Bioremediation of an oxyfluorfen-polluted soil using biostimulants obtained by fermentation processes: Effect on biological properties.

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Highlights

- Oxyfluorfen herbicide decreases soil biochemical activity.
- Oxyfluorfen decreased the relative abundance of the Firmicutes and Acidobacteria phyla.
- Biostimulants increased the relative abundance of the Bacteroidetes, Chloroflexi and Proteobacteria phyla.
- The low molecular weight protein of biostimulants increased the degradation of herbicide.

Abstract

We report a study of the bioremediation of two biostimulants obtained from sewage sludge by fermentation processes in a oxyfluorfen-polluted soil over a 72-day period under laboratory conditions. The effects thereof on enzymatic activities, bacterial community and the evolution of oxyfluorfen in soil are determined. At the end of the experiment, and compared with the non-polluted soil, dehydrogenase, urease, β-glucosidase, and phosphatase activities in the oxyfluorfen-polluted soil decreased by 58.3%, 30.4%, 44.7%, and 48%. The application of oxyfluorfen decreased the relative abundance of the Firmicutes (24.5%) and Acidobacteria (8.3%) phyla, and increased the relative abundance of the Gaiellales order (Actinobacteria phylum). The application of both biostimulants to the soil increased the relative abundance of the Bacteroidetes, Chloroflexi and Proteobacteria phyla and decreased the relative abundance of the Acidobacteria and Actinobacteria phyla. The relative abundance of the Chloroflexi, Firmicutes and Proteobacteria phyla increased when applying the experimental biostimulants in the polluted soil. Application of both biostimulants decreased soil oxyflurofen concentration, suggesting that the use of both biostimulants with higher amounts of low molecular weight proteins and peptides could be very useful on the remediation of oxyfluorfen-polluted soils.

Keywords

OxyfluorfenSewage sludge biostimulantFermentation processSoil enzymatic activitiesDNA metabarcoding

1. Introduction

Pesticides are used extensively in agriculture with the objective of eliminating possible pests caused by weeds, insects or pathogens that may cause a negative effect on the development of crops and consequently, on crop yield (Undabeytia et al., 2013; Rosenheim et al., 2020; Nehra et al., 2021). However, the continuous use of these pesticides is a serious environmental problem due to the resulting pollution of soil and aquifers (Duttagupta et al., 2020; Wołejko et al., 2020; Manjarres-López et al., 2021). Some authors have suggested that the environmental problems caused by pesticides depend on their persistence degree. Thus, the lower the degradation of the pesticide and, consequently, the greater its persistence, the higher the risk of environmental contamination (Barba et al., 2017).

Oxyfluorfen is a commonly used pre- or post-emergent diphenyl ether herbicide for broad leaved and grassy weeds in olive farming, principally in Southern European agricultural soils (Hermosin et al., 2013; Rodríguez-Morgado et al., 2014). Oxyfluorfen is currently considered a highly toxic herbicide with a medium to high soil persistence, and a field life of approximately 37 to 172 days (Sondhia, 2009; Mantzos et al., 2014; Rodríguez-Morgado et al., 2014). According to the Pesticide Properties Data Base (PPDB, 2020), the degradation rate (DT50) of oxyfluorfen in soil obtained in laboratory conditions at 20 °C is 138 days, indicating the persistent character of this herbicide in soils.

The persistent use this herbicide is causing serious ecological problems in terms of safety and risk to health (Zhang et al., 2018, Zhang et al., 2018). In this sense, several authors have highlighted the contamination of surface and groundwater as a result of drift and runoff (Harrison et al., 2019; Lupi et al., 2019; Tudi et al., 2021). On the other hand, residual oxyfluorfen could also bioaccumulate in crops (Sondhia,

2010). The elimination of residual oxyfluorfen is therefore an environmental aspect of great importance.

In recent years, several organic wastes with high organic matter content, such as municipal solid waste, sheep manure, biomixtures containing organic wastes generated in olive orchards and the olive oil agroindustry, different edaphic biostimulants (BSs) obtained from rice brain, sewage sludge and chicken feathers have been used in order to eliminate/reduce the toxic effects of oxyfluorfen in soil (Gómez et al., 2014; Rodríguez-Morgado et al., 2014; Delgado-Moreno et al., 2017).

Gómez et al. (2014) suggest that by increasing the content of low molecular weight peptides (<300 Daltons) of these organic compounds, the degradation rate of oxyfluorfen increases due to the fact that these low molecular weight peptides are easily absorbed by soil microorganisms that are tolerant to oxyfluorfen, causing a stimulation of these microorganisms and accelerating the rate of degradation of the herbicide.

Rodríguez-Morgado et al. (2019) obtained different BS from sewage sludge by fermentation processes using the Bacillus licheniformis. Application of these biostimulants to the soil resulted in stimulation of the microorganisms in that soil. Consequently, these results could suggest that these new biostimulants may be useful for the bioremediation of soils polluted by oxyfluorfen.

It is know that soil microorganisms are very sensitive to change of the ecosystem (Starke et al., 2019; Zhou et al., 2020). Therefore, the study of biological parameters such as enzymatic activities and microbial biodiversity is very useful when studying such changes in the soil ecosystem (Rodríguez-Morgado et al., 2014, Rodríguez-Morgado et al., 2019). Soil microbial enzymes are involved in the C, N, P and S

cycles, and provide information on the metabolic capacity of the soil (Shaw and Burns, 2006). On the other hand, metabarcoding is currently one of the most common molecular methods for species identification and, as such, has generated a lot of interest in the study of soil biodiversity (Creer et al., 2016; Zhang et al., 2018, Zhang et al., 2018). This technique is based on amplification and sequencing of the 16S rRNA gene amplicon and is commonly used to characterize soil microbial communities (Orgiazzi et al., 2015; Parlapani et al., 2018; Bukin et al., 2019). Therefore, the study of the soil enzymatic activities as well as its bacterial community through the metabarcoding technique could be very useful to understand how the biology of the soil responds to any toxin applied to said soil.

No studies using BSs obtained from sewage sludge after fermentation processes to remediate oxyfluorfen-polluted soil have been reported. We hypothesize that BSs with different chemical composition applied to soil contaminated with oxyfluorfen stimulate soil microorganisms, thus accelerating the degradation of said herbicide and thus decreasing its toxicity. As such, the purpose of this research was to investigate the behavior of this type of biostimulant in a soil polluted with oxyfluorfen and its influence on the soil's biological properties.

2. Material and methods

2.1. Soil characteristics

The experimental soil was an agricultural sandy clay loam soil from southern Spain classified as Arenic Calcaric Regosol (WRB, 2014). This soil has 611 ± 39 g kg−1 sand, 123 ± 22 g kg−1 silt, and 266 ± 17 g kg−1 clay. The soil pH was 7.6 ± 0.1, 17.7 ± 1.0 g kg−1 organic matter, and 0.87 ± 0.14 g kg−1 N. The methodology used to determine these soil parameters is described in Rodríguez-Morgado et al. (2014).

2.2. Biostimulants and herbicide characteristics

Two BSs derived from sewage sludge were used (Table 1). Table 1 shows the chemical composition of the sewage sludge and the experimental BSs. The methods used to determine the chemical properties of these BSs are detailed in Rodríguez-Morgado et al. (2019). The sewage sludge was provided by the CENTA (Seville, Spain). The BSs used were as follows:

Table 1. Chemical characteristics and protein molecular weight distribution (mean ± standard error, n = 3) of sewage sludge and biostimulants obtained by fermentation processes.

	SS	BS1	BS2
Dry matter (%)	5.3a ± 0.7	5.6a ± 0.2	5.4a ± 0.2
pH	6.4a ± 0.3	8.0b ± 0.3	8.3b ± 0.2
Organic matter (g kg^{-1})	477a ± 17	475a ± 11	468a ± 19
N (g kg^{-1})	29.2a ± 6.3	31.4a ± 4.7	29.6a ± 5.2
P (g kg^{-1})	10.9a ± 1.8	11.7a ± 1.9	12.5a ± 1.9
K (g kg^{-1})	5.8a ± 1.3	6.0a ± 1.0	6.3a ± 1.5
S (g kg^{-1})	14.9a ± 2.0	18.4a ± 2.0	15.7a ± 1.8
Ca (g kg^{-1})	41.0a ± 3.6	45.9a ± 3.8	42.8a ± 2.7
Mg (g kg^{-1})	6.6a ± 1.3	6.9a ± 1.7	7.5a ± 1.2
Fe (g kg^{-1})	16.3a ± 1.9	16.8a ± 1.1	18.1a ± 1.6
Si (g kg^{-1})	22.7a ± 2.1	27.8a ± 1.9	26.1a ± 2.1
Protein molecular weight distribution (Da)			
>10,000	98.8b ± 1.3	40.0a ± 2.1	42.8a ± 2.7
10,000–5000	0.0a ± 0.0	15.6b ± 2.1	13.8b ± 1.6
5000–1000	1.2a ± 0.5	11.8b ± 1.9	11.7b ± 1.3
1000–300	0.0a ± 0.0	1.6b ± 0.4	2.0b ± 0.5
<300	0.0a ± 0.0	31.0b ± 2.5	29.7b ± 3.2

Files followed by the same letter(s) are not significantly different according to the Tukey test ($p < 0.05$).
SS: sewage sludge; BS1: biostimulant 1; BS2: biostimulant 2.

BS1: obtained by treating sewage sludge with Bacillus licheniformis ATCC 21415 in a fermentation process and basically comprising bacteria + enzymes + hydrolyzed organic matter.

BS2: obtained by treating sewage sludge by Bacillus licheniformis ATCC 21415 in a fermentation process and basically comprising hydrolyzed organic matter.

The fermentation processes used are detailed in Rodríguez-Morgado et al. (2019).

Soil was polluted with oxyfluorfen at a rate of 4 L ha^{-1} (recommended application rate). The commercial formulation was Fenfen (24% p v^{-1}, 240 g l^{-1}).

2.3. Experimental layout

Three hundred grams of dried and sieved (<2 mm) soil was preincubated according to Tejada (2009) criteria. Subsequently, the soil was mixed with oxyfluorfen in 1-L glass bottles and the two experimental BSs at a rate of 1%. Thus, and to apply the similar amount of organic matter with each BS, the soil was mixed with 57.4 g of BS1 or 59.3 g of BS2. Since BSs were liquid, both organic compounds were solubilized in distilled water (500 L ha^{-1}) before applying.

The jars were kept at 25 ± 0.5 °C for 72 days and soil moisture was kept constant during the experiment by adding distilled water at 60% of the water-holding capacity. The treatments were established in replicates (n = 3) and are detailed as follows: (i) C, control soil, non-polluted soil and non-organically amended; (ii) B1, non-polluted soil and amended with BS1; (iii) B2, non-polluted soil and amended with BS2; (iv) O, oxyfluorfen-polluted soil and non-organically amended; (v) OB1, oxyfluorfen-polluted soil and amended with BS1; and (vi) OB2, oxyfluorfen-polluted soil and amended with BS2.

For each experimental treatment, dehydrogenase, urease, β-glucosidase and alkaline phosphatase activities were measured in triplicate on days 2, 6, 9, 14, 21, 35, 50 and 72 during the incubation experiment.

Soil bacterial community composition was determined at the end of the incubation period and for each treatment.

2.4. Soil analysis

2.4.1. Soil biological properties

The methodology used to determine each enzyme activity is detailed in Tejada et al. (2006).

Soil bacterial community was determined according to Caballero et al. (2020) criteria. For each treatment, 2 g of soil subsamples were pooled. Soil genomic DNA was extracted using the DNeasy PowerSoil DNA Isolation kit from Qiagen (Hilden, Germany) according the manufacturer's instructions.

For libraries preparation the V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using the primers Bakt 341F (5′ CCTACG GGN GGC WGC AG 3′)/Bakt 805R (5′ GAC TAC HVG GGTATC TAA TCC 3′) (Herlemann et al., 2011) as the forward and reverse. PCR reactions were performed as described by Macías-Benitez et al. (2020) and Caballero et al. (2020), and PCR products were sequenced on the Illumina Miseq PE300 platform.

Raw sequence reads were de-multiplexed, and pre-processed using FLASH and CUTADAPT software 1.3 (Magoc and Salzberg, 2011; Martin, 2011), and the FASTQ files were processed using the bioinformatic tool QIIME, 1.9.0 (Caporaso et al., 2010), following the criteria described by Caballero et al. (2020).

Chimeric sequences were detected and removed by implementing the UCHIME algorithm (Edgar et al., 2011) in VSEARCH, using the Greengenes database as reference (DeSantis et al., 2006). Then, sequences were clustered into OTUs with ≥100% identity threshold using the de novo approach, singleton OTUs were removed, and the representative sequences for each OTU were assigned to a bacterial taxon with a confidence threshold of 97% using the RDP classifier (Wang et al., 2007).

In order to analyze the complexity of species diversity of each sample, the alpha diversity indices Chao, Good's coverage, Simpson, Shannon, and Faith's phylogenetic diversity (PD_whole_tree) were calculated.

To process and visualise results, OTU data files generated by QIIME were imported into R version 3.5.1 (R Core Team, 2018) using phyloseq R package (McMurdie and Holmes, 2013).

Rarefaction waves, relative abundance plots, and a principal coordinate analysis (PCoA) based on Bray Curtis dissimilarity metrics were generated using Vegan (Oksanen et al., 2018) and ggplot2 (Wickham, 2016) R packages.

The original sequence data for this study have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB43935.

2.4.2. Soil oxyfluorfen

Oxyfluorfen in soil was extracted and determined on days 2, 6, 9, 14, 21, 35, 50 and 72 after the start of incubation.

Soil oxyfluorfen extraction was done using the Anastassiades et al. (2003) method. Oxyfluorfen was determined using a tandem mass spectrometer and electron impact. The chromatographic conditions and the MS/MS parameters are described in Rodríguez-Morgado et al. (2014).

2.5. Statistical analysis

Two-way analysis of variance (ANOVA) with Tukey post hoc testing was used to evaluate the differences between soil enzymatic activities and oxyfluorfen concentration. The differences were considered statistically significant when the calculated P-value was <0.05. Statgraphics Plus 2.1 software package was used for data analysis.

3. Results and discussion

3.1. Persistence of oxyfluorfen in soil

The application of both BSs decreased the soil herbicide concentration (Fig. 1). At the end of the incubation period and compared with the O treatment, the herbicide concentration significantly decreased by 37.5% in the O + B1 treatment and by 25% in the O + B2 treatment.

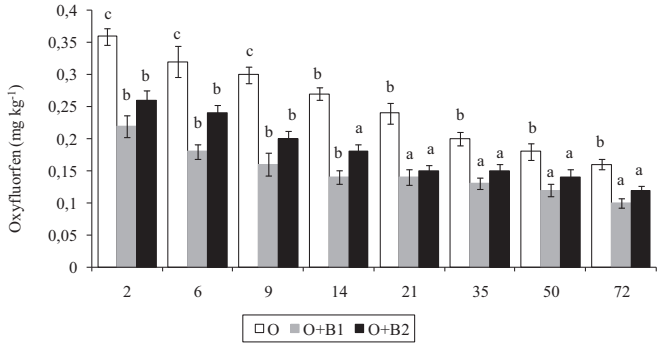


Fig. 1. Evolution of oxyfluorfen (mean ± standard error, n = 3) in soils during the experimental period. Columns followed by the same letter(s) are not significantly different ($p > 0.05$).

These results suggest that the application of both BSs increased the degradation of oxyfluorfen in soil possibly due to stimulation of oxyfluorfen-tolerant microorganisms in soil. Rodríguez-Morgado et al. (2014) also observed a significant degradation of oxyfluorfen in soil amended with BSs obtained from sewage sludge by enzymatic hydrolysis processes. However, it must be taken into consideration that the degradation percentage of this herbicide in soils amended with the BSs obtained by fermentation processes was lower than that obtained by Rodríguez-Morgado et al. (2014).

3.2. Effects on soil enzymatic activities

During the first days of experiment, the application of both BSs to the soil only significantly stimulated the dehydrogenase activity (Table 2). Thus, compared with the control treatment and for the B1 and B2 treatments, the dehydrogenase activity increased significantly ($p < 0.05$) by 73.5% and 64.3%, respectively, at 2 days after the beginning of the experiment. This activity then began to gradually decrease. At the end of the incubation period, all treatments studied showed similar values. In contrast, the urease, β-glucosidase and phosphatase activities were not stimulated when both BSs were applied to the non-polluted soil (Table 3).

Table 2. Evolution of dehydrogenase and urease activities (mean ± standard error, n = 3) in soils amended with the experimental edaphic biostimulants and polluted with oxyfluorfen during the experimental period.

	2	6	9	14	21	35	50	72
Dehydrogenase activity (μg INTF g^{-1} h^{-1})								
C	3.5b ± 0.7	3.4b ± 0.4	3.2b ± 0.4	3.4b ± 0.3	3.4b ± 0.4	3.3b ± 0.2	3.5b ± 0.3	3.6b ± 0.3
B1	13.2c ± 1.3	11.2c ± 1.6	5.2b ± 0.9	3.6b ± 0.4	3.6b ± 0.5	3.5b ± 0.4	3.6b ± 0.4	3.6b ± 0.3
B2	9.8c ± 1.6	7.8bc ± 1.5	4.5b ± 1.0	3.8b ± 0.5	3.5b ± 0.4	3.4b ± 0.3	3.6b ± 0.2	3.7b ± 0.4
O	1.6a ± 0.3	1.4a ± 0.2	1.5a ± 0.3	1.6a ± 0.1	1.5a ± 0.2	1.4a ± 0.1	1.4a ± 0.2	1.5a ± 0.3
O + B1	9.5c ± 1.4	8.0c ± 1.4	6.1bc ± 0.6	4.2b ± 1.0	3.0ab ± 0.3	2.5a ± 0.3	2.5a ± 0.3	2.3a ± 0.3
O + B2	7.6bc ± 1.7	5.0b ± 1.1	4.1b ± 1.0	3.5b ± 0.7	2.5a ± 0.2	2.3a ± 0.3	2.3a ± 0.2	2.1a ± 0.2
Urease activity (μg NH_4^+ g^{-1} h^{-1})								
C	2.3b ± 0.5	2.4b ± 0.2	2.3b ± 0.2	2.3b ± 0.3	2.2b ± 0.3	2.3b ± 0.4	2.2b ± 0.3	2.3b ± 0.3
B1	2.2b ± 0.4	2.2b ± 0.3	2.3b ± 0.2	2.4b ± 0.2	2.4b ± 0.2	2.2b ± 0.3	2.3b ± 0.2	2.3b ± 0.2
B2	2.2b ± 0.3	2.3b ± 0.2	2.3b ± 0.4	2.4b ± 0.3	2.4b ± 0.2	2.2b ± 0.2	2.3b ± 0.2	2.4b ± 0.2
O	1.4a ± 0.2	1.6a ± 0.2	1.6a ± 0.2	1.4a ± 0.1	1.7a ± 0.3	1.5a ± 0.2	1.6a ± 0.1	1.6a ± 0.3
O + B1	1.3a ± 0.1	1.4a ± 0.3	1.4a ± 0.3	1.5a ± 0.2	1.6a ± 0.3	1.7a ± 0.3	1.7a ± 0.2	1.6a ± 0.3
O + B2	1.4a ± 0.2	1.4a ± 0.2	1.6a ± 0.1	1.6a ± 0.1	1.6a ± 0.2	1.4a ± 0.3	1.5a ± 0.2	1.7a ± 0.3

Columns followed by the same letter(s) are not significantly different ($p > 0.05$).
INTF: 2-*p*-iodo-3-nitrophenyl formazan.

Table 3. Evolution of β-glucosidase and phosphatase activities (mean ± standard error, n = 3) in soils amended with the experimental edaphic biostimulants and polluted with oxyfluorfen during the experimental period.

	2	6	9	14	21	35	50	72
β-Glucosidase activity (μmol PNP g^{-1} h^{-1})								
C	0.46b ± 0.07	0.48b ± 0.07	0.49b ± 0.09	0.50b ± 0.05	0.48b ± 0.06	0.49b ± 0.05	0.50b ± 0.05	0.47b ± 0.04
B1	0.48b ± 0.08	0.47b ± 0.09	0.49b ± 0.07	0.49b ± 0.02	0.46b ± 0.07	0.50b ± 0.07	0.48b ± 0.06	0.49b ± 0.02
B2	0.46b ± 0.07	0.46b ± 0.06	0.47b ± 0.1	0.48b ± 0.04	0.47b ± 0.05	0.49b ± 0.03	0.48b ± 0.03	0.48b ± 0.05
O	0.23a ± 0.02	0.25a ± 0.02	0.26a ± 0.04	0.23a ± 0.03	0.26a ± 0.04	0.25a ± 0.02	0.24a ± 0.02	0.26a ± 0.03
O + B1	0.31a ±0.07	0.32a ±0.07	0.34a ±0.06	0.31a ± 0.06	0.34a ± 0.08	0.35a ± 0.04	0.31a ± 0.06	0.34a ± 0.07
O + B2	0.33a ± 0.05	0.32a ± 0.04	0.30a ± 0.05	0.33a ± 0.07	0.33a ± 0.05	0.33a ± 0.06	0.35a ± 0.04	0.33a ± 0.05
Phosphatase activity (μmol PNP g^{-1} h^{-1})								
C	2.3b ± 0.3	2.5b ± 0.4	2.4b ± 0.3	2.5b ± 0.4	2.4b ± 0.3	2.5b ± 0.1	2.4b ± 0.3	2.5b ± 0.3
B1	2.5b ± 0.1	2.4b ± 0.2	2.5b ± 0.3	2.6b ± 0.3	2.5b ± 0.2	2.4b ± 0.3	2.5b ± 0.4	2.6b ± 0.4
B2	2b ± 0.3	2.5b ± 0.3	2.4b ± 0.4	2.5b ± 0.4	2.4b ± 0.3	2.6b ± 0.4	2.5b ± 0.1	2.5b ± 0.4
O	1.5a ± 0.2	1.4a ± 0.1	1.4a ± 0.2	1.3a ± 0.3	1.4a ± 0.3	1.6a ± 0.3	1.5a ± 0.2	1.3a ± 0.2
O + B1	1.8a ± 0.2	1.8a ± 0.3	1.7a ± 0.1	1.6a ± 0.1	1.9a ± 0.3	1.8a ± 0.3	1.9a ± 0.3	1.7a ± 0.2
O + B2	1.7a ± 0.1	1.8a ± 0.2	1.6a ± 0.2	1.8a ± 0.3	1.8a ± 0.2	1.6a ± 0.1	1.7a ± 0.1	1.8a ± 0.1

Columns followed by the same letter(s) are not significantly different ($p > 0.05$).
PNP: *p*-nitrophenol.

Rodríguez-Morgado et al. (2019) have suggested that the main reason why only dehydrogenase activity is stimulated is a consequence of the process for producing these biostimulants. During this biochemical process, and in order to obtain energy, the B. licheniformis bacterium excretes a large number of enzymes, which degrade organic compounds. For this reason, when applying these biostimulants to the soil, soil microorganisms are capable of directly absorbing these degraded compounds, without needing to excrete any type of enzyme to produce them. On the other hand, and according to Rodríguez-Morgado et al. (2019), the reason why the dehydrogenase activity was higher in the soil amended with BS1 than with BS2 is due to the presence/absence of live bacteria and enzymes in these products.

These results are different from those obtained when biostimulants obtained by enzymatic hydrolysis processes are applied to the soil. Thus, after application to the soil a significant increase in intra- and extracellular enzymatic activities was observed during the first few days in the latter case. We believe that this difference between biostimulants obtained by fermentation processes with Bacillus licheniformis and biostimulants obtained by enzymatic hydrolysis processes is due to the fact that, in the former, the bacterium is able to degrade any existing organic substrate during the biochemical process carried out in the bioreactor. In edaphic biostimulants, however, not all organic substrates are degraded by the action of proteolytic enzymes.

Application of the herbicide to soil significantly decreased the enzymatic activities during the experimental period (Table 2, Table 3). Thus, at the end of the incubation period, and in comparison with the control treatment, in the O treatment the dehydrogenase activity significantly decreased (58.3%, $p < 0.05$), the urease activity decreased significantly (30.4%, $p < 0.05$), the β-glucosidase activity decreased

significantly (44.7%, $p < 0.05$) and the phosphatase activity also decreased significantly (48%, $p < 0.05$). These results are similar with those reported by Nadijer et al. (2013), Gómez et al. (2014) and Franco-Andreu et al. (2016), who highlighted the toxic effect of oxyfluorfen on soil biochemical activity. Franco-Andreu et al. (2016) suggest that the decrease in soil biochemical activity caused by oxyfluorfen could be due to the fact that this herbicide could suppress the microbial populations involved in the nutrient cycle and/or that could hinder the interaction between the enzymatic active sites and soluble substrates, causing an opposite effect on the enzyme activity expression.

The application of both BSs to the polluted soil decreased the inhibition of dehydrogenase activity during the first days of incubation (Table 2). At the end of the experimental period and compared with the O treatment, the dehydrogenase activity concentration decreased by 34.7% in the O + B1 treatment and by 28.6% in the O + B2 treatment. With regard to the urease, β-glucosidase and phosphatase activities, the application of both BSs to the herbicide-polluted soils did not produce any significant changes in the values of said enzymatic activities (Table 2, Table 3). These results contrast with those reported by Rodríguez-Morgado et al. (2014), who observed a stimulation in extracellular enzymatic activities in a soil polluted with the same dose of oxyfluorfen and amended with a biostimulant also obtained from sewage sludge but obtained using enzymatic hydrolysis processes.

The application of both BSs decreased the soil herbicide concentration (Fig. 1). At the end of the experiment and compared with the O treatment, the herbicide concentration decreased significantly by 37.5% in the O + B1 treatment and by 25% in the O + B2 treatment. Rodríguez-Morgado et al. (2014) also observed a decrease in the concentration of oxyfluorfen in a soil amended with BSs obtained from

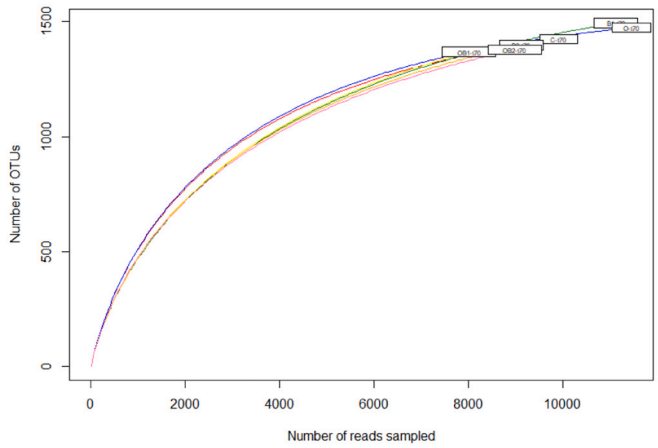
sewage sludge by enzymatic processes. However, the percentage oxyfluorfen degradation obtained in the study carried out by Rodríguez-Morgado et al. (2014) was greater than in our experiment, which suggests that the use of sewage sludge biostimulants obtained using fermentation processes has a lower effect on oxyfluorfen degradation than sewage sludge biostimulants obtained using enzymatic processes. This is possibly due to the fact that the percentage of low molecular weight peptides (<300 Da) in the BSs obtained by fermentation processes is lower than in the BSs obtained by enzymatic hydrolysis processes, which causes less stimulation of the soil microorganisms and consequently less degradation of oxyfluorfen.

3.3. Effects on soil bacterial community diversity

After the different quality filtrates mentioned above, a total of 58,632 sequences were obtained, with between 8014 and 11,464 sequences per sample. To perform the subsequent analysis each sample was normalized using a minimum sequence depth of 8014.

The rarefaction curves tended to reach saturation (Fig. 2) and coverage rates were higher than 94% in all samples, thus indicating that our analysis captured most of the bacterial diversity present in them (Table 4). The diversity and wealth indices did not show large differences between the treatments. Similarly, application of the contaminant or of the BSs also failed to produce significant changes in these parameters.

(A)



(B)

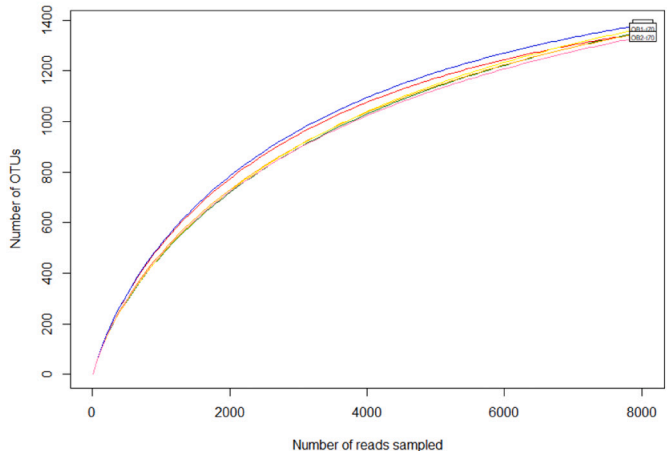


Fig. 2. Rarefaction curves of the complete dataset of sequences (A) and the randomly chosen subset of 8014 sequences (B).

Table 4. α-Diversity indices of soil samples. Coverage nonparametric coverage estimator, Observed OTUS observed operational taxonomic units, Shannon nonparametric Shannon diversity index, Simpson nonparametric Simpson diversity index, PD_whole_tree Faith's Phylogenetic Diversity index, Chao1 richness of the Chao1 estimator.

Treatment	Coverage	Observed OTUs	Shannon	Simpson	PD_whole_tree	Chao1
C	0.959	1349.800	9.152	0.994	60.234	1541.254
B1	0.945	1353.800	8.914	0.994	62.962	1679.628
B2	0.946	1351.900	9.020	0.995	62.077	1687.983
O	0.955	1382.700	9.200	0.995	60.715	1602.427
O + B1	0.943	1368.900	8.963	0.995	62.940	1751.047
O + B2	0.946	1335.300	8.985	0.995	61.747	1684.172

The PCoA based on the distance dissimilarity of Bray Curtis, which explained 56.9% (PCoA1) and 13.7% (PCoA2) of the total genetic variance revealed changes in the composition of bacterial communities upon application of BS1 and BS2 (Fig. 3). This representation showed three perfectly differentiated clusters, thus indicating which showed that while oxifluorfen did not modify the composition of the bacterial populations of the samples with respect to the C treatment, the effect of BS1 and BS2 produced changes in the composition that resulted in populations different from those of the control and from each other.

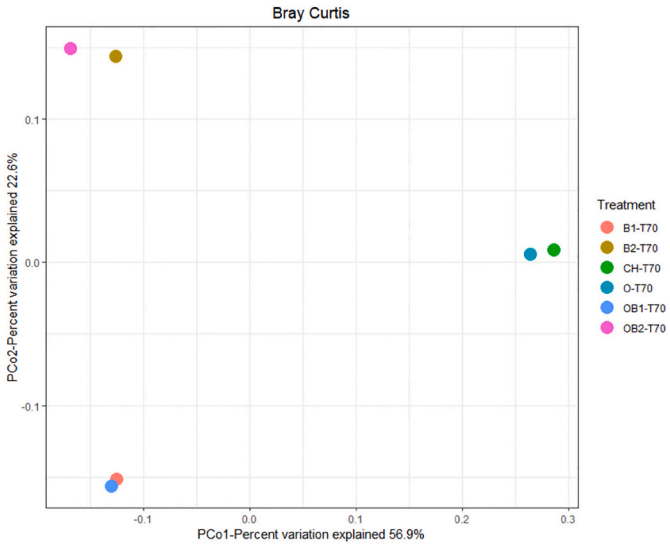


Fig. 3. Principal coordinate analysis (PCoA) based on the Bray Curtis distance.

Thus, in the experimental soil, the most abundant phyla were Actinobacteria (38.5%), Proteobacteria (28.1%), Acidobacteria (9.6%), Chloroflexi (9.5%) and Firmicutes (5.3%), which together represented >90% of all bacteria. At the end of the experiment, the taxonomic composition of the oxyfluorfen-polluted soil was

different from that of C treatment. These results suggest that the application of oxyfluorfen to the soil modified its microbial structure, resulting in an increase or decrease in certain groups of bacteria. Singh et al. (2020) have suggested that herbicides have variable effects on different soil populations depending on the different concentrations of herbicides and microbial species present. The fact that the population of a certain group of microorganisms decreases in the soil is due to the fact that they are not capable of tolerating the concentration of said herbicide. In contrast, an increase in the microorganism population is a consequence of the fact that these microorganisms can use the herbicide as a source of carbon and energy (García-Delgado et al., 2019; Liang et al., 2020; Pertile et al., 2021).

At the end of the incubation period, the Firmicutes phylum was found to show the greatest change with respect to the C treatment, with a decrease of 24.5% in its relative abundance being observed (Fig. 4, Supplementary Table). In this phylum, the Bacilli class was the most representative, with a 24.5% decrease in relative abundance in the polluted soil compared to treatment C (Fig. 5, Supplementary Table). The most representative genus of this class was Bacillus, which decreased by 20% in the soil polluted by the herbicide compared to the control soil, thus suggesting that this bacterium is very sensitive to oxyfluorfen in soil. These results do not agree with those reported by Mohamed et al. (2011), who found an increase in the Bacillus population in soil contaminated by oxyfluorfen. According to these authors, this bacterium plays a fundamental role in the degradation of oxyfluorfen in soil. The reason that our results do not agree with those obtained by Mohamed et al. (2011) is possibly due to the exposure time of the herbicide in the soil. These authors carried out their study of microbial diversity 21 days after contaminating the

soil with oxyfluorfen, whereas in our experiment, the soil microbial diversity analysis was performed at 72 days. According to the soil herbicide persistence, the oxyfluorfen concentration at 21 days was higher than at 72 days.

On the other hand, it should be noted that our results show the effect of oxyfluorfen on Bacillus only at the genus level, without being able to detect any species. The increase in the Bacillus population proposed by Mohamed et al. (2011) in oxyfluorfen polluted soils suggests that there may be specific species of this genus that may be resistant to this herbicide and consequently suppose an increase in the population of this bacterium.

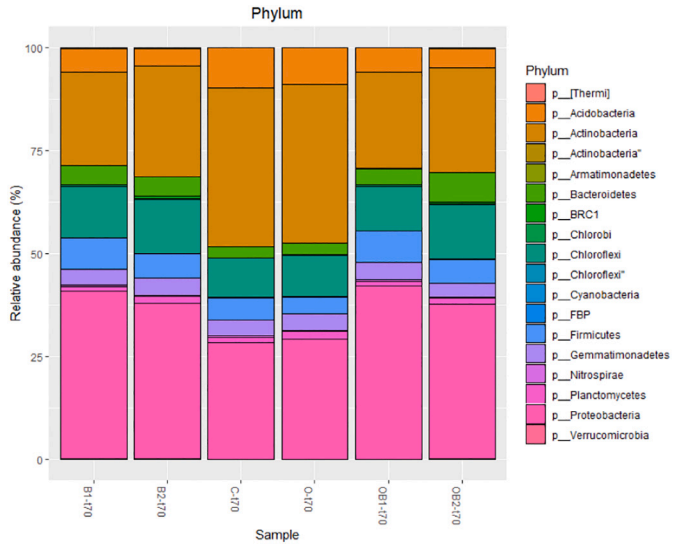


Fig. 4. Bacterial community composition at phylum level.

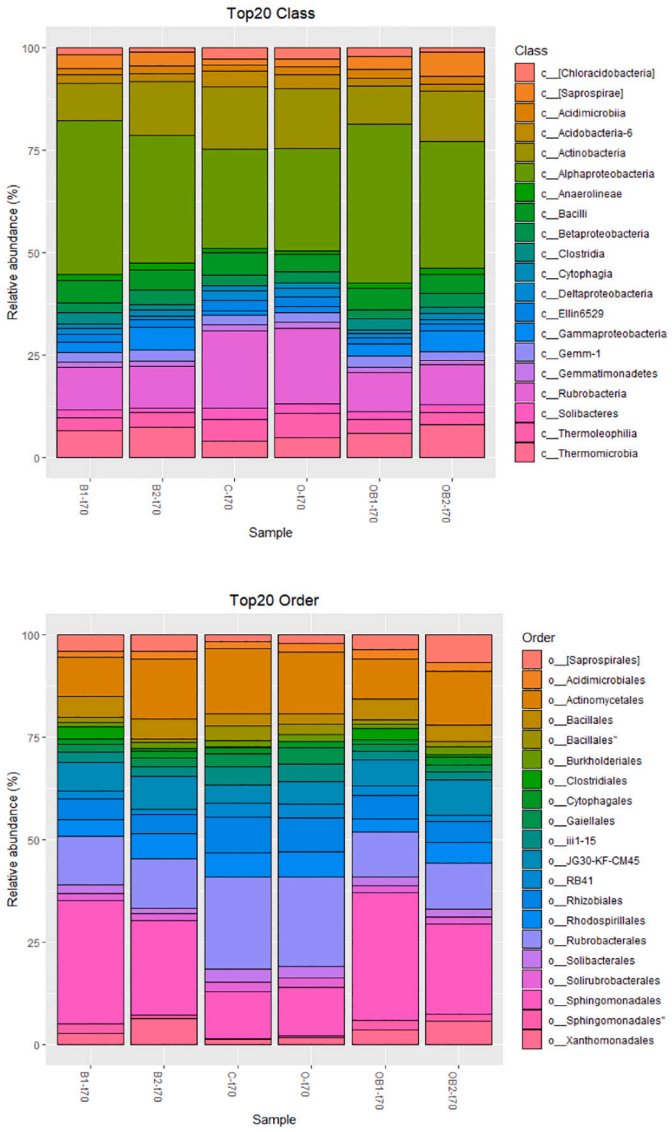


Fig. 5. Top 20 most-abundant identified bacterial class and order.

Wu et al. (2018) found that the number of OTUs belonging to the Actinobacteria phylum increased in soil polluted with the herbicide fomesafen, which has a very similar chemical structure and mechanism of action to oxyfluorfen. Our experiment resulted in an increase in Actinobacteria, generally those belonging to the Gaiellales order (Fig. 3, Fig. 4, Supplementary Table). This increase in Actinobacteria phylum is possibly due to the fact that these bacteria play an important role in the degradation of pesticides, due to the fact that they have a high capacity to produce

extracellular enzymes capable of degrading complex and recalcitrant pollutants in different environments (Duran et al., 2015; Rachedi et al., 2018).

With regard to Proteobacteria phylum, our results showed an increase of 89.5% in the relative abundance of the Sphingomonadales order (Alphaproteobacteria class) (Fig. 3, Fig. 4, Supplementary Table). Keum et al. (2008) have suggested that Sphingomonas (Sphingomonadales order, Alphaproteobacteria class) has a marked ability to degrade the oxyfluorfen herbicide due to its ability to adapt in this contaminated environment. Despite this, we observed that the number of OTUs for this genus was similar to that for the control treatment and for the oxyfluorfen-polluted soil. This is possibly due to the fact that, in our experiment, the microbial biodiversity study was carried out 72 days after applying oxyfluorfen to the soil, when the levels of this herbicide in the soil were lower.

The application of both BSs changed the soil bacteria population. Thus, an increase in the Bacteroidetes phylum population, mainly the Flavisolibacter genus (Chitinophagaceae family), was observed (Fig. 3, Supplementary Figs. 1 and 2, Supplementary Table). These results are similar with those reported by Fierer et al. (2012), who highlighted that the nitrogen content of the soil influenced the population of this bacteria group.

In addition, and compared to the C treatment, the application of organic matter to the soil increased the number of OTUs belonging to the Chloroflexi phylum (24% for B1 treatment and 28% for B2 treatment), with the Ardenscatena genus (Ardenscatenaceae family) being the most abundant within said phylum (Fig. 3, Supplementary Figs. 1 and 2, Supplementary Table). These results are similar with those obtained by Freeman et al. (2009), Eisenlord and Zak (2010) and Zhang et al. (2017), who observed an increase in the population of bacteria belonging to the

Chloroflexi phylum, especially the Ardenscatena genus, after the incorporation of organic matter into the soil. According to Fierer et al. (2012), the growth of these copiotrophic bacteria is a consequence of an increase in nutrients in the soil and their ready availability for said microorganisms.

The application of both BSs in the non-polluted soil also changed the bacterial population belonging to the Proteobacteria phylum. Thus, compared with the C treatment, the population of this phylum increased by 30.8% in B1 treatment and 25.3% in B2 treatment (Fig. 3, Supplementary Figs. 1 and 2, Supplementary Table). Wang et al. (2019) also observed an increase in the population of this phylum after the addition of farmyard manure to the soil. According to Francioli et al. (2016), this group of copiotrophic bacteria are usually abundant in environments rich in organic matter. Moreover, Zeng et al. (2016) and Zhou et al. (2017) observed an increase in this group of bacteria in soils fertilized with nitrogen. The most representative genera that increased in Proteobacteria phylum were Kaistobacter and Sphingomonas (Alphaproteobacteria class). Also, Zhou et al. (2017) found an increase in the population of Sphingomonas in soils after the addition of nitrogen.

As regards the Firmicutes phylum, only the application of B1 to the soil caused a change in its population, with an increase of 43% in the Bacillus genus being found at the end of the experiment (Fig. 3, Supplementary Figs. 1 and 2, Supplementary Table). Several authors have reported an increase in the relative abundance of Firmicutes after the application of organic matter to the soil (Pascault et al., 2013; Whitman et al., 2016). The fact that this only occurs for B1 treatment in our experiment is possibly due to the fact that BS1 comprises bacteria from the Bacillus genus. We believe that the reason why we did not detect the Bacillus genus in B2 treatment is due to the chemical composition of the applied organic matter. BS2

contains no bacteria and is a biostimulant comprising low molecular weight peptides that are rapidly absorbed by soil microorganisms in a short period of time. This means that the residence time of this organic source in the soil is limited. The sources of organic matter used by Pascault et al. (2013) and Whitman et al. (2016) were remains of wheat and alfalfa, which generally tend to be made up of high molecular weight peptides that need to be degraded by soil microorganisms and, consequently, increases the permanence of these organic sources in the soil.

Unlike the previous phyla and compared to the control treatment, the bacteria population belonging to the Actinobacteria phylum decreased by 41.3% in treatment B1 and by 30.4% in treatment B2 (Fig. 3, Supplementary Figs. 1 and 2, Supplementary Table). The most representative genus in said phylum was Rhodococcus (Pseudonocardiaceae family), which decreased by 50% in soils amended with B1 and B2 treatments. Wang et al. (2019) have suggested that Actinobacteria play an important role in the degradation of organic matter by way of various extracellular enzymes. As mentioned previously, the fermentation process used to produce the biostimulants resulted in the degradation of all organic substrates, thus meaning that, after application of the experimental biostimulants, the bacteria do not excrete extracellular enzymes that degrade organic C. This is possibly the reason why the OTUs for Actinobacteria decreased in the soils amended with the two biostimulants studied.

In addition, and in comparison with the C treatment, the Acidobacteria population decreased in the soils amended with both biostimulants, with a decrease of 40.6% in B1 treatment B1 and 55.2% in B2 treatment B2 (Fig. 3, Supplementary Figs. 1 and 2, Supplementary Table). Francioli et al. (2016) also observed a decrease in the population of these bacteria in soils fertilized with nitrogen. According to Fierer et

al. (2012), Ramirez et al. (2012) and Trivedi et al. (2013), the increase in soil nutrients upon fertilization could stimulate the growth of copiotrophic bacteria and, at the same time, affect the growth of oligotrophic microorganisms.

Finally, and in comparison with O treatment, the soil bacterial population for O + B1 and O + B2 treatments also showed changes. Thus, the relative abundance of the Bacteroidetes, Chloroflexi, Firmicutes and Proteobacteria phyla increased for O + B1 and O + B2 treatments with respect to the O treatment, thus highlighting the importance of these biostimulants in the bioremediation of soils polluted by oxyfluorfen.

4. Conclusions

Oxyfluorfen causes a negative effect on soil biology, decreasing its biochemical activity of the soil as well as in the relative abundance of the Firmicutes and Acidobacteria phyla. The application of both biostimulants obtained from sewage sludge upon fermentation with Bacillus licheniformis increased the degradation of oxyfluorfen in soil, increasing the relative abundance of these phyla. These results therefore suggest the fundamental role of said biostimulants in the bioremediation of soils polluted by the oxyfluorfen herbicide.

Declaration of competing interest

The authors declare the source of funding for the manuscript. On the other hand, there is no conflict of interest.

Acknowledgments

This work was supported by the Ministerio de Economía y Competitividad (Spain), Plan Estatal 2013-2016, reference CTM2015-64354-C3-3-R, the Ministerio de Ciencia, Innovación y Universidades (Spain), Plan Estatal 2017-2020, reference RTI2018-097425-B-I00, and Junta de Andalucia (Consejería de Economía y

Educación), Proyectos I + D + i FEDER Andalucía 2014–2020, with reference US-1263885.

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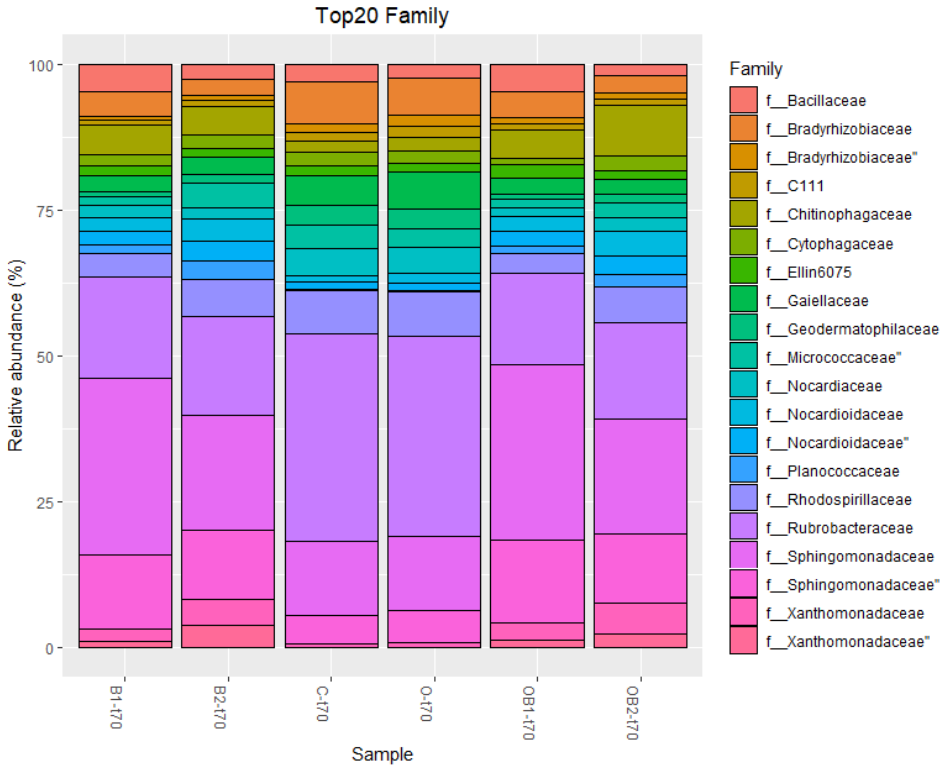
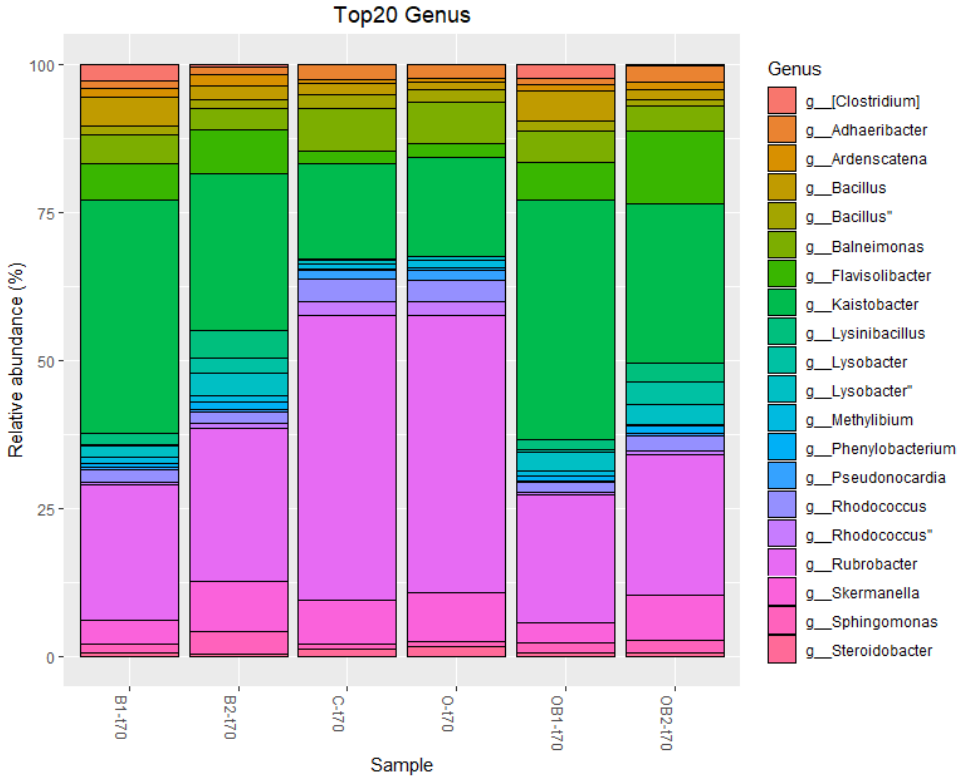


Supplementary Figure 1. Top 20 most-abundant identified bacterial family



Supplementary Figure 2. Top 20 most-abundant identified bacterial geneus

Supplementary Table 1. Taxonomic categories for experimental treatments at the end of the incubation period.

Taxonomic Categories					Samples					
Phylum	**Class**	**Order**	**Family**	**Genus**	**CH_t70**	**B1_t70**	**B2_t70**	**O_t70**	**OB1_t70**	**OB2_t70**
Acidobacteria	Acidobacteria-6	iii1-15	mb2424	Unidentified	0,76	0,46	0,42	0,71	0,29	0,30
			Unidentified		2,67	1,51	1,42	2,48	1,34	1,15
		Unidentified			0,60	0,20	0,27	0,54	0,29	0,22
	Acidobacteriia	Acidobacteriales	Koribacteraceae	*Candidatus Koribacter*	0,11	0,12	0,05	0,10	0,09	0,04
	Solibacteres	Solibacterales	Solibacteraceae	*Candidatus Solibacter*	0,07	0,00	0,00	0,09	0,01	0,00
			Unidentified		2,57	1,83	1,02	2,11	1,91	1,71
	Sva0725	Sva0725	Unidentified		0,15	0,04	0,06	0,11	0,04	0,07
	[Chloracidobacteria]	RB41	Ellin6075	Unidentified	0,75	1,02	0,79	0,69	1,30	0,82
			Unidentified		1,91	0,54	0,27	2,00	0,64	0,29
Actinobacteria	Acidimicrobiia	Acidimicrobiales	AKIW874	Unidentified	0,35	0,05	0,19	0,40	0,26	0,14
			C111	Unidentified	0,71	0,51	0,61	0,89	0,57	0,56
			EB1017	Unidentified	0,06	0,09	0,07	0,11	0,05	0,11
			Iamiaceae	*Iamia*	0,01	0,07	0,11	0,06	0,15	0,11
			Unidentified		0,24	0,52	0,74	0,30	1,09	0,89
	Actinobacteria	Actinomycetales	Actinosynnemataceae	Unidentified	0,11	0,01	0,05	0,09	0,00	0,04
			Bogoriellaceae	*Georgenia*	0,00	0,00	0,01	0,00	0,00	0,00
			Cellulomonadaceae	*Actinotalea*	0,00	0,00	0,04	0,00	0,01	0,10
				Cellulomonas	0,14	0,06	0,06	0,11	0,06	0,06
			Geodermatophilaceae	*Geodermatophilus*	0,35	0,06	0,26	0,36	0,12	0,16
				Modestobacter	0,07	0,01	0,07	0,09	0,00	0,00
				Unidentified	1,81	0,74	0,97	1,77	0,76	0,95
			Gordoniaceae	*Gordonia*	0,00	0,00	0,01	0,00	0,05	0,05
			Intrasporangiaceae	*Phycicoccus*	0,16	0,17	0,22	0,19	0,27	0,30

				Unidentified	0,16	0,15	0,42	0,20	0,15	0,69
			Kineosporiaceae	Unidentified	0,17	0,06	0,04	0,11	0,05	0,04
			Microbacteriaceae	*Agromyces*	0,21	0,15	0,22	0,25	0,22	0,19
				Unidentified	0,14	0,11	0,12	0,26	0,12	0,11
			Micrococcaceae	Unidentified	2,21	1,04	2,45	1,71	0,87	1,46
			Micromonosporaceae	*Actinoplanes*	0,05	0,05	0,01	0,07	0,01	0,01
				Dactylosporangium	0,02	0,01	0,00	0,01	0,00	0,00
				Virgisporangium	0,16	0,07	0,04	0,10	0,06	0,01
				Unidentified	0,89	0,44	0,30	0,60	0,44	0,32
			Mycobacteriaceae	*Mycobacterium*	0,39	0,10	0,06	0,35	0,15	0,16
			Nocardiaceae	*Nocardia*	0,02	0,00	0,02	0,05	0,01	0,00
				Rhodococcus	2,18	1,09	1,06	2,12	0,86	1,19
			Nocardioidaceae	*Aeromicrobium*	0,01	0,01	0,00	0,02	0,02	0,01
				Kribbella	0,09	0,04	0,04	0,15	0,02	0,01
				Nocardioides	0,06	0,22	0,24	0,15	0,14	0,21
				Pimelobacter	0,01	0,17	0,05	0,01	0,25	0,17
				Unidentified	1,01	2,17	3,67	1,16	2,36	3,62
			Promicromonosporaceae	*Promicromonospora*	0,04	0,00	0,06	0,01	0,01	0,00
			Propionibacteriaceae	Unidentified	0,64	0,22	0,22	0,42	0,17	0,19
			Pseudonocardiaceae	*Actinomycetospora*	0,06	0,01	0,01	0,02	0,01	0,02
				Pseudonocardia	0,60	0,12	0,11	0,64	0,14	0,14
				Saccharopolyspora	0,01	0,02	0,00	0,10	0,05	0,04
				Unidentified	0,00	0,02	0,01	0,02	0,00	0,01
			Sporichthyaceae	Unidentified	0,02	0,07	0,02	0,09	0,02	0,06
			Streptomycetaceae	*Streptomyces*	0,09	0,04	0,04	0,12	0,07	0,07
				Unidentified	0,47	0,12	0,25	0,31	0,27	0,10
			Streptosporangiaceae	*Streptosporangium*	0,01	0,01	0,00	0,00	0,04	0,01

			Williamsiaceae	*Williamsia*	0,00	0,01	0,01	0,00	0,00	0,00
			Unidentified		0,99	0,40	0,44	0,92	0,55	0,49
		Micrococcales	Unidentified		0,89	0,57	0,42	0,87	0,41	0,35
	MB-A2-108	0319-7L14	Unidentified		0,39	0,25	0,30	0,54	0,37	0,34
	Nitriliruptoria	Euzebyales	Euzebyaceae	*Euzebya*	0,00	0,01	0,02	0,01	0,02	0,02
	Rubrobacteria	Rubrobacterales	Rubrobacteraceae	*Rubrobacter*	17,31	9,28	9,26	16,66	8,61	8,73
				Unidentified	0,27	0,21	0,16	0,37	0,22	0,22
	Thermoleophilia	Gaiellales	Gaiellaceae	Unidentified	2,51	1,46	1,66	3,14	1,51	1,45
			Unidentified		0,01	0,01	0,00	0,02	0,00	0,00
		Solirubrobacterales	Conexibacteraceae	Unidentified	0,01	0,05	0,01	0,07	0,06	0,00
			Patulibacteraceae	Unidentified	0,07	0,06	0,04	0,05	0,10	0,01
			Solirubrobacteraceae	Unidentified	0,41	0,31	0,24	0,69	0,40	0,31
			Unidentified		1,85	1,09	1,32	1,50	1,05	1,04
	Unidentified				0,04	0,05	0,05	0,09	0,02	0,02
Armatimonadetes	0319-6E2	Unidentified			0,00	0,02	0,00	0,00	0,04	0,02
BRC1	PRR-11	Unidentified			0,00	0,02	0,51	0,00	0,04	0,49
Bacteroidetes	At12OctB3	Unidentified			0,02	0,19	0,15	0,04	0,19	0,20
	Bacteroidia	Bacteroidales	Porphyromonadaceae	Unidentified	0,00	0,05	0,00	0,00	0,02	0,00
	Cytophagia	Cytophagales	Cytophagaceae	*Adhaeribacter*	0,90	0,59	0,50	0,81	0,40	1,00
				Pontibacter	0,01	0,04	0,30	0,05	0,05	0,12
				Rhodocytophaga	0,09	0,16	0,21	0,09	0,12	0,19
				Unidentified	0,22	0,29	0,31	0,17	0,12	0,11
	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Pedobacter*	0,00	0,00	0,01	0,00	0,00	0,05
				Unidentified	0,00	0,00	0,06	0,00	0,00	0,10
	[Saprospirae]	[Saprospirales]	Chitinophagaceae	*Flavisolibacter*	0,71	2,53	2,65	0,87	2,52	4,59
				Unidentified	0,55	0,69	0,44	0,62	0,42	0,75
			Saprospiraceae	Unidentified	0,16	0,02	0,02	0,19	0,01	0,04

Chlorobi	OPB56	Unidentified			0,05	0,22	0,17	0,16	0,26	0,15
	Unidentified				0,02	0,11	0,00	0,04	0,10	0,02
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	*Anaerolinea*	0,00	0,09	0,04	0,00	0,01	0,37
				Longilinea	0,00	0,01	0,00	0,00	0,01	0,00
		Ardenscatenales	Ardenscatenaceae	*Ardenscatena*	0,22	0,52	0,62	0,24	0,40	0,45
		Caldilineales	Caldilineaceae	*Caldilinea*	0,05	0,11	0,06	0,04	0,04	0,02
				Unidentified	0,11	0,29	0,40	0,16	0,32	0,42
		SBR1031	A4b	Unidentified	0,01	0,01	0,01	0,02	0,02	0,05
		envOPS12	Unidentified	Unidentified	0,47	0,35	0,27	0,27	0,35	0,11
		Unidentified			0,00	0,00	0,00	0,00	0,01	0,00
	C0119	Unidentified			0,95	0,75	0,64	0,92	0,82	0,64
	Chloroflexi	AKIW781	Unidentified		0,62	0,60	0,47	0,62	0,42	0,54
		Chloroflexales	Chloroflexaceae	Unidentified	0,00	0,02	0,01	0,01	0,01	0,01
			Unidentified		0,01	0,05	0,01	0,06	0,04	0,04
		Herpetosiphonales	Unidentified		0,14	0,05	0,04	0,09	0,00	0,02
		[Roseiflexales]	[Kouleothrixaceae]	Unidentified	0,05	0,00	0,02	0,04	0,05	0,02
			Unidentified	Unidentified	0,12	0,14	0,16	0,12	0,21	0,11
		Unidentified			0,05	0,05	0,05	0,06	0,05	0,06
	Ellin6529	Unidentified			2,25	1,72	1,80	2,03	1,36	1,55
	Gitt-GS-136	Unidentified			0,46	1,36	1,42	0,50	0,94	0,92
	S085	Unidentified			0,04	0,06	0,04	0,01	0,07	0,05
	TK10	AKYG885	Dolo_23	Unidentified	0,01	0,00	0,06	0,01	0,04	0,04
		B07_WMSP1	Unidentified		0,01	0,02	0,02	0,05	0,02	0,01
		Unidentified			0,02	0,01	0,02	0,00	0,00	0,02
	TK17	mle1-48	Unidentified		0,04	0,14	0,09	0,00	0,05	0,11
		Unidentified			0,04	0,02	0,05	0,02	0,04	0,00
	Thermomicrobia	AKYG1722	Unidentified		0,20	0,52	0,71	0,19	0,37	0,52

		JG30-KF-CM45	Unidentified		3,59	5,62	6,14	4,39	5,15	6,99
	Unidentified				0,09	0,02	0,01	0,05	0,00	0,01
Cyanobacteria	Chloroplast	Chlorophyta	Unidentified		0,02	0,00	0,00	0,01	0,00	0,00
		Stramenopiles	Unidentified		0,01	0,02	0,00	0,05	0,01	0,04
		Streptophyta	Unidentified		0,00	0,04	0,00	0,04	0,00	0,01
	Nostocophycideae	Nostocales	Nostocaceae	Unidentified	0,01	0,00	0,00	0,01	0,00	0,00
	Oscillatoriophycideae	Oscillatoriales	Phormidiaceae	*Phormidium*	0,00	0,00	0,01	0,00	0,00	0,00
FBP	Unidentified				0,01	0,01	0,02	0,00	0,00	0,02
Firmicutes	Bacilli	Bacillales	Alicyclobacillaceae	*Alicyclobacillus*	0,39	0,16	0,19	0,40	0,10	0,11
			Bacillaceae	*Bacillus*	1,52	2,66	1,40	1,20	2,71	1,04
				Unidentified	0,19	0,12	0,19	0,17	0,16	0,29
			Paenibacillaceae	*Ammoniphilus*	0,07	0,15	0,11	0,09	0,26	0,07
				Aneurinibacillus	0,00	0,01	0,00	0,00	0,00	0,01
				Brevibacillus	0,00	0,01	0,00	0,00	0,00	0,01
				Paenibacillus	0,01	0,00	0,01	0,01	0,01	0,01
				Unidentified	0,01	0,01	0,01	0,01	0,00	0,00
			Planococcaceae	*Lysinibacillus*	0,07	0,80	1,73	0,02	0,76	1,24
				Paenisporosarcina	0,02	0,00	0,00	0,00	0,01	0,00
				Solibacillus	0,00	0,00	0,01	0,00	0,00	0,00
				Sporosarcina	0,00	0,00	0,00	0,00	0,00	0,02
				Unidentified	0,00	0,11	0,10	0,05	0,02	0,35
			Unidentified		2,88	0,89	0,81	2,01	0,94	1,10
		Turicibacterales	Turicibacteraceae	*Turicibacter*	0,00	0,04	0,00	0,00	0,02	0,01
	Clostridia	Clostridiales	Caldicoprobacteraceae	*Caldicoprobacter*	0,00	0,01	0,00	0,00	0,01	0,00
			Clostridiaceae	*Alkaliphilus*	0,00	0,01	0,01	0,00	0,01	0,01
				Caloramator	0,01	0,19	0,02	0,00	0,11	0,05
				Clostridium	0,00	0,11	0,04	0,01	0,09	0,01

				Proteiniclasticum	0,00	0,00	0,00	0,00	0,00	0,05
				SMB53	0,00	0,12	0,04	0,00	0,09	0,02
				Unidentified	0,02	0,19	0,07	0,00	0,14	0,14
			Gracilibacteraceae	*Lutispora*	0,00	0,01	0,00	0,00	0,01	0,00
			Peptostreptococcaceae	*Tepidibacter*	0,00	0,04	0,00	0,00	0,07	0,02
				[Clostridium]	0,01	1,11	0,16	0,00	1,00	0,12
			Symbiobacteriaceae	*Symbiobacterium*	0,02	0,56	0,09	0,00	0,76	0,11
			[Tissierellaceae]	*Sedimentibacter*	0,00	0,01	0,02	0,00	0,01	0,01
			Unidentified		0,00	0,16	0,85	0,01	0,25	0,86
Gemmatimonadetes	Gemm-1	Unidentified			2,16	2,13	2,47	2,21	2,51	1,98
	Gemm-3	Unidentified			0,02	0,04	0,04	0,07	0,05	0,16
	Gemm-5	Unidentified			0,02	0,10	0,10	0,07	0,16	0,05
	Gemmatimonadetes	Ellin5290	Unidentified		0,04	0,02	0,00	0,02	0,00	0,00
		Gemmatimonadales	Ellin5301	Unidentified	0,49	0,55	0,55	0,55	0,44	0,29
			Gemmatimonadaceae	*Gemmatimonas*	0,09	0,01	0,07	0,07	0,01	0,00
			Unidentified		0,01	0,00	0,00	0,00	0,00	0,00
		KD8-87	Unidentified		0,01	0,02	0,05	0,01	0,02	0,02
		N1423WL	Unidentified		0,27	0,11	0,05	0,22	0,05	0,07
		Unidentified			0,62	0,79	0,97	0,72	0,97	0,92
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira*	0,30	0,26	0,12	0,24	0,25	0,14
				Unidentified	0,11	0,07	0,00	0,09	0,02	0,00
Planctomycetes	Phycisphaerae	WD2101	Unidentified		1,06	0,60	0,66	1,36	0,71	0,52
	Planctomycetia	B97	Unidentified		0,07	0,02	0,01	0,09	0,00	0,00
		Gemmatales	Gemmataceae	*Gemmata*	0,16	0,17	0,15	0,24	0,10	0,12
			Isosphaeraceae	Unidentified	0,04	0,12	0,25	0,14	0,19	0,35
		Pirellulales	Pirellulaceae	*A17*	0,01	0,01	0,01	0,00	0,01	0,00
				Unidentified	0,02	0,05	0,34	0,02	0,09	0,26

		Planctomycetales	Planctomycetaceae	*Planctomyces*	0,04	0,14	0,31	0,01	0,12	0,27
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas*	0,00	0,02	0,01	0,00	0,01	0,00
				Mycoplana	0,01	0,00	0,16	0,00	0,02	0,12
				Phenylobacterium	0,11	0,34	0,50	0,21	0,29	0,45
				Unidentified	0,00	0,80	0,11	0,02	0,61	0,11
		Ellin329	Unidentified	Unidentified	0,00	0,01	0,00	0,00	0,02	0,00
		Rhizobiales	f__Beijerinckiaceae	Unidentified	0,79	0,42	0,44	0,90	0,52	0,27
			Bradyrhizobiaceae	*Balneimonas*	2,65	1,92	1,31	2,48	2,16	1,52
				Bradyrhizobium	0,62	0,14	0,10	0,42	0,14	0,10
				Unidentified	0,95	0,55	0,65	1,20	0,75	0,61
			Hyphomicrobiaceae	*Devosia*	0,11	0,09	0,05	0,09	0,06	0,10
				Hyphomicrobium	0,00	0,02	0,01	0,00	0,01	0,01
				Pedomicrobium	0,10	0,01	0,02	0,02	0,01	0,00
				Rhodoplanes	0,30	0,15	0,07	0,20	0,10	0,02
				Unidentified	0,21	0,11	0,10	0,19	0,12	0,14
			Methylobacteriaceae	Unidentified	0,11	0,05	0,12	0,12	0,07	0,14
			Methylocystaceae	*Methylosinus*	0,00	0,00	0,00	0,00	0,04	0,00
				Pleomorphomonas	0,10	0,10	0,10	0,12	0,11	0,41
				Unidentified	0,01	0,01	0,02	0,04	0,02	0,01
			Phyllobacteriaceae	*Mesorhizobium*	0,05	0,05	0,05	0,09	0,02	0,06
				Phyllobacterium	0,05	0,01	0,01	0,00	0,01	0,00
				Unidentified	0,00	0,02	0,02	0,00	0,01	0,00
			Rhizobiaceae	*Agrobacterium*	0,09	0,00	0,00	0,11	0,01	0,01
				Kaistia	0,00	0,04	0,00	0,00	0,02	0,00
				Rhizobium	0,02	0,01	0,01	0,02	0,01	0,01
				Sinorhizobium	0,05	0,10	0,26	0,02	0,11	0,46
				Unidentified	0,00	0,05	0,00	0,05	0,05	0,00

			Rhodobiaceae	*Afifella*	0,07	0,05	0,05	0,07	0,04	0,01
			Xanthobacteraceae	*Labrys*	0,17	0,11	0,05	0,16	0,02	0,05
				Unidentified	0,06	0,00	0,01	0,01	0,02	0,00
			Unidentified		2,03	0,44	0,49	1,82	0,50	0,41
		Rhodobacterales	Rhodobacteraceae	*Paracoccus*	0,01	0,00	0,35	0,00	0,00	0,30
				Rubellimicrobium	0,20	0,16	0,07	0,21	0,19	0,21
		Rhodospirillales	Acetobacteraceae	*Roseomonas*	0,04	0,01	0,05	0,00	0,05	0,04
				Unidentified	0,31	0,34	0,46	0,42	0,34	0,30
			Rhodospirillaceae	*Azospirillum*	0,04	0,02	0,01	0,02	0,01	0,02
				Skermanella	2,60	1,63	3,08	2,91	1,39	2,85
				Unidentified	1,17	0,77	0,75	1,06	0,70	0,47
			Unidentified		0,39	0,34	0,44	0,45	0,22	0,37
		Sphingomonadales	Erythrobacteraceae	Unidentified	0,24	0,19	0,35	0,24	0,34	0,39
			Sphingomonadaceae	*Kaistobacter*	5,84	16,03	9,58	5,90	16,21	9,87
				Novosphingobium	0,02	0,04	0,04	0,02	0,07	0,10
				Sphingomonas	0,35	0,62	1,36	0,32	0,70	0,77
				Sphingopyxis	0,00	0,02	0,00	0,00	0,00	0,00
				Unidentified	2,38	7,04	6,63	2,75	7,90	6,50
			Unidentified		0,19	1,87	0,66	0,35	1,87	1,35
	Betaproteobacteria	ASSO-13	Unidentified	Unidentified	0,00	0,02	0,00	0,00	0,02	0,01
		Burkholderiales	Alcaligenaceae	*Achromobacter*	0,00	0,00	0,00	0,00	0,01	0,00
				Unidentified	0,10	0,02	0,04	0,07	0,01	0,04
			Comamonadaceae	*Hydrogenophaga*	0,00	0,06	0,02	0,00	0,04	0,00
				Methylibium	0,25	0,40	0,35	0,42	0,41	0,14
				Ramlibacter	0,05	0,01	0,12	0,01	0,02	0,09
				Variovorax	0,00	0,00	0,00	0,02	0,00	0,00
				Unidentified	0,69	0,29	0,54	0,85	0,31	1,00

			Oxalobacteraceae	*Cupriavidus*	0,00	0,04	0,02	0,00	0,01	0,20
				Janthinobacterium	0,01	0,00	0,00	0,01	0,00	0,00
				Unidentified	0,02	0,02	0,02	0,04	0,01	0,00
			Unidentified		0,31	0,09	0,39	0,17	0,15	0,40
		Ellin6067	Unidentified	Unidentified	0,09	0,04	0,02	0,11	0,05	0,00
		Hydrogenophilales	Hydrogenophilaceae	*Thiobacillus*	0,00	0,04	0,02	0,00	0,02	0,00
		MND1	Unidentified	Unidentified	0,21	0,05	0,04	0,10	0,02	0,02
		Methylophilales	Methylophilaceae	Unidentified	0,17	0,71	0,89	0,41	0,39	0,66
		Nitrosomonadales	Nitrosomonadaceae	Unidentified	0,04	0,27	0,57	0,00	0,27	0,45
		SC-I-84	Unidentified	Unidentified	0,06	0,02	0,05	0,06	0,06	0,00
		Unidentified			0,27	0,14	0,15	0,24	0,17	0,12
	Deltaproteobacteria	Myxococcales	Cystobacteraceae	*Cystobacter*	0,01	0,05	0,02	0,00	0,02	0,10
			Haliangiaceae	Unidentified	0,31	0,14	0,14	0,27	0,19	0,07
			Myxococcaceae	*Anaeromyxobacter*	0,01	0,05	0,04	0,02	0,07	0,30
				Myxococcus	0,00	0,02	0,00	0,07	0,00	0,01
			Polyangiaceae	Unidentified	0,00	0,19	0,16	0,01	0,09	0,07
			Unidentified		1,09	0,54	0,05	0,97	0,22	0,12
		Syntrophobacterales	Syntrophobacteraceae	Unidentified	0,57	0,21	0,21	0,41	0,21	0,17
		[Entotheonellales]	[Entotheonellaceae]	*Candidatus Entotheonella*	0,00	0,01	0,01	0,02	0,02	0,02
				Unidentified	0,21	0,16	0,15	0,19	0,11	0,15
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	0,00	0,01	0,02	0,00	0,01	0,00
				Perlucidibaca	0,00	0,00	0,00	0,00	0,00	0,01
			Pseudomonadaceae	*Pseudomonas*	0,00	0,01	0,00	0,04	0,00	0,00
		Xanthomonadales	Sinobacteraceae	*Steroidobacter*	0,49	0,25	0,19	0,62	0,26	0,26
				Unidentified	0,27	0,20	0,09	0,32	0,20	0,15
			Xanthomonadaceae	*Arenimonas*	0,00	0,05	0,11	0,01	0,11	0,22
				Lysobacter	0,26	0,85	2,26	0,25	1,40	2,61

				Pseudoxanthomonas	0,00	0,14	0,00	0,00	0,11	0,00
				Thermomonas	0,02	0,09	0,17	0,06	0,09	0,02
				Unidentified	0,06	0,67	2,12	0,09	0,75	1,31
		Unidentified			0,02	0,00	0,00	0,04	0,01	0,02
Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	*Opitutus*	0,04	0,00	0,00	0,04	0,02	0,00
	[Pedosphaerae]	[Pedosphaerales]	Ellin517	Unidentified	0,11	0,14	0,16	0,06	0,06	0,12
	[Spartobacteria]	[Chthoniobacterales]	[Chthoniobacteraceae]	Unidentified	0,01	0,05	0,02	0,02	0,06	0,10
[Thermi]	Deinococci	Deinococcales	Deinococcaceae	*R18-435*	0,00	0,10	0,07	0,01	0,04	0,25
			Trueperaceae	*Truepera*	0,07	0,06	0,07	0,02	0,04	0,05
				Unidentified	0,02	0,02	0,02	0,02	0,02	0,02
Unidentified	0,35	0,34	0,26	0,35	0,31	0,25					