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via DNA-Stable Isotope Probing: comparison with
enrichment culture**

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Culture-independent analysis of linuron mineralizing microbiota and
functions in on-farm biopurification systems via DNA-Stable Isotope
Probing: comparison with enrichment culture

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15 Abstract

16 Our understanding of the microbiota involved in *in situ* biodegradation of xenobiotics like pesticides in
17 natural and engineered environments is poor, taking into account the large fraction of uncultivables. On-
18 farm biopurification systems (BPSs) treating farm-produced pesticide-contaminated waste water are
19 interesting environments to study microbial community adaptation. DNA-stable isotope probing (DNA-
20 SIP) identified *Ramlibacter* and *Variovorax* as the main *in situ* assimilators of the phenylurea herbicide
21 linuron in the BPS matrix. While *Variovorax* strains are common linuron-degrading isolates, *Ramlibacter*
22 was never before implicated in linuron degradation. Known catabolic genes for linuron hydrolysis and
23 aromatic ring conversion were enriched in the ^{13}C -DNA fractions, as well as mobile genetic elements
24 previously linked to linuron catabolic genes, suggesting that *in situ* linuron assimilation related to
25 known catabolic modules which are prone to horizontal gene transfer. Soil free culturing of linuron
26 degraders from the BPS sample resulted in a community dominated by *Variovorax* while *Ramlibacter*
27 was not recovered. Our work provides evidence for the role of the common linuron degrading isolate
28 *Variovorax* in *in situ* linuron biodegradation but also shows that culturing only provides a partial picture
29 of the *in situ* linuron degrading microbiota in the BPS environment.

30

31 INTRODUCTION

32 The toxic properties of pesticides often extend beyond their intended targets and impose a major
33 environmental and health concern. Point source contamination, i.e., the uncontrolled release of
34 pesticides at high concentrations, often originates at the farm yard during the cleaning and filling of
35 spraying equipment¹. On-farm biopurification systems (BPSs), consisting of an excavation or container
36 that contains a matrix composed of soil, lignocellulosic material and humified organic substrate such as
37 peat, treat pesticide contaminated wastewater at farm yards through biodegradation and sorption¹⁻⁴.

Besides their application in water treatment, BPSs are considered interesting environments for studying genetic adaptation to pesticides and organic xenobiotics. They are operated on a long-term and continuous base with high pesticide loads, thereby imposing a strong selective pressure for the evolution and proliferation of pesticide degrading bacteria^{5,6}. A well-studied pesticide in the context of the bacterial ecology of xenobiotic biodegradation, is the phenylurea herbicide linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methyl urea)⁷. Culture-dependent studies, mainly targeting agricultural soil, indicate that biodegradation is performed by either individual bacterial strains or consortia. Strains of the genus *Variovorax* seem to play a crucial role by either performing the complete mineralization, or by performing at least the initial hydrolysis step in consortia^{8–11}. Various genes involved in linuron catabolism have been identified. *libA*¹² and *hylA*¹³, identified in *Variovorax*, encode for enzymes that hydrolyze linuron to 3,4-dichloroaniline (DCA), while *dcaQTA1A2BR* and *ccdRCFDE* encode for the conversion of DCA to chlorocatechol and of chlorocatechol to oxoadipate, respectively. These catabolic functions are often located on mobile genetic elements, indicating that horizontal gene transfer contributes to their evolution and dissemination^{14,15}. Initial studies using targeted qPCR approaches, indicated that *Variovorax* and the above mentioned catabolic genes are also relevant for linuron degradation in BPS. However, these studies intentionally used agricultural soil inhabited by linuron degrading *Variovorax* strains as an ingredient of an artificial BPS matrix to assess bioaugmentation with linuron acclimated materials^{16–19}. Recent studies on an operational BPS environment showed that *Variovorax* associated linuron catabolic genes are present on mobile genetic elements (MGEs)^{6,20} but their indigenous hosts remain elusive.

While various pesticide degrading isolates have been reported, knowledge about the microbiota responsible for *in situ* pesticide degradation is scarce and the contribution of isolates to the *in situ* process is not clear. Stable Isotope Probing (SIP) and in particular Nucleic acid based Stable Isotope Probing (RNA/DNA-SIP) holds a great promise to culture-independently link identity with *in situ*

functionality in complex communities and reveal uncultivated micro-organisms and the catabolic functions that govern biodegradation in natural and engineered habitats^{21–23}. Only few studies identified *in situ* degraders of pesticides using SIP and none of those have taken into account functionality. Moreover, most studies examined agricultural soil, but the engineered environment of a bioremediation system, and in particular the complex BPS environment, have yet to be scrutinized. In addition, only few pesticide compounds were targeted and none of them belong to the environmentally important group of phenylurea herbicides²³.

In this study, DNA-SIP combined with 16S rRNA gene amplicon sequencing was used to identify *in situ* degraders of linuron in the complex environment of a BPS material with a history of linuron exposure, in a microcosm setup. In addition to 16S rRNA gene amplicon sequencing, PCR targeting known linuron catabolic genes as well as genes for MGEs, was used to assess their implication in *in situ* linuron biodegradation. Two time points were considered, spanning a period of 440 days, to determine robustness. Moreover, to examine whether DNA-SIP and culturing identified the same linuron catabolic organisms and gene functions, and hence to estimate the effect of culturing bias, an enrichment culture (EC) derived from the BPS material was likewise examined regarding community composition and functionality.

MATERIALS AND METHODS.

Chemicals used. Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methyl urea] (purity, 99.5%) was purchased from Sigma Aldrich (St. Louis, MO) and ¹⁴C linuron ([phenyl-U-14C]-linuron) (16.93 mCi/mM, radiochemical purity >95%) from Izotop (Hungary). ¹³C linuron ([phenyl-U-13C] linuron) was synthesized in house based on a protocol modified from²⁴. The procedure of in-house synthesis is provided in the supporting material.

Microcosm setup. Microcosms were set up in glass cylinders of 4 cm diameter and 10 cm height and equipped with glass filters as described²⁵. The cylinders were filled to a height of 7 cm with sieved (2 mm) BPS matrix material. The BPS material originated from a BPS installed in 2013 at the Inagro agricultural research center (Kortrijk, Belgium) and was composed of 50% (v/v) coconut husks, 40% (v/v) potting soil and 10% (v/v) agricultural soil. Prior to sampling, the BPS received wastewater contaminated with a pesticide mixture that included linuron, for 2 years. The list of pesticides used at Inagro during that period is provided in the supporting material. Around 2 kg BPS matrix was collected in October 2015 from the upper 10 cm layer of matrix in the top container of the system, at positions where the wastewater was distributed over the matrix surface. Samples were mixed and used in the set up of triplicate microcosms for two treatments. The first treatment consisted of an irregular irrigation (three times per week) with 1 mL of sterile water containing 20 mg L⁻¹ linuron (BPS⁺) while in the second treatment the microcosms were irrigated with 1 mL of sterile water without linuron (BPS⁻). The microcosms were incubated at 25 °C in the dark.

DNA-SIP analysis. DNA-SIP experiments were set up in 20 mL pyrex glass-tubes containing material from the microcosms taken after 180 days (SIP-1) and 440 days (SIP-2) of treatment. SIP-1 was set up by spiking 0.5 gram of material taken from one BPS⁺ and one BPS⁻ microcosm, with 0.5 ml MS medium²⁶ containing either 100 mg L⁻¹ of ¹³C-ring labeled linuron or 100 mg L⁻¹ non-labeled linuron, in addition to 11.65 µg l⁻¹ (~80 bq ml⁻¹) ¹⁴C ring-labeled linuron. The vials were incubated at 20 °C on a rotary shaker at 120 rpm in the dark. The vials were equipped with CO₂-traps that were sampled regularly and the radioactivity was determined to generate cumulative mineralization curves as reported²⁷. Six and three replicates were initiated using BPS⁺ and BPS⁻ material, respectively. Assay vials without microcosm material were included as negative controls for mineralization.

SIP-2 was set up identically to SIP-1, except that the used material consisted of pooled material from all BPS⁺ or BPS⁻ microcosms and that no ¹⁴C ring-labeled linuron was spiked. Instead, an identically ¹⁴C-ring labeled linuron mineralization-assay setup was conducted in parallel.

After incubation, DNA was extracted from all replicate SIP-1 and SIP-2 BPS⁺ systems following the SDS-based method described by Zhou *et al.*²⁸ with proportional adaptations in the solution volumes to accommodate 0.25 g material. No DNA was extracted from BPS⁻ material since linuron was not mineralized. The DNA was dissolved in 10 mM Tris-HCl buffer (pH 8). In case of SIP-1, DNA extracts of 5 replicates were pooled for each tested condition and the pooled DNA and residual non-pooled DNA extract were analyzed separately. Similarly, SIP-2 DNA extracts were pooled and the pooled DNA analyzed.

In both SIP-1 and SIP-2, isopycnic ultracentrifugation on a linear CsCl gradient and collection of fractions after centrifugation were performed as described²⁹. The buoyant density of the fractions was determined through measurement of the refractive index using a AR200 Digital Refractometer (Reichert, Depew, NY)³⁰. The DNA in the fractions was precipitated using a PEG6000 solution (30%), washed with ethanol (70%), eluted in 10 mM Tris-HCl buffer (pH 8) and stored at -20 °C.

Culture-dependent enrichment of linuron degraders. Two hundred mg of BPS material was incubated in 100 mL Erlenmeyer flasks containing 10 mL of MMO medium⁸ (containing ammonium to avoid N-limitation) amended with 20 mg L⁻¹ linuron as the sole carbon source. Linuron concentrations were determined over time as explained³¹. When the linuron concentration was below detection (3 µg L⁻¹), 1 mL of the culture was transferred to fresh linuron (20 mg L⁻¹) containing MMO medium. Transfer of the culture was conducted twice. The second transfer was conducted in triplicate to obtain sufficient biomass for DNA extraction. Upon linuron depletion, the three replicate cultures were pooled and DNA was extracted using a CTAB (cetyl trimethylammonium bromide) based DNA extraction method³²

PCR and denaturing gradient gel electrophoresis (DGGE) analysis.

131 The sequences of the primers used for PCR and the PCR conditions are reported in supplementary table
132 S1. PCR reactions were performed in a Biometra thermocycler (Analytik Jena, Germany) or Eppendorf
133 Mastercycler (Eppendorf, Germany) apparatus. PCR products were visualized after agarose gel
134 electrophoresis (1.5%) in Tris-acetate/EDTA buffer (90 V for 1 h) using GelRed. 16S rRNA gene fragments
135 for DGGE were amplified by PCR using bacterial primer sets 63F and 518R³³ or 8F and 533R³⁴. Forward
136 primers contained a GC clamp sequence (5'-CGCCCGCCG CGCGCGGCGGGCGGGGCGGGGCGGGGGCACGGGGGG-
137 3') at the 5' end. In case of insufficient amplification, a nested PCR approach was implemented using the
138 bacterial 16S rRNA gene primers GM3F and 908R in the first PCR³⁴. DGGE was performed in a C.B.S
139 scientific (Del Mar, CA) DGGE system using 8% acrylamide gels with a linear denaturing gradient ranging
140 from 35% to 65% of urea and formamide and was operated at 120 V for 16 h as described³⁵.

141 **16S rRNA gene amplicon sequencing and data analysis.** For 16S rRNA gene amplicon sequencing a
142 nested PCR was performed. The first PCR targeted the V1-V5 region of the bacterial 16S rRNA gene using
143 primers GM3F and 908R³⁴. PCR conditions are reported in supplementary table S1. The obtained V1-V5
144 amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and used as
145 template for the second PCR. The second PCR targeted the V4 region using primers A519F and 802R³⁴
146 amended with overhang adapter sequences. The amplicons were obtained and purified according to the
147 Illumina "16S Metagenomic Sequencing Library Preparation" protocol (Illumina, San Diego, CA). Library
148 preparation, sequencing on the Illumina MiSeq platform and sequencing analysis were performed as
149 described³⁶. Quality control and sequencing analysis resulted in 83.531 reads per sample after
150 subsampling according to the sample with the lowest number of valid reads. Phylogeny was assigned
151 using Silva Database V.128^{37,38} and sequences grouped in Operational Taxonomic Units (OTUs) at an
152 identity threshold of 97%. Rarefaction analysis and Good's coverage determination were done as
153 described³⁶. Sequence analysis was performed on Bioline 8³⁹.

Cloning and Sanger sequencing of 16S rRNA gene amplicons. 16S rRNA gene amplicons, obtained using primer set 27F and 1492R or 8F and 533R using the PCR conditions described in supplementary table S1, were cloned into *E. coli* (Mix & go Competent Cells – Strain Zymo 5α, Zymo Research, Irvine, CA) using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Cloned 16S rRNA gene fragments were sequenced using the 27F and 1492R or 8F and 533R primers on a Sanger platform (GATC Biotech AG, Germany), retrieving around 1300 bp or 520 bp long 16S rRNA gene sequences, respectively. Sequence similarity was determined using NCBI Blast⁴⁰. Maximum likelihood phylograms of relevant 16S rRNA gene sequences were obtained after alignment using Muscle v3.8.31⁴¹ and implementing an 1000x bootstrap analysis using RAxML v.8.2⁴².

RESULTS

Linuron mineralization activity in BPS material and selection of time points for DNA-SIP analysis.

DNA-SIP experiments were initiated at day 180 (SIP-1) and day 440 (SIP-2) from BPS⁺ and BPS⁻ microcosm materials. Mineralization was monitored based on CO₂ production from ¹⁴C-labeled linuron. Linuron was clearly mineralized, but only in the BPS⁺ microcosms, with a 35% to 50% conversion to CO₂, with the residual carbon likely assimilated into biomass (Figure S1). The assumption that mineralization was accompanied by growth was supported by the mineralization kinetics, which fitted the Gompertz kinetic model⁴³. Kinetics of linuron mineralization differed between SIP-1 and SIP-2. The lag phase was shorter in SIP-2 as compared to SIP-1, likely because the material of the BPS⁺ microcosms contained a higher number of linuron-degraders due to the longer feed with linuron^{16,19,25,44}. Furthermore, more CO₂ was produced in SIP-1 compared to SIP-2. Differences in CO₂ from linuron mineralization were reported previously⁴⁴. In order to avoid cross-labeling of peripheral populations, it was decided to take samples for DNA-SIP in the late exponential phase, when most linuron was likely mineralized, maximum growth was attained and no decay of the linuron mineralizers was expected yet.

DNA-SIP: microbial community analysis. For both SIP-1 and SIP-2, heavy ^{13}C -labeled DNA fractions that showed distinct DGGE bands compared to other DNA fractions of the ^{13}C -linuron amended incubations, as well as to the non-labeled DNA fractions of corresponding density, were identified (Figure S2). In SIP-1, two clearly separated unique bands were identified (Figure S2 panel A) while SIP-2 showed two closely located unique bands (Figure S2 panel B). DGGE analysis that compared relevant SIP-1 and SIP-2 fractions using the same primer set for 16S rRNA gene amplification, revealed that the lower unique band in the ^{13}C -DNA fraction of SIP-1 consisted of two closely migrating bands that migrated similarly to the two unique bands found in the ^{13}C -DNA fractions of SIP-2. The upper unique band in the ^{13}C -DNA fraction of SIP-1 consisted of two closely migrating bands but with no counterpart in the SIP-2 ^{13}C -DNA fractions (Fig S2 panel C). Based on the DGGE results, relevant fractions (see Figure S2 panel B) obtained from the ^{13}C -linuron amended samples and corresponding samples from the unlabeled linuron amended samples, were selected for 16S rRNA gene amplicon sequencing. In case of SIP-2, the respective fractions were pooled prior to sequencing. 16S rRNA gene amplicons obtained from non-fractionated DNA of SIP-1 was likewise sequenced.

Rarefaction curves of the sequence reads showed full asymptotes or at least 98% coverage, indicating robust taxonomic sampling (Figure S3, Table S2). A clearly increased relative abundance of an OTU assigned to the genus *Ramlibacter* was observed in the heavy fractions of the SIP-1 ^{13}C -linuron amended samples (37%-39% relative abundance), compared to its proportion in the light density DNA fractions of the ^{13}C -labeled linuron amended samples (0.08% - 0.09%) and in the non-fractionated DNA from ^{13}C -linuron amended samples (0.8% - 0.95%). The relative abundance of *Ramlibacter*-associated sequences was also significantly lower in both heavy (0.62% - 1.3%) and light (0.17% - 0.5%) DNA fractions of the ^{12}C -linuron amended samples and in DNA of the non-labeled linuron amended samples prior to centrifugation (0.57% - 0.95%) (Figure 1 panel A). Highly similar results were found for the non-pooled replicate DNA extracts (Figure S4), suggesting *Ramlibacter* as the organism assimilating the carbon from

linuron in the BPS. However, *Ramlibacter* is closely related to *Variovorax* and we found that the 16S rRNA gene of *Variovorax* type strains can exhibit up to 99.27% sequence similarity in the V4 region with *Ramlibacter* species, implying that *Variovorax* sequences could have been mistakenly assigned to the *Ramlibacter* OTU. Manual inspection of the individual sequences assigned to the *Ramlibacter* OTU, revealed that a sequence 100% identical to the V4 region of various *Variovorax* strains and in particular of a clade of strains that includes most of the linuron degrading *Variovorax* sp. strains (such as strains WDL1, PBS-H4 and PBL-H6), contributed significantly (i.e., $10.11 \pm 1.59\%$) to the *Ramlibacter* OTU. Both *Ramlibacter* and *Variovorax* sequences showed a high impact of the ^{13}C -labeling on relative abundances, i.e., the *Ramlibacter* sequences showed a 70-fold and 59-fold increase in the heavy ^{13}C -DNA fractions compared to the heavy non-labeled DNA fractions and the samples prior to centrifugation, respectively, while the *Variovorax* associated sequences displayed an 11-fold increase and 34 fold increase, respectively. We conclude that next to *Ramlibacter*, also *Variovorax* and especially strains belonging to its “linuron degrading” clade assimilated the carbon from linuron in SIP-1.

The OTU related to *Ramlibacter* was also the only enriched OTU in the ^{13}C -linuron amended fractions in SIP-2. The OTU showed a 18- and 17-fold higher abundance in the heavy ^{13}C -DNA fractions (4.7%) compared to its abundance in the total community DNA prior to centrifugation (0.26%) and to the corresponding fractions of the non-labeled linuron amended systems (0.27%), respectively (Figure 1 panel B). *Variovorax* associated sequences were not recovered in the heavy DNA fractions of the ^{13}C -labeled sample.

The observation that *Variovorax* sequences were erroneously assigned to the *Ramlibacter* OTU and the knowledge that *Variovorax* is a well-known linuron degrader while *Ramlibacter* is not, prompted us to obtain more sequence evidence for the involvement of *Ramlibacter* in linuron assimilation in the BPS environment. To this end, almost full 16S rRNA gene sequences were obtained from the ^{13}C -DNA fractions of SIP-1 by random cloning and sequencing of 16S rRNA gene amplicons recovered using

primers 27F and 1492R. Three cloned 16S rRNA gene fragments showed dual banding patterns on DGGE that corresponded to the unique two lower DGGE bands found in the ^{13}C -DNA fractions of SIP-1 and SIP-2 (represented by clone 3.1, Figure 2) and their sequences phylogenetically most related to the *Ramlibacter* genus showing 98.4% – 98.7% identity to *Ramlibacter* type species 16S rRNA sequences and 95.8% – 96.1% to *Variovorax* sequences (Figure 3). However, none of the clones corresponded to the two other (higher) unique bands observed in ^{13}C -DNA fractions of SIP-1. Therefore, these bands were cut from the gel and cloned. Two clones (DGGE 12 and DGGE 18) displayed the dual band migration at the expected location in the DGGE gel (Figure S5). As found for clone 3.1, the 520 bp sequences of the DGGE-clones 12 and 18, which do not include the V4 region, related most to *Ramlibacter* (Figure S6). Interestingly, while the almost full length 16S rRNA clones identified as *Ramlibacter* showed 99.8% - 100% identity to one another in that region, they showed only 97.3% - 98.3% identity to the DGGE clones. The clones obtained from the DGGE bands show a seemingly identical DGGE migration pattern as the linuron degrading isolate *Variovorax* sp. WDL1, whose V4 region of the 16S rRNA sequence is identical to the sequence of the SIP-1 *Variovorax* subpopulation. However, comparison of the sequences through BLAST alignment supported the assignment of those clones to the *Ramlibacter* genus (97.2 – 97.8% identity) rather than the *Variovorax* genus (94.8% – 94.9% identity to type species, 95.4% – 96% identity to linuron degrading strains), which was further supported by phylogram calculation (Figure S6). Cloning of the SIP-1 DNA-fractions produced no clones that related to *Variovorax*, while cloning of the SIP-2 DNA-fractions did not produce any clones derived from *Ramlibacter* or *Variovorax*, likely due to its relatively low abundance compared to other OTUs.

DNA-SIP: functional gene analysis. The DNA-SIP fractions were tested by PCR for the presence of marker genes related to linuron catabolism, i.e., *hlyA*, *libA* and *dcaQ* as well as to horizontal gene transfer (HGT) and MGEs, i.e., the IncP1-plasmid marker gene *trfA* and the insertion sequence *IS1071* that were previously associated with linuron catabolic genes^{6,14,15}. In SIP-1, the three catabolic markers

and the two MGE markers, were enriched in the heavy fractions of the ^{13}C -linuron amended samples while little or no amplification occurred in the low density fractions of the ^{13}C -linuron amended samples and in the corresponding fractions of the non-labeled linuron amended systems. Instead, amplification was observed in the lower density DNA fractions of the non-labeled linuron amended systems (Figure 4 panel A). The SIP-2 fractions exhibited similar results with few exceptions. *libA* was not amplified in any of the DNA fractions (Figure 4 panel B). Moreover, the shift of *IS1071* towards the heavy fractions of the ^{13}C -linuron amended sample, was less pronounced than in SIP-1 and *trfA* showed an even distribution across all fractions derived from both the ^{13}C -linuron and non-labeled linuron amended sample.

Cultivation-dependent isolation: enrichment of linuron degraders from BPS material. A soil-free EC that rapidly degraded linuron was obtained from the original BPS sample material (Figure S7). Rarefaction analysis of the 16S rRNA gene amplicon reads suggested robust sampling with high coverage (Figure S3, Table S2). The EC was dominated by 3 major OTUs identified as *Pseudomonas* (31%), *Variovorax* (19%) and an unclassified genus associated with the *Comamonadaceae* family (17%) (Figure 5). The *Variovorax* OTU related to the clade of linuron degrading *Variovorax* isolates and the unclassified *Comamonadaceae* to *Acidovorax*. Other notable OTUs were associated with *Hyphomicrobium* (7%), *Comamonas* (4%), and a second unclassified *Comamonadaceae* OTU (4%). The latter OTU was comprised of 50% *Rhodoferrax* and 50% *Variovorax* sequences of which the *Variovorax* sequences most related to *Variovorax boronicumulans*, suggesting the presence of at least two distinct *Variovorax* populations in the EC. No *Ramlibacter* associated sequences were recovered (Figure 5). To acquire further information on the phylogeny of the EC organisms, almost full 16S rRNA genes were cloned, sequenced and phylogenetically analyzed as well as analyzed by DGGE. The sequencing of the 16S rRNA gene clones agreed with the amplicon sequencing results. Two clones not only shared 98.85% sequence similarity in the near full-length 16S rRNA sequence with one another, but also identically matched the V4 region of the *Variovorax* sequences recovered through amplicon sequencing. Phylogenetically the

clones related most to the reported *Variovorax* clade containing the linuron degraders (Figure 3). A third clone showed 99.6% similarity to the *Comamonas* OTU and corresponded to the 16S rRNA gene sequence of a *Comamonas* strain previously isolated from linuron degrading consortia^{8,10} (Figure 3), while the two remaining clones most related to *Acidovorax*, matching the *Acidovorax* OTU consensus sequence (99.6% and 100%). Clear differences were evident between the EC community DGGE profile and the profiles obtained from the SIP-1 and SIP-2 ¹³C-fractions, most notably the absence of the *Ramlibacter* associated band represented by SIP-1 clone 3.1, in the EC community. Note that the band derived from EC clone 1.10 migrated to the same height as the (upper) unique DGGE band identified in the SIP-1 ¹³C-DNA fraction (identified as *Ramlibacter*), migrated to the same height as the band derived from EC clone 1.10, despite having different sequences in the region used for DGGE analysis. The EC exhibited PCR amplification of all tested catabolic and MGE marker genes (data not shown).

DISCUSSION

Relevance of the selected sampling time points for identification of *in situ* linuron degraders through

DNA-SIP. In the present study, we identified the organisms responsible for linuron *in situ* mineralization in a BPS environment through application of DNA-SIP. The time point of DNA extraction after the addition of ¹³C-labeled linuron was chosen in the late logarithmic growth phase of the mineralizing bacteria, as indirectly determined through monitoring ¹⁴CO₂ production from ¹⁴C-labeled linuron. The cumulative mineralization curves showed a logarithmic profile and fit the Gompertz kinetic model, indicating that the compound was mineralized along with growth and that no growth/assimilation on/of decaying cells occurred. Indeed, in case of the latter, deviations in the mineralization profile, such as a dual mineralization phase, would be expected. Moreover, the observation that only a limited number of genera was enriched in the ¹³C-DNA fractions, including one known linuron degrading genus along with known linuron catabolic genes, suggests that true linuron assimilators were targeted without cross-

labeling of peripheral populations. Therefore, we conclude that the selected time points for SIP analysis were relevant to identify microbiota directly involved in *in situ* linuron assimilation in the examined BPS.

DNA-SIP identifies *Variovorax* and *Ramlibacter* as contributors to *in situ* linuron degradation in BPS.

The SIP approach suggested that members of the *Comamonadaceae* family, more specifically of the genera *Ramlibacter* and, to a lesser degree, *Variovorax* are implicated in the *in situ* assimilation of ^{13}C derived from linuron. *Variovorax* strains have often been isolated as linuron degraders and several studies have indicated their involvement in *in situ* biodegradation in agricultural soil and in the BPS environment^{14,16–19}. However, this study for the first time unequivocally links the genus *Variovorax*, and in particular the *Variovorax* clade harboring most linuron degrading *Variovorax* isolates, to *in situ* linuron degradation, emphasizing the ecological relevance of this clade for linuron biodegradation. Other linuron degrading isolates belong to genera like *Hydrogenophaga* and *Diaphorobacter*, in the *Comamonadaceae* family, *Mycobacterium* and *Arthrobacter* (*Actinobacteria*) and *Achromobacter* (*Alcaligenaceae*)^{10,45–47} but none of those could be linked with *in situ* linuron assimilation in the studied BPS environment. Instead, in addition to *Variovorax*, the genus *Ramlibacter* was found as being involved in the *in situ* degradation of linuron. Moreover, the organism even dominated the linuron assimilating population and likely involved at least two different genotypes as indicated by the DGGE analysis. In contrast to *Variovorax*, no *Ramlibacter* member has yet been isolated as a linuron degrader, but the genus was recently reported to be enriched in another linuron-fed BPS environment along with other genera like *Variovorax*, *Hydrogenohaga* (*Comamonadaceae*) and *Hyphomicrobium* (*Alphaproteobacteria*)¹⁴. Interestingly, *Ramlibacter* has been associated with the biodegradation of organic contaminants including natural occurring aromatic compounds, i.e., biphenyl⁴⁸, *m*-xylene⁴⁹ and phenol⁵⁰ as well as xenobiotics, i.e., the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D)⁵¹ and the plasticizer di-(2 ethylhexyl) phthalate (DEHP)⁵² but these only involve culture-independent studies that make use of either DNA-SIP or metagenomics. The *Ramlibacter* genus currently includes only few

322 isolated species obtained from various environments like soil and a cooling water system^{53–59}. The SIP-2
323 experiment suggested *Ramlibacter* as the only organism involved in linuron assimilation, while
324 *Variovorax* was not detected. This, however, does not necessarily imply its complete absence, but
325 instead might be due to a reduced abundance below detection within the background community,
326 despite robust sampling. This is supported by the observation that *Ramlibacter* was also significantly
327 lower in relative abundance in SIP-2 compared to its abundance in SIP-1. A plausible explanation is that
328 less linuron was degraded and hence assimilated, also causing the observed decreased CO₂ production
329 compared to SIP-1. However, CO₂ production and carbon assimilation can vary depending on a plethora
330 of factors⁶⁰. Alternatively, the decreased relative abundance might involve changes in background
331 community composition and more specifically the fact that some OTUs increased considerably in
332 absolute numbers, changing the relative contribution of other OTUs. A change in background microbial
333 community composition was indeed observed between SIP-1 and SIP-2. The reasons for this change is
334 unknown, but the BPS environment provides a variety of carbon substrates which likely vary in
335 biodegradability and as such could sustain successional colonization by different communities and
336 involve complex interactions of both degraders and non-degraders.

337 **Known linuron catabolic genes drive linuron biodegradation in BPS and appear linked to presence of**
338 **MGEs.** A set of genes, representing essential steps in the canonical pathway for linuron metabolism⁸
339 were enriched in the ¹³C-DNA fractions, consistent with the observed assimilation of linuron carbon into
340 cellular material, suggesting that this well-known pathway is being followed *in situ*. *hylA* and *libA* encode
341 two iso-functional linuron hydrolases that belong to two different hydrolase families (amidohydrolase
342 and amidase signature family, respectively), showing the involvement of redundant linuron hydrolysis
343 functionalities in the BPS. The catabolic genes show distinct dynamics as indicated by the differences
344 between SIP-1 and SIP-2. Notably, the lack of detectable *libA* in SIP-2 was striking, indicating that its
345 hosts were less competitive in the system at day 440. Previously, we have shown dynamics of both *hylA*

346 and *libA* as well as of *dcaQ* by means of qPCR in linuron impacted BPS material and agricultural soil ¹⁹.
347 The examined catabolic genes were previously identified in *Variovorax* but also in other β -
348 proteobacteria especially in the *Comamonadaceae* family, which includes *Ramlibacter*, therefore their
349 association with *Ramlibacter* is plausible. Other catabolic genes linked to linuron biodegradation were
350 not tested and hence we cannot exclude their involvement in linuron degradation in the BPS. These
351 genes include *puhA* and *puhB* that encode for broad substrate range phenylurea hydrolases belonging to
352 the amidohydrolase family, but those were identified in phenylurea degrading *Actinobacteria* and were
353 never linked with linuron metabolism^{45,46}. Recently, *phh* (amidohydrolase related to PuhA and PuhB) and
354 *tccA2* (amidase family) were identified as encoding for two new broad substrate phenylurea hydrolases
355 in another member of the *Comamonadaceae* family, *Diaphorobacter* sp. strain LR2014-1, belonging to a
356 linuron mineralizing consortium⁴⁷. Apart from known linuron catabolic genes, MGE marker genes were
357 also enriched in the ¹³C-DNA fraction indicating that these genes were present in the linuron assimilating
358 organisms that carry the detected linuron biodegradation genes and that they are prone to HGT.
359 Although we do not have evidence that the catabolic genes are linked physically with the MGE, this is
360 likely the case. Genome sequences of linuron degrading organisms, including *Variovorax*, show the
361 presence of *IS1071* in conjunction with most, if not all of the catabolic genes (like *hyla*, *libA*, *dcaQ*)^{15,61}.
362 The presence of *IS1071* and its conjunction with linuron degradation genes in the BPS environment was
363 shown before^{5,6} and likely *IS1071* contributes significantly to the genetic adaptation to degrade
364 pesticides in the BPS environment⁶. Similarly, linuron catabolic genes like *hyla* and *dcaQ* have been
365 linked to IncP-1 plasmids in isolates from agricultural soils, as well as to plasmids exogenously derived
366 from a BPS environment^{14,20}. On those plasmids, the linuron catabolic genes are commonly embedded in
367 an *IS1071* associated composite transposon which might explain the co-enrichment in the ¹³C-fraction of
368 both *trfA* and *IS1071* in SIP-1. Apart from IncP-1 plasmids, *IS1071* composite transposons carrying
369 linuron catabolic genes can be transferred to other replicons^{6,15}. This might explain why the shift of *trfA*

370 towards the heavy ^{13}C -labeled DNA fractions was not observed in SIP-2. Instead, the presence of IncP-1
371 plasmids in all SIP-2 fractions might be linked to the promiscuity and high transferability of IncP-1
372 plasmids⁶².

373 **Assigning linuron biodegradation activity to DNA-SIP-identified populations.** Despite the enrichment of
374 *Ramlibacter* and *Variovorax* and the identification of genes known to be involved in linuron assimilation,
375 the functions cannot be unequivocally assigned to either organism, especially since linuron is
376 metabolized by both single strains and consortia. In consortia, linuron is hydrolyzed to 3,4-DCA and *N,O*-
377 dimethylhydroxylamine (*N,O*-DMHA) by a first organism, often *Variovorax*, and 3,4-DCA is further
378 mineralized by other organisms while *N,O*-DMHA is utilized by methylotrophic bacteria like
379 *Hyphomicrobium*⁸. The fact that multiple microbiota were labeled through DNA-SIP makes the
380 degradation by consortia plausible. In our study, linuron was ^{13}C -labeled in the ring structure, and hence
381 microbiota that assimilated the aromatic ring carbon are expected to be labeled and not necessarily
382 those that hydrolyze linuron. However, the observation that typical linuron hydrolysis genes like *hylA*
383 and *libA* were enriched in the ^{13}C -DNA suggests that either (i) degradation of the compound in parallel
384 by several individual strains or (ii) that linuron hydrolyzers that perform complete linuron mineralization,
385 leak a part of the produced DCA to other bacteria. Recently, a more complex, not yet fully elucidated,
386 carbon flow network in linuron degrading consortia was suggested, where *Variovorax* performs the
387 unproductive hydrolysis step, and despite lacking the *dca* cluster, receives a substantial portion of
388 carbon. The mechanism is currently unknown, but could involve transfer of linuron derived carbon from
389 organisms that degrade the aromatic structure, back to the linuron hydrolyzer¹⁵. The observation that
390 the catabolic genes are likely linked to MGEs, further complicates the situation since the genes could be
391 (temporally) exchanged between different members of a consortium and as such change the catabolic
392 properties continuously. Interestingly, the disappearance of *libA* in SIP-2 compared to SIP-1 was
393 congruent with the disappearance of *Variovorax* in the labeled community in SIP-2 as well as with the

disappearance of one of the *Ramlibacter* associated bands in the DGGE profiles. This might suggest that at least one of them carried *libA* and that the remaining *Ramlibacter* carried both *hylA* and *dcaQ*, likely enabling it to perform the complete pathway on its own, at least at day 180.

Community composition of the linuron degrading enrichment culture suggests robustness of enrichment approaches while showing differences to DNA-SIP approach. The OTU analysis of the linuron degrading EC from the BPS, showed near to no similarity to results obtained through the DNA-SIP approach and instead identified genera previously isolated as members of linuron degrading consortia, such as *Variovorax*, *Comamonas*, *Hyphomicrobium* and *Pseudomonas*^{8,10}. The phylogenetic and functional similarity of this consortium, which represents the first linuron degrading consortium enriched from a BPS environment, to previously reported linuron degrading consortia from arable soil, is striking and suggests strong robustness in enriching such consortia independent of the target environment, at least under the implemented conditions. We purposefully utilized minimal media and culture conditions that were similar to previous studies, but we cannot exclude the possibility of enriching differently composed consortia when alternate culture approaches are used. Recently, a substantially different consortium including *Diaphorobacter* and *Achromobacter* was described⁴⁷. However, no consortium which includes *Ramlibacter* as a prominent linuron assimilator has been described to date.

In the here described EC, all examined catabolic and functional genes were found to be present, including *libA*. Interestingly, the sequence of the dominant *Variovorax* type matches the sequence of the *Variovorax* subpopulation identified in SIP-1, suggesting that in the cultivation process, *Variovorax* has completely replaced *Ramlibacter* in its function. The mechanisms that define the non-competitive nature of *Ramlibacter* in the EC and its competitive nature in the BPS are unknown. The BPS environment, containing solid surfaces and various other nutrient sources, differs significantly from the suspended culture system in defined media. Moreover, the complex BPS environment might impact

linuron and nutrient bioavailability. While the medium used in the EC differs in nitrogen availability, it is unlikely to be the ultimate cause of the difference, since the BPS material itself contains other nitrogen sources. Additional consideration can be given to the fact that the EC was initiated directly from the BPS material prior to linuron treatment in microcosms, while the SIP incubations were initiated using BPS material enriched with linuron. However, it is again unlikely that this can cause the observed differences, as several other enrichments that were since initiated from linuron treated BPS, resulted in highly similar communities (data not shown). The implication of several transfers in the EC might contribute to the observed differences, since these will especially benefit rapidly growing organisms. Interestingly, slow growth and necessity for additional growth factors were reported for several *Ramlibacter* isolates, suggesting poor fitness under standard culturing conditions^{53,54}. Although the community composition strongly differs, all tested linuron catabolic genes and MGE markers were found to be present in the organisms identified by DNA-SIP and in the EC. This is partly explained by the presence of *Variovorax* in both the SIP-1 system and in the EC and might be further explained by the apparent mobile character of the catabolic genes. Overall, our study provides evidence of the environmental role of the common linuron degrading isolate *Variovorax* in *in situ* linuron biodegradation, but also that culturing approaches might only provide a partial picture of the populations responsible for *in situ* linuron biodegradation.

Figures

439
440 **Figure 1.** 16S rRNA gene amplicon diversity (genus level) in selected high and low fractions density DNA fractions in
441 the SIP-1 (A) and SIP-2 (B) experiment. OTUs were assigned with a 97% identity threshold. For SIP-1 two separate
442 heavy (H) and light (L) fractions are depicted for both ¹³C-labeled and non-labeled (¹²C) DNA. For SIP-2 three heavy

(H) and three light (L) fractions were pooled prior to sequencing. DNA samples of the respective SIP-incubations prior to ultracentrifugation were analyzed as well for both SIP-1 and SIP-2 (indicated as PC).

Figure 2. 16S rRNA gene DGGE profiling showing community fingerprints of relevant DNA-SIP density fractions originating from ^{13}C -linuron amended BPS⁺ material in SIP-1 and SIP-2, as well as of the linuron degrading enrichment culture (EC). Corresponding amplicons originating from almost full 16SrRNA gene clones obtained from SIP-1 and EC DNA are included along with the corresponding 16S rRNA gene fragment of the linuron degrading *Variovorax* sp. WDL1. Primer set 8F and 533R was used for 16S rRNA gene amplification for all samples. Red arrows indicate DGGE bands associated with ^{13}C -DNA fraction SIP-1 16S rRNA gene clone 3.1 and the corresponding bands in the ^{13}C -DNA fraction SIP-1 and SIP-2 profiles. Blue arrows indicate bands associated with EC clone 1.10, with *Variovorax* sp. WDL1 and the corresponding bands in the EC profile.

Figure 3. Phylogram showing nearly complete 16S rRNA gene sequences recovered by cloning from the ¹³C-DNA fraction of SIP-1 (green) and from the linuron enrichment culture (red), as well as 16S rRNA gene sequences from previously reported linuron degrading strains (blue) and other bacteria implicated in linuron degrading consortia (purple). Bacterial 16S rRNA sequences of type strains related to the afore mentioned bacteria were included to enable correct branch formation.

Figure 4. PCR analysis of linuron-catabolic and MGE marker genes in ^{13}C and ^{12}C -DNA after fractionation in the SIP-1 (A) and SIP-2 experiment (B). The fraction numbers in the density range expected to contain ^{13}C -DNA are indicated in red for DNA derived from BPS⁺ material amended with ^{13}C -linuron and in blue for DNA derived from the BPS material amended with non-labeled linuron.

Figure 5. 16S rRNA gene diversity (genus level) in the EC as compared to this of the ^{13}C -DNA of the SIP-1 and SIP-2 experiment. OTUs were assigned with a 97% identity threshold.

ASSOCIATED CONTENT**Supporting Information**

List of pesticides used at Inagro during operational period of the sampled BPS;

Method description of in-house synthesis of [phenyl-U-¹³C] linuron; Information regarding PCR methodology, primer sequences and used PCR programs; ¹⁴C-linuron mineralization kinetics of SIP-1 and SIP-2 samples including their fit to the Gompertz kinetic model; 16S rRNA gene amplicon DGGE community fingerprints of DNA fractions of SIP-1 and SIP-2 samples; Rarefaction analysis of the SIP-1, SIP-2 and EC samples sequenced on a Illumina MiSeq platform; Good's coverage of the SIP-1, SIP-2 and EC samples sequenced on a Illumina MiSeq platform; OTU graph displaying 16S rRNA gene diversity of the SIP-1 replicate samples; 16S rRNA gene amplicon DGGE fingerprints of DGGE clones 12 and 18 derived from DNA retrieved from the upper unique DGGE bands observed in the SIP-1 heavy ¹³C-DNA fractions in comparison to EC clone 1.10 (*Variovorax*); Phylogram showing V1-3 region sequences of clones 12 and 18, recovered by cloning from the upper unique DGGE bands identified in the ¹³C-DNA fraction of SIP-1 in comparison to relevant sequences; Degradation activity of the linuron EC over the enrichment period.

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Notes

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