in situ response of the linuron degradation potential to linuron application in an agricultural field

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Abstract:

To assess the involvement of the genus *Variovorax* and the linuron hydrolase gene *libA* in *in situ* linuron degradation in agricultural fields, changes in *Variovorax* community size and composition, in *libA* abundance and in linuron mineralization capacity were monitored in field soil plots either treated or not with a linuron-containing herbicide mixture. Changes in *Variovorax* community composition and increases in *libA* gene copy numbers corresponded to increases in linuron mineralization capacity in the plot treated with the herbicide mixture, suggesting that *Variovorax* and *libA* proliferated as a response to linuron in the field and their contribution to *in situ* linuron degradation. The response of the *Variovorax* community composition was especially due to the proliferation of a *Variovorax* phylotype (phylotype D) that differed from *Variovorax* phylotypes previously associated with linuron degradation and *libA* abundance in the same soil, suggesting the occurrence of horizontal gene transfer of *libA* in the examined field. The involvement of *Variovorax* phylotype D and *libA* in linuron degradation in the examined soil was supported by lab soil microcosm experiments that showed further increase or decrease of both the population size of phylotype D and *libA* numbers when linuron was added or omitted, respectively. Attempts to enrich in suspended cultures and isolate the corresponding organism from the soil were unsuccessful. In those enrichments, *Variovorax* phylotype D was replaced by other *Variovorax* phylotypes showing that linuron-degrading strains isolated by liquid enrichment cultures are not always representatives of those responsive to linuron in the field, although the genus specificity of linuron degradation was retained.

Introduction

Microbial degradation is considered as an important mechanism in the dissipation of crop protection products in the environment. Various bacterial strains and consortia that degrade or mineralize crop protection products such as linuron (7, 9, 32, 38), atrazine (17, 31, 41), isoproturon (39) or 2,4-dichlorophenoxyacetic acid (10) have been isolated from pesticide-treated agricultural soils by means of long-term enrichment in suspended cultures in which the pesticide of interest is provided as sole carbon source. However, little is known about the involvement and contribution of these organisms to the *in situ* degradation of the respective compounds and concerns exist about the occurrence of biases during the enrichment process. Indeed, in several independent studies, alternative isolation procedures resulted in different culture compositions (6, 22, 30). Enrichment in suspended culture can result in biases because of several reasons. First, enrichment results in proliferation of the fastest growing organisms under the applied conditions, at the expense of slow-growing species (44). Second, the medium composition and the incubation conditions are often not representative for *in situ* conditions. Third, xenobiotic degradation genes are often located on mobile genetic elements and the risk of their transfer from the indigenous host to a new host by horizontal gene transfer (HGT) during enrichment exists (29). As such, enrichment can result in the isolation of species different from the *in situ* relevant populations. This is also suggested from studies that make use of stable isotope probing (SIP) (24) and micro-arrays (29).

On the other hand, in case of certain pesticides, enrichment almost exclusively results in isolation of the same microbial guilds, despite the geographical isolation of the source environments. For instance, enrichment for isoproturon- and carbofuran-degrading organisms almost exclusively results in organisms belonging to the genus *Sphingomonas* (14, 25, 39), while enrichment for linuron degraders almost exclusively leads to members of the genus

Variovorax (7, 9, 11, 13, 32, 38). For unknown reasons, these bacterial genera seem to be specialized in the degradation of particular crop protection products and the hypothesis can be made that they are also *in situ* involved in degradation of the respective compounds. An approach to link specific bacterial guilds to degradation of a xenobiotic in a given environment is to associate the size or diversity dynamics of corresponding biomarkers in that environment with changes in biodegradation capacity as a response to application of the xenobiotic and this in comparison with a non-treated control. Increases in biomarker numbers or changes in its composition concomitant with an increase in biodegradation capacity in the treated system compared to non-treated control systems would indicate that the organisms associated with the biomarkers proliferate at the expense of the added compound and hence would suggest their involvement in its degradation. Accordingly, we previously (3, 36) showed the occurrence of a particular shift in the *Variovorax* community composition in agricultural soil microcosms (SMs) as a response to exposure to linuron. This shift coincided with an increase in linuron mineralization capacity and in abundance of the linuron hydrolase gene *libA*, strongly suggesting that linuron-degrading *Variovorax* strains carrying *libA* proliferated at the expense of linuron in the SMs (4) and supporting the hypothesis that the genus *Variovorax* is involved in linuron degradation in the examined linuron-treated agricultural soil. However, no data on the response of a microbial community to linuron application and the actual identity of *in situ* linuron degraders in field settings exist.

Therefore, in order to understand the contribution of the genus *Variovorax* to *in situ* linuron degradation, the *in situ* dynamics of the *Variovorax* community, the *libA* gene pool and the linuron mineralization capacity of a soil microbial community as a response to linuron application in an agricultural field were monitored. The studied agricultural field had a history

of linuron treatment and was the location from which the soil used in the SM study reported by Bers et al. (3) originated.

Materials and methods

Pesticides used. Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea] (99.5%) was purchased from Sigma Aldrich, Belgium. [Phenyl-U-14C] Linuron (16.93 mCi mmol_1, radio-chemical purity 95%) was obtained from Izotop, Hungary. Acetone was used as a carrier to dissolve linuron in water and bacterial growth media. Acetone carrying linuron was left to evaporate in a sterile bottle after which the sterile liquid was added to the linuron cristals. 24 hours of constant agitation were required to completely dissolve the cristals.

Experimental field set-up and sampling. The experimental field site used in this study is situated in Halen (Belgium). The local soil is a sandy loam soil with a total carbon content of 0.8 % and a total nitrogen content of 0.06 % (7). The field has been cultivated with rotating potato, wheat and sugar beet crops throughout the years and has a unrecorded history of linuron treatment. Previously, it was shown that the soil harbors a linuron degradation capacity and several linuron-mineralizing consortia have been isolated from it by enrichment (7). Two experimental field plots of 15 m^2, located next to each other inside the agricultural field, were planted with potatoes on April 23, 2010. One plot ('P0') was designed as a control plot and not treated with any herbicide, the other plot ('PF') was treated with a herbicide mixture consisting of 450 g linuron ha^{-1}, 2400 g prosulfocarb ha^{-1} and 800 g pendimethalin ha^{-1} applied as Afalon SC (Makhteshim Agan), Defi (SYNGENTA) and Stomp 400SC (BASF), respectively. The formulations were applied on May 21, 2010 before emergence of the potato plants. Soil samples were taken from each plot at three positions (marked as A, B and C in

plot ‘PF’ and C, D and E in plot ‘P0’) from the top 10 cm soil layer and stored at 4 °C until use (maximum two days). At each sampling time, samples were taken at the same positions. The soil samples were sieved (< 2 mm) and for both plots 50g of each position was combined and homogenized after which appropriate amounts were used for DNA-extraction and molecular analysis (day 0, 20, 34 and 48), dry weight measurement (day 0, 20, 34 and 48) and to conduct ^{14}C-linuron mineralization assays (day 0, 7, 20, 34, 48 and 62) to determine the linuron mineralization capacity. To determine dry weight and moisture content, soil samples (~500 mg) were weighed before and after overnight dehydration at 105 °C. No significant differences in moisture content were observed (data not shown). All analyses were always performed on three replicates of the homogenized soil samples except for day 20 when only two replicates were analyzed in case of the three samples taken from plot ‘P0’.

Soil microcosms. Soil (a homogenous mixture of equal amounts collected at positions A, B and C in field plot ‘PF’ and postitions D, E and F in field plot ‘P0’ 176 days after pesticide treatment) was sieved (2mm) and used to set up six SMs in glass columns (height: 10 cm, diameter: 5 cm) with a glass filter installed at 8 cm depth and packed with soil. Three columns were irrigated with sterile tap water as a control, and three columns were irrigated with sterile tap water containing linuron (60 mg L^{-1}). Columns were incubated at 25°C for 245 days. Irrigation was performed discontinuously as described previously with an average irrigation of 0.5 mL day^{-1} (3). After 78 days of incubation, irrigation was ceased for 31 days but was resumed at day 110. At day 159, linuron supply was ceased for one of the columns previously irrigated with linuron. Soil samples for DNA extraction were taken after 28, 110, 149 and 245 days of incubation and used to perform bacterial and *Variovorax*-specific real-time qPCR and bacterial and *Variovorax*-specific PCR-DGGE. Linuron mineralization capacity was

determined at day 0, day 28 and day 245 according to the linuron mineralization assay reported below.

Isolation of phylotype D *Variovorax* strains by plating. At day 157, 1 g of soil from the columns irrigated with linuron was suspended in 9 mL 0.9 w/v% NaCl by mixing the solution for one hour. Soil particles in the mixture were left to settle for 30 min and *Variovorax*-specific PCR-DGGE and *libA*-specific PCR was performed on DNA extracts of the supernatant. A ten-fold diluton series of the supernatant (10^{0}-10^{-10}) was plated on R2A agar-plates supplemented with linuron (20 mg L^{-1}), as described previously (7). After incubation at 25°C, representative colonies were picked on the basis of colony morphology to perform a *Variovorax*-specific colony-PCR. The amplicons were analyzed by DGGE.

Enrichment of linuron-degrading bacteria in liquid cultures. Enrichment in liquid cultures for linuron degraders from the examined soil was initiated on two occasions. On the first occasion, enrichment was started from soil sampled at the field from plot PF at day 20. 240 mg of soil was suspended in 6 mL of mineral medium MMO (prepared as described previously (9)) containing linuron (20 mg L^{-1}) as sole source of carbon. This was performed in duplicate for each soil sample. In one of the two replicates ~ 745 dpm mL^{-1} ^{14}C-linuron was added. The cultures were incubated on a shaker in the dark at 25 °C. Mineralization of linuron was monitored as described previously (37). When 80 % of linuron was mineralized, 500 µl of the cultures containing only cold linuron were transferred to 25 mL of fresh mineral medium MMN (prepared as described previously (9)) with linuron (20 mg L^{-1}). For the next two months, the cultures were regularly transferred to the same medium when more than 60 % of linuron was degraded as measured by HPLC (as described previously (7)). After these

two months, the enrichment cultures were maintained by alternating adding a fresh amount of linuron (10 mL MMN with 60 mg L^{-1} linuron) or transfering the enrichment culture to fresh medium each 7 days. Samples for DNA extraction were taken from the initial enrichment cultures (day 4) and after 4 transfers (at day 17), 15 transfers (at day 61) and 21 transfers (at day 122). Undiluted DNA extracts were used to perform bacterial and *Variovorax*-specific PCR-DGGE and real-time qPCR. In addition, the capacity to mineralize linuron was measured after 4, 15 and 21 transfers, by inoculating 50 µl of culture to 5 mL of MMO containing 20 mg L^{-1} linuron and ~ 745 dpm mL^{-1} of ^{14}C-linuron. At day 4, samples were taken from the initial enrichment culture and ten-fold dilution series were plated on R2A agar plates supplemented with linuron (20 mg L^{-1}), as described previously (7). After incubation at 25°C, individual colonies were picked on the basis of different colony morphologies and used to perform a colony-PCR for *Variovorax*-specific PCR-DGGE. On the second occasion, enrichment was initiated from soil samples taken at day 134 from soil microcosms (see below) treated with linuron. For each soil column, two enrichment cultures were started by suspending 500 mg of soil in 25 mL of mineral medium MMO containing linuron (20 mg L^{-1}) as sole source of carbon. 500 µL of the enriched cultures was transferred to 25 mL of fresh medium when at least 50 % of the linuron in the medium was degraded, as evaluated by HPLC. Samples for DNA extraction were taken from the first enrichment culture at day 4, after eight transfers (day 28) and after 16 transfers (day 56).

^{14}C-mineralization assay. ^{14}C-linuron mineralization experiments were performed and mineralization lag times and maximum mineralization rates were determined as described previously (37), by transfering 200 mg of soil to 5 mL of MMO containing 20 mg L^{-1} linuron and ~745 dpm mL^{-1} ^{14}C-linuron. Background activities detected in the mineralization assays

ranged between 27 and 64 dpm. In all mineralization assays 40-65% of activity could be recovered within 46 days. Mineralization lag times were subjected to repeated measures two-way ANOVA on a significance level of 0.01 to assess the effects of pesticide addition. Repeated measures one-way ANOVA (significance level 0.01) was used to analyze significancies in differences in lag times between sampling times for each treatment and between treatments at each sampling time.

Molecular methods. DNA extraction from soil samples (500 mg wet weight) was performed using the E.Z.N.A. Soil DNA kit (Omega Bio-Tek) as described by the manufacturer. DNA-extraction of liquid bacterial culture samples (2 mL) was performed using the Dneasy® blood and tissue kit (Qiagen) with an adapted protocol. The protocol as provided by the manufacturer was supplemented with an extra mechanical lysis step after enzymatic lysis. Mechanical lysis was performed by adding 0.3 g of glass beads to the sample, vortexing at full speed for 30 s and heating the sample for 1h at 60 °C. Real-time PCR was performed in a Rotor Gene Real-Time Centrifugal DNA Amplification Apparatus (Corbett Research, Australia). Real-time PCR targeting the bacterial 16S rRNA gene was performed as reported by Haest et al. (19). Real-time PCRs targeting *Variovorax* and *libA* were performed as described previously (3) (4). Population sizes were subjected to two-way repeated measures ANOVA on a significance level of 0.01 to assess the effects of application of the herbicide mixture or linuron. One-way repeated measures ANOVA (significance level 0.01) was used to analyze differences in population sizes between sampling times for each treatment and between treatments at each sampling time. The bacterial 16S rRNA genes of soil microbial communities were amplified through PCR by means of primerpair GC63F/518R (12) and a PCR reaction mixture and protocol previously described by Moreels et al. (26). β-

211 proteobacterial soil communities were amplified by means of a nested PCR with primer pairs
212 F948β/R1494 (18, 45) and F984GC/R1378 (21) as previously described (16). *Variovorax*-
213 specific PCR was performed as described (3). DGGE based on the method cited by Muyzer et
214 al. (27) was performed with the bacterial, β-proteobacterial and *Variovorax*-specific PCR-
215 products according to the specific DGGE-protocols described previously by El Fantroussi et
216 al. (12), Gelsomino et al. (16) and Bers et al. (3), respectively. Similarity and cluster analysis
217 of DGGE fingerprints was based on the Pearson product-moment correlation coefficient using
218 UPGMA as described previously (42). Cloning of amplification products was performed
219 using the PCR2.1 TOPO cloning kit (Invitrogen NV) as described by the manufacturer.
220 Extraction of PCR products out of a DGGE polyacrylamide gel was performed by submerging
221 the gel slice containing the band of interest in 50 µl of sterile mQ water and incubating the
222 sample for 4 h at 4 °C. Afterwards the water, containing the DNA from the gel slice, was
223 collected, checked by *Variovorax*-specific PCR-DGGE and the amplicons were cloned.
224 Shotgun sequencing was performed using the BigDyeTM Terminator Cycle Sequencing Kit
225 (Applied Biosystems) as described by the manufacturer. The sequencing reaction was
226 performed on a thermocycler UNOII (Biometra, Germany). ClustalX (40) and BlastN (1)
227 were used to, respectively, align and identify the retrieved nucleotide sequences.
228
229 **Results**
230 **Response of the soil microbial community on application of the linuron-containing**
231 **herbicide mixture in the field.** The linuron mineralization lag times and maximum
232 mineralization rates were comparable for samples taken from plot 'P0' (9.52 ± 0.44 days and
233 6.38 ± 0.86 % ^{14}C-linuron day^{-1}, respectively) and plot 'PF' (8.62 ± 0.77 days and 5.76 ± 1.03
234 % ^{14}C-linuron day^{-1}, respectively) before applying the herbicide mixture to plot 'PF'. Seven

235 days after application of the pesticides on plot 'PF' no significant difference in linuron
236 mineralization lag time between plot 'P0' (9.43 ± 2.07 days) and plot 'PF' (8.14 ± 0.48 days)
237 was observed. Twenty days after treatment of plot "PF" with the herbicide mixture, a
238 significant lower linuron mineralization lag time was observed for the samples of plot 'PF'
239 (5.24 ± 0.65 days) compared to the samples of plot 'P0' (8.98 ± 1.35 days), indicating an
240 increase of size of the linuron-mineralizing microbial soil community (37) in plot 'PF' as a
241 response to the application of the herbicide-mixture. Moreover, at day 20, coinciding with the
242 increase of linuron mineralization capacity, the *Variovorax* community structure in plot 'PF'
243 changed and became different from plot 'P0' (Fig. 1 and further illustrated by UPGMA
244 cluster analysis of *Variovorax*-specific DGGE fingerprints (Fig. S1)). The five most dominant
245 *Variovorax* populations (corresponding to DGGE-bands V1, V2, V4, V5 and V7) present
246 before the application of the herbicide mixture remained, but a previously not observed
247 population (corresponding to DGGE-band V6 and designated as phylotype D) appeared in the
248 *Variovorax* community of plot 'PF' (Fig. 1). Moreover, the percentage of *libA* copy numbers
249 in relation to the bacterial 16S rRNA gene numbers which initially was under the detection
250 limit in all samples and remained like that during the experimental period for samples taken
251 from plot 'P0', increased till above the detection limit 20 days after applying the herbicide
252 mixture ((1.13 ± 0.63) x 10^{-1} %, corresponding to (5.49 ± 3.15) x 10^{3} *libA* copies (g^{-1} dry wt
253 soil)) in plot 'PF'. The *libA* PCR product from two samples was cloned and four clones were
254 sequenced. All retrieved nucleotide sequences showed 100% identity with the corresponding
255 *libA* nucleotide sequence of *Variovorax* sp. SRS16 (2). Two weeks later (34 days after
256 application of the herbicide mixture) the linuron mineralization capacity of plot 'PF'
257 decreased to a level comparable to the linuron mineralization capacity of plot 'P0', the
258 original *Variovorax* community structure was restored and *libA* numbers dropped again below

the detection limit. After day 34 no more significant changes in linuron mineralization lag time and hence mineralization capacity, *Variovorax* community structure and *libA* abundance were recorded. Throughout the experimental period, no differences in maximum linuron mineralization rate between plot 'P0' and plot 'PF' were observed (data not shown).

Since we previously never observed *Variovorax* phylotype D, the V6-band was cloned and sequenced. Fig. 2 shows the nucleotide sequence of the amplicon of V6 aligned with corresponding 16S rRNA gene nucleotide sequences of *Variovorax* strains belonging to relevant *Variovorax* phyloptypes. The 16S rRNA gene amplicon of *Variovorax* phylotype D showed only one nucleotide difference with the corresponding 16S rRNA gene sequence of the phylotype to which DGGE-band V5 belongs and 96% identity with the corresponding 16S rRNA gene sequence of *Variovorax* sp. SRS16 (phylotype A, band V3), DSM66 (phylotype C, band V2) and WDL1 (phylotype B, band V1). Real-time qPCR detected neither significant changes in the total bacterial community size nor in the *Variovorax* community size during the experimental period (data not shown). Moreover, as well the bacterial as the β-proteobacterial community structure, as evaluated by 16S rRNA gene specific PCR-DGGE, were identical in plots 'PF' and 'P0' and did not show changes over time (data not shown).

Reponse of the soil microbial community to linuron application in soil microcosms. In order to acquire more evidence whether the observed changes in the treated plot regarding linuron mineralizaton capacity, *Variovorax* community and *libA* abundance at day 20 was due to linuron application and not to other factors such as the application of the other two pesticides or indirectly due to changes in the local environment induced by the pesticide application, a SM experiment was initiated. Six microcosms containing a mixture of fresh soil samples of plots 'P0' and 'PF', collected 176 days after applying the herbicide mixture, were

283 incubated under controlled conditions. Three soil columns were irrigated with water, while
284 the remaining three columns were irrigated with linuron dissolved in water. At the start of the
285 incubation (day 0), all microcosms showed a similar linuron mineralization lag time (Table 1),
286 a similar *Variovorax* community composition (Fig. 3) and similar percentages of *libA* copy
287 numbers in relation to the bacterial 16S rRNA gene numbers (Table 1). At day 28, the lag
288 time recorded with samples taken from the linuron-fed columns however decreased
289 significantly to 3.07 ± 0.06 days while it remained unchanged in the water-fed columns
290 (Table 1). Concomitantly, an additional phylotype corresponding to DGGE-band V6 and
291 hence phylotype D emerged (Fig. 3) while the percentage of *libA* copy numbers in relation to
292 the bacterial 16S rRNA gene copy number increased up to $(7.90 \pm 2.25) \times 10^{-3}$ % (Table 1). In
293 contrast, no changes in *Variovorax* community composition neither in *libA* abundance
294 occurred in the water-fed columns (Fig. 3 and Table 1). At day 110, no linuron mineralization
295 data were collected but phylotype D remained dominant in the *Variovorax* community (Fig.
296 3) and *libA* numbers (Table 1) remained relatively high in the linuron-fed microcosms despite
297 the stop of the irrigation for 31 days. After resuming irrigation from day 110 on, a significant
298 increase in *libA* abundance in both linuron- and water-fed SMs was observed by day 149
299 (Table 1). However, the percentages of *libA* gene copies in relationship to the bacterial 16S
300 rRNA gene copies in linuron-fed SMs ($(1.61 \pm 4.41) \times 10^{-1}$ %) remained significantly higher
301 than in the water-fed SMs ($(1.91 \pm 0.85) \times 10^{-2}$ %). Moreover, in the linuron-fed SMs a
302 significant increase in overall *Variovorax* community size was observed by day 149 (Table 1).
303 No linuron mineralization data were collected at day 149. From day 159 on, it was decided to
304 cease linuron supply for one of the linuron-fed columns to further acquire evidence that
305 linuron application was effecting phylotype D proliferation and linuron mineralization
306 capacity. It was expected that in this SM, the linuron mineralization capacity would decrease

307 concomitant with a decrease in *libA* number and lowered dominancy of phylotype D. This
308 was indeed the case. At day 245, a significantly higher linuron mineralization lag time was
309 recorded in the SM in which linuron feeding was stopped compared to the two SMs for which
310 linuron supply was continued (Table 1). This decrease in linuron mineralization coincided
311 with a decrease in *libA* abundance and *Variovorax* 16S rRNA gene copies (Table 1) and a
312 decrease in abundance of phylotype D compared to the two SMs for which linuron supply was
313 continued. The decrease in abundance of phylotype D was illustrated by UPGMA analysis.
314 The *Variovorax* community DGGE profile of that SM clustered with those of the other two
315 linuron-fed SMs (showing 90 % similarity) at day 149 but clustered with those of the SMs
316 which did not receive linuron at day 245 (Fig. 3). Furthermore, at day 245, as at day 149, all
317 microcosms still showed a higher *libA* abundance compared with this recorded at day 28.
318 Moreover, a decreased linuron mineralization lag time was observed for all microcosms
319 compared with this recorded at day 28. However, *libA* abundance and linuron mineralization
320 capacity, as measured by the linuron mineralization lag time, of the linuron-fed columns
321 remained significantly higher, compared to those measured for the water-fed columns at day
322 245 (see Table 1). The increased mineralization capacity and *libA* abundance that was also
323 observed in water-fed columns after day 110, can be attributed to resuming irrigation with
324 water after the SMs had experienced a period of 32 days without irrigation (from day 78 till
325 day 110). The positive effect of a wetting after a drought period on *libA* gene abundance and
326 linuron mineralization capacity has been observed previously (3, 4). The bacterial community
327 composition as evaluated by bacterial 16S rRNA gene-specific PCR-DGGE did not change
328 during the entire experiment (data not shown).
329

Isolation of members of *Variovorax* phylotype D and enrichment of the linuron-mineralizing soil microbial community. Since previously, no isolated *Variovorax* strains belonging to phylotype D have been associated with linuron degradation, several attempts were taken to isolate and culture the organism associated with phylotype D to confirm its ability to degrade linuron. Therefore, enrichment cultures in liquid medium were initiated with (i) soil samples taken from plot 'PF' 20 days after application of the herbicide mixture and with (ii) soil samples derived from the strongly linuron enriched SMs at day 134. For both types of inocula, linuron degradation capacity increased significantly during enrichment (data not shown). In contrast to what was observed in the field and the SMs, *Variovorax* phylotype D (DGGE-band V6) rapidly lost its dominance as shown by *Variovorax*-specific DGGE (Fig. S2). Instead, a *Variovorax* community developed which was clearly different from the original community and whose composition varied in time and between the different cultures. Those observations suggest that the *Variovorax* population linked with DGGE-band V6 is not dominantly involved in linuron mineralization in the enrichment cultures. However, in one of the enrichment cultures initiated with soil taken from the linuron fed SMs (enrichment culture B1), phylotype D was temporarily enriched after eight transfers (day 28) but then also disappeared (Fig. S2). Moreover, in almost all enrichments, percentages of *libA* copy numbers in relation to the bacterial 16S rRNA gene copy numbers decreased significantly (data not shown), suggesting that the liquid enrichment of the linuron-degrading soil microbial community is concomitant with the enrichment of linuron-degrading bacteria that use a linuron hydrolase gene different from *libA*. The exception was one of the six enrichment cultures (culture B2) which were initiated with soil samples taken from the linuron-fed SMs that showed an increase in *libA* copy number. In another attempt to isolate members of *Variovorax* phylotype D, the soil microbial community in the linuron-fed SMs at day 158 was

plated on linuron-containing R2A agar medium. *Variovorax*-specific PCR-DGGE and *libA*-specific PCR confirmed the presence of phylotype D and *libA* in the suspension washed from the soil of the SMs. However, although several colonies that were growing on R2A supplemented with linuron could be identified as *Variovorax* (i.e. corresponding to phylotypes A, B, C and DGGE-band V4), none of them showed a DGGE profile similar to V6.

Discussion

To the best of our knowledge, this study is the first report on the in field response of the microbial community of an agricultural soil to the application of a phenylurea herbicide in relation to its biodegradation. The response to linuron application was monitored by PCR-detection of specific biomarkers that have been previously associated with linuron biodegradation and by assaying changes in linuron mineralization potential. It was found that, in response to the application of a herbicide mixture containing linuron, a decrease in linuron mineralization lag time was observed with samples taken from the treated plot as compared to samples taken from the non-treated plot, suggesting an increase in the size of a linuron-mineralizing microbial soil population in the treated plot. This growth coincided with an increase in *libA* abundance, and the appearance of a new phylotype D within the *Variovorax* community. An identical response was observed in SMs containing soil from the field plots when only linuron was added. The data were largely in accordance with other data previously obtained in soil microcosms (4). Moreover, a clear correlation similar to the one described by Bers et al. (4) was found between *libA* numbers and mineralization lag times displayed in the current SM experiment (data not shown) implying that *libA* is connected with linuron mineralization potential. The parallel dynamics of the three markers imply the specific response of a particular *Variovorax* phylotype carrying *libA* to the application of linuron in

the field and its proliferation at the expense of linuron. This strongly suggests that these organisms degraded linuron in the field and converted it into biomass, although we do not have actual data on the in field linuron removal. The latter can be done but it is impossible to differentiate between the contribution of biotic and abiotic factors in the eventual loss of linuron. However, we can still not fully exclude that the observed responses in the field are due to other factors than linuron or that other factors contributed to the observed response. A possibility is that the in field response was (also) related with the application of prosulfcarb and/or pendimethalin which were applied in addition to linuron in the field experiment. However, this is very unlikely taking into account (i) the previously identified association between linuron degradation, *libA* and *Variovorax* (2-4, 35, 36) and (ii) the knowledge that both prosulfocarb (a thiocarbamate herbicide) and pendimethalin (a dinitroaniline herbicide) have a chemical structure that is considerably different from linuron and hence are very unlikely substrates for LibA that showed a high substrate specificity for linuron (2). Moreover, an identical response as in the field was obtained in the SM experiment where the soils were only fed with linuron. Alternatively, the difference in *in situ* response between the treated and non-treated plots can be indirectly due to changes in soil matrix after addition of the pesticide mixtures. This is also unlikely taking into account the specificity of the response, i.e., a response that could be clearly related to biodegradation of linuron. In addition, at day 20, the soil matrix appeared not different between the treated and non-treated plots. On day 20, potato plants on both plots only just appeared and were highly similar in size and health appearance. Also, neither on plot 'P0' nor on plot 'PF' weeds were growing yet and soil moisture contents did not differ significantly between both plots. Moreover, in the SM experiment operated under controlled conditions, an identical response was found as in the field.

The pattern of the in field response to pesticide treatment consisted of an increase in linuron-mineralizing population till around day 20 followed by a decrease afterwards. The latter is insinuated by the increase in linuron mineralization lag phase, the decrease in *libA* numbers and the loss of phyotype D in the *Variovorax* community after day 20. This apparent decay can be explained by the loss of selective advantage of the linuron-degrading population once linuron was degraded and removed in the field. Similar dynamics have been observed previously in controlled SM experiments in which the soil was treated with a single dose of linuron, equivalent at one field dose (3, 4). An apparent decay of the linuron-mineralizing population was also found in the SM experiment in the current study once linuron was omitted in the feed. It suggests that linuron-degrading populations started to decay once linuron is omitted in the treatment. Similar observations have been done previously in field situations for atrazine but not for phenylurea herbicides (5, 8).

The results obtained in the current study are largely in accordance with those reported in a previous SM study using the same soil (3), i.e., an increase in *libA* number, a change in *Variovorax* community structure and an increase in linuron mineralization potential upon treatment of the soil with linuron. The only exception is that a different *Variovorax* phylotype became dominant. The soil used in both studies originated from the same field but in case of the study of Bers et al. (3), it was a mixture of several samples collected on different locations within the field. Moreover, those samples were taken two years before the start of the field experiment. The experimental plots used in the current study, were located next to each other at one dedicated location within the field. The observed difference might be due to spatial variation in linuron-degrading identities within the field. Franklin et al. (15) previously suggested that individual populations of a soil microbial community can respond differently to different soil properties. Therefore, soil microbial community composition can show spatial

426 heterogenity within a field. Rasmussen et al. (28) suggested that intrinsic soil parameters
427 (such as pH, available potassium or C_{total}/N_{total} ratio) affected the actively linuron-
428 metabolising bacterial community in soil. Shi et al. (34) observed dominance of different
429 *Sphingomonas* phylotypes that could be linked to isoproturon degradation at different
430 locations in the examined agricultural field. The type of dominant *Sphingomonas* phylotype
431 could be linked to the local pH values. As such, heterogeneity within the field might have
432 caused the observed differences in *Variovorax* community structure and response to linuron
433 between this field trial and the SM experiment previously described by Bers et al. (3). Next to
434 spatial, also temporal changes in soil microbial community structure/composition might be
435 responsible for the observed differences in linuron-degrading entities between the two
436 experiments. Between the two sampling campaigns, field management might have resulted
437 into a change in bacterial community structure. Schutter et al. (33) reported that although
438 major determinants of soil microbial community structure were season- and field-dependent
439 factors, shifts in community structure can occur as soil physical and chemical properties
440 change in response to a change in agricultural practice. For instance, Jackson et al. (23)
441 reported about an immediate change in microbial community structure of a soil after tillage.
442 Interestingly, as well in the previously described SM experiment as in the current study, *libA*
443 gene abundance increased as a response to linuron and a similar relationship between linuron
444 mineralization lag time and *libA* abundance was found. However, either *Variovorax* phylotype
445 A or *Variovorax* phylotype D proliferated. This suggests that *libA* in the soil might have been
446 transfered from members of phylotype A to members of phylotype D by means of HGT and
447 that phylotype D is a more competitive strain than phylotype A under the current soil
448 conditions. Indications for in field HGT of catabolic genes between microorganisms in
449 contaminated soils was reported previously (20, 43).

In two attempts to isolate *Variovorax* phylotype D in liquid enrichments, prolonged enrichment of linuron-degrading bacteria in suspended culture resulted into a linuron-mineralizing community with a *Variovorax* community composition which was clearly different from that proliferating in the field and in the SMs. In the liquid enrichment cultures, *Variovorax* phylotype D disappeared rapidly from the *Variovorax* community except from one culture initiated from a sample taken from the linuron-fed SM that contained soil that was strongly enriched for *Variovorax* phylotype D. In that culture, a temporal proliferation of phylotype D and *libA* occurred which further supports the hypothesis that the corresponding organism is able to grow on linuron and is carrying *libA*. The SMs compose a more realistic mimic of the *in situ* bacterial linuron degradation process than the liquid enrichment culture. This points towards the occurrence of enrichment bias in suspended cultures and shows that the result of an enrichment experiment is not always representative for the *in situ* degrading culture. Moreover, it shows that two organisms belonging to the same genus and with apparent similar specialized catabolic features display different fitness under different environmental conditions. The possibility of such enrichment "biases" has been implied from other studies in which the use of different procedures to enrich organisms for a specified function resulted into different isolates. However, the outcome was often not related with *in situ* biodegradation (6, 22, 30) except for a study reported by Rhee et al. (29). The latter authors showed that naphthalene amended enrichments in suspension resulted into the isolation of members of the gram-positive genus *Rhodococcus* while addition of naphthalene to the soil used as inoculum for the enrichment cultures resulted into growth of *Betaproteobacteria*. This is in contrast with our study where the genus-specificity of the catabolic function was retained. In contrast with biodegradation of naphthalene, a function that is widespread between various microbial guilds, degradation of pesticides is often

474 associated with a specific microbial group as in the case of linuron degradation (7, 9, 11, 13,
475 32, 38). Also studies that used a DNA/RNA SIP approach to identify organisms related with a
476 specific catabolic function, pointed towards the occurrence of such biases but none of those
477 studies actually compared SIP data with the outcome of an enrichment procedure for the same
478 environmental sample (24). As such, this is the first report that compares the outcome of an *in*
479 *situ* response with this of an isolation procedure. The occurrence of such biases can be
480 explained in different ways. Phylotype D might represent an organism specialized to live in an
481 unsaturated soil environment with relatively low water activity. Otherwise, bacteria belonging
482 to phylotype D need association with soil particles, what makes it difficult to culture them in
483 liquid culture. Dunbar et al. (10, 24) previously suggested that enrichment without solid
484 support tends to select for bacteria which grow rapidly and as such outcompete slow-growing
485 micro-organisms. Also Breugelmans et al. (6) observed different compositions of linuron-
486 degrading cultures enriched in the presence or absence of soil particles from soil originating
487 from the same field. Alternatively, the medium composition of the suspended cultures might
488 have been unsuitable for the growth of phylotype D. Interestingly, in case of the liquid
489 enrichment initiated from soil samples from the linuron enriched soil microcosms, *libA* copy
490 numbers remained high in one of the six enrichments, despite the disappearance of phylotype
491 D from the *Variovorax* community structure, suggesting the occurrence of HGT during the
492 enrichment process and as such, the mobile character of the *libA* gene.
493
494 **Conclusions**
495 This paper shows for the first time that degradation of linuron can be associated with
496 *Variovorax* in a field setting and the possible involvement of *Variovorax* in *in situ* phenylurea
497 herbicide natural attenuation. Our results furthermore suggest that *libA* can be shared between

498 different *Variovorax* populations within a microbial soil community suggesting the
499 occurrence of intragenus HGT of *libA* within that community. Moreover, although our data
500 show that LibA is of major importance for linuron degradation in the field, also other linuron
501 hydrolases appear to contribute to the flexibility of the linuron degradation potential.
502 Furthermore, it was illustrated that previously reported linuron-degrading isolates originating
503 from suspended enrichment cultures do not necessarily represent the most active *in situ*
504 linuron degraders in the soil inoculum although the genus specificity of the linuron
505 degradation function was retained.
506

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510

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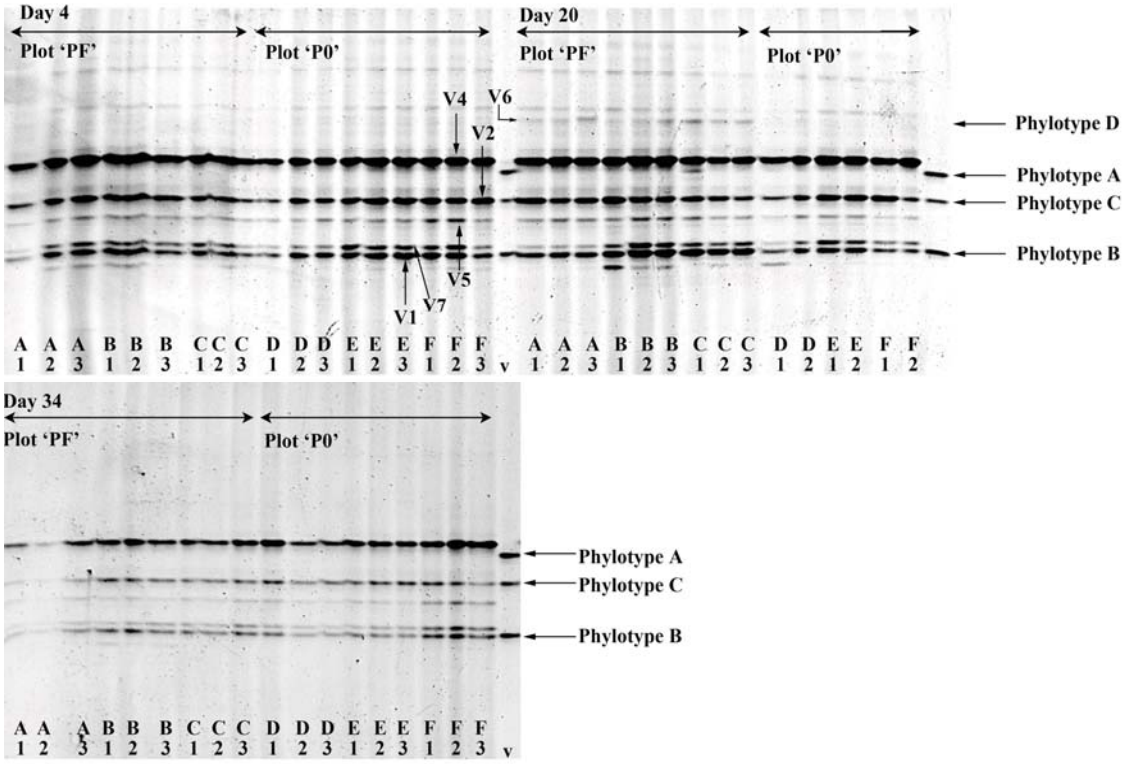

Fig. 1 Dynamics of the *Variovorax* community composition in the experimental field plots as revealed by *Variovorax*-specific PCR-DGGE. Plot 'PF' was treated with a mixture of three herbicides including linuron and plot 'P0' was left untreated. The lanes marked by 'v' show the *Variovorax* 16S rRNA gene DGGE marker. V1 (36), V2 (36), V3 (36), V4 (36), V5, V6 and V7 indicate bands associated with different *Variovorax* populations. Corresponding *Variovorax* phylotypes are indicated. The letters in the lane markers correspond to the sampling positions, i.e., A, B and C in plot 'PF' and D, E and F in plot 'P0' from which the template DNA for PCR was extracted. The numbers 1, 2 and 3 indicate the replicate DNA extracts. At day 20, only two replicate DNA-extracts were prepared from samples collected at sampling positions D, E and F in plot 'P0'.

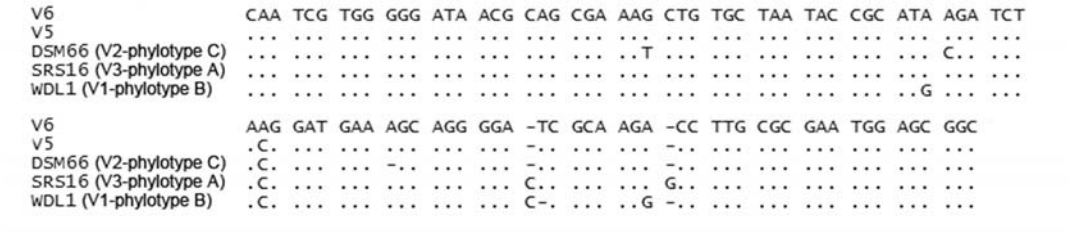

Fig. 2 Alignment of the VarF-VarR amplicon sequence of *Variovorax* phylotype V6 with the corresponding 16S rRNA gene region of other phylotypes. Sequences are shown for the *Variovorax* phylotype to which DGGE-band V5 belongs, *Variovorax* sp. WDL1 (representative of phylotype B), DSM66 (representative of phylotype C) and SRS16 (representative of phylotype A). Dots mark nucleotides identical to V6, dashes mark gaps.

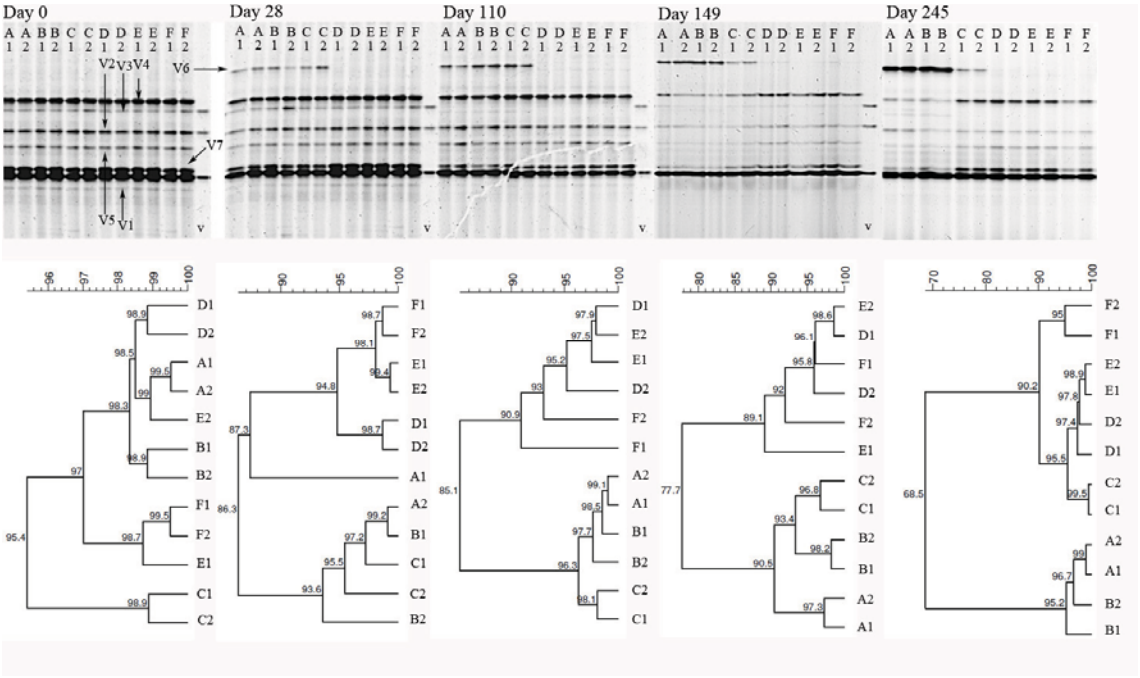

Fig. 3 Dynamics of the *Variovorax* community in the SMs as analyzed by *Variovorax*-specific PCR-DGGE. The lanes designated A1 – C2 show the *Variovorax* community of linuron-fed SMs while those designated D1 – F2 show the *Variovorax* community of water-fed SMs. Lanes C1 and C2 show the *Variovorax* community composition of the microcosms in which linuron supply was ceased after day 159. At all time points, of each SM, two replicate analyses (indicated with the numbers 1 and 2) were done. The lanes marked by 'v' show the *Variovorax* 16S rRNA gene DGGE marker. V1-V7 mark different DGGE-bands corresponding to the different *Variovorax* phylotypes as indicated. Corresponding UPGMA dendrograms of the community profiles are shown below each gel. The scale indicates the similarity level (0 - 100 %) and numbers at nodes indicate the degree of similarity. In the dendrograms, designation of profiles of the UPGMA clusters corresponds to the lane designation in the DGGE profile above each dendrogram.

Table 1 Percentages of *Variovorax* 16S rRNA and *libA* gene copies within the bacterial soil community of water-fed SMs (W SMs) and linuron-fed SMs (L SMs) as determined by *Variovorax*-specific and *libA*-specific real-time qPCR and recorded linuron mineralization lag times as determined by ^{14}C-linuron mineralization assays.

Sampling time	% *Variovorax*/Bacteria*		% *libA*/Bacteria*		Lag time (day)**	
	W SMs	L SMs***	W SMs	L SMs***	W SMs	L SMs***
0	0.30 ± 0.10	0.28 ± 0.20	(3.00 ± 1.50) x 10^{-4}	(3.20 ± 1.80) x 10^{-4}	6.91 ± 0.28	6.98 ± 0.40
28	0.32 ± 0.06	0.32 ± 0.18	(2.98 ± 1.99) x 10^{-4}	(7.90 ± 2.25) x 10^{-4}	6.88 ± 0.33	3.07 ±0.06
110	0.19 ± 0.06	0.33 ± 0.12	(2.75 ± 1.13) x 10^{-4}	(8.66 ± 4.14) x 10^{-3}	ND	ND
149	0.45 ± 0.15	0.87 ± 0.27	(1.91 ± 0.85) x 10^{-2}	(1.61 ± 4.41) x 10^{-1}	ND	ND
245	0.25 ± 0.10	0.76 ± 0.15	(6.78 ± 3.75) x 10^{-3}	(6.71 ± 1.72) x 10^{-1}	3.28 ± 0.83	0.73 ± 0.05
		[0.23 ± 0.02]		[(4.42 ± 1.03) x 10^{-2}]		[1.91 ± 0.90]

*Average values determined for duplicate samples of each SM, both measured in triplicate, are shown together with the SD.

**Average values determined for duplicate samples of each SM are shown together with the SD.

***At day 245 the numbers between square brackets show the values determined for the SM in which linuron supply was ceased after day 159.

ND: not done.