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Endophyte-enhanced phytoremediation of DDE-contaminated using Cucurbita pepo: A field trial Peer-reviewed author version

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2	trial
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21 Abstract

Although the use of the pesticide 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) was banned
from the mid-1970s, its most abundant and recalcitrant degradation product, 2,2-bis(*p*-chlorophenyl)1,1-dichloro-ethylene (DDE), is still present in terrestrial and aquatic ecosystems worldwide.

Zucchini (*Cucurbita pepo* ssp. *pepo*) has been shown to accumulate high concentrations of DDE and
was proposed for phytoremediation of contaminated soils. We performed a field trial covering a full
plant life cycle. *Cucurbita pepo* plants inoculated with the plant growth-promoting endophytic strains *Sphingomonas taxi UH1, Methylobacterium radiotolerans UH1, Enterobacter aerogenes UH1,* or a
consortium combining these three strains were grown on a DDE-contaminated field for 100 days. The
effects of these inoculations were examined at both the plant level, by evaluating plant weight and
plant DDE-content, and at the level of the cultivable and total endophytic communities.

Inoculating plants with *S. taxi UH1, M. radiotolerans UH1*, and the consortium increased plant weight. No significant effects of the inoculations were observed on DDE-concentrations in plant tissues. However, the amount of DDE accumulated by *C. pepo* plants per growing season was significantly higher for plants that were inoculated with the consortium of the three strains. Therefore, inoculation of *C. pepo* with DDE-degrading endophytes might be promising for phytoremediation applications.

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- 39

40 KEYWORDS: Pesticides, DDE, DDT, endophytes, field experiment

42 Introduction

The pesticide 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane (DDT) was used worldwide until the 43 1970s when its deleterious effects on human health and wildlife were recognized ¹. In soils, DDT 44 45 rapidly degrades to the persistent 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene (DDE) and DDEcontaminated soils can still be found worldwide^{2, 3}. A possible remediation strategy for these 46 contaminated soils is phytoremediation. Phytoremediation is an in situ natural remediation 47 technology that has been demonstrated to be successful in many cases ⁴⁻⁷. The strategy relies on plants 48 49 and their associated microorganisms to take up contaminants from the soil and metabolize or store them⁸. 50

A primary requirement for the efficient phytoremediation of contaminated soils is an appropriate plant. *Cucurbita pepo* plants were shown to accumulate several organic contaminants under field conditions, including chlordane ⁹, Dieldrin, Endrin ^{10, 11}, and hexachlorocyclohexanes (HCHs) ^{12, 13}. White et al. ¹⁴ also demonstrated the effective uptake of weathered DDE by *Cucurbita pepo ssp. pepo* cultivar Raven. This zucchini cultivar showed soil-to-plant bioconcentration factors up to 23.7 and is thus a suitable plant for phytoremediation of DDE-contaminated soils.

However, when envisaging efficient phytoremediation, plants are not the sole factor. Endophytic bacteria that reside inside plant tissues are known to play crucial roles in plant growth and development in general ¹⁵, as well as in the remediation of organic contaminants ¹⁶.

A collection of 585 bacterial endophytic strains that was established during earlier research ¹⁷ was investigated *in vitro* for plant growth-promoting traits and DDE-degradation potential. Suitable endophytes were selected and further identified through full genome sequencing¹⁸⁻²⁰. After verifying the lack of pathogenic traits, the 3 full genome sequenced endophytic strains (*Sphingomonas taxi UH1, Methylobacterium radiotolerans UH1, Enterobacter aerogenes UH1*) were applied in the field experiment described in this manuscript. The main objectives of this experiment were to verify if the selected strains could promote both plant growth and DDE removal from a contaminated soil in situ.

68 Materials and methods

69 Inoculation of seeds

The selected bacterial strains were cultivated by transferring 5 μ L of the bacterial stock into 30 mL of 869 medium (Mergeay et al., 1985). A consortium was created as well by combining the selected strains into one 50 mL tube. The bacterial suspensions were incubated at 30°C for 3 days.

Seeds of *Cucurbita pepo ssp. pepo* cultivar Raven (Johnny's Selected Seeds, Winslow, ME, USA)
 were surface sterilized for 1 minute in 1% NaOCl and put in the bacterial suspension (10⁹ cells mL⁻
 ¹) for overnight inoculation.

76

77 Field characteristics

The experimental field that was utilized for this trial is located on Lockwood Farm, owned by the
Connecticut Agricultural Experiment Station (N41,406786°; W72,906043°; Hamden, CT, USA).

The soil in the field was identified as being a Cheshire fine sandy loam soil containing 56% sand, 36% silt, and 8% clay. The percentage of organic carbon was 1.4 and an average pH of 6.7 was measured. The cation exchange capacity of the soil was 18.6 cmol kg⁻¹.

83

84 Growth and harvest of plants

The seeds were germinated between wet paper towels for 3 days at 30°C. The seedlings were 85 transferred to 750 mL plastic pots containing vermiculite. The bacterial suspension was again added 86 and plants watered daily with ¹/₄ Hoagland nutrient solution ²¹. After 7 days, the plants were of 87 sufficient size to be transferred to the field. Plants were set out in 5 rows covered with black plastic 88 to avoid weed growth. Each row containing 8 plants approximately 1 m apart (Figure 1). Holes were 89 made in the plastic foil to insert the plants. Soil samples were taken from each mound/planting site to 90 determine local DDE concentrations. The plants were watered continuously by a drip irrigation 91 system underneath the plastic. 92

Harvest ready fruits were collected 3 times per week. After a growing period of approximately 100
days, plants were harvested. Shoots were clipped and roots were excavated. All plant tissues were
transferred to the lab for further analysis.

Weights were determined separately for leaves, stems, roots, and fruits. All plant tissues were rinsed
thoroughly with tap water. Approximately 5 g of shoot and root tissue were separated for endophyte
isolation and 454 pyrosequencing, while the remainder of plant tissues were homogenized and stored
at 4°C for GCMS analysis to determine DDE concentrations.

100

101 DDE content in plant tissues

102 DDE was extracted from plant tissues using the QuEChERS method ²². Up to 15 g fresh tissue was added to a 50 mL centrifuge tube with 15 mL acetonitrile and 30 μ L o,p'-DDE from a 10 mg mL⁻¹ 103 solution as an internal standard. The tubes were incubated on a wrist-action shaker for 10 min after 104 which 6 g MgSO₄ and 1.5 g C₂H₃NaO₂ were added. The tubes were shaken for 30 min and afterwards 105 centrifuged for 10 min at 3000 rpm. New 15 mL tubes were filled with 1.5 g MgSO₄ and 0.5 g of 106 primary secondary amine (PSA). Two mL of toluene was added to wet the powders before 10 mL of 107 the primary extract was transferred to each tube. The tubes were shaken for 30 s and centrifuged for 108 10 min at 3000 rpm. Six mL of each extract was concentrated to 1 mL under nitrogen pressure. These 109 extracts were amended with 100 ng mL⁻¹ o,p'-DDE as an internal standard before the DDE 110 concentrations were determined using GCMS. 111

Standards of o,p'-DDE, p,p'-DDT, p,p'-DDD, and p,p'-DDE (Environmental Protection Agency National Pesticide Standard Repository, Fort Meade, MD) were weighed in toluene to prepare standards at 1,000 µg mL⁻¹, which were then diluted to obtain mixed calibration standards from 25-1,000 ng mL⁻¹. Each calibration was furthermore amended with 100 ng mL-1 o,p'-DDE as an internal standard since the breakdown of p,p'-DDT to p,p'-DDE/DDE in the GC inlet is unpredictable, total DDx was calculated.

The concentration of DDx in plant tissues and soil was determined on an Agilent (Avondale, PA, 118 USA) 7890B gas chromatograph (GC) with a 5977A mass selective detector (MSD). Two microliters 119 of sample were injected into a multi-mode inlet (MMI) in pulsed splitless mode at 250°C with He as 120 121 the carrier gas and then onto an Agilent HP-5MS 30-m column with 0.25mm ID and guard column. The GC oven initial temperature was 150°C for 1 min, then ramped to 250°C at a rate of 5°C min⁻¹, 122 and then ramped at a rate of 50°C min⁻¹ to a final temperature of 300°C which was held for 8 min. 123 After a 5 min solvent delay, the MSD detected analytes using scan mode at a mass to charge ratio 124 (m/z) 100-430. The instrument was calibrated with the standards described above, covering a range 125 of 25-1,000 ng mL⁻¹. 126

127

128 Cultivation-dependent isolation

129 Isolation of cultivable endophytes

At harvest, plant tissues were thoroughly washed with tap water in order to remove soil particles and plant weight was determined. Root and shoot tissues were incubated separately in 1% NaOCl for surface sterilization. Subsequently, tissues were rinsed 3 times in sterile distilled water (dH₂O) and dried on sterilized filter paper. In order to verify surface sterility, an aliquot (100 μ l) of the third rinsate was transferred to a Petri dish containing 869 medium ²³ (per liter: 0.35 g CaCl₂.2H₂O, 1.00 g Glucose D+, 5.00 g NaCl, 10.0 g Tryptone, 5.00 g Yeast Extract, 15 g Agar; adjusted to pH 7 with HCl or NaOH).

The surface sterilized tissues of three individual plants were transferred to sterilized mortars containing 5 mL sterile 10 mM MgSO₄ and were crushed. The crushed root and shoot tissues were transferred to obtain serial dilutions (0, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}) and each dilution (100μ I) was spread onto plates containing 1/10 diluted 869 medium ²³. All plates were prepared in triplicate and incubated at 30°C for 4 days. The colonies on the plates were counted and the number of colony forming units (cfu) per gram of fresh plant tissue were calculated. For each treatment, averages and standard errors were calculated of the 3 replicates. The colonies were purified and in total 585 isolated strains were stored in 15 $\%_w$ glycerol at -80°C.

145

146 Genotypic characterization of cultivable bacterial strains

The DNA of the 585 isolated strains was recovered with the Qiagen DNeasy blood and tissue kit 147 (Qiagen, Venlo, the Netherlands). A Nanodrop ND-1000 Spectrophotometer (Isogen Life Science, 148 149 De Meern, the Netherlands) was used to analyze the quality and quantity of the extracted DNA. The 150 DNA was used directly for 16S rDNA amplification with a universal 1392R primer (5primer ACGGGCGGTGTGTRC-3) and bacteria-specific 26F (5-151 a AGAGTTTGATCCTGGCTCAG-3)²⁴. The 16S products were digested and separated by gel 152 electrophoresis (1.5% agarose, 90V, 2h). The banding patterns were analyzed and 50 different DNA 153 fingerprints were distinguished. At least one representative of each pattern was selected and sent for 154 16S rDNA sequencing at Macrogen (Amsterdam, the Netherlands). Consensus sequences were 155 obtained with the Staden package and identification was acquired from the Ribosome Database 156 Project based on the most closely related species (http://rdp.cme.msu.edu/classifier/classifier.jsp)²⁵. 157 The Shannon-Wiener indices of all different communities were calculated to estimate the diversity 158 obtained after the different growth conditions ^{26, 27}. 159

160

161 *Phenotypic characterization of cultivable bacterial strains*

162 The bacterial strains were tested *in vitro* for their plant growth-promoting traits. The bacteria were 163 grown in liquid 869 medium ²³ at 30°C for 2 days, washed and resuspended in 1 mL sterile MgSO₄. 164 Twenty μ l of this suspension was used for inoculation of 96 well microplate assays to investigate the 165 production of IAA using Salkowski reagent ²⁸, siderophores using the Chrome Azurol S (CAS) assay 166 ²⁹, 1-aminocyclopropane-1-carboxylate (ACC) deaminase using 2,4-dinitrophenylhydrazine and 167 NaOH ³⁰, organic acids using Alizarin red S ³¹ and phosphate solubilization capacity using NBRIP 168 medium ³². For all plant growth promotion assays, the strains were assigned scores of + or -169 depending on the presence of a color in the colorimetric tests.

170 *Screening bacteria for DDE-degrading capacities*

171 An auxanography test was performed for all bacterial strains to screen them for potential DDE-172 degradation capability. Each bacterial strain was grown in liquid 869 medium ²³. After 4 days, 1 μ L 173 of each bacterial suspension was diluted in 999 μ L sterile 10 mM MgSO4.

174 One hundred µl of the bacterial suspension was plated on selective 284 medium. Immediately thereafter, 50 μ L of a sterile 50 mg L⁻¹ DDE or a 50 mg L⁻¹ DDE + 200 μ g g-1 CuNPs solution were 175 added to the surface and smeared out on ³/₄ of the outer circle of the plate (Figure 4). The plates were 176 177 incubated at 30° for 6 days. Plates with bacteria growing on the total surface were considered neutral, and were considered to be tolerant to the used concentrations of DDE and CuNPs. Plates on which 178 strains did not show growth on the outer circle containing DDE and CuNPs were considered negative, 179 since DDE was toxic to these bacterial strains. If the bacterial strains showed enhanced growth in the 180 area covered with DDE, the plates were considered positive and these strains are presumed to use 181 182 DDE as a carbon source while being tolerant to CuNPs.

183 *Statistical analysis*

184 The averages and standard deviations were calculated from three replicates from a mixed sample of 185 three plants. The samples were compared using a one way ANOVA with Dunn comparison test.

186

187 Cultivation-independent molecular analysis

188 *Extraction and sequencing*

The plant tissue samples were surface sterilized (3 min in sterile MilliQ water, 1.5 min ethanol 70%, 3 min NaOCl 1%, 1.5 min ethanol 70%, rinse 5 times in sterile MilliQ water) before being crushed in liquid nitrogen. All genomic DNA was extracted using an Invisorb spin plant mini kit (Stratec Biomedical, Germany). A template specific 967F primer (5- CAACGCGAAGAACCTTACC-3) was combined with 1391R primer (5- GACGGGGCGGTGWGTRCA-3) to target a 424 bp fragment in the

V3-V4 region. In a two-step PCR amplification, first the original primers were used, and then the 194 same primers with a 10 bp multiplex identifier (MID) were employed. PCR reactions contained 1x 195 Roche FastStart High Fidelity Reaction Buffer with 1.8 mM MgCl₂, 1.5U of Roche FastStart High 196 197 Fidelity DNA-polymerase, 0.2 mM Roche dNTP, 300 nM 341F, 100 nM 783Ra, 100 nM 783Rb, 100 nM 783Rc and 1 μ l of 1/20 diluted template DNA (1 ng μ l⁻¹) in a volume of 50 μ l. The PCR program 198 consisted of an initial denaturation step of 95°C for 2 min, followed by 25 cycles (1st PCR) or 10 199 cycles (2nd PCR) of denaturation at 95°C for 30 s, annealing at 53°C for 40 s, extension at 72°C for 1 200 201 min, with a final extension of 5 min at 72°C. The amplicons were purified from 1.5% agarose gels using a QIAquick gel extraction kit (Qiagen, Venlo, the Netherlands) and 1 µl was used for the second 202 203 PCR using MID-elongated primers. The PCR products were purified with a OIAquick PCR purification kit (Qiagen, Venlo, the Netherlands). The DNA was quantitated using a Quant-IT 204 PicoGreen dsDNA assay kit (Life Technologies Europe, Gent, Belgium), after which equimolar 205 concentrations of the barcoded amplicons were collected per library and diluted to 100 µl using TE 206 buffer. The library was unidirectionally sequenced using a Roche 454 GS-FLX Plus Life Sciences 207 208 Genome Sequencer at Macrogen (Seoul, South Korea).

209

210 Analysis of obtained pyrosequencing data

The FASTA files containing the raw pyrosequencing data were accessed using Mothur bioinformatics 211 software ³³ for processing and analysis according to Schloss et al. ³⁴. The obtained sequences were 212 denoised before barcodes and primers were removed. The remaining sequences were aligned and 213 classified along known sequences in the SILVA rRNA database ³⁵. Chimeric sequences, 214 mitochondrial and chloroplast sequences were deleted and the remaining sequences were grouped 215 into operational taxonomic units (OTUs) based on a 97% similarity criterion. Rarefaction curves were 216 starting to level off (Figure 5), but sequencing at a greater depth could have revealed more OTUs. 217 The similarity between samples and their resemblance to the cultivated communities were visualized 218 using Primer7 (Version 7.0.5, Primer-E Ltd.). Clustering of samples was based on S17 Bray-Curtis 219

similarity of the group average of the species after square root transformation of the samples. The
nMDS was based on S17 Bray-Curtis similarity as well, with square root transformation, Kruskall
stress formula 1 and minimum stress 0.01. ANOSIM (analysis of similarities) with 999 permutations
was used to test the spatial separation of the samples in nMDS.

224

225 **Results and discussion**

226 Plant weights

Plant weight was determined to evaluate the effects of inoculation with endophytic bacterial strains 227 on plant growth over the total life cycle (Figure 2); the weights were determined separately for leaves, 228 229 stems, roots, and fruits. Significant increases in weight were observed for stems and roots of plants inoculated with the consortium of all three strains. Furthermore, plants inoculated with S. taxi UH1 230 and *M. radiotolerans UH1 showed* tended to higher weights in comparison to non-inoculated control 231 plants. However, plants inoculated with *E. aerogenes UH1* showed a slightly diminished growth in 232 comparison to controls; weights for leaves, stem, and roots tended to be lower. This generally 233 234 improved weight underlines the plant growth-promoting effects of the endophytic strains.

235 Soil DDE concentrations

At the moment of planting, soil samples were taken from each mound to determine the soil DDE concentrations (table 1). No significant differences in soil DDE concentrations were observed. The plants from different treatments were randomly planted (Figure 1) for an optimal evaluation and comparison between the different inoculations.

240 DDE concentrations in plant tissues

After determining the DDE concentrations in different plant tissues (leaf, stem, root, and fruit), the bioconcentration factors (BCFs) could be calculated (Figure 3). The BCFs are the ratio of the concentration of DDE accumulated in the plant tissues to the concentration of DDE present in the soil. As expected, the BCFs of leaves are very low, ranging from 0.025 to 0.075; by comparison, BCFs of roots and stems range from 19 to 25. This difference can be explained by the difficult translocation of DDE in plant tissues due to the hydrophobic character of the molecule. Therefore, larger effects of DDE on the endophytic communities can be expected in roots, in comparison to shoots.

With both the DDE concentrations of each plant compartment and weight of the respective compartments known, the total amount of DDE that was extracted from the field per plant during the entire growth period could be calculated as follows (Figure 4): (DDE concentration in plant tissue) x (plant weight).

Although no significant differences were observed between inoculated plants and control plants for the DDE concentrations, the total amount of DDE that was removed from the soil in one growth cycle was significantly higher in plants that were inoculated with the consortium in comparison to noninoculated control plants.

By estimating the mound volume in which a *C. pepo* plant can grow, the total amount of DDE a plant can reach was assessed. The mound had a surface area of 1m by 0.5m and a depth of 0.25m. This gives a volume of 125,000 cm³ with a density of 1.14 g cm⁻³, totaling 142.5 kg of soil per plant. Table 2 presents the amounts of DDE that were present per mound, the amounts of DDE that were taken up by the plants and the percentage this amount represents in comparison to the soil DDE content.

262 Endophytic communities

263 *Genotypic identification*

The endophytic communities of the roots and shoots were investigated using both cultivationdependent and cultivation-independent techniques. The cultivation-dependent isolation of the endophytic communities produced 530 cultivable strains, belonging to 4 phyla and 34 genera (Table 3 and Figure 6).

The cultivation-independent molecular analysis was conducted using 454 pyrosequencing. Macrogen (Seoul, South Korea) delivered the raw data in 4 FASTA files. These files contained in total 573,227 strains with a mean length of 388.01 base pairs. The files were analyzed using Mothur ³³ and the

sequences were filtered using following criteria: length >200 bp, <8 homopolymers, <2 differences 271 with the primers, <1 difference with the barcodes, pre-clustered to reduce sequencing errors, removal 272 of chimeras, and more than 95% in the same range of genes. After this filtering, 207,128 sequences 273 274 remained, containing a total of 26,862 unique sequences. Subsequently, all sequences belonging to mitochondria, chloroplasts, archaea, and eukaryotes were deleted. After this step, 199,500 sequences 275 276 remained, implying that 3.66% of all sequences belonged to non-bacterial DNA. In the total of all 277 samples, 288 different genera were detected belonging to 20 different phyla, or subphyla in case of 278 the Proteobacteria (Figure 6). With a mean OTU length of 223.19 bp, identification to the species level was not possible. 279

Although 288 different genera were detected during the pyrosequencing process, many of them were only detected once across the different samples. Therefore, we focus on the 15 most abundant genera, which account for a large portion of the total bacterial communities (ranging from 64.83% to 79.66% of the total community).

Inoculating the plants with Sphingomonas taxi UH1, Methylobacterium radiotolerans UH1, 284 Enterobacter aerogenes UH1, or a consortium combining the three strains increased their abundancy 285 in the community in the case of S. taxi UH1 (Shoot), M. radiotolerans UH1, E. aerogenes UH1 (Root) 286 and the consortium but had no apparent effect on the presence of S. taxi UH1 (Root) and E. aerogenes 287 288 UH1 (Shoot) since even a slightly lower presence was detected in the inoculated plants (Figure 7). It is important to evaluate the inoculation efficiency since an inoculation can only be considered 289 290 successful in case the presence of a bacterial strain is enhanced in the communities. However, in field conditions, an effective establishment of the inoculated strain is not evident because of the large 291

292 number of bacterial genera competing for a position in the community.

The observed changes in abundance are larger for root communities of the plants inoculated with *M. radiotolerans UH1, E. aerogenes UH1,* and the consortium. This is not surprising since all three inoculated strains originated from roots of *C. pepo* plants exposed to DDE.

Several authors mentioned that, when isolating endophytes, many bacterial genera are unaccounted 296 for due to their inability to grow under laboratory conditions ^{36, 37}. A comparison of the number of 297 genera that are cultivable (n=34) and the number of genera present in the total bacterial communities 298 299 (n=288), seems to support this hypothesis. However, when comparing the percentages of the total bacterial communities that were recovered by the cultivation-dependent techniques, percentages 300 ranging between 42.50% and 58.16% were observed at the genus level. This implies that although a 301 302 significant part of the total communities was indeed cultivable under laboratory conditions, many 303 endophytes that were present in the plant tissues were not cultivable.

When analyzing the similarity of all communities using the Analysis of Similarity (ANOSIM) and Similarity Percentages (SIMPER) tools in Primer7, a clear clustering by the cultivation-dependent and cultivation-independent techniques is observed (Figure 8). Due to the low cultivability rate of the endophytic communities, the cultivable-dependent techniques show a lower similarity than the cultivation-independent techniques that account for all endophytes present, 20.34% and 65.63% average similarity, respectively. The samples inoculated in different conditions also cluster inside the treatments, demonstrating the effect of inoculating the plants on the endophytic communities.

311 *Phenotypic identification*

Many endophytic bacterial strains were shown to possess plant growth-promoting capacities ¹⁵. By 312 investigating the symbiotic relationships between plants and their associated microorganisms, they 313 might be exploited in phytoremediation processes ¹⁶. Only cultivable endophytic strains can be tested 314 for their plant growth-promoting capacities in vitro. Table 4 describes the percentages of bacteria that 315 scored positive in the different plant growth promotion assays. Of the 530 strains that were examined, 316 2.83% (n=15) showed no *in vitro* plant growth promotion in the 5 assays that were performed, while 317 4.34% (n=23) of all strains scored positive on all 5 assays. No differences were observed between the 318 plant growth-promoting capacities of the endophytic communities isolated from non-inoculated 319 control plants or from inoculated plants; the numbers of strains displaying *in vitro* plant growth 320 321 promotion were very similar and apparently not influenced by inoculation.

Along with their plant growth-promoting capacities, all strains were also assessed for their *in vitro* DDE-degradation potential (Table 5). A total of 39 strains or 7.36% showed DDE-degradation potential. Of these strains, 38 were isolated from plants that were previously inoculated with DDEdegrading endophytic strains, while the remaining strain was isolated from a non-inoculated control plant.

327 Conclusion

The effects of inoculating plants with *Sphingomonas taxi UH1*, *Methylobacterium radiotolerans UH1*, *Enterobacter aerogenes UH1*, or a consortium of all three strains were evaluated at different levels, plant growth, DDE-uptake, and endophytic communities.

Increasing tendencies were observed for the weight of plants inoculated with *S. taxi UH1, M. radiotolerans UH1*, and the consortium in comparison to the non-inoculated control plants. The effect of inoculation with the consortium was significant for roots and stems. In contrast, inoculation with *E. aerogenes UH1* led to a slight, yet statistically insignificant decrease of the plant weight.

The DDE concentrations in the soil were similar for the different treatments (non-inoculated control or inoculated plants). Inoculation with DDE-degrading endophytes did not affect the bioconcentration factors of DDE in the plant tissues. The ratio of the concentrations of DDE inside the plant tissues to the DDE concentrations in the soil did not change when plants were inoculated.

However, when the amounts were calculated of DDE that was removed from the soils per plant and per growth cycle, a significantly higher value was observed for plants inoculated with the consortium of the three endophytic strains. This increase is mainly due to the higher root and stem weights of the plants, these being the compartments containing the highest DDE concentrations.

When the total and cultivable endophytic communities were evaluated and compared over all treatments, no obvious differences were observed. However, when comparing the abundances of the endophytic strains that were used for inoculation were compared between the non-inoculated control and the inoculated plants, increases were observed for *S. taxi UH1* (shoot), *M. radiotolerans UH1* (root and shoot), *E. aerogenes UH1* (root), and the consortium (root and shoot). However, the

348	inoculated strains S. taxi UH1 and E. aerogenes UH1 were observed in slightly lower abundancies in
349	the shoot tissues of inoculated plants in comparison to the non-inoculated control plants. These
350	observations might be due to the fact that the endophytic strains were all originally isolated from roots
351	and thus predominantly or even exclusively colonized the roots of the inoculated plants.
352	When combining all these results, we can conclude that inoculation of <i>Cucurbita pepo</i> plants with a
353	consortium of Sphingomonas taxi UH1, Methylobacterium radiotolerans UH1, and Enterobacter
354	aerogenes UH1 can significantly (46%) increase the phytoremediation potential of the plants in DDE-

355

357 Acknowledgements

contaminated soils.

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- 360
- 361 **Conflict of interest**
- 362 None declared

363 TABLES

Treatment	Average DDE concentration		
	(in ng g ⁻¹ dry weight) \pm SD		
Non-inoculated control	149.7 ± 24.2		
Sphingomonas taxi UH1	172.8 ± 26.0		
Methylobacterium radiotolerans UH1	153.0 ± 34.8		
Enterobacter aerogenes UH1	147.0 ± 24.0		
Consortium	158.6 ± 50.1		

Table 1 Average DDE concentrations in soils at moment of planting

365 Control plants are not inoculated, other plants were inoculated with the indicated strains, or a

366 combination of the three strains for the consortium.

367

Table 2 Estimation of the amounts of DDE removed by *C. pepo* plants per mound during a full growth

369 cycle

	Control	St	Mr	Ea	Consortium
DDE concentration in	149.65	172.76	153.02	146.96	178.60
soil (µg kg ⁻¹)					
Amount of DDE per	21.667	25.012	22.154	21.277	25.858
mound (mg)					
Amount of DDE taken	0.81010	0.95981	0.83243	0.71163	1.4132
up by plant (mg)					
% of DDE removed by	3 74	3 83	4.03	3 34	5 47
plant	5.71	5.05	1.05		,

370

371 **Table 3** Cultivable bacterial genera isolated from *Cucurbita pepo*

Phylum	Class	Genus	
Actinobacteria	Actinobacteria	Arthrobacter	
		Curtobacterium	
		Frigoribacterium	
		Microbacterium	

		Nocardioides
		Plantibacter
		Streptomyces
		Terrabacter
Bacteriodetes	Flavobacteria	Chryseobacterium
		Flavobacterium
		Myroides
	Sphingobacteria	Chitinophaga
		Sphingobacterium
Proteobacteria	Alphaproteobacteria	Agrobacterium
		Brevundimonas
		Devosia
		Ensifer
		Methylobacterium
		Ochrobactrum
		Rhizobium
		Sphingomonas
	Betaproteobacteria	Variovorax
	Gammaproteobacteria	Acinetobacter
		Enterobacter
		Klebsiella
		Lysobacter
		Pectobacterium
		Pseudomonas
		Stenotrophomonas
		Vibrio
		Xanthomonas
Firmicutes	Bacilli	Bacillus
		Exiguobacterium
		Paenibacillus

Bacterial genera (phylum, class) that were isolated from *C. pepo* using cultivation-dependent

373 techniques.

Table 4 Percentages of bacterial strains showing plant growth-promoting capacities

		OA	IAA	Sid	ACC	P-sol
Control	Root	40%	44%	43%	47%	58%
	Shoot	39%	57%	54%	47%	50%
Inoculated	Root	42%	60%	64%	58%	62%
	Shoot	41%	49%	67%	60%	68%

Plant growth-promoting capacities of tested bacterial strains (control n=104, inoculated n=426). OA
= organic acids, IAA = Indole-3-acetic acid, Sid = Siderophores, ACC = ACC-deaminase, P-sol =
phosphate solubilization.

Table 5 Bacterial strains showing DDE-degrading potential

species	# positive	Isolated from
Arthrobacter sp.	2	Ea
Chitinophaga sp.	1	Ea
Chryseobacterium sp.	1	St
	2	Control
Enterobacter sp.	1	Ea
Exiguobacterium sp.	1	Cons
Microbacterium sp.	2	Ea
	2	Control
Pectobacterium sp.	1	Ea
Plantibacter sp.	3	Mr
Pseudomonas sp.	2	Mr
	3	Ea
Rhizobium sp.	1	Control
	1	Mr
Sphingomonas sp.	2	Mr
	1	Control
	1	Ea
Stenotrophomonas sp.	5	Control
	2	St
	2	Mr

Variovorax sp.	2	Control
Vibrio sp.	1	Cons

 $\overline{\text{# positive}} = \text{number of bacterial strains that scored positive on the auxanography, Control = not}$

- 382 inoculated, St = Sphingomonas taxi UH1, Mr = Methylobacterium radiotolerans UH1, Ea =
- *Enterobacter aerogenes UH1*, Cons = consortium.

FIGURE LEGENDS

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- Figure 1 Layout of the field experiment. One meter of space was between each plant inside rows as
 well as in between rows. Each row was covered in black plastic and a watering system was present.
- Figure 2 Average fresh weights ± SD of *Cucurbita pepo* plants after harvesting in grams. St = *Sphingomonas taxi UH1*, Mr = *Methylobacterium radiotolerans UH1*, Ea = *Enterobacter aerogenes UH1*, Cons = consortium, Red line indicates weight of control plants. a Leaf fresh weight, b Stem
 fresh weight, c Root fresh weight, d Fresh weight of all fruits harvested during the total growth period.
 * are significantly different from control plants, p<0.05.

396

Figure 3 Bioconcentration Factors (BCFs) of different plant tissues (dry weight) from different
treatments (average ± SD). BCF = DDE concentration in plant tissue/soil DDE concentration. St = *Sphingomonas taxi UH1*, Mr = *Methylobacterium radiotolerans UH1*, Ea = *Enterobacter aerogenes UH1*, Cons = consortium.

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Figure 4 Average amount of DDE (in mg) removed \pm SD per plant per growth season in milligram for the different treatments. St = *Sphingomonas taxi UH1*, Mr = *Methylobacterium radiotolerans UH1*, Ea = *Enterobacter aerogenes UH1*, Cons = consortium. * is significantly different from noninoculated control plants (p<0.05).

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Figure 5 Rarefaction curves of the different replicates and treatments of roots and shoots. The
rarefaction curves were assembled showing the numbers of observed OTUs, defined at a 97%
sequence similarity cut-off, relative to the total number of identified bacterial sequences.

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411 Figure 6 Comparison of the compositions of the cultivable bacterial communities and the total
412 bacterial communities for all treatments. Cultivable communities show all isolated species; total

communities show the top 15 of detected species. Top: isolations from root material, bottom:
isolations from shoot material. T= Total community, C= Cultivable community, Control= noninoculated, St= inoculation with *Sphingomonas taxi UH1*, Mr= inoculation with *Methylobacterium radiotolerans UH1*, Ea= inoculation with *Enterobacter aerogenes UH1*, Consortium= inoculation
with the consortium of *S. taxi UH1*, *M. radiotolerans UH1*, and *E. aerogenes UH1*.

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Figure 7 % increase in the relative abundancy of the inoculated bacterial strains in comparison to the
endophytic communities of the control plants. St S: *S. taxi UH1* Shoot, St R: *S. taxi UH1* Root, Mr
S: *M. radiotolerans UH1* Shoot, Mr R: *M. radiotolerans UH1* Root, Ea S: *E. aerogenes UH1* Shoot,
Ea R: *E. aerogenes UH1* Root, Co S: Consortium Shoot, Co R: Consortium Root.

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Figure 8 Cluster based on S17 Bray-Curtis similarity; cluster mode is the group average of the species present in the samples; square root transformation; cophenetic correlation 0.94306. Hollow circles= cultivable community root tissue, hollow squares = cultivable community shoot tissue, full circles= total community root tissue, full square= total community shoot tissue. Blue= control, green= inoculated with *S. taxi UH1*, red= inoculated with *M. radiotolerans UH1*, purple= inoculated with *E. aerogenes UH1*, and orange= inoculated with a consortium of *S. taxi UH1*, *M. radiotolerans UH1*, and *E. aerogenes UH1*.

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- 524 525

- 526 <u>Figures</u>
- **<u>Figure 4</u>**





534 Figure 7

<u>Figure 6</u>



<u>Figure 8</u>

