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Robust Linuron Degradation in On-Farm Biopurification Systems Exposed to Sequential Environmental Changes▽

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On-farm biopurification systems (BPS) treat pesticide-contaminated wastewater of farms through biodegradation. Adding pesticide-primed soil has been shown to be beneficial for the establishment of pesticide-degrading populations in BPS. However, no data exist on the response of pesticide-degrading microbiota, either endogenous or introduced with pesticide-primed soil, when BPS are exposed to expected less favorable environmental conditions like cold periods, drought periods, and periods without a pesticide supply. Therefore, the response of microbiota mineralizing the herbicide linuron in BPS microcosm setups inoculated either with a linuron-primed soil or a nonprimed soil to a sequence of such less favorable conditions was examined. A period without linuron supply or a drought period reduced the size of the linuron-mineralizing community in both setups. The most severe effect was recorded for the setup containing nonprimed soil, in which stopping the linuron supply decreased the linuron degradation capacity to nondetectable levels. In both systems, linuron mineralization rapidly reestablished after conventional operation conditions were restored. A cold period and feeding with a pesticide mixture did not affect linuron mineralization. The changes in the linuron-mineralizing capacity in microcosms containing primed soil were associated with the dynamics of a particular *Variovorax* phylotype that previously had been associated with linuron mineralization. This study suggests that the pesticide-mineralizing community in BPS is robust in stress situations imposed by changes in environmental conditions expected to occur on farms. Moreover, it suggests that, in cases where effects do occur, recovery is rapid after restoring conventional operation conditions.

The treatment of pesticide-contaminated wastewater in on-farm biopurification systems (BPS) is a low-cost and effortless solution for farmers to minimize the direct losses of pesticides to surface water (6). On-farm BPS operate as biofilters in which the pesticides are removed from the wastewater by biodegradation and sorption processes occurring in the biofilter matrix. The matrix in a BPS, designated a biomix, is composed of a mixture of soil, peat, and straw or other organic waste materials (6, 9). The addition of pesticide-primed soil to BPS has been proposed as an alternative for bioaugmentation with axenic cultures of specialized pesticide-degrading bacteria to accelerate pesticide degradation and avoid the production of toxic metabolites (7, 18). Sniegowski et al. (18) showed that bioaugmentation with a linuron-primed soil containing linuron-mineralizing microorganisms immediately resulted in the establishment of a linuron mineralization capacity in BPS microcosms. The size of the linuron-mineralizing populations in the system further increased when the microcosms were fed linuron. BPS microcosms inoculated with nonprimed soils also developed a linuron mineralization capacity but only after a much longer period of linuron supply. In the BPS microcosms containing primed soil, the increase in linuron mineralization capacity was concomitant with the appearance of a *Variovorax* 16S rRNA gene phylotype associated with cultured linuron-degrading *Variovorax* strains, suggesting the involvement of this phylotype in linuron degradation in those microcosms (18).

On-farm BPS are expected to operate efficiently for several years while enduring different season-associated changes in environmental conditions, such as dry-wet cycles likely to occur in the summer, cold periods in the winter, periods without pesticide supply between two spraying seasons, and periods in which BPS are fed different pesticide formulations. Such changes might induce stresses for specific pesticide-degrading populations in the BPS, resulting in changes in their population sizes and disturbance of BPS degradation performance (6). However, it is unknown how pesticide-degrading populations present in a BPS respond to periods of less favorable conditions, especially when they are sequentially imposed. The sensitivity of pesticide-degrading populations to less favorable conditions can be high because of the anticipated poor pesticide-degrading functional redundancy within a microbial community (11). Previous studies have reported the effect of a single environmental stress condition on pesticide-degrading microbial populations and their pesticide-degrading activity in soils and other ecosystems (12, 13, 17) but not in BPS, except for the effect of pesticide mixtures on pesticide degradation in biobed systems (9). Moreover, it is unknown whether or not such perturbations affect particular pesticide-degrading micropopulations added to BPS through bioaugmentation with pesticide-primed soil.

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TABLE 1. Overview of the different BM setups operated in this study

Setup[a]	Soil source	Linuron-primed soil	Linuron treatment	% Moisture content (wt/wt)	pH (±SD)
L^-	Agriculture	+	−	56.12	6.20 (0.12)
L^+	Agriculture	+	+	60.52	6.20 (0.12)
C^-	Construction site	−	−	48.00	6.05 (0.22)
C^+	Construction site	−	+	48.14	6.05 (0.22)

[a] Treatment with (+) or without (−) linuron as indicated.

This study examines whether the linuron-mineralizing populations that had been enriched in the BPS microcosms systems (BM) reported by Sniegowski et al. (18) were affected by sequentially imposed stress conditions caused by relevant changes in environmental conditions. It was further examined whether systems bioaugmented with pesticide-primed soil endured the imposed stresses better than BMs inoculated with nonprimed soil. The implemented environmental perturbations included a stop in pesticide supply, a cold period, a dry-wet cycle, and the addition of a pesticide mixture instead of a single pesticide. The effect of the stresses on the *Variovorax* community in the systems was assessed.

MATERIALS AND METHODS

Pesticides used. Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methyl urea] (purity, 99.5%), isoproturon [3-(4-isopropylphenyl)-1,1-dimethyl urea] (purity, 99.0%), and atrazine (2-chlor-4-ethylamino-6-isopropylamino-1,3,5-triazine) (purity, 98.0%) were purchased from Sigma-Aldrich, Belgium, and bentazon (3-isopropyl-2,1,3-benzothiadiazine-4-on-2,2-dioxide) (purity 98.8%) was from BASF, Germany. [Phenyl-U-^{14}C]linuron (16.93 mCi mmol^{-1}; radiochemical purity, >95%) was obtained from Izotop, Hungary.

Experimental setup and implemented stress conditions. The BMs used were those operated in the first experiment of Sniegowski et al. (18). Those BMs were set up in glass cylinders filled with 25% (vol/vol) cut straw (±0.5 cm^2), 25% (vol/vol) peat, and 50% (vol/vol) nonprimed soil C or linuron-primed soil L. Soil L, a sandy loam soil, was treated with linuron in the field on a long-term basis and was shown to contain a linuron mineralization capacity (3), while sandy loam soil C, originating from a building construction site, had no history of linuron treatment. BMs of setups C^- and L^- were irrigated during 12 weeks with sterile tap water, while BMs of setups C^+ and L^+ received during the same period tap water containing 60 mg liter^{-1} linuron with an average volume of 3.18 liter m^{-3} day^{-1}. Each week the solutions were manually applied using a micropipette on Monday (1 ml), Wednesday (1 ml), and Friday (1.5 ml). All four setups included triplicate BMs, which were incubated in the dark at 25°C. The initial moisture content, determined by a weight difference of ±0.5 g matrix material before and after dehydration for 2 days at 60°C, is shown in Table 1.

The BMs were subjected to sequential environmental changes from week 12 as illustrated in Fig. 1. The stress conditions implemented on BMs of setup C^+ and setup L^+ included a linuron supply stop (10 weeks of irrigation with sterile tap water without linuron), a cold period (14 weeks of incubation at 4°C without a supply of water), a drought period (5 weeks of incubation at 25°C without a supply of water), a rewetting period (2 weeks of incubation at 25°C with a supply of water without linuron), and a supply of a pesticide mixture containing, in addition to linuron (60 mg liter^{-1}), atrazine, isoproturon, and bentazon at concentrations of 20 mg liter^{-1} each during 4 weeks. In the latter case, the mixture was supplied to two of the three BMs within a setup, while the remaining BM received tap water with only linuron. After each imposed stress condition, initial standard conditions (2 weeks of supply of tap water with linuron and incubation at 25°C) were reestablished to study the potential recovery of the pesticide mineralization capacity. BMs of setups C^- and L^- were subjected to the same sequential perturbations but were never fed with linuron. The moisture content was monitored during the dry-wet cycle as described above. Samples for [^{14}C]linuron mineralization assays were resampled from the upper 1-cm layer from the same BM at weeks 17, 22, 28, 42, 49, 51, 55, and 60. At week 60, the mineralization assay was performed in the presence and absence of the other three pesticides, and a batch degradation assay (as described below) was performed in which the removal of all four pesticides was checked.

[^{14}C]linuron mineralization assay. [^{14}C]linuron mineralization assays were performed, and cumulative mineralization curves were established as described by Sniegowski et al. (18). Briefly, 0.2-g biomix samples were suspended in 15-ml Pyrex tubes, closed with Teflon-lined stoppers, and equipped with alkali [^{14}C]CO_2 traps in 5 ml mineral medium (18), and both unlabeled linuron (final concentration, 25 mg liter^{-1}) and ^{14}C-labeled linuron (final radioactivity of 213 Bq ml^{-1}, corresponding to a concentration of 31 μg liter^{-1}) as the only carbon and nitrogen sources. The vials were incubated at 20°C (±1°C) on a rotary shaker (125 rpm). Periodically, the NaOH solution was removed from the trap and replaced with fresh alkali. After mixing the removed NaOH solution with 5 ml of liquid scintillation cocktail (Ultima Gold; Perkin Elmer), the radioactivity was measured in a liquid scintillation counter (Tri Carb 2800 Tr; Perkin Elmer). The percentage of produced [^{14}C]CO_2 in reference to the initial amount of [^{14}C]linuron was calculated to establish cumulative [^{14}C]linuron mineralization curves. All assays were done in triplicate, and abiotic controls without soil were included. The mineralization rate and the lag time, defined as the period between initiating the experiment and the start of mineralization, were calculated as described by Sniegowski et al. (18). Average lag times were statistically compared by means of a t test assuming unequal variance ($P < 0.05$).

Batch pesticide degradation assay. Batch degradation assays were done in sterile 100-ml Erlenmeyer flasks containing 200 mg (wet weight) material from the mixed upper layer (1 cm) of the BM matrix and 50 ml of minimal medium (pH 7.0) containing linuron, atrazine, isoproturon, and bentazon at concentrations of 20 mg liter^{-1} each. Abiotic controls without biological activity were set up identically, but the pH of the medium was lowered to pH 3. The Erlenmeyer flasks were incubated on a horizontal shaker (125 rpm) at 25°C in the dark. Seven hundred-μl samples were analyzed by reverse-phase high-performance liquid chromatography (HPLC) (LaChrom; Merck Hitachi) after centrifugation at 12,000 rpm for 5 min as described by Breugelmans et al. (3). The compounds were separated by a gradient elution with CH_3CN and H_2O containing 0.05% H_3PO_4 (0 min, 80/20; 10 min, 70/30; 12 min, 70/30; 18 min, 50/50; 25 min, 50/50; 30 min, 80/20) at a flow rate of 1.0 ml min^{-1}.

Molecular techniques. DNA was extracted from the samples taken from the BMs (upper 1-cm layer) according to the time frame shown in Fig. 1. The composition of the *Variovorax* community was visualized by denaturing gradient gel electrophoresis (DGGE) fingerprints of *Variovorax* 16S rRNA gene fragments amplified by double PCR using *Variovorax*-specific primers as described by Bers et al. (2). The quantification of both the total bacterial population and the

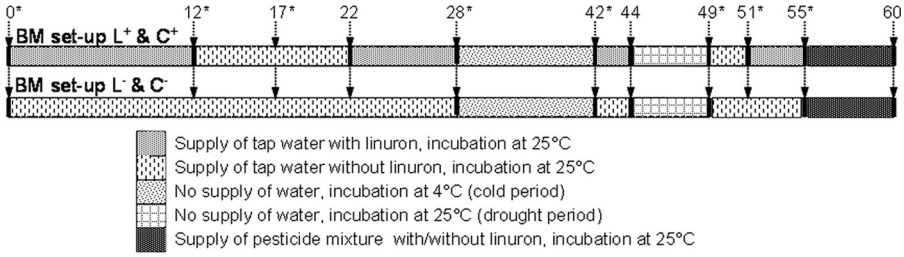

FIG. 1. Time scheme of the imposed sequential environmental perturbations applied to the different BM setups. Sampling times are marked with arrows, and the numbers of weeks after the initiation of the experiment are indicated. At sampling times marked with an asterisk, samples were taken to analyze the *Variovorax* community.

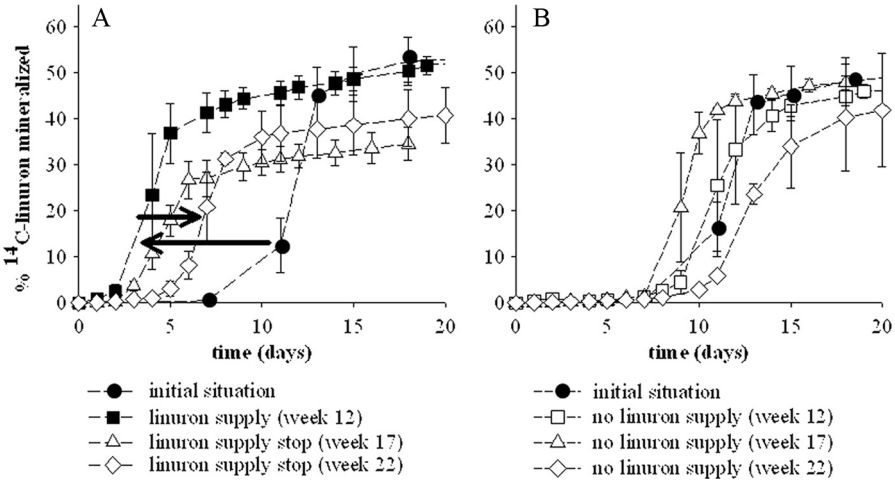

FIG. 2. Effect of a linuron supply stop on the linuron mineralization capacity of BMs of setup L^+ (A) and setup L^- (B) inoculated with linuron-primed soil. Samples were taken at 0, 12, 17, and 22 weeks after starting the treatment. The arrows indicate the changes in lag time. The data are average values with standard deviations from three replicates.

Variovorax population was based on real-time PCR amplification of targeted 16S rRNA gene fragments as described by Bers et al. (2). Averages of the 16S rRNA gene copy numbers of triplicate BM samples and significant differences between samples were analyzed by analysis of variance (ANOVA) ($P < 0.05$).

RESULTS

Effect of a stop in linuron feeding. Changes in pesticide mineralization capacity in the BMs were monitored by means of ^{14}C mineralization assays performed on BM samples. The observed lag time in the mineralization curves was previously shown to be indicative for the linuron mineralization capacity in the BMs and hence for the size of the linuron-mineralizing community (18). As reported by Sniegowski et al. (18), after 12 weeks of linuron feeding, the BMs of setup L^+ showed linuron mineralization with an average lag time of 0.46 ± 0.56 days, which was significantly shorter than the lag time of 9.9 ± 0.4 days recorded at week 0. In contrast, the lag times recorded for samples taken from BMs of setup L^-, irrigated with tap water without linuron, were similar at week 12 to those recorded at week 0. Moreover, BMs of setup C^+ also had acquired the capacity to mineralize linuron, since the average lag time recorded for samples taken from BMs of setup C^+ after 12 weeks of linuron feeding was 4.5 ± 1.8 days, while BMs of setup C^- did not show a linuron mineralization capacity. Stopping the linuron supply had a clear effect on the linuron mineralization capacity of setup L^+. As illustrated in Fig. 2, the lag time recorded for samples of BMs of setup L^+ increased from 0.46 ± 0.56 to 2.8 ± 0.6 days and 5.2 ± 0.5 days after 5 and 10 weeks, respectively. In contrast, the lag time recorded for samples of BMs of setup L^- (9.0 ± 0.1 days) was not significantly changed at week 22 compared to that at week 12. Stopping the supply of linuron had an even more severe effect on the linuron mineralization capacity of BMs of setup C^+. Linuron mineralization lag times recorded for samples taken from those BMs gradually increased when feeding with linuron was stopped. After 10 weeks, two out of three BMs of setup C^+ did not show any linuron mineralization capacity, while the third BM showed an increased lag time (21.8 days) compared to the lag time recorded at week 12 (Fig. 3). When the feed of linuron was restored for 6 weeks, the linuron mineralization capacity of BMs of setups L^+ and C^+ was fully restored, and associated lag times recorded for samples taken from those BMs were similar to

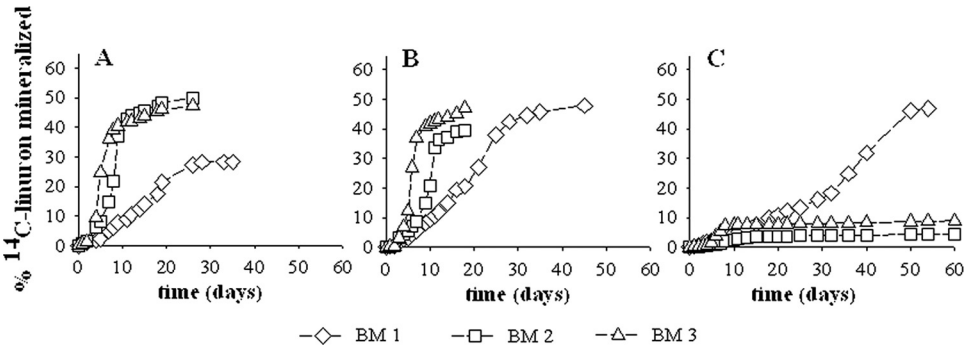

FIG. 3. Effect of a linuron supply stop on the linuron mineralization capacity of BMs from setup C^+, i.e., the situation after 12 weeks of linuron supply (A), 5 weeks of no linuron supply (B), and 10 weeks of no linuron supply (C). The graphs show the mineralization curves recorded for each replicate (BM 1, BM 2, and BM 3).

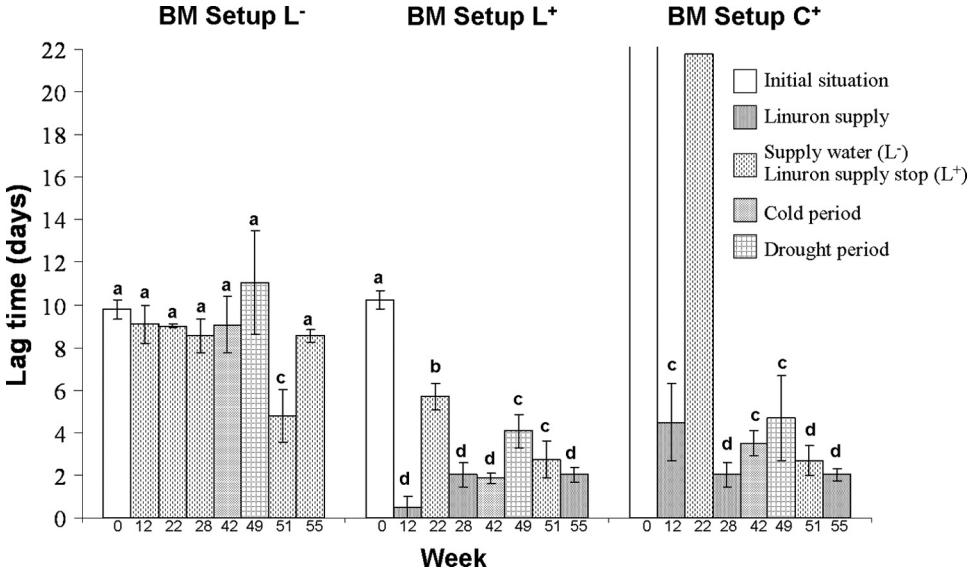

FIG. 4. Overview of the lag times recorded in the mineralization assays performed with samples taken from BMs of setups L^-, L^+, and C^+ after the implementation of the different (stress) conditions and recovery periods. The lag times are average values with indicated standard deviations from replicate BMs. Average lag times that significantly differ from each other are indicated with a different letter. Week 0, initial situation; week 12, after 12 weeks of water (setup L^-) or linuron supply (setups L^+ and C^+); week 22, after 10 weeks of linuron supply stop (all setups); week 28, after 6 weeks of water (setup L^-) or linuron supply (setups L^+ and C^+); week 42, after 14 weeks of a cold period (all setups); week 49, after 5 weeks of drought (all setups); week 51, after 2 weeks of rewetting (all setups); week 55, after 4 weeks of water (setup L^-) or linuron supply (setups L^+ and C^+). The lag time recorded in setup C^+ at week 22 is from the single BM replicate which maintained its linuron mineralization capacity. No lag times were calculated from the batch mineralization assays performed with samples taken from BMs of setup C^-, since no linuron mineralization was recorded.

those recorded at week 12. It shows that stopping the supply of linuron did not cause a permanent change to the linuron-mineralizing populations in any of the BM setups.

Effect of a cold period. Mineralization curves recorded for samples taken from the BMs at week 42, i.e., after 14 weeks of incubation at 4°C, did not significantly differ from those recorded before initiating the cold period (at week 20), i.e., no change in lag times and mineralization rates were observed (Fig. 4). This was the case for all setups, despite the fact that no water or linuron was supplied. The resupply of linuron and/or tap water and incubation at 25°C after the cold period did not affect the lag time and mineralization rate in any of the setups.

Effect of a dry-wet cycle. From week 44, the BMs were subjected to a drought period of 5 weeks by incubating the microcosms at 25°C without supplying water. During that period, the overall moisture content decreased from 53.20% ± 7.60% (wt/wt) to 2.12% ± 0.65% (wt/wt). Effects are shown in Fig. 4. All setups that had demonstrated a linuron mineralization capacity at week 12 still showed a mineralization capacity after the drought period. However, linuron mineralization lag times recorded for samples taken from BMs of setup L^+ were significantly increased, from 1.8 ± 0.2 to 4.1 ± 0.8 days. Mineralization lag times for samples taken from setup C^+ also were increased, from 2.8 ± 1.7 to 4.7 ± 2.0 days, but that difference was not significant. The drought period seems, therefore, to have a more severe effect on the linuron-mineralizing population of setup L^+ than a stop in linuron supply. Samples from BMs of setup L^-, which did not receive a linuron-containing solution, showed a small insignificant increase in lag time from 9.2 ± 0.1 to 11.1 ± 2.4 days. The drought period was succeeded by a rewetting phase of 2 weeks, in which all BMs were irrigated with tap water without linuron until the initial moisture content was reestablished. Mineralization curves recorded with samples from BMs of setups L^+ and C^+ taken after this period (at week 51) showed a decrease in lag time to 2.8 ± 0.9 and 2.7 ± 0.7 days, respectively (Fig. 4). Interestingly, samples from BMs of setup L^-, which previously never had shown a major change in lag time, showed a decrease in lag time from 11.1 ± 2.4 to 4.8 ± 1.2 days, indicating the growth of a linuron-mineralizing population in the BMs. This was not the case for setup C^-. After reestablishing standard conditions with a corresponding feed of tap water with or without linuron, samples from setups L^+ and C^+ (taken at week 55) showed a further decrease in linuron mineralization lag time to 1.5 ± 0.4 and 1.3 ± 0.3 days, respectively, which is lower than those recorded before the drought period. Mineralization curves recorded for samples taken from BMs of setup L^- showed a lag time similar to the lag time recorded directly after rewetting.

Effect of the supply of a pesticide mixture. Mineralization experiments performed with samples from setups L^-, L^+, and C^+ showed no significant change in lag time before and after the period in which the pesticide mixture was applied (Fig. 4). Moreover, the presence of bentazon, atrazine, and isoproturon did not affect the mineralization kinetics of linuron (data not shown). The degradation kinetics of linuron in the batch degradation assay monitored by HPLC were comparable to those recorded in the mineralization assay. Interestingly, a capacity to degrade atrazine developed in the BMs containing linuron-primed soil when fed with the pesticide mixture. Isoproturon was not degraded, and bentazon appeared to be removed by abiotic processes rather than biodegradation in the batch deg-

TABLE 2. 16S rRNA gene copy numbers of bacteria and *Variovorax* in BMs of setups L^-, L^+, C^-, and C^+ as determined at different time steps during the implementation of the stress conditions

Week[b]	Copy numbers for setup[a]:					
	L^-			L^+		
	Variovorax	Bacteria	*Variovorax*/bacteria (%)	*Variovorax*	Bacteria	*Variovorax*/bacteria (%)
0	$3.4 \times 10^7 \pm 1.4 \times 10^7$	$2.6 \times 10^{10} \pm 0.1 \times 10^{10}$	0.13 ± 0.06	$6.7 \times 10^7 \pm 4.6 \times 10^7$	$1.6 \times 10^{10} \pm 0.8 \times 10^{10}$	0.42 ± 0.35
12	$1.2 \times 10^8 \pm 0.7 \times 10^8$	$3.7 \times 10^{10} \pm 3.5 \times 10^{10}$	0.31 ± 0.22	$3.1 \times 10^8 \pm 1.4 \times 10^8$	$3.1 \times 10^{10} \pm 0.1 \times 10^{10}$	0.72 ± 0.16
17	$3.0 \times 10^8 \pm 2.9 \times 10^8$	$4.7 \times 10^{10} \pm 3.8 \times 10^{10}$	0.63 ± 0.61	$2.2 \times 10^8 \pm 1.7 \times 10^8$	$3.8 \times 10^{10} \pm 2.4 \times 10^{10}$	0.72 ± 0.16
28	$5.3 \times 10^7 \pm 0.7 \times 10^7$	$3.1 \times 10^{10} \pm 0.5 \times 10^{10}$	0.17 ± 0.02	$3.0 \times 10^7 \pm 4.5 \times 10^7$	$2.5 \times 10^{10} \pm 0.6 \times 10^{10}$	1.2 ± 0.18
42	$1.3 \times 10^8 \pm 1.0 \times 10^8$	$5.4 \times 10^{10} \pm 2.3 \times 10^{10}$	2.42 ± 1.8	$4.5 \times 10^8 \pm 1.3 \times 10^8$	$3.2 \times 10^{10} \pm 2.1 \times 10^{10}$	1.4 ± 0.40
49	$1.2 \times 10^8 \pm 1.1 \times 10^8$	$2.3 \times 10^{10} \pm 0.09 \times 10^{10}$	0.53 ± 0.47	$2.8 \times 10^8 \pm 0.5 \times 10^8$	$2.1 \times 10^{10} \pm 1.6 \times 10^{10}$	1.3 ± 0.25
51	$2.5 \times 10^7 \pm 2.3 \times 10^7$	$2.7 \times 10^{10} \pm 1.5 \times 10^{10}$	0.09 ± 0.09	$4.3 \times 10^7 \pm 3.9 \times 10^7$	$4.2 \times 10^{10} \pm 1.0 \times 10^{10}$	0.10 ± 0.09
55	$2.1 \times 10^7 \pm 1.1 \times 10^7$	$4.0 \times 10^{10} \pm 2.2 \times 10^{10}$	0.05 ± 0.02	$1.9 \times 10^8 \pm 0.5 \times 10^8$	$3.5 \times 10^{10} \pm 2.0 \times 10^{10}$	0.56 ± 0.15

[a] ND, not done. *Variovorax*/bacteria, percentage of the number of *Variovorax* 16S rRNA gene copies relative to the number of bacterial 16S rRNA gene copies; $n = 3$.
[b] Week 0, initial situation (all setups); week 12, situation after supply of water without linuron (setups L^- and C^-) and with linuron (setups L^+ and C^+); week 17, situation after stopping the linuron supply for 5 weeks (all setups); week 28, situation after 6 weeks of supply of water without linuron (setups L^- and C^-) or with linuron (setups L^+ and C^+); week 42, situation after 14 weeks of incubation at 4°C (all setups); week 49, situation after 5 weeks of drought (all setups); week 51, situation after 2 weeks of rewetting with water without linuron (all setups); week 55, situation after 4 weeks of supply of water without linuron (setups L^- and C^-) and with linuron (setups L^+ and C^+).

radation experiments, since the controls without inoculum showed the loss of bentazon.

Effect of stress conditions on the *Variovorax* community. The dynamics of the size of the *Variovorax* community and the bacterial community during stress conditions was monitored using targeted real-time PCR (Table 2). The bacterial community size in BMs of setup L^- and L^+ remained rather stable during the different implemented stress conditions. In BMs of setups C^- and C^+, significantly lower numbers of bacteria were detected at week 12 (in setup C^-) and week 51 (in setup C^+). Based on the fluctuations in the linuron mineralization capacity, a decline in the *Variovorax* community size in BMs of setup L^+ at week 17 (linuron supply stop) and week 49 (drought period) was expected. However, the number of *Variovorax* 16S rRNA gene copies remained unchanged except for a significant decline after the rewetting period, which is in contrast to the recorded highly enhanced linuron mineralization. The numbers of *Variovorax* 16S rRNA gene copies in BMs of setups L^-, C^-, and C^+ fluctuated more over time and showed an overall larger standard deviation than those of setup L^+. At week 42 (after the cold period), an unexpected significant increase was observed in the number of *Variovorax* 16S rRNA gene copies in BMs of setups L^- and C^-. However, because of the large variations in *Variovorax* numbers in these two setups, we cannot conclude that this was related to the cold period.

Variovorax 16S rRNA gene DGGE fingerprints were analyzed from samples taken at weeks 17, 28, 42, 49, 51, and 55.

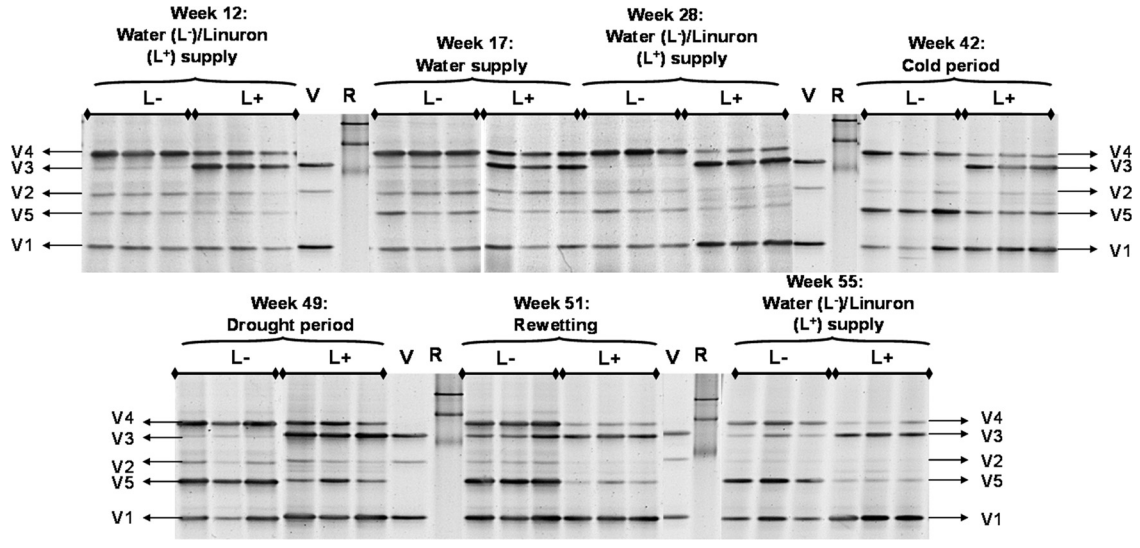

FIG. 5. Dynamics of the *Variovorax* community composition in BMs of setups L^- and L^+ during the imposed stress conditions as analyzed by *Variovorax* 16S rRNA gene DGGE. DGGE profiles shown are those obtained from the 3 BM replicates within the setup indicated above the lanes. Week 12, situation after water (setup L^-) or linuron supply (setup L^+); week 17, after linuron supply stop of 5 weeks (all setups); week 28, after water (setup L^-) or linuron supply (setup L^+) for 6 weeks; week 42, after a cold period of 14 weeks (all setups); week 49, after a drought period of 5 weeks (all setups); week 51, after a rewetting of 2 weeks (all setups); week 55, after 4 weeks of water (setup L^-) or linuron supply (setup L^+). DGGE fingerprints of the reference samples designated ref-nonVar and ref-Var are indicated with R and V, respectively.

TABLE 2—*Continued*

Copy numbers for setup[a]:					
C⁻			C⁺		
Variovorax	Bacteria	*Variovorax*/ bacteria (%)	*Variovorax*	Bacteria	*Variovorax*/ bacteria (%)
$5.4 \times 10^7 \pm 3.8 \times 10^7$	$3.3 \times 10^9 \pm 1.4 \times 10^9$	1.66 ± 1.11	$4.1 \times 10^7 \pm 3.8 \times 10^7$	$4.9 \times 10^9 \pm 1.4 \times 10^9$	0.84 ± 1.11
$1.3 \times 10^6 \pm 1.1 \times 10^6$	$0.3 \times 10^9 \pm 0.1 \times 10^9$	0.54 ± 0.49	$9.0 \times 10^6 \pm 0.8 \times 10^6$	$3.3 \times 10^9 \pm 3.0 \times 10^9$	0.28 ± 0.37
$1.6 \times 10^7 \pm 1.1 \times 10^7$	$5.0 \times 10^9 \pm 2.1 \times 10^9$	0.31 ± 0.22	$1.8 \times 10^7 \pm 1.3 \times 10^7$	$5.5 \times 10^9 \pm 1.2 \times 10^9$	0.34 ± 0.23
$3.5 \times 10^6 \pm 1.3 \times 10^6$	$3.2 \times 10^9 \pm 1.2 \pm 10^9$	0.11 ± 0.04	$2.5 \times 10^7 \pm 4.0 \times 10^7$	$2.8 \times 10^9 \pm 1.9 \times 10^9$	0.88 ± 1.4
$1.6 \times 10^8 \pm 0.6 \times 10^8$	$4.1 \times 10^9 \pm 1.5 \times 10^9$	4.0 ± 1.4	$1.0 \times 10^8 \pm 0.8 \times 10^8$	$1.7 \times 10^9 \pm 1.3 \times 10^9$	3.6 ± 4.2
$2.1 \times 10^7 \pm 1.8 \times 10^7$	$1.8 \times 10^9 \pm 1.4 \times 10^9$	1.2 ± 0.99	$1.9 \times 10^7 \pm 1.9 \times 10^7$	$2.3 \times 10^9 \pm 2.1 \times 10^9$	0.84 ± 0.82
ND	ND	ND	$1.1 \times 10^6 \pm 0.4 \times 10^6$	$0.4 \times 10^9 \pm 0.04 \times 10^9$	2.8 ± 1.0
$3.3 \times 10^6 \pm 1.9 \times 10^6$	$2.6 \times 10^9 \pm 2.2 \times 10^9$	0.13 ± 0.07	$2.8 \times 10^7 \pm 1.1 \times 10^7$	$3.7 \times 10^9 \pm 0.3 \times 10^9$	0.60 ± 0.21

Figure 5 shows the fingerprints obtained with samples from setups L^+ and L^-. As reported previously (18), BMs of all setups showed after 12 weeks of treatment the occurrence of four dominating bands at positions V1, V2, V4, and V5, except for the BMs of setup L^+, where an additional fifth band (at position V3) became dominant at week 12. This band is associated with *Variovorax* phylotype A, a subgroup within the *Variovorax* phylogeny which contains only linuron-degrading *Variovorax*, including *Variovorax* sp. SRS16 (3, 18, 19). In the fingerprints obtained with samples of the BMs from setup L^+ taken after week 12, no additional bands appeared. In general, the initial four bands in the DGGE profiles from setup L^+ did not diminish in intensity in the samples taken after the period of stopping the linuron supply (week 17). However, after the reestablishment of the linuron supply (week 28), bands at positions V1 and V3 clearly dominated. The band at position V1 corresponds to *Variovorax* phylotype B, which contains the linuron-degrading strain WDL1 (5). Interestingly, bands at positions V4 and V5 became more dominant in the DGGE profiles from setup L^+ after the drought period (week 49), indicating that the *Variovorax* populations related to strain SRS16 (band at position V3) and strain WDL1 (band at position 1) diminished in size during the drought period. However, after rewetting (week 51), the two bands at positions V1 and V3 dominated the profile again. DGGE profiles of BM samples of setup L^- consistently showed the four-banded profile with band positions V1, V2, V4, and V5. However, after rewetting the dry matrix, a band at position V3 became visible, which is in agreement with the observed increased linuron mineralization capacity in setup L^-. Furthermore, the *Variovorax* population associated with band position V5 clearly flourished after the temperature drop.

Figure 6 shows the *Variovorax* 16S rRNA gene DGGE fingerprints obtained with samples from setups C^- and C^+. In contrast to the fingerprints of setup L^+, the fingerprints of setup C^+ showed no clear dominating band in the profile

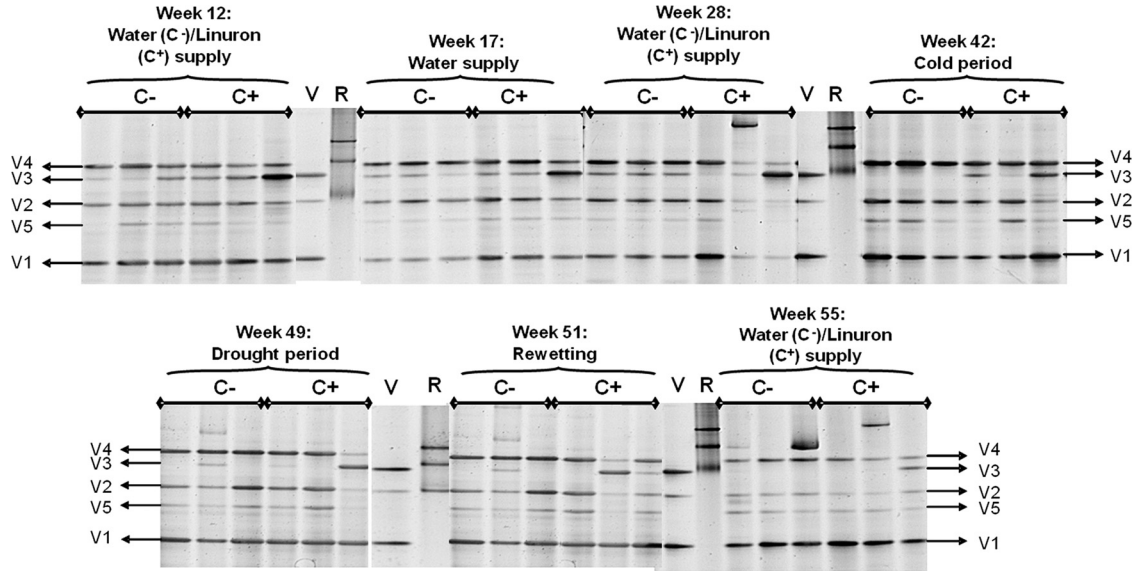

FIG. 6. Dynamics of the *Variovorax* community composition in BMs of setups C^- and C^+ during the imposed stress conditions as analyzed by *Variovorax* 16S rRNA gene DGGE. DGGE profiles shown are those obtained from the 3 BM replicates within the setup indicated above the lanes. Week 12, situation after water (setup C^-) or linuron supply (setup C^+); week 17, after linuron supply stop of 5 weeks (all setups); week 28, after water (setup C^-) or linuron supply (setup C^+) for 6 weeks; week 42, after a cold period of 14 weeks (all setups); week 49, after a drought period of 5 weeks (all setups); week 51, after a rewetting of 2 weeks (all setups); week 55, after 4 weeks of water (setup C^-) or linuron supply (setup C^+). DGGE fingerprints of the reference samples designated ref-nonVar and ref-Var are indicated with R and V, respectively.

compared to these of setup C^-. Although a band at position V3 appeared in the profile of this setup, this band also was regularly detected in setup C^-, which did not show any linuron-mineralizing capacity. Furthermore, although the fingerprint profiles generally showed bands at positions similar to those of the fingerprints of setups L^- and L^+, there were no changes in the profiles after most of the imposed stress conditions. On the other hand, more bands started to appear in the profiles of both setups C^- and C^+ after the drought period, but the profiles were not always the same for the triplicate BM.

DISCUSSION

The effect of stresses sequentially imposed by relevant environmental changes on the linuron-degrading microbial community of a BPS was examined in BMs. Control BMs in which no stress was applied were not included in this study. Nevertheless, the effects of the changing environmental conditions was clear, as shown by their dynamic character; i.e., effects on linuron mineralization capacity and *Variovorax* community were observed only after imposing the stress situation, and recovery toward normal activities/community composition was observed after lifting the stress situation.

A linuron supply stop and a drought period had the most severe effects on the linuron-mineralizing capacity in setups L^+ and C^+. Apparently in both setups the decay rate of the linuron-degrading community exceeded the growth rate under nonselective conditions, i.e., without pesticide feed, and the linuron-mineralizing communities lost their selective advantage. The linuron-mineralizing community in setup L^+, however, never decreased in size below the size recorded at week 0. Since in setup L^- the linuron-mineralizing capacity was never lost, even after 22 weeks without linuron feed, it is unlikely that in setup L^+ longer periods without linuron feed will result in the complete loss of the linuron-mineralizing capacity. It indicates that even under nonselective conditions, the linuron-mineralizing populations compete well with other bacteria and easily remain in the system. Decay rates under nonselective conditions in setup C^+ apparently were higher than those in setup L^+. This can be due to differences in sensitivity to environmental factors that determine decay rates, such as grazing by protozoa (14). Otherwise, the diversity within the linuron-mineralizing community might have been higher in setup L^+ than in setup C^+. More diverse communities have been suggested to be less sensitive to environmental perturbations, especially for specialized functions, such as pesticide degradation (11). Alternatively, bacterial xenobiotic degradation gene functions often show a high degree of instability under nonselective conditions, and linuron degradation functions might have been more easily lost in the C^+ populations (4).

The drought period clearly had a negative impact on the linuron-mineralizing community of setups L^+ and L^-. Previously, Shelton and Parkin (17) observed a decreased mineralization of carbofuran in soil after reducing the moisture content, while Issa and Wood (12) observed reduced atrazine degradation but increased isoproturon degradation in agricultural surface and subsurface soil undergoing desiccation. Moreover, rewetting the soil positively affected the linuron-mineralizing capacity in all setups. This is in contrast to Mercadier et al. (15), who reported a delay in the mineralization of metalaxyl-M and lufenuron in soil after a dry-wet cycle. In our study, the mineralization of linuron in setups L^+, L^-, and C^+ increased compared to the situation before the drought period. This increase occurred without the selective conditions of linuron in the feed. This was unexpected, since no report exists on the increase of the size of xenobiotic degrading bacterial populations without selective forces. In accordance with Franzluebbers et al. (10), we hypothesize that the drought period resulted in the release of easily degradable carbon from dead biomass, which enabled the proliferation of linuron degraders. It indicates again that in all setups, the linuron-mineralizing populations competed well with other microorganisms.

The cold period without linuron supply did not affect the linuron-mineralizing capacity in any of the setups. This was in strong contrast to the decreased linuron mineralization capacity observed after the period without linuron supply at 25°C. Lower temperatures are known to decrease bacterial activity and turnover time but do not necessarily result in changes in the population size (16). Also, in none of the setups was the linuron degradation capacity affected by mixtures of other pesticides. Martins and Mermoud (13) observed no decreased rates of degradation when three different pesticides were simultaneously added to soil, while effects in suspension were noted. Bazot and Lebeau (1), however, observed the simultaneous mineralization of diuron and glyphosate in both free and immobilized cell suspensions. In contrast, Fogg et al. (9) observed the decreased degradation of six different pesticides applied as a mixture in a biomix. Apparently, the effect of the presence of one pesticide on the degradation of the other depends on the type of pesticides and their bioavailability. Interestingly, the biodegradation of atrazine improved in setup L^+ during feeding with the pesticide cocktail but not in setups C^+, C^-, and L^-, indicating that the addition of linuron-primed soil and the feed with atrazine caused the proliferation of atrazine degraders in setup L^+. Soil L likely contained an atrazine-degrading population. The field from which soil L originated has been used for the cultivation of corn, which often implicates treatment with atrazine. This result supports the use of pesticide-primed soil for improving pesticide degradation in BPS.

A change in linuron mineralization capacity was not necessarily reflected in the number of bacterial and *Variovorax* 16S rRNA gene copies. As discussed previously (18), we believe that linuron degraders are only a small fraction of the bacterial and *Variovorax* community in the BMs, and that any changes remain unnoted within the bulk of the bacterial and *Variovorax* 16S rRNA gene pool. In contrast, in setup L^+, we observed the diminishment and reappearance of *Variovorax* phylotype A concomitantly with the diminishment and reappearance of the linuron mineralization capacity as a response to the stress conditions. It shows that solely quantifying the abundance of specific genera or higher taxa can overlook relationships between effects and microbial community structure, and that the simultaneous use of techniques which address diversity signatures of the studied community is beneficial for understanding such relationships. *Variovorax* phylotype A contains *Variovorax* sp. SRS16, a well-known linuron-degrading *Variovorax* strain (19). We sequenced the DGGE band associated with phylo-

type A at different sampling times, and in all cases 100% sequence identity to the SRS16 16S rRNA gene sequence was found (data not shown). Those observations support the hypothesis that the corresponding populations are involved in linuron degradation in setup L^+, as suggested by Sniegowski et al. (18). Further support for this hypothesis comes from the observation that in setup L^- the sudden increase in linuron mineralization capacity upon rewetting occurred concomitantly with the appearance of *Variovorax* phylotype A. Moreover, we observed that the dynamics of the linuron mineralization capacity and of the *Variovorax* phylotype A in setup L^+ was congruent with the increase and decrease in copy numbers of the gene responsible for linuron hydrolysis in *Variovorax* sp. SRS16 (K. Bers, K. Sniegowski, and D. Springael, unpublished results). Final evidence for the involvement of *Variovorax* phylotype A in linuron degradation requires other techniques, like DNA/RNA stable isotope probing (8). On the other hand, it cannot be excluded that other organisms were involved in linuron degradation. However, the DGGE profiling of bacterial 16S rRNA genes showed no differences between setups L^+ and L^- (data not shown).

In contrast to setup L^+, in setup C^+ no relationship existed between the proliferation of a specific *Variovorax* phylotype and changes in linuron mineralization capacity, suggesting that organisms different from *Variovorax* are responsible for linuron mineralization in setup C^+. Moreover, it might explain why stopping the linuron feed affected the linuron mineralization capacity in setups C^+ and L^+ differently.

In conclusion, our data suggest that pesticide-degrading populations proliferating in the matrix of a BPS, such as *Variovorax*, are quite robust and only moderately respond to environmental changes expected to occur in a BPS. No dramatic differences in stress response existed between pesticide-degrading populations added through pesticide-primed soil or nonprimed soil. In case effects occur, the restoration of the selective conditions seems to rapidly result into the reestablishment of the pesticide-degrading capacity. Those data are in support of the use of BPS for the treatment of pesticide-contaminated wastewater at farm yards (6).

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