RESEARCH ARTICLE

High prevalence of IncP-1 plasmids and IS*1071* insertion sequences in on-farm biopurification systems and other pesticide-polluted environments

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Abstract

Mobile genetic elements (MGEs) are considered as key players in the adaptation of bacteria to degrade organic xenobiotic recalcitrant compounds such as pesticides. We examined the prevalence and abundance of IncP-1 plasmids and IS*1071*, two MGEs that are frequently linked with organic xenobiotic degradation, in laboratory and field ecosystems with and without pesticide pollution history. The ecosystems included on-farm biopurification systems (BPS) processing pesticide-contaminated wastewater and soil. Comparison of IncP-1/IS*1071* prevalence between pesticide-treated and nontreated soil and BPS microcosms suggested that both IncP-1 and IS*1071* proliferated as a response to pesticide treatment. The increased prevalence of IncP-1 plasmids and IS*1071*-specific sequences in treated systems was accompanied by an increase in the capacity to mineralize the applied pesticides. Both elements were also encountered in high abundance in field BPS ecosystems that were in operation at farmyards and that showed the capacity to degrade/mineralize a wide range of chlorinated aromatics and pesticides. In contrast, IS*1071* and especially IncP-1, MGE were less abundant in field ecosystems without pesticide history although some of them still showed a high IS*1071* abundance. Our data suggest that MGE-containing organisms were enriched in pesticide-contaminated environments like BPS where they might contribute to spreading of catabolic genes and to pathway assembly.

Introduction

Many synthetic organic compounds, that is organic xenobiotics, have entered our environment due to anthropogenic activities. It has been shown that bacteria can develop novel enzymatic activities and metabolic pathways that enable them to use such organic xenobiotics as sole source of energy and carbon or as a nutrient source (van der Meer, 2008). Genes involved in the degradation of organic xenobiotics are often located on mobile genetic elements (MGEs), such as plasmids and transposons. These MGEs are considered as crucial agents in the evolution and adaptation of bacterial communities by controlling the intra- and interbacterial exchange of genetic material (Top & Springael, 2003; Heuer & Smalla, 2012). Among the best described plasmids that carry organic xenobiotic catabolic genes are the IncP-1 plasmids. For instance, various IncP-1 plasmids have been reported that carry catabolic gene clusters for the degradation of pesticides like 2,4-D and atrazine. This is in contrast with catabolic plasmids belonging to other plasmid groups like IncP-2, IncP-7, and IncP-9 plasmids, that mostly carry genes involved in the degradation of natural pollutants like hydrocarbons (Top & Springael, 2003; Nojiri *et al.*, 2004; Dennis, 2005). Plasmids belonging to the IncP-1 group are large low-copy broad-host range plasmids that are transferable between and replicate in most Gram-negative species (Musovic *et al.*, 2006). Five

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different IncP-1 subgroups (α, β, γ, δ and ε) have been described (Bahl *et al.*, 2009), and recently, a novel subgroup ζ has been added (Norberg *et al.*, 2011). Another MGE that is often reported as carrier of xenobiotic catabolic gene clusters are IS*1071* composite transposons (Top & Springael, 2003; Nojiri *et al.*, 2004; Dennis, 2005). The 3.2-kb-long insertion sequence IS*1071* consists of a transposase (*tnpA*) gene that is flanked by long inverted repeats of 110 bp (Nakatsu *et al.*, 1991). Based on the similarity of the *tnpA* gene, IS*1071* is the only insertion sequence that is categorized as a class II transposon although it does not contain a resolution function (Sota *et al.*, 2008). Interestingly, IS*1071* elements are often found on catabolic IncP-1 plasmids where they flank the catabolic gene clusters forming IS*1071* composite transposons. Schlüter *et al.* (2003) reported on a IncP-1 plasmid that contains a single IS*1071* element that is associated with antibiotic resistance genes instead of a catabolic function. IS*1071* elements have also been identified on plasmids different from IncP-1 [e.g. pMOL28 (Janssen *et al.*, 2010) and BYI23_E (Lim *et al.*, 2012)] and on bacterial chromosomes (e.g. in *Cupriavidus metallidurans* CH34; Janssen *et al.*, 2010) where they are associated with various gene functions.

Several studies report on the response of bacterial communities to the application of an organic xenobiotic pressure in laboratory experiments and in field experiments. For instance, the repeated application of pesticides in agricultural soils can result in an accelerated mineralization of the compound. This has been explained by the growth of organisms with degradative gene functions and/or the horizontal gene transfer of the responsible genes within the resident microbial community (Rousseaux *et al.*, 2001; Bers *et al.*, 2011b; Sniegowski *et al.*, 2011). The abundances of specific xenobiotic catabolic gene functions in polluted environments have been examined in several studies (Erb & Wagner-Döbler, 1993; Hallier-Soulier *et al.*, 1996; de Lipthay *et al.*, 2003; Martin-Laurent *et al.*, 2004; Bers *et al.*, 2012), leading to the conclusion that increased numbers of xenobiotic catabolic gene functions can be related to pollution. However, up to now, there is no report that examined the impact of an organic xenobiotic pressure on the prevalence of MGEs.

In this study, the presence of IncP-1 plasmids and IS*1071* was examined in microcosm and field environments with different pollution backgrounds and organic xenobiotic degradation capacities. The focus was on pesticide-treated environments such as soil and on-farm biopurification systems (BPS). BPS are used for the treatment of pesticide-contaminated wastewater at farms. They operate as a biofilter system in which pesticides are removed from the wastewater by sorption on and biodegradation in the filter matrix that consists of soil, peat, straw, and other agricultural wastes (De Wilde *et al.*, 2007). BPS receive high loads of pesticides at relatively high concentrations during a substantial time period of the year, creating a strong and long-term selective pressure for the evolution and growth of pesticide-degrading bacteria (Sniegowski *et al.*, 2011). First, the abundance, diversity, and dynamics of IncP-1 and IS*1071* were assessed in soil and BPS microcosm (BM) systems that received, or not, a pesticide contamination using PCR, real-time PCR, and sequence analysis of amplicon clone libraries. It was hypothesized that MGE abundance would increase in ecosystems that are treated with pesticides compared with nontreated systems. In a second part of the research, the presence, abundance, and diversity of IncP-1 and IS*1071* were determined in field ecosystems including BPS operating at farm yards and several soils contaminated with either pristine or contaminated with nonxenobiotic compounds. The *trfA* gene was used as a gene marker for IncP-1 (Bahl *et al.*, 2009) and *tnpA* for IS*1071* (Providenti *et al.*, 2006).

Materials and methods

Plasmids

Plasmids used in this study are presented in Table 1. Reference IncP-1 plasmids (representing the different IncP-1 subgroups α, β, γ, δ and ε) were used as positive controls for *trfA*-targeted conventional PCR and Southern blot hybridization. Plasmid pMS0252 containing a single IS*1071* copy originating from the IncP-1 plasmid pUO1 was used as positive control in *tnpA*-targeted conventional PCR and Southern blot hybridization, as template for *tnpA* probe preparation and for preparing standard series for *tnpA*-directed qPCR. The plasmids were propagated in either *Escherichia coli* at 28 °C or 37 °C in Luria–Bertani broth or *Alcaligenes xylosoxidans* at 30 °C in tryptic soy broth media. Fifteen gram per litre agar (Lab M, UK) was added in case solid media was used, and

Table 1. Plasmids used in this study

Plasmid	Host	Relevant characteristics	References
RP4	*E. coli*	Ap^R; Km^R; Tc^R; IncP-1α	Datta *et al.* (1971)
R751	*E. coli*	Tm^R; IncP-1β	Thorsted *et al.* (1998)
pQKH54	*E. coli*	IncP-1γ	Haines *et al.* (2006)
pEST4011	*A. xylosoxidans*	IncP-1δ	Vedler *et al.* (2004)
pKJK5	*E. coli*	Tc^R; IncP-1ε	Bahl *et al.* (2007)
pMS0252	*E. coli*	Tc^R; carries a cloned IS*1071* element	Sota *et al.* (2006)

antibiotics were added when appropriate with the following concentrations: ampicillin (Ap), 100 mg L^{-1}; tetracycline (Tc), 10 mg L^{-1}; kanamycin (Km), 50 mg L^{-1}, and trimethoprim (Tm), 20 mg L^{-1}.

Chemicals

Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methyl urea] (purity, 99.5%), atrazine [2-chloro-4ethylamine-6-isopropylamino-S-triazine] (99.5%), metamitron [4-amino-3-methyl-6-phenyl-1,2,4-triazin-5(4*H*)-one] (99.5%), 2-chlorobenzoic acid (2-CBA; 98%), 3-chlorobenzoic acid (3-CBA; > 99%), 4-chlorobenzoic acid (4-CBA; 99%), 2,3-dichlorobenzoic acid (2,3-DCBA; 97%), 2,4-dichlorobenzoic acid (2,4-DCBA; 98%), 2,5-dichlorobenzoic acid (2,5-DCBA; 97%), 2,6-dichlorobenzoic acid (2,6-DCBA; 98%), 3,4-dichlorobenzoic acid (3,4-DCBA; 99%), 3,5-dichlorobenzoic acid (3,5-DCBA; 97%), 2,3,5-trichlorobenzoic acid (2,3,5-TCBA; 97%), 3-chloroaniline (3-CA; 99%), 4-dichloroaniline (4-CA; 98%), and 3,4-dichloroaniline (3,4-DCA; 98%) were purchased from Sigma Aldrich (Belgium). 4-chlorophenol (4-CP; > 99%) was obtained from Avocado Research chemicals (UK). Radiolabeled pesticides [phenyl-U-14C] linuron (627 MBq $mmol^{-1}$, radiochemical purity > 95%), [phenyl-U-14C] atrazine (1395 MBq $mmol^{-1}$, radiochemical purity > 95%), [phenyl-U-14C] metamitron (522 MBq $mmol^{-1}$, radiochemical purity > 95%), and [phenyl-U-14C] isoproturon (445 MBq $mmol^{-1}$, radiochemical purity > 95%) were purchased from Izotop (Hungary).

Environmental samples

Environmental samples used in this study originated from a soil microcosm (SM) experiment, a BM experiment, several BPS operating at farm yards and several soils (Table 2). The SM experiment has been described before (Bers *et al.*, 2011b, 2012) and aimed at assessing the role

Table 2. Overview of microcosm and field environments used in this study and their characteristics

Ecosystem	Pollutant	Characteristics/remarks	References
Microcosm set-up			
SM-1	–	Control SM system irrigated with tap water without linuron	Bers *et al.* (2011b)
SM-2	Linuron	Irrigated with a single dose of linuron in tap water	Bers *et al.* (2011b)
SM-3	Linuron	Irrigated with multiple doses of linuron in tap water	Bers *et al.* (2011b)
BM-1	–	Simulated BPS matrix without pesticide-primed soil and irrigated with tap water	This study
BM-2	Pesticide mixture	Simulated BPS matrix without pesticide-primed soil and irrigated with a mixture of linuron, isoproturon, metamitron, and atrazine in tap water	This study
BM-3	–	Simulated BPS matrix containing a mixture of pesticide-primed soils irrigated with tap water without pesticides	This study
BM-4	Pesticide mixture	Simulated BPS matrix containing a mixture of pesticide-primed soils and irrigated with a mixture of linuron, isoproturon, metamitron, and atrazine in tap water	This study
Field BPS			
Pristine BPS	–	Simulated BPS matrix mixture consisting of straw (27.5 vol%), peat (38.5 vol%), coconut husk (27.5 vol%), manure (5.5 vol%)	This study
BPS Leefdaal	Pesticide mixture	On-farm BPS, continuously treated with a mixture of pesticides in operation at farm in Leefdaal (Belgium)	This study
BPS Pcfruit L	Pesticide mixture	On-farm BPS, continuously treated with a mixture of pesticides in operation at farm in St.-Truiden (Belgium)	This study
BPS Pcfruit R	Pesticide mixture	On-farm BPS, continuously treated with a mixture of pesticides in operation at farm in St.-Truiden (Belgium)	This study
BPS Lierde	Pesticide mixture	On-farm BPS, continuously treated with a mixture of pesticides in operation at farm in Lierde (Belgium)	This study
BPS Kortrijk	Pesticide mixture	On-farm BPS, continuously treated with a mixture of pesticides in operation at farm in Kortrijk (Belgium)	This study
BPS Koksijde	Pesticide mixture	On-farm BPS, continuously treated with a mixture of pesticides in operation at farm in Koksijde (Belgium)	This study
Field soils			
WL	–	Pristine woodland soil (Saint-Michel-des-Saints, Canada)	This study
Zeveren-1	–	Soil with background Zn concentration of 52 mg kg^{-1} zinc, 30 m from electricity transmission pylon (Zeveren, Belgium)	Mertens *et al.* (2006)
Zeveren-2	Zn	Soil near electricity transmission pylon and contaminated with 1233 mg kg^{-1} zinc (Zeveren, Belgium)	Mertens *et al.* (2006)

of *Variovorax* in linuron mineralization in agricultural soil. Briefly, SMs consisted of small glass columns filled with soil originating from a field with a history of linuron treatment designated as soil L. The experiment included three different set-ups in which soil L in the SMs was differently treated, that is, (1) a discontinuous irrigation with sterile tap water; (2) a single dose of 1 mL of sterile tap water containing 50 mg L^{-1} linuron at the start of the incubation period; and (3) a discontinuous irrigation with sterile tap water containing 50 mg L^{-1} linuron. During treatment, the SMs were incubated at 25 °C. Each set-up included three replicate SMs. The samples used in this study for analysis of IncP-1 and IS*1071* content were taken after 3 weeks of incubation. At that time point clear differences were observed in *Variovorax* community and linuron mineralization capacities between the SM set-ups with and without linuron treatment (Bers *et al.*, 2011b). The BM experiment was performed in a way similar to the BM experiment reported by Sniegowski *et al.* (2011). BMs consisted of the same glass columns used in the SM experiment, but were filled with either a mixture of 27.5 vol% cut straw, 38.5 vol% peat, 27.5 vol% coconut husk chips, and 5.5 vol% cow manure (set-ups without pesticide-primed soil) or a mixture of 25 vol% cut straw, 35 vol% peat, 25 vol% coconut husk chips, 5 vol% cow manure, and 10 vol% of a mixture of pesticide-primed soils (set-up with pesticide-primed soil). The soil mixture consisted of different soils that had been treated with the pesticides that were used for irrigating the BMs in the pesticide-treated set-ups (see below) and that contained corresponding pesticide-degrading bacterial populations. Triplicates of the two BM set-ups were treated with either sterile tap water or sterile tap water supplemented with 50 mg L^{-1} linuron, 50 mg L^{-1} isoproturon, 50 mg L^{-1} metamitron, and 30 mg L^{-1} atrazine. After 12 weeks of incubation at 25 °C, 200 mg samples of the BM matrix were taken to determine pesticide mineralization capacities (see below) and 400 mg to extract DNA and determine the prevalence of IncP-1 and IS*1071*. On-farm BPS matrix samples were taken from six different systems in operation at different farms situated at different locations in Belgium. From each BPS, fresh samples on the top layer of the matrix (0–10 cm) were collected from three different positions in the system and stored at 4 °C in the dark. Soil samples originated from a pristine Canadian woodland and from a meadow in Zeveren in Belgium. At the latter location, two samples were examined, that is, one taken in the direct neighborhood of a galvanized electricity transmission pylon and contaminated with corroded zinc at 1233 mg kg^{-1} and one at 30 m distance from the pylon containing background zinc concentrations (52 mg kg^{-1}) as described (Mertens *et al.*, 2006).

Total community DNA extraction

Extraction of total community DNA from soil samples taken in the SM experiment and from environmental ecosystem samples was performed using the E.Z.N.A.® Soil DNA kit (Omega Bio-Tek), as described by the manufacturer. Total DNA from samples taken in the BM experiment was extracted, as described by Uyttebroek *et al.* (2006).

Conventional end-point PCR detection and Southern blot hybridization

A 281-bp fragment of the IncP-1 backbone gene *trfA* was amplified from environmental DNA using the primer mixture described by Bahl *et al.* (2009). This primer mixture consists of three forward and three reverse primers that together amplify the *trfA* genes of the five IncP-1 plasmid subgroups (α, β, γ, δ, and ε). Reactions were performed in a total volume of 25 μL containing 0.625 U TrueStart Taq DNA polymerase and 1X buffer (Fermentas, Germany), 200 μM of each dNTP, 2.5 mM $MgCl_2$, 0.1 mg mL^{-1} bovine serum albumin (BSA), 0.6 μM of each primer, and 1 μL of template DNA. PCR consisted of an initial denaturation step of 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 20 s at 60 °C, and 20 s at 72 °C and a final elongation step of 5 min at 72 °C. PCR amplification of a 179-bp *tnpA* fragment of IS*1071* was performed using primers tnpA-F and tnpA-R, described by Providenti *et al.* (2006). The reactions were performed in a total volume of 25 μL containing 0.625 U Taq DNA polymerase and 1X PCR buffer (Qiagen, Belgium), 200 μM of each dNTP, 0.1 mg mL^{-1} BSA, 0.1 μM of each primer, and 1 μL of template DNA. The PCR started with a denaturation step of 15 min at 95 °C, followed by 35 cycles of 20 s at 94 °C, 20 s at 62 °C, and 20 s at 72 °C and ended with an elongation step of 2 min at 72 °C. PCR products were separated by agarose gel electrophoresis (1.2–2%) in Tris-acetate/EDTA buffer and visualized using ethidium bromide. Afterward, the products were blotted onto Hybond-N+ membranes (Amersham) by capillary transfer using 20X SSC buffer (0.3 M sodium citrate, 3 M NaCl, pH 7.0) during 16 h (Sambrook & Russell, 2001). The PCR fragments were fixed to the membrane by 2-h incubation at 80 °C and hybridized with appropriate probes for identity confirmation. Digoxigenin (DIG)-labeled hybridization probes were generated from the appropriate reference plasmids (Table 1) by means of PCR using the DIG-PCR labeling kit (Roche Applied Science, Germany), and the primer pairs and reaction conditions reported above except that DIG-labeled nucleotides (Roche Applied Science) were used. For verifying the identity of IncP-1 amplicons by

DNA–DNA hybridization, a mixture of *trfA* probes targeting the five IncP-1 subgroups (α, β, γ, δ, and ε) were used. For each of the subgroups, *trfA*-labeled amplicons were first generated separately and mixed afterward. Hybridizations were performed in middle stringent hybridization buffer (5X SSC, 20% formamide, 2% blocking agent, 0.1% sodium lauroylsarcosine, and 0.02% SDS). The membranes were washed twice in low-stringent solution (2X SSC, 0.1% SDS; 5 min, room temperature) and twice in high-stringent solution (0.1X SSC, 0.1% SDS; 15 min, 68 °C), before chemiluminescent detection with CPD-star (Roche Applied Science), according the manufacturer's recommendations.

Cloning and sequencing of PCR amplicons

PCR amplification products of the 281-bp IncP-1 *trfA* and 179-bp *tnpA* fragments were cloned into pCR2.1 TOPO® vector using the TOPO TA Cloning kit (Invitrogen) and transformed into *E. coli* TransforMax™EC100™ cells (Epicentre) using a MicroPulser™ from Biorad. Randomly chosen clones were sequenced with the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) using the universal M13 primers in accordance with the manufacturer's recommendations. The sequences were analyzed for nucleotide homology to available sequences in the nonredundant NCBI database using BLASTN (Altschul *et al.*, 1997; Zhang *et al.*, 2000).

Real-time PCR

Quantification of bacterial 16S rRNA gene abundance was performed according to the SYBRgreen real-time PCR approach, as described by Haest *et al.* (2011). Taqman real-time PCR was used to determine the copy number of IS*1071* based on the *tnpA* gene. To quantify IS*1071*, the *tnpA*-specific primer set was used as described by Providenti *et al.* (2006), and a dual-labeled Taqman probe, tp_tnpA (5′-FAM-TCTTGAAGCCTTTGCTGG CCAGAGTA-TAMRA-3′), was designed. The reaction was performed in a Rotor-Gene centrifugal real-time cycler (Corbett Research, Australia) in a total reaction volume of 25 μL containing 1.25-U TrueStart Taq DNA polymerase and 1X buffer (Fermentas), 200 μM of each dNTP, 2.5 mM $MgCl_2$, 0.1 mg mL^{-1} BSA, 0.3 μM of each primer, and 0.15-μM probe tp_tnpA. The real-time PCR consisted of an initially denaturation step of 10 min at 95 °C followed by 45 cycles of 15 s at 95 °C and 60 s at 60 °C with detection of the fluorescence signal after each cycle. The detection limit of the method is 1000 *tnpA* copies per μL template DNA. The abundance of *tnpA* relative to the 16S rRNA gene copy number was calculated, and values were expressed as relative *tnpA* abundances. Measured absolute copy numbers of the 16S rRNA gene per gram soil in microcosm and field ecosystems are presented in Supporting Information, Table S1. Significant differences in relative abundances between samples were examined by analysis of variance (ANOVA; $P < 0.05$).

Pesticide mineralization assays

^{14}C-pesticide mineralization assays were performed in triplicate to determine pesticide mineralization capacities, as described by Sniegowski *et al.* (2011). The assay was performed in 15-mL Pyrex tubes by suspending 200 mg (wet weight) of environmental sample in 5 mL of minimal medium MMO adjusted to pH 5.8 (prepared as described by Dejonghe *et al.*, 2003) containing 20 mg L^{-1} of unlabeled pesticide and ^{14}C-labeled pesticide with a final radioactivity of 238 Bq mL^{-1}, corresponding to 94,5 mg mL^{-1} ^{14}C-linuron (2.52 MBq mg^{-1}), 36.8 mg mL^{-1} ^{14}C-atrazine (6.46 MBq mg^{-1}), 92.2 mg mL^{-1} ^{14}C-metamitron (2.58 MBq mg^{-1}), or 0.11 mg mL ^{14}C-isoproturon (2.16 MBq mg^{-1}). The Pyrex tubes were closed with Teflon-lined stoppers, equipped with alkaline CO_2 traps and incubated at 20 °C in the dark on a rotary shaker (125 r.p.m.). At regular intervals, the CO_2 traps were sampled and replenished with fresh NaOH solution (0.5 M). Radioactivity in the NaOH samples was measured in a liquid scintillation counter (Tri-Carb 2800TR; Perkin Elmer), as described by Breugelmans *et al.* (2007). The percentage of produced $^{14}CO_2$ of the initial added amount of ^{14}C-pesticide was calculated to establish cumulative mineralization curves, from which lag phase, mineralization extent, and maximal mineralization rate were calculated, as described by Sniegowski *et al.* (2009). Significance of differences between pesticide-treated and pesticide-untreated samples was analyzed using ANOVA ($P < 0.05$).

Batch chloroaromatic degradation assays

Batch chloroaromatic degradation assays were performed in triplicate in 15-mL reaction tubes by suspending 200 mg (wet weight) of sample in 5 mL of minimal media MMO (pH 7.0) that contained a mixture of chloroaromatic compounds. Three different mixtures were used. Mixture 1 consisted of 20 mg L^{-1} 2-CBA, 20 mg L^{-1} 4-CBA, 20 mg L^{-1} 2,4-DCBA, 30 mg L^{-1} 4-CA, and 30 mg L^{-1} 3,4-DCA. Mixture 2 consisted of 20 mg L^{-1} 3-CBA, 20 mg L^{-1} 2,6-DCBA, 20 mg L^{-1} 4-CP, and 20 mg L^{-1} 2,3,5-TCBA; and mixture 3 consisted of 20 mg L^{-1} 2,3-DCBA, 20 mg L^{-1} 2,5-DCBA, 20 mg L^{-1} 3,4-DCBA, 20 mg L^{-1} 3,5-DCBA, and 30 mg L^{-1} of 3-CA. Abiotic controls that contained sterilized soil that had been autoclaved three times were included.

The reaction tubes were incubated on a horizontal shaker (125 r.p.m.) at 20 °C in the dark. After 8 weeks of incubation, samples of 700 μL were taken and centrifuged at 20 000 ***g*** for 5 min. The supernatants were analyzed by reverse-phase high-performance liquid chromatography (HPLC, LaChrom; Merck Hitachi) equipped with a 250-mm-long Alltima HP C18 silica column (Grace, Belgium) using a 40/60 mobile phase consisting of CH_3CN/H_3PO_4 buffer (pH 2.5) at a flow rate of 0.8 mL min^{-1}. The individual compounds were identified and quantified based on retention times and peak areas derived from corresponding standard mixtures with known compound concentrations. For each sample and each compound, the ratio of the concentration in the biotic system and abiotic control system was calculated. To signify differences between the biotic system and abiotic control system, ANOVAS ($P < 0.05$) were performed.

Accession numbers

The sequences of cloned *trfA* amplicons were deposited in the GenBank under accession numbers KC285547–KC285585 and KC295449.

Results

Presence and abundance of IncP-1 and *IS1071* in pesticide-treated microcosm experiments

The presence of *trfA* and *tnpA*, as genetic markers for IncP-1 and IS*1071*, respectively, was examined by means of *trfA*- and *tnpA*-specific PCR on DNA extracted from the SM and BM set-ups that were either pesticide treated or not. The identity of the amplicons was checked afterwards by DNA–DNA hybridization using PCR-labeled gene probes generated from appropriate reference plasmids (Table 1), as explained in the Materials and methods section. As shown in Fig. 1, *trfA* amplicons were observed in all SM set-ups, but the intensities of the signals were clearly higher for the pesticide-treated SMs (in set-ups SM-2 and SM-3) compared with the nontreated SMs (set-up SM-1), indicating a higher abundance of IncP-1 in pesticide-treated SM systems compared with nontreated SM systems. Hybridization with the mixture of *trfA* probes confirmed the identity of the amplicons (Fig. 1). PCR targeting the *tnpA* gene clearly showed the presence of IS*1071* in two of the replica microcosms of set-up SM-3 that received multiple doses of linuron (Fig. 2). Hybridization using *tnpA* as a probe confirmed the identity of the amplicons as IS*1071,* but also revealed the presence of IS*1071* in the third SM-3 replica microcosm as well as in the single-dose linuron-treated microcosms of set-up SM-2 (Fig. 2). *tnpA*-based real-time PCR confirmed these results (Fig. 3). In the microcosms without pesticide treatment (set-up SM-1), *tnpA* relative abundances were below the detection limit of 1000 *tnpA* copies per μl template DNA. SMs that received a single dose of linuron showed a 4.2-fold higher average relative *tnpA* abundance (8.24E-04 ± 1.88E-05 *tnpA* rrn^{-1}) excluding the one sample which was below the detection limit, while SMs amended with multiple doses showed a 38-fold higher value (value of 3.14E-02 ± 2.45E-02 *tnpA* rrn^{-1}).

Results obtained in the BM experiment were similar to those obtained in the SM experiment. *trfA* amplicons were recovered from all set-ups (Fig. 1) except from the BM systems without pesticide-primed soil and treated with tap water (BM-1). The intensities of the *trfA* PCR products were clearly increased in the pesticide-treated systems (BM-2 and BM-4) compared with the nontreated systems (BM-1 and BM-3). The intensity of PCR signals were similar in pesticide-treated BM set-ups containing pesticide-primed soil (BM-4) and those without primed soil (BM-2), except for one replicate of set-up BM-2 that showed a lower intensity. Hybridization with *trfA* probes confirmed the identity of the *trfA* PCR products and demonstrated that IncP-1 plasmids are also present in the three replicas of the set-up without pesticide-primed soil and treated only with water (BM-1; Fig. 1). From the set-ups BM-3 and BM-4 containing pesticide-primed soils, 10 cloned *trfA* PCR products of each set-up were sequenced and revealed that the IncP-1 δ subgroup was predominantly present (98–99% nucleotide identity) in both BM set-ups with and without pesticide treatment. Only one clone originating from the set-up treated with pesticides (BM-4) showed a higher identity to *trfA* of another IncP-1 plasmid group, that is, the IncP-1β subgroup (100% identity). Pesticide-treated microcosms (BM-2 and BM-4) showed also more intense *tnpA* amplicon PCR signals compared with nontreated systems (BM-1 and BM-3) although one BM-2 sample showed only a weak signal (Fig. 2). *tnpA* PCR signals obtained from the pesticide-treated set-ups showed, however, higher intensities for the set-up containing pesticide-primed soils (BM-4) compared with the set-up without primed materials (BM-2). DNA–DNA hybridization with the *tnpA* probe confirmed the identity of the *tnpA* amplicons, but also revealed weak *tnpA* signals and hence low IS*1071* abundances in microcosms of the nontreated BM-3 set-up containing pesticide-primed soil (Fig. 2). The *tnpA* PCR products recovered from the pesticide-treated set-up BM-4 were cloned and sequenced. The sequences showed 99–100% nucleotide identity to IS*1071* sequences reported in the NCBI nonredundant database (data not shown). Real-time PCR confirmed the *tnpA* PCR results (Fig. 3). High average relative *tnpA* abundances were found for the

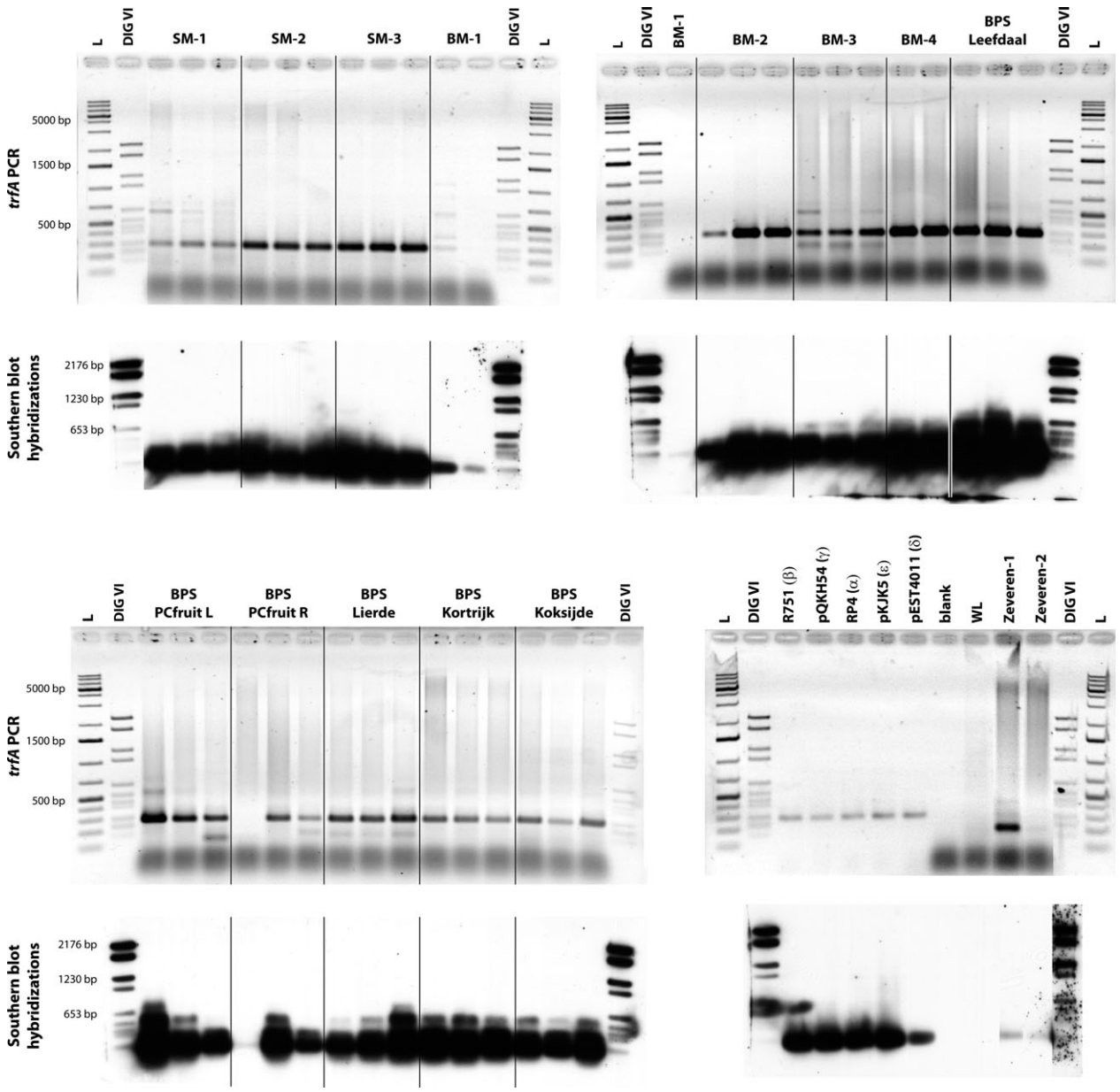

Fig. 1. Detection of IncP-1 plasmids in total community DNA derived from microcosm and field ecosystems by PCR and Southern blot hybridizations targeting the *trfA* gene. The three or two lanes below each indicated ecosystem represent the three or two replicates. Exposure time to X-ray film after hybridization was 15 min. for the SM and BM ecosystems. For the field BPS ecosystems, the exposure time was 4 min. For the Canadian woodland (WL) and the low (Zeveren-1) and high (Zeveren-2) zinc-contaminated Zeveren soils, an exposure time of 15 min was used. RP4, R751, pQKH54, pEST4011, and pKJK5 were used as positive controls representing the five IncP-1 subgroups.

pesticide-treated BMs set-ups whether they contained pesticide-primed soil (BM-4) or not (BM-2), that is, 3.14E-02 ± 2.45E-02 *tnpA* rrn^{-1} and 3.26E-02 ± 2.94E-02 *tnpA* rrn^{-1}, respectively. In the pesticide-treated BM-2 sample for which only a weak *tnpA* signal was observed by conventional PCR and hybridization, the *tnpA* copy number as determined by qPCR was below the detection limit. In the nontreated microcosms without pesticide-primed soil (set-up BM-1), *tnpA* copy numbers were below the detection limit while a low average relative abundance of 2.80E-04 ± 3.46E-04 *tnpA* rrn^{-1} was recorded in nontreated microcosms containing pesticide-primed soil (BM-3), which was about 700-times lower than the number recorded in the corresponding pesticide-treated system (BM-4). ANOVA on the SM and BM samples showed that there was a significant difference ($P = 0.032$) between the

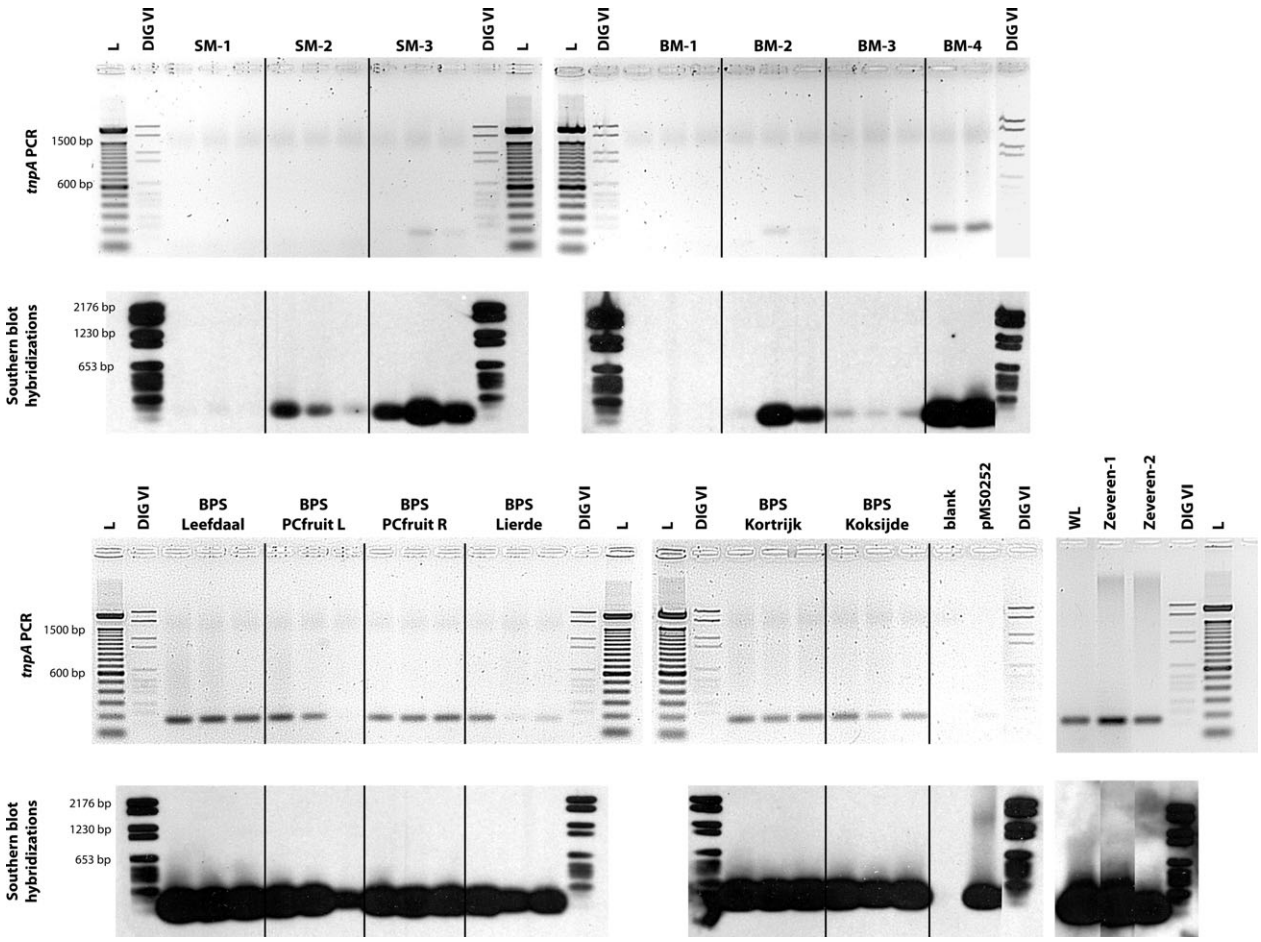

Fig. 2. Detection of IS*1071* elements in total community DNA of microcosm and field ecosystems by PCR and Southern blot hybridizations targeting the *tnpA* gene. The three or two lanes below each indicated ecosystem represent the three or two replicates. Exposure time to X-ray film after hybridization was 10 min. for the SM and BM ecosystems. For the field BPS ecosystems, the exposure time was 2 min. For the Canadian woodland (WL) and the low (Zeveren-1) and high (Zeveren-2) zinc-contaminated Zeveren soils, an exposure time of 2 min was used. pMS0252 containing IS*1071* was used as a positive control.

average relative *tnpA* abundance in the pesticide-treated BM set-ups and this in the nontreated BM set-ups.

To associate MGE abundance with pesticide degradation capacity in the microcosm set-ups, samples were taken from the microcosms to assess their pesticide mineralization potential before and after incubation. The data on the dynamics of linuron mineralization capacity in the SM experiment was reported before and showed a decreased lag time in the linuron-treated set-ups compared with the set-ups that only received tap water, suggesting an increase in linuron mineralization capacity upon linuron treatment (Bers *et al.*, 2011b). At the start of the BM experiment, only the BM systems containing pesticide-primed materials (BM-3 and BM-4), showed the capacity to mineralize linuron, metamitron, atrazine, and isoproturon but with relatively long lag times of, respectively, 18.64 ± 2.07, 26.16 ± 3.98, 44.36 ± 16.71, 51.97 ± 18.55 days (Table 3). The set-ups without pesticide-primed soil (BM-1 and BM-2) did not show any initial mineralization capacity. After 12 weeks of pesticide treatment, increased mineralization capacities were observed in both pesticide-treated systems (BM-2 and BM-4) for linuron and metamitron, with lag times that were significantly shorter than those recorded initially, that is, the lag time for linuron mineralization was reduced to 0.40 ± 0.19 and 0.17 ± 0.16 days for set-up BM-4 and set-up BM-2, respectively, and for metamitron mineralization to 2.84 ± 0.02 and 0.65 ± 0.03 days, respectively (Table 3). In contrast, BM microcosms containing pesticide-primed soil treated with only tap water (BM-3) showed only a slight improvement of mineralization capacity with lag times of 13.3 ± 1.99 days for linuron mineralization and 13.99 ± 3.15 days for metamitron mineralization. No improvement of the pesticide mineral-

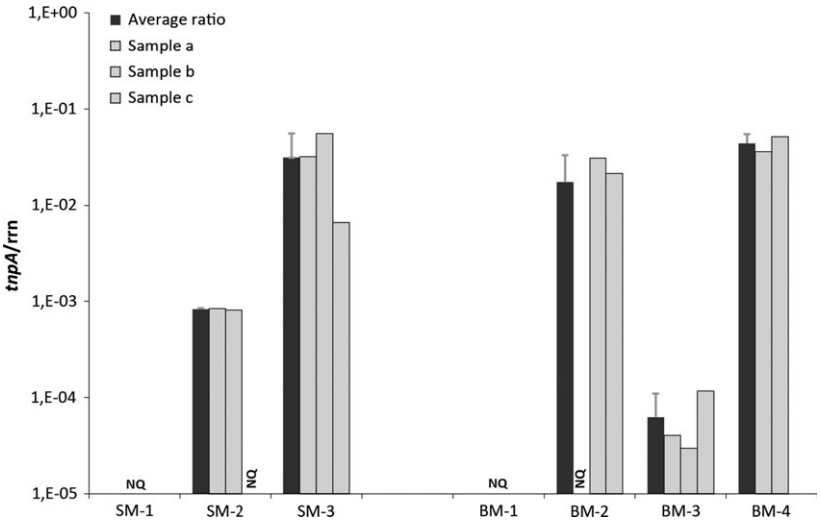

Fig. 3. Abundances of IS*1071* transposase gene *tnpA* relative to the 16S RNA gene copy number (rrn) in the BM and SM microcosm with and without pesticide treatment. The dark gray bars represent the average values with their standard deviation calculated from samples that gave values above the detection limit, while the light gray bars are the relative *tnpA* rrn^{-1} for each of the individual samples. All microcosms were operated in triplicate, except BM-4 that was only performed in twofold. NQ: no quantification (below the detection limit).

Table 3. Overview of the lag times in pesticide mineralization in the BM experiment at the start of the experiment (W0) and after 12 weeks (W12) of treatment with and without pesticides. Different BPS matrix compositions and treatments were used. Set-ups BM-1 en BM-2 included BMs in which the matrix was composed entirely of nonpesticide-contaminated materials. Set-ups BM-3 and BM-4 included BMs in which the matrix contained pesticide-primed soils. BMs of set-up BM-1 and BM-3 were treated with sterile tap water without pesticides, while BMs of set-up BM-2 and BM-4 received tap water containing a mixture of linuron, metamitron, atrazine, and isoproturon on a regular base. All values are average values calculated from three replicates ($n = 3$) with indicated standard deviations. A (–) sign indicates that no mineralization was observed during the assay

	^{14}C-pesticide mineralization lag times (days)							
	Linuron		Metamitron		Atrazine		Isoproturon	
	W0	W12	W0	W12	W0	W12	W0	W12
BM-1	–	–	–	–	–	–	–	–
BM-2	–	0.17 ± 0.16	–	0.65 ± 0.03	–	–	–	–
BM-3	18.64 ± 2.07	13.3 ± 1.99	26.16 ± 3.98	13.99 ± 3.15	44.36 ± 16.71	42.56 ± 2.49	51.97 ± 18.55	–
BM-4	18.64 ± 2.07	0.40 ± 0.19	26.16 ± 3.98	2.84 ± 0.02	44.36 ± 16.71	0.86 ± 0.20	51.97 ± 18.55	22.5 ± 4.40

ization capacity was recorded in the control BM set-up without pesticide-primed materials (Table 3). For atrazine and isoproturon, an increase in mineralization capacity compared with the initial mineralization capacity was only recorded for the BMs with pesticide-primed soil (BM-4), with respective lag times of 0.86 ± 0.20 and 22.5 ± 4.40 days (Table 3).

Prevalence of IncP-1 and *IS1071* in environmental ecosystems with different pollution history

Conventional PCR suggested that almost all samples taken from the six BPS in operation at farms contained high numbers of IncP-1 and IS*1071* because *trfA* and *tnpA* conventional PCR signals were for all samples very strong (Figs 1 and 2). In the investigated soils, no (Canadian woodland soil) or week aspecific (both Zeveren soils) *tfrA* signals (Fig. 1), but highly intense *tnpA* signals (all soils; Fig. 2) were recorded. Hybridization confirmed in all cases the identity of the amplicons as either *trfA* or *tnpA*. In the case of the Zeveren soils, the aspecific *trfA* PCR products did not hybridize. The *trfA* PCR products recovered from BPS Leefdaal and BPS Kortrijk and *tnpA* amplicons recovered from BPS Leefdaal, BPS Kortrijk, Zeveren–1, and the Canadian woodland soil were cloned and sequenced. All 10 cloned *trfA* amplicons recovered from BPS Leefdaal and 8 of the 10 *trfA* amplicons recovered from BPS Kortrijk showed highest nucleotide identity to *trfA* of IncP-1ε plasmid pKJK5 (> 97.9%). The other two cloned *trfA* amplicons of BPS Kortrijk showed the highest identity with *trfA* of the IncP-1β subgroup (> 91.5%). Cloned *tnpA* amplicons showed high identity with *tnpA* of IS*1071* of plasmid pUO1 with identities of more than > 99.4% for BPS Leefdaal amplicons, more than 97.8% for BPS Kortrijk amplicons, more than 97.8%

for the Canadian woodland amplicons, and more than 98.9% for the zinc-polluted Zeveren amplicons (data not shown). Real-time PCR data on *tnpA* abundance confirmed the *tnpA*-targeted conventional PCR data (Fig. 4). All samples from all six BPS showed relative IS*1071 tnpA* abundances which were higher than those observed in the pesticide-treated BM microcosm studies (> 1E-02 *tnpA* rrn^{-1}). As suggested by the conventional PCR results, *tnpA* and hence IS*1071* relative abundances were also quite high in the soil samples and similar to those recorded in various BPS samples, that is, 2.46E-02 *tnpA* rrn^{-1} in the Canadian woodland soil sample, 2.52E-02 *tnpA* rrn^{-1} in the zinc-polluted Zeveren soil and 1.05E-02 *tnpA* rrn^{-1} in the noncontaminated Zeveren soil. Nevertheless, BPS samples, originating from BPS Leefdaal (4.45E-01 ± 2.05E-01 *tnpA* rrn^{-1}), BPS Pcfruit L (2.49E-01 ± 3.51E-01 *tnpA* rrn^{-1}), and BPS Pcfruit R (1.01E-01 ± 3.91E-04 *tnpA* rrn^{-1}) showed 10-fold higher *tnpA* average relative abundances than the soils. In some of the BPS, high variances in abundances were obtained for samples taken from different locations in the same BPS.

To examine whether the observed high abundances of IncP-1 and IS*1071* biomarkers could be related to organic xenobiotic biodegradation activities, organic xenobiotic degradation activity screens were performed for the BPS samples, the woodland and the zinc-polluted Zeveren soil and a so-called pristine BPS matrix that was composed of fresh materials that are commonly used in BPS. The activity screens included mineralization of three different ^{14}C-radiolabeled pesticides that are commonly applied in agriculture and degradation of 14 different chlorinated aromatic compounds including chlorinated phenols, chlorinated anilines, and chlorinated benzoic acids.

Metamitron was efficiently mineralized in samples taken from most of the systems except in the pristine BPS sample and the Canadian woodland sample (Fig. 5a). Most systems showed a very short lag time of around 2.7 days. The highest extent of metamitron mineralization (56.43 ± 1.06%) and the fastest mineralization rate (8.73 ± 0.48% day^{-1}) was recorded with samples taken from BPS Kortrijk (Table 4). Linuron mineralization was observed with all samples of the tested BPS ecosystems except with the three samples of BPS Leefdaal and one sample of BPS Lierde (Fig. 5b). Linuron mineralization lag times were very different for the different BPS and ranged between 2.45 ± 0.10 days for BPS Kortrijk and 16.89 ± 8.11 days for BPS Koksijde (Table 4). Because of the high spatial differences in atrazine mineralization, only the individual samples are presented in Fig. 5c. Mineralization of atrazine was observed with all samples of BPS Kortrijk, one sample of BPS Leefdaal and to a lesser extent with one sample of BPS Lierde (Table 4).

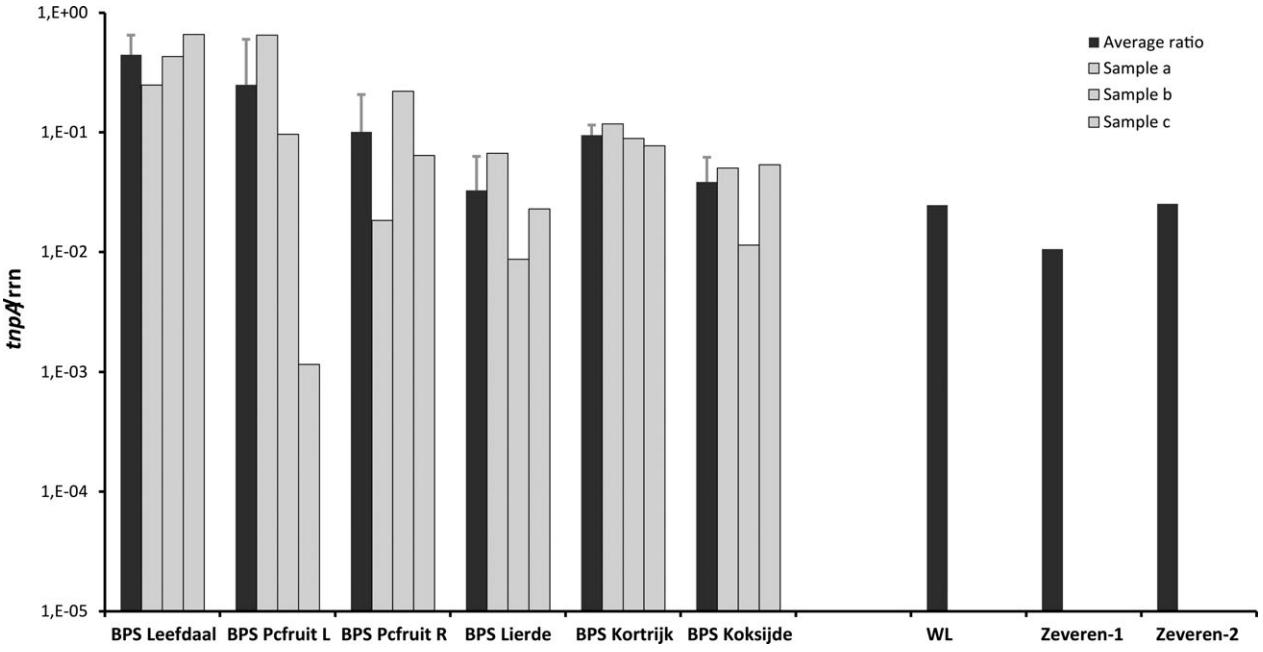

Fig. 4. Abundances of IS*1071* transposase gene *tnpA* relative to the 16S RNA gene copy number (rrn) in different on field BPS in operation and in field soils. The dark gray bars represent the average values with their standard deviation, while the light gray bars are the relative *tnpA* rrn^{-1} for each of the individual samples. From the Zeveren soils and the Canadian woodland soil, only one sample was analyzed.

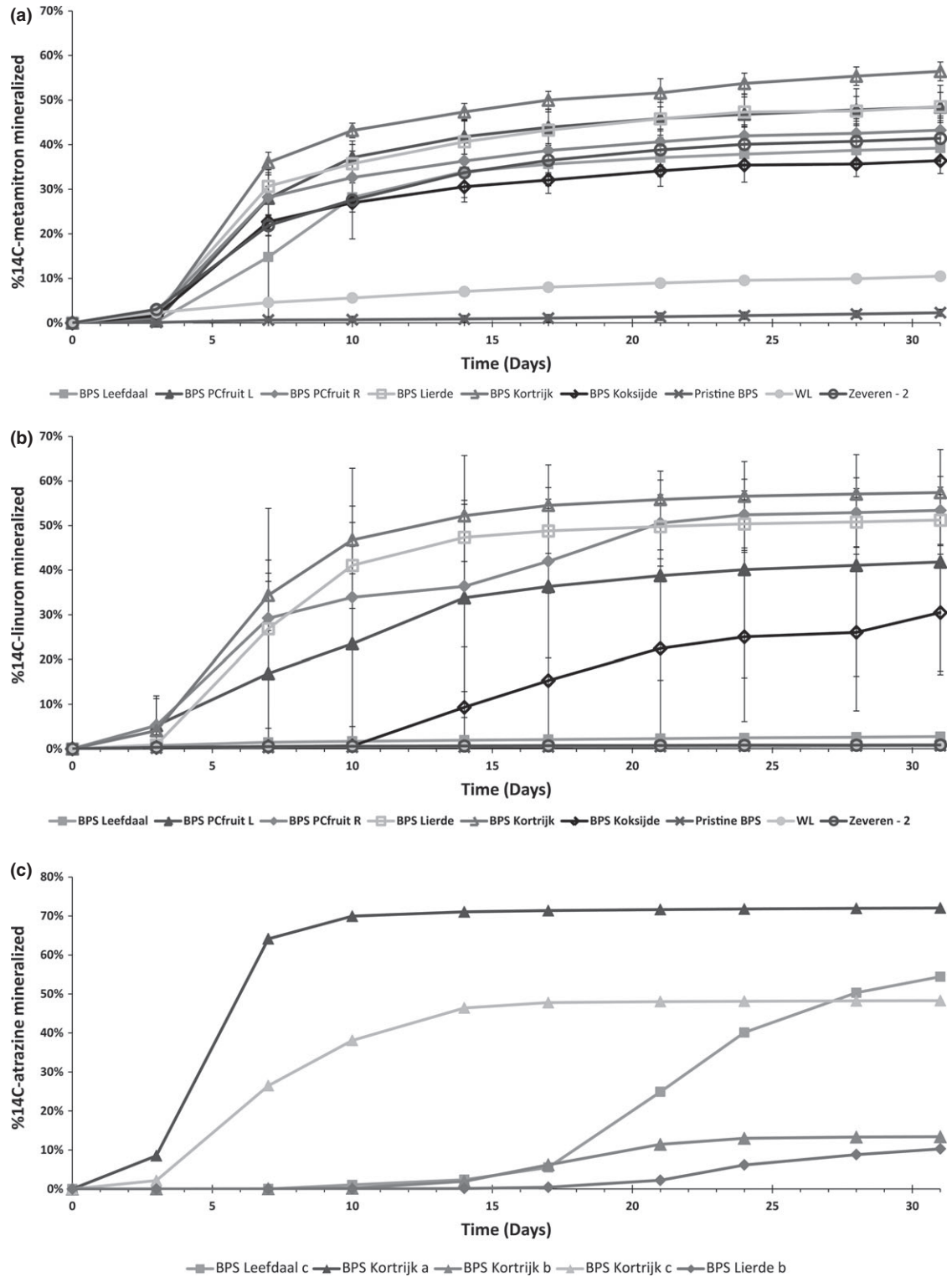

Fig. 5. Pesticide mineralization kinetics recorded with samples taken from the field ecosystems. Cumulative $^{14}CO_2$ production curves are presented for mineralization of either ^{14}C-labeled metamitron (a), linuron (b), or atrazine (c). Data in (a) and (b) are average values from values recorded for three replicate samples with the corresponding standard deviation. In one sample of BPS Lierde, no linuron mineralization was observed, and therefore, this sample was excluded from the graph. For atrazine, a high variation in mineralization capacity was recorded, and therefore, only the graphs recorded with the individual samples are shown in (c).

Table 4. Overview of pesticide mineralization extents, mineralization rates, and lag times in pesticide mineralization assays performed on samples from the field ecosystems. Average values and standard deviations were calculated from tree replicates ($n = 3$). When no mineralization rate and/or lag time could be calculated from the mineralization curves a (–) sign is used

	Metamitron			Linuron			Atrazine		
	ME (%)	MR (% day^{-1})	Lag time (days)	ME (%)	MR (% day^{-1})	Lag time (days)	ME (%)	MR (% day^{-1})	Lag time (days)
BPS Leefdaal	39.23 ± 5.7	6.54 ± 1.43	4.99 ± 1.77	2.76 ± 0.97	–	–	54.43*	5.08*	16.1*
BPS Pcfruit L	48.44 ± 4.87	6.99 ± 1.95	3.02 ± 0.15	41.82 ± 25.23	5.00 ± 3.51	3.22 ± 4.12	0.13 ± 0.04	–	–
BPS Pcfruit R	43.25 ± 3.17	6.54 ± 1.24	2.69 ± 0.05	53.4 ± 7.61†	7.97 ± 2.12†	6.05 ± 6.92†	0.06 ± 0.00	–	–
BPS Lierde	48.55 ± 3.17	7.07 ± 0.92	2.66 ± 0.07	51.22 ± 5.71	4.48 ± 4.2	3.01 ± 0.12	10.30*	1.31*	19.3*
BPS Kortrijk	56.43 ± 2.12	8.73 ± 0.48	2.87 ± 0.06	57.39 ± 1.22	7.57 ± 1.81	2.45 ± 0.1	44.59 ± 29.5	7.13 ± 6.3	5.88 ± 5.84
BPS Koksijde	36.40 ± 0.82	5.27 ± 0.4	2.69 ± 0.03	30.48 ± 13.07	4.56 ± 1.24	16.89 ± 8.11	0.07 ± 0.03	–	–
Pristine BPS	2.30 ± 0.8	–	–	0.70 ± 0.27	–	–	0.24 ± 0.12	–	–
WL	10.49 ± 0.95	0.77 ± 0.25	–	0.75 ± 0.06	–	–	0.04 ± 0.01	–	–
Zeveren-2	41.43 ± 4.54	4.7 ± 0.46	2.35 ± 0.07	0.88 ± 0.09	–	–	0.12 ± 0.03	–	–

ME, mineralization extent; MR, maximal mineralization rate.
*Data based on one sample showing mineralization.
†Data based on two samples showing mineralization.

Residual chloroaromatic concentrations (in %) recorded in the chloroaromatic degradation assays after 8 weeks incubation are presented in Table 5. The chlorinated benzoic acids, 2,3-DCBA and 2,6-DCBA did not show degradation in any of the tested samples, while 3-CBA was degraded in all samples. 2-CBA, 4-CBA, 2, 4-DCBA, and 3,5-DCBA were degraded in most of the BPS environments. 3,4-DCBA was degraded only in BPS Leefdaal and BPS Koksijde and 2,5-DCBA only in BPS Kortrijk, BPS Pcfruit L, and BPS Koksijde. The latter two BPS did only show a slight degradation (< 20% relative to the abiotic control) of 2,5-DCBA, while in BPS Kortrijk, two samples showed complete degradation of 2,5-DCBA. Only in BPS Koksijde, some removal of 2,3, 5-TCBA was seen, although compared with the abiotic control still more than 95% was present after 8 weeks. The soils showed considerable less chloroaromatic degradation capacities. The zinc-polluted Zeveren soil showed removal of 4-CBA, 3,5-DCBA, and 3,4-DCBA. Neither the Canadian woodland soil nor the pristine BPS showed degradation of chlorobenzoic acids besides 3-CBA. The chlorinated phenol, 4-CP, was degraded in all ecosystems, but the Canadian woodland soil still contained more than 50% of the compound after the 8 weeks of incubation. Degradation of the chlorinated anilines, 3-CA, 4-CA, and 3,4-DCA, was only observed in the BPS environments except for 4-CA whose degradation was also recorded in the zinc-polluted Zeveren soil.

Discussion

This study is the first report that examines the relationship between the presence/abundance of MGE, organic xenobiotic pollution, and xenobiotic degradation capacity. Focus was on IS*1071* and IncP-1 plasmids that have been often reported as MGEs associated with bacterial organic xenobiotic-degradative gene functions and on pesticide-polluted environments. The controlled SM and BM experiments showed that both IncP-1 plasmids and IS*1071* increased in abundance as a response to pesticide pollution, while the environmental systems with anthropogenic pesticide pollution display abundances that are similar or even higher than those recorded in the controlled experiments. Relative abundances of *IS1071*, as quantified by qPCR, were on average at least 38–700-fold higher in SM and BM set-ups exposed to multiple doses of pesticides compared with set-ups that were not exposed to pesticides. This difference cannot be explained by differences in bacterial community composition due to pesticide application and therefore possible differences in numbers of 16S rRNA gene copies per cell, because 16S rRNA gene copy numbers vary only between 1 and 15. (Lee *et al.*, 2009). Moreover, we have shown previously that exposure

Table 5. Overview of residual chloroaromatic concentrations in chloroaromatic degradation assays performed with samples taken from the field ecosystems. Degradation of fourteen different chlorinated aromatic compounds (chlorobenzoic acids, chloroanilines, and chlorophenol) was tested in batch degradation experiments performed as reported in the Materials and methods section. Residual values are those recorded after 8-week incubation and are in% of the initial chloroaromatic concentration. Values of as well the biotic set-ups as sterile abiotic control set-ups are reported. The values presented are averages of 3 replicate incubations. Values for the biotic set-ups are at the left site of the slash symbol and in bold, values for the abiotic are at the right site of the slash symbol. Ecosystems and chloroaromatic compounds for which significant differences (ANOVA, n=3, $p < 0.05$) between the biotic and abiotic series were recorded are marked in gray

	2-CBA	3-CBA	4-CBA	2,3-DCBA	2,4-DCBA	2,5-DCBA	2,6-DCBA	3,4-DCBA	3,5-DCBA	2,3,5-TCBA	3-CA	4-CA	3,4-DCA	4-CP
BPS Leefdaal	**94**/116	**5**/98	**12**/108	**84**/98	**107**/107	**111**/103	**97**/86	**0**/94	**0**/96	**103**/100	**13**/29	**8**/19	**0**/20	**10**/82
BPS Pcfruit L	**0**/97	**24**/96	**1**/94	**104**/96	**0**/98	**72**/85	**107**/87	**90**/86	**56**/83	**100**/93	**0**/16	**0**/17	**0**/17	**5**/80
BPS Pcfruit R	**36***/108	**8**/103	**0**/104	**104**/97	**0**/107	**70**/93	**112**/97	**63**/89	**0**/90	**110**/106	**0**/12	**0**/13	**0**/13	**0**/81
BPS Lierde	**40***/113	**1**/99	**0**/108	**101**/107	**43**/111	**82**/94	**107**/95	**97**/102	**0**/106	**107**/109	**10**/24	**9**/30	**9***/30	**4**/70
BPS Kortrijk	**1**/115	**6**/114	**2**/117	**109**/82	**7**/126	**26***/91	**114**/105	**96**/93	**26***/96	**112**/117	**0**/47	**0**/44	**0**/38	**5**/80
BPS Koksijde	**101**/119	**0**/109	**0**/115	**111**/99	**0**/118	**83**/104	**104**/105	**0**/102	**0**/106	**102**/111	**0**/32	**0**/37	**0**/38	**0**/89
Pristine BPS	**81**/88	**2**/92	**84**/90	**98**/97	**97**/89	**93**/90	**87**/94	**90**/94	**93**/96	**81**/86	ND	**0**/7	**2**/3	**0**/63
WL	**104**/100	**12**/92	**102**/98	**105**/104	**102**/97	**105**/103	**94**/90	**103**/102	**105**/103	**95**/89	**35**/29	**14**/13	**24**/19	**40**/79
Zeveren-2	**106**/104	**28**/86	**0**/104	**107**/109	**103**/103	**107**/109	**94**/84	**0**/107	**0**/109	**90**/80	**53**/51	**15**/37	**41**/37	**0**/83

ND, not done.
*Two replicates showed complete degradation.

to linuron had no impact on overall bacterial community structure in BM (Sniegowski *et al.*, 2011). Also in the BM experiment reported in the current study, no differences in bacterial community structure were observed between microcosms exposed and nonexposed to pesticides (data not shown). Similarly, addition of linuron had no effect on overall bacterial community structure, neither when added as a single dose nor as multiple doses in the SM experiment (Bers *et al.*, 2011b), suggesting that also in the single-dose SM system (SM-2) changes in relative *tnpA* abundance cannot be explained by changes in community structure. Copy numbers of IncP-1 plasmids were not measured since at the time of experimentation, no method was available that allowed to detect all IncP-1 groups. Recently, Jechalke *et al.* (2013) reported a real-time PCR method for quantification of IncP-1 plasmids based on the *korB* gene that is conserved among all IncP-1 plasmid groups. The method was applied on samples taken from BPS Kortrijk and showed relative *korB* numbers of 2E-03 rrn^{-1}, which is lower than the values found for IS*1071* but which still indicates a high abundance of IncP-1 in the microbial community (Heuer *et al.*, 2012). The observations suggest that IncP-1- and IS*1071*-bearing bacteria proliferated as a response to pesticide exposure and emphasize on the ecological role of those MGE. The data are reminiscent to data reported by Heuer *et al.* (2012) who demonstrated that in agriculture soils, a positive correlation exists between antibiotic exposure and the presence of IncP-1ε plasmids. As for xenobiotic-degradative gene functions, antibiotic resistance genes, and their distribution between different bacteria have been often associated with IncP-1 plasmids (Schlüter *et al.*, 2007). On the other hand, we cannot conclude whether the observed increased abundance of IncP-1 and IS*1071* specific sequences is related to bacterial proliferation or actual gene transfer.

In both SM and BM set-ups, the increased abundance of IncP-1 plasmids and IS*1071* elements corresponded with an increased capacity to mineralize pesticides insinuating a relationship between number of IS*1071*/IncP-1-bearing organisms and pesticide mineralizing individuals. In the SM experiment, a correlation existed between the linuron mineralization capacity and the copy number of *libA* that encodes the first step in linuron degradation in bacteria of the genus *Variovorax* (Bers *et al.*, 2012). A similar correlation ($R^2 = 0.84$) exists between *tnpA* numbers and *libA* numbers (Fig. 6). This correlation suggests that IS*1071* composite transposons might carry catabolic functions for linuron degradation in the SM microcosm systems but does not necessarily imply that the *libA* marker is associated with IS*1071*. In the genome of the linuron degrading *Variovorax* sp. SRS16 in which *libA* was identified, no direct linkage was found between *libA* and

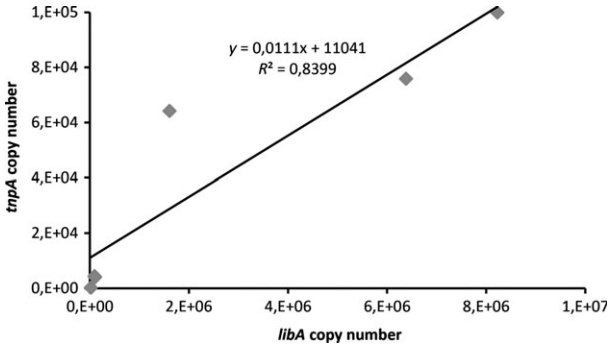

Fig. 6. Correlation between the abundance of the linuron hydrolase gene *libA* and the IS*1071* transposase gene *tnpA* in the SM experiment. Data for *libA* abundance were retrieved from Bers *et al.* (2012).

IS*1071* (Bers *et al.*, 2011a). In contrast, the gene cluster for degradation of 3,4-DCA, the prime metabolite of linuron hydrolysis, is bordered by IS*1071* elements in strain SRS16 (Bers *et al.*, 2011a). Similarly, other 3,4-DCA catabolic gene clusters encoded by the IncP-1 plasmids pWDL7 and pNB8c are flanked by IS*1071* elements (Król *et al.*, 2012). In the BM experiment, especially the mineralization capacity for atrazine, metamitron, and linuron were improved upon pesticide exposure. The genes for bacterial isoproturon and metamitron degradation are currently unknown, but atrazine catabolic gene clusters have been identified that are flanked by IS*1071* elements on IncP-1 plasmids (Martinez *et al.*, 2001). Devers *et al.* (2008) reported on the importance of insertion sequences in the adaptation of bacteria to degrade atrazine. Similarly, the high abundances of IncP-1- and IS*1071*-specific sequences in the BPS in operation were clearly accompanied with the capacities to mineralize various pesticides including linuron and atrazine for which the pristine BPS and the tested pristine soils did not show mineralization. Moreover, a wider range of chloroaromatic compounds were degraded by the BPS samples compared with the range degraded by microbial communities in pristine BPS and in the soils tested. Indeed, 57–71% of the tested compounds were degraded in the BPS, while 14% in the pristine BPS. For the tested soils, 14% of the compounds were degraded in the woodland soil and 43% in the zinc-polluted Zeveren soil. Genes involved in degradation of choroaromatic compounds like 2-CBA, 3-CBA, 4-CBA, 2,5-DCBA, 3,4-DCBA, 3-CA, 3,4-DCBA, and 4-CP have been previously coupled with IncP-1 plasmids and/or IS*1071* elements in various chloroaromatic-degrading bacteria (Springael *et al.*, 1993; Nakatsu *et al.*, 1995; Di Gioia *et al.*, 1998; Ledger *et al.*, 2006; Jencova *et al.*, 2008; Król *et al.*, 2012).

IncP-1ε and IncP-1β appeared to be the dominating IncP-1 subgroups in the on-farm BPS in operation. In the BM microcosms, especially IncP-1 plasmids of the IncP-1δ subgroup appeared to be dominant although also amplicons associated with IncP-1β plasmids were recovered. IncP-1β subgroup plasmids have been the most frequently reported IncP-1 plasmids associated with organic xenobiotic degradation (Dennis, 2005). IncP-1ε plasmids have been especially regarded as carriers of antibiotic resistance genes (Heuer *et al.*, 2012), but recent data showed the occurrence of IncP-1ε that carry genes for 2,4-dichlorophenoxyacetic acid degradation (Sen *et al.*, 2011). Other IncP-1 plasmids that contain 2,4-dichlorophenoxyacetic acid catabolic gene clusters are IncP-1δ plasmids like pEST4011 (Vedler *et al.*, 2004) and pIJB1 (accession number JX847411). These findings suggest that organic xenobiotic catabolic gene clusters cannot be associated with a particular IncP-1 subgroup. Two subtypes of IS*1071* have been reported sharing 81% nucleotide similarity (Nakatsu *et al.*, 1991; Ma *et al.*, 2007). The two subtypes are often found together, each flanking one site of the catabolic gene cluster. The primers used in the study only targets the first IS*1071* subtype, and sequence data confirmed that the *tnpA* amplicons belonged all to this subtype.

An unexpected high abundance of IS*1071* was recorded in the Canadian woodland and the soils from Zeveren, suggesting that this insertion sequence is more omnipresent than expected. The catabolic activity screenings showed that limited degradative capacities are present in the Canadian woodland, while in the Zeveren soil as well mineralization of metamitron as an extended removal of chlorinated compounds compared with the pristine BPS and the Canadian woodland was observed. It is of interest to examine in these soils with which genes IS*1071* is associated.

In conclusion, our data show that pesticide treatment creates a selective environment leading to increased abundances of IncP-1 plasmids and IS*1071* elements. Along with the increased presence of MGEs also an increase in xenobiotic-degradative capacity was observed. While IncP-1 plasmids appear to be more strictly correlated with xenobiotic pollution, IS*1071* appears to be more omnipresent than expected in the environment. The high abundance of IncP-1 and IS*1071* elements in BPS together with an extreme high variety in catabolic capacities further suggests that those environments can be considered as natural laboratories of evolution that are of interest for discovering new catabolic enzymes and plasmid groups. Our focus is currently on studying the genes that are associated with IS*1071* elements and IncP-1 plasmids in BPS.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Overview of recorded absolute numbers of 16S rRNA gene (rrn) copies in the investigated microcosms (SM and BM experiment) and field ecosystems.