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Unravelling the plastisphere-soil and plasticplane microbiome of plastic mulch residues in agricultural soils

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Abstract:	Advances in molecular techniques have recently uncovered microbial communities associated with plastic debris. The term "plastisphere," was originally used for microbial communities on marine plastic. In terrestrial systems, this term has been extended to the plastic-soil interface, encompassing microbes from the surrounding soil. Although some studies have revealed differences in microbial composition and diversity between plastisphere and bulk soil, high-resolution spatial analyses of microbial communities on the immediate plastic surface (plasticplane) and in the attached soil (plastisphere-soil), are still lacking. In this study, a methodology was developed to disentangle the bacterial populations associated with the plastisphere-soil of weathered plastic mulch from agricultural fields from those on the plasticplane by using culture-based and High-Throughput sequencing approaches. A significantly higher number of colony-forming units were cultured from the plastisphere-soil compared to the plasticplane. Main genera isolated from the plasticplane by culturing included Arthrobacter, Pseudarthrobacter, Priestia, Massilia, Microbacterium, Bacillus, and Kocuria genera, some of which are known plastic-degraders. High-throughput sequencing analysis revealed higher bacterial richness in plastisphere-soil, while beta diversity showed main significant differences among field plots. Core taxa significantly associated to the plasticplane included Bacillus, Sphingomonas, Nocardioides, and Solirubrobacter. This study provides a pioneering description of a methodology to differentially analyze microbial communities at different soil-plastic interfaces, particularly on a small spatial scale using samples from plastic mulch residues in agricultural soils. This methodology may lay a foundation for future research to isolate and identify microbial plastic degraders, contributing to efforts against mitigating plastic pollution.
Suggested Reviewers:	Andersol Abel de Souza Machado machadoaas@gmail.com
	Jie Wang jiewangcau@cau.edu.cn
	Linda Amaral-Zettler amaral@mbl.edu
Response to Reviewers:	1) Reviewer #1: In this manuscript, the authors separated the plastisphere-soil and plas-ticplane samples using two-steps washing methods. They found that the plasticplane mi-crobiome were less diverse than the plastisphere-soil communities,

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and distinct communi-ties were observed. This topic is important. Previous studies have declared that the soil plastisphere should include the microbiome on the microplastics and the soil influenced by microplastics. One important question is the distance that microplastics can influence. Although this study did not answer this question, they still give some novelty points. This study can be published after revision.
1. The figures in this manuscript are not clear enough and need to be improved for clarity and readability.
ANSWER: DONE
All figures have been updated following the required format and size.
2. Line 21: The full name of "NGS approaches" should be written in the part of abstract.
ANSWER: DONE
We have changed it to high-throughput sequencing as it is explained in the introduction, and the full name was written (Line 22).
3. Some paragraphs in this manuscript have a different indentation distance at the beginning of the paragraph, so authors are supposed to pay attention to the stand-ardization of the formatting.
ANSWER: DONE
Standardization of the formatting was updated for each of the paragraphs needed, as well as the headings.
4. Line 51: In the second paragraph of introduction, please add references behind the "… of soil-borne pathogens".
ANSWER: DONE
References were added to the paragraph as recommended (Line 52).
5. Lines 112-114: In the concluding sentence of the introduction, the description of the research implications of the paper is too macro.
ANSWER: DONE
The introduction has been restructured and rewritten with the mentioned paragraph streamlined by removing details about less central objectives and methodologies. The re-search implications are briefly summarized and emphasized (Lines 123-124; 123-132).
6. In the section of 2.3, would the first suspension contain bacteria from the surface of the plastic? Did this lead to an increase in the diversity of plastisphere-soil bacte-ria?
ANSWER: DONE
The first suspension was obtained after softly shaking them for just two minutes at 250 rpm using a rotatory shaker which is a method to suspend soil particles without dis-turbing microbial cells that are more tightly attached to surfaces such as plastic debris. Typically, ultrasonic treatments and vigorously shaking are needed to detach microbial cells/biofilms from surfaces. We have explained this methodology better in section 2.3 and also added a reference in which we described that we have tried to adapt the conventional protocols developed to isolate and differentiate rhizosphere and rhizoplane bacteria from roots (Lines 171-172, 176-177).
7. Line 177: Please provide the specific composition of the "R2A agar plates" medi-um in the supplementary material.
ANSWER: DONE
The specific composition of the R2A media was added. (Lines 196-199).
8. Please harmonize "ml" to "mL", "minute" to "min" throughout the manuscript.
ANSWER: DONE
(Lines175,176,178,179,184,185,186,188)
9. Line 194: "7 min at 50 pulses s−1" should be "7 min at 50 pulses s^{-1}".
ANSWER: DONE
Superscript was updated (Line 216).
10. Lines 289-297: The figure of the section 3.2 placed in the supplementary materi-al may lead to a lack of persuasion. Please add more content as necessary in this paragraph.
ANSWER: DONE
A more detailed description of the figure, emphasizing its importance, has been included. (Lines 323-328)
11. Line 346: "Class_ASV511" can not be found in the figure 6.
ANSWER: DONE
The figure referring to this "Class_ASV511" and Skermanella were indeed missing, and it has been added to the Supplementary Material (Figure S5) and cited in the main text (Line 376).
12. Lines 369-373: "This study addresses this gap by developing a methodology for ... at a small spatial scale" , the authors are supposed to revise this sentence for clari-ty.

	ANSWER: DONE The sentence has been reviewed and changes have been made in the paragraph for clarity and avoiding repetition. (Lines 396,398,399,402). 13. Line 452: " It should be remark that" should be " It should be remarked that". ANSWER: DONE Line 452 – Couldn't find this sentence in the paragraph, probably was referring to the phrase "It should be taken into account that" – and it was updated to "It should be re-marked that" (Line 481). 2) Reviewer #2: The manuscript titled "Unravelling the plastisphere-soil and plas-ticplane microbiome of plastic mulch residues in agricultural soils" was reviewed. In general, the manuscript described a novel way to differentiate the microbial communities associated with soil near plastic and adhere to plastic surface, i.e. plastisphere-soil and plasticplane. This manuscript is well written generally, and I have few suggestions to dis-cuss with the authors. 1. It's indeed a pioneering work to distinct different compartments in such fine scale, but the importance to do so is vaguely stated. Is it possible to clearly explain why it's crucial to differentiate microbial communities attached to plastisphere-soil and plasticplane in only one or several sentence? ANSWER: DONE Plastic debris are anthropogenic physical particles associated with a range of chemical compounds, which are completely distinct from natural soil particles. Given the persistent nature of plastic contaminants and their increasing accumulation in the environment, as-sessing and comparing the microbial communities associated with plastic particles to those living on natural soil particles is of significant interest. Most previous research has focused on evaluating the microbial communities colonizing plastic surfaces in comparison to bulk soil. However, it is crucial to consider the extreme heterogeneity of soils. Most studies on the plastisphere have come from aquatic environ-ments, which are more homogeneous, making it reasonable to compare microbial com-munities attached to plastic surfaces with those in bulk, non-influenced regions. In con-trast, soil ecosystems are highly heterogeneous, and observed differences in microbial communities may simply be a result of natural spatial constraints. Thus, as emphasized in previous review by Rillig et al., 2023, there is still a lack of in-formation on plastisphere microbial communities at the microscale habitat and in which the compartmentalized and heterogeneous nature of soil should not be ignored. This high-lights the importance of this research, which assesses and compares at a finer spatial scale the microbial communities on plastic surfaces and on the attached- soil. To emphasize the points explained above in our article, some of these comments were added in the Introduction section (Lines 69-72;83-84;86-90;97-101) 2. The Introduction part need to be reorganized. It's too long and the key points were not clear. ANSWER: DONE. The introduction has been reorganized and rewritten to emphasize the key points and make it more concise. 3. Please check again typos and spelling errors. For instance, in Line 194, -1 should be given in superscript. ANSWER: DONE All typos and spelling errors have been double checked/corrected. Correction of -1 in superscript has also been done (Line 216).

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Dear editors of Applied Soil Ecology journal,

We are pleased to present our manuscript "**Unravelling the plastisphere-soil and plasticplane microbiome of plastic mulch residues in agricultural soils**" to be considered for publication as a research article in the special issue Entitled '*Soil Health II: Advances and Mordern Challenges*', of the Applied Soil Ecology journal.

Soil health, defined as the capacity of living soil to sustain plants, animals, and humans, has been threatened by human activity. Plastic pollution, for instance, has been reported worldwide due to the increased use and mismanagement of plastic materials such as greenhouse covers and plastic mulch. However, the impacts of plastic on soil health are still not well understood. While plastic mulch offers benefits, significant accumulation of plastic debris in soil over time has been observed, leading to fragmentation and representing one of the main sources of macro- and microplastic pollution in agricultural soils.

This study offers a pioneering description of microbial communities at the soil-plastic interface, particularly on a small spatial scale. Plastic mulch debris from agricultural fields was collected, and a methodology was developed to differentiate the recovery of microbial communities associated with the soil (plastisphere-soil) to the ones adhering to the plastic surface (plasticplane). Core microbial taxa were identified, differences in the composition and abundance of bacterial communities across the two compartments were assessed. The study also highlighted both enrichment and depletion of specific bacterial taxa, offering insights into the ecological dynamics at the soil-plastic interface.

The study is also particularly notable for its assessment of environmentally relevant materials, as samples were collected from natural settings, offering a more realistic perspective compared to traditional laboratory experiments. This approach enhances the ecological validity of the findings, allowing for a better understanding of microbial community dynamics in real-world agricultural environments.

Furthermore, this work lays a foundation for future research on potential microbial degraders. The isolated species could be further evaluated for their capacity to degrade polymer-based materials, contributing to global efforts to combat plastic pollution.

We declare that none of the work contained in this manuscript is published or accepted in any other journal, and there are no conflicts of interest to declare.

We would like to thank the editor and reviewers for their constructive comments and suggestions on our manuscript. We have carefully considered the reviewers' feedback and made all necessary revisions to address their concerns. Both a clean and a tracked version of the revised manuscript are submitted for your consideration. Please take into consideration that the line numbers of the changes mentioned in this document refer to the tracked version of the revised manuscript

Yours sincerely and on behalf of all co-authors,

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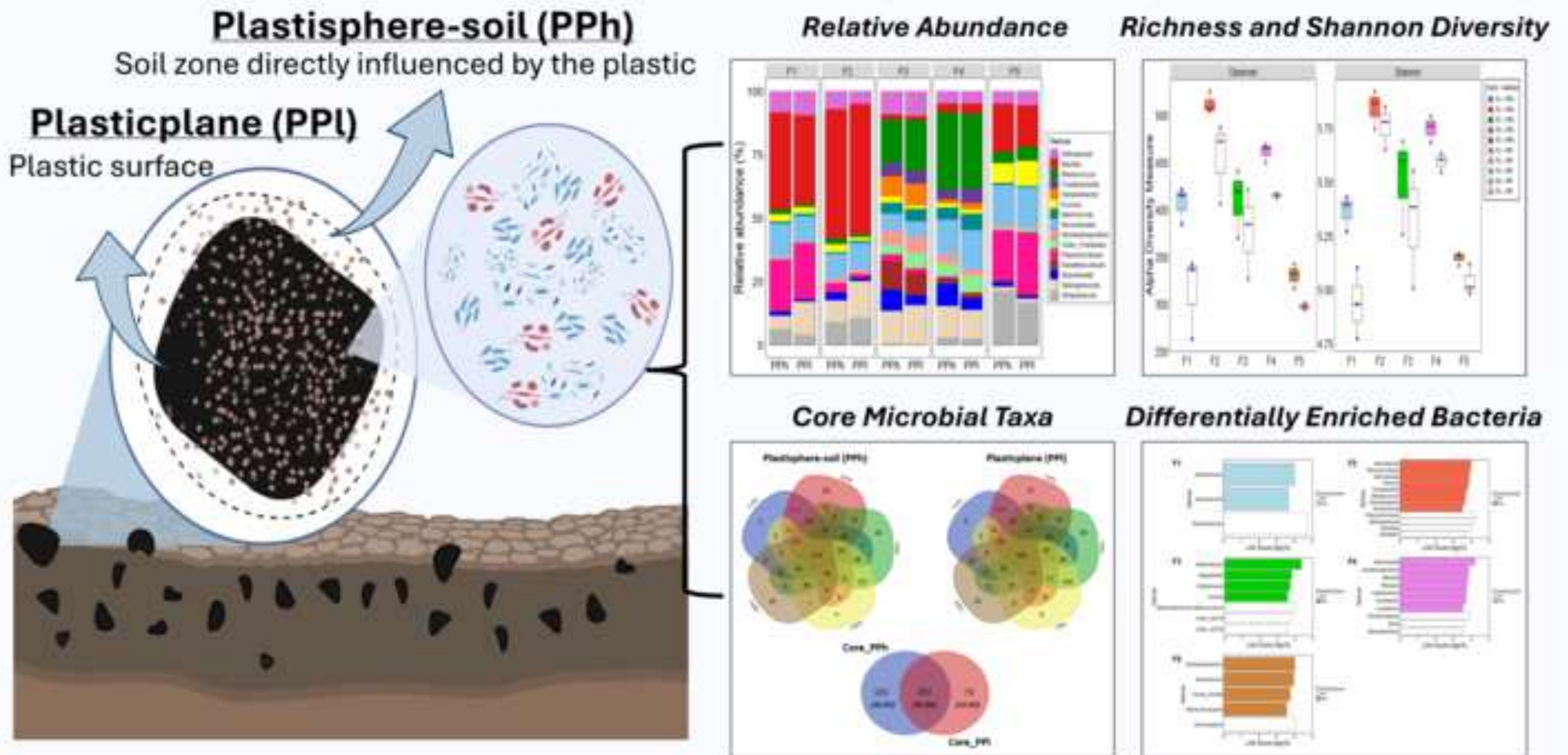

Unravelling the plastisphere-soil and plasticplane microbiome of plastic mulch residues in agricultural soils

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Highlights:

- Method developed to assess microbial communities on plastic-associated compartments.
- Key plastic-associated bacteria from natural samples were isolated and identified.
- Higher species richness in the plastisphere-soil compared to the plastic surface.
- Plastic additives could influence the associated microbial communities.
- A core plasticplane bacterial community was identified across all field sites.

1 **Unravelling the plastisphere-soil and plasticplane microbiome of plastic mulch residues in**
2 **agricultural soils**

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13 **Abstract**
14 Advances in molecular techniques have recently uncovered microbial communities associated with
15 plastic debris. The term "plastisphere," was originally used for microbial communities on marine plas-
16 tic. In terrestrial systems, this term has been extended to the plastic-soil interface, encompassing mi-
17 crobes from the surrounding soil. Although some studies have revealed differences in microbial com-
18 position and diversity between plastisphere and bulk soil, high-resolution spatial analyses of microbial
19 communities on the immediate plastic surface (plasticplane) and in the attached soil (plastisphere-soil),
20 are still lacking. In this study, a methodology was developed to disentangle the bacterial populations
21 associated with the plastisphere-soil of weathered plastic mulch from agricultural fields from those on
22 the plasticplane by using culture-based and High-Throughput sequencing approaches. A significantly
23 higher number of colony-forming units were cultured from the plastisphere-soil compared to the plas-
24 ticplane. Main genera isolated from the plasticplane by culturing included *Arthrobacter, Pseudarthro-*
25 *bacter, Priestia, Massilia, Microbacterium, Bacillus*, and *Kocuria* genera, some of which are known
26 plastic-degraders. High-throughput sequencing analysis revealed higher bacterial richness in plas-
27 tisphere-soil, while beta diversity showed main significant differences among field plots. Core taxa
28 significantly associated to the plasticplane included *Bacillus*, *Sphingomonas*, *Nocardioides,* and *Soli-*
29 *rubrobacter*. This study provides a pioneering description of a methodology to differentially analyze

microbial communities at different soil-plastic interfaces, particularly on a small spatial scale using samples from plastic mulch residues in agricultural soils. This methodology may lay a foundation for future research to isolate and identify microbial plastic degraders, contributing to efforts against mitigating plastic pollution.

Keywords: *agricultural fields; core bacteria; environmental pollution; microbial diversity; plastisphere; soil health.*

1. Introduction

The use of plastic mulch plays an important role in crop production by improving water-use efficiency and soil temperature, decreasing weed pressure, and providing higher yields and earlier harvests. However, associated drawbacks, including its disposal and environmental pollution, have gained significant attention in recent years (FAO, 2021; Zhang et al., 2020). The process of removing plastic mulch from the soil is considered a time-consuming and laborious activity. Frequently, the complete retrieval of this plastic is not achievable, leading to a significant accumulation of plastic debris in the soils that can be further fragmented, resulting in one of the major sources of microplastic (particles smaller than 5 mm) pollution in agricultural soils (Bläsing & Amelung, 2018; FAO, 2021; Huang et al., 2020; van Schothorst et al., 2021). Available studies have already highlighted notable levels of macro- and microplastic pollution in agricultural fields continuously exposed to plastic mulch worldwide. This reveals a pervasive and persistent issue, raising concerns about the potential effects on soil health and ecosystem services, and consequently, it emphasizes the need for further research in this field (Huang et al.,2020; Meng et al., 2023; Van Schothorst et al.,2021; Ya et al., 2022).

Microbial communities are crucial for ecosystem functionality, with soil microorganisms playing key roles in nutrient cycling, organic matter decomposition, soil structure maintenance, and regulation of soil-borne pathogens (Garbeva et al., 2004; Saccá et al., 2017). Current molecular techniques including High-Throughput Sequencing (HTS) combined with advanced microscopy have recently unveiled the occurrence of diverse microbial communities associated with different plastic debris from different environments (Amaral-Zettler et al., 2020; Luo et al., 2023; Schlundt et al., 2020; Vethaak & Leslie, 2016; Zettler et al., 2013). Zettler et al. (2013) first introduced the term 'plastisphere' to describe the unique microbial community colonizing plastic marine debris. However, the application of this term to terrestrial environments necessitates careful consideration to avoid potential confusion or misinterpretation. While the term is sometimes used in strict adherence to its original definition, referring

60 specifically to the microbial communities closely associated with plastic surfaces, in other instances, it
61 is applied more broadly, akin to the concept of the ‘rhizosphere'. This broader usage encompasses the
62 entire soil-plastic interface, thus including both microbes attached to the plastic and those in the inter-
63 acting soil (Rüthi et al., 2020). This expanded interpretation serves to highlight the complex interac-
64 tions within the plastisphere in terrestrial ecosystems.

65 Given the persistent nature of plastic contaminants and their increasing accumulation in the envi-
66 ronment, assessing and comparing the microbial communities associated with plastic particles to those
67 living on natural soil particles is of significant interest. However, it must be taken into consideration
68 the fact that plastic are anthropogenic materials, chemically and physically distinct from natural soil
69 particles (De Souza Machado et al., 2018). Thus, the microbial community residing on the plastic sur-
70 face can differentiate from the one found in the attached soil. In this context, our study aims to develop
71 a methodology that allows to distinguish the main compartments that microbes can occupy in terrestrial
72 agroecosystems polluted with plastics. Therefore, we propose the adoption of the term 'plastisphere-
73 soil’ to describe the soil zone directly influenced by plastic. Additionally, we suggest using 'plas-
74 ticplane' as a more specific term to describe the surface of the plastic where specific microbial coloni-
75 zation can also occur akin to the concept of “rhizoplane” terminology, broadly adopted in soil micro-
76 biology studies (Foster, 1986; Y. Li et al., 2023; Rashid Mwajita et al., 2013; Wieland et al., 2001).

77 Most previous research has focused on evaluating the microbial communities colonizing plastic
78 surfaces in comparison to bulk soil, reporting notable differences in composition, diversity, and rich-
79 ness compared to those associated with plastic debris (Gkoutselis et al., 2021; Yu et al., 2021). How-
80 ever, it is crucial to consider the extreme heterogeneity of soils. Most studies on the plastisphere have
81 come from aquatic environments, which are more homogeneous, making it reasonable to compare mi-
82 crobial communities attached to plastic surfaces with those in bulk, non-influenced regions. In contrast,
83 soil ecosystems are highly heterogeneous, and observed differences in microbial communities may
84 simply be attributed to natural spatial constraints.

85 As emphasized by Rillig et al. (2023), there is still a lack of information on plastisphere microbial
86 communities at the microscale, where the compartmentalized and heterogeneous nature of soil must
87 not be overlooked. This highlights the importance of this research, which isolated and compared mi-
88 crobial communities across adjacent plastic-associated microenvironments at a small spatial scale, us-
89 ing both culture-dependent and culture-independent approaches.

90 Plastic mulch debris was collected from agricultural fields, providing a more realistic perspective
91 compared to traditional laboratory experiments. A methodology was developed to differentiate the re-
92 covery of the microbial communities associated with the soil adhering to the plastic (referred to as
93 plastisphere-soil) and those closely associated with the plastic surface itself (termed plasticplane). This
94 study has also identified core microbial taxa consistently associated with plastic mulch debris, as well
95 as isolated bacteria species that could be further evaluated for their potential to degrade polymer-based
96 materials contributing to global efforts to mitigate plastic pollution.

97 ## 2. Material and methods

98 ### 2.1 Study site and field sampling

99 Weathered plastic mulch debris was collected from five agricultural fields located at Baza (Gra-
100 nada), southern Spain (Figure S1). The fields were characterized by intensive horticultural production
101 and historical use of plastic at least twice a year over the last ten years. At each sampling site, a total
102 of ten weathered plastic mulch debris samples measuring from 5-10 x 5-10 cm, were randomly col-
103 lected from the topsoil. Samples were gently shaken to remove the loosely attached soil particles,
104 placed in sampling bags, and stored at 4ºC until further analysis.

105 ### 2.2 Plastic characterization by Raman and Fourier infrared (FTIR) spectroscopy

106 Prior to chemical characterization, plastic films were thoroughly washed with warm water (40ºC),
107 followed by a 20-min ultrasonication in deionized water. Subsequently, the films were rinsed and
108 wiped using cotton wool in distilled water and 70% isopropanol to remove residual soil and potential
109 bio-contaminants.

110 The chemical characterization of the plastic film surfaces was performed using Raman and FTIR.
111 FTIR is especially sensitive to observing oxidative products resulting from weathering, while Raman
112 spectra can provide insights into the polymer backbone, crystallinity, and the presence of inorganic
113 additives undetectable by FTIR.

114 Raman analysis was performed using a Renishaw InVia microspectrometer (Renishaw plc, New
115 Mills, Wotton under Edge, UK) equipped with a 532 nm laser (15 mW at the sample) and a 50x objec-
116 tive lens (numerical aperture NA 0.50). Raman spectra were collected in the 100-3500 cm^{-1} range using
117 a 2400 lines per millimeter grating, resulting in a spectral resolution of approximately 1 cm^{-1}. All

118 measurements were conducted with 5 accumulations at 5% laser power. After collection, each spec-
119 trum underwent cosmic ray removal (Wire 4.2), baseline correction, and normalization at the 2851 cm^{-1}
120 in Spectragryph version 1.2.15. Given the heterogeneous nature of the plastic surface, spectra were
121 collected at ten different positions on each plastic sample. The fraction of trans (α_t) and amorphous (α_a)
122 conformers were calculated using I_{1298} and I_{1305} intensities of the Raman bands at 1298 and 1305 cm^{-1},
123 respectively according to Hiejima et al.(2018).

124 Alongside Raman spectroscopy, a Cary 630 FTIR (Agilent Technologies Inc., Danbury, CT, USA)
125 with an attenuated total reflection (ATR accessory, diamond substrate) was used to identify plastics
126 and potential weathering effects on the polymer surface functional groups (penetration depth~2 µm at
127 1700 cm^{-1}). FTIR spectra were gathered in the 650–4000 cm^{-1} range, with 64 accumulated scans and
128 a 2 cm^{-1} spectral resolution. After collection, each spectrum underwent baseline correction and nor-
129 malization at the 2912 cm^{-1} peak in Spectragryph.

130 **2.3 Assessment of microbial communities associated with plastic mulch debris**

131 To assess and distinguish the microbial community of the plastisphere-soil (PPh) from the one
132 tightly attached to the plastic surface (plasticplane (PPl)) the collected plastic samples were subjected
133 to sequential washing steps (Figure S2) by using a modified protocol mimicking those developed to
134 sample and isolate rhizosphere and rhizoplane bacteria (Barillot et al., 2013).

135 Plastic mulch samples collected from each field were cut into small pieces (2 x 1.5 cm) with sterile
136 scissors and pooled into a single composite sample. A total of 50 mg of composite plastic samples was
137 placed in a falcon tube containing 10 mL of sterile distilled water. Collectively, three replicates of
138 plastic samples were processed per field plot. Falcon tubes were softly shaken for two min at 250 rpm
139 in a horizontal rotatory shaker, obtaining a first suspension that contained the attached soil fraction
140 (plastisphere-soil). From this soil suspension, 1-mL aliquots were taken for isolation of cultivable bac-
141 teria whereas 1.5-mL aliquots were taken for DNA extraction. Specifically, the 1.5-mL aliquots were
142 centrifuged at 14,000 rpm for 1 min. Then, the supernatant was discarded, and this process was repeated
143 six times to recover a soil pellet that was stored at -20°C until DNA extraction.

144 On the other hand, the washed plastic pieces from the first suspension were subjected to a second
145 intermediate washing step (Figure S2). Plastic debris was recovered and transferred to another falcon
146 tube that was shaken for 2 min with 20 mL of sterile water. Then, the washed plastic mulch films

147 (plasticplane) were transferred to a new 15-mL tube with 5 mL of sterile water, and it was sonicated
148 for 5 min and vigorously vortexed for 2 min. A 1-mL aliquot of the resulting suspension, containing
149 bacteria from the plasticplane, was taken for isolation of cultivable bacteria (see below), whereas the
150 washed plastics were transferred to a 2-mL tube and kept at -20 °C until DNA extraction.

151 Additionally, samples from the plastic surfaces at each consecutive washing step were observed
152 under a stereomicroscope (Leica, M165C, Leica Microsystems, Germany) to assess the efficacy of the
153 washing process in removing soil particles attached to the plastic surfaces, and the same washed pieces
154 were subjected to SEM-EDX analysis described below to corroborate the efficacy of the different
155 washing steps to differentiate plastisphere-soil and plasticplane interfaces.

156 2.3.1 Isolation and characterization of culturable bacteria

157 Aliquots of plastisphere-soil and plasticplane suspensions were subjected to serial dilutions and
158 100-μl aliquots were plated in triplicate on R2A (Difco, Detroit, MI, USA) agar plates which contains
159 yeast extract (0.5 g L^{-1}), proteose peptone No. 3 (0.5 g L^{-1}), casamino acids (0.5 g L^{-1}), dextrose (0.5
160 g L^{-1}), soluble starch (0.5 g L^{-1}), sodium pyruvate (0.3 g L^{-1}), dipotassium phosphate (0.3 g L^{-1}), mag-
161 nesium sulfate (0.05 g L^{-1}), and agar (15 g L^{-1}), with a final pH of 7.2.

162 The number of colonies forming units (CFU) was assessed after a 2-day incubation period at 28ºC.
163 Subsequently, a representative number of distinct colonies from the plasticplane were selected based
164 on morphology (color, shape, margin, and texture). Then, selected colonies were isolated and purified
165 (three cloning steps) and kept in 40 % glycerol stocks at -80ºC.

166 For bacteria identification, DNA was extracted using the DNeasy kit (QIAGEN, Madrid, Spain).
167 The near-complete 16S rDNA gene was amplified using 8f (5′-AGAGTTTGATCCTGGCTCAG-3′)
168 and 1492r (5′-ACGGCTACCTTGTTACGACTT-3′) primers (Weisburg et al., 1991) following the
169 protocol outlined in Anguita-Maeso et al. (2022). Amplicons were sequenced by Sanger sequencing
170 with the same primers used for the PCR at STABvida sequencing facilities (Caparica, Portugal). Se-
171 quences were assembled and manually corrected using DNASTAR software version 15.3.0.66 (Madi-
172 son, WI, USA). The identification of isolates to the genus/species level was carried out by comparing
173 their sequences with reference 16S rRNA gene sequences in the GenBank "nt" database using the
174 BLAST algorithm as described by Altschul et al. (1997).

175 2.3.2 DNA Extraction and 16S rRNA Gene Amplicon Library Preparation

176 DNA from plastisphere-soil and plasticplane samples was extracted using the DNeasy PowerSoil
177 Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions with small modifications.
178 Briefly, samples were homogenized with the lysis buffer for 7 min at 50 pulses s^{-1} with the Tissuelyser
179 LT (QIAGEN) and then were incubated for 1 h at 60 °C to increase cell lysis. DNA was eluted in a
180 final volume of 50 µL of sterilized distilled water and its purity was determined using a NanoDrop®156
181 ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). This DNA
182 was used as a template for amplicon library preparation as described previously in Anguita-Maeso et
183 al., (2022). Shortly, the V5-V6 region of the bacterial 16S rRNA gene was amplified with the primers
184 799F (5'- AACMGGATTAGATACCCKG-3') and 1115R (5'-AGGGTTGCGCTCGTTG-3'). Bar-
185 codes indexes were added to the amplicons using Fluidigm barcodes (Access Array Barcode Library
186 for Illumina® Sequencers kit). Next, barcoded PCR products were purified by using Agencourt AM-
187 Pure XP (Beckman Coulter Inc., Brea, CA, USA), following the manufacturer's instructions. Purified
188 PCR products were quantified by using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo
189 Fisher Scientific) and a TECAN SAFIRE microplate reader (Tecan Group, Männedorf, Switzerland).
190 Equimolecular amounts from each individual sample were added to a single tube; the pooled library
191 was quantified by using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), and was purified again
192 if primer dimers were still evident. Finally, the library was sequenced on the Illumina MiSeq platform
193 (V2; PE 2× 250 bp) at the Genomics Unit at the Madrid Science Park Foundation, Madrid, Spain. The
194 ZymoBIOMICS microbial standard (Zymo Research Corp., Irvine, CA, USA) and water (no template
195 DNA) were used as internal positive and negative controls, respectively, for library construction and
196 sequencing.

197 2.4 Scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX) of Plastic 198 Surfaces

199 Scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX) was utilized to eval-
200 uate the plastic surface morphology, determine the elemental composition of the plastic, and assess the
201 efficacy of the washing steps in removing soil particles adhered to the plastic. Distinctly colored plastic
202 mulch samples (black and light grey) were specifically chosen from each sampling site. These samples
203 underwent fixation with 2% glutaraldehyde, dehydration through an ethanol series, and gold coating.
204 The scanning electron microscopy analysis was conducted using a JEOL JSM 7800 F scanning micro-
205 scope (JEOL Ltd., Peabody, MA, USA) equipped with energy-dispersive X-ray spectrometer (EDX)
206 at Central Service of Research Support (SCAI) of the University of Cordoba, Spain.

207 **2.5 Statistical and Bioinformatics Analysis**

208 Statistical analysis of culturable bacteria was performed using R statistical software (R Core Team,
209 2013). The significant effect of field location and soil-plastic habitat was tested using a factorial anal-
210 ysis of variance (ANOVA) followed by Tukey's multiple comparison test ($P< 0.05$). The Shapiro-Wilk
211 test was used to determine whether the data were normally distributed whereas the homogeneity of
212 variance was tested using Levene's test. Data from bacteria enumeration were log-transformed to fulfill
213 the assumptions of homogeneity and normality.

214 TrimGalore v.0.6.6 tool was employed for quality control and adapter trimming of the demulti-
215 plexed raw fastq files. In this process, the first 10 bp of all reads were trimmed, and a truncation length
216 of 240 base pairs for forward bacterial reads and 200 base pairs for reverse bacterial reads was applied
217 to achieve a satisfactory Phred quality score ($Q > 30$). Subsequently, the high-quality reads underwent
218 analysis using the DADA2 method to identify the amplicon sequence variants (ASVs) present in the
219 samples (Callahan et al., 2016). Taxonomic classification was carried out using the Silva SSU v.138
220 database, and singleton ASVs were excluded from taxonomy assignment and statistical analysis. Dif-
221 ferences in bacterial communities were assessed using alpha-diversity indexes (Richness and Shannon)
222 at the ASV level. The non-parametric Scheirer–Ray–Hare test ($P < 0.05$), implemented with the rcom-
223 panion v.2.4.1 package in R (Mangiafico, 2022), was employed to evaluate the effects of the sampled
224 field and habitat (plastisphere-soil or plasticplane) and their interaction on alpha-diversity indexes.
225 Beta-diversity was determined through principal coordinate analysis (PCoA) of weighted UniFrac dis-
226 tance matrices. Additionally, the Permutational multivariate analysis of variance using distance matri-
227 ces (ADONIS function) from the vegan package in R (with 999 permutations) was employed to test
228 the effects ($P < 0.05$) of the sampled plot, habitat, and their interaction. To achieve parity in the total
229 number of counts between samples, alpha- and beta-diversity analyses were performed after
230 resampling the abundance values to the minimum number of reads observed. Moreover, a linear dis-
231 criminant analysis effect size (LEfSe) (Segata et al., 2011), based on the microbiomeMarker package
232 in R (Cao et al., 2022), was employed to identify differences in microbiota composition at the genus
233 level among the various treatments ($P < 0.05$).

234 **3. Results**

235 **3.1 Plastic characterization**

236 The most representative spectra, showcasing the overall plastic composition and the influences of
237 weathering is shown in Figure 1. Both Raman and FTIR analyses confirmed that the plastic mulch
238 collected from the five fields was composed of polyethylene (Table S1).

239 Raman spectra analysis of the plastic samples displayed distinct peaks corresponding to the native
240 bonds in the LDPE polymer, found in the C-C stretching (1040–1200 cm^{-1}), -CH_2- twisting and bend-
241 ing (1300–1500 cm^{-1}), and C-H stretching (2800-3000 cm^{-1}) regions (Fig. 1). The latter is sensitive to
242 subtle structural changes due to intermolecular interactions and crystallinity. The peaks at 2882 and
243 2851 cm^{-1} indicated the presence of LDPE's amorphous and crystalline regions, respectively. Plastic
244 samples from Field 1 had a significant decrease in the intensity of the 2882 cm^{-1} peak relative to the
245 2851 cm^{-1} peak, compared to the plastic samples obtained from other fields, indicating its lower crys-
246 tallinity.

247 Additional Raman signals linked to LDPE's crystallinity appeared at peaks 1305 and 1298 cm^{-1},
248 associated with the CH_2 twisting modes of the amorphous and trans (consecutive trans chains) con-
249 formers, respectively (Meier, 2002). The highest amorphous fraction was found in the plastic samples
250 from Field 1, being 45.39 ± 0.03%, while the fractions obtained from Fields 3, 4, and 5 were 36.4 ±
251 0.1%, 33.19 ± 0.06%, and 34.28 ± 0.06% respectively. Field 2 was excluded from the calculations due
252 to a broad additive band at 1360 cm^{-1}. Often, plastic degradation, especially due to photodegradation,
253 results in increased crystallinity (decreased amorphous phase), leading to material embrittlement and
254 fragmentation.

255 The lower Raman spectral region (100-600 cm^{-1}) indicated the presence of inorganic compounds.
256 In this case, the presence of titanium oxide (TiO_2) and the blue pigment Lazurite-a sodium silico alu-
257 minate in a sulfur matrix were confirmed in plastic samples from Field 2 (Figure S3 A). This was
258 evidenced by the presence of bands corresponding to anatase TiO_2 (143 cm^{-1}) and rutile TiO_2 (233,
259 442, 610 cm^{-1}), and the characteristic bands of Lazurite (257, 547, 805, 1094, 1644, and 2183 cm^{-1},
260 (Figure S3 B). These findings align with the SEM-EDX data, which also revealed the presence of
261 titanium (0.12%wt), silicon (2.76%wt), calcium (1.87%wt), sulfur (0.15%wt), and aluminum
262 (1.01%wt) in plastic samples from Field 2, while no sulfur and titanium were detected in plastics from
263 other fields (Table S2).

264 Interestingly, plastic samples from Fields 1, 3, and 4 exhibited a noticeable presence of carbonyl
265 (C=O) groups in their FTIR spectra, potentially formed during plastic oxidation. Samples from Field

266 3 and Field 4 displayed unique peaks due to ketone carbonyl at 1717 cm^{-1}, while those from Field 1
267 showed a peak due to ester carbonyl at 1740 cm^{-1}. The carbonyl index, a common indicator of oxidation
268 level, which is calculated as the ratio of peak intensity between 1717 (or 1740) cm^{-1} and 1465 cm^{-1},
269 was 0.13, 0.20, and 0.28 for samples from Fields 1, 3, and 4, respectively, indicating a higher concen-
270 tration of carbonyl in plastic samples from Field 4.

271 **3.2 Microscopic evaluation of plastic surface: scanning electron microscopy and optical mi-**
272 **croscope analysis**

273 Visual inspection using light images from a stereomicroscope (Figures S4 A and B) and SEM (Fig-
274 ure S4 C) was performed to assess the efficacy of the sequential washing steps in removing soil parti-
275 cles from the plastic surface and enabling independent sampling of plastisphere-soil and plasticplane-
276 associated microorganisms. The image shows the condition of the plastic surface immediately after
277 field sampling and after each successive washing steps described in Figure S2.

278 Initial attempts revealed that even after two washing steps, some soil particles remained, which
279 could lead to misleading results in the differential extraction of microorganisms associated with the
280 soil particles rather than the plastic surface. Therefore, an additional washing step was introduced to
281 remove the soil particles more effectively (Figure S2). This modification included in our protocol en-
282 sured a clear distinction between the plastisphere-soil and plastic-plane compartments, facilitating
283 more accurate characterization of their respective microbial communities.

284 **3.3 Characterization of the culturable bacterial population**

285 Both plastic-debris compartments (plastisphere-soil and plasticplane) and location (field) factors
286 significantly affected the population density of culturable bacteria ($P<0.001$) (Figure 2). The bacterial
287 density found on the plastisphere-soil was significantly greater than that obtained on the plastic surface
288 for all sampled fields. Thus, the bacterial density isolated from the plastisphere-soil fraction ranged
289 from log 7.1 to log 7.7 CFU g^{-1} plastic, whereas the bacterial population on the plastic surface ranged
290 from log 6.2 to 6.9 CFU g^{-1} plastic.

291 A total of 74 bacteria from the plasticplane were isolated, cultivated, and taxonomically identified
292 by 16S rRNA gene sequencing. Isolated bacteria were assigned to four phyla, five classes, six orders,
293 nine families, and 19 genera (Table S3A). The most abundant genera included *Arthrobacter* (16.2% of

294 isolates) followed by *Priestia* (14.9% of isolates), *Pseudarthrobacter* (13.5% of isolates), *Massilia* and
295 *Microbacterium* (9,5% each), *Bacillus* (8,1%) and *Kokuria* (6.8%) (Table S3B). On the other hand,
296 less frequent bacteria included *Frondihabitans, Lysinibacillus, Nocardioides, Paenarthrobacter, Pae-*
297 *nibacillus, Planococcus, Planomicrobium, Rossellomorea,* and *Rufibacter.* Plasticplane samples from
298 Fields 3 and 5 showed a higher diversity of bacterial genera (8 and 10 respectively), whereas in those
299 from the remaining field plots, a total of seven bacterial genera were identified.

300 **3.4 Composition and diversity of bacterial communities in the plastisphere-soil and plas-**
301 **ticplane**

302 Illumina sequencing yielded a total of 351,265 good-quality reads after removal of chimeras, un-
303 assigned, or mitochondrial reads. A total of 1,701 amplicon sequence variants (ASVs) were identified
304 among all treatments, with 1,682 ASVs being retained for alpha- and beta-diversity analysis after rar-
305 efying all data to the minimum number of reads and singleton removal. The Scheirer–Ray–Hare test
306 indicated significant differences ($P < 0.05$) for the Richness and Shannon alpha-diversity indices ac-
307 cording to the field plot ($H = 19.63$, $P = 0.001$, and $H = 21.54$, $P = 0.001$, respectively) whereas plastic
308 -associated compartments resulted significant for Richness ($H = 4.13$, $P = 0.04$), but not for Shannon
309 ($H = 3.25$, $P = 0.07$) diversity. Furthermore, there was no significant interaction between the plastic
310 fraction and field plot for both alpha-diversity indices ($H < 0.77$, $P > 0.94$) (Figure 3).

311 Principal coordinate analysis of beta-diversity weighted UniFrac distances differentiated bacterial
312 communities mainly according to the field location. Thus, there was a clear tendency to group bacterial
313 communities according to the sampled field along Axis 1, which explained 49.9% of the variation
314 (Figure 4). In fact, ADONIS analysis supported the results described above and indicated a significant
315 main effect of the field plot ($R^2 = 0.79$, $P < 0.001$). However, the bacterial habitat (plastisphere-soil or
316 plasticplane) resulted not significant ($R^2 = 0.01$, $P > 0.196$) nor their interaction ($R^2 = 0.03$, $P > 0.424$).

317 A total of 24 phyla, 60 classes, 173 orders, 321 families, and 723 genera of bacteria were taxonom-
318 ically identified by illumine sequencing. Most abundant bacterial genera in all the plastisphere-soil
319 samples belonged to *Bacillus* (23.3%), *Nocardioides* (11.7%), *Planomicrobium* (10.4), *Blastococcus*
320 (10.1%) and *Streptomyces* (8.5%) genera, whereas those from plasticplane included *Blastococcus*
321 (15.6%), *Sphingomonas* (10.7%), *Arthrobacter* (10.1%), *Bacillus* (9.3%) and *Hymenobacter* (7.4%).
322 In Field 1 and 2, there was a prevalence of *Bacillus* (41.3% and 51.8%, respectively), whereas, in Fields
323 3 and 4, *Blastococcus* was the dominant genus (18.9% and 30.7%, respectively) (Figure 5). When

324 evaluations were made within each field, comparing the microbial community associated with the plas-
325 ticplane with the one from the plastisphere-soil, there were no evident changes in the proportion of the
326 most abundant genera.

327 In line with these results, Linear Discriminant Analysis Effect Size (LEfSe) analysis was used to
328 identify key bacterial genera differentially associated with the plasticplane or plastisphere-soil fractions
329 of plastic mulch debris sampled at different field locations. Globally, LEfSe identified a bacterial en-
330 richment of *Skermanella* and Class_ASV511 enriched in the plastisphere-soil (Figure S5). However,
331 when applying LEfSe to each field independently, field plot F2 showed a higher number of differen-
332 tially abundant genera. In particular, genera such as *Polycyclovorans*, *Sphingomonas,* and *Cellvibrio*,
333 were enriched in the plasticplane, while genera such as *Arthrobacter, Planomicrobium*, and *Sker-*
334 *manella* were significantly more abundant in the plastisphere-soil than in the plasticplane (Figure 6).

335 **3.5 Core community analysis**

336 The Venn Diagram (Figure S6) analysis revealed the shared and unique bacterial genera in the
337 different plastic-associated habitats. Notably, in all evaluated fields, between 62.5% and 88.4% of the
338 bacterial genera were shared between both habitats. Interestingly, on plastic debris recovered from field
339 plots F3 and F5, approximately 13% of bacterial genera were exclusively found on the plasticplane,
340 while in the other fields, these values were lower.

341 The core microbial taxa consistently found only in the plastisphere-soil of all evaluated samples
342 recovered from the different fields were represented by 220 bacterial ASVs, while 79 ASVs repre-
343 sented the core taxa on the plasticplane (Figure 7). The majority of bacteria consistently found on the
344 plasticplane belonged to the genera *Bacillus*, *Sphingomona*s, *Nocardioides,* and *Solirubrobacter*. A
345 complete list identifying the core plasticplane bacterial community can be found in Supplementary
346 Table S4.

347 **4. Discussion**

348 While some studies have compared the plastisphere microbiome with bulk soil, highlighting sig-
349 nificant differences in their microbial community composition and structure, there is still a need for a
350 more comprehensive understanding of microbial communities at a finer spatial definition within the
351 plastic-soil interface (Bandopadhyay et al., 2020; Gkoutselis et al., 2021; Rillig et al., 2023; Rüthi et

352 al., 2020). This study addresses this gap by developing a methodology to differentially sample and
353 compare the bacterial community composition and diversity of plastic surfaces (plasticplane) with
354 those found on natural soil particles interacting with this anthropogenic material (plastisphere-soil)

355 Polyethylene polymers are commonly regarded as recalcitrant materials, known for their resistance
356 to degradation (Brown et al., 2022; El-Sherif et al., 2022). However, research suggests that weathered
357 polyethylene plastics, previously exposed to UV radiation, can be colonized and undergo partial me-
358 tabolism facilitated by specific microbial taxa capable of secreting enzymes such as esterase, lipases,
359 peroxidases, and oxidoreductases (El-Sherif et al., 2022; Sharma & Neelam, 2023). Consequently, it
360 was expected that, as observed, the microbial community richness on the surface of weathered poly-
361 ethylene might experience a reduction or selection of specific bacterial taxa compared to soil particles.
362 This difference can be attributed to the easily assimilated carbon sources provided by the associated
363 organic matter in soil particles, contrasting with the resistant nature of polyethylene, which is not con-
364 sidered a straightforward or efficient carbon source for most bacteria. Interestingly, these results are
365 similar to those described for the rhizosphere soil and rhizoplane-root interfaces, where the secretion
366 of root exudates favors the growth of specific bacteria that are better adapted to this environment,
367 leading to a decrease in overall bacterial richness (Philippot et al., 2013).

368 Furthermore, our results revealed similarities in the microbial communities' composition retrieved
369 according to the field of origin of the plastic debris. Thus, the tendency to group bacterial communities
370 according to the sampled field, and the observed similarities between Fields 3 and 4, which are geo-
371 graphically closer to each other, highlight the role of environmental and geographic constraints in shap-
372 ing the soil microbial community at the plastic-soil interface. Additionally, the plastic debris collected
373 from Field 2 exhibited a higher number of differentially abundant taxa on the plasticplane compared
374 to the plastisphere-soil. Interestingly, this plastic was unique in its color, appearing light grey instead
375 of the commonly found black plastic mulch of the remaining field sites. Analysis of the plastic's chem-
376 ical properties by SEM-EDX and Raman spectroscopy revealed the presence of titanium dioxide (TiO_2)
377 on this specific mulch film, a pigment commonly found in white plastics due to its high light scattering
378 efficiency, inertness, thermal stability, dispersibility, and cost-effectiveness (Puglisi et al., 2019;
379 Turner & Filella, 2023).

380 Some previous studies have demonstrated the effects of TiO_2 on soil bacterial communities (Ge et
381 al., 2011, 2012, 2013). Ge et al. (2012), for instance, found that the presence of this chemical compound

382 can significantly reduce certain bacterial genera, including *Actinoplanes, Balneimonas, Blastococcus,*
383 *Bradyrhizobium*, and *Skermanella.* The toxicity of this material to some bacteria can be related to ox-
384 idative damage to bacterial cell walls, leading to membrane disorganization and permeability. Our re-
385 sults also indicate a reduction in the *Blastococcus* genus on the plastic surface compared to the plas-
386 tisphere-soil. In contrast, the genera *Polycyclovarans* and *Cellvibrio* were enriched on the plasticplane
387 compared to the plasticphere. These findings suggest that plastic additives and dyes may influence the
388 assemblage of plastic-associated bacterial communities, underscoring the need for further research fo-
389 cusing on this topic.

390 Differing from most contaminants found in the soil, plastic debris represents a potential threat to
391 the soil environment as it constitutes an external anthropogenic particle with a distinct shape, size, and
392 volume compared to natural soil particles (Rillig et al., 2023). Additionally, it contains a series of
393 additives, such as pigments, plasticizers, and antioxidants, which can leach over time and could interact
394 with the microbial community (Macan et al., 2024). Furthermore, these materials can adsorb contami-
395 nants such as pesticides commonly applied in agricultural fields, as well as heavy metals (K. Li et al.,
396 2023; Rillig et al., 2023). Consequently, all these factors may play a role in selecting specific taxa more
397 adapted to colonize the plasticplane.

398 Isolating, cultivating, and identifying key species from the plasticplane is particularly noteworthy,
399 as they present promising candidates for further assessments as plastic degraders. Both molecular and
400 culturable methods revealed a prevalent presence of *Arthrobacter* genus in plastic-soil interface. Nu-
401 merous reports in the literature highlight its significant role in degrading various carbon sources, em-
402 phasizing its active hydrolytic enzyme production and the ability to break down persistent conventional
403 plastics (Gobbetti & Rizzello, 2014; Han et al., 2020). Furthermore, members of the *Priestia* genus
404 were isolated and cultured from the majority of plasticplane samples. This genus is recognized as an
405 environmental bacterium extensively used in biotechnology and bioremediation due to its ability to
406 produce several enzymes (Dhaka et al., 2022; Shwed et al., 2021). Other culturable bacterial genera
407 isolated and identified, such as *Bacillus, Terribacillus, Paenibacillus, and Kocuria*, have also been
408 previously associated with plastics according to existing literature and could be further explored as key
409 genera in plastic biodegradation assays (Anwar et al., 2016; Bardají et al., 2019; Harshvardhan & Jha,
410 2013; Vidal-Verdú et al., 2022). More specifically, *Priestia megaterium* and *Bacillus pumilus,* for
411 instance, were some of the cultivated and identified species in this study which have also been previ-

412 ously isolated from the plastic debris and assessed for their biodegradation abilities on a range of pol-
413 ymer types such as polyethylene (PE), polypropylene (PP) and also poly(lactic acid) (PLA) and poly(3-
414 hydroxybutyrate) (PHB) biodegradation (Jeszeová et al., 2018; Sangeetha Devi et al., 2019; Takaku et
415 al., 2006; Wróbel et al., 2023).

416 HTS of 16S rRNA gene allowed the identification of a high diversity of bacteria, many of which
417 were not isolated by using cultured-based approaches. The results showed that certain bacterial taxa,
418 including species from the genus *Bacillus, Sphingomonas, Nocardioides, Solirubrobacter, Nitro-*
419 *sospira,*and *Paenibacillus*, were consistently present on the plasticplane of all plastic samples recov-
420 ered from the different fields. This analysis identified these taxa as core genera associated with the
421 plastic surface of LDPE-based plastic mulches. *Bacillus*, for instance, emerged as one of the most
422 dominant genera in the core plasticplane. The literature indicates that this genus is considered a prom-
423 inent bacterial taxon involved in plastic biodegradation (Priya et al., 2022). Specific *Bacillus* species
424 have been evaluated for their plastic degradation abilities, including LDPE, in different studies. For
425 instance, Yao et al. (2022) observed a 3.49% and 2.82% weight loss of LDPE films after exposure to
426 different *Bacillus* strains, after 30 days of incubation. Moreover, *Nocardioide* is a taxon characterized
427 by its ability to thrive under low-nutrient conditions, being able to degrade a range of pollutants as a
428 source of carbon and nitrogen (Ma et al., 2023; Zhao et al., 2023). *Nitrospira* spp. have also been
429 recently isolated and assessed as potential degrader of various plastic materials, including LDPE, while
430 *Paenibacillus* and *Sphingomonas* have also been previously characterized as key taxa of the plas-
431 tisphere-associated microbiome (Bardají et al., 2019; Di Pippo et al., 2020; P. Wang et al., 2023; Wu
432 et al., 2022).

433 It should be remarked that, in this study, assessments were conducted using commercial samples
434 of environmental relevance collected from the natural environment, offering a more realistic perspec-
435 tive compared to laboratory incubation experiments. Additionally, all the assessed plastic samples were
436 characterized as polyethylene. Further studies should also be conducted considering a wider range of
437 plastic types (e.g., biodegradable plastic mulches), and in a more diverse range of agricultural fields.
438 Biodegradable plastic mulch, for instance, is starting to be widely adopted in the field as an alternative
439 to conventional LDPE plastic and it could play a more prominent role in shaping the microbial com-
440 munity by selecting specific taxa able to metabolize it and thus having a greater interference in the soil
441 microbial communities (Zhang et al., 2024). Bandopadhyay et al. (2020), for instance, showed that

some bacterial genera such as *Methylobacterium, Arthrobacter*, and *Sphingomonas* were enriched on biodegradable plastic mulch in comparison to conventional LDPE mulches.

5. Conclusion

This study has significantly contributed to expanding our understanding of the plastic-associated microbial communities in agricultural systems. It stands by providing a detailed methodology to disentangle the microbial communities at the soil-plastic interface, particularly at a small spatial scale, through the distinct assessment of the 'plasticplane' and 'plastisphere' microbiomes.

This research focused on developing a methodology to unravel and compare the assemblage of the plasticplane and plastisphere-soil microbiome, emphasizing taxonomic profiling, with less attention given to functional characterization. Nevertheless, this methodology could be applied to develop further research focusing on functional approaches, which can offer valuable insights into microbial mechanisms, metabolic activities, and enzymatic functions associated with plastic biodegradation.

Our findings reveal that the plastic surface can host taxa that are consistently present on the plasticplane across different field sites. This suggests the existence of a core microbial community with a strong affinity for plastic surfaces, regardless of the specific field site. Moreover, it has been shown that geographical constraints can play a significant role in shaping both the microbial community of the soil and therefore that of the plastic interacting with it. Additives can also influence the associated microbial community, prompting the need for further detailed evaluations.

Finally, this study serves as a foundational basis for future research, particularly in the characterization of potential microbial degraders. Thus, some key bacterial genera closely associated with the plastic debris identified in this study included *Bacillus, Nocardioides, Solirubrobacter,* and *Sphingomonas*. These bacterial species could be pivotal in assessing their ability to degrade not only LDPE plastics but also various other polymer-based materials, thereby contributing to the global effort to tackle plastic pollution.

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Response to Reviewers

Title: Unravelling the plastisphere-soil and plasticplane microbiome of plastic mulch residues in agricultural soils

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Journal: Applied Soil Ecology

Manuscript Number: APSOIL-D-24-02313

We would like to thank the editor and reviewers for their constructive comments and suggestions on our manuscript. We have carefully considered the reviewers' feedback and made all necessary revisions to address their concerns. Both a clean and a tracked version of the revised manuscript are submitted for your consideration. Please take into consideration that the line numbers of the changes mentioned in this document refer to the tracked version of the revised manuscript.

1) Reviewer #1: *In this manuscript, the authors separated the plastisphere-soil and plasticplane samples using two-steps washing methods. They found that the plasticplane microbiome were less diverse than the plastisphere-soil communities, and distinct communities were observed. This topic is important. Previous studies have declared that the soil plastisphere should include the microbiome on the microplastics and the soil influenced by microplastics. One important question is the distance that microplastics can influence. Although this study did not answer this question, they still give some novelty points. This study can be published after revision.*

1. The figures in this manuscript are not clear enough and need to be improved for clarity and readability.

ANSWER: DONE

All figures have been updated following the required format and size.

2. Line 21: The full name of "NGS approaches" should be written in the part of abstract.

ANSWER: DONE

We have changed it to high-throughput sequencing as it is explained in the introduction, and the full name was written (Line 22).

3. Some paragraphs in this manuscript have a different indentation distance at the beginning of the paragraph, so authors are supposed to pay attention to the standardization of the formatting.

ANSWER: DONE

Standardization of the formatting was updated for each of the paragraphs needed, as well as the headings.

4. Line 51: In the second paragraph of introduction, please add references behind the "… of soil-borne pathogens".

ANSWER: DONE

References were added to the paragraph as recommended (Line 52).

5. Lines 112-114: In the concluding sentence of the introduction, the description of the research implications of the paper is too macro.

ANSWER: DONE

The introduction has been restructured and rewritten with the mentioned paragraph streamlined by removing details about less central objectives and methodologies. The research implications are briefly summarized and emphasized (Lines 123-124; 123-132).

6. In the section of 2.3, would the first suspension contain bacteria from the surface of the plastic? Did this lead to an increase in the diversity of plastisphere-soil bacteria?

ANSWER: DONE

The first suspension was obtained after softly shaking them for just two minutes at 250 rpm using a rotatory shaker which is a method to suspend soil particles without disturbing microbial cells that are more tightly attached to surfaces such as plastic debris. Typically, ultrasonic treatments and vigorously shaking are needed to detach microbial cells/biofilms from surfaces. We have explained this methodology better in section 2.3 and also added a reference in which we described that we have tried to adapt the conventional protocols developed to isolate and differentiate rhizosphere and rhizoplane bacteria from roots (Lines 171-172, 176-177).

7. Line 177: Please provide the specific composition of the "R2A agar plates" medium in the supplementary material.

ANSWER: DONE

The specific composition of the R2A media was added. (Lines 196-199).

8. Please harmonize "ml" to "mL", "minute" to "min" throughout the manuscript.

ANSWER: DONE

(Lines175,176,178,179,184,185,186,188)

9. Line 194: "7 min at 50 pulses s−1" should be "7 min at 50 pulses s−1".

ANSWER: DONE

Superscript was updated (Line 216).

10. Lines 289-297: The figure of the section 3.2 placed in the supplementary material may lead to a lack of persuasion. Please add more content as necessary in this paragraph.

ANSWER: DONE

A more detailed description of the figure, emphasizing its importance, has been included. (Lines 323-328)

11. Line 346: "Class_ASV511" can not be found in the figure 6.

ANSWER: DONE

The figure referring to this "Class_ASV511" and *Skermanella* were indeed missing, and it has been added to the Supplementary Material (Figure S5) and cited in the main text (Line 376).

12. Lines 369-373: "This study addresses this gap by developing a methodology for ... at a small spatial scale" , the authors are supposed to revise this sentence for clarity.

ANSWER: DONE

The sentence has been reviewed and changes have been made in the paragraph for clarity and avoiding repetition. (Lines 396,398,399,402).

13. Line 452: " It should be remark that" should be " It should be remarked that".

ANSWER: DONE

Line 452 – Couldn't find this sentence in the paragraph, probably was referring to the phrase "It should be taken into account that" – and it was updated to "It should be remarked that" (Line 481).

<u>2) Reviewer #2:</u> *The manuscript titled "Unravelling the plastisphere-soil and plasticplane microbiome of plastic mulch residues in agricultural soils" was reviewed. In general, the manuscript described a novel way to differentiate the microbial communities associated with soil near plastic and adhere to plastic surface, i.e. plastisphere-soil and plasticplane. This manuscript is well written generally, and I have few suggestions to discuss with the authors.*

1. It's indeed a pioneering work to distinct different compartments in such fine scale, but the importance to do so is vaguely stated. Is it possible to clearly explain why it's crucial to differentiate microbial communities attached to plastisphere-soil and plasticplane in only one or several sentence?

ANSWER: DONE

Plastic debris are anthropogenic physical particles associated with a range of chemical compounds, which are completely distinct from natural soil particles. Given the persistent nature of plastic contaminants and their increasing accumulation in the environment, assessing and comparing the microbial communities associated with plastic particles to those living on natural soil particles is of significant interest.

Most previous research has focused on evaluating the microbial communities colonizing plastic surfaces in comparison to bulk soil. However, it is crucial to consider the extreme heterogeneity of soils. Most studies on the plastisphere have come from aquatic environments, which are more homogeneous, making it reasonable to compare microbial communities attached to plastic surfaces with those in bulk, non-influenced regions. In contrast, soil ecosystems are highly heterogeneous, and observed differences in microbial communities may simply be a result of natural spatial constraints.

Thus, as emphasized in previous review by Rillig et al., 2023, there is still a lack of information on plastisphere microbial communities at the microscale habitat and in which the compartmentalized and heterogeneous nature of soil should not be ignored. This highlights the importance of this research, which assesses and compares at a finer spatial scale the microbial communities on plastic surfaces and on the attached- soil.

To emphasize the points explained above in our article, some of these comments were added in the Introduction section (Lines 69-72;83-84;86-90;97-101)

2. The Introduction part need to be reorganized. It's too long and the key points were not clear.

ANSWER: DONE.

The introduction has been reorganized and rewritten to emphasize the key points and make it more concise.

3. Please check again typos and spelling errors. For instance, in Line 194, -1 should be given in superscript.

ANSWER: DONE

All typos and spelling errors have been double checked/corrected. Correction of -1 in superscript has also been done (Line 216).

Revised manuscript (with changes marked) Click here to view linked References

Unravelling the plastisphere-soil and plasticplane microbiome of plastic mulch residues in agricultural soils

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Abstract

Advances in molecular techniques have recently uncovered microbial communities associated with plastic debris. The term "plastisphere," was originally used for microbial communities on marine plastic. In terrestrial systems, this term has been extended to the plastic-soil interface, encompassing microbes from the surrounding soil. Although some studies have revealed differences in microbial composition and diversity between plastisphere and bulk soil, high-resolution spatial analyses of microbial communities on the immediate plastic surface (plasticplane) and in the attached soil (plastisphere-soil), are still lacking. In this study, a methodology was developed to disentangle the bacterial populations associated with the plastisphere-soil of weathered plastic mulch from agricultural fields from those on the plasticplane by using culture-based and High-Throughput sequencing ~~Next-Generation Sequencing~~ approaches. A significantly higher number of colony-forming units were cultured from the plastisphere-soil compared to the plasticplane. Main genera isolated from the plasticplane by culturing included *Arthrobacter, Pseudarthrobacter, Priestia, Massilia, Microbacterium, Bacillus*, and *Kocuria* genera, some of which are known plastic-degraders. High-throughput sequencing analysis revealed higher bacterial richness in plastisphere-soil, while beta diversity showed main significant differences among field plots. Core taxa significantly associated to the plasticplane included *Bacillus*, *Sphingo-*

*mona*s, *Nocardioides,* and *Solirubrobacter*. This study provides a pioneering description of a methodology to differentially analyze microbial communities at different soil-plastic interfaces, particularly on a small spatial scale using samples from plastic mulch residues in agricultural soils. This methodology may lay a foundation for future research to isolate and identify microbial plastic degraders, contributing to efforts against mitigating plastic pollution.

Keywords: *agricultural fields; core bacteria; environmental pollution; microbial diversity; plastisphere; soil health.*

1. Introduction

The use of plastic mulch plays an important role in crop production by improving water-use efficiency and soil temperature, decreasing weed pressure, and providing higher yields and earlier harvests. However, associated drawbacks, including its disposal and environmental pollution, have gained significant attention in recent years (FAO, 2021; Zhang et al., 2020). The process of removing plastic mulch from the soil is considered a time-consuming and laborious activity. Frequently, the complete retrieval of this plastic is not achievable, leading to a significant accumulation of plastic debris in the soils that can be further fragmented, resulting in one of the major sources of microplastic (particles smaller than 5 mm) pollution in agricultural soils (Bläsing & Amelung, 2018; FAO, 2021; Huang et al., 2020; van Schothorst et al., 2021). Available studies have already highlighted notable levels of macro- and microplastic pollution in agricultural fields continuously exposed to plastic mulch worldwide. This reveals a pervasive and persistent issue, raising concerns about the potential effects on soil health and ecosystem services, and consequently, it emphasizes the need for further research in this field (Huang et al.,2020; Meng et al., 2023; Van Schothorst et al.,2021; Ya et al., 2022).

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Microbial communities are crucial for ecosystem functionality, with soil microorganisms playing key roles in nutrient cycling, organic matter decomposition, soil structure maintenance, and regulation of soil-borne pathogens (Garbeva et al., 2004; Saccá et al., 2017). ~~Consequently, these microorganisms are vital for crop production. In this context, understanding the composition of microbial communities at the plastic-soil interface is of significant relevance from both microbial ecology and agricultural perspectives (Yu et al., 2021).~~

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Current molecular techniques including ~~high~~High-~~throughput~~ Throughput ~~sequencing~~ Sequencing ~~technologies~~ (HTS) combined with advanced microscopy have recently unveiled the occurrence of

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diverse microbial communities associated with different plastic debris from different environments (Amaral-Zettler et al., 2020; Luo et al., 2023; Schlundt et al., 2020; Vethaak & Leslie, 2016; Zettler et al., 2013). Zettler et al. (2013) first introduced the term 'plastisphere' to describe the unique microbial community colonizing plastic marine debris. However, the application of this term to terrestrial environments necessitates careful consideration to avoid potential confusion or misinterpretation. While the term is sometimes used in strict adherence to its original definition, referring specifically to the microbial communities closely associated with plastic surfaces, in other instances, it is applied more broadly, akin to the concept of the 'rhizosphere'. This broader usage encompasses the entire soil-plastic interface, thus including both microbes attached to the plastic and those in the interacting soil (Rüthi et al., 2020). This expanded interpretation serves to highlight the complex interactions within the plastisphere in terrestrial ecosystems.

Given the persistent nature of plastic contaminants and their increasing accumulation in the environment, assessing and comparing the microbial communities associated with plastic particles to those living on natural soil particles is of significant interest. However, it must be taken into consideration the fact that plastic ~~particles~~ are anthropogenic materials, chemically and physically distinct from natural soil particles (De Souza Machado et al., 2018). Thus, the microbial community residing on the plastic surface can differentiate from the one found in the attached soil. In this context, our study aims to develop a methodology that ~~may~~ allows to distinguish the main compartments that microbes can occupy in terrestrial agroecosystems polluted with plastics. Therefore, we propose the adoption of the term 'plastisphere-soil' to describe the soil zone directly influenced by plastic. Additionally, we suggest using 'plasticplane' as a more specific term to describe the surface of the plastic where specific microbial colonization can also occur akin to the concept of "rhizoplane" terminology, broadly adopted in soil microbiology studies (Foster, 1986; Y. Li et al., 2023; Rashid Mwajita et al., 2013; Wieland et al., 2001).

~~Bulk soil constitutes a distinct ecological niche that, as demonstrated by several studies, can host microbial communities with~~ Most previous research has focused on evaluating the microbial communities colonizing plastic surfaces in comparison to bulk soil, reporting notable differences in composition, diversity, and richness compared to those associated with plastic debris (Gkoutselis et al., 2021; Yu et al., 2021). However, it is crucial to consider the extreme heterogeneity of soils. Most studies on the plastisphere have come from aquatic environments, which are more homogeneous, making it rea-

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88 sonable to compare microbial communities attached to plastic surfaces with those in bulk, non-influ-
89 enced regions. In contrast, soil ecosystems are highly heterogeneous, and observed differences in mi-
90 crobial communities may simply be attributed to natural spatial constraints.

91 ~~-it has been suggested that these differences may be primarily attributed to spatial constraints re-~~
92 ~~sulting from sampling at relatively large spatial scales. This observation underscores the need for high-~~
93 ~~resolution spatial analyses to accurately compare microbial communities across adjacent microenvi-~~
94 ~~ronments, such as the 'plasticplane' (the immediate plastic surface) and 'plastisphere soil'. These spe-~~
95 ~~cific compartments, particularly in their relation to plastic-associated microbial communities, remain~~
96 ~~underexplored (Rillig et al., 2023).~~

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97 As emphasized by Rillig et al. (2023), there is still a lack of information on plastisphere microbial
98 communities at the microscale, where the compartmentalized and heterogeneous nature of soil must
99 not be overlooked. This highlights the importance of this research, which isolated~~assesses~~ and com-
100 pared~~s~~ microbial communities across adjacent plastic-associated microenvironments at a small spatial
101 scale, using both culture-dependent and culture-independent approaches.

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102 ~~Furthermore, the existence of a 'core' plastisphere community, defined as a set of microbial taxa~~
103 ~~consistently associated with terrestrial plastic debris, remains uncertain being a topic that still needs to~~
104 ~~be further studied (Rillig et al., 2023; C. Wang et al., 2022). Identifying these taxa could facilitate the~~
105 ~~selection of keystone-associated microorganisms that could promote plastic degradation. Agostini et~~
106 ~~al. (2021) for instance, identified a core microbiome in an aquatic environment, composed of specific~~
107 ~~taxa from microbial families such as Oleiphilaceae, Rhizobiaceae, and Hyphomonadaceae, which were~~
108 ~~characterized by their hydrocarbon metabolizing capabilities, enabling them to colonize the plas-~~
109 ~~tisphere effectively.~~

110 ~~The interaction between plastic and microorganisms can be explained by different mechanisms.~~
111 ~~In certain scenarios, plastic represents a suitable surface for microbial attachment where microorgan-~~
112 ~~isms keep retrieving the nutrients needed from the surrounding soil. Alternatively, plastic can act as a~~
113 ~~habitat and substrate for microbial growth by also providing a suitable carbon source (Puglisi et al.,~~
114 ~~2019; M. Zhang et al., 2019). However, additional clarification is required regarding the different phys-~~
115 ~~icochemical aspects of plastics that might selectively harbor certain microorganisms in contrast to other~~
116 ~~natural particles found in the soil environment. It is crucial to consider that plastics are not purely~~

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~~composed of polymers but consist of a base polymer and various additives, i.e., plasticizers, antioxidants, light stabilizers, flame retardants, and dyes that may also have an impact on the microbial community (Puglisi et al., 2019; Rillig et al., 2023).~~

~~Our study aimed to characterize the microbial communities present at the soil-plastic interface and to identify the occurrence of a 'core' plastisphere community, using both culture-dependent and culture-independent approaches.~~

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Plastic mulch debris was collected from agricultural fields, providing a more realistic perspective compared to traditional laboratory experiments.~~and a~~ A methodology was developed to differentiate the recovery of the microbial communities associated with the soil adhering to the plastic (referred to as plastisphere-soil) and those closely associated with the plastic surface itself (termed plasticplane). ~~Additionally, plastic properties were assessed using vibrational spectroscopies including Fourier transformed infrared (FTIR) and Raman spectroscopies to characterize the plastics sampled under study and to evaluate how plastic composition might influence the shaping of microbial communities.~~ This study has also identified core microbial taxa consistently associated with plastic mulch debris, as well as isolated bacteria species that could be further evaluated for their potential to degrade polymer-based materials ~~Our results may also contribute to the identification and isolation of potential plastic-degrading microorganisms associated with plastics, thereby supporting~~ contributing to global efforts to mitigate plastic pollution ~~worldwide~~.

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2. ~~2.~~ Material and methods

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2.1 Study site and field sampling

Weathered plastic mulch debris was collected from five agricultural fields located at Baza (Granada), southern Spain (Figure S1). The fields were characterized by intensive horticultural production and historical use of plastic at least twice a year over the last ten years. At each sampling site, a total of ten weathered plastic mulch debris samples measuring from 5-10 x 5-10 cm, were randomly collected from the topsoil. Samples were gently shaken to remove the loosely attached soil particles, placed in sampling bags, and stored at 4ºC until further analysis.

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2.2 Plastic characterization by Raman and Fourier infrared (FTIR) spectroscopy

Prior to chemical characterization, plastic films were thoroughly washed with warm water (40ºC), followed by a 20-min ultrasonication in deionized water. Subsequently, the films were rinsed and wiped using cotton wool in distilled water and 70% isopropanol to remove residual soil and potential bio-contaminants.

The chemical characterization of the plastic film surfaces was performed using Raman and FTIR. FTIR is especially sensitive to observing oxidative products resulting from weathering, while Raman spectra can provide insights into the polymer backbone, crystallinity, and the presence of inorganic additives undetectable by FTIR.

Raman analysis was performed using a Renishaw InVia microspectrometer (Renishaw plc, New Mills, Wotton under Edge, UK) equipped with a 532 nm laser (15 mW at the sample) and a 50x objective lens (numerical aperture NA 0.50). Raman spectra were collected in the 100-3500 cm^{-1} range using a 2400 lines per millimeter grating, resulting in a spectral resolution of approximately 1 cm^{-1}. All measurements were conducted with 5 accumulations at 5% laser power. After collection, each spectrum underwent cosmic ray removal (Wire 4.2), baseline correction, and normalization at the 2851 cm^{-1} in Spectragryph version 1.2.15. Given the heterogeneous nature of the plastic surface, spectra were collected at ten different positions on each plastic sample. The fraction of trans (α_t) and amorphous (α_a) conformers were calculated using I_{1298} and I_{1305} intensities of the Raman bands at 1298 and 1305 cm^{-1}, respectively according to Hiejima et al.(2018).

Alongside Raman spectroscopy, a Cary 630 FTIR (Agilent Technologies Inc., Danbury, CT, USA) with an attenuated total reflection (ATR accessory, diamond substrate) was used to identify plastics and potential weathering effects on the polymer surface functional groups (penetration depth~2 µm at 1700 cm^{-1}). FTIR spectra were gathered in the 650–4000 cm^{-1} range, with 64 accumulated scans and a 2 cm^{-1} spectral resolution. After collection, each spectrum underwent baseline correction and normalization at the 2912 cm^{-1} peak in Spectragryph.

2.3 Assessment of microbial communities associated with plastic mulch debris

To assess and distinguish the microbial community of the plastisphere-soil (PPh) from the one tightly attached to the plastic surface (plasticplane (PPl)) the collected plastic samples were subjected to sequential washing steps (Figure S2) by using a modified protocol mimicking those developed to sample and isolate rhizosphere and rhizoplane bacteria (Barillot et al., 2013).

Plastic mulch samples collected from each field were cut into small pieces (2 x 1.5 cm) with sterile scissors and pooled into a single composite sample. A total of 50 mg of composite plastic samples was placed in a falcon tube containing 10 mL of sterile distilled water. Collectively, three replicates of plastic samples were processed per field plot. Falcon tubes were softly shaken for two min at 250 rpm in a horizontal rotatory shaker, obtaining a first suspension that contained the attached soil fraction (plastisphere-soil). From this soil suspension, 1-mL aliquots were taken for isolation of cultivable bacteria whereas 1.5-mL aliquots were taken for DNA extraction. Specifically, the 1.5-mL aliquots were centrifuged at 14,000 rpm for 1 min. Then, the supernatant was discarded, and this process was repeated six times to recover a soil pellet that was stored at -20°C until DNA extraction.

On the other hand, the washed plastic pieces from the first suspension were subjected to a second intermediate washing step (Figure S2). Plastic debris was recovered and transferred to another falcon tube that was shaken for 2 min with 20 mL of sterile water. Then, the washed plastic mulch films (plasticplane) were transferred to a new 15-mL tube with 5 mL of sterile water, and it was sonicated for 5 min and vigorously vortexed for 2 min. A 1-mL aliquot of the resulting suspension, containing bacteria from the plasticplane, was taken for isolation of cultivable bacteria (see below), whereas the washed plastics were transferred to a 2-mL tube and kept at -20 °C until DNA extraction.

Additionally, samples from the plastic surfaces at each consecutive washing step were observed under a stereomicroscope (Leica, M165C, Leica Microsystems, Germany) to assess the efficacy of the washing process in removing soil particles attached to the plastic surfaces, and the same washed pieces were subjected to SEM-EDX analysis described below to corroborate the efficacy of the different washing steps to differentiate plastisphere-soil and plasticplane interfaces.

2.3.1 Isolation and characterization of culturable bacteria

Aliquots of plastisphere-soil and plasticplane suspensions were subjected to serial dilutions and 100-µl aliquots were plated in triplicate on R2A (Difco, Detroit, MI, USA) agar plates which contains yeast extract (0.5 g L^{-1}), proteose peptone No. 3 (0.5 g L^{-1}), casamino acids (0.5 g L^{-1}), dextrose (0.5 g L^{-1}), soluble starch (0.5 g L^{-1}), sodium pyruvate (0.3 g L^{-1}), dipotassium phosphate (0.3 g L^{-1}), magnesium sulfate (0.05 g L^{-1}), and agar (15 g L^{-1}), with a final pH of 7.2.

The number of colonies forming units (CFU) was assessed after a 2-day incubation period at 28ºC. Subsequently, a representative number of distinct colonies from the plasticplane were selected based

202 on morphology (color, shape, margin, and texture). Then, selected colonies were isolated and purified
203 (three cloning steps) and kept in 40 % glycerol stocks at -80ºC.

204 For bacteria identification, DNA was extracted using the DNeasy kit (QIAGEN, Madrid, Spain).
205 The near-complete 16S rDNA gene was amplified using 8f (5′-AGAGTTTGATCCTGGCTCAG-3′)
206 and 1492r (5′-ACGGCTACCTTGTTACGACTT-3′) primers (Weisburg et al., 1991) following the
207 protocol outlined in Anguita-Maeso et al. (2022). Amplicons were sequenced by Sanger sequencing
208 with the same primers used for the PCR at STABvida sequencing facilities (Caparica, Portugal). Se-
209 quences were assembled and manually corrected using DNASTAR software version 15.3.0.66 (Madi-
210 son, WI, USA). The identification of isolates to the genus/species level was carried out by comparing
211 their sequences with reference 16S rRNA gene sequences in the GenBank "nt" database using the
212 BLAST algorithm as described by Altschul et al. (1997).

213 **2.3.2 DNA Extraction and 16S rRNA Gene Amplicon Library Preparation**

214 DNA from plastisphere-soil and plasticplane samples was extracted using the DNeasy PowerSoil
215 Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions with small modifications.
216 Briefly, samples were homogenized with the lysis buffer for 7 min at 50 pulses s^{-1} with the Tissuelyser
217 LT (QIAGEN) and then were incubated for 1 h at 60 °C to increase cell lysis. DNA was eluted in a
218 final volume of 50 µL of sterilized distilled water and its purity was determined using a NanoDrop®156
219 ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). This DNA
220 was used as a template for amplicon library preparation as described previously in Anguita-Maeso et
221 al., (2022). Shortly, the V5-V6 region of the bacterial 16S rRNA gene was amplified with the primers
222 799F (5'- AACMGGATTAGATACCCKG-3') and 1115R (5'-AGGGTTGCGCTCGTTG-3'). Bar-
223 codes indexes were added to the amplicons using Fluidigm barcodes (Access Array Barcode Library
224 for Illumina® Sequencers kit). Next, barcoded PCR products were purified by using Agencourt AM-
225 Pure XP (Beckman Coulter Inc., Brea, CA, USA), following the manufacturer's instructions. Purified
226 PCR products were quantified by using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo
227 Fisher Scientific) and a TECAN SAFIRE microplate reader (Tecan Group, Männedorf, Switzerland).
228 Equimolecular amounts from each individual sample were added to a single tube; the pooled library
229 was quantified by using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), and was purified again
230 if primer dimers were still evident. Finally, the library was sequenced on the Illumina MiSeq platform
231 (V2; PE 2× 250 bp) at the Genomics Unit at the Madrid Science Park Foundation, Madrid, Spain. The

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ZymoBIOMICS microbial standard (Zymo Research Corp., Irvine, CA, USA) and water (no template DNA) were used as internal positive and negative controls, respectively, for library construction and sequencing.

2.4 Scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX) of Plastic Surfaces

Scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX) was utilized to evaluate the plastic surface morphology, determine the elemental composition of the plastic, and assess the efficacy of the washing steps in removing soil particles adhered to the plastic. Distinctly colored plastic mulch samples (black and light grey) were specifically chosen from each sampling site. These samples underwent fixation with 2% glutaraldehyde, dehydration through an ethanol series, and gold coating. The scanning electron microscopy analysis was conducted using a JEOL JSM 7800 F scanning microscope (JEOL Ltd., Peabody, MA, USA) equipped with energy-dispersive X-ray spectrometer (EDX) at Central Service of Research Support (SCAI) of the University of Cordoba, Spain.

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2.5 Statistical and Bioinformatics Analysis

Statistical analysis of culturable bacteria was performed using R statistical software (R Core Team, 2013). The significant effect of field location and soil-plastic habitat was tested using a factorial analysis of variance (ANOVA) followed by Tukey's multiple comparison test ($P < 0.05$). The Shapiro-Wilk test was used to determine whether the data were normally distributed whereas the homogeneity of variance was tested using Levene's test. Data from bacteria enumeration were log-transformed to fulfill the assumptions of homogeneity and normality.

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TrimGalore v.0.6.6 tool was employed for quality control and adapter trimming of the demultiplexed raw fastq files. In this process, the first 10 bp~~pb~~ of all reads were trimmed, and a truncation length of 240 base pairs for forward bacterial reads and 200 base pairs for reverse bacterial reads was applied to achieve a satisfactory Phred quality score (Q > 30). Subsequently, the high-quality reads underwent analysis using the DADA2 method to identify the amplicon sequence variants (ASVs) present in the samples (Callahan et al., 2016). Taxonomic classification was carried out using the Silva SSU v.138 database, and singleton ASVs were excluded from taxonomy assignment and statistical analysis. Differences in bacterial communities were assessed using alpha-diversity indexes (Richness and Shannon) at the ASV level. The non-parametric Scheirer–Ray–Hare test ($P < 0.05$), implemented

with the rcompanion v.2.4.1 package in R (Mangiafico, 2022), was employed to evaluate the effects of the sampled field and habitat (plastisphere-soil or plasticplane) and their interaction on alpha-diversity indexes. Beta-diversity was determined through principal coordinate analysis (PCoA) of weighted UniFrac distance matrices. Additionally, the Permutational multivariate analysis of variance using distance matrices (ADONIS function) from the vegan package in R (with 999 permutations) was employed to test the effects ($P < 0.05$) of the sampled plot, habitat, and their interaction. To achieve parity in the total number of counts between samples, alpha- and beta-diversity analyses were performed after resampling the abundance values to the minimum number of reads observed. Moreover, a linear discriminant analysis effect size (LEfSe) (Segata et al., 2011), based on the microbiomeMarker package in R (Cao et al., 2022), was employed to identify differences in microbiota composition at the genus level among the various treatments ($P < 0.05$).

3. Results

3.1 Plastic characterization

The most representative spectra, showcasing the overall plastic composition and the influences of weathering is shown in Figure 1. Both Raman and FTIR analyses confirmed that the plastic mulch collected from the five fields was composed of polyethylene (Table S1).

Raman spectra analysis of the plastic samples displayed distinct peaks corresponding to the native bonds in the LDPE polymer, found in the C-C stretching (1040–1200 cm^{-1}), -CH_2- twisting and bending (1300–1500 cm^{-1}), and C-H stretching (2800-3000 cm^{-1}) regions (Fig. 1). The latter is sensitive to subtle structural changes due to intermolecular interactions and crystallinity. The peaks at 2882 and 2851 cm^{-1} indicated the presence of LDPE's amorphous and crystalline regions, respectively. Plastic samples from Field 1 had a significant decrease in the intensity of the 2882 cm^{-1} peak relative to the 2851 cm^{-1} peak, compared to the plastic samples obtained from other fields, indicating its lower crystallinity.

Additional Raman signals linked to LDPE's crystallinity appeared at peaks 1305 and 1298 cm^{-1}, associated with the CH_2 twisting modes of the amorphous and trans (consecutive trans chains) conformers, respectively (Meier, 2002). The highest amorphous fraction was found in the plastic samples from Field 1, being 45.39 ± 0.03%, while the fractions obtained from Fields 3, 4, and 5 were 36.4 ±

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0.1%, 33.19 ± 0.06%, and 34.28 ± 0.06% respectively. Field 2 was excluded from the calculations due to a broad additive band at 1360 cm^{-1}. Often, plastic degradation, especially due to photodegradation, results in increased crystallinity (decreased amorphous phase), leading to material embrittlement and fragmentation.

The lower Raman spectral region (100-600 cm^{-1}) indicated the presence of inorganic compounds. In this case, the presence of titanium oxide (TiO_2) and the blue pigment Lazurite-a sodium silico aluminate in a sulfur matrix were confirmed in plastic samples from Field 2 (Figure S3 A). This was evidenced by the presence of bands corresponding to anatase TiO_2 (143 cm^{-1}) and rutile TiO_2 (233, 442, 610 cm^{-1}), and the characteristic bands of Lazurite (257, 547, 805, 1094, 1644, and 2183 cm^{-1}, (Figure S3 B). These findings align with the SEM-EDX data, which also revealed the presence of titanium (0.12%wt), silicon (2.76%wt), calcium (1.87%wt), sulfur (0.15%wt), and aluminum (1.01%wt) in plastic samples from Field 2, while no sulfur and titanium were detected in plastics from other fields (Table S2).

Interestingly, plastic samples from Fields 1, 3, and 4 exhibited a noticeable presence of carbonyl (C=O) groups in their FTIR spectra, potentially formed during plastic oxidation. Samples from Field 3 and Field 4 displayed unique peaks due to ketone carbonyl at 1717 cm^{-1}, while those from Field 1 showed a peak due to ester carbonyl at 1740 cm^{-1}. The carbonyl index, a common indicator of oxidation level, which is calculated as the ratio of peak intensity between 1717 (or 1740) cm^{-1} and 1465 cm^{-1}, was 0.13, 0.20, and 0.28 for samples from Fields 1, 3, and 4, respectively, indicating a higher concentration of carbonyl in plastic samples from Field 4.

3.2 Microscopic evaluation of plastic surface: scanning electron microscopy and optical microscope analysis

~~SEM and optical microscope image analysis (Figure S4) indicated that the sequential washing protocol that we designed to sample independently plastisphere soil and plasticplane-associated microorganisms effectively removed soil particles. The design of additional washing steps during the procedure was needed to eliminate the soil particles tightly attached to the plastic surface. By adding this step to our protocol, we could guarantee the distinction between the successive compartments of the plastisphere soil and plasticplane, allowing the subsequent accurate characterization of their respective microbial communities.~~Visual inspection using light images from a stereomicroscope (Figures S4 A and B) and SEM (Figure S4 C) was performed to assess the efficacy of the sequential washing steps in

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removing soil particles from the plastic surface and enabling independent sampling of plastisphere-soil and plasticplane-associated microorganisms. The image shows the condition of the plastic surface immediately after field sampling and after each successive washing steps described in Figure S2~~1~~.

Initial attempts revealed that ~~E~~even after two washing steps, some soil particles remained, which could lead to misleading results in the differential extraction ~~and identification~~ of microorganisms associated with the soil particles rather than the plastic surface. Therefore, an additional washing step was introduced to remove the soil particles more effectively (Figure S2~~1~~). This modification included it~~on~~ our protocol ensured a clear distinction between the plastisphere-soil and plastic-plane compartments, facilitating more accurate characterization of their respective microbial communities.

3.3 Characterization of the culturable bacterial population

Both plastic-debris compartments (plastisphere-soil and plasticplane) and location (field) factors significantly affected the population density of culturable bacteria ($P<0.001$) (Figure 2). The bacterial density found on the plastisphere-soil was significantly greater than that obtained on the plastic surface for all sampled fields. Thus, the bacterial density isolated from the plastisphere-soil fraction ranged from log 7.1 to log 7.7 CFU g^{-1} plastic, whereas the bacterial population on the plastic surface ranged from log 6.2 to 6.9 CFU g^{-1} plastic.

A total of 74 bacteria from the plasticplane were isolated, cultivated, and taxonomically identified by 16S rRNA gene sequencing. Isolated bacteria were assigned to four phyla, five classes, six orders, nine families, and 19 genera (Table S3A). The most abundant genera included *Arthrobacter* (16.2% of isolates) followed by *Priestia* (14.9% of isolates), *Pseudarthrobacter* (13.5% of isolates), *Massilia* and *Microbacterium* (9,5% each), *Bacillus* (8,1%) and *Kokuria* (6.8%) (Table S3B). On the other hand, less frequent bacteria included *Frondihabitans, Lysinibacillus, Nocardioides, Paenarthrobacter, Paenibacillus, Planococcus, Planomicrobium, Rossellomorea,* and *Rufibacter.* Plasticplane samples from Fields 3 and 5 showed a higher diversity of bacterial genera (8 and 10 respectively), whereas in those from the remaining field plots, a total of seven bacterial genera were identified.

3.4 Composition and diversity of bacterial communities in the plastisphere-soil and plasticplane

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Illumina sequencing yielded a total of 351,265 good-quality reads after removal of chimeras, unassigned, or mitochondrial reads. A total of 1,701 amplicon sequence variants (ASVs) were identified among all treatments, with 1,682 ASVs being retained for alpha- and beta-diversity analysis after rarefying all data to the minimum number of reads and singleton removal. The Scheirer–Ray–Hare test indicated significant differences ($P < 0.05$) for the Richness and Shannon alpha-diversity indices according to the field plot ($H = 19.63$, $P = 0.001$, and $H = 21.54$, $P = 0.001$, respectively) whereas plastic -associated compartments resulted significant for Richness ($H = 4.13$, $P = 0.04$), but not or Shannon ($H = 3.25$, $P = 0.07$) diversity. Furthermore, there was no significant interaction between the plastic fraction and field plot for both alpha-diversity indices ($H < 0.77$, $P > 0.94$) (Figure 3).

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Principal coordinate analysis of beta-diversity weighted UniFrac distances differentiated bacterial communities mainly according to the field location. Thus, there was a clear tendency to group bacterial communities according to the sampled field along Axis 1, which explained 49.9% of the variation (Figure 4). In fact, ADONIS analysis supported the results described above and indicated a significant main effect of the field plot ($R^2 = 0.79$, $P < 0.001$). However, the bacterial habitat (plastisphere-soil or plasticplane) resulted not significant ($R^2 = 0.01$, $P > 0.196$) nor their interaction ($R^2 = 0.03$, $P > 0.424$).

A total of 24 phyla, 60 classes, 173 orders, 321 families, and 723 genera of bacteria were taxonomically identified by illumine sequencing. Most abundant bacterial genera in all the plastisphere-soil samples belonged to *Bacillus* (23.3%), *Nocardioides* (11.7%), *Planomicrobium* (10.4), *Blastococcus* (10.1%) and *Streptomyces* (8.5%) genera, whereas those from plasticplane included *Blastococcus* (15.6%), *Sphingomonas* (10.7%), *Arthrobacter* (10.1%), *Bacillus* (9.3%) and *Hymenobacter* (7.4%). In Field 1 and 2, there was a prevalence of *Bacillu*s (41.3% and 51.8%, respectively), whereas, in Fields 3 and 4, *Blastococcus* was the dominant genus (18.9% and 30.7%, respectively) (Figure 5). When evaluations were made within each field, comparing the microbial community associated with the plasticplane with the one from the plastisphere-soil, there were no evident changes in the proportion of the most abundant genera.

In line with these results, Linear Discriminant Analysis Effect Size (LEfSe) analysis was used to identify key bacterial genera differentially associated with the plasticplane or plastisphere-soil fractions of plastic mulch debris sampled at different field locations. Globally, LEfSe identified a bacterial enrichment of *Skermanella* and Class_ASV511 enriched in the plastisphere-soil (Figure S5). However,

when applying LEfSe to each field independently, field plot F2 showed a higher number of differentially abundant genera. In particular, genera such as *Polycyclovorans*, *Sphingomonas,* and *Cellvibrio*, were enriched in the plasticplane, while genera such as *Arthrobacter, Planomicrobium*, and *Skermanella* were significantly more abundant in the plastisphere-soil than in the plasticplane (Figure 6).

3.5 Core community analysis

The Venn Diagram (Figure S6~~5~~) analysis revealed the shared and unique bacterial genera in the different plastic-associated habitats. Notably, in all evaluated fields, between 62.5% and 88.4% of the bacterial genera were shared between both habitats. Interestingly, on plastic debris recovered from field plots F3 and F5, approximately 13% of bacterial genera were exclusively found on the plasticplane, while in the other fields, these values were lower.

The core microbial taxa consistently found only in the plastisphere-soil of all evaluated samples recovered from the different fields were represented by 220 bacterial ASVs, while 79 ASVs represented the core taxa on the plasticplane (Figure 7). The majority of bacteria consistently found on the plasticplane belonged to the genera *Bacillus*, *Sphingomona*s, *Nocardioides,* and *Solirubrobacter*. A complete list identifying the core plasticplane bacterial community can be found in Supplementary Table S4.

4. ~~4.~~ Discussion

While some ~~reports~~ studies have compared the plastisphere microbiome with bulk soil, highlighting significant differences in their microbial community composition and structure, there is still a need for a more comprehensive understanding of microbial communities at a ~~small~~finer ~~-~~spatial definition within the plastic-soil interface (Bandopadhyay et al., 2020; Gkoutselis et al., 2021; Rillig et al., 2023; Rüthi et al., 2020). This study addresses this gap by developing a methodology to differentially ~~assess~~ sample and compare ~~for investigating the microbial communities associated with different compartments of the plastic interface, enabling to establish differences between the~~ the bacterial community composition and diversity of plastic surfaces (‘plasticplane)’ with those found on natural soil particles interacting with this anthropogenic material (‘plastisphere-soil)’ ~~at a small spatial scale.~~

Polyethylene polymers are commonly regarded as recalcitrant materials, known for their resistance to degradation (Brown et al., 2022; El-Sherif et al., 2022). However, research suggests that weathered

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405 polyethylene plastics, previously exposed to UV radiation, can be colonized and undergo partial me-
406 tabolism facilitated by specific microbial taxa capable of secreting enzymes such as esterase, lipases,
407 peroxidases, and oxidoreductases (El-Sherif et al., 2022; Sharma & Neelam, 2023). Consequently, it
408 was expected that, as observed, the microbial community richness on the surface of weathered poly-
409 ethylene might experience a reduction or selection of specific bacterial taxa compared to soil particles.
410 This difference can be attributed to the easily assimilated carbon sources provided by the associated
411 organic matter in soil particles, contrasting with the resistant nature of polyethylene, which is not con-
412 sidered a straightforward or efficient carbon source for most bacteria. Interestingly, these results are
413 similar to those described for the rhizosphere soil and rhizoplane-root interfaces, where the secretion
414 of root exudates favors the growth of specific bacteria that are better adapted to this environment,
415 leading to a decrease in overall bacterial richness (Philippot et al., 2013).

416 Furthermore, our results revealed similarities in the microbial communities' composition retrieved
417 according to the field of origin of the plastic debris. Thus, the tendency to group bacterial communities
418 according to the sampled field, and the observed similarities between Fields 3 and 4, which are geo-
419 graphically closer to each other, highlight the role of environmental and geographic constraints in shap-
420 ing the soil microbial community at the plastic-soil interface. Additionally, the plastic debris collected
421 from Field 2 exhibited a higher number of differentially abundant taxa on the plasticplane compared
422 to the plastisphere-soil. Interestingly, this plastic was unique in its color, appearing light grey instead
423 of the commonly found black plastic mulch of the remaining field sites. Analysis of the plastic's chem-
424 ical properties by SEM-EDX and Raman spectroscopy revealed the presence of titanium dioxide (TiO_2)
425 on this specific mulch film, a pigment commonly found in white plastics due to its high light scattering
426 efficiency, inertness, thermal stability, dispersibility, and cost-effectiveness (Puglisi et al., 2019;
427 Turner & Filella, 2023).

428 Some previous studies have demonstrated the effects of TiO_2 on soil bacterial communities (Ge et
429 al., 2011, 2012, 2013). Ge et al. (2012), for instance, found that the presence of this chemical compound
430 can significantly reduce certain bacterial genera, including *Actinoplanes, Balneimonas, Blastococcus,*
431 *Bradyrhizobium*, and *Skermanella.* The toxicity of this material to some bacteria can be related to ox-
432 idative damage to bacterial cell walls, leading to membrane disorganization and permeability. Our re-
433 sults also indicate a reduction in the *Blastococcus* genus on the plastic surface compared to the plas-
434 tisphere-soil. In contrast, the genera *Polycyclovarans* and *Cellvibrio* were enriched on the plasticplane
435 compared to the plasticphere. These findings suggest that plastic additives and dyes may influence the

assemblage of plastic-associated bacterial communities, underscoring the need for further research focusing on this topic.

Differing from most contaminants found in the soil, plastic debris represents a potential threat to the soil environment as it constitutes an external anthropogenic particle with a distinct shape, size, and volume compared to natural soil particles (Rillig et al., 2023). Additionally, it contains a series of additives, such as pigments, plasticizers, and antioxidants, which can leach over time and could interact with the microbial community (Macan et al., 2024). Furthermore, these materials can adsorb contaminants such as pesticides commonly applied in agricultural fields, as well as heavy metals (K. Li et al., 2023; Rillig et al., 2023). Consequently, all these factors may play a role in selecting specific taxa more adapted to colonize the plasticplane.

Isolating, cultivating, and identifying key species from the plasticplane is particularly noteworthy, as they present promising candidates for further assessments as plastic degraders. Both molecular and culturable methods revealed a prevalent presence of *Arthrobacter* genus in plastic-soil interface. Numerous reports in the literature highlight its significant role in degrading various carbon sources, emphasizing its active hydrolytic enzyme production and the ability to break down persistent conventional plastics (Gobbetti & Rizzello, 2014; Han et al., 2020). Furthermore, members of the *Priestia* genus were isolated and cultured from the majority of plasticplane samples. This genus is recognized as an environmental bacterium extensively used in biotechnology and bioremediation due to its ability to produce several enzymes (Dhaka et al., 2022; Shwed et al., 2021). Other culturable bacterial genera isolated and identified, such as *Bacillus, Terribacillus, Paenibacillus, and Kocuria*, have also been previously associated with plastics according to existing literature and could be further explored as key genera in plastic biodegradation assays (Anwar et al., 2016; Bardají et al., 2019; Harshvardhan & Jha, 2013; Vidal-Verdú et al., 2022). More specifically, *Priestia megaterium* and *Bacillus pumilus*, for instance, were some of the cultivated and identified species in this study which have also been previously isolated from the plastic debris and assessed for their biodegradation abilities on a range of polymer types such as polyethylene (PE), polypropylene (PP) and also poly(lactic acid) (PLA) and poly(3-hydroxybutyrate) (PHB) biodegradation (Jeszeová et al., 2018; Sangeetha Devi et al., 2019; Takaku et al., 2006; Wróbel et al., 2023).

HTS of 16S rRNA gene allowed the identification of a high diversity of bacteria, many of which were not isolated by using cultured-based approaches. The results showed that certain bacterial taxa,

including species from the genus *Bacillus, Sphingomonas, Nocardioides, Solirubrobacter, Nitrosospira,*and *Paenibacillus*, were consistently present on the plasticplane of all plastic samples recovered from the different fields. This analysis identified these taxa as core genera associated with the plastic surface of LDPE-based plastic mulches. *Bacillus*, for instance, emerged as one of the most dominant genera in the core plasticplane. The literature indicates that this genus is considered a prominent bacterial taxon involved in plastic biodegradation (Priya et al., 2022). Specific *Bacillus* species have been evaluated for their plastic degradation abilities, including LDPE, in different studies. For instance, Yao et al. (2022) observed a 3.49% and 2.82% weight loss of LDPE films after exposure to different *Bacillus* strains, after 30 days of incubation. Moreover, *Nocardioide* is a taxon characterized by its ability to thrive under low-nutrient conditions, being able to degrade a range of pollutants as a source of carbon and nitrogen (Ma et al., 2023; Zhao et al., 2023). *Nitrospira* spp. have also been recently isolated and assessed as potential degrader of various plastic materials, including LDPE, while *Paenibacillus* and *Sphingomonas* have also been previously characterized as key taxa of the plastisphere-associated microbiome (Bardají et al., 2019; Di Pippo et al., 2020; P. Wang et al., 2023; Wu et al., 2022).

It should be ~~taken into account~~ remarked that, in this study, assessments were conducted using commercial samples of environmental relevance collected from the natural environment, offering a more realistic perspective compared to laboratory incubation experiments. Additionally, all the assessed plastic samples were characterized as polyethylene. Further studies should also be conducted considering a wider range of plastic types (e.g., biodegradable plastic mulches), and in a more diverse range of agricultural fields. Biodegradable plastic mulch, for instance, is starting to be widely adopted in the field as an alternative to conventional LDPE plastic and it could play a more prominent role in shaping the microbial community by selecting specific taxa able to metabolize it~~,~~ and thus having a greater interference in the soil microbial communities (Zhang et al., 2024). Bandopadhyay et al. (2020), for instance, showed that some bacterial genera such as *Methylobacterium, Arthrobacter*, and *Sphingomonas* were enriched on biodegradable plastic mulch in comparison to conventional LDPE mulches.

5. ~~5.~~ Conclusion

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This study has significantly contributed to expanding our understanding of the plastic-associated microbial communities in agricultural systems. It stands by providing a detailed methodology to disentangle the microbial communities at the soil-plastic interface, particularly at a small spatial scale, through the distinct assessment of the 'plasticplane' and 'plastisphere' microbiomes.

This research focused on developing a methodology to unravel and compare the assemblage of the plasticplane and plastisphere-soil microbiome, emphasizing taxonomic profiling, with less attention given to functional characterization. Nevertheless, this methodology could be applied to develop further research focusing on functional approaches, which can offer valuable insights into microbial mechanisms, metabolic activities, and enzymatic functions associated with plastic biodegradation.

Our findings reveal that the plastic surface can host taxa that are consistently present on the plasticplane across different field sites. This suggests the existence of a core microbial community with a strong affinity for plastic surfaces, regardless of the specific field site. Moreover, it has been shown that geographical constraints can play a significant role in shaping both the microbial community of the soil and therefore that of the plastic interacting with it. Additives can also influence the associated microbial community, prompting the need for further detailed evaluations.

Finally, this study serves as a foundational basis for future research, particularly in the characterization of potential microbial degraders. Thus, some key bacterial genera closely associated with the plastic debris identified in this study included *Bacillus, Nocardioides, Solirubrobacter,* and *Sphingomonas*. These bacterial species could be pivotal in assessing their ability to degrade not only LDPE plastics but also various other polymer-based materials, thereby contributing to the global effort to tackle plastic pollution.

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528 **Crispin Halsall**: Writing – review & editing, Supervision, Project administration, Funding acquisi-
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530 quisition.
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Unravelling the plastisphere-soil and plasticplane microbiome of plastic mulch residues in agricultural soils

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Abstract

Advances in molecular techniques have recently uncovered microbial communities associated with plastic debris. The term "plastisphere," was originally used for microbial communities on marine plastic. In terrestrial systems, this term has been extended to the plastic-soil interface, encompassing microbes from the surrounding soil. Although some studies have revealed differences in microbial composition and diversity between plastisphere and bulk soil, high-resolution spatial analyses of microbial communities on the immediate plastic surface (plasticplane) and in the attached soil (plastisphere-soil), are still lacking. In this study, a methodology was developed to disentangle the bacterial populations associated with the plastisphere-soil of weathered plastic mulch from agricultural fields from those on the plasticplane by using culture-based and High-Throughput sequencing approaches. A significantly higher number of colony-forming units were cultured from the plastisphere-soil compared to the plasticplane. Main genera isolated from the plasticplane by culturing included *Arthrobacter, Pseudarthrobacter, Priestia, Massilia, Microbacterium, Bacillus*, and *Kocuria* genera, some of which are known plastic-degraders. High-throughput sequencing analysis revealed higher bacterial richness in plastisphere-soil, while beta diversity showed main significant differences among field plots. Core taxa significantly associated to the plasticplane included *Bacillus*, *Sphingomonas*, *Nocardioides,* and *Solirubrobacter*. This study provides a pioneering description of a methodology to differentially analyze

30 microbial communities at different soil-plastic interfaces, particularly on a small spatial scale using
31 samples from plastic mulch residues in agricultural soils. This methodology may lay a foundation for
32 future research to isolate and identify microbial plastic degraders, contributing to efforts against miti-
33 gating plastic pollution.

34 **Keywords:** *agricultural fields; core bacteria; environmental pollution; microbial diversity; plas-*
35 *tisphere; soil health.*

36 ## 1. Introduction

37 The use of plastic mulch plays an important role in crop production by improving water-use effi-
38 ciency and soil temperature, decreasing weed pressure, and providing higher yields and earlier harvests.
39 However, associated drawbacks, including its disposal and environmental pollution, have gained sig-
40 nificant attention in recent years (FAO, 2021; Zhang et al., 2020). The process of removing plastic
41 mulch from the soil is considered a time-consuming and laborious activity. Frequently, the complete
42 retrieval of this plastic is not achievable, leading to a significant accumulation of plastic debris in the
43 soils that can be further fragmented, resulting in one of the major sources of microplastic (particles
44 smaller than 5 mm) pollution in agricultural soils (Bläsing & Amelung, 2018; FAO, 2021; Huang et
45 al., 2020; van Schothorst et al., 2021). Available studies have already highlighted notable levels of
46 macro- and microplastic pollution in agricultural fields continuously exposed to plastic mulch world-
47 wide. This reveals a pervasive and persistent issue, raising concerns about the potential effects on soil
48 health and ecosystem services, and consequently, it emphasizes the need for further research in this
49 field (Huang et al.,2020; Meng et al., 2023; Van Schothorst et al.,2021; Ya et al., 2022).

50 Microbial communities are crucial for ecosystem functionality, with soil microorganisms playing
51 key roles in nutrient cycling, organic matter decomposition, soil structure maintenance, and regulation
52 of soil-borne pathogens (Garbeva et al., 2004; Saccá et al., 2017). Current molecular techniques in-
53 cluding High-Throughput Sequencing (HTS) combined with advanced microscopy have recently un-
54 veiled the occurrence of diverse microbial communities associated with different plastic debris from
55 different environments (Amaral-Zettler et al., 2020; Luo et al., 2023; Schlundt et al., 2020; Vethaak &
56 Leslie, 2016; Zettler et al., 2013). Zettler et al. (2013) first introduced the term 'plastisphere' to describe
57 the unique microbial community colonizing plastic marine debris. However, the application of this
58 term to terrestrial environments necessitates careful consideration to avoid potential confusion or mis-
59 interpretation. While the term is sometimes used in strict adherence to its original definition, referring

60 specifically to the microbial communities closely associated with plastic surfaces, in other instances, it
61 is applied more broadly, akin to the concept of the ‘rhizosphere'. This broader usage encompasses the
62 entire soil-plastic interface, thus including both microbes attached to the plastic and those in the inter-
63 acting soil (Rüthi et al., 2020). This expanded interpretation serves to highlight the complex interac-
64 tions within the plastisphere in terrestrial ecosystems.

65 Given the persistent nature of plastic contaminants and their increasing accumulation in the envi-
66 ronment, assessing and comparing the microbial communities associated with plastic particles to those
67 living on natural soil particles is of significant interest. However, it must be taken into consideration
68 the fact that plastic are anthropogenic materials, chemically and physically distinct from natural soil
69 particles (De Souza Machado et al., 2018). Thus, the microbial community residing on the plastic sur-
70 face can differentiate from the one found in the attached soil. In this context, our study aims to develop
71 a methodology that allows to distinguish the main compartments that microbes can occupy in terrestrial
72 agroecosystems polluted with plastics. Therefore, we propose the adoption of the term 'plastisphere-
73 soil’ to describe the soil zone directly influenced by plastic. Additionally, we suggest using 'plas-
74 ticplane' as a more specific term to describe the surface of the plastic where specific microbial coloni-
75 zation can also occur akin to the concept of “rhizoplane” terminology, broadly adopted in soil micro-
76 biology studies (Foster, 1986; Y. Li et al., 2023; Rashid Mwajita et al., 2013; Wieland et al., 2001).

77 Most previous research has focused on evaluating the microbial communities colonizing plastic
78 surfaces in comparison to bulk soil, reporting notable differences in composition, diversity, and rich-
79 ness compared to those associated with plastic debris (Gkoutselis et al., 2021; Yu et al., 2021). How-
80 ever, it is crucial to consider the extreme heterogeneity of soils. Most studies on the plastisphere have
81 come from aquatic environments, which are more homogeneous, making it reasonable to compare mi-
82 crobial communities attached to plastic surfaces with those in bulk, non-influenced regions. In contrast,
83 soil ecosystems are highly heterogeneous, and observed differences in microbial communities may
84 simply be attributed to natural spatial constraints.

85 As emphasized by Rillig et al. (2023), there is still a lack of information on plastisphere microbial
86 communities at the microscale, where the compartmentalized and heterogeneous nature of soil must
87 not be overlooked. This highlights the importance of this research, which isolated and compared mi-
88 crobial communities across adjacent plastic-associated microenvironments at a small spatial scale, us-
89 ing both culture-dependent and culture-independent approaches.

90 Plastic mulch debris was collected from agricultural fields, providing a more realistic perspective
91 compared to traditional laboratory experiments. A methodology was developed to differentiate the re-
92 covery of the microbial communities associated with the soil adhering to the plastic (referred to as
93 plastisphere-soil) and those closely associated with the plastic surface itself (termed plasticplane). This
94 study has also identified core microbial taxa consistently associated with plastic mulch debris, as well
95 as isolated bacteria species that could be further evaluated for their potential to degrade polymer-based
96 materials contributing to global efforts to mitigate plastic pollution.

97 **2. Material and methods**

98 **2.1 Study site and field sampling**

99 Weathered plastic mulch debris was collected from five agricultural fields located at Baza (Gra-
100 nada), southern Spain (Figure S1). The fields were characterized by intensive horticultural production
101 and historical use of plastic at least twice a year over the last ten years. At each sampling site, a total
102 of ten weathered plastic mulch debris samples measuring from 5-10 x 5-10 cm, were randomly col-
103 lected from the topsoil. Samples were gently shaken to remove the loosely attached soil particles,
104 placed in sampling bags, and stored at 4ºC until further analysis.

105 **2.2 Plastic characterization by Raman and Fourier infrared (FTIR) spectroscopy**

106 Prior to chemical characterization, plastic films were thoroughly washed with warm water (40ºC),
107 followed by a 20-min ultrasonication in deionized water. Subsequently, the films were rinsed and
108 wiped using cotton wool in distilled water and 70% isopropanol to remove residual soil and potential
109 bio-contaminants.

110 The chemical characterization of the plastic film surfaces was performed using Raman and FTIR.
111 FTIR is especially sensitive to observing oxidative products resulting from weathering, while Raman
112 spectra can provide insights into the polymer backbone, crystallinity, and the presence of inorganic
113 additives undetectable by FTIR.

114 Raman analysis was performed using a Renishaw InVia microspectrometer (Renishaw plc, New
115 Mills, Wotton under Edge, UK) equipped with a 532 nm laser (15 mW at the sample) and a 50x objec-
116 tive lens (numerical aperture NA 0.50). Raman spectra were collected in the 100-3500 cm^{-1} range using
117 a 2400 lines per millimeter grating, resulting in a spectral resolution of approximately 1 cm^{-1}. All

118 measurements were conducted with 5 accumulations at 5% laser power. After collection, each spec-
119 trum underwent cosmic ray removal (Wire 4.2), baseline correction, and normalization at the 2851 cm^{-1}
120 in Spectragryph version 1.2.15. Given the heterogeneous nature of the plastic surface, spectra were
121 collected at ten different positions on each plastic sample. The fraction of trans (α_t) and amorphous (α_a)
122 conformers were calculated using I_{1298} and I_{1305} intensities of the Raman bands at 1298 and 1305 cm^{-1},
123 respectively according to Hiejima et al.(2018).

124 Alongside Raman spectroscopy, a Cary 630 FTIR (Agilent Technologies Inc., Danbury, CT, USA)
125 with an attenuated total reflection (ATR accessory, diamond substrate) was used to identify plastics
126 and potential weathering effects on the polymer surface functional groups (penetration depth~2 µm at
127 1700 cm^{-1}). FTIR spectra were gathered in the 650–4000 cm^{-1} range, with 64 accumulated scans and
128 a 2 cm^{-1} spectral resolution. After collection, each spectrum underwent baseline correction and nor-
129 malization at the 2912 cm^{-1} peak in Spectragryph.

130 **2.3 Assessment of microbial communities associated with plastic mulch debris**

131 To assess and distinguish the microbial community of the plastisphere-soil (PPh) from the one
132 tightly attached to the plastic surface (plasticplane (PPl)) the collected plastic samples were subjected
133 to sequential washing steps (Figure S2) by using a modified protocol mimicking those developed to
134 sample and isolate rhizosphere and rhizoplane bacteria (Barillot et al., 2013).

135 Plastic mulch samples collected from each field were cut into small pieces (2 x 1.5 cm) with sterile
136 scissors and pooled into a single composite sample. A total of 50 mg of composite plastic samples was
137 placed in a falcon tube containing 10 mL of sterile distilled water. Collectively, three replicates of
138 plastic samples were processed per field plot. Falcon tubes were softly shaken for two min at 250 rpm
139 in a horizontal rotatory shaker, obtaining a first suspension that contained the attached soil fraction
140 (plastisphere-soil). From this soil suspension, 1-mL aliquots were taken for isolation of cultivable bac-
141 teria whereas 1.5-mL aliquots were taken for DNA extraction. Specifically, the 1.5-mL aliquots were
142 centrifuged at 14,000 rpm for 1 min. Then, the supernatant was discarded, and this process was repeated
143 six times to recover a soil pellet that was stored at -20°C until DNA extraction.

144 On the other hand, the washed plastic pieces from the first suspension were subjected to a second
145 intermediate washing step (Figure S2). Plastic debris was recovered and transferred to another falcon
146 tube that was shaken for 2 min with 20 mL of sterile water. Then, the washed plastic mulch films

(plasticplane) were transferred to a new 15-mL tube with 5 mL of sterile water, and it was sonicated for 5 min and vigorously vortexed for 2 min. A 1-mL aliquot of the resulting suspension, containing bacteria from the plasticplane, was taken for isolation of cultivable bacteria (see below), whereas the washed plastics were transferred to a 2-mL tube and kept at -20 °C until DNA extraction.

Additionally, samples from the plastic surfaces at each consecutive washing step were observed under a stereomicroscope (Leica, M165C, Leica Microsystems, Germany) to assess the efficacy of the washing process in removing soil particles attached to the plastic surfaces, and the same washed pieces were subjected to SEM-EDX analysis described below to corroborate the efficacy of the different washing steps to differentiate plastisphere-soil and plasticplane interfaces.

2.3.1 Isolation and characterization of culturable bacteria

Aliquots of plastisphere-soil and plasticplane suspensions were subjected to serial dilutions and 100-µl aliquots were plated in triplicate on R2A (Difco, Detroit, MI, USA) agar plates which contains yeast extract (0.5 g L^{-1}), proteose peptone No. 3 (0.5 g L^{-1}), casamino acids (0.5 g L^{-1}), dextrose (0.5 g L^{-1}), soluble starch (0.5 g L^{-1}), sodium pyruvate (0.3 g L^{-1}), dipotassium phosphate (0.3 g L^{-1}), magnesium sulfate (0.05 g L^{-1}), and agar (15 g L^{-1}), with a final pH of 7.2.

The number of colonies forming units (CFU) was assessed after a 2-day incubation period at 28ºC. Subsequently, a representative number of distinct colonies from the plasticplane were selected based on morphology (color, shape, margin, and texture). Then, selected colonies were isolated and purified (three cloning steps) and kept in 40 % glycerol stocks at -80ºC.

For bacteria identification, DNA was extracted using the DNeasy kit (QIAGEN, Madrid, Spain). The near-complete 16S rDNA gene was amplified using 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-ACGGCTACCTTGTTACGACTT-3′) primers (Weisburg et al., 1991) following the protocol outlined in Anguita-Maeso et al. (2022). Amplicons were sequenced by Sanger sequencing with the same primers used for the PCR at STABvida sequencing facilities (Caparica, Portugal). Sequences were assembled and manually corrected using DNASTAR software version 15.3.0.66 (Madison, WI, USA). The identification of isolates to the genus/species level was carried out by comparing their sequences with reference 16S rRNA gene sequences in the GenBank "nt" database using the BLAST algorithm as described by Altschul et al. (1997).

2.3.2 DNA Extraction and 16S rRNA Gene Amplicon Library Preparation

176 DNA from plastisphere-soil and plasticplane samples was extracted using the DNeasy PowerSoil
177 Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions with small modifications.
178 Briefly, samples were homogenized with the lysis buffer for 7 min at 50 pulses s^{-1} with the Tissuelyser
179 LT (QIAGEN) and then were incubated for 1 h at 60 °C to increase cell lysis. DNA was eluted in a
180 final volume of 50 µL of sterilized distilled water and its purity was determined using a NanoDrop®156
181 ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). This DNA
182 was used as a template for amplicon library preparation as described previously in Anguita-Maeso et
183 al., (2022). Shortly, the V5-V6 region of the bacterial 16S rRNA gene was amplified with the primers
184 799F (5'- AACMGGATTAGATACCCKG-3') and 1115R (5'-AGGGTTGCGCTCGTTG-3'). Bar-
185 codes indexes were added to the amplicons using Fluidigm barcodes (Access Array Barcode Library
186 for Illumina® Sequencers kit). Next, barcoded PCR products were purified by using Agencourt AM-
187 Pure XP (Beckman Coulter Inc., Brea, CA, USA), following the manufacturer's instructions. Purified
188 PCR products were quantified by using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo
189 Fisher Scientific) and a TECAN SAFIRE microplate reader (Tecan Group, Männedorf, Switzerland).
190 Equimolecular amounts from each individual sample were added to a single tube; the pooled library
191 was quantified by using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), and was purified again
192 if primer dimers were still evident. Finally, the library was sequenced on the Illumina MiSeq platform
193 (V2; PE 2× 250 bp) at the Genomics Unit at the Madrid Science Park Foundation, Madrid, Spain. The
194 ZymoBIOMICS microbial standard (Zymo Research Corp., Irvine, CA, USA) and water (no template
195 DNA) were used as internal positive and negative controls, respectively, for library construction and
196 sequencing.

197 2.4 Scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX) of Plastic 198 Surfaces

199 Scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX) was utilized to eval-
200 uate the plastic surface morphology, determine the elemental composition of the plastic, and assess the
201 efficacy of the washing steps in removing soil particles adhered to the plastic. Distinctly colored plastic
202 mulch samples (black and light grey) were specifically chosen from each sampling site. These samples
203 underwent fixation with 2% glutaraldehyde, dehydration through an ethanol series, and gold coating.
204 The scanning electron microscopy analysis was conducted using a JEOL JSM 7800 F scanning micro-
205 scope (JEOL Ltd., Peabody, MA, USA) equipped with energy-dispersive X-ray spectrometer (EDX)
206 at Central Service of Research Support (SCAI) of the University of Cordoba, Spain.

2.5 Statistical and Bioinformatics Analysis

Statistical analysis of culturable bacteria was performed using R statistical software (R Core Team, 2013). The significant effect of field location and soil-plastic habitat was tested using a factorial analysis of variance (ANOVA) followed by Tukey's multiple comparison test ($P < 0.05$). The Shapiro-Wilk test was used to determine whether the data were normally distributed whereas the homogeneity of variance was tested using Levene's test. Data from bacteria enumeration were log-transformed to fulfill the assumptions of homogeneity and normality.

TrimGalore v.0.6.6 tool was employed for quality control and adapter trimming of the demultiplexed raw fastq files. In this process, the first 10 bp of all reads were trimmed, and a truncation length of 240 base pairs for forward bacterial reads and 200 base pairs for reverse bacterial reads was applied to achieve a satisfactory Phred quality score ($Q > 30$). Subsequently, the high-quality reads underwent analysis using the DADA2 method to identify the amplicon sequence variants (ASVs) present in the samples (Callahan et al., 2016). Taxonomic classification was carried out using the Silva SSU v.138 database, and singleton ASVs were excluded from taxonomy assignment and statistical analysis. Differences in bacterial communities were assessed using alpha-diversity indexes (Richness and Shannon) at the ASV level. The non-parametric Scheirer–Ray–Hare test ($P < 0.05$), implemented with the rcompanion v.2.4.1 package in R (Mangiafico, 2022), was employed to evaluate the effects of the sampled field and habitat (plastisphere-soil or plasticplane) and their interaction on alpha-diversity indexes. Beta-diversity was determined through principal coordinate analysis (PCoA) of weighted UniFrac distance matrices. Additionally, the Permutational multivariate analysis of variance using distance matrices (ADONIS function) from the vegan package in R (with 999 permutations) was employed to test the effects ($P < 0.05$) of the sampled plot, habitat, and their interaction. To achieve parity in the total number of counts between samples, alpha- and beta-diversity analyses were performed after resampling the abundance values to the minimum number of reads observed. Moreover, a linear discriminant analysis effect size (LEfSe) (Segata et al., 2011), based on the microbiomeMarker package in R (Cao et al., 2022), was employed to identify differences in microbiota composition at the genus level among the various treatments ($P < 0.05$).

3. Results

3.1 Plastic characterization

236 The most representative spectra, showcasing the overall plastic composition and the influences of
237 weathering is shown in Figure 1. Both Raman and FTIR analyses confirmed that the plastic mulch
238 collected from the five fields was composed of polyethylene (Table S1).

239 Raman spectra analysis of the plastic samples displayed distinct peaks corresponding to the native
240 bonds in the LDPE polymer, found in the C-C stretching (1040–1200 cm^{-1}), -CH_2- twisting and bend-
241 ing (1300–1500 cm^{-1}), and C-H stretching (2800-3000 cm^{-1}) regions (Fig. 1). The latter is sensitive to
242 subtle structural changes due to intermolecular interactions and crystallinity. The peaks at 2882 and
243 2851 cm^{-1} indicated the presence of LDPE's amorphous and crystalline regions, respectively. Plastic
244 samples from Field 1 had a significant decrease in the intensity of the 2882 cm^{-1} peak relative to the
245 2851 cm^{-1} peak, compared to the plastic samples obtained from other fields, indicating its lower crys-
246 tallinity.

247 Additional Raman signals linked to LDPE's crystallinity appeared at peaks 1305 and 1298 cm^{-1},
248 associated with the CH_2 twisting modes of the amorphous and trans (consecutive trans chains) con-
249 formers, respectively (Meier, 2002). The highest amorphous fraction was found in the plastic samples
250 from Field 1, being 45.39 ± 0.03%, while the fractions obtained from Fields 3, 4, and 5 were 36.4 ±
251 0.1%, 33.19 ± 0.06%, and 34.28 ± 0.06% respectively. Field 2 was excluded from the calculations due
252 to a broad additive band at 1360 cm^{-1}. Often, plastic degradation, especially due to photodegradation,
253 results in increased crystallinity (decreased amorphous phase), leading to material embrittlement and
254 fragmentation.

255 The lower Raman spectral region (100-600 cm^{-1}) indicated the presence of inorganic compounds.
256 In this case, the presence of titanium oxide (TiO_2) and the blue pigment Lazurite-a sodium silico alu-
257 minate in a sulfur matrix were confirmed in plastic samples from Field 2 (Figure S3 A). This was
258 evidenced by the presence of bands corresponding to anatase TiO_2 (143 cm^{-1}) and rutile TiO_2 (233,
259 442, 610 cm^{-1}), and the characteristic bands of Lazurite (257, 547, 805, 1094, 1644, and 2183 cm^{-1},
260 (Figure S3 B). These findings align with the SEM-EDX data, which also revealed the presence of
261 titanium (0.12%wt), silicon (2.76%wt), calcium (1.87%wt), sulfur (0.15%wt), and aluminum
262 (1.01%wt) in plastic samples from Field 2, while no sulfur and titanium were detected in plastics from
263 other fields (Table S2).

264 Interestingly, plastic samples from Fields 1, 3, and 4 exhibited a noticeable presence of carbonyl
265 (C=O) groups in their FTIR spectra, potentially formed during plastic oxidation. Samples from Field

266 3 and Field 4 displayed unique peaks due to ketone carbonyl at 1717 cm^{-1}, while those from Field 1
267 showed a peak due to ester carbonyl at 1740 cm^{-1}. The carbonyl index, a common indicator of oxidation
268 level, which is calculated as the ratio of peak intensity between 1717 (or 1740) cm^{-1} and 1465 cm^{-1},
269 was 0.13, 0.20, and 0.28 for samples from Fields 1, 3, and 4, respectively, indicating a higher concen-
270 tration of carbonyl in plastic samples from Field 4.

271 **3.2 Microscopic evaluation of plastic surface: scanning electron microscopy and optical mi-**
272 **croscope analysis**

273 Visual inspection using light images from a stereomicroscope (Figures S4 A and B) and SEM (Fig-
274 ure S4 C) was performed to assess the efficacy of the sequential washing steps in removing soil parti-
275 cles from the plastic surface and enabling independent sampling of plastisphere-soil and plasticplane-
276 associated microorganisms. The image shows the condition of the plastic surface immediately after
277 field sampling and after each successive washing steps described in Figure S2.

278 Initial attempts revealed that even after two washing steps, some soil particles remained, which
279 could lead to misleading results in the differential extraction of microorganisms associated with the
280 soil particles rather than the plastic surface. Therefore, an additional washing step was introduced to
281 remove the soil particles more effectively (Figure S2). This modification included in our protocol en-
282 sured a clear distinction between the plastisphere-soil and plastic-plane compartments, facilitating
283 more accurate characterization of their respective microbial communities.

284 **3.3 Characterization of the culturable bacterial population**

285 Both plastic-debris compartments (plastisphere-soil and plasticplane) and location (field) factors
286 significantly affected the population density of culturable bacteria ($P<0.001$) (Figure 2). The bacterial
287 density found on the plastisphere-soil was significantly greater than that obtained on the plastic surface
288 for all sampled fields. Thus, the bacterial density isolated from the plastisphere-soil fraction ranged
289 from log 7.1 to log 7.7 CFU g^{-1} plastic, whereas the bacterial population on the plastic surface ranged
290 from log 6.2 to 6.9 CFU g^{-1} plastic.

291 A total of 74 bacteria from the plasticplane were isolated, cultivated, and taxonomically identified
292 by 16S rRNA gene sequencing. Isolated bacteria were assigned to four phyla, five classes, six orders,
293 nine families, and 19 genera (Table S3A). The most abundant genera included *Arthrobacter* (16.2% of

294 isolates) followed by *Priestia* (14.9% of isolates), *Pseudarthrobacter* (13.5% of isolates), *Massilia* and
295 *Microbacterium* (9,5% each), *Bacillus* (8,1%) and *Kokuria* (6.8%) (Table S3B). On the other hand,
296 less frequent bacteria included *Frondihabitans, Lysinibacillus, Nocardioides, Paenarthrobacter, Pae-*
297 *nibacillus, Planococcus, Planomicrobium, Rossellomorea,* and *Rufibacter.* Plasticplane samples from
298 Fields 3 and 5 showed a higher diversity of bacterial genera (8 and 10 respectively), whereas in those
299 from the remaining field plots, a total of seven bacterial genera were identified.

300 **3.4 Composition and diversity of bacterial communities in the plastisphere-soil and plas-**
301 **ticplane**

302 Illumina sequencing yielded a total of 351,265 good-quality reads after removal of chimeras, un-
303 assigned, or mitochondrial reads. A total of 1,701 amplicon sequence variants (ASVs) were identified
304 among all treatments, with 1,682 ASVs being retained for alpha- and beta-diversity analysis after rar-
305 efying all data to the minimum number of reads and singleton removal. The Scheirer–Ray–Hare test
306 indicated significant differences ($P < 0.05$) for the Richness and Shannon alpha-diversity indices ac-
307 cording to the field plot ($H = 19.63$, $P = 0.001$, and $H = 21.54$, $P = 0.001$, respectively) whereas plastic
308 -associated compartments resulted significant for Richness ($H = 4.13$, $P = 0.04$), but not for Shannon
309 ($H = 3.25$, $P = 0.07$) diversity. Furthermore, there was no significant interaction between the plastic
310 fraction and field plot for both alpha-diversity indices ($H < 0.77$, $P > 0.94$) (Figure 3).

311 Principal coordinate analysis of beta-diversity weighted UniFrac distances differentiated bacterial
312 communities mainly according to the field location. Thus, there was a clear tendency to group bacterial
313 communities according to the sampled field along Axis 1, which explained 49.9% of the variation
314 (Figure 4). In fact, ADONIS analysis supported the results described above and indicated a significant
315 main effect of the field plot ($R^2 = 0.79$, $P < 0.001$). However, the bacterial habitat (plastisphere-soil or
316 plasticplane) resulted not significant ($R^2 = 0.01$, $P > 0.196$) nor their interaction ($R^2 = 0.03$, $P > 0.424$).

317 A total of 24 phyla, 60 classes, 173 orders, 321 families, and 723 genera of bacteria were taxonom-
318 ically identified by illumine sequencing. Most abundant bacterial genera in all the plastisphere-soil
319 samples belonged to *Bacillus* (23.3%), *Nocardioides* (11.7%), *Planomicrobium* (10.4), *Blastococcus*
320 (10.1%) and *Streptomyces* (8.5%) genera, whereas those from plasticplane included *Blastococcus*
321 (15.6%), *Sphingomonas* (10.7%), *Arthrobacter* (10.1%), *Bacillus* (9.3%) and *Hymenobacter* (7.4%).
322 In Field 1 and 2, there was a prevalence of *Bacillu*s (41.3% and 51.8%, respectively), whereas, in Fields
323 3 and 4, *Blastococcus* was the dominant genus (18.9% and 30.7%, respectively) (Figure 5). When

324 evaluations were made within each field, comparing the microbial community associated with the plas-
325 ticplane with the one from the plastisphere-soil, there were no evident changes in the proportion of the
326 most abundant genera.

327 In line with these results, Linear Discriminant Analysis Effect Size (LEfSe) analysis was used to
328 identify key bacterial genera differentially associated with the plasticplane or plastisphere-soil fractions
329 of plastic mulch debris sampled at different field locations. Globally, LEfSe identified a bacterial en-
330 richment of *Skermanella* and Class_ASV511 enriched in the plastisphere-soil (Figure S5). However,
331 when applying LEfSe to each field independently, field plot F2 showed a higher number of differen-
332 tially abundant genera. In particular, genera such as *Polycyclovorans*, *Sphingomonas,* and *Cellvibrio*,
333 were enriched in the plasticplane, while genera such as *Arthrobacter, Planomicrobium*, and *Sker-*
334 *manella* were significantly more abundant in the plastisphere-soil than in the plasticplane (Figure 6).

335 **3.5 Core community analysis**

336 The Venn Diagram (Figure S6) analysis revealed the shared and unique bacterial genera in the
337 different plastic-associated habitats. Notably, in all evaluated fields, between 62.5% and 88.4% of the
338 bacterial genera were shared between both habitats. Interestingly, on plastic debris recovered from field
339 plots F3 and F5, approximately 13% of bacterial genera were exclusively found on the plasticplane,
340 while in the other fields, these values were lower.

341 The core microbial taxa consistently found only in the plastisphere-soil of all evaluated samples
342 recovered from the different fields were represented by 220 bacterial ASVs, while 79 ASVs repre-
343 sented the core taxa on the plasticplane (Figure 7). The majority of bacteria consistently found on the
344 plasticplane belonged to the genera *Bacillus*, *Sphingomona*s, *Nocardioides,* and *Solirubrobacter*. A
345 complete list identifying the core plasticplane bacterial community can be found in Supplementary
346 Table S4.

347 **4. Discussion**

348 While some studies have compared the plastisphere microbiome with bulk soil, highlighting sig-
349 nificant differences in their microbial community composition and structure, there is still a need for a
350 more comprehensive understanding of microbial communities at a finer spatial definition within the
351 plastic-soil interface (Bandopadhyay et al., 2020; Gkoutselis et al., 2021; Rillig et al., 2023; Rüthi et

352 al., 2020). This study addresses this gap by developing a methodology to differentially sample and
353 compare the bacterial community composition and diversity of plastic surfaces (plasticplane) with
354 those found on natural soil particles interacting with this anthropogenic material (plastisphere-soil)

355 Polyethylene polymers are commonly regarded as recalcitrant materials, known for their resistance
356 to degradation (Brown et al., 2022; El-Sherif et al., 2022). However, research suggests that weathered
357 polyethylene plastics, previously exposed to UV radiation, can be colonized and undergo partial me-
358 tabolism facilitated by specific microbial taxa capable of secreting enzymes such as esterase, lipases,
359 peroxidases, and oxidoreductases (El-Sherif et al., 2022; Sharma & Neelam, 2023). Consequently, it
360 was expected that, as observed, the microbial community richness on the surface of weathered poly-
361 ethylene might experience a reduction or selection of specific bacterial taxa compared to soil particles.
362 This difference can be attributed to the easily assimilated carbon sources provided by the associated
363 organic matter in soil particles, contrasting with the resistant nature of polyethylene, which is not con-
364 sidered a straightforward or efficient carbon source for most bacteria. Interestingly, these results are
365 similar to those described for the rhizosphere soil and rhizoplane-root interfaces, where the secretion
366 of root exudates favors the growth of specific bacteria that are better adapted to this environment,
367 leading to a decrease in overall bacterial richness (Philippot et al., 2013).

368 Furthermore, our results revealed similarities in the microbial communities' composition retrieved
369 according to the field of origin of the plastic debris. Thus, the tendency to group bacterial communities
370 according to the sampled field, and the observed similarities between Fields 3 and 4, which are geo-
371 graphically closer to each other, highlight the role of environmental and geographic constraints in shap-
372 ing the soil microbial community at the plastic-soil interface. Additionally, the plastic debris collected
373 from Field 2 exhibited a higher number of differentially abundant taxa on the plasticplane compared
374 to the plastisphere-soil. Interestingly, this plastic was unique in its color, appearing light grey instead
375 of the commonly found black plastic mulch of the remaining field sites. Analysis of the plastic's chem-
376 ical properties by SEM-EDX and Raman spectroscopy revealed the presence of titanium dioxide (TiO_2)
377 on this specific mulch film, a pigment commonly found in white plastics due to its high light scattering
378 efficiency, inertness, thermal stability, dispersibility, and cost-effectiveness (Puglisi et al., 2019;
379 Turner & Filella, 2023).

380 Some previous studies have demonstrated the effects of TiO_2 on soil bacterial communities (Ge et
381 al., 2011, 2012, 2013). Ge et al. (2012), for instance, found that the presence of this chemical compound

382 can significantly reduce certain bacterial genera, including *Actinoplanes, Balneimonas, Blastococcus,*
383 *Bradyrhizobium*, and *Skermanella.* The toxicity of this material to some bacteria can be related to ox-
384 idative damage to bacterial cell walls, leading to membrane disorganization and permeability. Our re-
385 sults also indicate a reduction in the *Blastococcus* genus on the plastic surface compared to the plas-
386 tisphere-soil. In contrast, the genera *Polycyclovarans* and *Cellvibrio* were enriched on the plasticplane
387 compared to the plasticphere. These findings suggest that plastic additives and dyes may influence the
388 assemblage of plastic-associated bacterial communities, underscoring the need for further research fo-
389 cusing on this topic.

390 Differing from most contaminants found in the soil, plastic debris represents a potential threat to
391 the soil environment as it constitutes an external anthropogenic particle with a distinct shape, size, and
392 volume compared to natural soil particles (Rillig et al., 2023). Additionally, it contains a series of
393 additives, such as pigments, plasticizers, and antioxidants, which can leach over time and could interact
394 with the microbial community (Macan et al., 2024). Furthermore, these materials can adsorb contami-
395 nants such as pesticides commonly applied in agricultural fields, as well as heavy metals (K. Li et al.,
396 2023; Rillig et al., 2023). Consequently, all these factors may play a role in selecting specific taxa more
397 adapted to colonize the plasticplane.

398 Isolating, cultivating, and identifying key species from the plasticplane is particularly noteworthy,
399 as they present promising candidates for further assessments as plastic degraders. Both molecular and
400 culturable methods revealed a prevalent presence of *Arthrobacter* genus in plastic-soil interface. Nu-
401 merous reports in the literature highlight its significant role in degrading various carbon sources, em-
402 phasizing its active hydrolytic enzyme production and the ability to break down persistent conventional
403 plastics (Gobbetti & Rizzello, 2014; Han et al., 2020). Furthermore, members of the *Priestia* genus
404 were isolated and cultured from the majority of plasticplane samples. This genus is recognized as an
405 environmental bacterium extensively used in biotechnology and bioremediation due to its ability to
406 produce several enzymes (Dhaka et al., 2022; Shwed et al., 2021). Other culturable bacterial genera
407 isolated and identified, such as *Bacillus, Terribacillus, Paenibacillus, and Kocuria*, have also been
408 previously associated with plastics according to existing literature and could be further explored as key
409 genera in plastic biodegradation assays (Anwar et al., 2016; Bardají et al., 2019; Harshvardhan & Jha,
410 2013; Vidal-Verdú et al., 2022). More specifically, *Priestia megaterium* and *Bacillus pumilus,* for
411 instance, were some of the cultivated and identified species in this study which have also been previ-

412 ously isolated from the plastic debris and assessed for their biodegradation abilities on a range of pol-
413 ymer types such as polyethylene (PE), polypropylene (PP) and also poly(lactic acid) (PLA) and poly(3-
414 hydroxybutyrate) (PHB) biodegradation (Jeszeová et al., 2018; Sangeetha Devi et al., 2019; Takaku et
415 al., 2006; Wróbel et al., 2023).

416 HTS of 16S rRNA gene allowed the identification of a high diversity of bacteria, many of which
417 were not isolated by using cultured-based approaches. The results showed that certain bacterial taxa,
418 including species from the genus *Bacillus, Sphingomonas, Nocardioides, Solirubrobacter, Nitro-*
419 *sospira,*and *Paenibacillus*, were consistently present on the plasticplane of all plastic samples recov-
420 ered from the different fields. This analysis identified these taxa as core genera associated with the
421 plastic surface of LDPE-based plastic mulches. *Bacillus*, for instance, emerged as one of the most
422 dominant genera in the core plasticplane. The literature indicates that this genus is considered a prom-
423 inent bacterial taxon involved in plastic biodegradation (Priya et al., 2022). Specific *Bacillus* species
424 have been evaluated for their plastic degradation abilities, including LDPE, in different studies. For
425 instance, Yao et al. (2022) observed a 3.49% and 2.82% weight loss of LDPE films after exposure to
426 different *Bacillus* strains, after 30 days of incubation. Moreover, *Nocardioide* is a taxon characterized
427 by its ability to thrive under low-nutrient conditions, being able to degrade a range of pollutants as a
428 source of carbon and nitrogen (Ma et al., 2023; Zhao et al., 2023). *Nitrospira* spp. have also been
429 recently isolated and assessed as potential degrader of various plastic materials, including LDPE, while
430 *Paenibacillus* and *Sphingomonas* have also been previously characterized as key taxa of the plas-
431 tisphere-associated microbiome (Bardají et al., 2019; Di Pippo et al., 2020; P. Wang et al., 2023; Wu
432 et al., 2022).

433 It should be remarked that, in this study, assessments were conducted using commercial samples
434 of environmental relevance collected from the natural environment, offering a more realistic perspec-
435 tive compared to laboratory incubation experiments. Additionally, all the assessed plastic samples were
436 characterized as polyethylene. Further studies should also be conducted considering a wider range of
437 plastic types (e.g., biodegradable plastic mulches), and in a more diverse range of agricultural fields.
438 Biodegradable plastic mulch, for instance, is starting to be widely adopted in the field as an alternative
439 to conventional LDPE plastic and it could play a more prominent role in shaping the microbial com-
440 munity by selecting specific taxa able to metabolize it and thus having a greater interference in the soil
441 microbial communities (Zhang et al., 2024). Bandopadhyay et al. (2020), for instance, showed that

442 some bacterial genera such as *Methylobacterium, Arthrobacter*, and *Sphingomonas* were enriched on
443 biodegradable plastic mulch in comparison to conventional LDPE mulches.

444 **5. Conclusion**

445 This study has significantly contributed to expanding our understanding of the plastic-associated
446 microbial communities in agricultural systems. It stands by providing a detailed methodology to dis-
447 entangle the microbial communities at the soil-plastic interface, particularly at a small spatial scale,
448 through the distinct assessment of the 'plasticplane' and 'plastisphere' microbiomes.

449 This research focused on developing a methodology to unravel and compare the assemblage of the
450 plasticplane and plastisphere-soil microbiome, emphasizing taxonomic profiling, with less attention
451 given to functional characterization. Nevertheless, this methodology could be applied to develop fur-
452 ther research focusing on functional approaches, which can offer valuable insights into microbial mech-
453 anisms, metabolic activities, and enzymatic functions associated with plastic biodegradation.

454 Our findings reveal that the plastic surface can host taxa that are consistently present on the plas-
455 ticplane across different field sites. This suggests the existence of a core microbial community with a
456 strong affinity for plastic surfaces, regardless of the specific field site. Moreover, it has been shown
457 that geographical constraints can play a significant role in shaping both the microbial community of
458 the soil and therefore that of the plastic interacting with it. Additives can also influence the associated
459 microbial community, prompting the need for further detailed evaluations.

460 Finally, this study serves as a foundational basis for future research, particularly in the characteri-
461 zation of potential microbial degraders. Thus, some key bacterial genera closely associated with the
462 plastic debris identified in this study included *Bacillus, Nocardioides, Solirubrobacter,* and *Sphingo-*
463 *monas*. These bacterial species could be pivotal in assessing their ability to degrade not only LDPE
464 plastics but also various other polymer-based materials, thereby contributing to the global effort to
465 tackle plastic pollution.

466

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Figure 1. Characterization of the plastic film samples from each of the fields. **A**. Normalized Raman spectra. **B**. Normalized FTIR spectra

Figure 2. Bacterial population density (log of the mean CFU g^{-1}) in the plastisphere-soil (PPh) and plasticplane (PPl) of plastic mulch debris sampled in different field plots. Means followed by different letters are significantly different (P<0.001) according to Tukey Test. Uppercase letters are related to the plastisphere, while lowercase letters are related to the plasticplane. The asterisks indicate significant (P<0.001) differences between the sampled plastic-associated compartments (plastisphere soil, PPh and plasticplane, PPl) at each field plot.

Figure 3. Boxplots of Richness (observed) and Shannon diversity indices for bacterial communities at ASV level in the plastisphere soil (PPh) or plasticplane (PPl) compartments of plastic mulch debris sampled at different field locations. The boxes represent the interquartile range, while the horizontal line within the box defines the median and whiskers represent the lowest and highest values of four values for each treatment combination.

Figure 4. Principal Coordinates Analysis (PCoA) of bacterial communities based on Weighted UniFrac distances at the ASV taxonomic level in the plastisphere (PPh) or plasticplane (PPl) compartments of plastic mulch debris sampled at different field locations. Points are colored by field location and shaped by habitat type.

Figure 5. Barplots showing the relative abundance of the 15 most abundant bacterial taxa at the genus level in the plastisphere (PPh) and plasticplane (PPl) compartments of plastic mulch debris sampled at different field plots (F1 to F5).

Figure 6. Linear discriminant analysis Effect Size (LEfSe) analysis of differentially enriched bacterial genera present in the plastisphere-soil (PPh) or plasticplane (PPl) compartments of plastic mulch debris sampled at different field locations. Horizontal bars represent the effect size for each taxon. Only significant taxa ($P < 0.05$) with a LDA Score (log10) higher than 3.5 are shown.

Figure 7. Venn diagrams comparing the observed shared and unique ASVs on the plastisphere soil (PPh) and plasticplane (PPl) found in each of the evaluated field plots and plastic compartments. Colors represent the field from which the samples were collected, and the numbers represent the number of shared or unique ASVs found.

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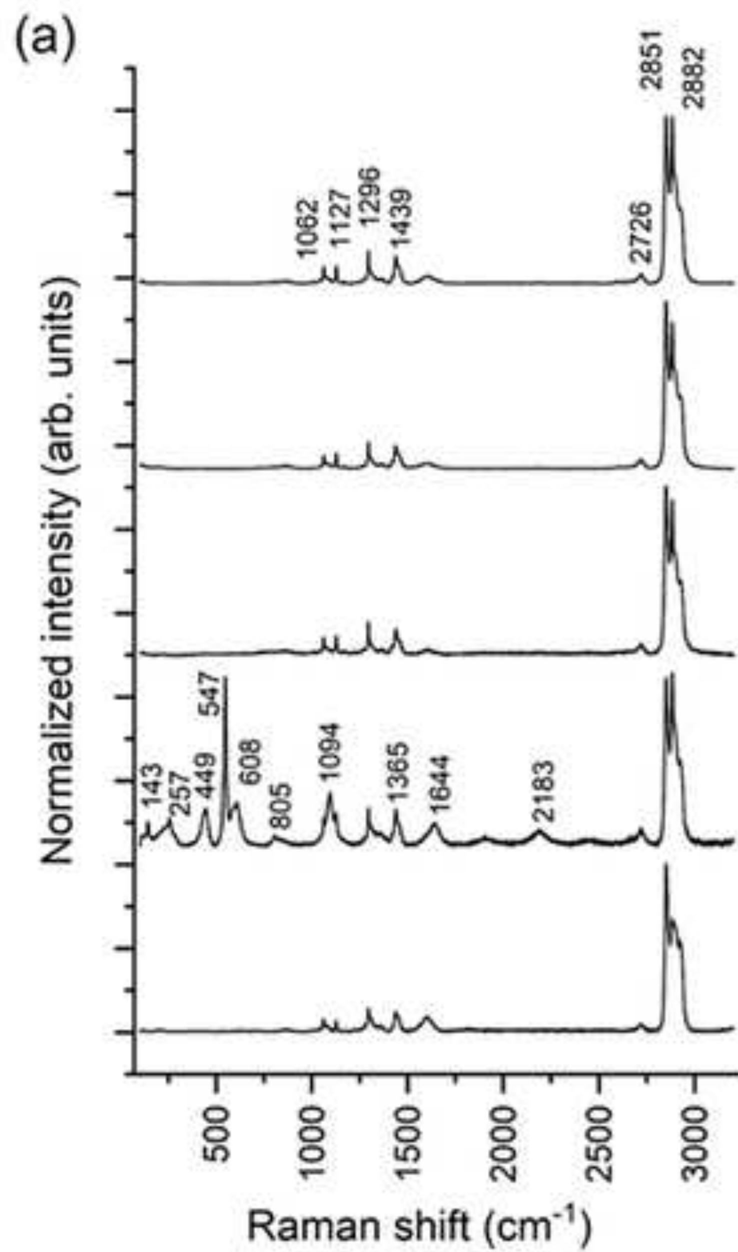

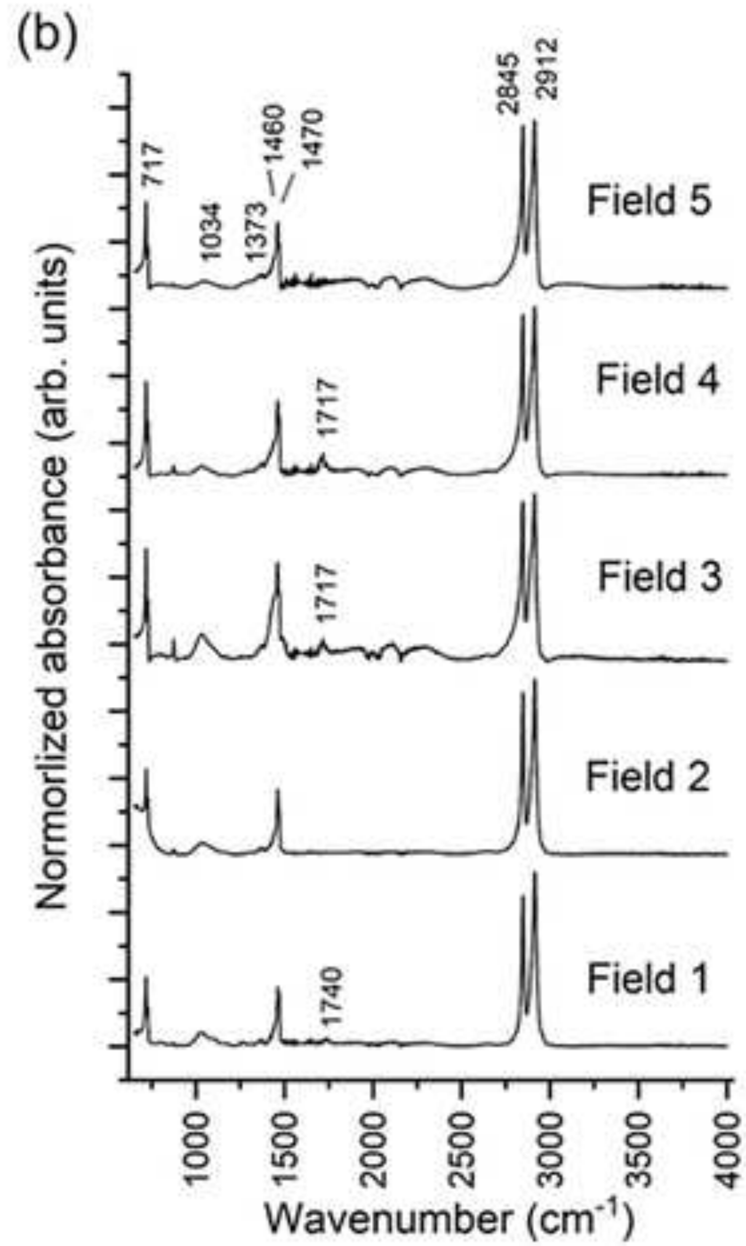

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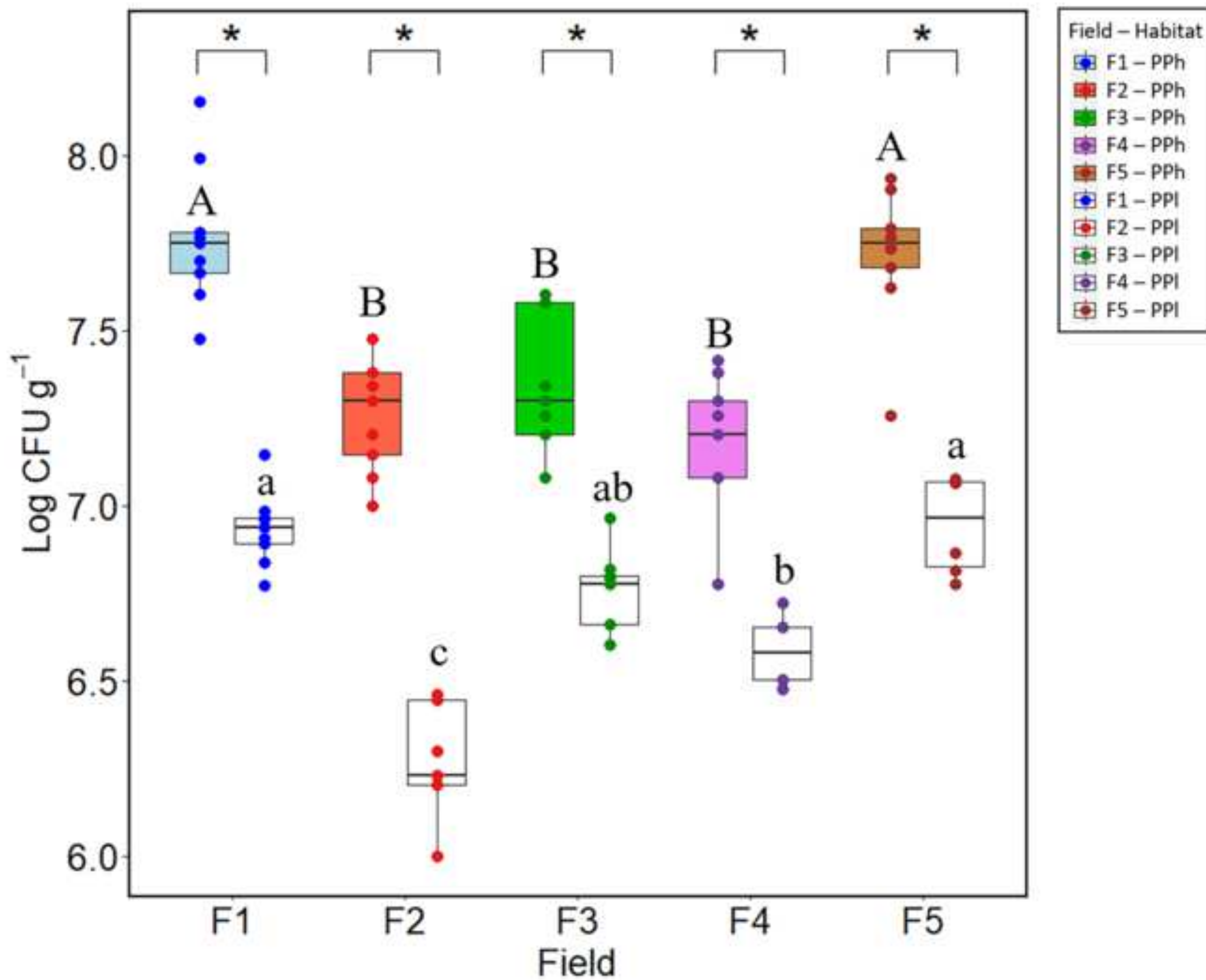

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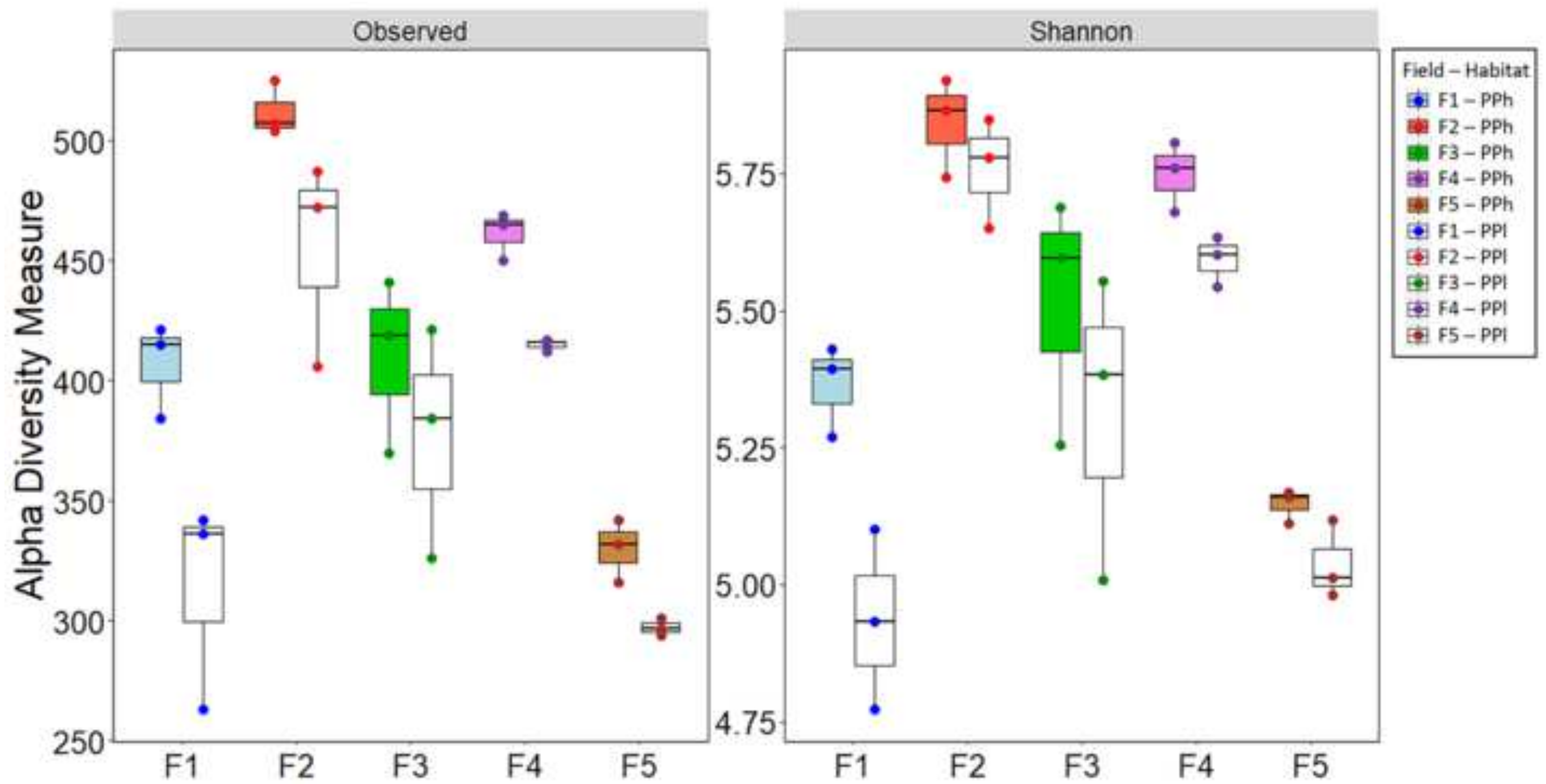

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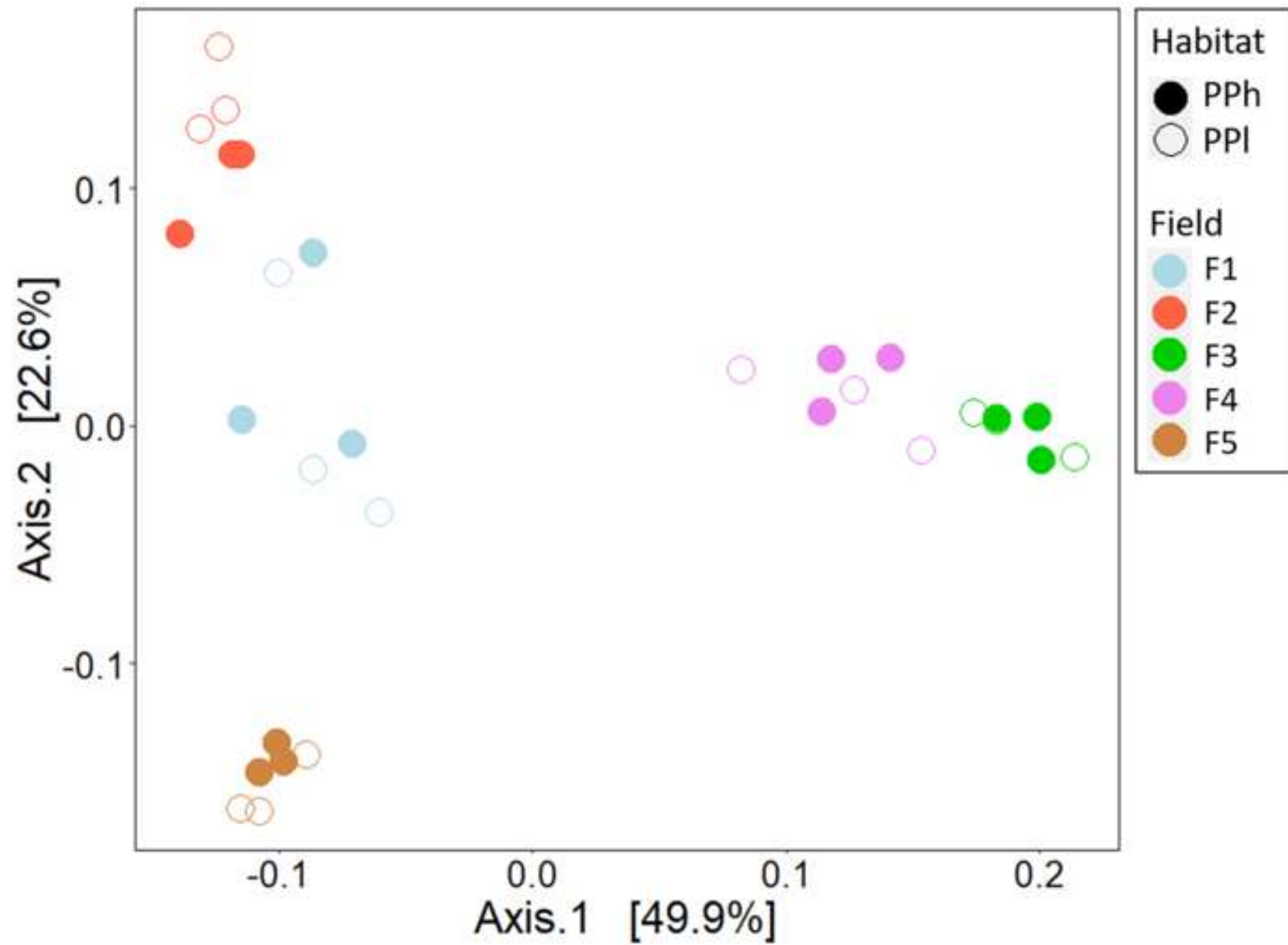

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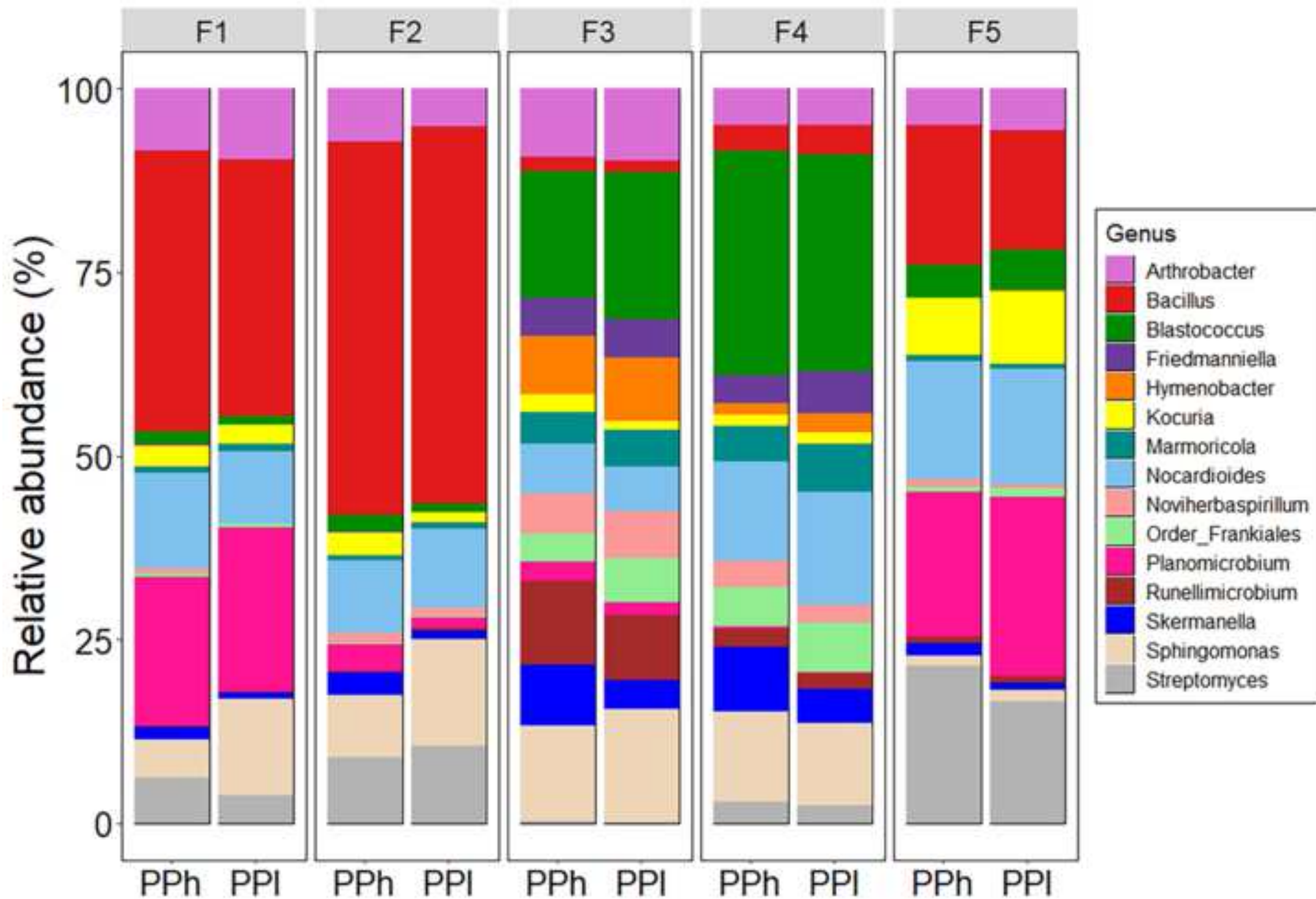

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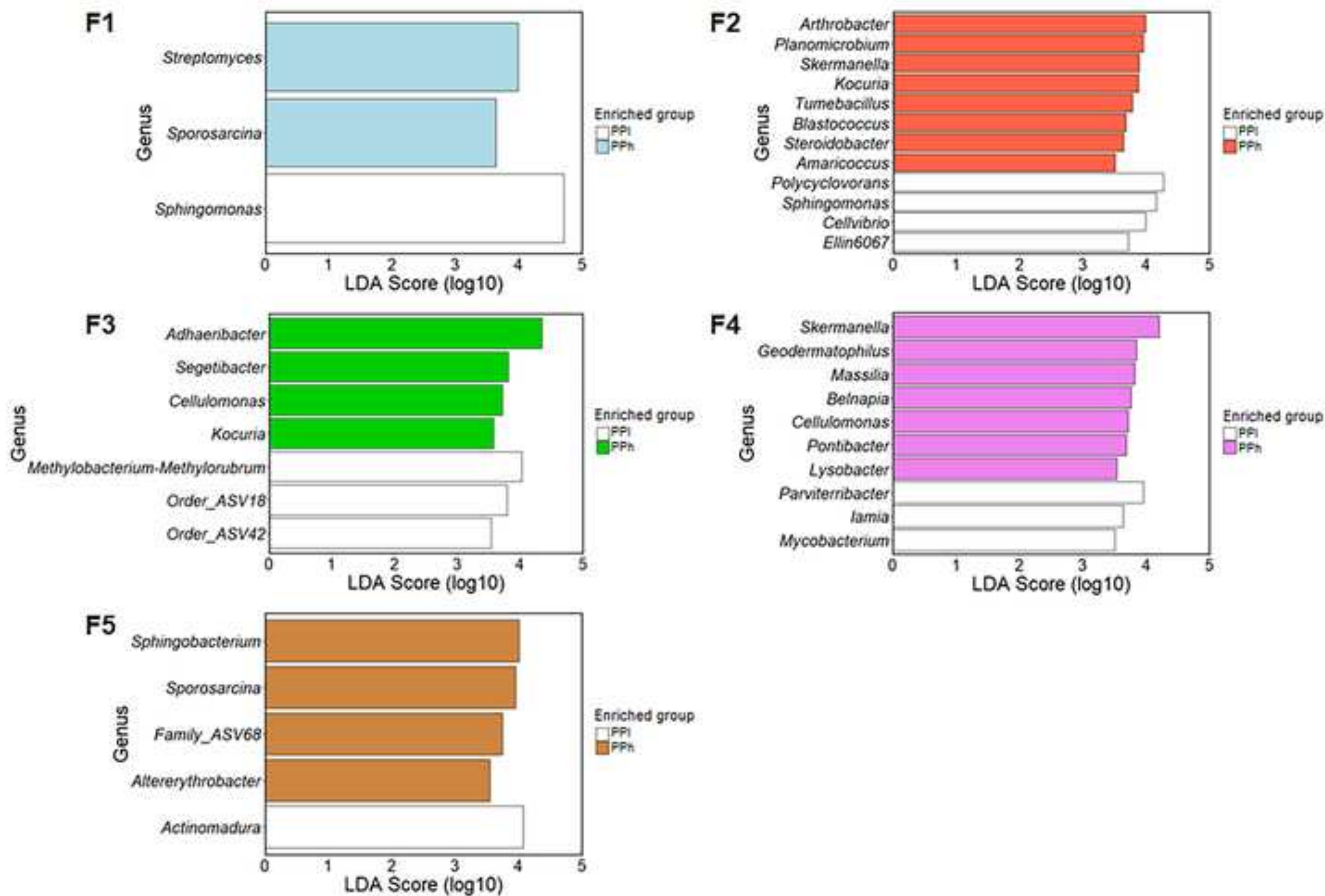

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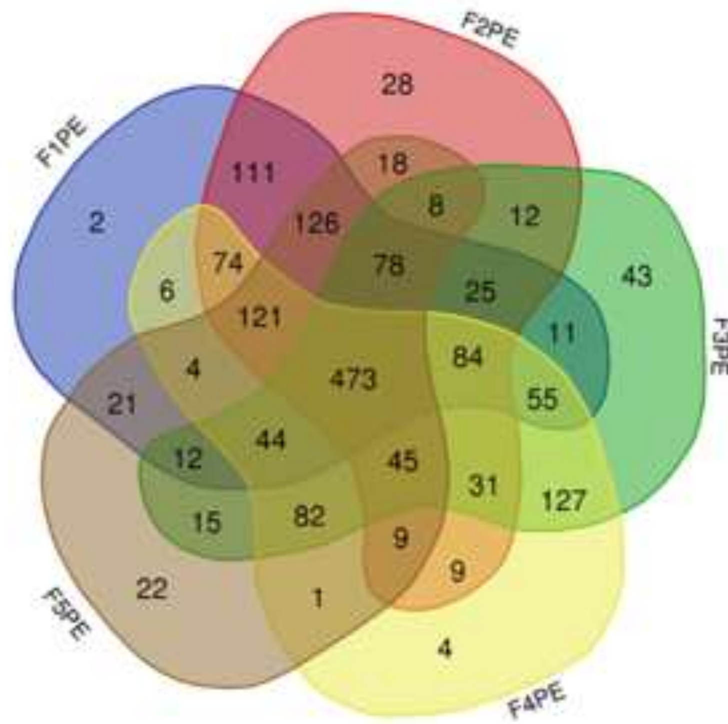

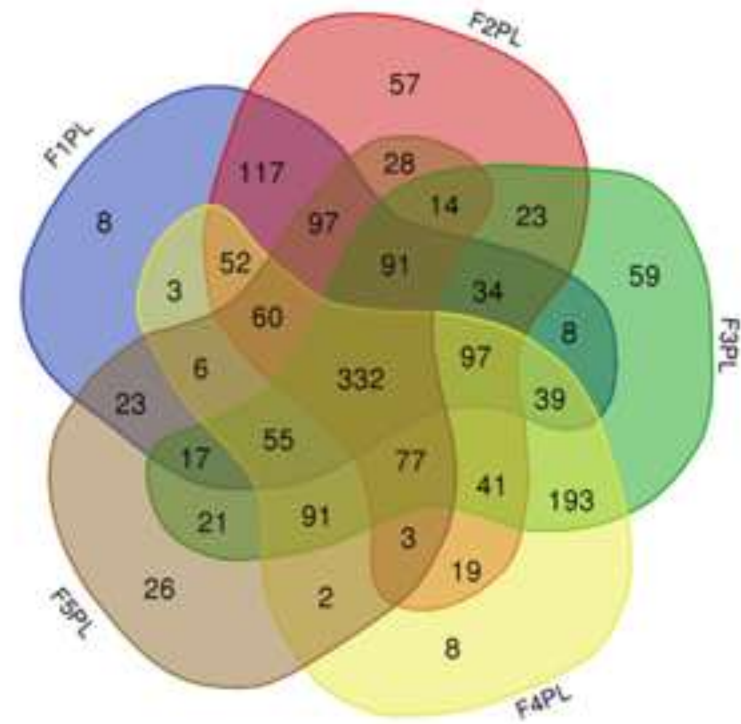

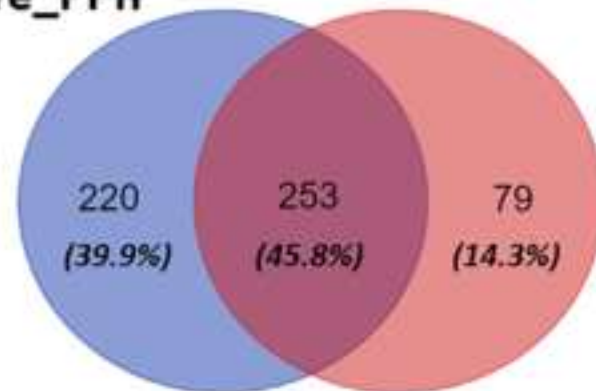

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Declaration of interests

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

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