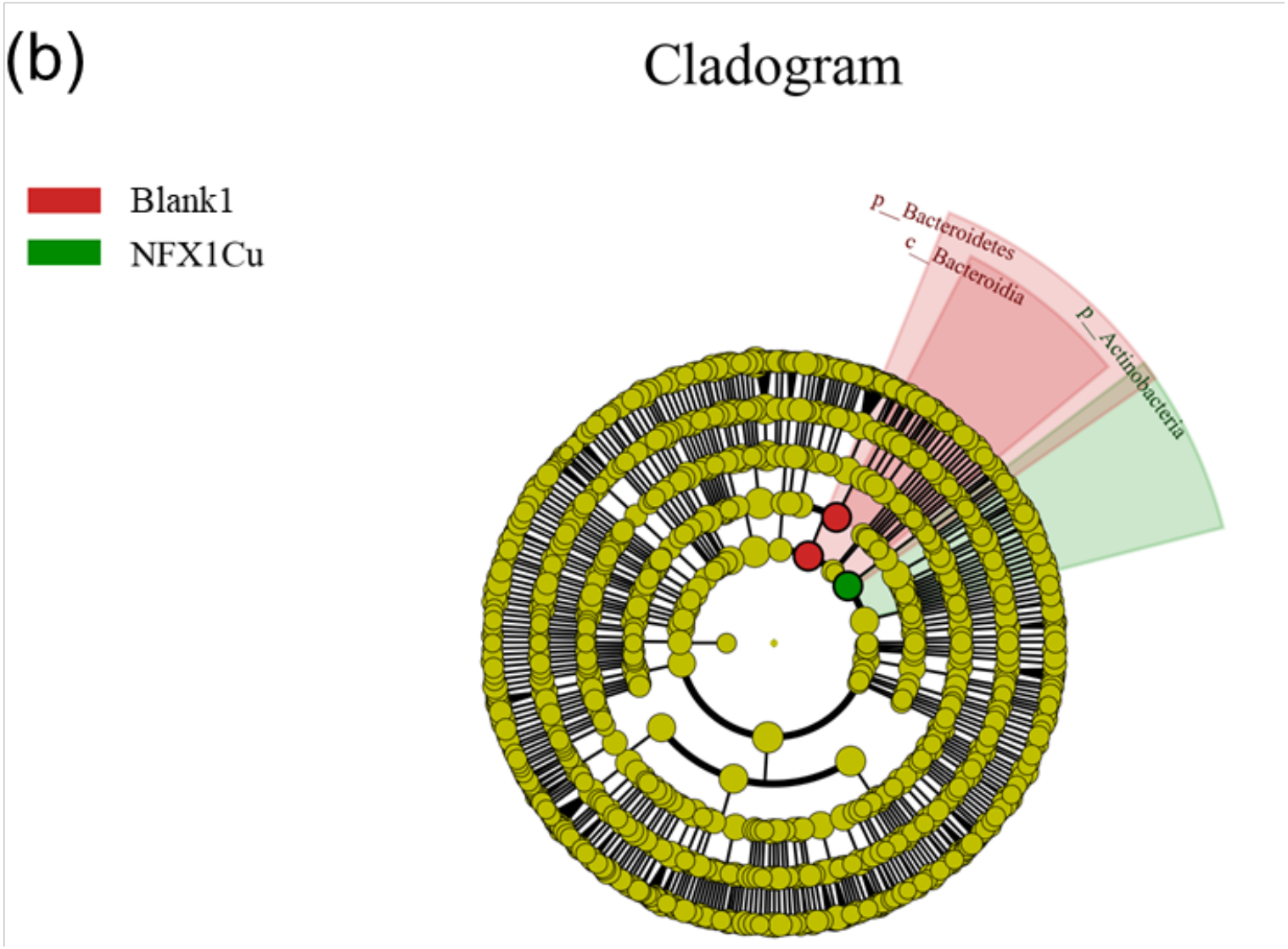
Notes: (a) for data shown in Fig. 1a; (b) the data did not follow homogeneity and Games-Howell's test was used instead; (c) independent samples *t*-test was used; for data shown in Fig. 1b; (d) equal variances assumed; (e) equal variances not assumed; (f) for data shown in Fig. 1c.

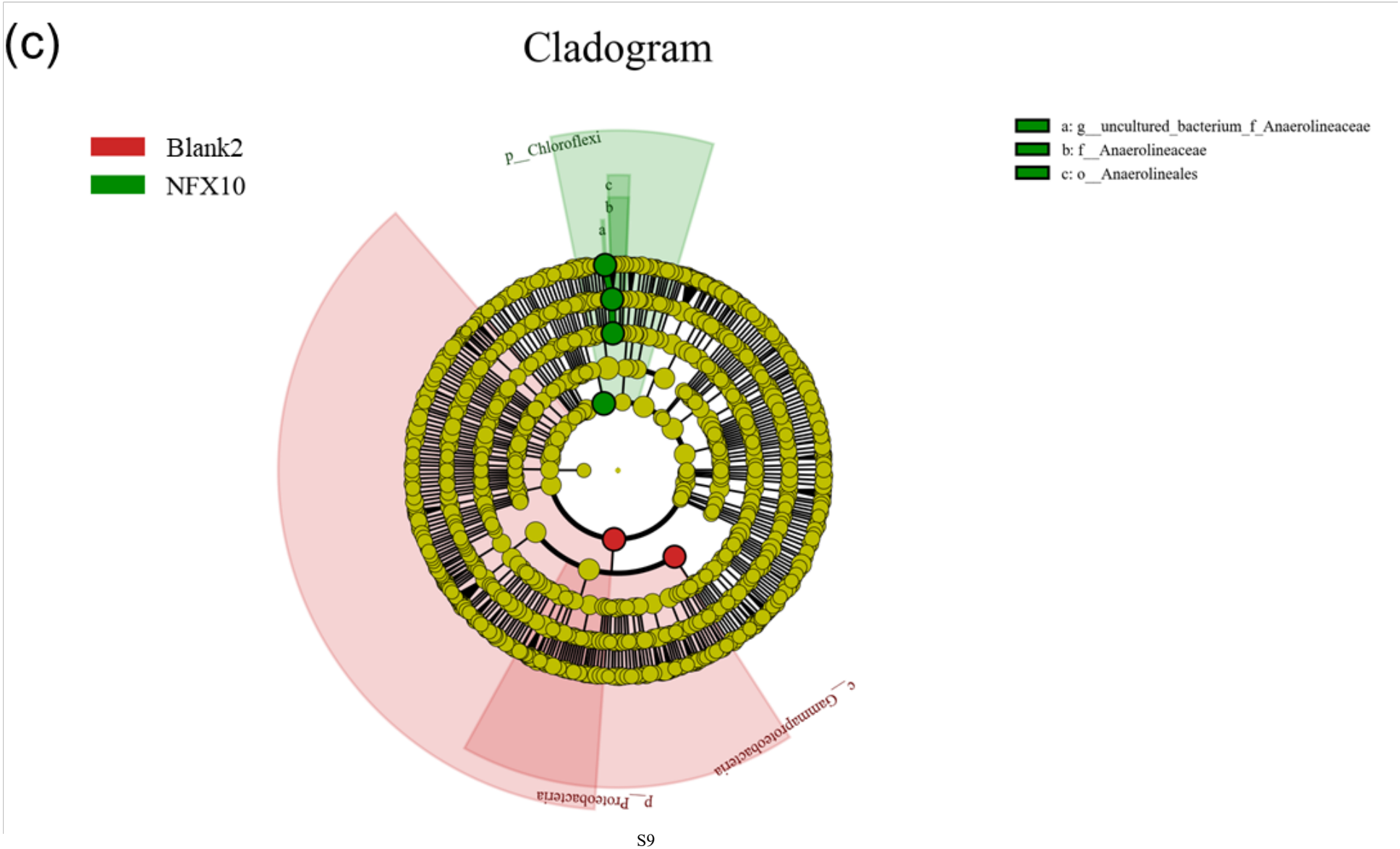
Table S5 Microbial abundance and diversity from OTU numbers and α-diversity indices.

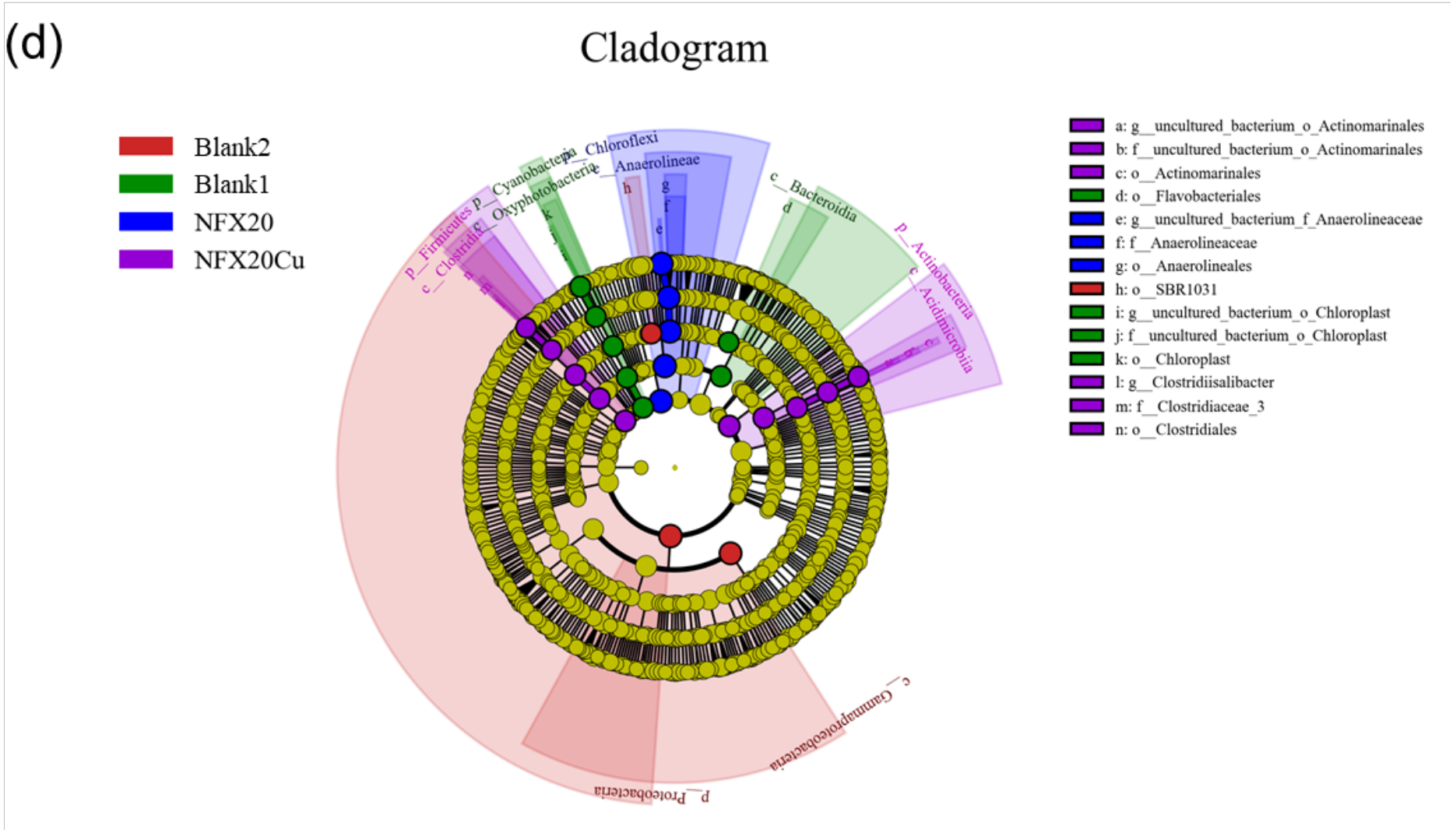
	No. of sequences	OTU	ACE	Chao 1	Shannon	Coverage
Blank 1	37357	1805	1821	1825	9.43	99.8%
Blank 2	35069	1797	1818	1825	9.36	99.8%
NFX1	35806	1813	1830	1837	9.23	99.8%
NFX10	36030	1810	1829	1832	9.21	99.8%
NFX20	39435	1811	1823	1824	9.12	99.8%
Cu	37266	1806	1820	1824	9.22	99.8%
NFX1Cu	42165	1814	1827	1829	9.18	99.8%
NFX10Cu	45246	1817	1821	1831	9.15	99.8%
NFX20Cu	45218	1758	1789	1805	8.84	99.6%

(a)


Cladogram
Blank1
Blank2
NFX10
NFX20
p__Chloroflexi
c__KD4_96
p__Gemmatimonadetes
p__Nitrospirae
c__Nitrospira
c__Gammaproteobacteria
a: f__Flavobacteriaceae
b: o__Flavobacteriales
c: g__uncultured_bacterium_f_Anaerolineaceae
d: f__Anaerolineaceae
e: o__Anaerolineales
f: g__uncultured_bacterium_c_Anaerolineae
g: f__uncultured_bacterium_c_Anaerolineae
h: o__uncultured_bacterium_c_Anaerolineae
i: g__uncultured_bacterium_c_KD4_96
j: f__uncultured_bacterium_c_KD4_96
k: o__uncultured_bacterium_c_KD4_96
l: g__Nitrospira
m: f__Nitrospiraceae
n: o__Nitrospirales
o: g__uncultured_bacterium_f_Methylophilaceae
p: f__Methylophilaceae
q: o__Betaproteobacteriales
r: g__Methylophaga
s: g__uncultured_bacterium_f_Methylophagaceae
t: f__Methylophagaceae
u: o__Nitrosococcales
v: g__Woeseia
w: f__Woeseiaceae
x: o__Steroidobacterales
y: o__Verrucomicrobiales


(b)
Cladogram
Blank1
NFX1Cu
p__Bacteroidetes
c__Bacteroidia
p__Actinobacteria


(c)
Cladogram
Blank2
NFX10
a: g__uncultured_bacterium_f_Anaerolineaceae
b: f__Anaerolineaceae
c: o__Anaerolineales
p__Chloroflexi
c
b
a
c__Gammaproteobacteria
p__Proteobacteria


(d)
Cladogram
Blank2
Blank1
NFX20
NFX20Cu
p__Chloroflexi
p__Cyanobacteria
c__Oxyphotobacteria
c__Anaerolineae
p__Firmicutes
c__Clostridia
c__Bacteroidia
p__Actinobacteria
c__Acidimicrobiia
c__Gammaproteobacteria
p__Proteobacteria
a: g__uncultured_bacterium_o_Actinomarinales
b: f__uncultured_bacterium_o_Actinomarinales
c: o__Actinomarinales
d: o__Flavobacteriales
e: g__uncultured_bacterium_f_Anaerolineaceae
f: f__Anaerolineaceae
g: o__Anaerolineales
h: o__SBR1031
i: g__uncultured_bacterium_o_Chloroplast
j: f__uncultured_bacterium_o_Chloroplast
k: o__Chloroplast
l: g__Clostridiisalibacter
m: f__Clostridiaceae_3
n: o__Clostridiales

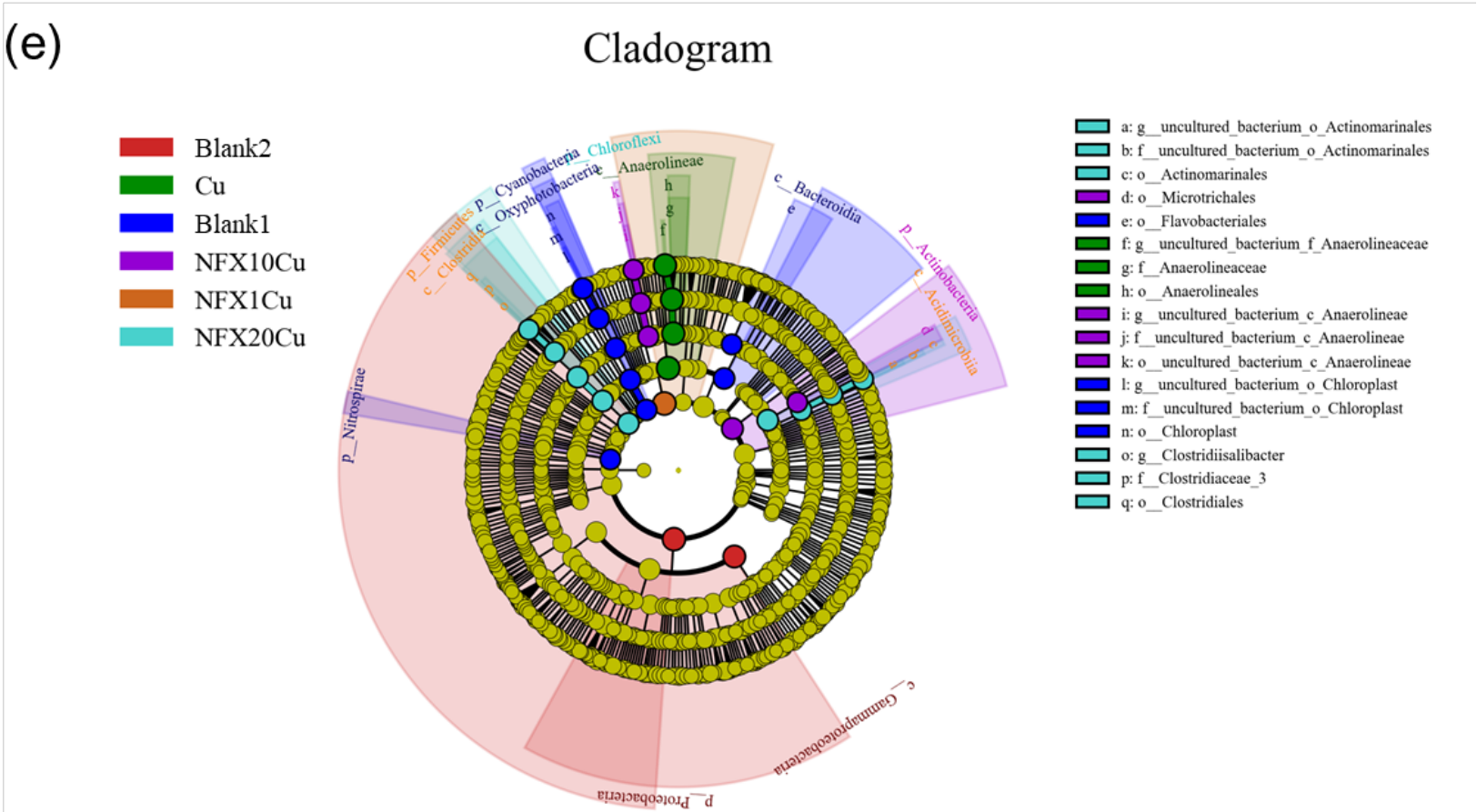
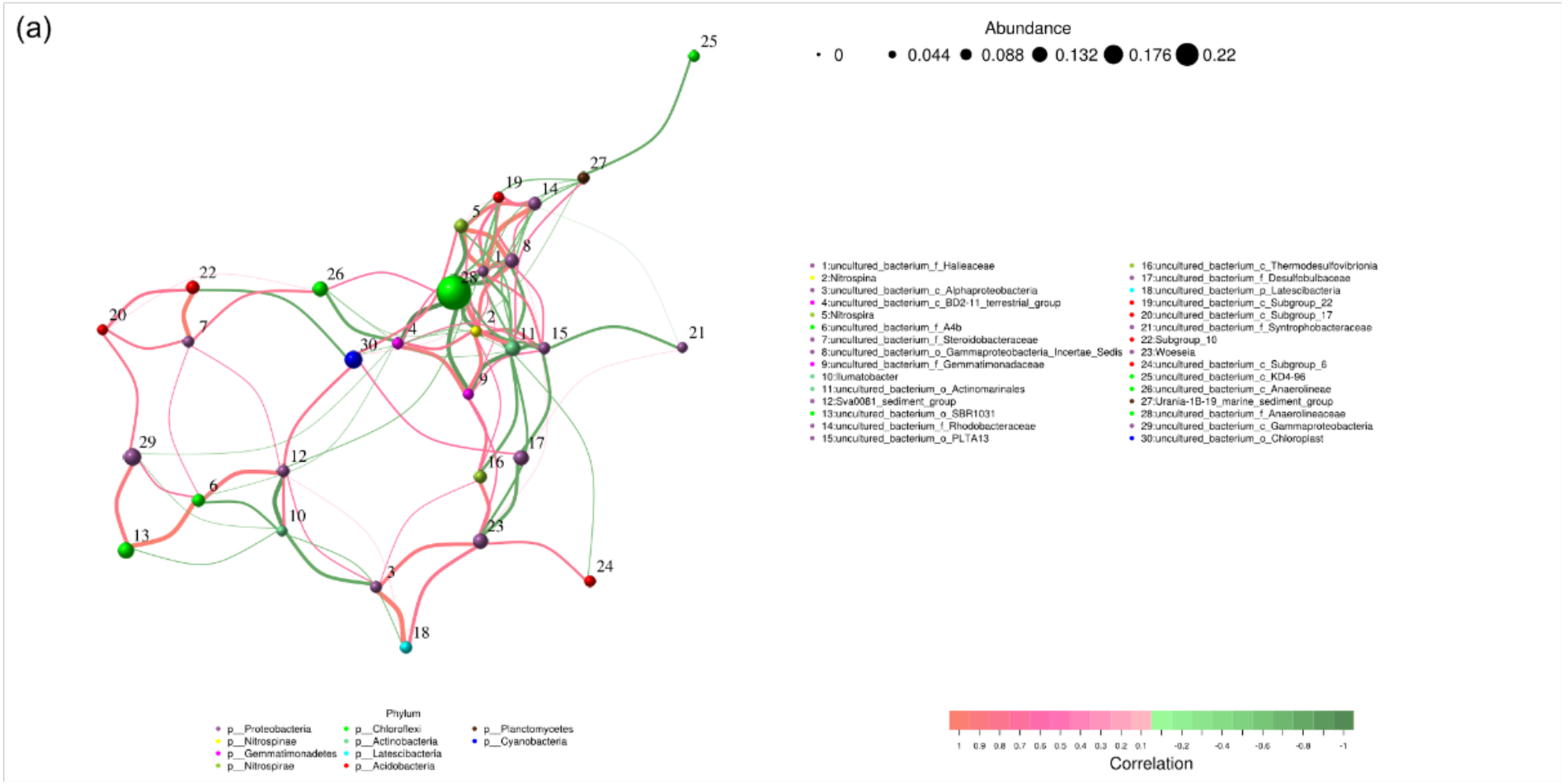
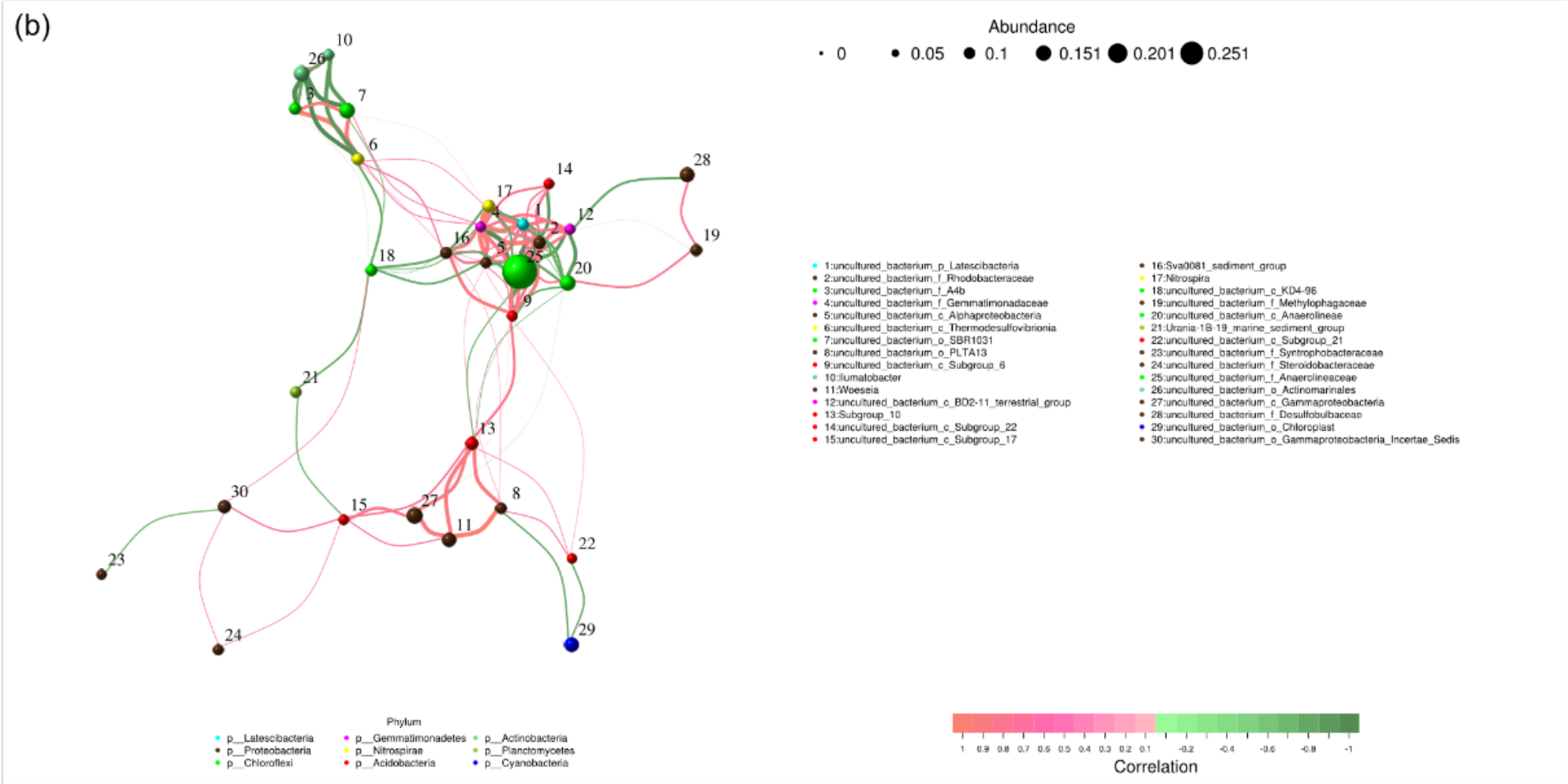
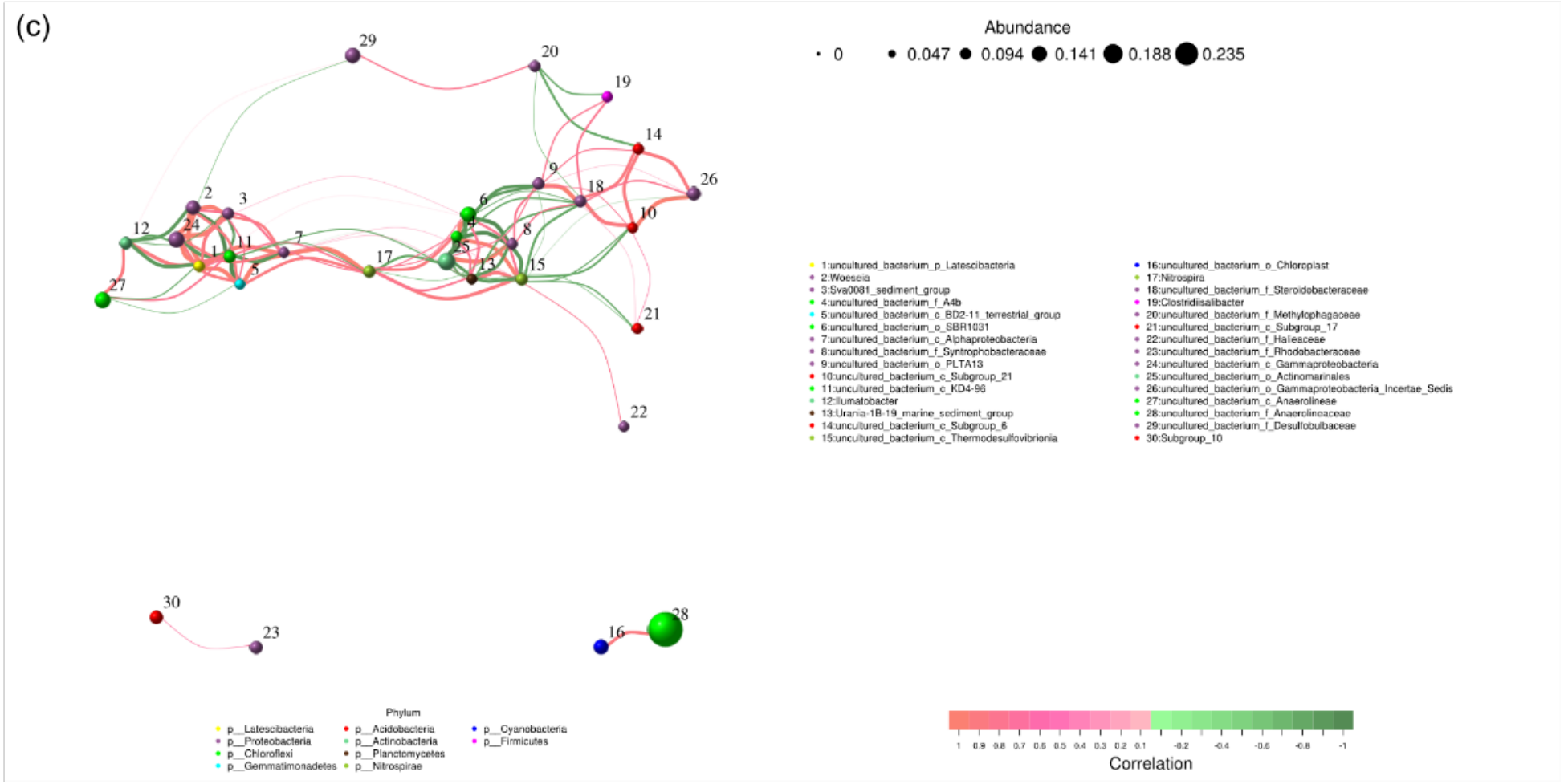


Figure S1. LEfSe Cladograms of biomarkers among different treatments in comparisons among (a) Blank 1, Blank 2, NFX1, NFX10 and NFX20; (b) Blank 1, NFX1, NFX1Cu, Cu and Blank 2; (c) Blank 1, NFX10, NFX10Cu, Cu and Blank 2; (d) Blank 1, NFX20, NFX20Cu, Cu and Blank 2; (e) Blank 1, Blank 2, Cu, NFX1Cu, NFC10Cu and NFX20Cu. LEfSe was conducted at levels from phylum to genus with an LDA threshold of 4.0, taxa with significant differences as highlighted in colored circles and shadings.


(a)
Abundance
0
0.044
0.088
0.132
0.176
0.22
1:uncultured_bacterium_f_Halieaceae
2:Nitrospina
3:uncultured_bacterium_c_Alphaproteobacteria
4:uncultured_bacterium_c_BD2-11_terrestrial_group
5:Nitrospira
6:uncultured_bacterium_f_A4b
7:uncultured_bacterium_f_Steroidobacteraceae
8:uncultured_bacterium_o_Gammaproteobacteria_Incertae_Sedis
9:uncultured_bacterium_f_Gemmatimonadaceae
10:Ilumatobacter
11:uncultured_bacterium_o_Actinomarinales
12:Sva0081_sediment_group
13:uncultured_bacterium_o_SBR1031
14:uncultured_bacterium_f_Rhodobacteraceae
15:uncultured_bacterium_o_PLTA13
16:uncultured_bacterium_c_Thermodesulfovibrionia
17:uncultured_bacterium_f_Desulfobulbaceae
18:uncultured_bacterium_p_Latescibacteria
19:uncultured_bacterium_c_Subgroup_22
20:uncultured_bacterium_c_Subgroup_17
21:uncultured_bacterium_f_Syntrophobacteraceae
22:Subgroup_10
23:Woesela
24:uncultured_bacterium_c_Subgroup_6
25:uncultured_bacterium_c_KD4-96
26:uncultured_bacterium_c_Anaerolineae
27:Urania-1B-19_marine_sediment_group
28:uncultured_bacterium_f_Anaerolineaceae
29:uncultured_bacterium_c_Gammaproteobacteria
30:uncultured_bacterium_o_Chloroplast
Phylum
p__Proteobacteria
p__Nitrospinae
p__Gemmatimonadetes
p__Nitrospirae
p__Chloroflexi
p__Actinobacteria
p__Latescibacteria
p__Acidobacteria
p__Planctomycetes
p__Cyanobacteria
1 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 -0.2 -0.4 -0.6 -0.8 -1
Correlation


(b)
Abundance
0
0.05
0.1
0.151
0.201
0.251
1:uncultured_bacterium_p_Latescibacteria
2:uncultured_bacterium_f_Rhodobacteraceae
3:uncultured_bacterium_f_A4b
4:uncultured_bacterium_f_Gemmatimonadaceae
5:uncultured_bacterium_c_Alphaproteobacteria
6:uncultured_bacterium_c_Thermodesulfovibrionia
7:uncultured_bacterium_o_SBR1031
8:uncultured_bacterium_o_PLTA13
9:uncultured_bacterium_c_Subgroup_6
10:Ilumatobacter
11:Woeseia
12:uncultured_bacterium_c_BD2-11_terrestrial_group
13:Subgroup_10
14:uncultured_bacterium_c_Subgroup_22
15:uncultured_bacterium_c_Subgroup_17
16:Sva0081_sediment_group
17:Nitrospira
18:uncultured_bacterium_c_KD4-96
19:uncultured_bacterium_f_Methylophagaceae
20:uncultured_bacterium_c_Anaerolineae
21:Urania-1B-19_marine_sediment_group
22:uncultured_bacterium_c_Subgroup_21
23:uncultured_bacterium_f_Syntrophobacteraceae
24:uncultured_bacterium_f_Steroidobacteraceae
25:uncultured_bacterium_f_Anaerolineaceae
26:uncultured_bacterium_o_Actinomarinales
27:uncultured_bacterium_c_Gammaproteobacteria
28:uncultured_bacterium_f_Desulfobulbaceae
29:uncultured_bacterium_o_Chloroplast
30:uncultured_bacterium_o_Gammaproteobacteria_Incertae_Sedis
Phylum
p__Latescibacteria
p__Proteobacteria
p__Chloroflexi
p__Gemmatimonadetes
p__Nitrospirae
p__Acidobacteria
p__Actinobacteria
p__Planctomycetes
p__Cyanobacteria
1 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 -0.2 -0.4 -0.6 -0.8 -1
Correlation


(c)
Abundance
0
0.047
0.094
0.141
0.188
0.235
1:uncultured_bacterium_p_Latescibacteria
2:Woeseia
3:Sva0081_sediment_group
4:uncultured_bacterium_f_A4b
5:uncultured_bacterium_c_BD2-11_terrestrial_group
6:uncultured_bacterium_o_SBR1031
7:uncultured_bacterium_c_Alphaproteobacteria
8:uncultured_bacterium_f_Syntrophobacteraceae
9:uncultured_bacterium_o_PLTA13
10:uncultured_bacterium_c_Subgroup_21
11:uncultured_bacterium_c_KD4-96
12:Ilumatobacter
13:Urania-1B-19_marine_sediment_group
14:uncultured_bacterium_c_Subgroup_6
15:uncultured_bacterium_c_Thermodesulfovibrionia
16:uncultured_bacterium_o_Chloroplast
17:Nitrospira
18:uncultured_bacterium_f_Steroidobacteraceae
19:Clostridiisalibacter
20:uncultured_bacterium_f_Methylophagaceae
21:uncultured_bacterium_c_Subgroup_17
22:uncultured_bacterium_f_Halieaceae
23:uncultured_bacterium_f_Rhodobacteraceae
24:uncultured_bacterium_c_Gammaproteobacteria
25:uncultured_bacterium_o_Actinomarinales
26:uncultured_bacterium_o_Gammaproteobacteria_Incertae_Sedis
27:uncultured_bacterium_c_Anaerolineae
28:uncultured_bacterium_f_Anaerolineaceae
29:uncultured_bacterium_f_Desulfobulbaceae
30:Subgroup_10
Phylum
p__Latescibacteria
p__Proteobacteria
p__Chloroflexi
p__Gemmatimonadetes
p__Acidobacteria
p__Actinobacteria
p__Planctomycetes
p__Nitrospirae
p__Cyanobacteria
p__Firmicutes
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
-0.2
-0.4
-0.6
-0.8
-1
Correlation

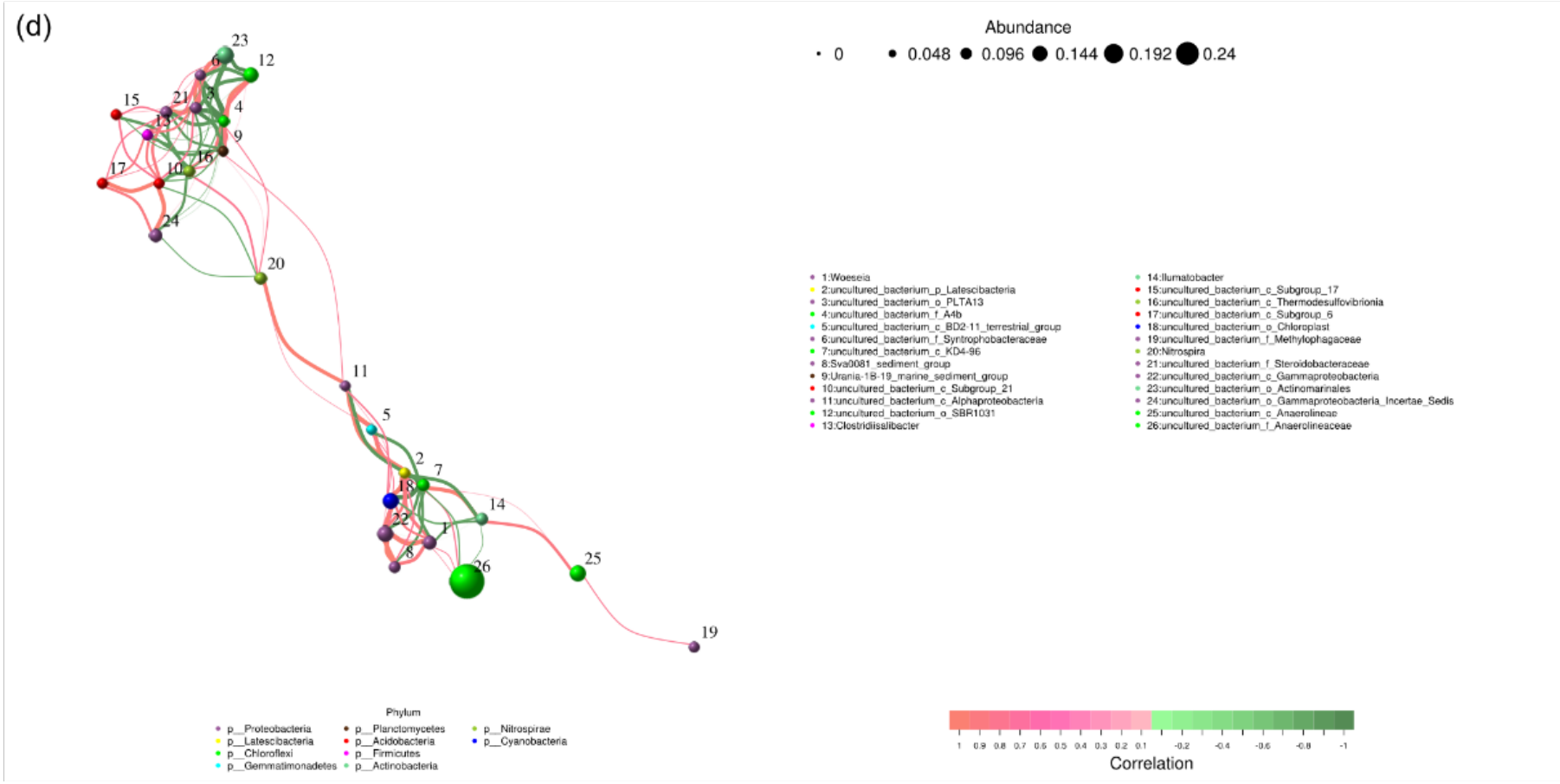


Figure S2. Network analysis among top 30 abundant genera in comparisons among (a) Blank 1, Blank 2 and Cu; (b) Blank 2, NFX1, NFX10 and NFX20; (c) Blank 2, NFX1Cu, NFX10Cu and NFX20Cu; (d) Cu, NFX1Cu, NFX10Cu and NFX20Cu; the green line represents negative correlation, orange as positive, and the thicker the line, the greater the correlation; the circle size represents the relative abundance of each genus.

Co-occurrence between sediment microbiomes (Network analysis) has been conducted among top 30 abundant genera. As shown in Fig. S2a, the network analysis of groups Blank 1, Blank 2 and Cu indicated that 28-day period and Cu contamination exhibited strong positive correlations between 3 pairs of genera ($r>0.9$, $p<0.001$): *uncultured-bacterium-f-Halieaceae* and *uncultured-bacterium-f-Rhodobacteraceae*, *uncultured-bacterium-c-Alphaproteobacteria* and *uncultured-bacterium-p-Latescibacteria*, and *Nitrospira* and *uncultured-bacterium-o-Gammaproteobacteria-Incertae-Sedis*, while negative relations between 2 other pairs ($r<-0.9$, $p<0.001$): *uncultured-bacterium-c-BD2-11-terrestrial-group* and *uncultured-bacterium-f-Anaerolineaceae*, and *Nitrospina* and *uncultured-bacterium-o-Actinomarinales*. Fig. S2b presented the NFX treatment strongly ($|r|>0.9$, $p<0.0001$) resulted in positive connections between 2 pairs of genera (*uncultured-bacterium-p-Latescibacteria* and *uncultured-bacterium-f-Rhodobacteraceae*, and *uncultured-bacterium-f-Gemmatimonadaceae* and *uncultured-bacterium-p-Latescibacteria*) and negative between another 2 pairs (*uncultured-bacterium-f-Rhodobacteraceae* and *uncultured-bacterium-f-Anaerolineaceae*, and *uncultured-bacterium-f-A4b* and *uncultured-bacterium-o-Actinomarinales*). As for comparisons among the control group Blank 2 and the co-contaminants treated groups (Fig. S2c), the positive correlation between genera *uncultured-bacterium-f-A4b* and *uncultured-bacterium-o-Actinomarinales* was also found, as well as in the comparisons among Cu and the combined treatments of NFX and Cu (Fig. S2d). Fig. S2c and Fig. S2d displayed the overlap of 11 pairs of genera with strong correlations ($|r|>0.85$, $p<0.05$), including 3 negative pairs and 8 positive pairs, suggesting that NFX played a more influential role compared to Cu in the sediment microbial community.

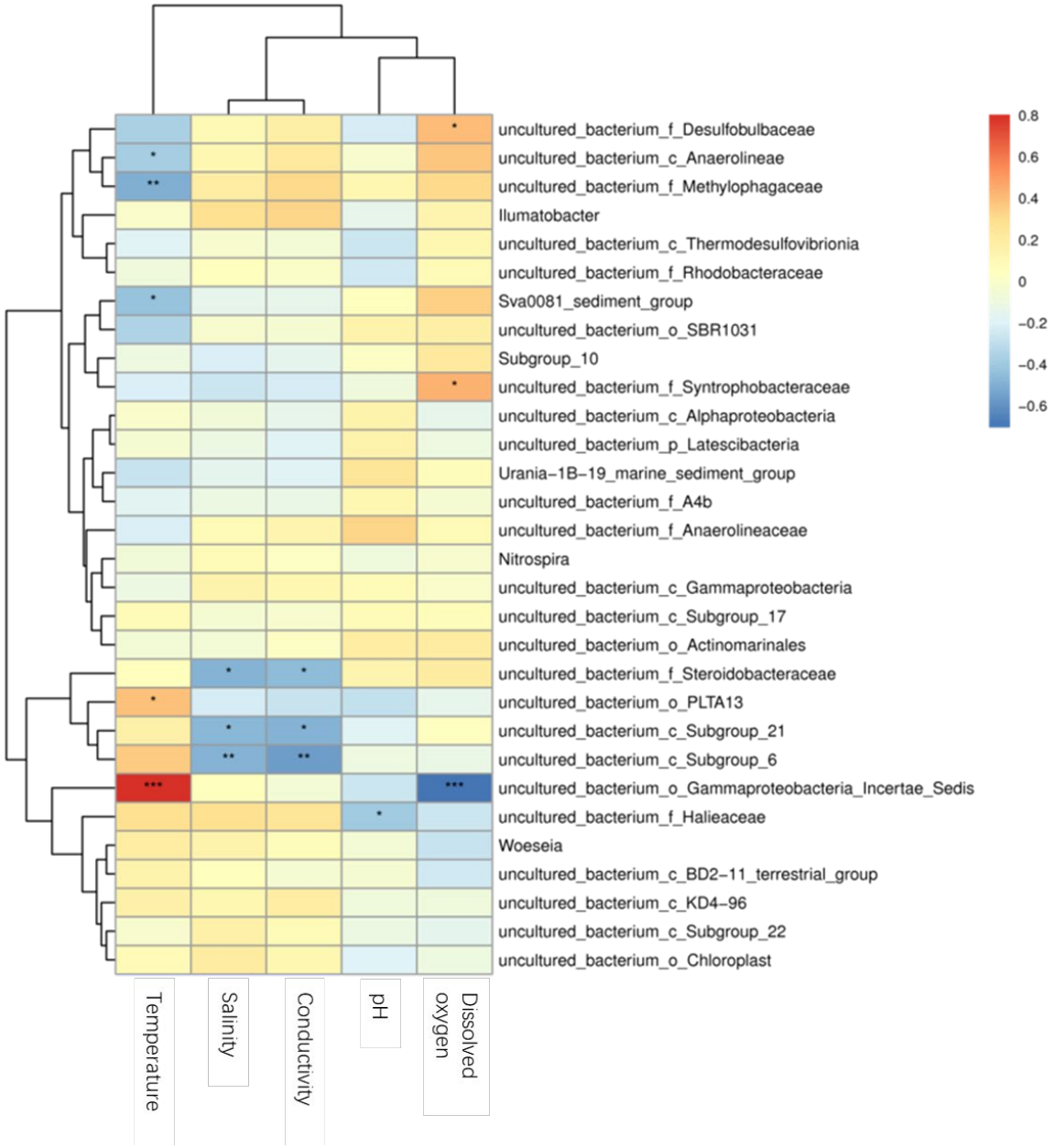


Figure S3. Correlation heatmaps of genera in sediment samples affected by environmental factors; *significant at $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig. S3 displayed the correlation heatmap of genera affected by environmental factors; *uncultured-bacterium-o-Gammaproteobacteria-Incertae-Sedis* presented notably negative correlation with dissolved oxygen ($r<-0.7$, $p<0.0001$), which was also positively related to temperature ($r>0.8$, $p<0.0001$), indicating that the genus is possibly anaerobic and mesophilic.

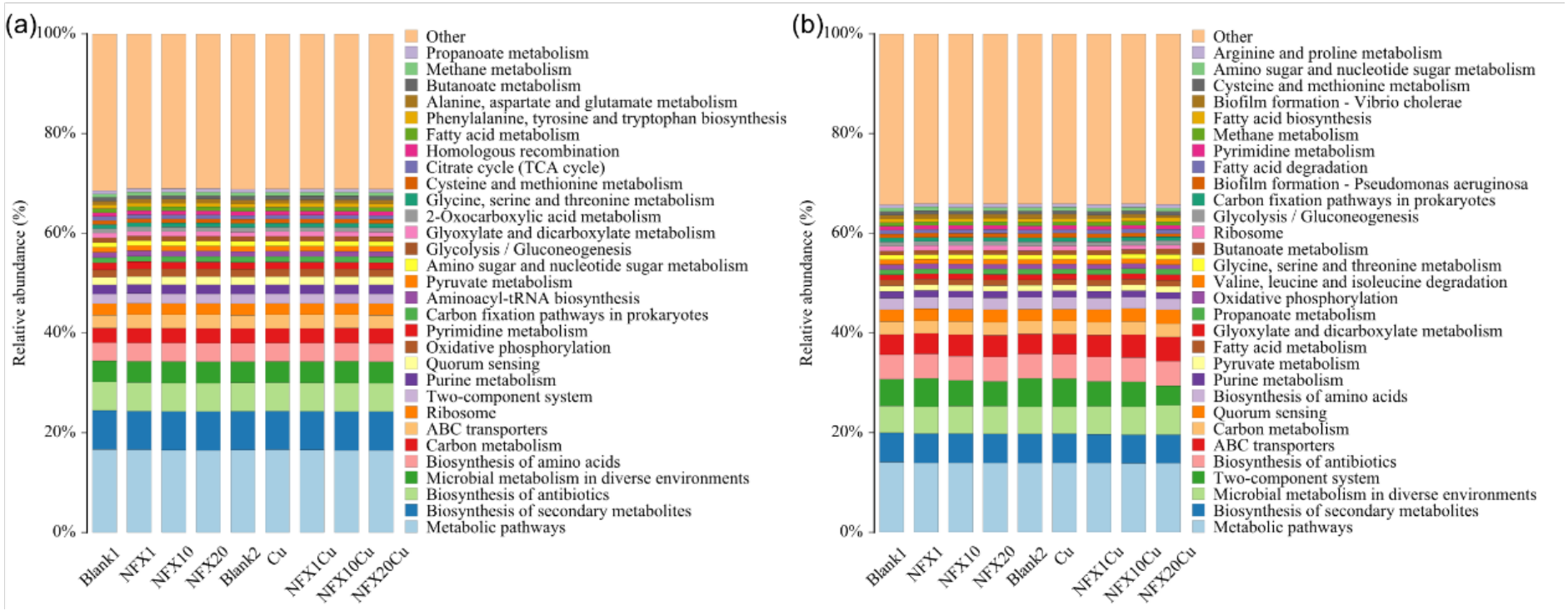
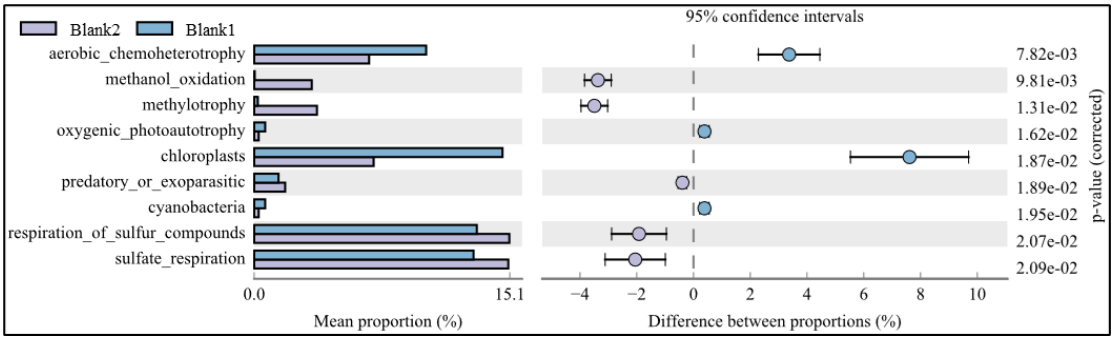
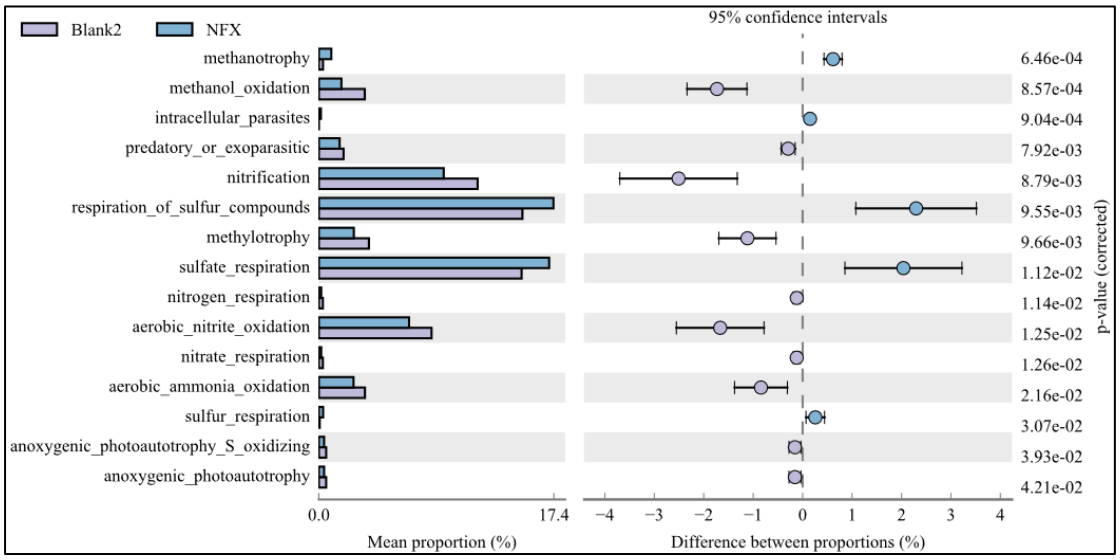
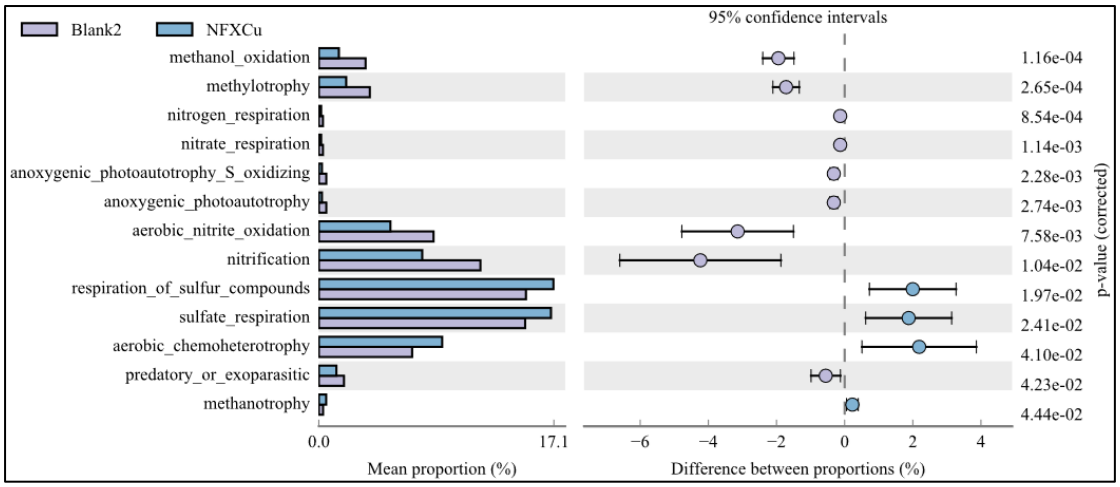


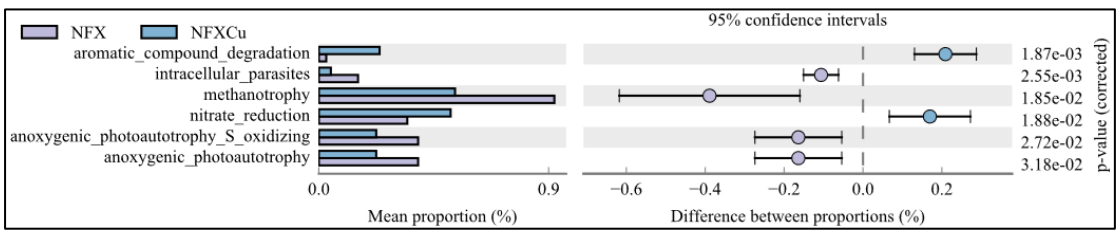
Figure S4. Function prediction of sediment samples with different treatment at the functional classification of level 3 by Picrust2 based on the KEGG database (a) and Tax4Fun (b).

Fig. S4 displayed the top 30 abundant functions predicted according to KEGG database by Picrust2 and Tax4Fun, and the differences among various treatments were inconspicuous, which implied that the microbial communities were relatively stable in regulating the major functions, with substantial overlap in functional diversity of different microbial species in sediment microbial community.


Blank2
Blank1
95% confidence intervals
aerobic_chemoheterotrophy
methanol_oxidation
methylotrophy
oxygenic_photoautotrophy
chloroplasts
predatory_or_exoparasitic
cyanobacteria
respiration_of_sulfur_compounds
sulfate_respiration
7.82e-03
9.81e-03
1.31e-02
1.62e-02
1.87e-02
1.89e-02
1.95e-02
2.07e-02
2.09e-02
p-value (corrected)
0.0
15.1
Mean proportion (%)
−4
−2
0
2
4
6
8
10
Difference between proportions (%)


Blank2
NFX
95% confidence intervals
methanotrophy
methanol_oxidation
intracellular_parasites
predatory_or_exoparasitic
nitrification
respiration_of_sulfur_compounds
methylotrophy
sulfate_respiration
nitrogen_respiration
aerobic_nitrite_oxidation
nitrate_respiration
aerobic_ammonia_oxidation
sulfur_respiration
anoxygenic_photoautotrophy_S_oxidizing
anoxygenic_photoautotrophy
6.46e-04
8.57e-04
9.04e-04
7.92e-03
8.79e-03
9.55e-03
9.66e-03
1.12e-02
1.14e-02
1.25e-02
1.26e-02
2.16e-02
3.07e-02
3.93e-02
4.21e-02
p-value (corrected)
0.0
17.4
Mean proportion (%)
−4
−3
−2
−1
0
1
2
3
4
Difference between proportions (%)


Blank2
NFXCu
95% confidence intervals
methanol_oxidation
methylotrophy
nitrogen_respiration
nitrate_respiration
anoxygenic_photoautotrophy_S_oxidizing
anoxygenic_photoautotrophy
aerobic_nitrite_oxidation
nitrification
respiration_of_sulfur_compounds
sulfate_respiration
aerobic_chemoheterotrophy
predatory_or_exoparasitic
methanotrophy
1.16e-04
2.65e-04
8.54e-04
1.14e-03
2.28e-03
2.74e-03
7.58e-03
1.04e-02
1.97e-02
2.41e-02
4.10e-02
4.23e-02
4.44e-02
p-value (corrected)
0.0
17.1
Mean proportion (%)
−6
−4
−2
0
2
4
Difference between proportions (%)


NFX
NFXCu
95% confidence intervals
aromatic_compound_degradation
intracellular_parasites
methanotrophy
nitrate_reduction
anoxygenic_photoautotrophy_S_oxidizing
anoxygenic_photoautotrophy
1.87e-03
2.55e-03
1.85e-02
1.88e-02
2.72e-02
3.18e-02
p-value (corrected)
0.0
0.9
Mean proportion (%)
−0.6
−0.4
−0.2
0.0
0.2
Difference between proportions (%)

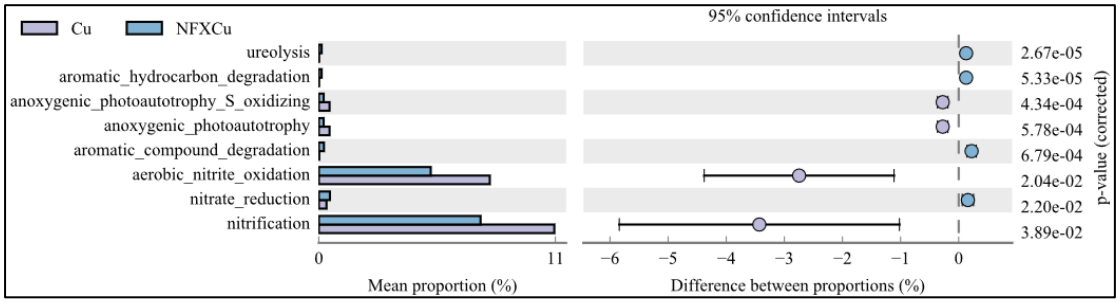


Figure S5. Significant differences in FAPROTAX function predictions between treatment groups.