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# Decay of low-density polyethylene by bacteria extracted from earthworm's guts: A potential for soil restoration

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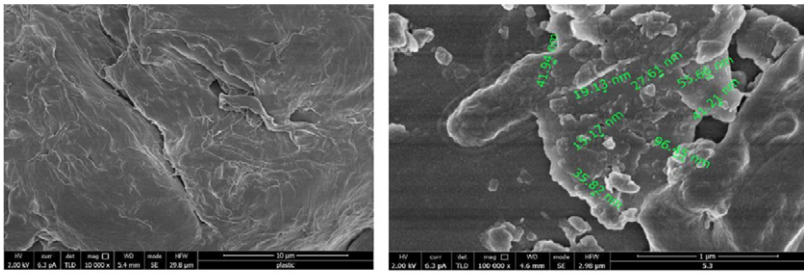
## HIGHLIGHTS

- 60% of microplastics content is decayed with isolated bacteria from *L. terrestris* gut.
- Microplastics size is reduced by the bacteria and nanoplastics are produced.
- Different volatiles were emitted in the treatments with bacteria and microplastics.

## GRAPHICAL ABSTRACT



LDPE without bacteria

LDPE with bacteria
Presence of nanoplastics

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## ABSTRACT

Low-density polyethylene (LDPE) is the most abundant source of microplastic pollution worldwide. A recent study found that LDPE decay was increased and the size of the plastic was decreased after passing through the gut of the earthworm *Lumbricus terrestris* (Oligochaeta). Here, we investigated the involvement of earthworm gut bacteria in the microplastic decay. The bacteria isolated from the earthworm's gut were Gram-positive, belonging to phylum Actinobacteria and Firmicutes. These bacteria were used in a short-term microcosm experiment performed with gamma-sterilized soil with or without LDPE microplastics (MP). We observed that the LDPE-MP particle size was significantly reduced in the presence of bacteria. In addition, the volatile profiles of the treatments were compared and clear differences were detected. Several volatile compounds such as octadecane, eicosane, docosane and tricosane were measured only in the treatments containing both bacteria and LDPE-MP, indicating that these long-chain alkanes are byproducts of bacterial LDPE-MP decay.


## 1. Introduction

Plastic pollution is recognized as a serious global problem. Whereas (micro)plastic pollution in aquatic systems is widely recognized, research concerning plastic pollution in the terrestrial ecosystem began just a few years ago (Huerta Lwanga et al., 2016; Huerta Lwanga et al.,

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2017; Maas et al., 2017; Rillig et al., 2017). Long-term assessments of plastic pollution in soils treated with plastic mulch or with urban sludge containing microplastics are non-existant (Rillig, 2012; Nizzetto and Langaas, 2016). However, there is strong evidence that agricultural soils treated with sludge or plastic mulch are suffering from severe plastic pollution thus monitoring and developing restoration techniques are urgently needed (Steinmetz et al., 2016; Zheng et al., 2017).

Low-density polyethylene (LDPE) is the most common petroleum-based plastic used for mulch in agricultural fields. It is a linear hydrocarbon polymer consisting of long chains of ethylene monomers $(C_2H_4)n$. Recently, Huerta Lwanga et al. (2016) revealed that the earthworm *Lumbricus terrestris* exposed to microplastic LDPE (<150 μm) could uptake LDPE and decrease its particle size (to <50 μm in the earthworm cast) thus facilitating the decay of LDPE as it passes through the gut. We hypothesized that the gut microbiome of *Lumbricus terrestris* contributed to this decay. Although, it is known that microbes isolated from the gut of the larvae of *Plodia interpunctella* (mealmoth) can partially biodegrade PE (Yang et al., 2014) nothing is known so far about the ability of the microbes from earthworm to degrade LDPE.

Therefore, the aim of the present study was to isolate and identify bacteria from the gut of *Lumbricus terrestris* and to test their effect on LDPE microplastic (LDPE-MP) decay in the soil microcosm.

## 2. Material and methods

### 2.1. Bacteria extraction from earthworm gut

Earthworms (*Lumbricus terrestris* species) were exposed to microplastic treatments for 60 days. The treatments consisted of surface microplastics mixed with *Populus nigra* litter w/w as described in Huerta Lwanga et al. (2016).

Six worms were collected from the 7% w/w microplastic treatment. Each worm was rinsed thoroughly with Ethanol and dried carefully with paper tissue. Earthworms were then frozen at −18 °C. Under sterilize conditions, the earthworms were defrosted and opened from the ventral side of the body. The opening was made carefully with the help of sterile chirurgical scissors from the prostomial ring (first ring of the body) to the last ring of the body. The incision was intentionally shallow in order to cut only the epithelium of the worm and keep the gut intact. The epithelium was carefully pushed to one side with the help of sterile entomological nails. Each earthworm was treated separately and the process of gut extraction was done on one worm at the time. The gut of each earthworm was extracted carefully by cutting the internal under tissue, which keeps the epithelium attached to the gut. Once the whole gut was extracted, it was immediately deposited inside a sterile container (one container per gut), which was sealed and stored at −18 °C until the bacterial extraction procedure could be carried out. The bacterial extraction was performed under sterile conditions by adding 3 ml of sterile phosphate-buffer to the container with the earthworm gut and mixing at 20 °C for 45 min. Aliquots of 100 μL of the mixture were used for making series of dilutions. From each dilution, 150 μL was spread in triplicate over 1/10 strength Tryptic Soy Broth agar (TSBA) (Garbeva and de Boer, 2009). The plates were then incubated for seven days at 20 °C.

### 2.2. Bacterial sequencing and identification

PCR amplification of the 16S rRNA genes from the bacterial isolates was performed with isolated DNA using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. The PCR mix contained 0.6 μM each of primers pA and 1492r (Edwards et al., 1989), 1 μL template and 1× GoTaq G2 Green Master Mix (Promega) containing GoTaq G2 DNA polymerase, reaction buffer (pH 8.5), 3 mM MgCl2 and 400 μM of each dNTP. The thermal protocol was as follows: initial denaturation at 95 °C for 2 min and 34 subsequent cycles at 95 °C for 30 s, at 55 °C for 60 s, and 72 °C for 45 s. The final elongation was at 72 °C for 10 min. The 16S rRNA PCR product of each bacterial isolate was purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and sent for sequencing to Macrogen (Amsterdam).

Sequences were trimmed and aligned by using DNAbaser Sequence Assembler v4.36 and NCBI nucleotide database and BLASTn algorithm (Altschul et al., 1990).

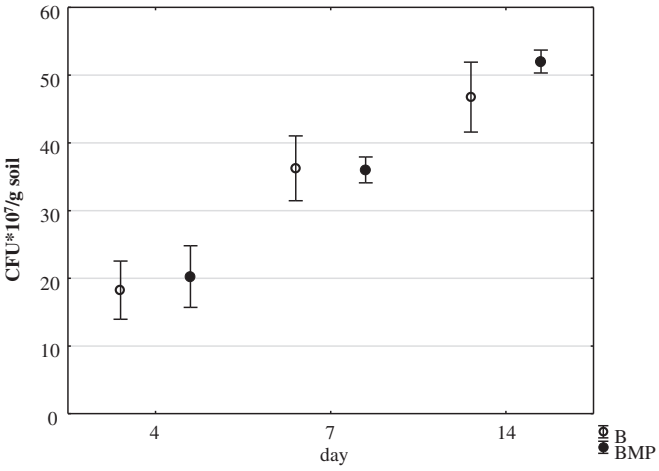


**Fig. 1.** Bacteria growth (CFU per gram soil) during the experiment.

### 2.3. Exposure of bacteria to microplastics and bacterium enumeration

Sandy soil with a low carbon-content and a low amount of mineral nitrogen (0.2 mg/kg nitrite and nitrate) and phosphate (1.1 mg/kg) was collected from an old river dune site near the village of Bergharen (51°10′N, 05°40′E) in the Netherlands. The soil was dried, sieved (ø 2 mm), and gamma-sterilized by Synergy Health Ede B.V. (Netherlands, Schulz-Bohm et al., 2017).

150 μm of low density Polyethylene (LDPE) particles were mixed with 20 g of gamma sterile soil (sandy soil with pH of 5.5 and 0.33% organic matter) at a concentration of 1% (w/w).

The isolated bacteria were inoculated into this soil mixture at a concentration of $10^5$ CFU per g soil for each strain. A control treatment was also established (mixture without bacteria). Four replicas per treatment

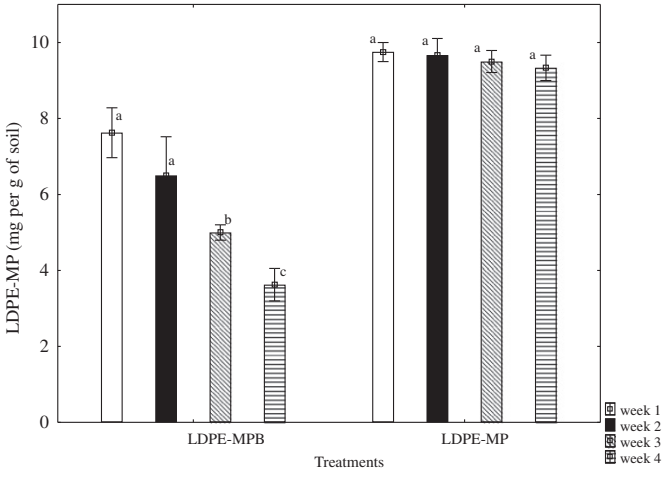


**Fig. 2.** LDPE-MP decay during 4 weeks with (LDPE-MPB) and without bacteria (LDPE-MP).

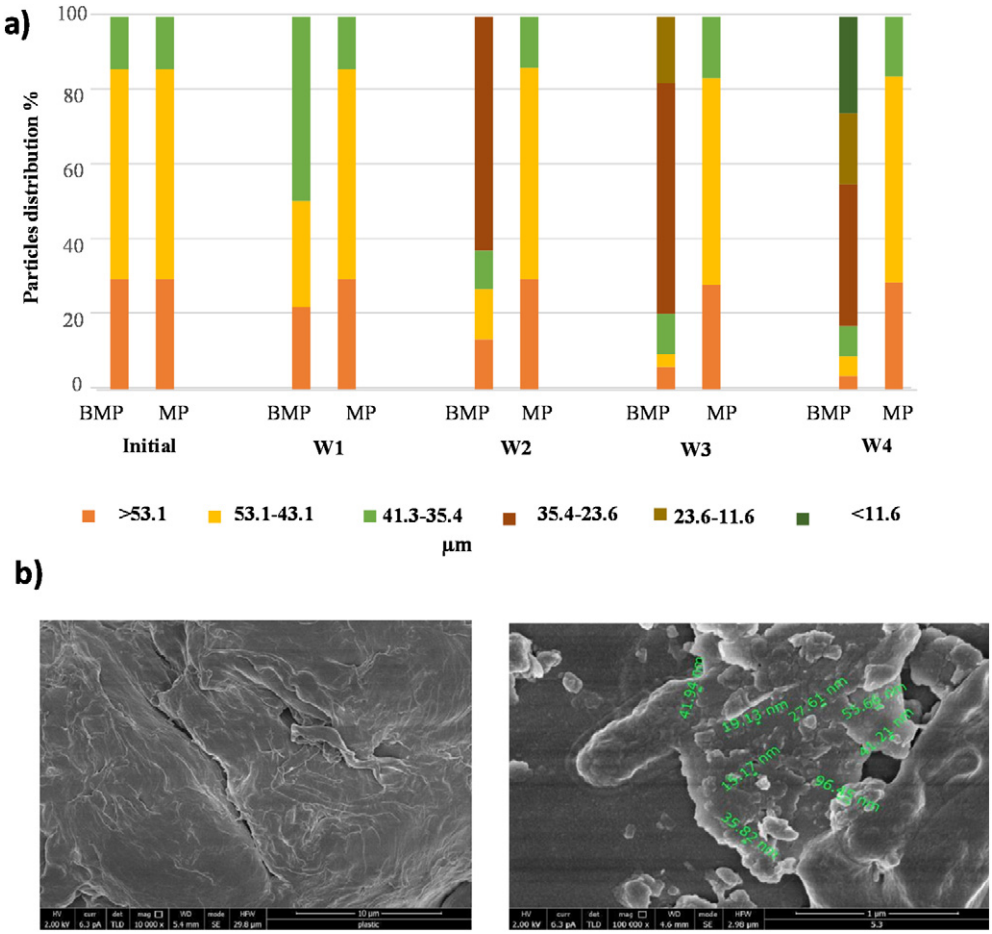


**Fig. 3.** LDPE-MP size during the experiment; a) size distribution with the presence of bacteria (BMP) and without bacteria (MP) b) nanoplastics detection; left, electronic microscope photography of LDPE-MP at the beginning of the experiment, no nanoplastics detected; right, electronic microscope photography at the end of experiment, nanoplastics well detected at day 21.

were used (16, 20 g containers were used per treatment). All treatments were incubated at 20 °C for a period of 21 days.

The bacterial CFU enumeration was carried out every week under sterile conditions. For this, 0.5 g of soil from each glass bottle (experimental unit) was collected and mixed with 1 ml of sterile phosphate-buffer. Subsequently, serial dilutions were prepared and plated in triplicate on 1/10 strength Tryptic Soy Broth agar (TSBA) as described previously by Garbeva and de Boer (2009) and Tyc et al. (2015).

### 2.4. Volatile trapping and measurement

For the collection of volatiles, prior to analysis, PDMS-silicone laboratory tubes (Carl Roth, www.carlroth.com) were prepared in batches as described by Kallenbach et al. (2014). Volatiles were collected for 20 min on the 3, 7 and 14 day. The PDMS tubes were stored at −20 °C until analysis could be carried out. Incubations, including controls without bacteria or without low density Polyethylene (LDPE), were done in four replicates. Trapped volatiles were desorbed using an automated thermodesorption unit (model UnityTD-100, Markes International Ltd., United Kingdom) at 210 °C for 12 min (Helium flow 50 ml/min) and trapped on a cold trap at −10 °C. The trapped volatiles were introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, USA) by heating the cold trap for 3 min to 280 °C. Split ratio was set to 1:20, and the column used was a 30 × 0.25 mm ID RXI-5MS, film thickness 0.25 µm (Restek 13424-6850, USA). The temperature program used was as follows: 39 °C for 2 min, from 39 °C to 95 °C at 3,5 °C/min, then to 165 °C at 6 °C/min, to 250 °C at 15 °C/min and finally to 300 °C at 40 °C/min and held for 20 min. The VOCs were detected using MS operating at 70 eV in EI mode. Mass-spectra's were extracted with MassHunter Qualitative Analysis Software V B.06.00 Build 6.0.633.0 (Agilent Technologies, USA) using the GC-Q-TOF qualitative analysis module. The obtained mass spectra's were exported as mzData files for further processing in MZmine. The files were imported to MZmine V2.14.2 (Pluskal et al., 2010) and compounds were identified via their mass spectra using the deconvolution function (Local-Maximum algorithm) in combination with two mass-spectral-libraries: NIST 2014 V2.20 (National Institute of Standards and Technology, USA http://www.nist.gov) and Wiley 9th edition mass spectral libraries and by their linear retention indexes (LRI). The LRI values were calculated using an alkane calibration mix in combination with AMDIS 2.72 (National Institute of Standards and Technology, USA). The calculated LRI were compared with those found in the NIST and in the in-house NIOO-KNAW LRI database. After deconvolution and mass identification, peak lists containing the mass features of each treatment (MZ-value/Retention time and the peak intensity) were created and exported as CSV files for statistical processing via MetaboAnalyst V3.0 (www.metaboanalyst.ca).

### 2.5. Microplastics decay determination

The size and amounts of microplastics were measured every week. A homogenized sample of 5 g was collected from each container once the

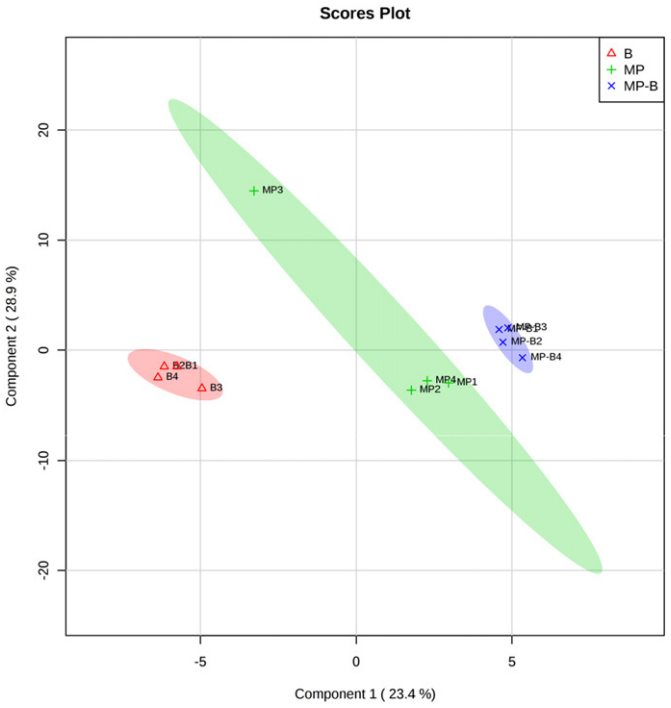


**Fig. 4.** Partial least square-discriminant analysis (PLS-DA) score plots of volatile profiles measured with GC-QTOF-MS at soil with: bacteria (B); with microplastics (MP) and with both bacteria and microplastics (MP-B).

sample was completely dried at 40 °C. The microplastics (LDPE-MP) were separated from the soil as follow: 1) sieving (<150 μm) and 2) flotation (ionized water). The sterile sandy soil had 90% sand and 0.33% organic matter which is a condition that helped with the microplastic separation. Due to the lighter density (<1) of microplastics, they floated and were collected and settled onto a petri dish, for their further examination. Four subsamples from each floating sample were collected and examined with the help of an optic microscope (Laborlux S type microscope, Leitz Wetzlar Germany) and microplastic sizes and amounts were determined.

Nanoplastics were detected at the end of the experiment using transmission electron microscopy (JEOL JEM 2100, with an acceleration voltage of 200 kV and 16 megapixel digital camera). After magnification, the image was captured by the digital camera and thus photos were made.

### 2.6. Statistics

Normal distribution of the data was evaluated by Levene's Test and the samples with normal distribution followed a one-way ANOVA and a Tukey test for identifying significant differences concerning bacteria growth and microplastics decay using Statistica 13.2 software.

Statistical analysis on volatolomic data was performed using MetaboAnalyst V3.0 (Xia et al., 2012; Xia et al., 2015). To identify significant mass features, One-way-ANOVAs with post-hoc Tukey tests (HSD-tests) were performed. To identify important mass features, PLS-D analysis was performed. Mass features were considered to be statistically relevant if p- and FDR-values were ≤0.05. Statistically relevant mass features were further used for compound identification.

## 3. Results

All bacteria isolated from the earthworm's gut were Gram-positive, belonging to phylum Actinobacteria (*Microbacterium awajiense*, *Rhodococcus jostii*, *Mycobacterium vanbaalenii* and *Streptomyces fulvissimus*) and Firmicutes (*Bacillus simplex* and *Bacillus* sp.). No significant differences in bacterial CFU per g soil were observed between the two treatments (Fig. 1).

However, 60% of the LDPE-MP initially present decayed after 21 days in the presence of bacteria (MP-B, Fig. 2). In the gamma-sterilized soil without bacteria, no decay took place during the same period (Fig. 2). The particle size distribution of MP-B was significantly reduced, showing a high number of smaller particles measuring an average of 53.1–41.3 to 35.4–23.6 μm (Fig. 3a). Even nanoparticles were detected at the end of the experiment (Fig. 3b). Clear differences among volatile profiles were revealed between treatments with bacteria and LDPE-MP (Fig. 4). Several volatile compounds such as octadecane, eicosane, docosane and tricosane were detected only in the soil treatment that included both LDPE-MP and bacteria (MP-B) (Fig. 5).

## 4. Discussion

The potential of the polyethylene-degrading microorganisms have become a research focus for more than a decade. Plastic degrading bacteria and fungi were isolated from various environments such as soil rhizospheres, polyethylene contaminated sites, and soil dumping sites (reviewed in Kale et al., 2015). The Gram-positive bacteria *Brevibacillus borstelensis* and *Rhodococcus ruber* were reported to have the capacity to degrade the CH2 backbone of plastics and use polyethylene as their sole carbon source (Hadad et al., 2005). So far, most experiments with polyethylene-degrading microorganisms were performed for longer periods of time, often ranging from 3 to 9 months. However, our results revealed that bacterial consortium isolated from the gut of the earthworm *Lumbricus terrestris* were able to significantly reduced the size of LDPE-MP within 4 weeks.

Several volatile compounds such as octadecane, eicosane, docosane and tricosane were detected only in the soil treatment that included both LDPE-MP and bacteria (MP-B). This indicates that these long chain alkanes are byproducts of LDPE-MP decay since they were not detected in sterile soil with LDPE-MP or in soils without bacteria. This formation of alkanes can be seen as a product of the break down the long C chains of the LDPE-MP. Recently, the same volatile compounds were reportedly produced by *Pseudomonas* spp. during polythene degradation (Kyaw et al., 2012). Thus, volatile compounds are important constituents of LDPE-MP degradation. In soil, volatile compounds play a major role in belowground microbe-microbe and plant-microbe interactions and fulfil important ecosystem functions (Schmidt et al., 2015; van Dam et al., 2016). However, the effect of volatile compounds produced as a result of microbial LDPE-MP decay on soil (micro) organisms and their interactions remains largely unknown.

## 5. Conclusion

LDPE has long been thought of and sold on the market as non-biodegradable. Our results revealed that bacterial consortium were able to significantly reduce the size of LDPE and are very promising with respect to the development of restoration techniques for LDPE-polluted soils.

## Acknowledgement

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## Author contributions

EHL, BT and PG did laboratory work. TS and HG helped in technical issues in laboratory. EH wrote paper together with PG, VG, XY and BT.

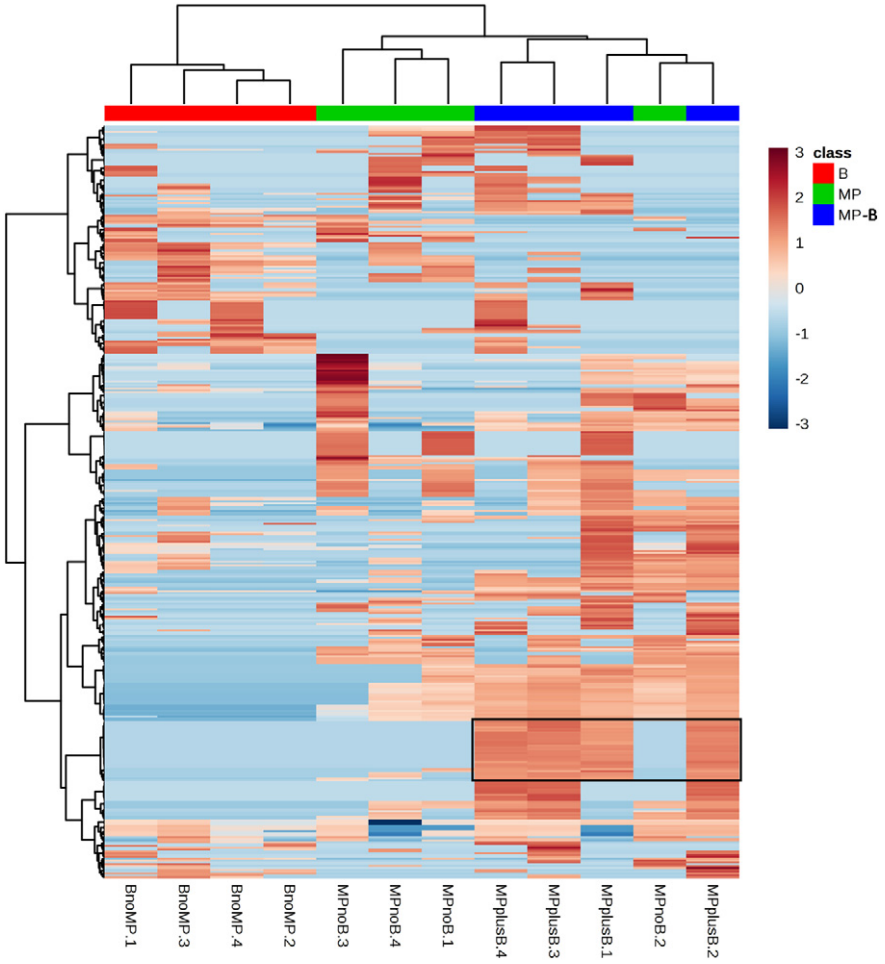


**Fig. 5.** Heat-map analyses of the volatile profiles. Columns represent three replicate measurements per treatments (soil with: bacteria (B); with microplastics (MP) and with both bacteria and microplastics (MP-B)). Each colored cell on the map corresponds to a concentration value per compound (blue, low abundance; red, high abundance). The black box indicates volatiles detected only in treatment MP-B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## Competing financial interests

There is no conflict of interest.

This is publication 6446 of the NIOO-KNAW.

## References


Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Edwards, U., Rogall, T., Blocker, H., Emde, M., Bottger, E.C., 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. 17 (19), 7843–7853.

Garbeva, P., de Boer, W., 2009. Inter-specific interactions between carbon-limited soil bacteria affect behavior and gene expression. Microb. Ecol. 58, 36–46.

Hadad, D., Geresh, S., Sivan, A., 2005. Biodegradation of polyethylene by the thermophilic bacterium *Brevibacillus borstelensis*. J. Appl. Microbiol. 98, 1093–1100.

Huerta Lwanga, E., Gertsen, H., Gooren, H., Peters, P., Salanki, T., Van der Ploeg, M., et al., 2016. Microplastics in the terrestrial ecosystem: implications for Lumbricus terrestris (Oligochaeta, Lumbricidae). Environ. Sci. Technol. 50, 2685–2691.

Huerta Lwanga, E., Gertsen, H., Gooren, H., Peters, P., Salanki, T., Van der Ploeg, M., et al., 2017. Incorporation of microplastics from litter into burrows of *Lumbricus terrestris*. Environ. Pollut. 220, 523–531.

Kale, K.S., Deshmukh, A.G., Dudhare, M.S., Patil, V.B., 2015. Microbial degradation of plastic: a review. J. Biochem. Technol. 6 (1), 952–961.

Kallenbach, M., Oh, Y., Eilers, E.J., Veit, D., Baldwin, I.T., Schuman, M.C., 2014. A robust, simple, high-throughput technique for time-resolved plant volatile analysis in field experiments. Plant J. 78, 1060–1072.

Kyaw, M.B., Champakalakshmi, R., Sakharkar, M.K., Lim, C.S., Sakharkar, K.R., 2012. Biodegradation of low density polyethylene (LDPE) by *Pseudomonas* species. Indian J. Microbiol. 52 (3), 411–419.

Maas, S., Daphi, D., Lehmann, A., Rillig, M.C., 2017. Transport of microplastics by two collembolan species. Environ. Pollut. 225, 456–459.

Nizzetto, L., Langaas, S., Futter, M., 2016. Pollution: do microplastics spill on to farm soils? Nature 537, 488.

Pluskal, T., Castillo, S., Villar-Briones, A., Oresic, M., 2010. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinformatics 11, 395.

Rillig, M.C., 2012. Microplastic in terrestrial ecosystems and the soil? Environ. Sci. Technol. 46, 6453–6454.

Rillig, M., Ziersch, L., Hempel, S., 2017. Microplastic transport in soil by earthworms. Sci. Rep. 7, 1362.

Schmidt, R., Cordovez, V., de Boer, W., Raaijmakers, J., Garbeva, P.V., 2015. Volatile affairs in microbial interactions. ISME J. 9, 2329–2335.

Schulz-Bohm, K., Geisen, S., Wubs, J., Song, C., de Boer, W., Garbeva, P., 2017. The prey' s scent – Volatile organic compound mediated interactions betweens oil bacteria and their protist predators. ISME J. 11, 817–820.

Steinmetz, Z., Wollmann, C., Schaefer, M., Buchmann, C., David, J., Troger, J., et al., 2016. Plastic mulching in agriculture. Trading short-term agronomic benefits for long-term soil degradation? Sci. Total Environ. 550, 690–705.

Tyc, O., Wolf, A.B., Garbeva, P., 2015. The effect of phylogenetically different bacteria on the fitness of Pseudomonas fluorescens in sand microcosms. Plos One 1–11.

van Dam, N.M., Weinhold, A., Garbeva, P.V., 2016. Calling in the dark: the role of volatiles for communication in the rhizosphere. In: Blande, J.D., Glinwood, R. (Eds.), Deciphering Chemical Language of Plant Communication. Springer, Bonn, pp. 175–210.

Xia, J., Mandal, R., Sinelnikov, I.V., Broadhurst, D., Wishart, D.S., 2012. MetaboAnalyst 2.0–a comprehensive server for metabolomic data analysis. Nucleic Acids Res. 40, W127–W133.

Xia, J., Sinelnikov, I.V., Han, B., Wishart, D.S., 2015. MetaboAnalyst 3.0-making metabolomics more meaningful. Nucleic Acids Res. 43, W251–W257.

Yang, J., Yang, Y., Wu, W.-M., Zhao, J., Jiang, L., 2014. Evidence of polyethylene biodegradation by bacterial strains from the guts of plastic-eating waxworms. Environ. Sci. Technol. 48, 13776–13784.

Zheng, W., Wen, M., Zhao, Z., Liu, J., Wang, Z., Zhai, B., et al., 2017. Black plastic mulch combined with summer cover crop increases the yield and water use efficiency of apple tree on the rainfed Loess Plateau. PLoS One 1–17.