Increased methane production associated with community shifts towards *Methanocella* in paddy soils with the presence of nanoplastics

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Supplemental materials and methods

1.1 LDPE nanoplastics and soil sampling

Low-Density Polyethylene (LDPE) (density: 0.91 g/cm^3) nanoparticles (NPs) with size of 50 nm were purchased from ZhongXin Plastic (Guangdong, China). Nanoplastics were sterilized with methanol, dried at 40 °C, and stored at 4 °C for further use [1]. Soil samples were collected in August 2019 from two typical rice-growing areas that differ in their soil types, defined as Black soil (BS) and Red soil (RS) in China. At each sampling site, we collected five soil cores with a depth of 0-20 cm during the drainage period of rice grouting. All soil cores were air-dried and sieved through 2 mm meshes to remove visible plant residues, roots, and stones.

1.2 Process measurements

The liquid and soil samples were immediately shock-frozen using liquid nitrogen and stored at −80 °C for molecular analysis. One subsample of each microcosm was freeze-dried and used for the extraction of dissolved organic carbon (DOC). A soil sample (2 g) from each of the 36 microcosms (two soil types × two time points × three treatments × three biological replicates) were subjected to DOC extraction using 1:10 (w/v) soil/ultrapure water. The soil microcosms were shaken for 1 min at 25 °C, placed statically for 20 min, and the supernatant was further filtered through a pre-rinsed 0.22 μm cellulose acetate membrane filter [1, 2]. Then, the DOC concentrations were determined using a total organic carbon analyzer (Elementar, Langenselbold, Germany).

A trace amount of the sample was affixed to the plate using conductive adhesive. Subsequently, samples were coated with platinum using a Quorum SC7620 sputter coater to establish the conductivity of the samples. A field emission scanning electron microscope (SEM; TESCAN MIRA LMS, Czech) was used to characterize the surface of recovered nanoplastic particles. The primary SEM parameters were 10,000 × magnification and 3 kV acceleration voltage. The solid-phase extraction of dissolved organic matter (DOM) from soil samples were conducted using Fourier transform ion cyclotron resonance (FT-ICR-MS) [1]. Three replicates of each treatment (control, 0.5% NPs. 5% NPs) were merged to produce one composite sample for the analysis of the black soil and the red soil. The van Krevelen diagram was performed to cross-plot hydrogen-to-carbon (H/C) and oxygen-to-carbon (O/C) element ratios. To assess DOM lability, the molecular lability boundary (MLB_L) was calculated by the number of formulas with H/C ≥ 1.5 divided by the total number of formulas in the sample [1]. All formulas were subdivided into four major classes as follows: CHO (containing C, H, and O); CHON (containing C, H, O, and N); CHOS (containing C, H, O, and S); and CHONS (containing C, H, O, N, and S). The formulas plotted in the van Krevelen diagram could be categorized into the following seven groups based on H/C and O/C ratios: (i) lipid-like (H/C, 1.5−2.0; O/C, 0−0.3); (ii) protein/amino sugar-like (H/C, 1.5−2.2; O/C, 0.3−0.67); (iii) carbohydrate-like (H/C,1.5−2.2; O/C, 0.67−1.2); (iv) highly unsaturated and phenolic-like (H/C, 0.7−1; O/C, 0−0.1); (v) lignin-like (H/C,

0.7−1.5; O/C, 0.1−0.67); (vi) tannin-like (H/C, 0.5−1.5; O/C, 0.67−1.2); and (vii) condensed aromatic-like compounds (H/C, 0.2−0.7; O/C, 0−0.67) [1].

1.3 DNA and RNA extraction

Total soil DNA was extracted using the PowerSoil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, USA) from 0.25 g wet soil following the manufacturer's instructions. The purity and concentration of DNA extracts were checked by NanoDrop spectrophotometry (NanoDrop Technologies Inc., Wilmington, DE, USA). The DNA extracts were stored at −20°C until molecular analysis (quantitative PCR and metagenomics).

Total RNA was isolated from the soil slurries using a previously described method [3]. In brief, 0.5 g of wet soil was mixed with glass beads and suspended in TPM buffer (consisting of 0.5 M Tris pH 7.0, 1.7% polyvinylpyrrolidone, and 0.2 M $MgCl_2$). Then the mixture was thoroughly vortexed and centrifuged at 20,000 × *g* for 5 minutes. The pellet was resuspended in PBL buffer comprising 0.05 M Tris pH 7.0, 0.05 M Na_2EDTA, 0.1% SDS w/v, and 6% v/v phenol. The resulting supernatant was extracted with phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol. The RNA was precipitated with isopropanol and resuspended in 50 µl of TE buffer. Finally, genomic DNA was removed using the TURBO DNA-free kit (Ambion, USA) according to the manufacturer`s instructions. The total RNA was concentrated and purified using the RNeasy MinElute Kit (Qiagen) following the kit's protocol.

84 **1.4 Metagenomic analysis**

85 Metagenomic libraries were constructed using size selection to fragment lengths
86 ranging from 300 to 500 bp [4]. This fragment length range was validated using an
87 Agilent Technologies 2100 Bioanalyzer with a High Sensitivity DNA chip according to
88 the manufacturer's instructions (Agilent Technologies, Palo Alto, CA). Subsequently,
89 36 metagenomic libraries were generated and sequenced on an Illumina Hiseq 2000
90 instrument in a 2×150 bp paired-end mode at Novogene Bioinformatics Technology.
91 Quality control of the raw reads was conducted using Trimmomatic (version 0.35) [5],
92 including the removal of the adapter sequences, low-quality bases (quality scores < 20),
93 undetermined reads ("N" larger than 5%), and short reads (< 100 bp) [4]. The high-
94 quality paired-end reads from each sample were individually assembled into contigs
95 using MEGAHIT (version 1.1.3) with the recommended parameters (-k-min 21 -k-max
96 141 -k-step 20) [6]. The quality of the resulting contigs was assessed using QUAST 2.3.
97 The metagenomic contigs were queried in Prokka and BLAST against the NCBI non-
98 redundant (nr) protein database using Diamond with default settings [7, 8]. The
99 functional profiling of each metagenomic dataset was achieved by aligning its contigs
100 against clusters of the orthologous groups (COG) database using MEGAN6 Ultimate
101 Edition (version 6.20.5) [9, 10].

102 The functional annotations related to carbon metabolic categories were classified
103 into aromatic and carbohydrate carbon classes (Table S2) [11]. Taxonomic assignment

of the aromatic and carbohydrate genes was carried out using Kraken2 with default settings [12]. To obtain the taxonomic profiles of those genes functionally annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, this particular set of sequence reads was extracted and blasted against the NCBI's non-redundant (nr) protein database using Diamond with the default settings as described previously [3]. Parsing and downstream analysis of the BLAST outputs were performed using MEGAN6 Ultimate Edition [10].

1.5 Genome binning and annotation of MAGs

Genome binning of the assembled contigs was carried out using metaWrap with default settings [13]. The completeness and contamination of the metagenome-assembled genomes (MAGs) were evaluated using CheckM (Version 1.1.2) [14], and the MAGs with completeness > 70% and contamination < 10% were used for further taxonomic assignment and functional analysis. The taxonomic classification was done using GTDB-Tk (v.1.3.0) [15]. Open reading frames were predicted using Prokka (version 1.14.6) and searched against the NCBI-nr and KEGG databases using Diamond with an e-value threshold of 10^{-5} [7, 16]. Phylogenomic trees were constructed using the maximum-likelihood method with IQ-TREE software and adopting the LG+F+R10 model. The alignments comprised 120 bacterial and 122 archaeal marker genes using the GTDB-Tk tool. The maximum-likelihood tree was visualized in the online iTol platform (https://itol.embl.de/) [17]. CoverM was used to calculate the relative

abundances of each MAG with default parameters. The average nucleotide identity (ANI) values between MAGs were calculated based on the get_homologues package with default parameters [18].

1.6 Metatranscriptomic analysis

In total, three cDNA libraries were created from the following three treatments after an incubation period of 160 days: (i) red soil with 0 % NPs (RS-CK); (ii) red soil with 0.5 % NPs (RS-0.5% NPs); and (iii) black soil with 0.5 % NPs (BS-0.5% NPs). Slurry material of the three replicate microcosms of each treatment was mixed to produce a composite sample for cDNA library preparation, using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England BioLabs) according to the manufacturer`s manual. The cDNA yield was determined using a Qubit dsDNA HS assay kit (Qubit, ThermoFisher, USA). The Experion DNA 12K Analysis Kit (Experion, Bio-Rad, USA) was used to evaluate the cDNA quality. The three cDNA libraries were sequenced in paired-end mode (2 × 150 bp) with an Illumina Hiseq 2000 instrument at Novogene (Tianjin, China). A total of 20 Gbp of metatranscriptomic raw sequence data were generated for each sample.

All raw sequences were quality-trimmed using Trimmomatic [5]. The rRNA and tRNA sequences were then filtered using SortMeRNA to extract the mRNA reads [19]. These were blasted against the NCBI-nr database using DIAMOND with the parameters described previously [3]. The BLAST outputs were taxonomically assigned

144 by the “lowest common ancestor” method implemented in MEGAN6 Ultimate Edition,
145 using the top 10 percent of hits with e-value scores below 10^{-5} [10]. Functional
146 annotation of the mRNA reads was done using SEED subsystems and KEGG categories
147 (newly updated version) implemented in MEGAN6 Ultimate Edition. To determine
148 pathway- and gene-specific transcript abundances, mapping of the mRNA datasets onto
149 the methanogen MAGs was performed using Bowtie2 [20] and MEGAN6 Ultimate
150 Edition (version 6.20.5).

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Supplemental discussion

Soil-specific enhancement of CH_4 production in black and red soils

Both the black soil and the red soil harbor highly complex microbial communities that in correspondence to the distinct soil physico-chemical properties [21], majorly differ in their taxonomic composition. This is well evidenced by the soil-specific abundance shifts of the bacterial community in response to the addition of LDPE NPs (Additional File 2: Fig. S11). Indeed, the metagenomic analysis revealed that, dependent on the particular treatment conditions, the 20 most abundant family-level groups in the black soil and the red soil only cover between 7.6% and 24.1% of the total bacterial community.

A total of 391 MAGs were identified to be of high quality (completeness > 70% and contamination < 10%) (Fig. S9). Most of the high-quality MAGs were affiliated with bacterial lineages, including Bacteroidetes (152), Proteobacteria (60), Firmicutes (38), Actinobacteriota (32), Chloroflexota (19), Acidobacteria (17), Dadabacteria (16), Desulfobacterota (6), Elusimicrobiota (5), Planctomyceota (3), Patescibacteria (3), and Omnitrophota (2). Among the various anaerobic methane oxidisers, we only detected *Ca.* Methanoperedenaceae. While its relative metagenomic abundance was below 0.81% across all the black soil treatments, no significant abundance change was observed in response to the NPs addition. No anaerobic methane oxidisers were detected in the red soil.

Driven by both the concentration of NPs and the incubation time, the metagenomic shift towards a significant increase in the methanogen abundance showed a highly significant correlation to the enhancement of CH_4 production ($P < 0.001$). However, CH_4 production was not only in the 0.5% NPs treatments but also in the control treatments significantly lower in the black soil than in the red soil over the complete 160-day incubation period, thereby indicating that soil-inherent traits also had a major impact on the CH_4 production in the 0.5% NPs treatments (compare CH_4 production between the black soil and the red soil in Fig. 1b). In addition to a significantly lower amount of microbial accessible carbon ($P < 0.05$) (Additional File 2: Table S2), the lower CH_4 production in the black soil may be due to a greater bacterial accessibility to alternative electron acceptors, such as Fe(III), Mn(IV), NO_3^-, and SO_4^{2-}, which may have partly suppressed methanogenesis. This view is well corroborated by the fact that relative to the red soil, the microbiota in the black soil showed an extended duration of the lag phase in both the control treatment and the NPs treatments (0.5%, 5%) until CH_4 production occurred. Concomitantly, the transient accumulation of fermentation products, such as acetate and lactate, was delayed (Fig. 1b; Additional File 2: Fig. S5).

Obviously, the soil carbon utilization through the methanogenic food web majorly differed between the black soil and the red soil. In particular, the high abundance of the family Geobacteraceae after 30-day incubation in the black soil suggests an elongated initial reductive phase, which is characterized by utilizing the available, but with

192 incubation time limiting, alternative electron acceptors. LDPE NPs induced, relative to
193 the control, a significant increase in metagenomic abundance of the Geobacteraceae
194 during the first 30 days of incubation ($P < 0.05$) (Additional File 2: Fig. S11), which is
195 well explained by the significant increase in DOC (Fig. 1a). Members of the
196 Geobacteraceae are metabolically versatile. Their key feature is the ability to couple the
197 oxidation of acetate with the dissimilatory reduction of insoluble Fe(III) and Mn(IV),
198 but these anaerobic bacteria are also able to utilize a variety of other electron acceptors,
199 such as NO_3^-, NO_2^-, N_2O, sulfur, fumarate and malate. Depending on the *Geobacter*
200 species, these bacteria are able to oxidize hydrogen and various organic acids [22]. In
201 addition, some *Geobacter* spp. (e.g., *G. netallireducens)* have the capacity to oxidize
202 aromatic compounds, such as benzene and phenol [23, 24]). Accordingly, the
203 metagenomic abundance of the Geobacteraceae in the black soil NPs treatments was –
204 relative to the control (3.76%) - significantly increased to 7.03%-7.36% after the 30-
205 day incubation ($P < 0.05$), albeit no CH_4 (0.5% NPs) or only a low amount of CH_4 (5%
206 NPs) was detectable (compare CH_4 production between the black and red soils in Figs.
207 1b and Additional File 2: Fig. S2). In anoxic rice field soil, alternative electron acceptors
208 are increasingly exhausted with incubation time [25]. In consequence, the metagenomic
209 abundance of the Geobacteraceae in the black soil NPs treatments (0.5%, 5%) had
210 significantly decreased from 7.03%-7.36% after the 30-day incubation period to 1.30%
211 -2.17% after the 160-day incubation period ($P < 0.001$); with the latter being in the
212 abundance range observed for the Geobacteraceae in the red soil across all the

213 treatments (Additional File 2: Fig. S11).

214 The bacterial communities in the two soil types also differed in their putative
215 propionate-oxidizing bacteria. While Peptococcaceae were specifically enriched in the
216 black soil in response to the increasing NPs concentrations, the significant increase in
217 abundance of the Syntrophaceae in the red soil was not driven by NPs but by incubation
218 time ($P < 0.05$) (Additional File 2: Fig. S11). A genus-level analysis revealed the NPs-
219 induced enrichment of *Pelotomaculum* (Peptococcaceae) in the black soil, but the
220 enrichment of *Smithella*-affiliated bacteria (Syntrophaceae) in the red soil with
221 incubation time. Both genera have previously been demonstrated to syntrophically
222 oxidize propionate in rice paddy soils [3, 26-29]. *Syntrophus* spp. are known to
223 anaerobically degrade aromatics (benzoate) and fatty acids in syntrophic association
224 with hydrogenotrophic methanogens [30]. In addition, relative to the control, the
225 metagenomic abundance of Bacillaceae genes encoding H_2-evolving hydrogenases had
226 strongly increased in the LDPE NPs treatments after 160 days of incubation. Members
227 of the Bacillaceae are – contrary to Syntrophomonadaceae – not known to
228 syntrophically oxidize short-chain fatty acids (SCFAs), but are able to release hydrogen
229 as a substrate for hydrogenotrophic methanogens [31]. The increase in metagenomic
230 abundance of Bacillaceae H_2-evolving hydrogenase genes was associated in the NPs
231 treatments with a significant increase in the relative metagenomic abundance of total
232 H_2-evolving hydrogenase genes (compare Additional File 2: Figs. S12a and S12b).

Thus, the increase in metagenomic abundance of Bacillaceae genes encoding H_2-evolving hydrogenases was linked to an increased metagenomic potential for hydrogen production, which additionally may have favored hydrogenotrophic methanogenesis. Finally, as discussed in the main text, the family-level composition of genes encoding the degradation of complex carbohydrates and aromatics clearly differed between the black soil and the red soil (Additional File 2: Figs. S18, S19, S20).

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