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Adaptation and diversification of bacterial communities to pesticide contaminants in on-farm biopurification systems via mobile genetic elements

Dissertationen aus dem Julius Kühn-Institut

Julius Kühn-Institut
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Adaptation and diversification of bacterial communities to pesticide contaminants in on-farm biopurification systems via mobile genetic elements

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina zu Braunschweig

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Álvaro Rodrigues da Silva, my grandfather,

this is for you

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Abstract

Bacteria play important ecological roles and through biodegradation processes, using the pollutants as carbon source to survive, they are fundamental in "cleaning" environments contaminated by anthropogenic activities. We assumed that the main pathways for bacterial adaptation to new contaminants are catabolic genes carried often on plasmids such as IncP-1, IncP-7 and IncP-9 plasmids. To identify "hot spot" environments containing bacterial communities carrying these catabolic plasmids the first step was to understand and predict plasmid dissemination and their functions. A screening of different environments revealed that biopurification systems (BPS), used for the treatment of pesticide contaminated waste water at farms through biodegradation and sorption processes, were "hot spots" of plasmids potentially carrying catabolic genes as high abundances of IncP-1, IncP-7 and IncP-9 plasmids were detected. A suite of different molecular biology tools such as PCR-Southern blot hybridization based detection, denaturing gradient gel electrophoresis (DGGE), clone library and pyrosequencing were applied to analyze total community (TC) DNA or plasmid DNA aiming to unravel the response of bacterial communities to pesticides and to reveal how the degree of pollution influences the abundance and diversity of mobile genetic elements (MGEs) such as plasmids. In order to investigate the influence of high pesticide concentrations on the relative abundance of specific bacterial populations and plasmids likely carrying degradative genes, a microcosm with BPS material spiked with linuron or not was established and monitored over the time. Moreover, for the first time the effects of different pesticides on the abundance and diversity of MGEs and shifts of bacterial communities in an on-farm BPS were investigated over an entire agricultural season. Interesting results were obtained. An increase in pesticide contamination in BPS was associated with an increase of abundance and fluctuations in the diversity of MGEs specifically of IncP-1 plasmids, very "promiscuous" broad-host-range (BHR) often carrying catabolic genes. Through exogenous plasmid isolation, a cultivation-independent technique, several of IncP-1 and IncP-9 plasmids were captured from BPS into *Pseudomonas putida* recipient cells, allowing a deeper plasmid analysis by sequencing (still in progress). The 16S rRNA gene-based analyses revealed that particular bacterial groups in BPS are responding to pesticides contamination, such as *Betaproteobacteria* which increased in abundance, while other groups such as *Firmicutes* and *Bacteriodetes* had a selective disadvantage in such a polluted environment. Through the methodologies applied in the present work, the dynamics and plasticity of bacterial communities of BPS in response to pesticide exposure was revealed and taxa and plasmids potentially involved in the biodegradation process were identified.

Zusammenfassung

Bakterien spielen eine bedeutende Rolle in der Ökologie indem sie Schadstoffe abbauen, die sie als Kohlenstoffquelle zum Überleben nutzen, leisten sie einen wesentlichen Beitrag bei der ‚Reinigung' von Gebieten, die durch Einwirkung des Menschen mit Schadstoffen belastet sind. Wir gingen davon aus, dass die Anpassung der Bakterien an neuartige Schadstoffe vor allem über katabolische Gene stattfindet, die sich oft auf Plasmiden wie IncP-1, IncP-7 und IncP-9 befinden. Um sogenannte 'hot spots' von Bakteriengemeinschaften, die diese katabolischen Plasmide beherbergen, erkennen zu können, bestand der erste Schritt darin, die Verbreitung der Plasmide zu verstehen und vorherzusagen. Ein Screening verschiedener Umwelthabitate zeigte, dass u.a. Bioreinigungssysteme, die auf landwirtschaftlichen Höfen für die Reinigung von Pestizid-belasteten Abwässern durch biologischen Abbau und Absorption eingesetzt werden, ‚hot spots' von Plasmiden mit katabolischen Genen sind, da IncP-1, IncP-7 und IncP-9 Plasmiden in hoher Abundanz nachgewiesen wurden. Eine Reihe von molekularbiologischen Methoden – Detektion durch PCR Southern Blot-Hybridisierung, denaturierende Gradienten-Gelelektrophorese (DGGE), Klonierung-Sequenzierung oder Pyrosequenzierung von Amplikons (16S rRNA-Genfragmente, *trfA*) wurden genutzt, um DNA aus der Gesamtgemeinschaft oder Plasmid-DNA zu analysieren und so die Antwort der Bakteriengemeinschaften auf Pestizide zu charakterisieren und dahinterzukommen, wie sich das Ausmaß der Verunreinigung auf die Verbreitung und Diversität mobiler genetischer Elemente (MGE) wie Plasmide auswirkt. Um den Einfluss hoher Pestizid-Konzentrationen auf die relative Abundanz von spezifischen bakteriellen Populationen und Plasmiden, die vermutlich Abbau-Gene tragen, zu erforschen, wurde ein Mikrokosmos mit Material aus einem Bioreinigungssystem – mit und ohne Linuron – etabliert und zu drei Zeitpunkten analysiert. Darüber hinaus wurden erstmalig während einer vollen landwirtschaftlichen Saison die Auswirkungen verschiedener Pestizide auf die Verbreitung und Diversität von MGE und Veränderungen der Bakteriengemeinschaften in einem farmeigenen Bioreinigungssystem untersucht und interessante Ergebnisse gewonnen. Ein Anstieg in der Pestizid-Exposition im Bioreinigungssystem ging einher mit einer größeren Abundanz und größeren Fluktuationen in der Diversität von MGE, vor allem von IncP-1 Plasmiden, die durch einen weiten Wirtsbereich charakterisiert sind und die häufig katabolische Genen tragen. Mit Hilfe der exogenen Plasmid-Isolierung, einer kultivierungsunabhängigen Technik, wurden mehrere IncP-1 und IncP-9 Plasmide aus dem Bioreinigungssystem in *Pseudomonas putida*-Rezeptor-Zellen überführt, wodurch eine genauere Plasmid-Analyse durch Sequenzierung ermöglicht wird (Auswertung ist noch nicht abgeschlossen). Die auf dem 16S rRNA-Gen basierenden Analysen machten deutlich, dass bestimmte Bakteriengruppen im Bioreinigungssystem auf eine Verunreinigung mit Pestiziden reagieren, so nahm die Abundanz der *Betaproteobacteria* und der *Gammaproteobacteria* zu, während andere Gruppen, z.B. *Firmicutes* und *Bacteriodetes*, in einem derartig verunreinigten Umfeld einen selektiven Nachteil hatten. Durch den genutzten polyphasischen Ansatz konnte in dieser Arbeit die Dynamik und Plastizität von bakteriellen Gemeinschaften in Bioreinigungssystem bei Pestizid-Exposition gezeigt werden und mögliche am Abbau beteiligte Taxa und Plasmide identifiziert werden.

Project outline

This PhD thesis is a part of a cooperative project between eighteen different research partners from Europe and South America (Argentina, Belgium, Denmark, Finland, France, Germany, Italy, The Netherlands, Slovenia, Sweden and United Kingdom). The project, entitled "Metagenomics for Bioexploration - Tools and Application" was funded by the European Union Commission. The main goal was to detect novel enzymes of industrial interest by developing new metagenomics methodologies to access the environmental microbial and functional genes' diversity. The desired enzymes are involved in the biodegradation of recalcitrant and xenobiotic compounds such as novel chitinases, ligninases, aerobic and anaerobic dehalogenases (halogenases as a spin-off).

We assumed that the desired genes are often located on catabolic plasmids present in the mobilome, which by definition is the collective pool of mobile genetic elements (MGEs) such as plasmids carried by microbial communities from different environments. Therefore, the mobilome of various environments was intensively studied by different partners in this project. The Julius Kühn-Institut (JKI) where I have made my PhD research had the main task to provide information about the occurrence of plasmids with potential catabolic functions in various polluted environments, indicating "hot spots" of MGEs for further isolations and analyses of the associated bacterial community composition. Followed by a general introduction in chapter I, the results based on the tasks listed above are presented in chapters II, III, IV and V.

Summary and thesis outline

Mobile genetic elements (MGEs) such as plasmids are important vehicles of horizontal gene transfer between bacteria. Plasmids are considered the major players in the bacterial adaptation to the contaminants released into the environment. Expressing catabolic genes carried by plasmids, which can be rapidly spread between different bacterial hosts through horizontal gene transfers, bacteria can use the toxic substances present in the environment as carbon source. Therefore, the investigation of plasmids present in various polluted environments was the start point of the current research. Basically, first, reservoirs of potentially catabolic plasmids were identified through PCR-based detection combined with Southern-blot hybridization analysis. After that, more in-depth analyses into the abundance and diversity of the MGEs identified were performed. Different strategies were used to capture the MGEs present in the bacterial populations of biopurification systems (BPS), a hot-spot of catabolic plasmids such as IncP-1, IncP-7 and IncP-9 plasmids. The composition and shifts in the bacterial communities likely hosting the MGEs involved in catabolic pathways was investigated through a range of cultivation-independent techniques.

Based on the hypothesis that the frequency of occurrence of genes encoding the desired enzymatic activities is increased in the MGE gene pool (Dennis, 2005, Heuer & Smalla, 2012) a suitable reservoir rich of potentially catabolic plasmids was chosen. Therefore, an extensive screening of the TC or fosmid DNA from various environments for different catabolic plasmid replicons detection by using PCR-based detection specific-amplicons combined with hybridization was performed. The results indicated BPS located in Belgium were "hot spots" of potential catabolic plasmids, such as incP-1, IncP-7 and IncP-9 plasmids. These results are summarized in chapter II.

We had found a rich reservoir of potentially catabolic plasmids: BPS heavily contaminated with various pesticides. Then, the second step was to investigate how these pesticides influenced the bacterial communities carrying the desired plasmids. In order to better understand how the bacterial communities in such complex systems are affected by pesticides, a microcosm experiment containing BPS material from Belgium was either spiked or not with high concentrations of linuron, a herbicide which is carcinogenic to humans and toxic to aquatic organisms. Based on the hypothesis that the exposure of a pre-adapted BPS bacterial community to linuron might cause changes in the relative abundance of bacterial populations likely carrying catabolic plasmids such as IncP-1 plasmids, a suite of different cultivation-

independent techniques was applied. The results suggested that different bacterial populations, much more than supposed, might be involved in the complex foodweb directly or indirectly involved in the degradation of linuron, and IncP-1 plasmids carried by these populations seemed to be the major players contributing to the bacterial survival at high concentrations of linuron. A proof of the correlation between pollution and increase in the abundance of IncP-1 plasmids was provided. These results can be found in chapter III.

Therefore, we wanted to investigate in more detail the correlation of pollution and plasmid abundance provided by the linuron microcosms. Is there any MGE else increased in abundance due to the increase of pesticide contamination? Is the bacterial diversity also affected? In order to answer these questions, the bacterial composition and shifts of the mobilome in BPS exposed to different kinds and concentrations of pesticides over an agriculture season was investigated. Indeed, as presented in chapter IV, the abundance of different MGEs and pesticide pollution was found to be correlated, and different concentrations of pesticides influence not only the IncP-1 plasmid abundance (Jechalke *et al.*, 2013) but also the composition of different subgroups (chapter IV). Due to the considerable shifts in abundance and diversity of the mobilome to diverse pesticides introduced into an on-farm BPS over a year, we were seeking to investigate the impact of changing pesticide concentrations on the hosts of the mobilome: the bacterial communities. As demonstrated in chapter V, DGGE and amplicon pyrosequencing of the 16S rRNA gene fragments indicated that *Proteobacteria*, mainly *Gammaproteobacteria* seemed to be the main responders to increase in the concentration of pesticides in BPS material, while *Firmicutes* and *Bacteriodetes* seemed to be negatively affected.

The work summarized above showed an uncommonly high abundance and a high diversity of IncP-1 plasmids in on-farm BPS. Therefore, we wanted to capture as many of these plasmids as possible! How to do that? The partners involved in the mobilome research in this project used different techniques to capture different plasmids, attempted to uncover the "plasmid-pool". Different approaches such as exogenous plasmid isolation (Bale *et al.*, 1987), endogenous plasmid isolation (Lidstrom & Wopat, 1984), a technique using ethidium bromide density gradient centrifugation to isolate plasmids (Radloff *et al.*, 1967) used by the Argentinean partners from the University of La Plata, and a method focusing in investigations of the pool of circular genetic elements (the metamobilome) using 454 high-throughput (Li *et al.*, 2012) sequencing was applied by the Danish partners from the University of Copenhagen. Each of the methods applied has their own limitations and therefore complementary methods were used to

study the mobilome from BPS. All the different techniques to isolate plasmids directly or indirectly from the DNA captured different types of plasmids. A huge diversity of functions encoded by the different plasmids was likely obtained. The search for catabolic genes carried by the plasmids isolated is still in progress. Below the type of plasmids captured by endogenous and exogenous plasmid isolation, which was performed by me will be described.

Through endogenous isolation different IncP-9 plasmids, e.g., pWWO-like and pNL15-like were observed in different *Pseudomonas* isolates (chapter III). An independent cultivation technique, the so-called exogenous plasmid isolation was applied using different strategies in order to obtain a high diversity of plasmids. Different selective markers based on toxic pollutants such as the pesticides 2,4-D and linuron, the intermediate degradation product 3,4-DCA and $HgCl_2$ were applied and combined with different recipient cells in different mating assays. $HgCl_2$ used as mercury resistance was frequently observed on IncP-1 plasmids which can also carry pesticide degradation genes, e.g. pAKD4, pQK54 and pJP4. *Cupriavidus necator JMP 228r gfp*-labeled and *Pseudomonas putida KT2442 gfp*-labeled were used as recipient in different biparental assays, and *Pseudomonas B13 gfp*-labeled was applied in a triparental exogenous isolation assay. Interestingly, IncP-1 and IncP-9 were captured into the same *Pseudomonas putida KT2442 gfp*-labeled recipient cell using $HgCl_2$ as selective marker in a biparental assay. Mineralization tests which I have carried out in the laboratory of Dirk Springael (Belgium partner) indicated that two of the IncP-1 exogenously isolated into *Pseudomonas putida KT2442 gfp*-labelled using $HgCl_2$ as selective marker, are involved in linuron degradation. Pyrosequencing analysis based on the *trfA* region of IncP-1 plasmids and *rep* regions of IncP-9 plasmids revealed that the backbones of the plasmids exogenously captured from BPS belonged to IncP-1β plasmids and pM3 and pWWO IncP-9 plasmids (data not shown). Moreover two IncP-1ε plasmids and one still unknown plasmid which possess a laccase gene (identified by the partner group from Slovenia) were captured through a triparental mating (data not shown). The analysis of these plasmids are in process by the partner from Copenhagen.

Finally, I would like to say how much I wish that the current work provided helpful information for bachelor, master, PhD students and professionals involved in biodegradation and bioremediation research.

Chapter I: General introduction

1.1 Release of pesticides into the environment

According to EPA (U.S. Environmental Protection Agency) plant protection products (PPPs) or pesticides, such as bactericides, herbicides, fungicides and insecticides can be defined as a substance or a mixture of substances often applied in agriculture in order to prevent, destroy, repel or mitigate any pest in order to increase the crop yield and quality. During the 1940s with the emergence of World War II, a higher attention was given to the control of crop yields due to the food rationing period (European Crop Protection). In that time, in Germany for instance, a product containing tetraethyl pyrophosphate (TEPP) was developed. TEPP belongs to the organophosphorus group, which is a group of pesticide most widely used in agriculture nowadays representing 36% from the total of the pesticides global market (Kanekar *et al.*, 2004). Indeed, the use of pesticides in agriculture is an efficient solution applied by the farmers to guarantee crop protection against insect and pest damages, avoiding the waste of expensive investments, which is especially true for potato crops, for which losses without pesticides applications could reach up to 75% (European Crop Protection).

However, the wide use of pesticides increased concerns regarding both adverse effects on human health (Eddleston *et al.*, 2002) and environmental pollution. Pesticides were found in high concentrations in ground and surface water (Spanoghe *et al.*, 2004). The intense pesticides applications resulted in different pest resistances which led to the development of stronger substances and increased the concentrations of pesticides applied time-by-time. Nowadays several types of pesticides which are classified according to their chemical structure are in use: organochlorines, organophosphates, organophosphonates, s-triazines, triazinones, carbamates, acetanilides and sulfonylureas. 2,4 dichlorophenoxyacetic acid (2,4-D) belongs to the s-triazine group, is the most widely used herbicide in the world since 1940. Probably two ofthe reasons for the continued application of this pesticide to protect crops (mainly maize crops) are their short half-life and the easy biodegradation mediated by different bacterial populations (Fisher *et al.*, 1978). However, atrazine for example, is considered a high risk pollutant due to the long half-life, ranging between 4 and 57 weeks in the environment. Therefore, atrazine was banned in the European Union, although still in use in other parts of the world (Mojib *et al.*, 2011).

Pesticide pollution of ground and surface water can originate from diffuse or point contamination. A point source contamination is characterized as a pollution which came from

single contamination source while the diffuse or non-point contamination originate from different sources of contamination. However, the pollution of ground and surface water often come from point contaminations (Spanoghe *et al.*, 2004), e.g. the step of mixing preparation of the pesticide spraying apparatus and mainly from leaching and run-off (Kookana & Simpson, 2000). Other factors like the inappropriate treatment of pesticide packaging, leakages and accidental drainage can also contribute to environmental contamination, but the occurrence is less often. In order to minimize and control this pesticide contamination, a pollution control technique employing microorganism has been successfully applied: BPS (De Wilde *et al.*, 2009, Omirou*, et al.*, 2012).

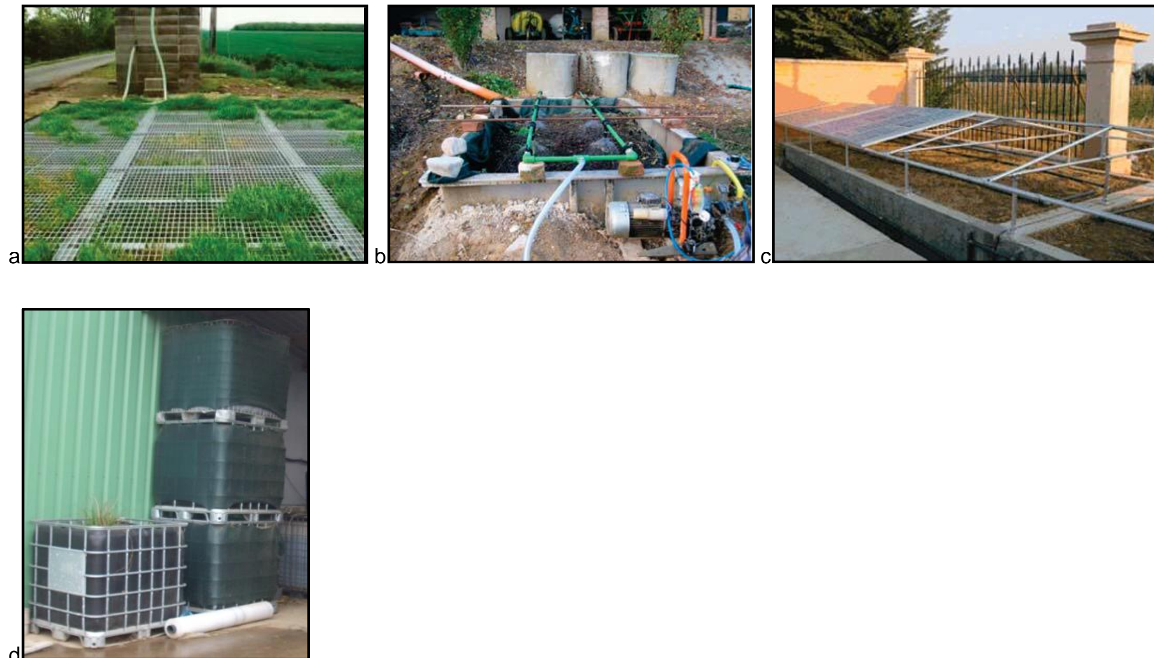
1.2 Biopurification systems (BPS)

Biopurification systems consist of a biobed composed of a mixture of different adsorbing materials such as peat, straw and topsoil, forming the so-called "biomix" which retains the pesticides in organic matter increasing the microbial degradation. Each of this adsorbing material has different functions: peat allows the formation of several sites for pesticide sorption, which contribute to the maintenance of aerobic conditions and the necessary humidity; the straw is used as carbon source for the microorganisms; the topsoil and compost provide microorganisms with biodegradative potential and furthermore the compost serves as nitrogen source for the microorganisms (De Wilde *et al.*, 2009). The ideal topsoil or soil-mixture applied as soil-manure mixture or soil-chitin mixture (Genot *et al.*, 2002) should originate from the local soil in order to increase the survival of the natural degrading-microorganisms presents in that specific environment (De Wilde *et al.*, 2009). This biomix has been applied as a pollution control technique using microorganisms to degrade pollutants present in contaminated waters with pesticides.

The first biobed model was originated from Sweden (von Wiren-Lehr *et al.*, 2001). It was composed of straw, peat and soil (50:25:25 vol %) and consisted of a cheap and simple construction able to degrade the pesticides originating from farms. After the building of the Swedish biobed, several countries, as Belgium, England, France and many others, have developed distinct types of biobeds, with modifications which have led to different nominations: biofilters (Debaer & Jaeken, 2006), biobac (Castillo *et al.*, 2008), biomassbed (Fait *et al.*, 2007), biotables (Castillo *et al.*, 2008) and Phytobac (Guyot & Chenivesse, 2006) (Fig. 1).

Fig. 1. Biopurification system variations

a) Biobeds in the UK (www.biobeds.info); b) Biomassbed in Italy (Fait *et al*., 2007); c) Biobac in France (www.biotisa.com); d) Biofilter in Belgium (Courtesy of Dirk Springael).



1.2.1 Phytobac

Phytobacs are quite large biobeds which do not include a grass layer, differently from the original Swedish biobed. In the Phytobac large volumes of water contaminated with pesticide often coming from tank waste and spillages during mixing and loading of pesticides, rinsing and cleaning of sprayers can be treated. The clean-up efficiency of the phytobac is ranging between 95%-99% (De Wilde *et al*., 2009). Phytobacs have a cheap, simple and relatively easy maintenance (Castillo *et al*., 2008). Variations in a composition of different Phytobacs can also be observed. For instance, the Phytobac in France developed by Bayer Crop-Science is composed of topsoil and chopped straw, while the biopurification system Phytobac from Belgium located in Kortrijk, Belgium (Fig. 2) studied in the present thesis is composed of coco chips, straw, soil and manure.

The Phytobac installed in Kortrijk has a large compartment (20 m x 1.2 m) which is receiving large volumes of water contaminated with pesticides composed of 37 different active compounds including 16 halogenated aromatics over the season (Jechalke *et al*., 2013a).

Fig. 2. Phytobac in Belgium, Kortrijk

1.3 Pesticide biodegradation

Although chemical and physical processes are important in the removal of pesticides from various environments (Cullington & Walker, 1999), microorganisms are pointed as major players in the pesticide degradation (Sørensen *et al.*, 2001, Bers *et al.*, 2011a, 2012). Pesticide biodegradation can be defined as the breaking down of toxic chemicals by microbial enzymes producing intermediate metabolites which can be nontoxic or even more toxic than the original active pesticide compound (Tixier *et al.*, 2001). When a toxic pollutant is released in the environment, bacteria normally do not produce a new enzyme, but rearrange their previously existing catabolic genes to get a pathway able to degrade the new pollutant. Through their catabolic pathways bacteria can survive by using the toxic compounds as carbon and nitrogen source at the same time the pollution is cleaned. After a repeated application of the same compound in the same field site, pre-adapted bacterial communities and increasing biodegradation rates can be observed (Arbeli & Fuentes, 2007).

Up to now, a broad range of different bacterial species able to degrade active pesticides compounds were isolated: *Pseudomonas* sp. BK8, diuron degrader (El-Deeb 2001); *Variovorax* sp., *Achromobacter* sp., *Mesorhizobium* sp., *Arthrobacter* sp, *Bradyrhizobium* and *Burkholderia* sp. are 2,4-dichlorophenoxyacetic acid (2,4-D) (Macur *et al.*, 2007); *Sphingomonas herbicidovorans,* a MCPA; 2,4-D; mecoprop and dichlorpropan (phenoxyalkanoic acid herbicides) degrader (Kohler, 1999); *Delftia acidovorans* CA28, a 3-chloroaniline degrader (Boon *et al.*, 2001); *Alcaligenes eutropha*, a 2,4-D degrader (Don & Pemberton, 1981); *Hyphomicrobium sulfonivorans* WDL6, a N,O-DMHA degrader (Breugelmans *et al.*, 2007), *Hyphomicrobium* sp. MAP-1, an insecticide methamidophos degrader (Wang *et al.*, 2010), *Comamonas testosteroni*, a linuron metabolite degrader and different *Variovorax* species involved in different steps of linuron degradation (Dejonghe *et al.*, 2003, Breugelmans *et al.*,

2007). *Variovorax* sp. SRS16 plays an important role in the phenyl-urea biodegradation. These bacteria can degrade both diuron and linuron (Sørensen *et al.*, 2005a, 2008). See an overview list of the major pesticide degrader isolates in Table 1.

Complete mineralization of pesticide compounds in the environment does not depend exclusively from the activity of catabolic enzymes produced by microorganisms. A range of other abiotic and biotic factors such as pH, temperature, moisture content, O_2 availability, organic matter, clay content and soil type affect the activity of the degrading bacteria and the bioavailability of the pollutant (Arbeli & Fuentes, 2007). The higher chlorphyritos degradation rates occur under alkaline pH, high clay contents and moisture content around 60% (Awasthi & Prakash, 1997). Metolachor can just be degraded under aerobic conditions and atrazine showed higher degradation rates under aerobic conditions (Accinelli *et al.*, 2001). Castillo & Torstensson (2007) showed that the degradation of different pesticides such as linuron and isoproturon has higher and faster degradation rates under higher temperatures, ranging between 10-20°C.

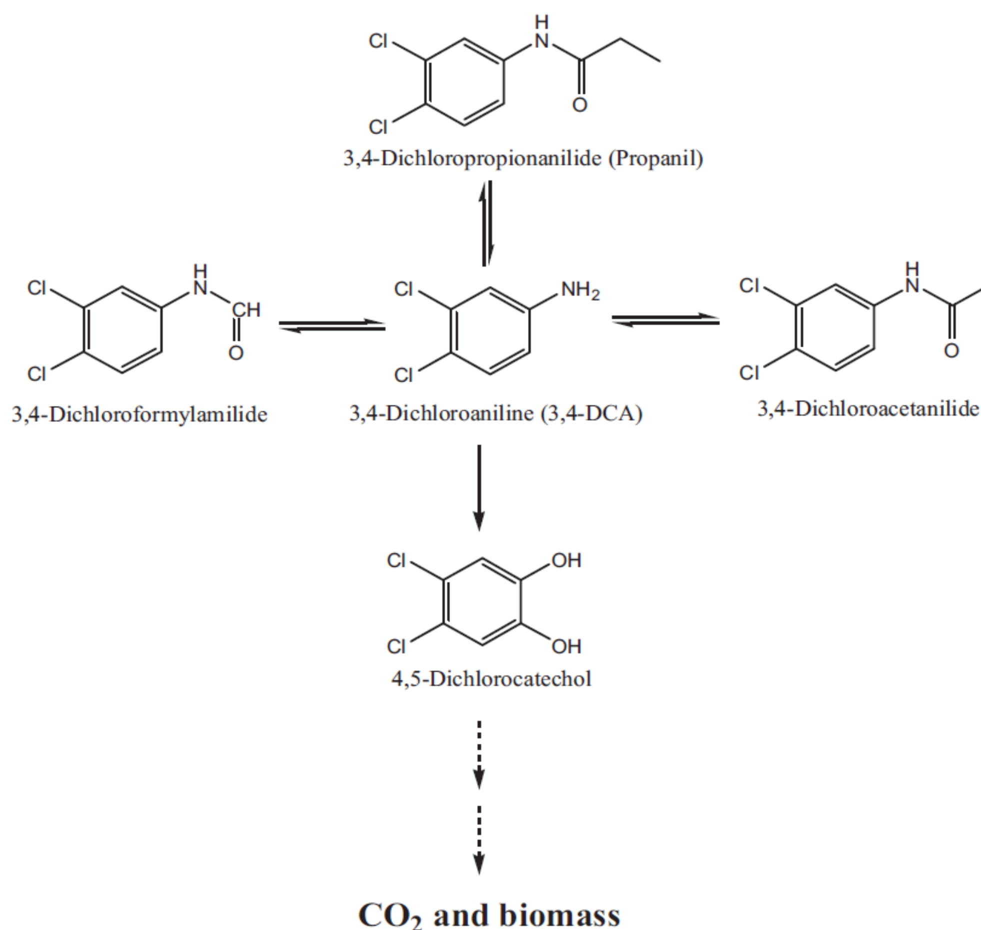
Table 1. Bacterial isolates involved in the degradation of different pesticides

Isolate	Pesticide/active compound	Reference
***Agrobacterium radiobacter* J14A**	atrazine	*(Struthers et al., 1998)*
Arthrobacter nicotinovorans	atrazine	*(Aislabie et al., 2005)*
***Arthrobacter globiformis* D47**	diuron	*(Turnbull et al., 2001)*
Arthrobacter atrocyaneus	glyphosate	*(Pipke & Amrhein, 1988)*
***Arthrobacter oxydans* P52**	phenmedipham	*(Pohlenz et al., 1992)*
***Achromobacter* sp**	2,4-D	*(Macur et al., 2007)*
Alcaligenes eutropha	2,4-D	*(Don & Pemberton, 1981)*
Aminobacter aminovorans	atrazine	*(Rousseaux et al., 2001)*
Achromobacter xylosoxidans	2,4-D; Linuron; 3,4-DCA	*(Vedler et al., 2004, Breugelmans et al., 2007)*
***Arthrobacter* sp.**	2,4-D	*(Macur et al., 2007)*
Afipia genosp	3,4-DCA	*(Breugelmans et al., 2007)*
Bradyrhizobium	2,4-D	*(Macur et al., 2007)*
Burkholderia	2,4-D	*(Macur et al., 2007)*
Chelatobacter heintzii	atrazine	*(Rousseaux et al., 2001)*
Cupriavidus* sp. *strain PBS-E1	3,4-DCA	*(Bers et al., 2011a)*
***Delftia acidovorans* CA28**	3-chloroaniline degrader	*(Boon et al., 2001)*
***Delftia acidovorans* WDL34**	3,4-DCA	*(Dejonghe et al., 2003; Bers et al., 2011a)*
***Comamonas testosteroni* WDL7**	3,4-DCA	*(Bers et al., 2011a)*
***Hydrogenophaga* sp. strain PBL-H3**	Linuron; 3,4-DCA	*(Breugelmans et al., 2009)*
***Hyphomicrobium sulfonivorans* WDL6**	N,O-DMHA	*(Breugelmans et al., 2007)*
Klebsiella pneumoniae	S-Metolachor	*(Martins et al., 2007)*
***Mesorhizobium* sp**	2,4-D	*(Macur et al., 2007)*
***Paracoccus* sp.**	S-Metolachor	*(Martins et al., 2007)*
***Paracoccus* sp. FLY-8**	S-Metolachor	*(Zhang et al., 2011a)*
***Pseudomonas strain* YAYA6**	atrazine	*(Yanze-Kontchou & Gschwind, 1994)*
***Pseudomonas* sp. BK8**	diuron	*(El-Deeb et al., 2001)*
***Rhodococcus* sp. strain NI86721**	atrazine	*(Nagy et al., 1995)*
***Sphingomonas sp.* Y57**	Isopropuron, diuron and propanil	*(Zhang et al., 2011b)*
Sphingomonas herbicidovorans	MCPA, 2,4-D, mecoprop and dichlorpropan	*(Kohler, 1999)*
Stenotrophomonas maltophilia	Atrazine	*(Rousseaux et al., 2001)*
***Variovorax* sp. WDL1**	Linuron; 3,4-DCA	*(Dejonghe et al., 2003)*
***Variovorax* sp. SRS16**	Linuron; diuron; 3,4-DCA	*(Sørensen et al., 2005a; 2008)*
***Variovorax* sp. strain PBL-E5**	Linuron; 3,4-DCA	*(Breugelmans et al., 2007)*
***Variovorax* sp. strain PBS-H4**	Linuron	*(Breugelmans et al., 2008)*
***Variovorax* sp. strain RA8**	Linuron	*(Satsuma, 2010)*
Variovorax paradoxus	3,4-DCA	*(Breugelmans et al., 2007)*
***Variovorax* sp.**	2,4-D	*(Macur et al., 2007)*

1.3.1 Pesticide biodegradation chemical pathway

The human activities producing toxic compounds are so diverse nowadays that it would be almost impossible to illustrate all pesticide catabolic pathways found in different bacterial populations. Thus, few examples of catabolic pathways to degrade two groups of herbicides used in agriculture all over the world will be discussed more in detail: phenylurea herbicides such as diuron, isoproturon and linuron and anilide herbicides such as propanil. For instance, from the first hydrolyzed linuron and propanil, a common harmful metabolite is produced: 3,4-DCA (Stangroom *et al.*, 1998). The initial hydrolysis of linuron is mediated by enzymes present in some bacterial populations such as different *Variovorax* species, producing 3,4-DCA and N,O-DMHA (Dejonghe *et al.,* 2003). The N,O-DMHA is easily and fast degraded by bacteria as *Hyphomicrobium* sp. (Breugelmans *et al.,* 2007). However, 3,4-DCA is more recalcitrant to degradation. Few bacterial groups are able to degrade this metabolite producing CO_2 and H_2O: *Variovorax* sp. WDL1; *Comamonas* sp. WDL7 and *Delfia acidovorans* WDL 34 (Dejonghe *et al.,* 2003). The initial hydrolysis of propanil can be mediated by an enzyme produced by a recently isolated bacterium, a *Sphingomonas* sp. Y57 (Zhang *et al.*, 2011b), one of the products of which is 3,4-DCA (Fig. 3). The metabolism which converts 3,4-DCA to 4,5-Dichlorocatechol producing CO_2 is also accomplished by the enzymes produced by the same bacterium *Sphingomonas* sp. Y57.

Fig. 3. Proposed pathway for degradation of propanil and 3,4-DCA in *Sphingomonas* sp. Y57 (Zhang *et al*., 2011b)



Linuron [N-(3,4-dichloro-phenyl)-N-methoxy-N-methylurea] is a widely used phenylurea herbicide. The linuron degradation starts with the amide hydrolysis of linuron, producing two intermediates: N,O-dimethylhydroxylamine (N,O-DMHA) and 3,4-dichloroaniline (DCA) (Fig. 3). Only few bacterial groups described up to now can initiate this first enzymatic step: *Variovorax* WDL1 (Dejonghe *et al*., 2003), *Variovorax* SRS16 (Sørensen *et al*., 2005a), *Variovorax* PBL-H6 (Breugelmans *et al*., 2007) and *Variovorax* RA8 (Satsuma, 2010) are able to initiate the hydrolysis that converts linuron to 3,4-dichloroaniline (DCA) and N,O-DMHA. They can also participate in the degradation of the linuron intermediates as well as other bacterial groups, for example: *Comamonas testosteroni* WDL7 produces enzymes that degrade 3,4-dichloroaniline (DCA) while *Hyphomicrobium sulfonivorans* strain WDL6 was shown to degrade N,O-DMHA (Dejonghe *et al*., 2003).

Bacterial species such as *Variovorax* sp. pointed as important players in pesticide degradation are those which grow faster on plates and therefore, do not necessarily represent the major pesticide degraders in the environment. This means that other bacteria which are still unknown due to the limitations of the classical cultivation methods could be involved in biodegradation and have even a more important ecological role. Interestingly, recent pyrosequence analysis of 16S rRNA bacteria gene amplicons from a microcosm experiment using BPS material enrichment with linuron showed that the results obtained from the cultivation are not so far away from those obtained by next generation sequence analysis of 16S rRNA gene fragments amplified from total community DNA (chapters III and V). The most significant bacteria communitie changes in response to pesticide additions were observed in the *Comamonadaceae* family which includes the different species of *Acidovorax* sp. and *Variovorax* sp. Another group indicated as linuron responder was *Polaromonas* sp. JS666 (also affiliated to the family *Comamonadaceae*), which was previously described as hydrocarbon, heavy metal and xenobiotic degrader (Mattes *et al.,* 2008). *Hyphomicrobium* sp. MC1, a degrader of halogenated pollutants (Vuilleumier *et al*., 2011) and *Pelagibacterium halotolerans* B2, a marine halotolerant bacterium (Xu *et al*., 2011), both belonging to the family *Hyphomicrobiaceae* were also indicated as linuron responders. Another interesting finding was the presence of responders belonging to the *Methylophilaceae* and *Trueperaceae* families which possess several uncultivated species (Prokofeva *et al*., 2005) (chapter III). However, the role of these taxa significantly increased in abundance in response to linuron needs still to be elucidated.

1.3.2 Genetic bases of pesticide biodegradation

The catabolic genes involved in the pesticide degradation are generally located on plasmids (preferentially large degradative plasmids), transposons or chromosomes. See a general overview of different pesticides degradative genes and their location in Table 2. Many of the pesticide degradative genes in isolates were found on IncP-1 plasmids, which therefore attracted a lot of attention (Dennis, 2005, Shintani *et al*., 2010). Genes encoding for enzymes such as the phenylurea hydrolase (*puh*) are able to degrade more than one pesticide belonging to the same group, such as chlorotoluron, isoproturon and linuron (Khurana *et al*., 2009). *puhA*, isolated from *Arthrobacter globiformis* (Turnbull *et al*., 2001) and *puhB*, isolated from *Mycobacterium brisbanense* (Khurana *et al*., 2009) were originally described to be responsible for diuron degradation. (Pesce *et al*., 2013) verified that the abundance of *puhB* significantly increased in response to diuron in soil. A hydrolase propanil gene nominated *prpH* is able to degrade propanil and the correspondent intermediate 3,4-DCA (Zhang *et al*., 2011b).

Bers *et al*. (2011a, 2012) revealed the *libA* genes play an important role in the linuron degradation, *dcaQTA*$_1$*A*$_2$*BR* gene cluster, which converts 3,4-DCA to chlorocatechol, such as 4,5-dichlorocatechol and *ccdRCFDE* gene cluster are involved in the further chlorocatechol product degradation. *puh* genes and arylacyl amidase gene, isolated from *Bacillus sphaericus* (Engelhardt *et al*., 1971), are some other genes involved in the conversion of linuron to 3,4-DCA. 2,4-D degradation genes are a good example of multi genes involved in the same pesticide degradation. The gene clusters *tfdCEBKA, tfdF,* found in the IncP-1 pEST4011 (Velder *et al*., 2004); *tfdABCDEF* on the IncP-1 plasmid pJP4 (Trefault *et al*., 2004); *tfdARCGEFB/tfdABC* on the IncP-1 plasmids pEMT1/pEMT3, respectively (Top *et al*., 2005); *tfdA* gene on the IncP-1 plasmids pKA2/pKA4 (Ka & Tiedje, 1994) and another type of *tfd* gene on the plasmid pIJB1 (Ka & Tiedje, 1994) are all involved in the 2,4-D degradation.

Table 2. Location of pesticide degradation genes on different mobile genetic elements (MGEs)

Pesticides or metabolites	Genes	Location	References
Atrazine	*aztABCDEF*	IncP-1 β pADP-1	*(Martinez et al., 2001, de Souza et al., 1998b)*
	tfd-like genes	Chromosomes/transposons / IncP-1	*(Velder et al., 2004, Trefault et al., 2004)*
	tfdCEBKA, tfdF	pEST4011	*(Velder et al., 2004)*
2,4-D	*tfdABCDEF*	pJP4	*(Trefault et al., 2004)*
	tfdARCGEFB / tfdABC	pEMT1 / pEMT3	*(Top et al., 2005)*
	tfd	pIJB1	*(Xia et al., 2001)*
	tfdA	pKA2 / pKA4	*(Ka & Tiedje, 1994)*
Chlorotoluron/diuron/linuron/isoproturon	*puhA/puhB/ arylacyl amidase*	*-	*(Turnbull et al., 2001, Khurana et al., 2009, Pesce et al., 2013)*
Linuron/3,4-DCA	*libA/dcaQTA1A2BR and ccdRCFDE*	chromosomes / IncP-1	*(Bers et al., 2011a, 2012)*
Propanil	*prpH*	*-	*(Zhang et al., 2011b)*

** gene location not confirmed yet*

1.3.3 Mixed cultures - an efficient pesticide removal "team"

Often the rate of pesticide degradation from a single strain is much slower than the degradation rates from mixed-cultures. Dejonghe *et al.* (2003) demonstrated that a consortium containing *Variovorax* sp. strain WDL1, was able to mineralize linuron; *Delfia acidovorans* WDL34, a 3,4-DCA degrader; *Hyphomicrobium sulfonivorans* WDL6, a N,O-DMHA degrader and *Pseudomonas* sp. strain WDL5, which contribute to the linuron degradation, showed a significantly higher rate of linuron degradation compared with the single *Variovorax* WDL1 degradation rates. *Sphingomonas* sp. strain SRS2 is able to degrade the pesticides isopropuron and carbofuran, however, a significantly enhanced degradation rate was observed when this strain was co-cultured with other different types of bacteria (Sørensen *et al.*, 2001). Katsuyama *et al*. (2009) showed that a mutualistic consortium compost of a *Sphingomonas* sp. and a *Burkholderia* sp. were able to perform the complete degradation of the pesticide fenitrothion. Interestingly, when one of these bacteria was inoculated alone, both were unable to mineralize fenitrothion. De Souza *et al*. (1998a) demonstrated the high efficiency of atrazine degradation by complex bacterial consortium from compost with different members of *Clavibacter* and *Pseudomonas*. The importance of a bacterial consortium in biodegradation is not exclusively related to pesticide compounds. Several studies indicated a significantly enhanced biodegradation of other types of pollutants such as polycyclic aromatic hydrocarbons (PAH) by a consortium of bacteria in comparison to single PAH degrader strains (Mandri & Lin, 2007).

The interactions between microorganisms in a consortium such as co-metabolism, synergism or antagonism are very important for the complete mineralization of an organic compound (Bauer & Capone, 1988) and may explain the enhancements in biodegradation by consortia. These interactions between different bacterial populations produce a "pool of enzymes" which considerably increases the rates of degradation. Different enzymes acting together produce a "metabolic complementation" or so-called "mutual functions complementation" which leads to the complete mineralization e.g. of a pesticide. Moreover, some microorganisms that are not directly involved in the degradation process are fundamental in the consortium because they may produce micronutrients or can excrete metabolites as essential growth factors, necessary for other degrading microorganisms (Katsuyama *et al.*, 2009).

1.4. Mobile genetic elements (MGEs)

Bacterial communities can adapt to, and survive in changing environments through different mechanisms: gene mutation, genetic rearrangement, inactivation or differential regulation of existing genes, which cause changes in the DNA allowing their survival (Thomas & Nielsen, 2005). Rearrangement and combination of pre-existing catabolic genes lead to the acquisition of new degradation pathways producing novel enzymes able to degrade the new pollutants released into the environment. These catabolic genes can be rapidly transferred among the bacterial populations through vertical gene transfer or more often, by horizontal gene transfer (HGT) (Heuer & Smalla, 2012). The HGT can be mediated through three mechanisms: transformation, which consists in the capture of a "free" DNA from the extern environment; conjugation, which consists in the transfer of DNA from cell-to-cell, and transduction, mediated by bacteriophages (Thomas & Nielsen, 2005). However, the HGT of catabolic genes mediated by mobile genetic elements (MGEs) during conjugation is assumed to be the major mechanism of bacterial adaptation to the changes in the environment (Top *et al.*, 2002, Sørensen *et al.*, 2005b).

MGEs can be defined as all elements able to move within and / or between the genomes of bacteria belonging to the same species or among different species. For example, plasmids, transposons, integrons and insertion sequences (IS) are important MGEs involved in the adaptation of bacterial populations to the environmental changes (Schlüter *et al.*, 2007).

1.4.1 Plasmids

Plasmids can be defined as extra-chromosomal linear or double-stranded DNA able to auto-replicate themselves independently from the chromosomal DNA replication from their hosts and can be transferred to other cells, so-called recipient cells. Every plasmid must have an origin of replication region. Plasmids are assumed to be essential players in an adaptation of a bacteria community to the pollutants released into the environment (Barkay & Wagner-Döbler, 2005). They are considered the major vehicles of gene transfer between bacteria, enhancing the genetic diversity (Thomas & Nielsen, 2005). The resistance genes carried by plasmids can be transferred by conjugation to various bacterial groups present in different environments: agricultural soil (Sen *et al.*, 2011), estuarine waters (Oliveira *et al.*, 2012), manure (Binh *et al.*, 2008, Heuer *et al.*, 2009) mangroves (Gomes *et al.*, 2010) plant surfaces (Bjorklof *et al.*, 1995), rhizosphere (Kroer *et al.*, 1998) and wastewater (Heuer *et al.*, 2002, Schlüter *et al.*, 2007, Bahl *et al.*, 2009). Plasmids belong to various incompatibility groups (Inc), such as IncN, IncW, IncP,

IncQ and the most recently discovered *PromA* group plasmids have received a lot of research inputs for their characterization and isolation due to their notable capacity of transfer to a broad-host-range which includes almost all Gram-negative genera (Schlüter *et al.*, 2007, Binh *et al.*, 2008, Van der Auwera Auwera *et al.*, 2009, Akiyama *et al.*, 2010, Moura *et al.*, 2010).

1.4.1.1 Incompatibility (Inc) plasmid groups

The classification of plasmids in different groups is based on different aspects, such as restriction patterns profile, incompatibility testing and plasmids sequence (Smalla *et al.*, 2000a). Incompatibility testing is based on the inability of plasmids from the same group co-existing in the same host cell. When two plasmids are incompatible (belonging to the same incompatibility groups), one of them is "expulsed" from the host cell as a strategy to avoid unnecessary cost of energy for plasmid maintenance. Based on the incompatibility, plasmids have been classified in 40 different incompatibility groups

(Couturier *et al.*, 1988). The plasmids belonging to different incompatibility groups can be divided into narrow-host-range plasmids (can transfer and replicate in a narrow range of hosts) such as: IncF, IncH and IncI and in broad-host-range (can transfer and replicate in a broad range of hosts) such as: IncN, IncP, IncQ and IncW (enterobacterial classification system; Suzuki *et al.*, 2010) and plasmids found into *Pseudomonas* group, such as IncP-1 (corresponding to IncP), IncP-4 (corresponding to IncQ), IncP-7 and IncP-9 plasmids (Thomas & Haines, 2004). Currently, 14 incompatibility plasmids groups classified in *Pseudomonas* (Thomas & Haines, 2004). Among these 14 plasmids, 3 are described as most relevant involved in enzymatic pathways and resistance to antibiotics: IncP-1, IncP-7 and IncP-9 (Dennis, 2005, Heuer & Smalla, 2012).

The structure of incompatibility plasmids can be divided into backbone genes and accessory genes. The backbone genes are correspondent to the essential genes necessary for the basic plasmid functions as replication, regulation, stable inheritance and gene transfer while the accessory genes are encoding various functions such as pathogenicity, virulence, antibiotic resistance, symbiosis, degradation of nature compounds and xenobiotics (Heuer & Smalla, 2012). These accessory genes are often located on MGEs inserted into the plasmid backbone, such as gene cassettes, insertion sequence (IS) elements, integrons and transposons. Conjugative plasmids can transfer their accessory genes such as antibiotic resistance genes, metal resistance genes and catabolic genes to different bacterial hosts. For instance, the "backbone" structure of all IncP-1 plasmids described up to now is rather conserved, whereas

the "accessory gene" portions have a high variability and are often located on one or two specific regions between the *oriV* and *trfA* and/or between *tra* and *trb* region (Schlüter *et al.*, 2007). Two regions are essential for the initial of replication: the origin of replication (*oriV*) and the *trfA* region. The *trfA* gene product (replication initiating protein) is responsible for the activation of the *oriV* region, initiating the replication (Pansegrau *et al.*, 1994).

1.4.1.2 IncP-1 plasmids

The IncP-1 plasmids (*Pseudomonas* classification) or IncP plasmids (*Enterobacteriaceae* classification) are considered the most "promiscuous" plasmids of the incompatibility group. They can replicate and maintain themselves in almost all Gram-negative bacteria, such as *Acinetobacter* spp., *Alcaligenes* spp., *Azotobacter* spp., *Hyphomicrobium* spp., *Neisseria* spp., *Paracoccus denitrificans, Pseudomonas* spp., *Rhizobium* spp., *Rhodospirillium* spp., *Vibrio cholera, Xanthomonas* spp. (Thomas & Helinski, 1989) and in a few Gram-positive bacteria (Adamczyk & Jagura-Burdzy, 2003, Musovic *et al.*, 2006, Leão *et al.*, 2013). The only Gram-negative genera unable to maintain IncP-1 plasmids are *Bacterioides* and *Myxococcus*. For instance, the reason for inability of IncP-1 plasmids' maintenance in *Bacterioides* seems to be the inability of conjugal transfer system and antibiotic resistance gene expression in these hosts (Thomas & Helinski, 1989). IncP-1 plasmids were first isolated from clinical specimen and were later observed in a wide range of different habitats: biogas plant, biopurification systems, compost, mangrove, manure, marine sponges, sediment, sewage, soil and water (Smalla *et al.,* 2006, Gomes *et al.,* 2010, Sen *et al.*, 2011, Heuer *et al.,* 2012, Oliveira *et al.,* 2012, Jechalke *et al.,* 2013a & chapter II). They are carrying antibiotic resistance genes, metal resistance and/or catabolic genes (Sen *et al.*, 2011). The ability of IncP-1 plasmids to transfer antibiotic resistance genes to different types of hosts contributed to the increase of antibiotic resistance in hospitals and in the environment. Moreover, different mutations and genetic rearrangements have been occurring in the IncP-1 plasmids, which led to the increasing diversity of antibiotic resistance genes (Top *et al.,* 2002; Heuer *et al.*, 2012). A big concern in the hospital is related to the evidences of the horizontal antibiotic resistance gene transfer from the environment reservoirs to the clinical context, contributing to the "crisis" in the controlling of infection diseases (Dantas & Sommer, 2012). Nowadays the idea is more acceptable of antibiotic resistance genes being present in the clinical context originated from environmental bacteria which are producing the antibiotics for survival issues in their habitat. This horizontal gene transfer from bacteria in the environment to bacteria in the hospital can be mediated by plasmids such as IncP-1 plasmids.

Other studies pointed out an important role of IncP-1 plasmids in the removal of contaminants (mainly man-made compounds) from the environment. They are carrying a broad range of xenobiotic degradative genes. Several pesticide degradation genes were detected and isolated from IncP-1 plasmids, such as *aztABCDEF* gene clusters, which are involved in atrazine degradation (Martinez *et al.*, 2001); different *tfd* gene clusters such as *tfdARCGEFB*/*tfdABC* (Top *et al.*, 1995); *tfdCEBKA* (Vedler *et al.*, 2004) and *tfdABCDEF* (Trefault *et al.*, 2004), all genes involved in the 2,4-dichlorophenoxyacetic acid (2,4-D) degradation. Moreover, studies indicated and proved a correlation between the abundance of IncP-1 plasmids and environment pollution (Smalla *et al.*, 2006, Heuer *et al.*, 2012). Moreover, IncP-1 plasmids are often carrying an insertion sequence (IS) so-called IS*1071*, a class II transposon, which plays an important role in the degradation of organic xenobiotics such as pesticides (Dunon *et al.*, 2013). The localization of IS*1071*, near to xenobiotics degradation genes carried by the "promiscuous" IncP-1 plasmids indicates the importance of IS*1071* elements in the spread of catabolic genes among various bacterial hosts, enhancing bacterial survival in polluted environments (Sota *et al.*, 2006a). In order to assess catabolic genes in the BPS of Kortrijk, Belgium, the laboratory of Dirk Springael (Belgium partner of the present project), through quantitative PCR (qPCR) by using specific primers targeting the *tnpA* genes (involved in the transposition of IS*1071* elements) and Taqman probe (used to increase the specificity of the amplification) showed that IS*1071* abundance was correlated with increase of pesticide pollution in the BPS present studied. IS*1071* elements are often carrying xenobiotic catabolic genes (Nojiri *et al.*, 2004), which might explain they high abundance in environments polluted with organic xenobiotics, such as pesticides.

The detection of IncP-1 specific sequences in the environment was mainly made by PCR-based detection combined with Southern blot hybridization (Götz *et al.,* 1996). The first IncP-1 primer system developed was based on primers targeting the sequences of the IncP1-α and IncP-1 β *trfA* region (Götz *et al.,* 1996). Later on, a new IncP-1 primer system was developed, also targeting the *trfA* region, but then with a much broader spectrum of detection, able to amplify five IncP-1 subgroups: α; β; γ; δ and ε (Bahl *et al.*, 2009). A new IncP-1 subgroup was recently described: IncP-1 ζ (Norberg *et al.*, 2011). Therefore, a new primer system able to target the *trfA* gene region of all six IncP-1 subgroups was developed for a PCR endpoint reaction (Jechalke, *et al.,* 2013a).

However, it is not enough to merely detect the presence of IncP-1. Due to their importance in the clinical context and for the environment, and knowing their worldwide

dissemination, a system to quantify, to measure their abundance was urgently needed. However, according to Jechalke *et al.* (2013a) the *trfA* portion is not the most conserved and suitable for qPCR detection. Other regions were found be more conserved than the *trfA* region: *trbC*, *trbE*, *traG*, *traI*, and *korB* (Herman *et al.*, 2011). The *korB* gene was considered the most suitable for a qPCR primer system design, because it provides more conserved sites for detection compared with the *trfA* region.

1.4.1.3 IncP-7 plasmids

IncP-7 plasmids are important players in the degradation of organic pollutants. They are often harboring genes involved in the degradation of natural contaminants and are considered as narrow-range-host because up to now they were exclusively found in *Pseudomonas* species (Dennis, 2005). However, interestingly, the transfer of an IncP-7 plasmid (pCAR1) into a non-*Pseudomonas* host such as *Stenotrophomonas maltophila* was demonstrated (Shintani *et al.*, 2008). The catabolic plasmid pCAR1 is so far the best studied IncP-7 plasmid and is completely sequenced (Maeda *et al.*, 2003). Plasmid pCAR1 was isolated from *Pseudomonas resinovorans* CA10 (Ouchiyama *et al.*, 1993) and is involved in the degradation of carbazole (*car* genes) and anthranilate (*ant* genes). The presence of pCAR1 was shown to increase the survival of *Pseudomonas* species in polluted areas (Shintani *et al.*, 2011). Different from IncP-1, which possess a rather conserved plasmid backbone, IncP-7 plasmids do not have a well conserved backbone, indicating several modifications during the gene transfer process over their evolution (Dennis, 2005). This might be problematic for the detection and identification of IncP-7 plasmids. Therefore, IncP-7 plasmids might have a much higher diversity than detected up to now. Moreover, IncP-7 plasmids can be found in a very high abundance in polluted environments such as biopurification systems contaminated with pesticides and river sediments contaminated with oil (chapter II). IncP-7 plasmids can be detected by specific-amplicon PCR in combination with Southern blot hybridization (Götz *et al.*, 1996, Izmalkova *et al.*, 2005).

1.4.1.4 IncP-9 plasmids

As *Pseudomonas* sp. are considered to be the major hosts of IncP-9 plasmids (Dennis, 2005), their endogenous isolation from *Pseudomonas* sp. obtained after growth on semi-selective media is relatively easy. They are important vehicles carrying degradative genes and consequently important players in the degradation of organic contaminants such as PAH (Flocco *et al.*, 2009, Gomes *et al.*, 2010). The plasmids pWW0, isolated from *Pseudomonas putida* (Williams & Murray, 1974) and NAH7 (Dunn & Gunsalus, 1973) SAL (Chakrabary, 1972)

are so far the best studied catabolic IncP-9 plasmids. Antibiotic resistance genes such as R2, pMG18 and pM3 plasmids, mercury resistance and UV resistance genes were also found in IncP-9 plasmids (Greated *et al.*, 2002, Sota *et al.*, 2006b). Currently, the complete sequences of four IncP-9 plasmids are available: pWW0 (Greated *et al.,* 2002), pDTG1 (Dennis & Zylstra, 2004), pNAH20 (Heinaru *et al.*, 2009) and pNAH7 (Sota *et al.,* 2006b), which is extremely helpful to better understand the evolution and functional aspects of this plasmid group which is likely of great importance for the adaptation of *Pseudomonas* populations to the pollution.

Plasmids belonging to the IncP-9 group were isolated from various geographic regions of the world (Gomes *et al.,* 2010, Jutkina *et al.,* 2011). A high abundance of these plasmids were found in biopurification systems contaminated with pesticides (chapters II and IV). However, a lack in the study of IncP-9 is observed and their diversity seems to be much broader than detected up to now (Sevastsyanovich *et al.*, 2008). Most of the knowledge about these plasmids is based on isolates. Sevastayanovich *et al.* (2008) proposed the division of IncP-9 plasmids in nine subgroups, based in the *repA* and *oriV* IncP-9 sequences divergence, where two major clusters were defined: pWW0 and pDTG1 branches. Recently, a new primer targeting conserved stretches in the *rep-oriV* region of IncP-9 plasmids showed a high abundance and diversity of IncP-9 plasmids in different environment samples (chapter II).

1.4.2 Integron-gene cassette system

Although the detection of antibiotic resistance genes was not the focus of the present thesis, a brief description of the genetic elements often carrying these genes, such as *integrons* and *gene cassettes*, was considered suitable here as class 1 and class 2 integrons were found in a high abundance in the BPS analyzed and are often carried by IncW, IncN plasmids and by those extensively studied in this work, i.e., IncP-1 plasmids (Liebert *et al.*, 1999, Heuer *et al.*, 2012).

Integrons are genetic elements present in Gram-negative bacteria around 30% belong to the *Gammaproteobacteria* (Moura *et al.*, 2007) and few in Gram-positive bacteria (Shi *et al.*, 2006). Integrons have a site-specific recombination system able to capture and mobilize antibiotic resistance genes located in gene cassettes, allowing their integration, excision and DNA rearrangements (Hall & Collis, 1995). This integron-gene cassette system is considered as the most relevant mechanism of antibiotic resistance gene acquisition between Gram-negative bacteria (Cambray *et al.*, 2010). Therefore, the integrons and gene cassettes are extremely important for the adaptation and survival of bacteria in environments exposed to antibiotics.

They are often associated with insertion sequence (IS) or as part of transposons, which may be located on plasmids, preferentially large conjugative plasmids, or in the chromosome (Moura *et al.*, 2012).

The structure of integrons can be divided in an *intI* gene that codes for the integron integrase; *Pint* promoter, responsible for expression of the integrase gene; *attI* recombination site; *Pc1* promoter responsible for the expression of gene cassette array; and sometimes also a second promoter *(Pc2)* is present that generally enhances the expression of gene cassette arrays (Jove *et al.*, 2010). A special class of integrons - so-called multiresistance integrons (MRIs) - represents the major players involved in the spreading of antibiotic multi-resistance gene cassettes between Gram-negative bacteria through horizontal gene transfer. Up to now, more than 130 gene cassettes, containing multiple antibiotic resistance genes were described (Partridge *et al.*, 2009). They can possess up to eight gene cassettes encoding antibiotic resistance and are divided in five groups based in the integrase coding sequence similarity (Mazel, 2006). Their detection can be performed through PCR amplification of *intI1*, *intI2*, *intI3*, *intI4* and *intI5* integrases, respectively (Jove *et al.*, 2010).

1.4.2.1 ***Integrons class 1* and *2***

Class 1 integrons are the best studied integrons and seem to be the major vehicle for antibiotic resistance gene dissemination during the last 50 years (Berg *et al.*, 2005, Moura *et al.*, 2010). They were first isolated in clinical context and related with nosocomial infections (Leverstein-van Hall *et al.*, 2003), often associated with the transposon Tn*402*-like (Stokes *et al.*, 2006). Nowadays it is clear that they are not only restricted to the clinical context but are also wide-spread in different environments such as wastewater (Gaze *et al.*, 2011, Moura *et al.*, 2012) and manure (Heuer *et al.*, 2011), containing several types of antibiotic resistance genes. Especially the integrons belonging to the classes 1 and 2 are often associated with environments exposed to antibiotics (Moura *et al.*, 2012).

The presence of *intI1*, an integrase facilitating the integration of gene cassettes into the *att* site into MGEs as transposons and conjugative plasmids contributed to the widespread dissemination of class 1 integrons to a broad range of different hosts from different environments. Many integrons contain an *aadA* gene cassette (Heuer *et al.*, 2012), encoding streptomycin–spectinomycin resistance which can also be found in a very high abundance in polluted environments as BPSs (chapter IV). The *sul1* genes are often associated with class 1 integrons and can be carried by IncP-1ε plasmids (Binh *et al.*, 2008, Heuer *et al.*, 2012), while

sul2 are often located on small non-conjugative plasmids such as IncQ plasmids (Sköld, 2000) or large conjugative plasmids such as the LowGC plasmids recently discovered by Heuer *et al.* (2009).

The integrons belonging to class 2 have a similar function as the class 1 integrons, however, they possess only a small portion of the resistance gene cassettes found in the class 1 integron, such as *aadA* gene cassettes and contain different transposons such as Tn7, Tn1825, Tn1826 (Ploy *et al.*, 2000).

1.4.2.2 *Gene cassettes*

Gene cassettes are normally found as linear sequences integrated into integrons localized on plasmids or bacterial chromosomes. They are often encoding antibiotic resistance genes, most of the time carrying only a single gene and only in few cases carrying two resistance genes. They have a recombination site located downstream of the gene and confer gene mobility (Hall & Collins, 1998). Often they do not possess their own promoter, depending on the promoters located in the integron to express their genes (Fluit & Schmitz, 2004).

1.5 Methods to assess the plasmid diversity in the environment

1.5.1 DNA extraction and purity

First of all, in order to get a more accurate detection of MGEs such as plasmids from environmental samples the quality of the DNA extraction is crucial. Nowadays a good quality of DNA extracted from the environment can be obtained through commercial kits for total DNA extraction from soil, as the FastDNA SPIN Kit for soil DNA extraction (Q-Biogene, Carlsbad, CA, USA) used in the research presented in this thesis. The commercial kits for total DNA extraction from environment samples often guarantee the lysis of the bacterial cells when combined with a mechanical bead beating process, providing sufficient DNA yield for further analysis. After extraction the DNA must be purified by using commercial kits, for instance the GENECLEAN SPIN Kit (Qbiogene, Heidelberg, Germany) or simply diluted, in order to get the DNA free of from inhibitors of PCR amplification reaction as humic acids (Götz *et al.,* 1996). After purification and verification of the DNA quality in an agarose gel PCR amplification is normally checked by amplifying 16S rRNA gene fragments.

1.5.2 PCR-based detection and Southern blot hybridization

Polymerase Chain Reaction (PCR) (Mullis, 1990) is a very sensitive technique used to amplify specific DNA sequences. Specific sequences derived from catabolic and antibiotic resistance genes as well as MGEs like integrons, transposons and plasmids can be detected by PCR amplification from environmental DNA or plasmid DNA. It is a very useful technique and was essential in the beginning of the project "Metaexplore European Union Project – Metagenomics for Bioexploration Tools and Application" for screening different environmental habitats for the presence of plasmids supposed to carry degradative genes. Using this approach it was possible to analyze large numbers of samples from different environments and to identify "hot spots" with high abundance of bacteria carrying MGEs (chapter II).

The success of PCR amplification depends on the specificity of the primers. To achieve a better performance it is necessary to optimize the concentrations of the reagents as well as the PCR reaction conditions. The denaturation, annealing and extension time and temperature must be adjusted in order to increase the specificity and sensitivity of the primer and the yield of the PCR product. Normally DNA denaturation occurs between 94C°-95C°. However, it is important to keep in mind that higher GC content primers need higher denaturation temperatures. The annealing step is critical for the primers to anneal to the template. Relatively low GC content (<50%) primers normally anneal only at temperatures lower than 55°C. The disadvantage of low annealing temperatures is the increase of nonspecific amplification. High GC content primers require higher annealing temperatures. The extension time depends on the length of the sequence that will be amplified. Normally the time of 1 min per kb product length must be sufficient to complete the extension (Gelfand & White, 1990).

However, the PCR technique has its limitations. The detection range of a specific sequence in a PCR reaction is limited to the primers' spectrum of detection. A solution to overcome this problem is based on the design of new primers which can detect a wider spectrum of target sequences. For instance, in the present work a new IncP-9 primer system targeting conserved stretches in the *rep-oriV* region, based on 28 *oriV* and *rep* sequences of IncP-9 plasmids was applied.

Another problem is that PCR reaction can also amplify non-specific products. In order to overcome this problem, besides the development of more specific primers, a PCR-based detection combined with Southern blot hybridization should be applied. This combination with Southern blot hybridization increases the sensitivity and specificity of detection. PCR-based

detection combined with Southern blot hybridization has been often used to detect broad host range plasmids (Götz *et al.,* 1996) present in different environments (Smalla *et al.*, 2000a, Heuer *et al.*, 2009). This combination is a suitable strategy to have insights into the prevalence and diversity of plasmids in a larger numbers of samples.

1.5.3 Detection of the relative abundance of IncP-1 plasmids in environmental samples

Quantitative real-time PCR (qPCR) is a suitable tool for an accurate and fast quantification of specific DNA sequences in environmental samples, allowing the measurement of taxonomic and functional genes such as 16S rRNA and *korB* genes, which are used to quantify the abundance of bacteria and of IncP-1 plasmids in the environment, respectively. Usually the copy numbers of a functional gene are given as relative abundance normalized by the abundance of 16S rRNA genes to consider differences in DNA extraction and amplification efficiency between samples. Differently from the traditional PCR, in which PCR product detection is done by end-point analysis, in the qPCR the measurements of amplicons are realized in real time during each cycle of the PCR. The main advantage of real-time PCR in comparison to conventional PCR is the accuracy and sensitivity in determining the starting template copy number, which allows a quantitative DNA analysis. A fluorescent reporter molecule is used to monitor the qPCR reaction, interacting with the amplified PCR-product and the measured fluorescence is equivalent to the copy number. SYBR Green 1 is the most often fluorescent DNA-binding dye applied in a qPCR reaction. It is a simple and cheap way to quantify a DNA sequence during amplification. However, due to the fact that SYBR Green 1 binds to double stranded DNA, unspecific DNA can also be amplified, producing biases. In order to overcome this problem, melting curves are used to verify the specificity of the amplification. A more specific DNA quantification can be obtained by means of a *Taq*Man probe, which was used for example to quantify the IncP-1 *korB* copy numbers in BPSs (Jechalke *et al.,* 2013a). This probe has a fluorescent reporter attached to the 5` end and a quencher dye in the 3´. When the target sequence is present, the primers and the probe bind to this target sequence. During the extension step, the Taq-polymerase used has 5‘ 3‘ exonuclease activity which degrades the probe releasing the dye and quencher, leading to a fluorescence signal which is equal to the number of amplified PCR-products. The measurement of the DNA amplification is realized by the accumulation of the fluorophore during the extension stage of each PCR cycle (Smith & Osborn, 2009).

1.5.4 Assessing the diversity of IncP-1 plasmids by cloning and sequencing of plasmid-specific sequences amplified from total community DNA (TC-DNA)

Traditionally insights into the diversity of plasmid-specific sequences amplified from the TC-DNA are obtained by cloning of PCR fragments into a cloning vector (Rastogi & Sani, 2011). PCR products of specific replicon-sequences such as *trfA* sequences of IncP-1 plasmids are inserted into a vector and transferred to a competent *Escherichia coli* cell, becoming so-called transformed cells. The selection of transformed cells containing the desired insert is made by a white-blue screening in which colonies are visualized on plate containing an antibiotic selective for the vector, a β-galactosidase (*lacZ*) inducer and a substrate for the enzyme (X-gal). When the transformed cell contains the insert, the β-galactosidase activity will be interrupted by the presence of the insert in the portion of the vector encoding for the *α* subunit of *lacZ*, producing white colonies. In the case of insert absence, the colonies will become blue due to the β-galactosidase activity. Therefore, different white colonies are selected and the inserts are identified by Sanger sequencing, generating a clone library likely with a high number of different sequences (Röling & Head, 2005). Nowadays several different commercial cloning kits with different vectors and competent cells are available, for example the QIAGEN cloning Kit (Hilden, Germany). The PCR-fragments are cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into competent cells such as *Escherichia coli* JM109 (Promega, Madison, WI, USA) and the blue/white selection is used to identify transformants carrying a vector with insert. Afterwards, the sequences of cloned fragments can be compared with known sequences available in databases such as GenBank. Indeed, the generation of clone libraries is a useful tool to get an idea about the diversity of different plasmids in the environment. However, a clone library do not necessary reflects the "real picture" from specific plasmids present in a bacterial population. Less abundant sequences might not be selected in this procedure, revealing a limited number of sequences. An increase in the number of clones for sequencing is an attempt to overcome this limitation. However, the increase in number of clones also increases the costs of sequencing and the work becomes even more time-consuming (Röling & Head, 2005).

1.5.5 High throughput sequencing of PCR-amplified plasmid specific sequences

Next generations sequencing (NGS) technologies such as 454, Illumina, SoLiD can produce thousands or millions of DNA sequences at a low price. These technichies are intensively applied in microbial ecology studies. In general, the amount of sequences acquired

via NGS is huge, which allows detecting rare populations that might have an important ecological role. These techniques were also used to study mobile genetic elements such as plasmids, which are important for the adaptations of bacteria to hostile environments. For example, several new plasmids present in polluted environments, such as activated sludge, are identified (Zhang *et al.,* 2011b).

In Metagenomics for Bioexploration - Tools and Application project, GS FLX Titanium PicoTiterPlate using the GS FLX pyrosequencing system (Roche, Basel, Switzerland) was applied to assess plasmid diversities in a range of diverse environments (chapters III and IV). PCR amplicons of *trfA* gene specific for IncP-1 plasmids and *rep* regions of IncP-7 and IncP-9 plasmids were generated respectively. The amplicons were sequenced with GS FLX Titanium systems. Compared to clone library, the data set acquired by pyrosequencing is huge, providing chances for in-depth analysis on the diversities of targeted plasmids. However, due to the high numbers of reads produced, the data evaluation is a challenge. For example, an elevated number of noisy reads is also observed. Therefore, noise removal algorithms must be applied, in order to distinguish the "real" plasmid diversity from sequence noise (Quince *et al*., 2011). PyroNoise is an algorithm that can be used to remove errors from pyrosequence and also errors generated from PCR amplification (Quince *et al*., 2009). Each time, more sophisticated algorithms have been faster developed in order to minimize the errors occurred during the pyrosequence process and in the further phylogenetic analysis. For instance, AmpliconNoise is even more sensitive than PyroNoise described above. The sequences are filtered and denoised by removing errors from sequencing and PCR single base errors (Quince *et al*., 2011) and chimeric sequences are removed *de novo* by using uChime in uSearch 5.2.32 (Edgar *et al*., 2011) (chapters II and IV).

1.5.6 Plasmid isolation from various environmental habitats

1.5.6.1 Endogenous plasmid isolation

Endogenous plasmid isolation is a classical cultivation-dependent approach used to isolate plasmids from environmental isolates. The samples from the environment are plated on either nonselective or selective media, which can be supplemented with antibiotics, heavy metal or other selective markers as source of carbon, e.g., xenobiotic compounds. The bacteria are isolated as pure cultures. However, their ability to grow under a certain selective marker can be either due to the chromosomally or plasmid-encoded genes. The first isolations of catabolic plasmids from the environment were performed from cultivable bacteria endogenously isolated

(Lidstrom & Wopat, 1984). Several antibiotic resistance, mercury resistance and catabolic plasmids isolated by cultivation-dependent technique were also completed sequenced, e.g., pEST1107 (Vedler *et al.*, 2004), pJP4 (Ledger *et al.*, 2006), pWWO (Greated *et al.*, 2002) and NAH7 (Sota *et al.*, 2006b).

The biggest advantage of the endogenous plasmid isolation approach is the possibility to identify the original plasmid host (Heuer & Smalla, 2012). However, in this approach, the cultivability of the bacteria host plasmid is required. Since it is well-known that most of the bacteria (about 95-99%) are still not cultivable by classical methods of microbiology, the capture of plasmids from the environment is also limited to the cultivation of their original hosts. Another limitation of the endogenous plasmid isolation is the abundance of the bacteria in the environment. When a specific bacterial group is present only in a low abundance, the plasmids carried by them will likely not be isolated. The isolation of bacteria favors those groups present in a higher numerical abundance or growing faster under cultivation conditions (Vartoukian *et al.*, 2010).

1.5.6.2 Exogenous plasmid isolation

A cultivation-independent technique (Bale *et al.*, 1987) has been applied to exogenously isolate plasmids from various environment samples through biparental and triparental assays (Hill *et al.*, 1992, Smalla *et al.*, 2006, Bahl *et al.*, 2007, 2008). A notable advantage of exogenous plasmid isolation is the ability of capture plasmids independently from uncultivable bacteria (Heuer & Smalla, 2012). In order to enhance the number of plasmids captured, a solid media should be used. For instance, it was demonstrated that the transfer capacity of the RP4 plasmid (IncP-1 plasmid) is several orders of magnitude higher on solid media than in liquid media, which might be due to the short rigid *pili* on solid surface (Bradley, 1980). When using the exogenous plasmid isolation the original host still being unknown this might not be relevant if we are just interested in capturing and characterizing the plasmids. However, when the identification of the host is desired, the exogenous plasmid isolation can be combined with the classical endogenous isolation. Another possibility of plasmid host identification *in situ* is the reporter-gene technology using *gfp* (green fluorescent protein) as fluorescent marker (Musovic *et al.*, 2006).

Many factors such as temperature, pH, nutrients, water availability, moisture, minerals, organic material and recipient cell type influence the frequency of plasmid transfer (Richaume *et al.*, 1989). Therefore, adjustments in the exogenous plasmid protocols can be done. For

instance, when the material used as donor contains high concentrations of a toxic pollutant, which might kill many recipient cells, an increasing of washing steps during the mating preparation is necessary to minimize the harmful effects. An increase in the transfer frequencies has been observed with a pre-enrichment of the environment sample used as donor (Top *et al.*, 1996). The enrichment depends on what we are looking for: if we want to capture plasmids carrying pesticide degradative genes, a mix of pesticides can be added into a minimal medium to be inoculated with the environment sample containing the desired plasmids. Moreover, in order to increase the diversity of the plasmids captured by exogenous isolation, different host cells can be applied in both, biparental and triparental matings. Depending on the recipient strain used, different plasmid types can be obtained. For instance, when we are looking for pesticide degradative plasmids, a biparental mating can be performed using recipient cells such as *Cupriavidus necator* as MCPA[20] and DCA[20] with mineral salt medium (MSM) (Margesin & Schinner, 1997), *Pseudomonas putida, Pseudomonas putida KT 2442 gfp*-labelled in a minimum mineral medium with 2,4 D[20] and a biparental mating using *Pseudomonas B13 gfp*-labelled (MSM) with linuron[20] as selective pressure in order to capture different pesticide degradation (chapter III).

1.5.6.2.1 Exogenous plasmid isolation through biparental assay

In exogenous plasmid isolation by biparental mating (Smalla *et al.,* 2006) the plasmids are captured directly from the bacterial fraction from the environment sample (donor) into a suitable recipient cell (Fig. 4). In this approach the exogenous plasmid isolation is based on the efficiency of the plasmids (present in the bacterial fraction) transferring under mating conditions of choice and their capacity of replication into the recipient cell applied and express respective selective markers or degradation abilities. To differentiate the recipient cells from the donors on agar plate, the recipient cells must have a selective marker. Rifampicin is often used as selective marker in the recipient cell, due to the low abundance (background) of naturally resistant bacteria found in soil, which can avoid false positive transconjugants (Smalla *et al.*, 2000b). A genetic modification of the recipient by inserting a *gfp* (green fluorescent protein) cassette is also very convenient to identify the transconjugants by the fluorescence produced which can be visualized in contact with UV light.

Different selective markers can be used in order to capture plasmids with different functions in a biparental mating. The choice depends on the goal. For instance, if the intention is to capture pesticide degradation plasmids, different pesticides can be used as selective marker,

such as mineral salt medium (MSM) supplemented with linuron, 2,4-D or MCPA or lindane (γ-HCH) (Anjum *et al.*, 2012) . An alternative to capture plasmids harboring pesticide degradation genes is the biparental mating using mercury chloride ($HgCl_2$) as selective marker. Several plasmids harboring pesticide degradation genes like pJP4 (involved in 2,4-D degradation) (Ledger *et al.*, 2006) can also carry mercury resistance genes (Neilson *et al.*, 1994, Sen*, et al.*, 2011). For instance, Sen *et al.* (2011) isolated four (at least three plasmids involved in degradation of chlorinated aromatic compounds) catabolic plasmids from soil using mercury as selective marker.

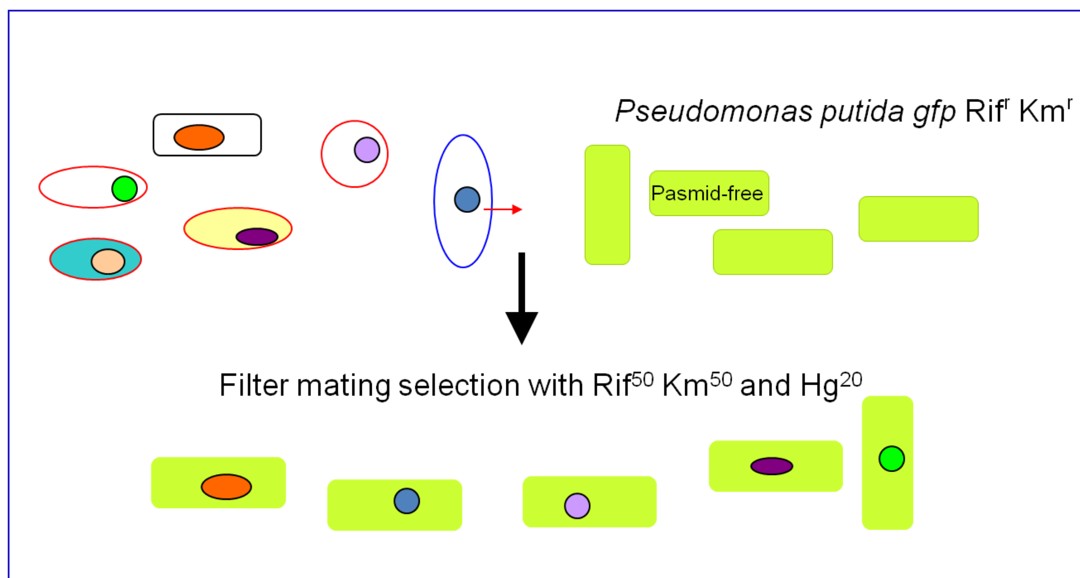


Fig. 4. Biparental mating from soil (donor) using *Pseudomonas putida gfp* as recipient cell and Hg^{20} as selective marker for catabolic plasmids isolation.

1.5.6.2.2 Which selective markers can be used to capture catabolic plasmids from BPS by biparental mating?

First of all it is important to get the information about the chemicals which were applied in the BPS. This information were were provided by the farmers. Moreover, the chemical analysis of pesticides from the BPS performed was extremely important in order to get an insight into which pesticides are being faster degraded, and which of them are being more recalcitrant to degradation.

For instance, in the BPS from Kortrijk, Belgium, previously described in this introduction, the pesticides S-metacholor and bentazon were detected in a very high concentration over the

agriculture season (chapter IV), indicating that these pesticides are recalcitrant or at least have a very slow rate of degradation (Munoz *et al.*, 2011). Consequently, they should not be used as a selective marker in biparental assays. On the other hand, pesticides such as MCPA, linuron, 2,4-D, which were not detected by chemical analysis but were known to be applied in this BPS (chapter IV), are good candidates of selective markers to capture catabolic plasmids by biparental exogenous isolation.

1.5.6.2.3 Exogenous plasmid isolation through triparental assay

In exogenous plasmid isolation by the triparental approach (Hill *et al.,* 1992) two donors, the bacterial fraction from the environment sample and the strain carrying a non-self transferable but mobilizable plasmid and a suitable recipient cell are required. For example, the strain *Pseudomonas B13 gfp*-labelled can be used as recipient cell, and *E. coli* C600 (pIE 723) carrying a mobilizable plasmid, such as IncQ plasmids (Top *et al.*, 1994, Götz & Smalla, 1997), as second donor (Fig. 5). The conjugative plasmids present in the environment sample such as IncP-1 plasmids are co-transferred into the recipient cell, such as *Pseudomonas* B13 *gfp*-labelled, during the mobilization of IncQ plasmids. *Pseudomonas* B13 *gfp*-labeled was previously used by (Dröge*, et al.*, 1999) to capture major IncP-1 plasmids from activated sewage sludge and most of them belonged to IncP-1 β subgroup. One of the advantages of triparental as well as of biparental matings is that the capturing of plasmids is independent of the cultivation from their original host. And different from the biparental approach, in a triparental mating the marker of the mobilizable plasmid are selected.The plasmid isolation in this case just depends on their capacity to mobilize plasmids such as IncQ plasmids (tra^-/mob^+ plasmids) and the capacity of the conjugative plasmids to be transferred into and replicated in the host (Thomas, 2000).

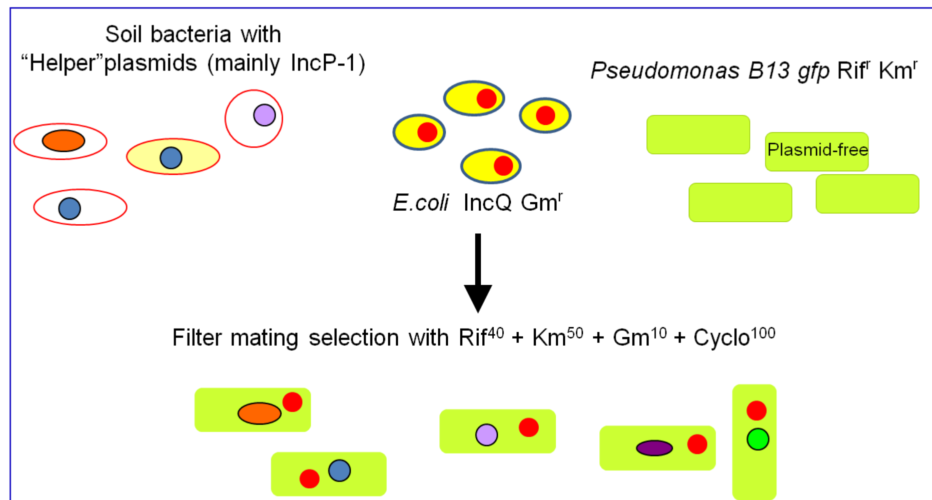


Fig. 5. Triparental mating from soil (donor) using *Pseudomonas* B13 *gfp* as recipient and *E. coli* C600 carrying pIE 723 (IncQ) as second donor.

1.6 Methods to assess the bacterial diversity trough 16S rRNA gene based analysis in the environment

1.6.1 Why is it important to evaluate the bacterial diversity?

Bacteria are involved in several ecological functions and it is assumed that high diversity is important for them to maintain the proper ecological role. Catabolic plasmids such as IncP-1, IncP-7 and IncP-9 plasmids are carried by different bacterial hosts. Although the genes carried by such plasmids can be a burden for the host due to the high cost of energy, they also present important benefits, enhancing the bacterial survival in polluted areas. The environmental conditions to which bacterial populations carrying catabolic plasmids are submitted is extremely important for the bacterial metabolism, maintenance and activity, directly influencing the stability, maintenance and transferability of catabolic plasmids carried by them. Consequently, to study the bacteria's community structure, diversity and shifts in their composition under stress conditions is certainly important to understand and predict the plasmid dissemination and its functions.

1.6.2 Insights into the bacterial community's dominant population: DGGE fingerprint technique

DGGE (denaturing gradient gel electrophoresis) (Muyzer *et al.*, 1993) of bacterial 16S rRNA gene fragments PCR amplified from total community DNA is a fingerprinting technique

applied to analyze shifts in the microbial community structure. The approach is fast, cheap and allows a simultaneous and reproducible analysis of many samples (Muyzer & Smalla, 1998). DGGE consists basically in an electrophoretic separation of PCR amplicons of the same length in a polyacrylamide gel with a gradient of denaturants. The separation of the DNA is based in the differences in sequences causing different melting temperature. The melting temperature of a DNA fragment is determined by its different G+C content. Therefore, the fingerprinting pattern is built according to the melting behavior of the sequences along a linear denaturing gradient which is obtained using urea and formamide. In order to prevent the complete denaturation of the PCR products during the electrophoresis, a "GC-clamp" attached to the end of one of the primers is applied (Muyzer *et al.*, 1993).

The use of universal primers targeting the 16S rRNA region allows the detection of the dominant bacterial population (>1%). However, in this case, the less abundant bacteria population cannot be visualized. To overcome this problem, taxon-specific primers to amplify specific bacteria groups as *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, is applied which increases the resolution of the DGGE. A good quality of DGGE analysis is directly linked to the quality of the PCR reaction. DGGE limitations are related to biases from DNA extraction and to the PCR amplification.

1.6.3 Metagenome analysis

Metagenomes can be defined as the DNAs obtained from uncultured microorganisms, and the study of these DNAs is the so-called metagenomics (Wooley *et al.*, 2010). The metagenomic studies have been revolutionizing the assessment of microbial diversity and abundance as well as the access to functional genes and MGEs carried by microorganisms. Genomic information from uncultured microorganisms can be quickly obtained directly from their habitats. Now, thousands of different microbial species can be analyzed in the same single run (National Research Council, 2007).

1.6.4 *16S rRNA* gene pyrosequence analysis from the metagenome

The adoption of gene amplification by polymerase chain reaction (PCR), followed by cloning and Sanger sequencing brought a new conception of microbial ecology understanding. However, several disadvantages were presented using cloning and Sanger sequencing: high costs and extensive laboratory work and limited insights from analyzing only a low numbers of clones from a few numbers of samples. Consequently, the reproduction of the "real picture"

from the microbial diversity was still far. Only a small portion of the bacterial community could be revealed. Then amplicon pyrosequencing of bacterial 16S rRNA gene fragments came up and allowed a more rapid and faster characterization of microbial communities, generating hundreds of thousands of sequence reads in a single run (Sogin *et al*., 2006). Next-generation sequencing (the Sanger method is the so-called 'first-generation' sequencing) with Roche 454 and Illumina are very often applied in high throughput sequencing platforms nowadays. In a single run, a large number of sequence reads can be produced. To reduce the cost, several samples can be analyzed in the same run using barcoded amplicons. The biggest advantage of these methods is the detection of bacterial population of relative low-abundances. In addition to the amount, the length of the sequences is also important for taxonomic identification in microbial ecological studies. In the past, one limitation of the illumina was the short fragment produced. However, one of the third-generation sequencing technologies, so-called GS FLX Titanium, produces an average of more than 400 bp of reading length. The substantial increase in the length also improved the taxonomic assignment of sequences data sets as long sequences provide more taxonomic information.

Aims of the current research

The objectives of the present thesis were:

- ✓ To investigate the occurrence of mobile genetic elements (MGEs), such as IncP-1, IncP-7 and IncP-9 catabolic plasmids in different environments, indicating "hot spot" reservoirs of MGEs involved in degradation of xenobiotics compounds;
- ✓ To assess the MGEs present in the chose environment: biopurification systems (BPS) contaminated with pesticides;
- ✓ To capture the main MGEs involved in the survival of bacterial communities to pesticide contamination in BPS;
- ✓ To investigate the structure and dynamics of the bacterial communities of BPS and their mobilome in response to pesticide (linuron) pollution (chapter III);
- ✓ To investigate the effects of different concentrations of various pesticides in the bacterial community composition in BPS over a entire agricultural season;
- ✓ To investigate the effects of different concentrations of various pesticides in abundance and diversity of MGEs in BPS over a entire agricultural season;
- ✓ To demonstrate the correlation between plasmid abundance and pollution trough quantitative PCR detection combined, southern blot hybridization and high throughput sequence analysis of the *trfA* portion sequences from IncP-1 plasmids.

Attempts to reach each objective listed above are found in the chapter II to chapter V.

Chapter II: The bacterial mobilome in different environmental habitats: Cultivation-independent screening revealed hot spots of IncP-1, IncP-7 and IncP-9 plasmid occurrence

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Abstract

IncP-1, IncP-7 and IncP-9 plasmids often carry genes encoding enzymes involved in the degradation of man-made and natural contaminants, thus contributing to bacterial survival in polluted environments. However, the lack of suitable molecular tools often limits the detection of these plasmids in the environment. In this study, PCR followed by Southern blot hybridization detected the presence of plasmid-specific sequences in total community (TC) DNA or fosmid DNA from samples originating from different environments and geographic regions. A novel primer system targeting IncP-9 plasmids was developed and applied along with established primers for IncP-1 and IncP-7. Screening TC DNA from biopurification systems (BPS) which are used on farm for the purification of pesticide-contaminated water revealed high abundances of IncP-1 plasmids belonging to different subgroups as well as IncP-7 and IncP-9. The novel IncP-9 primer-system targeting the *rep* gene of nine IncP-9 subgroups allowed the detection of a high diversity of IncP-9 plasmid specific sequences in environments with different sources of pollution. Thus polluted sites are "hot spots" of plasmids potentially carrying catabolic genes

Introduction

The search for novel enzymes able to degrade recalcitrant natural contaminants such as chitins and lignins and man-made pollutants such as halogenated aliphatic and aromatic compounds motivated metagenomic exploration from various environments. It has been observed that the microbial metagenomes of open ecosystems, including soils and aquatic habitats, clearly offer rich reservoirs of genes that determine the desired enzymatic reactions in which chitinases, ligninases and dehalogenases are involved. By anthropogenic activities, recalcitrant compounds have also been released as environmental pollutants. While the typical metagenomic approaches employ genetic or activity screens of cloned large size DNA fragments from various environments, capturing mobile genetic elements (MGE) into suitable recipients might be an alternative and complementary approach to access genes coding for novel enzymes or even complete degradative pathways. Mobile genetic elements such as plasmids are assumed to play an important role in the adaptation of bacterial communities to changing and, due to pollutants, often challenging environmental conditions (Heuer & Smalla, 2012). Partial or complete degradative pathways were previously reported to be localized on plasmids belonging to the IncP-1, IncP-7 or IncP-9 group (Shintani *et al.*, 2010). The present study aimed to monitor various environments for the abundance of these plasmids by using a cultivation-independent total community (TC-) DNA based approach to select the most promising habitats for mining the mobilome potentially carrying genes coding for novel enzymes. We hypothesized that the frequency of occurrence of genes encoding the desired enzymatic activities is increased in the MGE gene pool (Dennis, 2005, Heuer & Smalla, 2012). Specially, plasmids belonging to the incompatibility groups (Inc) P-1, P-7 and P-9 are often carrying genes responsible for the degradation of xenobiotic (man-made) and natural organic pollutants, being essential players in the adaptation of bacterial communities to the new inputs of toxic compounds released in the environment (Barkay & Smets, 2005, Frost *et al.*, 2005, Heuer & Smalla, 2012). Therefore, selected natural or pretreated (enriched) environments were analyzed for the prevalence of plasmids belonging to the IncP-1, IncP-7 and IncP-9 group by means of a cultivation-independent approach. Some of these environments were enriched for the desired degradation function by adding the relevant substrates, i.e. chitin, lignin and/or organohalogens. The habitats sampled included multifold soils (chitin and/or lignin-treated soils, peat bogs), biopurification systems (BPS) for pesticide removal from contaminated water, biogas production plants, wastewater, as well as aquatic (river bank sediments, sponges) environments from a wide range of geographic regions. Total community DNA was extracted

after a harsh lysis and used as template for PCR amplification with IncP-1, IncP-7 and IncP-9 specific primers. The specificity of the amplicons was confirmed by Southern blot hybridization. While previously published primer systems were used for the detection of IncP-1 and IncP-7 plasmids (Izmalkova *et al.*, 2005, Bahl *et al.*, 2009), a novel primer system for the specific amplification of IncP-9 plasmids was developed in the present study. Digoxigenin labeled probes used for Southern blot hybridization were generated from reference plasmids of the known IncP-1, IncP-7 and IncP-9 plasmids. Southern blot hybridization using probes derived from reference plasmids belonging to different subgroups of IncP-1 plasmids provided new insights into their environmental dissemination. Our results showed a particularly widespread dissemination of IncP-1 plasmid-specific sequences. Different hot spots of plasmid occurrence were identified.

Materials and Methods

Extraction of total community DNA (TC-DNA) and metagenomic DNA from different environmental samples

The TC-DNA and/or metagenomic DNA from different environmental samples originating from various geographic regions were extracted using different methods. The protocols used for TC-DNA and/or metagenomic DNA of each sample type are given in Table 1.

16S rRNA gene PCR amplification and quantification

16S rRNA gene PCR amplification reaction was done as previously described by Heuer *et al.* (2009) (product size of 1506 bp). The quality of the PCR product was visualized after electrophoresis in 1% agarose gel stained with ethidium bromide under UV light in comparison with the 1-kb gene-rulerTM DNA ladder (Fermentas, St Leon-Rot, Germany). Quantitative PCR (qPCR) targeting the 16S rRNA gene was performed with the TaqMan system as described by Suzuki *et al.* (2000). The 16S rRNA gene qPCR standard was made through dilution series (10^{-3} to 10^{-7}) of the cloned 16S rRNA gene amplicons (1467 bp) from E. *coli.*

Southern blot-PCR based detection of IncP-1 plasmids

IncP-1 plasmids belonging to the α, β, γ, δ and ε subgroups were detected based on the amplification of the *trfA* region (product size of 281 bp) from TC-DNA and metagenomic DNA using the primers described by Bahl *et al.* (2009). Digoxygenin-labeled probes targeting different IncP-1 plasmids subgroups were generated from reference plasmids belonging to the IncP-1α, β, γ, δ and ε plasmids (Table 2). The IncP-1 mixed probe was prepared by mixing probes generated for the different subgroups. The random primed digoxigenin labeling of PCR amplicons excised from preparative agarose gels was done according to the Roche manufacturer's protocol (Boehringer, Mannheim, Germany).

Southern blot-PCR based detection of IncP-7

PCR amplification of the *rep* region of IncP-7 plasmids (product size of 524 bp) from TC-DNA and metagenomic DNA was performed as previously described by Izmalkova *et al.* (2005), and Southern blotted PCR amplicons were hybridized at medium stringency condition with the dig-labeled IncP-7 probe generated from the reference plasmid pCAR1 isolated from *Pseudomonas resinovorans* according to the manufacturer's instructions (QIAGEN® Plasmid

Mini Kit) (Table 2). The random primed digoxigenin labeling of PCR amplicons was done as described above.

Analyzing the diversity and abundance of IncP-9 plasmids by a novel PCR system targeting the *oriV-rep* region

To study the abundance and diversity of IncP-9 plasmids, a novel PCR system targeting the *oriV-rep* regions was developed and applied to detect IncP-9 plasmids in TC- and metagenomic DNA from all samples analyzed (Table 1).

Multiple alignments of 28 sequences of *oriV* (EU499619-EU499641, AF078924, AB237655, AJ344068, AB257759 and AF491307) and *rep* (EU499644-EU499666, AF078924, AB237655, AJ344068, AB257760 and AF491307) were performed with MEGA. Conserved regions of sequences belonging to nine IncP-9 subgroups (Sevastsyanovich *et al*., 2008) were used for the primer design. The selected primer system consists of 21-mer degenerated forward primer (5-GAG GGT TTG GAG ATC ATW AGA-3) and reverse primer (5-GGT CTG TAT CCA GTT RTG CTT-3). *In silico* analysis showed no mismatch for at least 12 bp at the 3'end of each primer and 1-4 mismatches for each sequence type at the 5' end (Fig. S1). The expected amplicon size is 610-637 bp. The primers were further tested with plasmid DNA from the reference plasmids summarized in Fig. S1. None of the plasmids belonging to other incompatibility groups was amplified while the reference plasmids were amplified. The reaction mixture (25 µl) contained 1 µl template DNA (1-5 ng), 1x Stoffel buffer (Applied Biosystems, Foster, CA), 0,2 mM dNTPs, 2,5 mM $MgCl_2$, 2 µg/µl bovine serum albumin, 0.2 µM of each primer, and 2.5 U TrueStartTaq DNA polymerase (Stoffel fragment, Applied Biosystems). Denaturation was carried out at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 53°C (primer annealing) and 2 min at 72°C and a final extension of 10 min at 72°C.

PCR amplicons of *oriV-rep* regions of nine IncP-9 subgroups IncP-9 plasmids (Table 2) were gel-purified and digoxigenin-labeled as described above. Southern blot hybridization of *oriV-rep* amplicons from different environmental samples listed above was performed with a mixture of these probes under medium stringency conditions following the manufacturer's instructions (Roche Diagnostic, Mannheim, Germany). PCR analysis revealed that *oriV-rep* amplicons were abundant in all replicates of three BPS, but not in other samples. Thus clone libraries were generated for these three BPS to confirm primer specificity. *oriV-rep* amplicons were gel-purified, ligated into pGEM vectors, and transformed into *E. coli* JM109 competent cells according to the instructions of the manufacturer. Clones containing the correct inserts were

selected for sequencing. BLAST-N analysis was used to identify *oriV-rep* sequences of IncP-9. All sequences analyzed share high similarity with IncP-9 *oriV* or *rep* sequences in NCBI. The sequences and those of known *oriV-rep* sequences in the data base were aligned and phylogenetic tree was calculated according to the neighbor-joining method and bootstrapping analysis by using Molecular Evolutionary Genetics Analysis (MEGA 4).

Results

Determination of bacterial 16S rRNA gene copies by qPCR

In order to estimate the bacterial density of the different environmental samples analyzed, 16S rRNA gene copies were determined by quantitative real-time PCR from the TC-DNA and metagenomic DNA. Most of the samples (Table 3) showed a high abundance of bacterial populations ranging from 10^8 to 10^9 16S rRNA gene copy numbers per gram of material. For few samples significantly lower 16S rRNA gene copy numbers per gram of material (Tukey´s test $p > 0.05$) were detected (Table 3).

Distribution of IncP-1 plasmids in different environments

In order to investigate the presence of IncP-1 plasmids in different habitats a Southern blot-PCR based detection system was applied. Hybridization signals of the expected size (251 bp) using the IncP-1 mixed probe from PCR products were detected in a very wide range of different habitats (Table 3), indicating that IncP-1 plasmids of different subgroups are widespread distributed. By using different IncP-1 specific probes for five different subgroups (α, β, γ, δ and ε), differences in the composition of IncP-1 plasmids according to the geographic area and sample type were observed. Strong hybridization signals of IncP-1α plasmids were only observed in one TC-DNA from Askö sediment (Sweden), in TC-DNA from a biogas production plant (Germany) and fosmid-DNA from Baltic Sea sediments. Strong hybridization signals were observed by using the IncP-1β specific probe in the TC-DNAs of all biopurification systems (BPS) samples from Belgium (Table 3, Fig. 1) and most of the sediment samples from Argentina, indicating that in these environments bacterial populations carrying IncP-1β plasmids are highly abundant. The highest IncP-1γ hybridization signal was observed in the TC-DNA of the BPS located in Kortrijk, Belgium. Less intense IncP-1γ hybridization signals were detected in the TC-DNA of other BPS from Belgium and in TC-DNA of sediments from Argentina. In all TC-DNAs of BPS from Belgium, strong IncP-1δ hybridization signals were observed and a weaker hybridization signal, compared to BPS TC-DNA, was detected in sediments TC-DNA from Argentina. Very strong IncP-1ε hybridization signals were again detected in all BPS TC-DNA from Belgium (Table 3, Fig. 2) and most of the sediments from Argentina. By using IncP-1 mixed-probe, strong hybridization signals were detected in soils from Argentina and soil treated with chitin from the UK, indicating a high abundance of IncP-1 plasmids.

Distribution of IncP-7 plasmids in different environments

In order to investigate the occurrence of IncP-7 plasmids in different environments, a PCR-based detection combined with Southern blot hybridization approach was applied. Strong hybridizations signals were observed in all TC-DNAs from BPS analyzed (Fig. 3), indicating a high abundance of IncP-7 plasmid carrying bacterial populations in BPS. Less intense hybridization signals were observed in the TC-DNAs of seven sediment river samples from Argentina and in the TC-DNA from soil amended with chitin from the United Kingdom. Hybridization signals using the IncP-7 specific plasmid amplicon probe were not detected in any of the other environmental samples analyzed (Table 3).

IncP-9 plasmid occurrence and diversity in different environmental samples

In order to verify the occurrence and diversity of IncP-9 plasmids in different habitats the new IncP-9 primer system developed in the present work was applied. Very strong hybridization signals were detected in all TC-DNAs of BPS samples, indicating that BPS are a reservoir of bacteria carrying IncP-9 plasmids. Less intense hybridization signals were observed in the TC-DNA of sediment samples from Argentina. A weaker hybridization signal was detected in the soil amended with chitin from the UK (Table 3).

To verify primer specificity and to gain insights into the IncP-9 plasmid diversity from BPS samples (indicated as a "hot spot" of IncP-9 plasmids), a clone library with PCR amplicons obtained with the primers targeting the *oriV-rep* region of IncP-9 plasmids from TC-DNA of three different BPS was generated and sequencing revealed the presence of different IncP-9 subgroups. As shown in the phylogenetic tree (Fig. 4) the different types of IncP-9 plasmids on BPS were similar to *oriV-rep* sequences of pWWO and pM3. However, several sequences could not be affiliated to previously known IncP-9 plasmid groups indicating an undiscovered diversity of this plasmid group.

Twenty *Pseudomonas* isolates from BPS material were tested for the presence of IncP-9 plasmids. From the 20 isolates, six showed a strong IncP-9 plasmid-specific Southern blot hybridization signal. The partial sequence of the *oriV-rep* IncP-9 fragment from the plasmids of these six *Pseudomonas* isolates indicated sequences similar to pWWO and pNL15 plasmids (data not shown).

Discussion

In the present study a screening using PCR-based detection combined with Southern blot hybridization allowed the detection of IncP-1, IncP-7 and IncP-9 plasmids in a wide range of different geographic areas and sample types, indicating a high abundance of these plasmids in environments with different sources of pollution. It is tempting to speculate that degradative genes localized on these plasmid groups might contribute to the bacterial degradation of various pollutants such as pesticides, due to the "metabolic complementation" produced by the combination of different genes among them: while IncP-1 plasmids are typically hosting genes associated with the degradation of man-made pollutants (xenobiotics) (Hill & Weightman, 2003, Krol *et al.*, 2012), IncP-7 and IncP-9 plasmids are often carrying genes responsible for natural contaminants, for instance, polyaromatic hydrocarbon degradation (Jutkina *et al.*, 2011). Screening TC-DNA revealed that IncP-1, IncP-7 and IncP-9 specific sequences are varying according to sample type and degree of pollution. IncP-1 plasmid specific sequences were detected in a wide range of environments: marine sponges, soils and sediments, Baltic Sea sediment fosmid library, biogas production plant, river sediments, chitin-treated soils and BPS contaminated with pesticides. Very strong hybridization signals for all different IncP-1 subgroups tested except for IncP-1α were specially observed in the BPS samples heavily contaminated with pesticides, indicating an unusual high abundance of bacterial populations carrying IncP-1 plasmids. Indeed, the use of BPS, defined as a pollution control technique employing microorganisms to degrade pesticides through biodegradation processes (Castillo *et al.*, 2008), in on-farm treatment of water contaminated with pesticide has substantiality increased and enhanced the degradations rates (Omirou *et al.*, 2012). Strong hybridization signals of IncP-1β and IncP-1ε plasmids observed in all BPS samples and in some sediments from Argentina contaminated with oil, suggested that IncP-1β and IncP-1ε plasmids are important players in the local adaptation to changing environmental conditions (Trefault *et al.*, 2004, Oliveira *et al.*, 2012). Strong IncP-1 plasmid hybridization signals observed in sediments from different regions: Warwick (UK), La Plata (Argentina) and sediments from Sweden indicate that IncP-1 plasmids might also have an important ecological role in the adaptation and biodegradation processes in sediments which was previously reported already for mercury contaminated sediments in Kazachstan (Smalla *et al.*, 2006). The indication of high abundance of IncP-1 plasmids in soils from different regions contaminated with different pollutants, such as soils from Argentina polluted with oil and soils from the UK enriched with chitin, also suggested that IncP-1 plasmids might substantially contribute to the adaptation and survival of the bacterial communities in

response to environmental pollutants in soils (Top & Springael, 2003, Krol *et al.*, 2012, Sen *et al.*, 2013, Dunon *et al.*, 2013). The results from several studies suggested a correlation between IncP-1 plasmids abundance and pollution as hypothesized by Smalla *et al.* (2006) and confirm previously published quantitative data on the abundance of IncP-1 plasmids in BPS samples from one BPS site by means of a qPCR targeting the *korB* gene. Obviously, the relative abundance of IncP-1 plasmids can only be precisely quantified by quantitative real-time PCR. However, the recently developed *korB* quantitative PCR system (Jechalke *et al.*, 2013a) cannot indicate the relative abundance of the different IncP-1 subgroups which was achieved with specific probes for different IncP-1 groups used in the present study in a semi-quantitative manner.

Already the study by Sevastsyanovich *et al.* (2008) showed that IncP-9 plasmid diversity is much broader than imagined before. In view of this huge plasmid diversity, a novel IncP-9 primer system was developed and established in the present work. Typically, IncP-9 plasmids are related to the degradation of natural pollutants as polyaromatic hydrocarbons (Gomes *et al.*, 2010). However, the detection of very strong IncP-9 hybridization signals mainly in BPS indicated that IncP-9 plasmids are also important players in the degradation of man-made pollutants or wood-derived aromatic compounds. IncP-9 plasmids often possess different aromatic-ring degradation genes. BPS typically contain wood chips but also various aromatic ring containing pesticides such as bentazon, epoxiconazol and diflufencian (Fetzner & Lingens, 1994), which could explain the high abundance of IncP-9 plasmids observed in BPS. Cloning and sequencing of amplicons obtained with the novel IncP-9 primers from BPS TC-DNA confirmed not only the specificity of the primers but also showed the presence of plasmids that were previously reported to carry degradative genes such as pWWO (Fig. 4) (Greated *et al.*, 2002, Sota *et al.*, 2006b). The presence of several sequences with high similarity to the *oriV-rep* sequence of pM3 an antibiotic resistance plasmid belonging to the IncP-9α subgroup in BPS 2 might be caused by manure additions as C-source in BPS material.

The indication of high abundance of IncP-9 plasmids in soils from Argentina contaminated with oil is not too surprising. IncP-9 plasmids are important vehicles for the dissemination of genes coding for enzymes involved in the degradation of polycyclic aromatic hydrocarbons (PAH) and are very often found in environments polluted with oil (Izmalkova *et al.*, 2006, Flocco *et al.*, unpublished).

PCR-Southern blot hybridization results showed that bacteria hosting IncP-7 plasmids were also highly abundant in BPS, indicating a role of these plasmids in the degradation of man-made pollutants such as pesticides. The high abundance of IncP-7 plasmids in soils enriched with chitin, suggested that bacterial carrying IncP-7 plasmids might play an important role in the degradation of chitin in soil.

It can be concluded that PCR-Southern blot hybridization detection of plasmid specific sequences from TC-DNA is a suitable and accurate but semi-quantitative approach to investigate the occurrence of plasmid-specific sequences in different environments and in a large number of samples. The detection of plasmids was possible independently of the cultivation of their original hosts (Heuer & Smalla, 2012) and indicated “hot spots” of IncP-1, IncP-7 and IncP-9 plasmids, such as BPS.

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Tables and Figures

Table 1. Description of environmental samples analyzed and TC-/Metagenomic DNA extraction applied.

Samples	Description of samples	TC-/Metagenomic DNA extraction method
A	Biogas production plant fermentation sample from Bielefeld, Germany	Zhou *et al*., 1996
B.1	Biopurification system (BPS) from Leefdaal, Belgium	Gomes *et al.,* 2007
B.2	BPS from Leefdaal, Belgium	Gomes *et al.,* 2007
B.3	BPS from Leefdaal, Belgium	Gomes *et al.,* 2007
C.1	BPS from Belgium (Pcfruit)	Gomes *et al.,* 2007
C.2	BPS from Belgium (Pcfruit)	Gomes *et al.,* 2007
C.3	BPS from Belgium (Pcfruit)	Gomes *et al.,* 2007
C.4	BPS from Belgium (Pcfruit)	Gomes *et al.,* 2007
C.5	BPS from Belgium (Pcfruit)	Gomes *et al.,* 2007
C.6	BPS from Belgium (Pcfruit)	Gomes *et al.,* 2007
D.1	BPS from Lierde, Belgium	Gomes *et al.,* 2007
D.2	BPS from Lierde, Belgium	Gomes *et al.,* 2007
D.3	BPS from Lierde, Belgium	Gomes *et al.,* 2007
E.1	BPS from Kortrijk, Belgium	Gomes *et al.,* 2007
E.2	BPS from Kortrijk, Belgium	Gomes *et al.,* 2007
E.3	BPS from Kortrijk, Belgium	Gomes *et al.,* 2007
F.1	BPS from Koksijde, Belgium	Gomes *et al.,* 2007
F.2	BPS from Koksijde, Belgium	Gomes *et al.,* 2007
F.3	BPS from Koksijde, Belgium	Gomes *et al.,* 2007
G.1	Soil from La Plata, Argentina	Jechalke *et al*., 2013a
G.2	Soil from La Plata, Argentina	Jechalke *et al*., 2013a
G.3	Soil from La Plata, Argentina	Jechalke *et al*., 2013a
H.1	Sediments from La Plata, Argentina	Jechalke *et al*., 2013a
H.2	Bordering soil from a water channel in La Plata, Argentina	Jechalke *et al*., 2013a
H.3	Bordering soil from a water channel in La Plata, Argentina	Jechalke *et al*., 2013a
J	Marginal river forest soil from La Plata, Argentina	Jechalke *et al*., 2013a
L.1	Bordering soil from a water channel in Buenos Aires, Argentina	Jechalke *et al*., 2013a
L.2	Bordering soil from a water channel in Buenos Aires, Argentina	Jechalke *et al*., 2013a
L.3	Bordering soil from a water channel in Buenos Aires, Argentina	Jechalke *et al*., 2013a
M	*Halichondria panicea* (marine sponge) from Oosterschelde, Netherlands	Sipkema *et al*., 2010
N	*Corticium candelabrum* (marine sponge) from Punta Santa Anna (Blanes), Spain	Sipkema *et al*., 2010
O	*Petrosia ficiformis* (marine sponge) from Punta Santa Anna (Blanes), Spain	Sipkema *et al*., 2010
P.1	Askö sediment from Baltic Sea Sweden (bottom fraction - anoxic)	Edlund *et al*., 2008
P.2	Askö sediment from Baltic Sea Sweden (middle fraction – mixed anoxic/oxic)	Edlund *et al*., 2008
P.3	Askö sediment from Baltic Sea Sweden (top fraction - oxic)	Edlund *et al*., 2008
Q	Pooled fosmid library, Askö sediment, Baltic Sea	Hårdeman & Sjöling, 2007
R	Landsort in Sweden	Edlund *et al*., 2008
S.1	Sediment from a river in Warwick, United Kingdom	Gomes *et al.,* 2007
S.2	Sediment from a river in Warwick, United Kingdom	Gomes *et al.,* 2007
T	Soil from Cuba amended with chitin (Test site 1)	Gomes *et al.,* 2007

Table 2. Probe generation for Southern blot hybridization

Probe	Reference plasmid	Plasmids host strain	Primers
IncP-1α	RP4	*E. coli*	Bahl *et al*., 2009
IncP-1β	R751	*E. coli CM544*	Bahl *et al*., 2009
IncP-1γ	pQKH54	*E. coli DH10B*	Bahl *et al*., 2009
IncP-1δ	pEST4011	*Alcaligenes xylosoxidans EST4002*	Bahl *et al*., 2009
IncP-1ε	p3-408	*E. coli cv601-GFP*	Bahl *et al*., 2009
IncP-7	pCAR1,	*Pseudomonas resinovorans CA10*	Izmalkova *et al.,* 2005
IncP-9 α	pM3	*Pseudomonas putida*	*This study*
IncP-9 β	pBS2	*Pseudomonas putida BS268*	*This study*
IncP-9 γ	pSN11	*Pseudomonas putida BS349*	*This study*
IncP-9 δ	pSN11	*Pseudomonas putida SN11*	*This study*
IncP-9 ε	pMG18	*Pseudomonas putida AC34*	*This study*
IncP-9 ζ	pNL60	*Pseudomonas* spp. *18d/1*	*This study*
IncP-9 η	pNL15	*E. coli C600*	*This study*
IncP-9 θ	pSVS15	*Pseudomonas fluorescens SVS15*	*This study*
IncP-9 ι	pNL22	*Pseudomonas* spp. *41a/2*	*This study*

Table 3. Bacterial density and PCR-Southern blot hybridization detection of plasmid replicon-specific sequences belong to IncP-1 five subgroups, IncP-7 and IncP-9. Hybridization signal: (+++) very strong, with exposure time up to five minutes; (++) strong, with exposure time up to one hour; (+) weak, with exposure time up to three hours; (-) none, with exposure time above to three hours; (/) not analyzed.

Sample	Description of samples	P-1	α	β	ε	γ	δ	P-7	P-9	16S log10/g
A	Biogas production plant from Bielefeld, Germany	+++	++	++	++	-	++	-	-	9,34
B.1	Biopurification system (BPS) from Leefdaal, Belgium	+++	-	++	+++	++	+++	+++	+++	9,32
B.2	BPS from Leefdaal, Belgium	+++	-	++	+++	++	+++	+++	+++	9,25
B.3	BPS from Leefdaal, Belgium	+++	-	++	+++	++	++	+++	+++	8,43
C.1	BPS from Belgium (Pcfruit)	+++	+	+++	+++	++	++	+++	+++	9,32
C.2	BPS from Belgium (Pcfruit)	+++	+	+++	++	++	+++	+++	+++	8,28
C.3	BPS from Belgium (Pcfruit)	++	-	++	++	-	+++	++	+	8,36
C.4	BPS from Belgium (Pcfruit)	+	-	++	+	+	+++	+++	+++	8,54
C.5	BPS from Belgium (Pcfruit)	+++	(+)	+++	+++	++	+++	+++	+++	8,66
C.6	BPS from Belgium (Pcfruit)	+	-	++	++	+	++	++	-	8,15
D.1	BPS from Lierde, Belgium	+++	-	++	-	-	+++	++	++	8,61
D.2	BPS from Lierde, Belgium	+++	-	++	++	-	+++	++	++	8,59
D.3	BPS from Lierde, Belgium	+++	-	++	+++	++	+++	++	++	8,31
E.1	BPS from Kortrijk, Belgium	+++	-	+++	+++	+++	+	+++	++	9,2
E.2	BPS from Kortrijk, Belgium	+++	-	+++	+++	+++	++	+++	+++	9,03
E.3	BPS from Kortrijk, Belgium	+++	-	++	++	+++	-	+++	+++	9,11
F.1	BPS from Koksijde, Belgium	++	(+)	++	+	-	+++	(+)	+++	9,01
F.2	BPS from Koksijde, Belgium	++	+++	++	-	-	++	-	+++	8,9
F.3	BPS from Koksijde, Belgium	++	(+)	++	+	-	+++	-	+++	8,95
G.1	Soil from La Plata, Argentina	+++	(+)	+	+	-	+++	+++	+++	8,55
G.2	Soil from La Plata, Argentina	+++	-	(+)	+	++	+	+++	+++	8,53
G.3	Soil from La Plata, Argentina	+++	-	++	-	++	+	-	(+)	8,22
H.1	Sediments from La Plata, Argentina	+++	++	+++	++	++	+++	+++	+++	8,96
H.2	Bordering soil from a water chanel in La Plata, Argentina	+++	++	++	++	++	+++	+	+++	8,49

Sample	Description of samples	P-1	α	β	ε	γ	δ	P-7	P-9	16S log10/g
H.3	Bordering soil from a water channel in La Plata, Argentina	+++	+	++	++	++	+++	-	+++	8,7
I	Sweet-water soil from a river in La Plata, Argentina	+++	+	++	++	++	++	+++	+++	7,91
J	Marginal river forest soil from La Plata, Argentina	++	-	-	-	-	-	-	-	8,32
L.1	Bordering soil from a water channel in Buenos Aires, Argentina	+++	-	++	+	-	++	-	-	8,29
L.2	Bordering soil from a water channel in Buenos Aires, Argentina	+++	+	+++	++	++	+++	-	(+)	8,6
L.3	Bordering soil from a water channel in Buenos Aires, Argentina	+++	-	++	++	++	+++	++	+++	7,66
M	*Halichondria panicea* (marine sponge) from Oosterschelde, Netherlands	++	-	++	-	-	+++	-	-	7,32
N	*Corticium candelabrum* (marine sponge) from Punta Santa Anna (Blanes), Spain	++	-	+	-	-	+	-	-	8,18
O	*Petrosia ficiformis* (marine sponge) from Punta Santa Anna (Blanes), Spain	++	-	++	-	-	+	-	-	8,4
P.1	Askö sediment from Baltic Sea Sweden (bottom fraction - anoxic)	++	-	-	-	-	+	-	-	8,34
P.2	Askö sediment from Baltic Sea Sweden (middle fraction – mixed anoxic/oxic)	++	++	+++	++	-	+	-	-	8,43
P.3	Askö sediment from Baltic Sea Sweden (top fraction - oxic)	+++	-	++	+++	-	+	-	-	8,09
Q	Pooled fosmid library, Askö sediment, Baltic Sea	+++	++	+	+	-	+	-	-	5,01
R	Landsort in Sweden	+++	++	-	-	-	-	-	-	8,16
S.1	Sediment from a river in Warwick, United Kingdom	+++	/	/	/	/	/	-	(+)	5,78
S.2	Sediment from a river in Warwick, United Kingdom	+++	/	/	/	/	/	++	-	6,26
T	Soil from Cuba amended with chitin (Test site 1)	+++	/	/	/	/	/	+++	++	6,95
negative control		-	-	-	-	-	-	-	-	
RP4 (IncP-1α)		+++	+++							
R751 (IncP-1β)		+++		+++						
pKJK5 (IncP-1ε)		++			++					
pQKH54 (IncP-1γ)		+++				+++				
pEST4011 (IncP-1δ)		+++					+++			
pCAR1 (IncP-7)								+++		
pNF 142 (IncP-9)									+++	

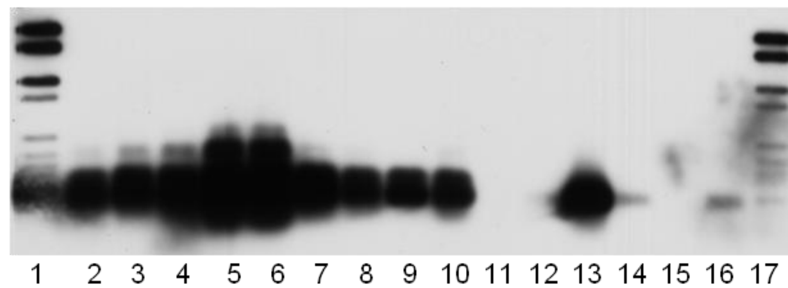


Fig. 1. Biopurification systems (BPS). Southern-blotted hybridization with PCR products with *trfA* primer system from TC-DNA of BPS (IncP-1β specific group). Lanes: 1; 17, dig ladder; lanes 2-4, BPS from Lierde, Belgium; lanes 5-8, BPS from Kortrijk, Belgium; lanes 8-10, BPS from Koksijde, Belgium; lane 11, negative control; lanes 12-16, IncP-1 positive controls RP4 (α), R751 (β), pKJK5 (ε), pQKH54 (γ) and pEST4011 (δ). Exposure time of 5 min.

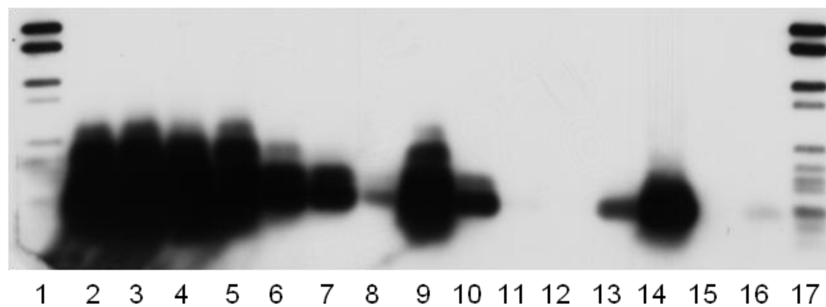


Fig. 2. Biopurification systems (BPS). Southern-blotted hybridization with PCR products with *trfA* primer system from TC-DNA of BPS with the IncP-1ε specific probe. Lanes: 1; 17, dig ladder; lanes 2-4 BPS from Lierde, Belgium; lanes 5-8, BPS from Kortrijk, Belgium; lanes 8-10, BPS from Koksijde, Belgium; lane 11, negative control; lanes 12-15, IncP-1 positive controls RP4 (α), R751 (β), pKJK5 (ε), pQKH54 (γ) and pEST4011 (δ). Exposure time of 5 min.

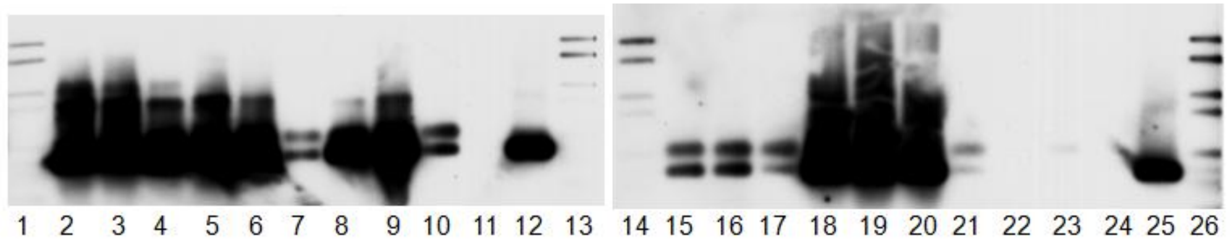
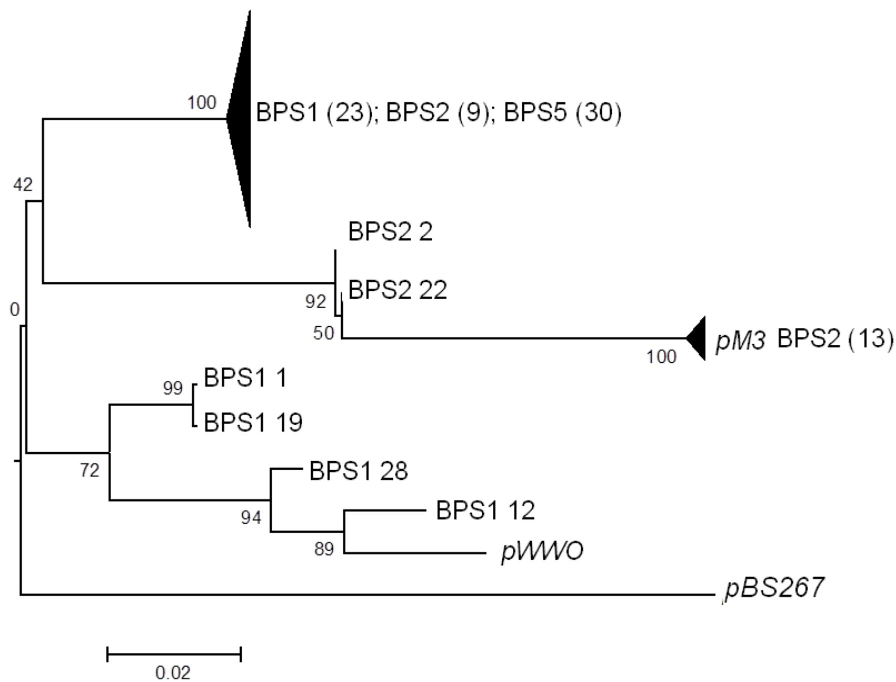


Fig. 3. Biopurification systems (BPS). Southern-blotted hybridization with PCR products with *rep* primer system from TC-DNA of BPS with the IncP-7 mixed-probe. Lanes: 1; 13 and 26, dig ladder; lanes 2-4, BPS from Leefdaal, Belgium; lanes 5-10, BPS from Belgium (Pcfruit); lanes 15-17, BPS from Lierde, Belgium; lanes 18-20, BPS from Kortrijk, Belgium; lanes 21-23, BPS from Koksijde, Belgium; lanes 11 and 24, negative control; lanes 12 and 25 IncP-7 positive controls pCAR-1. Exposure time of 5 min.

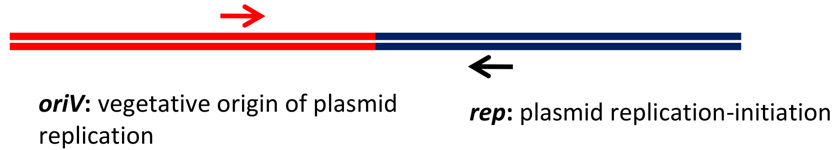
Fig. 4. Neighbor-Joining phylogenetic tree based on the multiple alignment of cloned amplicon sequences of the *oriV-rep* IncP-9 gene. Sequences from known IncP-9 plasmids have been included as references. Value at each node is percent bootstrap support of 1,000 replicates. BPS1; BPS2 and BPS5 correspondent to three different biopurification system (BPS), located in Belgium. Numbers in bracket correspond to number of clones and numbers not in bracket correspond to the clone designation.



Note: Waiting for accessory numbers of the sequences recentely submitted to GeneBank.

Supplement Material

Fig. S1. Development of primer system for endpoint IncP-9 PCR of plasmid-replicon sequences



Forward primer	G	A	G	G	G	T	T	T	G	G	A	G	A	T	C	A	T	W	A	G	A
pM80 alpha OriV	T	G	G	G	G	T	T	A	G	G	A	G	A	T	C	A	T	A	A	G	A
NPL-1 beta OriV	T	G	G	G	G	T	T	A	G	G	A	G	A	T	C	A	T	A	A	G	A
pBS268 gamma OriV	A	G	G	G	G	A	T	T	A	G	A	G	A	T	C	A	T	A	A	G	A
pMG18 epsilon OriV	A	A	G	C	G	T	T	A	G	G	A	G	A	T	C	A	T	A	A	G	A
pNL15 eta OriV	T	G	G	G	G	T	T	A	G	G	A	G	A	T	C	A	T	A	A	G	A
pBS216 Xi OriV	G	A	G	T	G	G	T	T	A	G	A	G	A	T	C	A	T	T	A	G	A
pNL60 zeta OriV	G	A	G	T	C	G	T	T	A	G	A	G	A	T	C	A	T	A	A	G	A
pSVS15 Theta OriV	G	A	G	T	G	G	T	T	A	G	A	G	A	T	C	A	T	A	A	G	A
Plasmid pM3	T	G	G	G	G	T	T	A	G	G	A	G	A	T	C	A	T	A	A	G	A
Plasmid pFKY1	G	A	G	T	G	G	T	T	A	G	A	G	A	T	C	A	T	T	A	G	A
plasmid NAH7	G	A	G	T	C	G	T	T	A	G	A	G	A	T	C	A	T	A	A	G	A
plasmid pWW0	T	G	G	G	G	T	T	A	G	G	A	G	A	T	C	A	T	A	A	G	A
plasmid pDTG1	G	A	G	T	G	G	T	T	A	G	A	G	A	T	C	A	T	T	A	G	A

Reverse Primer	G	G	T	C	T	G	T	A	T	C	C	A	G	T	T	R	T	G	C	T	T
pM80 alpha rep	G	G	T	C	T	G	G	A	T	C	C	A	G	T	T	G	T	G	C	T	T
p8C beta rep	G	G	T	C	T	G	A	A	T	C	C	A	G	T	T	G	T	G	C	T	T
pBS268 gamma rep	G	G	T	T	T	G	T	A	T	C	C	A	G	T	T	A	T	G	C	T	T
R2 epsilon rep	G	G	T	C	T	G	G	A	T	C	C	A	G	T	T	G	T	G	C	T	T
pMG18 epsilon rep	G	G	T	C	T	G	G	A	T	C	C	A	G	T	T	G	T	G	C	T	T
pNL15 eta rep	G	G	T	C	T	G	T	A	T	C	C	A	G	T	T	A	T	G	C	T	T
pBS216 Xi rep	G	G	T	T	T	G	A	A	T	C	C	A	G	T	T	G	T	G	C	T	T
pNL60 zeta rep	T	G	T	C	T	G	A	A	T	C	C	A	G	T	T	G	T	G	C	T	T
pSVS15 Theta rep	G	G	T	C	T	G	A	A	T	C	C	A	G	T	T	G	T	G	C	T	T
Plasmid pM3	G	G	T	C	T	G	G	A	T	C	C	A	G	T	T	G	T	G	C	T	T
plasmid pWW0	G	G	T	C	T	G	A	A	T	C	C	A	G	T	T	G	T	G	C	T	T
Plasmid pFKY1	G	G	T	T	T	G	A	A	T	C	C	A	G	T	T	G	T	G	C	T	T
plasmid pDTG1	G	G	T	T	T	G	A	A	T	C	C	A	G	T	T	G	T	G	C	T	T
plasmid NAH7	T	G	T	C	T	G	A	A	T	C	C	A	G	T	T	G	T	G	C	T	T

Chapter III: Bacterial community shifts and increase in abundance of IncP-1 plasmids in response to linuron application in material from an on-farm biopurification system

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Abstract

On-farm biopurification systems (BPS) are used for the treatment of pesticide contaminated wastewater at farms through biodegradation and sorption processes. However, information on the biological processes that lead to pesticide removal and the microbiota involved is scarce. Here we report on the response of BPS bacterial communities to linuron in a microcosm experiment. Both DGGE and pyrosequencing of 16S rRNA gene fragments amplified from community DNA indicated shifts in the bacterial community after linuron application. Responding populations were organisms that have been previously associated with linuron degradation such as *Hyphomicrobiaceae* and *Comamonadaceae*. In addition, numerous taxa with increased abundance were identified that have never been associated with linuron degradation. qPCR showed an increased relative abundance of IncP-1 *korB* copies in response to linuron application suggesting an increase in IncP-1 plasmids in the community. Amplicon pyrosequencing of the IncP-1 *trfA* gene revealed a high IncP-1 plasmid diversity and suggested that populations carrying IncP-1β plasmids increased in abundance while those carrying IncP-1ε plasmids decreased in number. IncP-1β plasmids were also exogenously isolated. Our data suggest the existence of a complex linuron degrading bacterial food web in the BPS and that IncP-1 plasmids might carry genes involved in the linuron degradation.

Introduction

On-farm biopurification systems (BPS) are increasingly used for the local treatment of pesticide contaminated wastewater at farms. In BPS, the contaminated water is percolated over a solid matrix, the so-called biomix, which is typically a homogenized mixture of local soil, chopped straw and peat, or composted material and in which the pesticides are removed by biodegradation and sorption processes. BPS are considered a simple, low cost, practical approach for farmers to treat pesticide contaminated water on farms (Castillo *et al.*, 2008). Although biodegradation is assumed to be an important process for pesticide removal in BPS, in contrast to agricultural soil, information about the involved microbiota and/or the microbial community response to pesticide application in a BPS environment is scarce. Often specialized bacteria that carry customized pesticide biodegradation pathways play a role in pesticide biodegradation in the environment. Mobile genetic elements (MGE), collectively referred to as the mobilome are assumed to promote bacterial adaptation to rapidly changing environments. Plasmids are considered to play a major role in the assembly and spread of pesticide biodegradative gene clusters in bacterial communities (Top & Springael, 2003, Dennis, 2005, Król *et al.*, 2012). Among the known degradative plasmids, plasmids belonging to the IncP-1 group are of particular importance as they have a broad host range and transfer efficiently in soil-like environments (Pukall *et al.*, 1996, Götz & Smalla, 1997, Musovic *et al.*, 2006, 2010, Mølbak *et al.*, 2007, Heuer & Smalla, 2012). Presently, the number of completely sequenced catabolic plasmids belonging to the IncP-1 group is rapidly increasing. Several IncP-1 plasmids have been identified that carry genes for the degradation of pesticides such as 2,4-dichlorophenoxyacetic acid (2,4-D) (Top *et al.*, 1995, Trefault *et al.*, 2004, Vedler *et al.*, 2004), atrazine (Martinez *et al.*, 2001) and chloroaniline (Król *et al.*, 2012). Sequencing has led to the description of a gene cluster in IncP-1β plasmid pAKD15 that might encode an unknown catabolic pathway (Sen *et al.*, 2011) pinpointing their important role in the removal of pesticides from the environment.

Recently, several studies have explored linuron biodegradation in BPS in microcosm systems. These studies showed the proliferation of *Variovorax,* an organism that has been often implicated with linuron degradation in soil. The proliferation of *Variovorax* was in accordance with an increase in linuron mineralizing capacity suggesting that *Variovorax* sp. also thrives on linuron in a BPS environment (Bers *et al.*, 2012, 2013). Moreover, an increase in the abundance of MGE such as IS*1071* and IncP-1 plasmids, accompanied by an increase in mineralization of various pesticides, was observed in BPS, indicating that microorganisms carrying those elements are involved in the degradation of pesticides such as linuron (Dunon *et al.*, 2013). However, those studies made use of pristine materials for composing the biomix matrix including a soil containing a linuron degrading bacterial community. Whether

organisms like *Variovorax* and mobile elements play a role in linuron degradation in an actual BPS environment and its community is not known.

To study this, response of pre-adapted bacterial communities and their IncP-1 content and diversity to linuron was examined in microcosms containing biomix material of a BPS that was in operation for several years at a farm in Kortrijk, Belgium. The biomix was either spiked or unspiked with linuron (5 mg g^{-1}) and its bacterial community monitored over 25 days. We hypothesized that the exposure of a pre-adapted BPS bacterial community to high concentrations of linuron will cause changes in the relative abundance of specific bacterial populations including organisms that grow at the expense of the added linuron and that the relative abundance of IncP-1 plasmids carrying degradative genes might increase due to the growth of bacterial cells carrying such IncP-1 plasmids and their spread through horizontal gene transfer. A range of different cultivation independent techniques was applied to test our hypothesis.

Materials and Methods

Linuron

Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methyl urea] (purity, 99.5%) was purchased from Sigma Aldrich (Belgium).

Sampling of the biopurification system (BPS)

The BPS in Kortrijk, Belgium, consists of soil and manure (straw and animal feces) and is used for cleaning water contaminated with different types of pesticides (mainly herbicides).The BPS (20 m x 1.2 m) was divided into three sections (left, middle and right). One composite sample taken from each section (ca. 5 kg of the top soil; 0-10 cm) was dried and sieved (2 mm). After sieving, the BPS material was stored at 4°C.

Microcosm set-up

Three replicates, representing the three BPS sections were used in the experiment. The microcosms were 50 mL Erlenmeyer flasks that contained either BPS biomix material amended with linuron (BPS^+ microcosms) or biomix material without linuron spike (BPS^- microcosms). Linuron spiked BPS material was prepared as follows. 25 mg linuron (Sigma Aldrich Belgium) was dissolved in 5 mL of acetone in the Erlenmeyer flask and mixed with 1 g BPS material. After overnight evaporation of the acetone, an additional 4 g BPS material was added to each replicate giving a final concentration of linuron of 5 mg g^{-1}. BPS^- microcosms were prepared similarly but 5 mL of acetone without linuron was first mixed with 1 g BPS material. The six microcosms were incubated in the dark, at approximately 20°C and the percentage of water in each sample was determined by weighing the samples before and after drying at 110 °C. In order to keep the moisture content constant, the samples were weekly weighed and water was added when necessary. Biomix samples were taken on days 1, 12 and 25 of the incubation. At each sampling time, the biomix material was mixed with a sterile spatula and 0.5 g material was taken for total community (TC) DNA extraction.

DNA extraction from microcosm samples

TC-DNA was extracted from 0.5 g microcosm material using the FastDNA® SPIN Kit for Soil (Bio101, Qbiogene, Carlsbad, CA) after a harsh lysis step by using FastPrep FP120 bead beating system for cell lysis. The DNA was purified by GENECLEAN Spin kit (Qbiogene), and the yield and quality were checked by electrophoresis in 1% (w/v) agarose gels under UV light after staining with ethidium bromide. The extracted TC-DNA was used for PCR-Southern blot hybridization, qPCR, DGGE and sequencing.

PCR amplification of 16S rRNA genes from TC-DNA

The absence of PCR inhibiting substances was checked by PCR amplification of 16S rRNA gene fragments from TC-DNA using the primers F27 and R1494 using the conditions previously described (Heuer *et al.*, 2009). Successful PCR amplification was checked by agarose gel electrophoresis.

PCR-Southern blot based detection of IncP-1 plasmids

The presence of IncP-1 plasmids was examined in all TC-DNA from BPS^+ and BPS^- replicates by PCR amplification of *trfA* gene fragments. The primers used targeted the *trfA* gene (281 bp) of the five IncP-1 subgroups (α, β, ε, γ and δ) (Bahl *et al.*, 2009). The reaction mixture (25 µL) contained 1 µL TC-DNA (1-5 ng), 1x True-Start buffer (Fermentas), 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 2 µg μL^{-1} bovine serum albumin, 0.2 µM of each primer, and 2.5 U TrueStartTaq DNA polymerase (Fermentas). Reaction conditions were as follows, i.e., 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 extension for 10 min at 72°C.

Southern blot hybridizations with a digoxygenin-labeled IncP-1 *trfA* probe mixture including *trfA* fragments of all IncP-1 subgroups, was done according to Götz *et al.* (1996). The probe mixture contained equal amounts of DIG-labeled PCR products generated by Random primer synthesis according to the manufacturer's instructions (Roche, Mannheim, Germany) from RP4 for IncP-1α, R751 for IncP-1β, pQKH54 for IncP-1γ, pEST4002 for IncP-1δ and pHH3-408 for IncP-1ε plasmids.

Detection of 16S rRNA and IncP-1 gene copies by quantitative real-time (q)PCR

The copy numbers of bacterial 16S rRNA genes were quantified as described by Suzuki *et al.* (2000), while *korB* copy number were quantified as described by Jechalke *et al.* (2013a) with the exception that the recently discovered IncP-1ζ plasmids were not targeted. *korB* gene copy numbers were related to 16S rRNA gene copy numbers.

Biparental exogenous isolation of plasmids

Capturing of mobile genetic elements conferring linuron degradation capacity

The rifampicin-resistant mutant, *Pseudomonas putida* KT2442 *gfp*, carrying the *gfp* gene coding for green fluorescence protein (GFP) (Heuer *et al.*, 2002) was used as recipient strain for biparental exogenous plasmid capturing. Potential transconjugants were selected on minimal salt medium (Dejonghe *et al.*, 2003) supplemented with linuron (20 µg mL^{-1}; Sigma-Aldrich) and rifampicin (50 µg $mL^{-1;}$ Serva Electrophoresis GmbH). The bacterial

fraction from BPS^- and BPS^+ material was obtained through shaking 3 g of material taken from each microcosm after 25 days of incubation in 25 mL of 1/10 tryptic soy broth (TSB; Merck KGaA, Darmstadt, Germany) at room temperature for 2 h. After settling for 1 h, the supernatant was transferred to a Falcon tube and settled again for 15 min. The supernatant was transferred to a new Falcon tube and centrifuged for 10 min at 500 x *g*. The recipient was obtained after overnight growth of *Pseudomonas putida* KT2442 *gfp* under shaking in 10 mL Luria Bertani (LB) broth containing kanamycin (50 µg mL^{-1}) and rifampicin (50 µg mL^{-1}) at 28°C. The cells were harvested by centrifugation for 10 min at 500 x *g* and resuspended in 1 mL TSB without antibiotics added. Five hundred µL of the cell pellet (corresponding to donor bacteria detached from 3 g of microcosm material) and 250 µL of resuspended recipient cells were mixed and centrifuged (5 min, 10,000 x *g*). After discarding the supernatant the cell pellet resuspended in 200 µL of 1/10 TSB was transferred to a filter placed on PCA as previously described (Heuer *et al.*, 2002). After overnight growth at 28°C, the filters were resuspended in 10 mL of sterile saline solution to dislodge the cells. Transconjugants were obtained by plating serial 10-fold dilutions of the cells resuspended from the filters onto a minimal salt medium (Dejonghe *et al.*, 2003) containing linuron (20 µg mL^{-1}) and cycloheximide (100 µg mL^{-1}; Serva Electrophoresis GmbH). The plates were incubated at 28°C and evaluated for the presence of transconjugants after 48 hours of incubation up to 20 days of incubation. The transfer frequencies are given as quotient of numbers of transconjugants and numbers of recipient.

Capturing of mobile genetic elements conferring mercury resistance

The rifampicin-resistant mutant, *Pseudomonas putida* KT2442 *gfp* was used again as recipient for biparental exogenous plasmid isolation. Cells were extracted from the microcosm material and used in the mating procedure as described above. Selection for transconjugants was perfomed by plating serial 10-fold dilutions on Mueller-Hinton (MH) agar plates (Merck KGaA, Darmstadt, Germany) containing $HgCl_2$ (20 µg mL^{-1}) and the same antibiotics as described above. After 48 h of incubation at 28°C, CFU were counted and GFP positive colonies were picked for further analysis.

Plasmid DNA isolation

Transconjugants were harvested from 5 mL overnight cultures in LB broth and plasmids extracted by phenol-chloroform extraction of the potassium acetate fraction (Smalla *et al.*, 2000a). The quality of the PCR products was visualized under UV light after agarose gel electrophoresis (1%, w/v) after staining of the gel with ethidium bromide.

Plasmid characterization

Plasmid DNA from transconjugants was screened for the presence of IncP-1, IncP-7 and IncP-9 by PCR (IncP-1 (Bahl *et al.*, 2009); IncP-7 (Izmalkova *et al.*, 2005); IncP-9 Dealtry *et al.*, chapter II). IncP-7 PCR products were hybridized with an IncP-7 mixed-probe (Dealtry *et al.*, chapter II). Plasmid DNA was digested with the enzymes Pst1 and BstZ17I (Fermentas GmbH, St. Leon Rot, Germany) and Southern blotted. Southern blotted plasmid restriction digests were hybridized with the IncP-1 mixed probe and the IncP-1β, IncP-1ε and the IncP-9 probes (Dealtry *et al.*, chapter II).

Pyrosequencing of bacterial 16S rRNA gene amplicons and IncP-1 *trfA* gene amplicons

Pyrosequencing of the 16S rRNA and IncP-1 *trfA* gene amplicons was performed on TC-DNA extracted from samples taken after 25 days incubation. A 480 bp region of the bacterial 16S rRNA gene was amplified with primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') (Hansen *et al.*, 2012) flanking the V3-V4 regions. A 281 bp region of the IncP-1 *trfA* gene was amplified using the three primer sets described by Bahl *et al.* (2009). 16S rRNA gene amplification and attachment of adapters and barcode tags was done as described (Masoud *et al.*, 2011). The attachment of adapters and barcodes to the *trfA* amplicons was done using the U-linker system described by (Holmsgaard *et al.*, in prep.). 35 cycles were run in the initial PCR amplification and the amplicon DNA was purified using the Montage Gel extraction kit (Millipore, Billerica, MA, USA). Sequencing was done at The Danish National High-Throughput DNA-Sequencing Centre on a GS FLX Titanium PicoTiterPlate using the GS FLX pyrosequencing system (Roche, Basel, Switzerland).

Analysis of *tfrA* amplicon sequences

Primers, barcode tags and adapters were trimmed away and the sequences quality filtered and denoised using AmpliconNoise (Quince *et al.*, 2011). Sequences of less than 231±3 bp in length were discarded (Holmsgaard *et al.*, in prep.). Chimeric sequences were removed *de novo* with uChime in uSearch 5.2.32 as described (Edgar *et al.*, 2011). Briefly, sequences were dereplicated and chimeric sequences removed *de novo* based on abundance. These non-chimeric sequences were then used as reference database to remove chimeric sequences in the standard uChime database mode. The cleaned amplicon sequences were assigned to the seven IncP-1 sub-groups by clustering them together with known IncP-1 *trfA* reference sequences (Table S1) at 89 % nucleotide sequence similarity

(Holmsgaard *et al.*, in prep.). Each cluster was given a number. Sequences from five large clusters, all containing reference sequences, were extracted. The sequences extracted from each of the clusters were then independently re-clustered at 97 % nucleotide sequences similarity. Each new cluster was given a number appended to the number of the cluster the sequences were extracted from. To calculate Neighbor-Joining trees a representative sequence from each of the original clusters (89% similarity) or each of new clusters (97% similarity) that contained ≥1 % of the sequences from the original cluster were used. Sequences were translated to amino acids, aligned using Muscle (8 iterations), back translate to nucleotides and used to build a neighbor-joining tree (1,000 bootstrap replicates) in MEGA5.0 (Tamura *et al.*, 2011). Significant differences ($p < 0.05$) between the relative number of sequences BPS^- and BPS^+ were tested using Student's t-test at both clustering levels. Data was log-transformed and variance homogeneity verified with Bartlett's test. Denoising and clustering was done with the QIIME 1.5.0 software package (http://qiime.org/, Caporaso *et al.*, 2010) using QIIME tools or QIIME wrappers of other tools. All other bioinformatics were done using Biopieces (www.biopieces.org, develop by Martin A. Hansen) and statistics were done in R 2.15.1 (R Core Team, 2012).

Analysis of bacterial 16S rRNA gene amplicon sequences

Pyrosequencing data from the 16S rRNA gene amplicons were trimmed, quality filtered and denoised as explained for the *trfA* sequences. Chimeric reads were removed with uChime in uSearch 5.2.32 (Edgar *et al.*, 2011) against the GOLD database (Pagani *et al.*, 2012). The analysis of sequences was done according to Ding *et al.* (2012). Briefly, sequences were used for BLASTN analysis against the SILVA database (Pruesse *et al.*, 2007). In order to compare the bacterial community structure in BPS^- and BPS^+ at day 25, cluster analyses were performed based on the pair-wise Pearson correlation that is suitable to compare samples with a different number of sequence reads. The reliability of clusters was tested by 500 times bootstrap analyses. Sequence clusters identified as belonging to the family *Comamonadaceae* were further analyzed for their possible membership to the *Variovorax* phylotypes. A representative sequence from each cluster was used to calculate a neighbor-joining tree (1,000 bootstrap replicates) together with the 16S rRNA gene sequences from 37 *Variovorax* and 13 *Acidovorax* strains. Sequences from the strains were trimmed to between primers 341F and 806R used for sequencing and aligned using Muscle in MEGA 5.0 (Tamura *et al.*, 2011).

PCR-DGGE analysis

PCR-DGGE analysis of community bacterial 16S rRNA gene was done as previously described (Gomes *et al.*, 2005). PCR-DGGE of *Variovorax* specific 16S rRNA genes was

performed as reported by (Bers *et al.*, 2011b). GelCompar 5.0 program was used to analyze the fingerprints. Clustering of patterns was calculated using unweighted pair group method with arithmetic mean (UPGMA) (Smalla *et al.*, 2001).

Nucleotide sequence accession numbers

Amplicon sequences have been submitted to NCBI SRA as study SRA072553 with 16S rRNA gene amplicons under accession numbers SRX259838, SRX259839, SRX259843-SRX259846 and IncP-1 *trfA* gene amplicons under accession numbers SRX262771-SRX262776.

Results

Response of bacterial communities to linuron as revealed by 16S rRNA gene based DGGE analysis and sequencing

DGGE analysis of bacterial and Variovorax communities

To evaluate the effect of linuron on the dynamics of the bacterial community, DGGE analysis of 16S rRNA genes amplified from TC-DNA extracted from the BPS^- and BPS^+ microcosm samples taken after 1, 12 and 25 day(s) of incubation was performed. The DGGE fingerprint (Fig. S1a) showed a high stability of the bacterial community over time with a low variation among replicates. UPGMA analysis based on Pearson correlation indices indicated a high similarity of the bacterial fingerprints among the samples over time (Fig. S1b, similarities varied between 77% and 88%. A temporal change was observed, in the BPS^+ microcosm containing material from the middle and right section of the BPS. In the fingerprints of these replicates one band with a stronger intensity on days 12 and 25 compared to day 1 was detected, and these four samples also formed a separate branch in the UPGMA dendrogram having 64% similarity with the fingerprints from the other samples. This indicates a shift in the bacterial community composition in response to linuron.

The effect of linuron on the dynamics of the *Variovorax* community was evaluated by means of *Variovorax* specific 16S rRNA gene DGGE analysis performed on TC-DNA extracted from the microcosms on days 1, 12 and 25. The *Variovorax* 16S rRNA gene DGGE fingerprints (Fig. S2a) showed a low variation of the *Variovorax* communities between the replicates from the BPS^- and BPS^+ over time and between treatments. A dominant band with an electrophoretic mobility similar to the corresponding 16S rRNA gene amplicon of *Variovorax* sp. WDL1 (*Variovorax* phylotype B) (Bers *et al.*, 2011b) was observed in all the BPS^- and BPS^+. On days 12 and 25 the intensity of a band with the same electrophoretic mobility as the corresponding 16S rRNA gene amplicon of *Variovorax* sp. SRS16 (*Variovorax* phylotype C) (Bers *et al.*, 2011b) became stronger in the BPS^+ microcosm with material from the middle section, indicating a temporal change of the relative abundance of this *Variovorax* phylotype in response to linuron in this replicate. Cluster analysis of the DGGEs based on Pearson correlation showed that the fingerprints of samples taken on days 12 and 25 from the BPS^+ microcosm containing material from the middle section formed a separate group which shared only 70% similarity with the other fingerprints (Fig. S2b).

Quantification of bacterial 16S rRNA gene copies

The addition of linuron did not significantly affect the 16S rRNA gene copy numbers between treatments and at different sampling times, although there was a trend towards

slightly increased copy numbers in the BPS^+ microcosms (Fig. 1). In both BPS^- and BPS^+ microcosms, the bacterial abundance was quite high with 10^9-10^{10} 16S rRNA gene copies per g BPS sample.

Bacterial 16S rRNA gene amplicon pyrosequencing

Altogether, 13,091 amplicon sequences of the 16S rRNA gene V3–V4 region were obtained from BPS^- and BPS^+ TC-DNA from day 25 samples. After a quality check between 1,698 and 2,605 sequences per sample were left with an average length of 353 nucleotides. The main effect of linuron in the BPS^+ was observed in a significant increase in the relative abundance of *Proteobacteria* and a decrease of *Bacteroidetes* (t-test, $p < 0.05$, data not shown). The positive responders in the BPS^+ on day 25 were the *proteobacterial* families *Comamonadaceae*, *Hyphomicrobiaceae*, *Methylophilaceae* and *Sphingomonadaceae*, but also *Microbacteriaceae* and *Trueperaceae* (Table 1). Taxa with a significantly decreased relative abundance in the BPS^+ were also found such as *Bacteroidetes* and *Firmicutes*. Sequences classified to the family *Comamonadaceae* were further analyzed by including the sequence in a neighbor-joining tree with sequences from known *Variovorax* and *Acidovorax* strains (Fig. S4). *Acidovorax* was included because they carry 16S rRNA genes highly similar to *Variovorax* making the two difficult to distinguish based on the sequence of the 16S rRNA gene stretch amplified. One cluster of sequences recognized as positive responders to linuron was identified as *Variovorax* phylotype A or C while another cluster was identified as *Acidovorax*. Linuron degraders are found in phylotype A that contain *Variovorax* sp. SRS16 (Sørensen *et al.*, 2005a), and phylotype B that contain *Variovorax* sp. WDL1 (Dejonghe *et al.*, 2003) and *Variovorax* sp. RA8 (Satsuma, 2010). No linuron degraders have been identified from phylotype C that contain all remaining *Variovorax.* Furthermore, a small cluster of sequences was identified as *Variovorax* phylotype B and another as phylotype C or A, and neither of these responded to linuron. The lowest Chao1 and Pielou's indices, corresponding to richness and evenness, respectively, were observed in BPS^+ microcosms (Fig. 2).

Effects of linuron spiking on IncP-1 plasmid abundance and diversity

Relative abundance of IncP-1 plasmids determined by korB TaqMan qPCR

The effect of linuron on the relative abundance of IncP-1 plasmids in the BPS^- and BPS^+ microcosms was evaluated by TaqMan qPCR using primers targeting the *korB* region of five IncP-1 subgroups (α, β, γ, δ and ε) (Jechalke *et al.*, 2013a). The BPS^- microcosms showed stable *korB* copy numbers per gram BPS^- material over time, with an abundance of *korB* relative to the 16S rRNA gene copy numbers of around 10^{-3} (Fig. 3). However, in the

BPS^+ a significant increase (Tukey´s test, $p < 0.05$) in relative *korB* abundance from 10^{-3} to 10^{-2} was detected after day 1, indicating a strong linuron effect on IncP-1 abundance.

Detection of IncP-1 plasmids by trfA PCR-Southern blot hybridization

Southern blot hybridization of the IncP-1 *trfA* gene PCR products suggested an increase of IncP-1 in BPS^+ microcosms over time. Strong hybridization signals from BPS^+ samples were especially noted on day 25. On day 12, a strong hybridization signal was also detected, but less intense in comparison to day 25 (data not shown). The intensity of the hybridization signal of BPS^- from the three sampling times as well as the BPS^+ at day 1 were equally weak compared to the other BPS^+ samples.

trfA based pyrosequence analysis

The effect of linuron on the diversity of IncP-1 plasmids and their relative abundance was evaluated by amplicon pyrosequencing of a region of the *trfA* gene. After denoising and chimera removal 45,955, 18,821 and 21,536 sequences were obtained from primer sets targeting IncP-1αβε, IncP-1γ and IncP-1δ, respectively. Sequences were assigned to the different IncP-1 subgroups by clustering at 89% nucleotide sequence similarity. 21 *trfA* sequences from known IncP-1 plasmids were included in the clustering as reference for the subgroups α, β-1, β-2, γ, δ, ε and ζ. 18 sequence clusters were identified, and the reference sequence was found in eight of the clusters (Fig. 4a). There was a small and almost negligible overlap in the IncP-1 subgroups targeted by the three primers sets. The largest overlap being the δ primer set also amplifying IncP-1β-1. However, the relative abundances of the *trfA* amplicons are primer specific and thus comparisons can not be made between sequences obtained with the different primer sets. Furthermore, the numbers of sequences are not related to the abundance of the different IncP-1 subgroups in the TC-DNA samples because of the method used for amplicon library construction. IncP-1α was only found in the BPS^- microcosms and composed only 0.01% of the sequences amplified with the αβε primer set. The sequences found in the clusters representing the β-1, β-2, γ, δ, and ε subgroups (Fig. 4a clusters 11, 19, 9, 21 and 18, respectively), and encompassing the large majority of sequences were extracted and re-clustered at 97% nucleotide sequence similarity to identify possible changes in abundance within the subgroups. After re-clustering the sequences from the original clusters split into many new clusters with reference sequences found in several of these (Fig. 4b). For instance, cluster 9 split into clusters 9.1 and 9.3. The 97% sequence similarity was chosen as a conservative estimate to avoid potential sequencing errors not removed by denoising and chimera checking. Pyrosequencing has an error-rate of about 1% (Huse *et al.*, 2007) and the sequences have a size of only 237 bp.

As expected, no sequences were obtained that clustered together with IncP-1ζ (cluster 2), the IncP-1γ plasmids pKS208 (cluster 12) and pMBUI1 (cluster 9.1) and IncP-1β-2 plasmid pB1 (cluster 19.7) as the primer sets do not target these plasmids. For β-1, the majority of amplicon sequences were found in cluster 11.101 together with most of the β-1 reference sequences. No sequences clustered together with pB136 (cluster 11.25, Fig. 4b). For β-2, almost all amplicon sequences were found in cluster 19.51 together with the β-2 reference sequences. The IncP-1ε sequences split into two major clusters with the majority of amplicon sequences clustering with pKJK5 in 18.64 and with pEMT3 in 18.74. Two small IncP-1ε clusters (18.49, 18.87) mainly with sequences from BPS^- samples were also found. These were most similar to pEMT3. All sequences identified in the γ subgroup clustered with pQKH54 (cluster 9.3) as expected since it is the only known IncP-1γ plasmid targeted by the primer set. A list of all reference plasmids used and the clusters is given in Table S1. The representative sequences from all clusters in Fig. 4b differed after translation into amino acids. The spiking of linuron to the BPS material resulted in significant changes in the abundance of the different IncP-1 subgroups with an increase of β-1 and a decrease of β-2 and ε subgroups (β-2 not significant, $p > 0.05$).

Exogenous plasmid isolation and characterization

In an attempt to capture plasmids conferring the ability to utilize linuron as carbon source, biparental exogenous isolation of plasmids into *Pseudomonas putida* KT2442 *gfp* was applied using minimal media containing linuron (20 μg mL^{-1}). However, no transconjugants were obtained. For that reason, an alternative approach to capture plasmids potentially involved in linuron degradation by biparental exogenous isolation was tried with $HgCl_2$ (20 μg mL^{-1}) as selective marker and once again using *Pseudomonas putida* KT2442 *gfp* as recipient cell. The frequency of plasmid transfer was about two orders of magnitude higher in BPS^- (10^{-6}) compared to the BPS^+ microcosms (10^{-8}). 6.2×10^{-6}, 3.8×10^{-5}, 5.0×10^{-6} in BPS^- (middle, right and left) and 5.9×10^{-8}, 2.2×10^{-8} in BPS^+ (right and left, no transconjugants were obtained from material of the middle section). The number of recipient cells in all BPS^- and BPS^+ replicates was quite high ranging between 10^9 to 10^{10} cells per gram. A total of 50 transconjugants (25 from both BPS^- and BPS^+) were picked, and out of them, 18 and 24 from BPS^- and BPS^+ respectively, grew on a fresh Mueller-Hinton (MH) agar plate (Merck KGaA, Darmstadt, Germany) containing $HgCl_2$ (20 μg mL^{-1}). Specific PCR amplifications for IncP-1, IncP-7 and IncP-9 plasmids indicated that IncP-9 plasmids were captured in all transconjugants from BPS^+ and BPS^- while IncP-1 plasmids were mainly found in the transconjugants from BPS^-. Only unspecific bands with the IncP-7 primers were observed (data not shown). In order to verify the PCR results, Southern blot hybridizations were applied to the digested plasmid DNA from each transconjugant by using IncP-1 mixed

probe, IncP-1β probe, IncP-1ε probe and IncP-9 mixed probe. Of the 42 transconjugants analyzed, 15 from BPS^- microcosms and six from BPS^+ carried IncP-1 plasmids. Southern blot hybridization using IncP-1β and IncP-1ε specific probes showed that all exogenously isolated IncP-1 plasmids belonged to the IncP-1β subgroup. Positive hybridization signals with the IncP-9 mixed probe were observed by Southern blot hybridization of BstI/PstI digested plasmids for all 42 transconjugants confirming that all transconjugants contained IncP-9 plasmids (data not shown).

To acquire a better understanding of the diversity of the IncP-1 and IncP-9 plasmids in the transconjugants, their plasmid DNA restriction patterns were compared. Five different patterns were generated (Fig. S3). While the plasmid DNA of transconjugants from BPS^- microcosms displayed five different restriction patterns only one pattern was observed in the transconjugants from BPS^+, indicating a higher diversity of IncP-1 plasmids in BPS^- samples. One restriction pattern type which was observed for all six IncP-1 containing transconjugants from the BPS^+ was also displayed by one of the IncP-1 positive transconjugants from BPS^-. Eighteen of the 24 transconjugants from BPS^+ carried only IncP-9 plasmids and three different restriction patterns were observed. Among the transconjugants from BPS^- nine carried only IncP-9 plasmids which displayed identical restriction patterns.

Discussion

To study the complex response of BPS bacterial communities and their mobilome to the phenylurea herbicide linuron, composite samples from the left, middle and right section of an on-farm BPS in Kortrijk were either spiked with linuron (BPS$^+$) or not (BPS$^-$). The BPS$^+$ and BPS$^-$ samples were incubated under identical conditions and sampled on days 1, 12 and 25 after spiking, making it possible to elucidate the effects of linuron on the bacterial community composition and the abundance of plasmids. Several previous studies pointed to an important role of IncP-1 plasmids in the adaptation of bacterial communities to new anthropogenic compounds released into the environment such as pesticides (Sen *et al.*, 2011, Król *et al.*, 2012). These plasmids are often carrying genes encoding catabolic functions and antibiotic resistances (Trefault *et al.*, 2004, Schlüter *et al.*, 2007) and are able to replicate in a broad range of hosts (Musovic *et al.*, 2006). Furthermore, they efficiently transfer in soils when sufficient nutrients are available (Pukall *et al.*, 1996, Götz & Smalla, 1997, Mølbak *et al.*, 2007). The results of the study by Dunon *et al.* (2013) and by Dealtry *et al.* (chapter II) showed an unusually high abundance of IncP-1 plasmids in BPS indicating that BPS seemed to be hot spots of bacterial populations carrying IncP-1 plasmids. Previously, IncP-1 plasmids were reported to occur in several environments such as contaminated soils, manure, sewage, mangrove and aquatic environments (Smalla *et al.*, 2006, Malik *et al.*, 2008, Gomes *et al.*, 2010, Oliveira *et al.*, 2012) and often a correlation of plasmid abundance and pollution was assumed but the experimental design was often not suitable to demonstrate this correlation. In the present study, pre-adapted bacterial communities of BPS material were exposed to rather high concentrations of linuron.

Both, Southern blot hybridization of *trfA* gene fragments amplified from TC-DNA and quantification of IncP-1 plasmids by means of the recently published *korB* qPCR system (Jechalke *et al.*, 2013a) showed that IncP-1 plasmids significantly increased in BPS$^+$ material compared to the BPS$^-$ material providing proof for the assumed correlation of IncP-1 plasmid abundance and pollution. This further validates what Dunon *et al.* (2013) have observed by hybridization with IS*1071* and IncP-1 plasmids. Interestingly, IncP-1 *korB* qPCR data and an increased abundance of *Variovorax* 16S rRNA genes on BPS$^+$, visualized by both DGGE and amplicon pyrosequencing (Fig. S2a, S4), provided vague indications that it might be possible that IncP-1 plasmids might be hosted by *Variovorax* species.

However, our attempts failed to exogenously capture IncP-1 plasmids with respective catabolic genes into *Pseudomonas putida* recipient strains by selecting transconjugants on minimal media containing linuron as sole carbon source. We speculate that likely linuron degradative genes were not expressed in the *Pseudomonas putida* background. Therefore, based on the knowledge that IncP-1 catabolic plasmids often carry mercury resistance genes

(Smalla *et al.*, 2006, Sen *et al.*, 2011), we tried to capture IncP-1 plasmids by selecting transconjugants on MH medium containing $HgCl_2$. Contrary to our expectations higher numbers of transconjugants were obtained from the BPS^- compared to BPS^+. Obviously the abundance of populations carrying IncP-1 plasmids conferring mercury resistance decreased in response to linuron while populations carrying IncP-1 plasmids with genes potentially involved in the degradation of linuron or its metabolites seemed not to confer mercury resistance. Although all IncP-1 plasmids captured from BPS^+ and BPS^- microcosms belonged to IncP-1β plasmid subgroup, based on the diversity of restriction patterns, a higher diversity of IncP-1β plasmids was captured from BPS^-. Five types of restriction patterns of IncP-1β plasmids were isolated from BPS^-, while only one restriction pattern of IncP-1β plasmids was captured from the BPS^+, indicating the influence of linuron on the composition of IncP-1 plasmids. Changes in the abundance of IncP-1 subgroups were also indicated by amplicon pyrosequencing of *trfA* genes amplified with three different primer systems. Analysis of the *trfA* gene amplicons from TC-DNA of BPS^- and BPS^+ taken on day 25 confirmed on the one hand, the high diversity of IncP-1 plasmids present, and on the other hand the differences in the abundance of different IncP-1 plasmids subgroups (Fig. 4a, b). As the three different primer systems described by Bahl *et al.* (2009) were used for amplicon sequencing, a comparison of the abundance was only possible for IncP-1α, IncP-1β and IncP-1ε as these amplicons were obtained with the same primer system. An impressive diversity based on IncP-1 *trfA* sequences was revealed and all subgroups targeted were detected though in different relative abundances. Although it is not well understood whether the different subgroups have evolved in different host backgrounds and likely have less burden in these hosts due to co-evolution, the presence of different IncP-1 subgroups in pre-adapted biofilter material is impressive. Considering the broad host range of IncP-1 plasmids, we suggest that a considerable proportion of degradative genes might be shared among BPS bacteria. Shifts in the abundance of the IncP-1β and IncP-1ε subgroups were observed in response to the linuron treatment. A significant higher number of sequences sharing high similarity (97%) to IncP-1β-1 plasmids were observed in the BPS^+. Several catabolic plasmids such as pADP-1, pUO1, pJP4 (Martinez *et al.*, 2001, Sota *et al.*, 2003, Ledger *et al.*, 2006) but also the antibiotic resistance plasmids R751 and pB10 and mercury resistance plasmid pTP6 belong to this group. In contrast, sequences with similarity to IncP-1β-2 plasmid such as pA81, pNB8c (Król *et al.*, 2012) and pPC1-1 were detected in a higher abundance in the BPS^-. Most remarkable was the highly significant decrease in abundance of plasmids with similarity to IncP-1ε. Populations carrying plasmids belonging to the IncP-1ε group with high similarity to pKJK5 and pEMT3 decreased in abundance as they were likely affected and the IncP-1ε plasmid did not provide a selective advantage in the presence of linuron. Sequences with high similarity to the IncP-1γ and IncP-1δ reference plasmids

pQKH54 and pEST4011, respectively, were detected in the BPS$^-$ and BPS$^+$ without variation in the relative abundance in response to linuron. The very low abundance of IncP-1α *trfA* sequences was surprising as IncP-1α plasmids were mainly reported in manure (Smalla *et al.*, 2000a). However, manure was previously added as nutrient source to BPS.

To better understand the response of the bacterial communities to the linuron spike, and to predict changes in the relative abundance of potential hosts of IncP-1 plasmid in the BPS$^-$ and BPS$^+$, 16S rRNA gene based analysis was performed. The 16S rRNA gene based DGGE fingerprints indicated a minor change of the dominant bacteria in response to linuron in the microcosms with material from the middle and right section of the BPS. This change was seen by the appearance of one strong band on day 12 and 25 and contradicts earlier studies which did not show effects of single or multiple applications of linuron on soil bacterial community structure in soil microcosms and in an agricultural field (Bers *et al.*, 2011a, 2013) and microcosms with a simulated BPS matrix (Sniegowski *et al.*, 2011a, 2011b). The minor change in the bacterial communities observed by the DGGE might be a result of the higher linuron concentration used in the present study. *Variovorax* was previously described to be involved in linuron degradation (Field *et al.*, 1997, Dejonghe *et al.*, 2003, Breugelmans *et al.*, 2007, Bers *et al.*, 2011a, 2011b, 2012, Sniegowski *et al.*, 2011a) and therefore, a *Variovorax* specific DGGE fingerprint described by Bers *et al.* (2011b) was employed to compare the response of *Variovorax* populations to linuron over the time. The constant presence of *Variovorax* phylotype B in all replicates is in line with previous observations of *Variovorax* diversity in microcosms and soil (Bers *et al.*, 2011b, Sniegowski *et al.*, 2011a, 2011b, Bers *et al.*, 2013) but the remaining phylotype observations are not. Where an increase in abundance of phylotype C in BPS$^+$ with material from the middle section is observed, previous studies have either observed a constant presence of phylotype C irrespectively of linuron treatment or a selection against it by the addition of linuron. Furthermore, the other studies found a similar abundance of phylotype A and B while the BPS$^+$ microcosms were only dominated by B. A slight increase in the abundance of phylotype A in BPS$^+$ observed is in accordance with the previous observations. The lower diversity of *Variovorax* phylotypes observed in comparison with field soil suggested that the *Variovorax* community was adapted to the conditions in the BPS. Furthermore, the positive selection for phylotype C has not been observed before and amplicon pyrosequencing was employed to, among other things, shed light on the matter.

In contrast to the DGGE analysis, pyrosequenced 16S rRNA gene amplicons revealed large shifts in bacterial communities in response to linuron spiking. This finding indicates the limitations of the resolution level of DGGE used in the present study and in previous work (Sekiguchi *et al.*, 2001, Kisand & Wikner, 2003). Overall, the richness and

evenness estimates (Fig. 2) showed that even though the total copy number of 16S rRNA genes in the BPS^+ did not change in relation to the BPS^- (Fig. 1), the addition of linuron resulted in a significant change (T-test, $p < 0.05$) in the community composition. The total number of operational taxonomic units (OTUs) decreased in the BPS^+ (drop in richness) while a few taxa became much more dominant (drop in evenness).

A more thorough analysis showed that in response to linuron spiking the highest increase in the relative abundance was found for the families *Hyphomicrobiaceae* and *Comamonadaceae* (Table 1). *Variovorax* belong to the *Comamonadaceae* but also *Hydrogenophaga* where a strain has been shown to degrade linuron (Breugelmans *et al.*, 2007). Several bacterial groups involved in linuron degradation have been identified in enrichment cultures by means of cultivation-dependent and -independent methods. These include different *Variovorax* species (Dejonghe *et al.*, 2003, Sørensen *et al.*, 2005a, 2005b, Breugelmans *et al.*, 2007, Satsuma, 2010), but also *Comamonas* spp. and *Hyphomicrobium* spp. WDL6 (Dejonghe *et al.*, 2003). Of the different bacterial species involved in linuron degradation, *Variovorax* species such as *Variovorax* sp. WDL1 (Dejonghe *et al.*, 2003), *Variovorax* sp. SRS16 (Sørensen *et al.*, 2005a), *Variovorax* sp. PBL-H6 (Breugelmans *et al.*, 2007) and *Variovorax* sp. RA8 (Satsuma, 2010) have received major attention as these bacteria initiate linuron degradation. SRS16 belong to *Variovorax* phylotype A while the others are phylotype B. This is interesting as one of the largest positive responders in the BPS^+ was an OTU identified as *Variovorax* phylotype A or C. It is highly possible that this is the same *Variovorax* phylotype C observed in the *Variovorax* specific DGGE (Fig. S2a) as the sequenced stretch of the 16S rRNA gene cannot clearly differentiate between phylotypes A and C. As previously mentioned, a positive selection by linuron for *Variovorax* phylotype C has not been observed in other studies and the sequence result adds further evidence that an adaptation of bacteria in BPS material to linuron degradation has taken place. For the OTU identified as phylotype B it is quite doubtful whether this is the same observed in the *Variovorax* specific DGGE as this OTU is less abundant than the other *Variovorax* OTUs identified to belong to other phylotypes and hence contradicts the DGGE results. In contrast to *Variovorax*, the other species involved in linuron degradation are mainly involved in the degradation of metabolites such as 3,4-dichloroaniline (DCA) and *N,O*-dimethylhydroxylamine (*N,O*-DMHA) originating from initial linuron hydrolysis by forming consortia with *Variovorax* (Breugelmans *et al.*, 2007). For instance, *Hyphomicrobium* spp. in linuron degrading enrichment cultures originating from linuron treated soil have been shown to degrade *N,O*-DMHA (Dejonghe *et al.*, 2003, Breugelmans *et al.*, 2007), a by-product of the initial hydrolysis of linuron performed by *Variovorax* (Engelhardt *et al.*, 1972). The increased abundance of *Hyphomicrobiaceae* to linuron application suggests that those organisms might also feed on *N,O*-DMHA originating from linuron hydrolysis in BPS and combined with the

constant presence of *Variovorax* phylotype B it appears that a linuron foodweb exists in the BPS that is similar to that observed in enrichment cultures with *Variovorax* sp. WDL1 (Dejonghe *et al.*, 2003). The increase in abundance of *Microbacteriaceae* (*Actinobacteria*), Gp16 (*Acidobacteria*) and *Truepera* (*Deinococcus-Thermus*) after linuron spiking has to our knowledge not been observed before. *Actinobacteria* and *Acidobacteria* are common and abundant phyla in both agricultural and contaminated soil (Holmsgaard *et al.*, 2011, Berg *et al.*, 2012, Li *et al.*, 2012) while *Deinococcus-Thermus* is rare. The responders from these three phyla make up around 1 % of the bacterial community and at the moment cultivation is not an easy approach. To date only one strain type of *Trueperaceae* has been described. This strain is radiation resistant and originates from a hot spring (Albuquerque *et al.*, 2005). No type strain exists for Gp16. Therefore we can only speculate on their involvement in linuron degradation if any. Although the stretch of the 16S rRNA gene sequenced does not make species characterization possible and only allows limited characterization of genera, our findings confirmed many of the previously identified degradative isolates from linuron contaminated soils, previously obtained by classical methods for isolation of bacteria (Breugelmans *et al.*, 2007).

The selection for *Variovorax* phylotype C in BPS^+ microcosms which contradicts previous findings might be the result of horizontal gene transfer between *Variovorax* phylotypes. The abundance of the linuron hydrolase gene *libA* has been shown to correlate with *Variovorax* abundance in linuron degrading communities, and it has been speculated that *libA* might be located on a transmissible element (Bers *et al.*, 2012, Bers *et al.*, 2013). Although we failed to isolate plasmids carrying genes for linuron degradation the possibility cannot be ruled out. DGGE, qPCR and amplicon pyrosequencing was only targeted at IncP-1 plasmids, and even though a significant increase in IncP-1β plasmids was observed, other mobilome elements could be involved.

In conclusion, it was demonstrated that IncP-1 plasmids might be important players involved in linuron degradation in BPS and their diversity is fluctuating when soils are spiked with linuron. Besides the known *Variovorax* and *Hyphomicrobium* responders to linuron, novel potential bacterial populations responding to linuron were indicated: taxa affiliated to *Methylophilaceae, Microbacteriaceae, Sphingomonadaceae* and *Trueperaceae*. A *Variovorax* phylotype previously not detected as a main linuron degrader was also observed. The present study contributes to a better understanding of bacteria probably involved in pesticide degradation and demonstrates that linuron degradation foodwebs previously observed in enrichment culture seem to play a role in linuron degradation in BPS material.

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Tables and Figures

Table 1. Relative abundance of *Bacteria* responding to spiking linuron analyzed by pyrosequencing of 16S rRNA gene amplicons from BPS^- and BPS^+ on day 25.

Phylum	Class	Order	Family	Genus	BPS^-	BPS^+
Proteobacteria (+)	*Alphaproteobacteria* (+)	*Rhizobiales* (+)	*Hyphomicrobiaceae* (+)	*Hyphomicrobium* (+)	1.7±0	4.1±1
		Sphingomonadales (+)	*Sphingomonadaceae* (+)		0.3±0	0.8±0
	Betaproteobacteria (+)	*Burkholderiales* (+)	*Comamonadaceae* (+)		0.4±0	3.4±1
		Methylophilales (+)	*Methylophilaceae* (+)		0±0	0.7±1
Bacteroidetes (-)	*Sphingobacteria* (-)	*Sphingobacteriales* (-)	*Cytophagaceae* (-)	*Adhaeribacter* (-)	2.1±0	0.8±0
				Pontibacter (-)	0.9±1	0.1±0
Actinobacteria (-)	*Actinobacteria* (-)	*Actinomycetales* (+)	*Microbacteriaceae* (+)		0.6±0	1.5±0
Firmicutes (-)	*Bacilli* (-)	*Bacillales* (-)	*Bacillaceae* (-)		9.6±0	7.6±1
	Clostridia (-)				3.8±0	2.6±1
Acidobacteria (+)	*Acidobacteria_Gp16* (+)			*Gp16* (+)	1.1±1	1.9±0
	Acidobacteria_Gp6 (-)				1.2±0	0.6±0
Deinococcus - Thermus (+)	*Deinococci* (+)	*Deinococcales* (+)	*Trueperaceae* (+)	*Truepera* (+)	0.3±0	0.9±0
Gemmatimonadetes (-)	*Gemmatimonadetes* (-)	*Gemmatimonadales* (-)	*Gemmatimonadaceae* (-)	*Gemmatimonas* (-)	1.6±0	0.8±0

Note: (+): taxa with significantly higher relative abundance in the BPS^+ samples; (-): taxa with significantly lower relative abundance in the BPS^+ samples

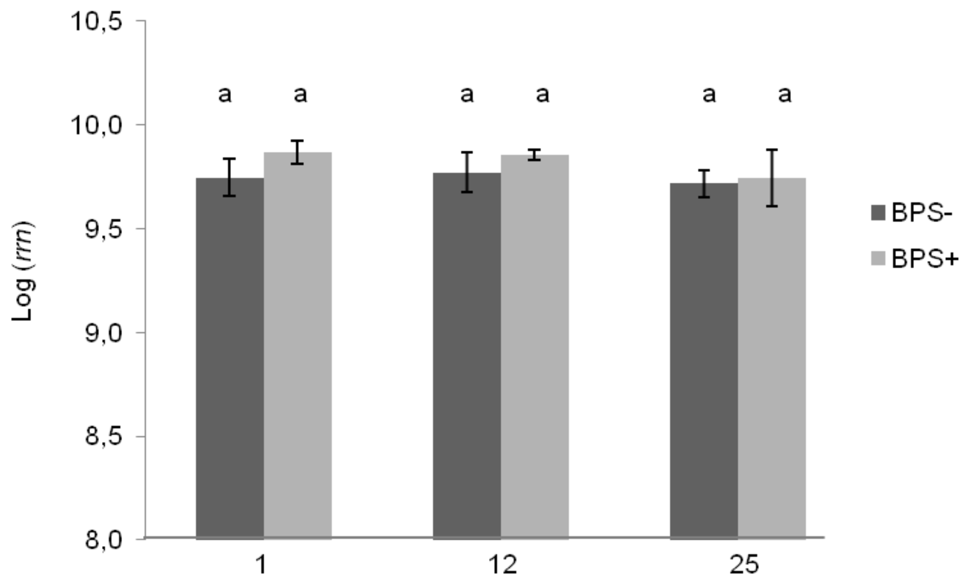


Fig. 1. Quantification of bacterial 16S rRNA gene copy numbers (*rrn*) in the BPS material untreated with linuron (BPS^-) or treated with linuron (BPS^+) sampled on days 1, 12 and 25. Different letters indicate significant differences (Tukey test). Error bars are standard deviation (n=3).

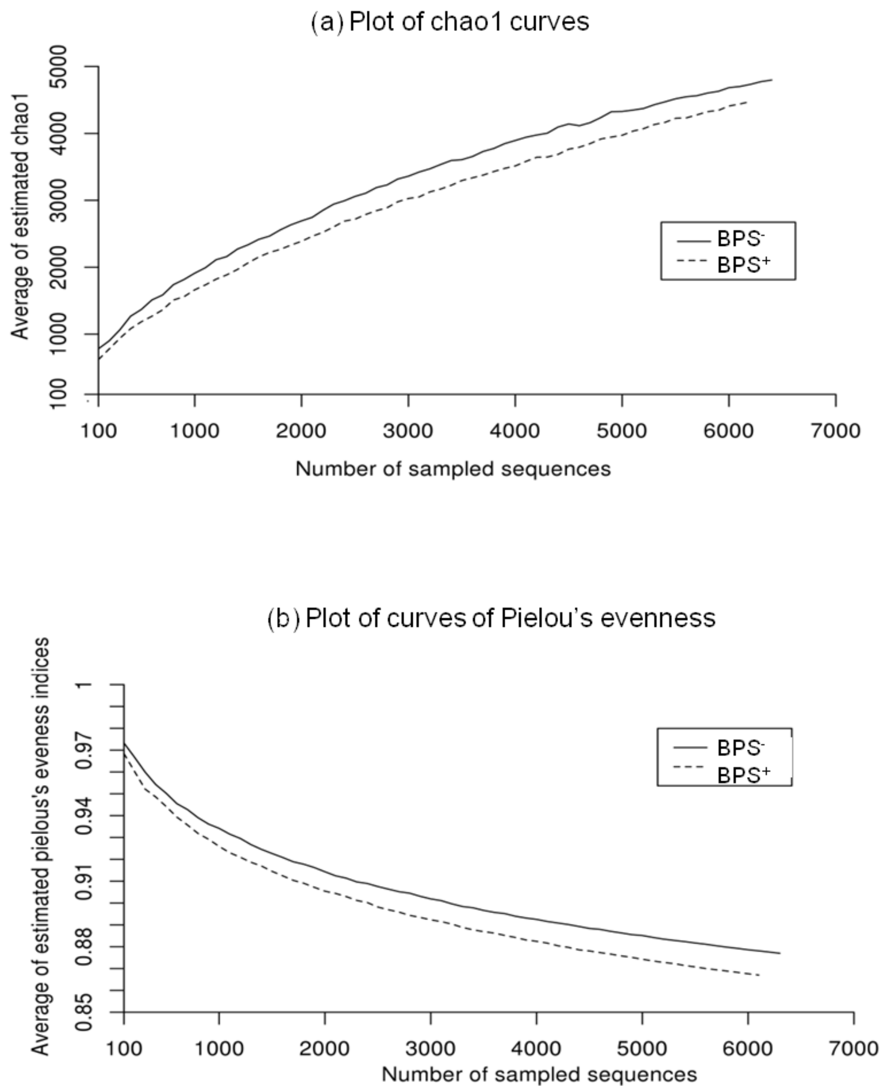


Fig. 2. Detection of (a) bacterial richness (chao1) and (b) Pielou's evenness in the BPS^- (control) and BPS^+ (linuron spiked). Average diversity indices were calculated by calculation of the index from a randomly sampled data set 100 times.

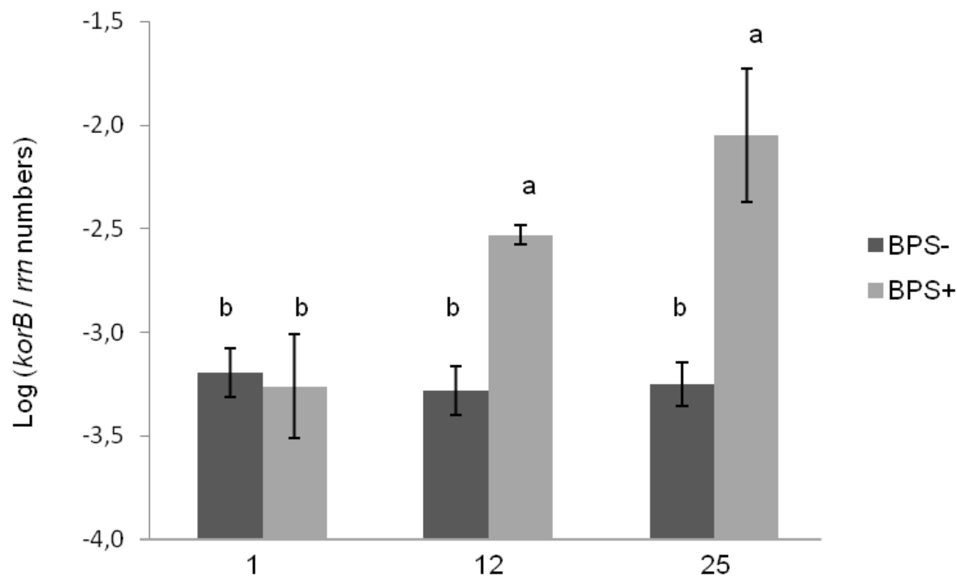
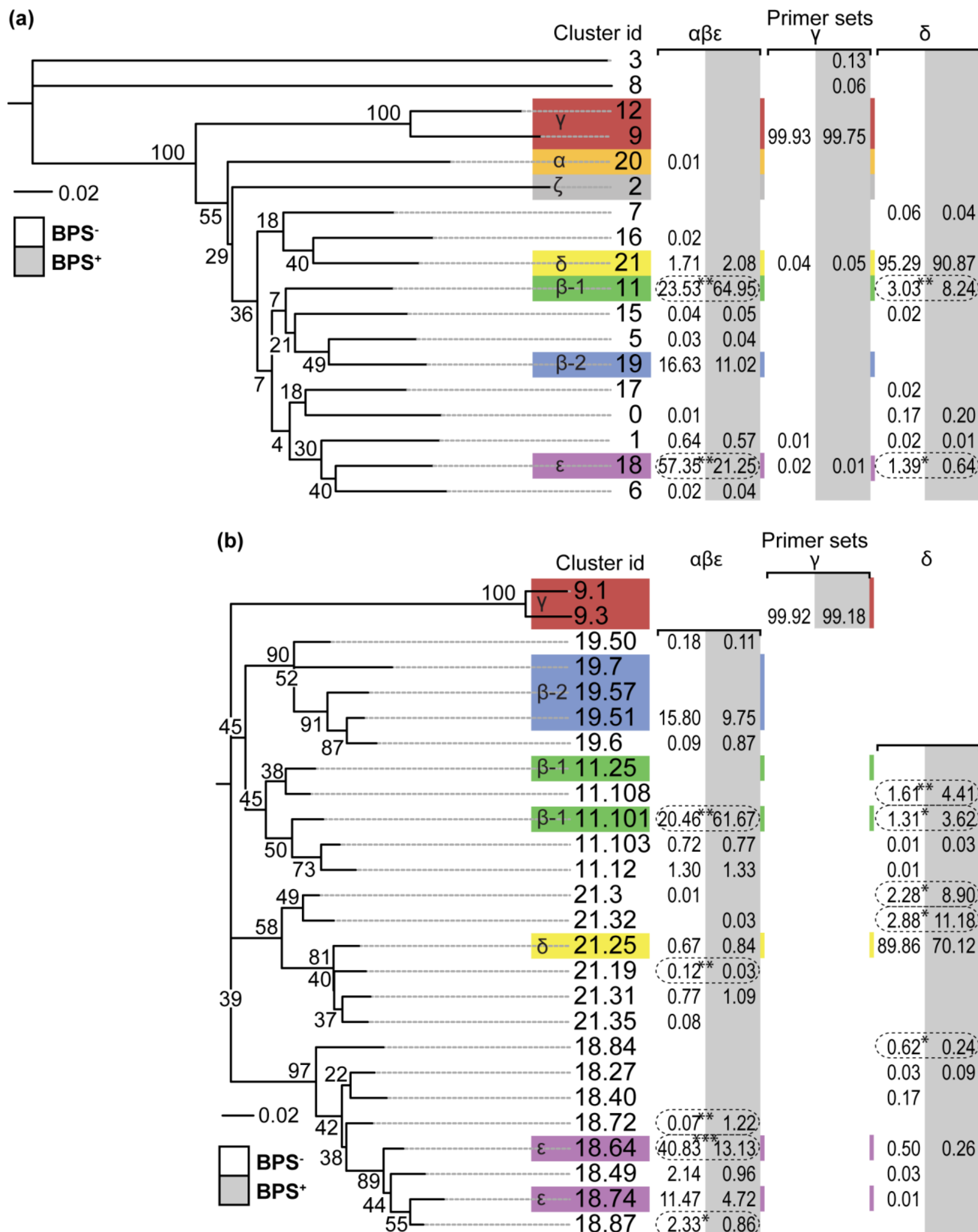


Fig. 3. Relative abundance of *korB* from IncP-1 plasmids in the BPS material untreated with linuron (BPS^-) or treated with linuron (BPS^+) sampled at days 1, 12 and 25. *korB* copy numbers were related to bacterial 16S rRNA gene copy numbers (*rrn*) quantified from the same sample. Different letters indicate significant differences (Tukey test). Error bars are standard deviation (n=3).

Fig. 4 Neighbor-joining trees of clusters of IncP-1 *trfA* amplicons sequences from BPS$^-$ and BPS$^+$ sampled on day 25. (a) Each branch represents one cluster of sequences with 89% sequences similarity. (b) Sequences from clusters 9, 11, 18, 19 and 21 in (a) re-clustered at 97% sequence similarity. Small clusters are not included except if they contain reference sequences. In both Figures known reference sequences were included in the clustering and their locations and subgroup names are indicated by colored boxes. The Tables show the mean relative abundance of sequences from each primer set. There is an overlap in targets between primer sets. Dotted circles indicate significant changes in abundance between BPS$^-$ and BPS$^+$ (Student's t-test, n = 3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). Numbers on branches are bootstrap-values in percent of 1,000 bootstraps.



Supplement Material

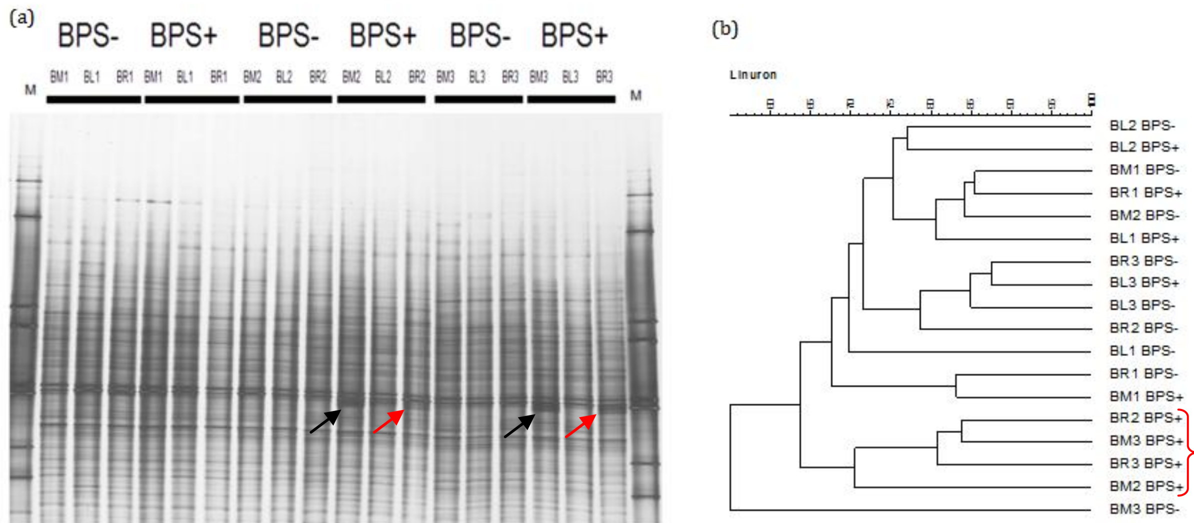


Fig. S1. DGGE fingerprint (a) *Bacteria* 16S rRNA gene fragments of BPS^{-} and BPS^{+} TC-DNAs on days 1, 12 and 25. Lanes marked by *M* are indicating the *Bacteria* 16S rRNA gene DGGE marker. Three replicates of BPS^{-} and BPS^{+} from each side on days 1, 12 and 25 are indicated by *BM1*, *BL1* and *BR1*; *BM2*, *BL2* and *BR2* and *BM3*, *BL3* and *BR3*, respectively. Arrows are indicating the extra band in BPS^{+} on days 12 and 25. The corresponding UPGMA dendrogram (b) based on Pearson´s correlations indices.

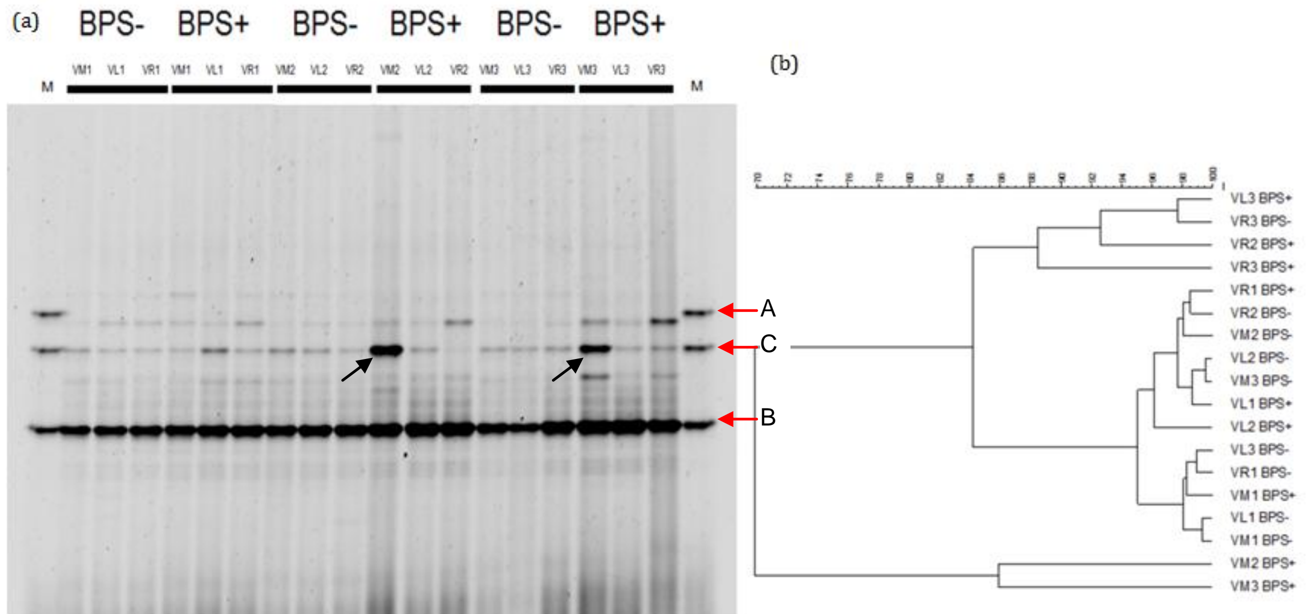


Fig. S2. DGGE fingerprint (a) *Variovorax*16S rRNA gene fragments of BPS$^-$ and BPS$^+$ TC-DNAs on days 1, 12 and 25. Lanes marked by *M* indicate the *Variovorax* 16S rRNA gene DGGE marker (Bers *et al.*, 2011b). From top to bottom markers are phylotype A (includes *Variovorax* spp. SRS16), C (includes *Variovorax* spp. DSM666) and B (includes *Variovorax* spp. WDL1). Three replicates of BPS$^-$ and BPS$^+$ from each side on days 1, 12 and 25 are indicated by *VM1*, *VL1* and *VR1*; *VM2*, *VL2* and *VR2* and *VM3*, *VL3* and *VR3*, respectively. Arrows are indicating the extra band in BPS$^+$ on days 12 and 25. The corresponding UPGMA dendrogram (b) based on Pearson´s correlations indices.

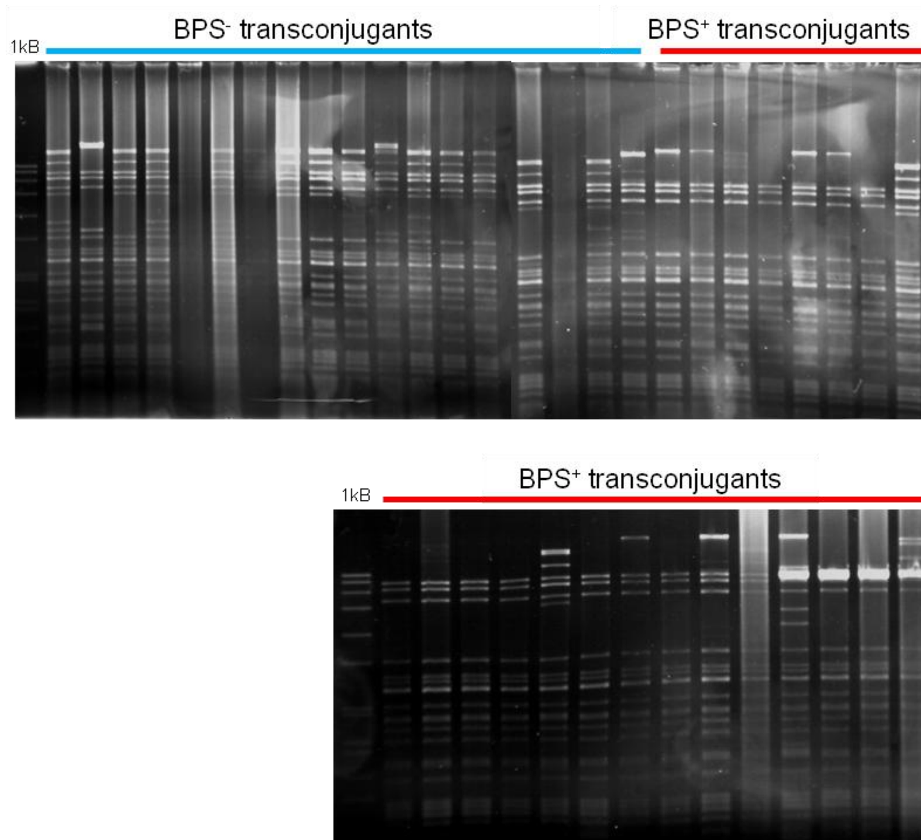


Fig. S3. Restriction patterns (Pst1 and BstZ17I enzymes) of plasmids exogenously isolated in *Pseudomonas putida* KT2442 from BPS^{-} and BPS^{+}.

Fig. S4.

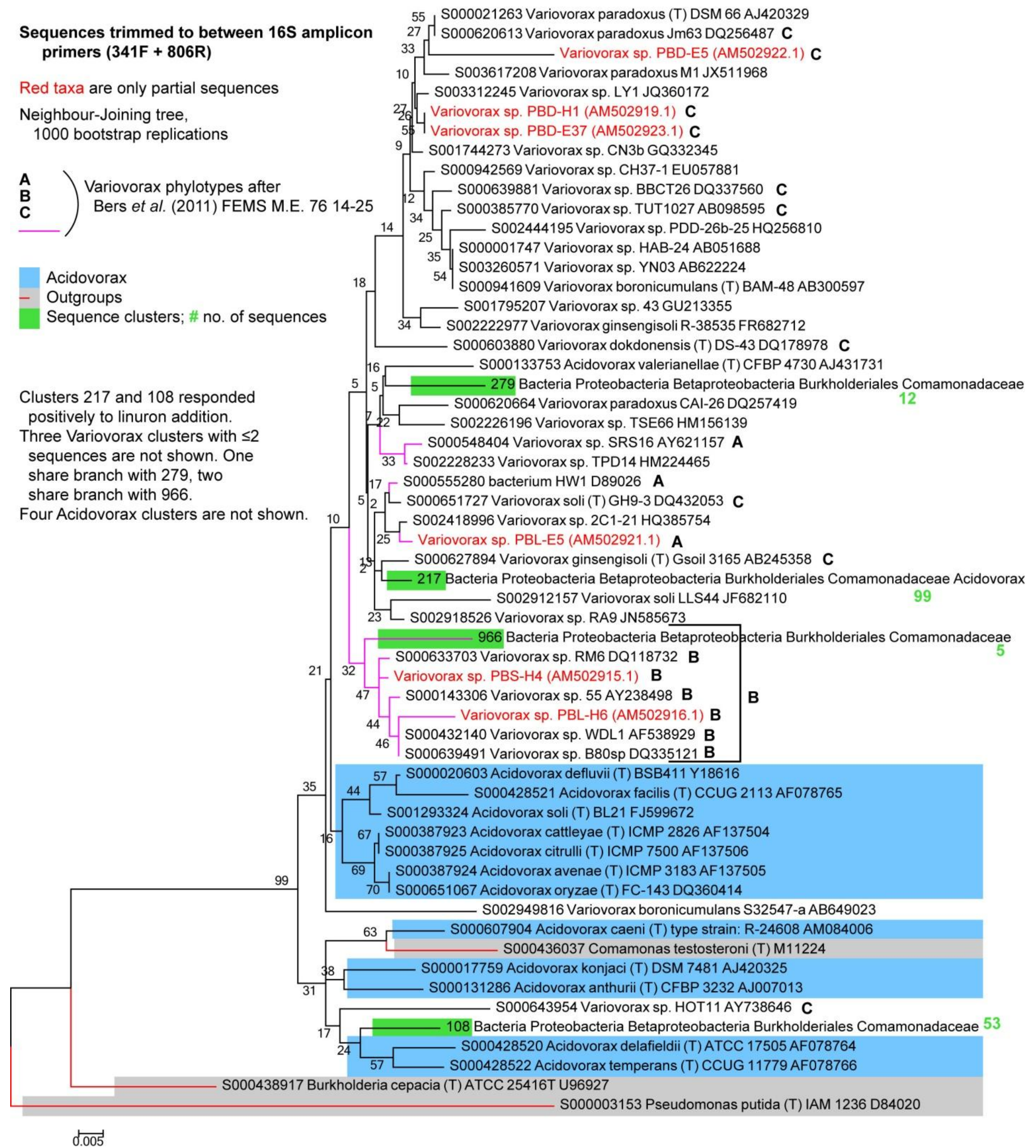


Table S1. IncP-1 plasmids whose *trfA* sequences were used as reference sequences and cluster numbers they were located in.

Subgroup	Cluster no.		Reference plasmids	Amplicon sequences[a]
	Fig. 4a	**Fig. 4b**		
Alpha	20	n/a	RK2[b]	Y
Beta-1	11	11.101	pB3, pUO1[c]	Y
	11	11.25	pB136	N
Beta-2	19	19.7	pB1	N
	19	19.57	pAOVO02	N
	19	19.51	pA1, pA81, pB12, pB4, pCNB[d], pRSB222, pYS1	Y
Gamma	12	n/a	pKS208	N
	9	9.1	pMBUI1	N
	9	9.3	pQKH54	Y
Delta	21	21.25	pAKD4, pEST4011[e]	Y
Epsilon	18	18.64	pKJK5[f]	Y
	18	18.74	pEMT3[g]	Y
Zeta	2	n/a	pMCBF1[h]	N

[a] Indicates with yes (Y) or no (N) if amplicon sequences clustered with the reference plasmid.

[b] The sequenced region of the *trfA* gene had 100% nucleotide sequence identity with IncP-1α plasmids pB11, pB5, pBS228, pG527, pSP21, pTB11 and pWEC911.

[c] The sequenced region of the *trfA* gene had 100% nucleotide sequence identity with IncP-1β-1 plasmids pADP-1, pAKD1, pAKD14, pAKD15, pAKD17, pAKD18, pAKD29, pAKD31, pAKD33, pALIDE02, pAMMD-1, pB10, pB8, pDS3, pIJB1, pJMP134-1, pJP4, pKS212, pKV29, pTP6, pTSA, pUO1 and R751.

[d] The sequenced region of the *trfA* gene had 100% nucleotide sequence identity with IncP-1β-2 plasmids pAKD26, pC1-1, pLME1, pNB8c, pRSB223 and pTB30.

[e] The sequenced region of the *trfA* gene had 100% nucleotide sequence identity with IncP-1δ plasmid pIJB1.

[f] The sequenced region of the *trfA* gene had 100% nucleotide sequence identity with IncP-1ε plasmids pHH128, pHH3408, pHH3414, pKS77.

[g] The sequenced region of the *trfA* gene had 100% nucleotide sequence identity with IncP-1ε plasmids pAKD16, pAKD25, pAKD34.

[h] The sequenced region of the *trfA* gene had 100% nucleotide sequence identity with IncP-1ζ plasmid pMCBF6.

Chapter IV: Shifts in abundance and diversity of mobile genetic elements to diverse pesticides introduced into an on-farm biopurification system over a year

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Abstract

Biopurification systems (BPS) are used on farms as pollution control technique to treat pesticide contaminated water. It is assumed that mobile genetic elements (MGEs) carrying degradative genes might contribute to the degradation of pesticides. Therefore, the composition and shifts of MGEs in particular of IncP-1 plasmids carried BPS bacterial communities exposed to various different pesticides were monitored over an agricultural season. PCR amplification of total community DNA using primers targeting the replication genes of different plasmid groups combined with Southern blot hybridization indicated a high abundance of plasmids belonging to IncP-1, IncP-7, IncP-9, IncQ and IncW while IncU and IncN plasmids were less or not detected. Furthermore, the integrase genes of class 1 and 2 integrons (*intI1*, *intI2*), genes endoding resistance to sulfonamides (*sul1*, *sul2*) and streptomycin (*aadA*) were detected and seasonality was revealed. Amplicon pyrosequencing of the IncP-1 *trfA* gene revealed a high IncP-1 plasmid diversity and an increase in abundance of IncP-1β and decrease of IncP-1ε over the time. The data of the chemical analysis showed increasing concentration of various pesticides over the agricultural season. The parallel increased relative abundance of bacteria carrying IncP-1β plasmids might point to their role in the degradation of many different pesticides.

Introduction

Biological biopurification systems (BPS) have often been applied on farms to control the environmental pollution caused by the release of water contaminated with pesticides (Omirou *et al.*, 2012). BPS are composed of a mixture of different adsorbing materials such as peat, straw and topsoil, and contain microorganisms carrying mobile genetic elements (MGEs) that harbor genes involved in biodegradation of xenobiotic compounds (De Wilde *et al.*, 2009). Although it is well known that biodegradation is the main process in the removal of xenobiotic compounds from the environment (Bers *et al.*, 2012), abiotic factors such as temperature and concentration of pesticides can substantially influence their degradation (Castillo & Torstensson, 2007). Accordingly, these abiotic factors might also influence the diversity and abundance of MGEs carrying genes coding for catabolic enzymes.

MGEs such as plasmids, phages, genomic islands and integrons are often associated with genes involved in the degradation of xenobiotic compounds and are responsible for the transfer of catabolic genes within bacterial communities (Heuer & Smalla, 2012). Plasmids belonging to the incompatibility groups IncP-1, IncN, IncQ, and IncW have a broad host range and therefore are of particular interest when studying antibiotic resistance genes or catabolic genes in the environment (Dennis, 2005, Fernandez-Lopez *et al.*, 2006, Revilla *et al.*, 2008, Shintani *et al.*, 2010). Specifically, IncP-1 plasmids are highly promiscuous plasmids and were reported to carry genes coding for enzymes involved in degradation of different xenobiotic compounds (Top *et al.*, 1995, Trefault *et al.*, 2004, Sen *et al.*, 2011). Correspondingly, it has been observed that prolonged exposure to pesticides enrich for bacterial populations carrying IncP-1 plasmids (Anjum *et al.*, 2011, Jechalke *et al.*, 2013a, Dealtry *et al.* chapter III).

In a PCR-Southern blot based screening of total community DNA from various environments and different geographic locations for the occurrence of potentially catabolic plasmids belonging to the IncP-1, IncP-7 and IncP-9 group BPS material was revealed as a "hot spot" for these plasmids (Dealtry *et al.*, chapter II). In accordance with previous findings (Jechalke *et al.*, 2013a), we hypothesize that temporal changes in the abundance and diversity of MGEs in an on-farm BPS might be caused by changes in the composition and concentration of the pesticides added to the BPS over the agricultural season. To test our hypothesis and to provide the first more comprehensive view on the mobilome of BPS bacterial communities different cultivation-independent techniques were applied to investigate shifts in the abundance and diversity of the MGEs of bacterial communities of an on-farm BPS located in Kortrijk, Belgium, over the agricultural season of 2011. During the agricultural season the BPS material was exposed to different pesticides with varying concentrations. The BPS was sampled three times over the agricultural season and PCR-

based screening of BPS total community (TC)-DNA was performed followed by Southern blot hybridization to check for the presence of broad-host-range, self-transferable and mobilizable plasmids and antibiotic resistance genes often located on class 1 and 2 integrons (*intI1* and *intI2*) and *aadA* gene cassettes. Additionally, the concentrations of a range of different pesticides found in the BPS were measured.

Materials and Methods

Biopurification systems (BPS) material and sampling

Samples were taken from a large BPS in Kortrijk, Belgium, composed of coco-chips, straw, manure and field soil. The BPS was used for water contaminated with different types of pesticides (see Table 2) from spillage and residue water collected when cleaning the spraying equipment. The BPS (20 m long by 1.2 m width) was divided into four compartments, representing four replicates, each of which was sampled three times over the agricultural season of 2011. From each compartment 12 cores of 4 cm diameter and 10 cm depth were collected, mixed, dried overnight in a clean bench and sieved (2 mm). Samples were collected before, during and after the pesticide spraying season (March, July andSeptember). After sieving, the BPS material was stored at 4°C for a few days until TC-DNA extraction. Aliquots of the 12 samples (four replicates from each sampling time) were used for chemical analysis to evaluate the concentration of pesticides present in the BPS.

BPS chemical analysis

Chemical analysis was performed at the Institut für Umweltforschung (INFU, Fakultät Chemie Lehrstuhl für Umweltchemie und Analytische Chemie, Technische Universität Dortmund, Dr. Sebastian Zühlke laboratory).

Total community DNA (TC-DNA) extraction

TC-DNA was extracted from 0.5 g of 2-mm-sieved BPS samples by using the FastPrep FP120 bead beating system for cell lysis and the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) and purified by using the GENECLEAN Spin Kit (MP Biomedicals). The DNA yield and quality was checked by 1% (w/v) agarose gel electrophoresis.

16S rRNA PCR amplification from the BPS TC-DNA

The absence of PCR inhibiting substances in the TC-DNA was checked by PCR amplification of 16S rRNA gene fragments by using the primers and conditions previously described (Heuer *et al.*, 2009) (product size of 1,506 bp). The PCR products were checked by electrophoresis as described above.

PCR-Southern blot hybridization detection of IncP-1, IncP-7, IncP-9, IncQ, IncN and IncW plasmids, and *intI1, intI2*, *sul1, sul2* and *aadA* genes

The primer systems (Götz *et al.*, 1996, Heuer & Smalla, 2007, Binh *et al.*, 2008, Bahl *et al.*, 2009, Moura *et al.*, 2010) used to detect IncP-1, IncP-7, IncP-9, IncQ, IncN and IncW

plasmids, *intI1, intI2*, *sul1*, *sul2* and *aadA* genes in the TC-DNA and reference strains used to generate the probes are listed in the Supplement Material (Table S1). Southern blotting and hybridization were performed as previously described by Binh *et al.* (2008).

Development of a new PCR-Southern blot hybridization system to detect IncU plasmids

A specific probe for Southern blot hybridization detection of IncU plasmids in TC-DNA was generated from the amplified 884 bp fragment of reference plasmid pRA3 using newly designed primers IncU_F3 (5'-TGGCTATCACARGCCGARTT-3') and IncU_R6 (5'-AGGTTGATCAGNGTGTCRTT-3') targeting conserved sequences of the replication region of IncU plasmids. Primers were designed based on an alignment of IncU plasmids (Table S.2). The PCR reaction mixture (25 µL) contained 1 µL template DNA (1-5 ng), 1 x Taq buffer, 0.2mM dNTPs, 2.5 mM $MgCl_2$, 0.2 µM of each primer, and 1.25 U EP-TaqPolymerase. Initial denaturation was carried out at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 59°C and 1 min at 72°C followed by a final extension step of 5 min at 72°C. The pRA3 PCR product was analyzed on 1% (w/v) agarose gel, cut from the gel and purified by using the QIAEX II Gel® Extraction kit. 10 µl of purified PCR product was labeled with dixogenin (DIG) according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). The probe was tested for specificity by the absence of cross hybridization to reference plasmids from IncP-1, IncP-7, IncP-9, IncQ, IncN and IncW (Table S2). Plasmid pRA3 was used as positive control for the detection of IncU plasmids in TC-DNA.

IncP-1 *trfA* amplicon pyrosequencing and analysis

The IncP-1 *trfA* gene was sequenced by amplicon pyrosequencing from TC-DNA from all 12 samples. A 281 bp region of the IncP-1 *trfA* gene was amplified using the three primer sets previously described (Bahl *et al.*, 2009) that were also used for Southern blot analysis. Amplification and addition of adapters and barcode tags was done using the U-linker system described by Holmsgaard *et al.* (in prep.). Briefly, the *trfA* region was PCR amplified using primers with U-linkers attached. Products were purified by agarose gel electrophoresis (Masoud *et al.*, 2011) using the Montage Gel extraction kit (Millipore, Billerica, MA, USA). A second round of PCR was used to add sequencing adapters and barcode tags. Approximately equal concentrations of tagged PCR products from each sample were used to ensure near-equal sample representation. Sequencing was done at The Danish National High-Throughput DNA-Sequencing Centre on a GS FLX Titanium PicoTiterPlate using the GS FLX pyrosequencing system (Roche, Basel, Switzerland).

Primers, U-linkers, barcode tags and adapters were trimmed away, sequences were quality filtered and denoised using AmpliconNoise (Quince *et al.*, 2011). Sequences outside 231 ± 3 bp in length were discarded (Holmsgaard *et al.*, in prep.). Chimeric sequences were removed *de novo* with uChime in uSearch 5.2.32 (Edgar *et al.*, 2011). In short, sequences were dereplicated and chimeric sequences removed *de novo* based on abundance. These non-chimeric sequences were then used as reference database to remove chimeric sequences in the standard uChime database mode. The cleaned amplicon sequences were assigned to the seven IncP-1 sub-groups by clustering them together with known IncP-1 *trfA* reference sequences at 89% sequence similarity (Holmsgaard *et al.*, in prep.). Sequences from five large clusters, all containing reference sequences, were extracted and independently re-clustered at 97% sequences similarity. A representative sequence from each new cluster that contained ≥1% of the sequences from the original cluster were aligned using Muscle and used to build a neighbor-joining tree (1,000 bootstrap replicates) in MEGA5.0 (Tamura *et al.*, 2011). Significant differences ($p < 0.05$) in the relative number of sequences between sampling times were tested by One-way ANOVA at both clustering levels. Data was $\log_{10}+1$-transformed and variance homogeneity verified with Bartlett's test.

Denoising and clustering were done with the QIIME 1.5.0 software package (Caporaso *et al.*, 2010) using QIIME tools or QIIME wrappers of other tools. All other bioinformatics were done using Biopieces (www.biopieces.org, developed by Martin A. Hansen) and statistics were done in R 2.15.2 (R Core Team, 2012).

Nucleotide sequence accession numbers

IncP-1 *trfA* amplicon sequences are submitted to NCBI SRA as study SRA072602 with accession numbers SRS428737, SRS428743, SRS428745, SRS428746, SRS428748, SRS428750 and SRS428751.

Results

Chemical analysis of BPS over the agricultural season

Table 1 lists the recovery concentrations of different pesticides found in the on-farm BPS at the three sampling times in March, July and September. A list of all pesticides applied to the BPS was provided by the farmers from Kortrijk, Belgium (Table 2). Of the 30 pesticides measured by chemical analysis, 15 were not detected: 2,4-D, 2,4-DP, amidosulfuron, atrazin, bromacil, desaminometamitron, desethylatrazin, dimethoat, fenpropidin, Ioxinyll, isoproturon, MCPA, MCPP, metazachlor and spiroxamin. The lack of detection of, for example, glyphosate, linuron and metamitronin the BPS samples likely indicates the efficiency of sorption and biodegradation in the BPS (Table 1). However, the concentrations added to the BPS were unknown. Bentazon (Wagner *et al.*, 1996) and S-Metalochlor (Munoz *et al.*, 2011), considered recalcitrant to degradation, were found in very high concentrations (µg/g). From March to September the concentration of S-Metalochlor increased indicating a slow, if any biodegradation process at all. However, the concentration of Bentazon decreased. The highest concentrations of about half of the pesticides detected were found in July, and more than half of the pesticides still had a higher concentration in September than initially measured in March.

Effects of different pesticide concentrations on abundance of broad-host-range (BHR) plasmids

To investigate the composition of MGEs in BPS bacteria and the influence of the different concentrations of pesticides applied to the BPS during the agricultural season on their abundance, PCR-Southern blot hybridization screenings of BHR plasmids belonging to the incompatibility groups IncP-1, IncP-7, IncP-9, IncQ, IncN, IncU and IncW were performed (Table 3). Strong IncP-1 hybridization signals obtained by using a mixed-probe targeting all IncP-1 subgroups indicated that bacterial populations carrying IncP-1 plasmids were very abundant at all sampling times. However, by using probes specific for the five different IncP-1 subgroups, a change in the composition of IncP-1 plasmids over different agricultural seasons was observed. Based on differences in the intensity of hybridization signals detected in the TC-DNA, the abundance of IncP-1β, IncP-1γ and IncP-1δ plasmids seemed to be affected by the changed concentrations of pesticides. Strong hybridization signals were obtained for the IncP-1β plasmids in March samples and the signal intensity increased over the year to 'very strong' in September. This indicated that bacterial populations carrying IncP-1β plasmids increased in abundance over the year. The signal intensity of IncP-1γ plasmids was weakest in March samples and the highest hybridization signal was observed in September. The highest signals of IncP-1δ plasmid hybridizations were observed in the

September samples. Strong hybridization signals of IncP-1ε plasmids were observed at all three sampling times, without variation over the season. Strong hybridization signals of IncP-9, IncQ and IncW plasmids were detected in all samples. IncP-7 plasmids were also detected in all replicates, with a slight increase in the hybridization signal from March to September (Table 3). Hybridization signals of IncU plasmids indicated a gradual decrease in the IncU plasmid abundance from March to September. IncN plasmids were not detected at all, indicating the absence or very low abundance (below Southern blot hybridization detection limits) of these plasmids in the BPS (Table 3).

Seasonal variations in IncP-1 plasmid diversity revealed by *trfA* gene amplicons

The diversity and relative abundance of IncP-1 plasmids were also evaluated by amplicon pyrosequencing of the *trfA* gene region of BPS TC-DNA over the agricultural season. After denoising and chimera removal 95,199, 49,308 and 57,011 sequences were obtained from primer sets targeting IncP-1αβε, IncP-1γ and IncP-1δ, respectively. After mixing the sequences from all three primer sets together with 21 known reference sequences from IncP-1 subgroups α, β, γ, δ, ε and ζ and clustering at 89% sequences similarity, 20 clusters were obtained. Eight of these clusters contained the reference sequences along with the majority of amplicon sequences (Fig. 1a). Only 0.03% of the sequences from primer set αβε belonged to the IncP-1α subgroup and all were from the March samples. No ζ sequences were found and no sequences clustered with γ pKS208. The clustering level of 89% was determined by clustering 66 known IncP-1 *trfA* genes trimmed to between the primers (237 bp) in steps of 1% from 84-100%. At 89% sequence similarity one cluster was formed for each IncP-1 subgroup except γ that split in two (Holmsgaard *et al.*, in prep.). Sequences from the five clusters representing β-1, β-2, γ, δ, ε and encompassing the large majority of sequences were extracted and re-clustered at 97% sequence similarity. 97% was chosen as a conservative estimate to avoid potential sequencing errors not removed by denoising and chimera checking. Pyrosequencing has an error-rate of about 1% (Huse *et al.*, 2007) and the size of the sequences is 237 bp. After re-clustering each original cluster split into many new clusters with reference sequences found in several of these (Fig. 1b). All β-1 reference sequences were found in cluster 11.101 except pB136 which was found in 11.25. For β-2, almost all amplicon and reference sequences were found in 19.51. Exceptions were pB1 and pAOVO02 which were found in 19.7 and 19.57, respectively. The IncP-1ε sequences split in two major groups with pKJK5 related plasmids and the large majority of sequences in 18.64 and pEMT3 related plasmids in 18.74. All γ sequences grouped with pQKH54 in cluster 9.3 and no sequences fell into the 9.1 cluster with pMBUI1. Except for the two γ clusters, all representative sequences differed after translation to amino acids. Significant changes in the relative abundance of the different IncP-1 subgroups were found

between the three sampling times. The β-1 and β-2 increased significantly in abundance from March to July (one-way ANOVA, $p < 0.05$) with no change between July and September. IncP-1ε on the other hand, significantly decreased in abundance from March to July and also showed no change between July and September. It is important to note that the relative abundances are primer specific and cannot be compared among sequences obtained with different primer sets. Furthermore, the numbers of sequences are not related to the abundance of the different IncP-1 subgroups in the TC-DNA samples because of the method used for amplicon library construction.

Seasonal variations in abundance of integrons and resistance genes

The influence of pesticides applied to the BPS on abundance of integrons and antibiotic resistance genes was investigated by PCR-Southern blot hybridization. Gene-specific sequences of *aadA, intI1, intI2, sul1* and *sul2* genes were present in all BPS samples. However, while the hybridization signals of *aadA* genes were stable over the season (Table 4), the *intI1, intI2*, *sul1* and *sul2* genes differed in hybridization signal intensity between sampling times. Hybridization signals indicated the highest abundance of *intI2* genes and the lowest abundance of *intI1* in July. The highest hybridization signal of *sul1* antibiotic resistance genes was found in March. A very strong signal indicated a very high abundance of *sul2* resistance genes in March and July, while only weak signals were detected in September.

Discussion

This is the first comprehensive study on the occurrence of various broad host and narrow host range plasmids, integrons and antibiotic resistance genes in on-farm BPS material exposed to different pesticides which was monitored with cultivation-independent approaches over the agricultural season 2011. The present study aimed at shedding some light on the mobilome harbored by bacteria in BPS material as mobile genetic elements are assumed to be important for the adaptation to and biodegradation of pollutants. The present work was motivated by two recent studies on BPS samples from various field sites in Belgium taken at single time points which showed a high abundance of IncP-1, IncP-7, IncP-9 plasmids and IS elements typically associated with degradative genes (Dunon *et al.*, 2013, Dealtry *et al.*, chapter II). Here we monitored fluctuations of a range of different plasmids, integrase genes of class 1 and class 2 integrons and sulfonamide and streptomycin resistance genes at three times during the agricultural season: before the pesticide spraying season (March), at the height of the spraying season (July) and after the season (September). During the year, the concentration of different pesticides in the BPS varied considerably and reflected both what the farmers used at different times of an entire agricultural season, and the rate of sorption and of biodegradation in the BPS. A semi-quantitative but highly sensitive and specific approach was used to detect plasmid replicon, integrase gene and antibiotic resistance gene specific sequences in TC-DNA. The strong PCR-Southern blot hybridization signals for IncP-1, IncP-7 and IncP-9-specific sequences amplified from TC-DNA indicated a high abundance of potentially catabolic plasmids in BPS bacterial communities throughout the agricultural season and thus confirmed previous observation made for single time points from different BPS (Dealtry *et al.*, chapter II). For the first time IncW, IncQ and IncU-specific sequences which typically represent antibiotic resistance plasmids were detected in the BPS TC-DNA for all three sampling times. Interestingly, plasmids belonging to the IncN were below the detection limit. The abundance of IncP-9, IncQ and IncW plasmids carrying bacterial populations in the BPS seemed to be both very high and rather stable over the season, independently of variations in the concentrations of pesticide present (Table 3). This result indicated that populations carrying these plasmids might be not affected by the different types and concentrations of pesticides added to the BPS over the agricultural season. IncP-9 plasmids might confer an advantage to the survival of the bacterial community in BPS exposed to pesticides or wood-derived polyaromatic hydrocarbons as IncP-9 plasmids were often found to carry genes encoding different aromatic-ring degrading enzymes (reviewed by Dennis, 2005, Sevastayanovich *et al.*, 2008) which might contribute to the degradation of the pesticides containing aromatic-ring in their chemical structure, such as bentazon, epoxiconazol and diflufencian.

The very high relative abundance of the promiscuous IncP-1 plasmids detected in the BPS is comparable to other “hot spots” of horizontal gene transfer such as manure, sewage or polluted river sediments (Heuer *et al.*, 2011, Gaze *et al.*, 2013). Our data confirm the results of two recent studies using TC-DNA from different BPS which also found an unusual high abundance of IncP-1 plasmids in the BPS (Dunon *et al.*, 2013, Dealtry *et al.*, chapter II). Interestingly, the abundance of IncP-1 plasmids seemed to be increased in BPS bacteria likely in response to the exposure to different types and concentrations of pesticides as recently reported by Jechalke *et al.* (2013a). An increased abundance of IncP-1 plasmids was also observed in microcosm experiments with material from BPS in Kortjik spiked with linuron or left unspiked (Dealtry *et al.*, chapter III). In the microcosm experiment with defined exposure to linuron within a rather short time the abundance of IncP-1 plasmids was increased as revealed by quantitative real-time PCR (Dealtry *et al.*, chapter III). Several other studies have pointed to the role of IncP-1 plasmids in the adaptation of the bacterial community to xenobiotic compounds such as pesticides released into the environment (revied by Top & Springael, 2003 and Dennis, 2005, Vedler, 2009, Sen *et al.*, 2011, Król *et al.*, 2012). The high abundance of the subgroups IncP-1β, γ, δ, ε revealed in the present study confirmed the findings by Dealtry *et al.* (chapter II). Although Southern blot hybridization do not provide quantitative information on specific genes copies numbers per gram of material as qPCR, changes in the abundance of the different subgroups could be indicated which were confirmed by amplicon pyrosequencing of the *trfA* amplicon obtained with the same primer sets as used for the PCR screening. Based on the exposure time of the identical blots hybridized with digoxygenin labeled probes generated from reference plasmids we proposed that bacteria carrying IncP-1β and IncP-ε plasmids were much more abundant in the BPS compared to IncP-1α. Based on the intensity of hybridization signal, the abundance ofIncP-1γ and IncP-δ plasmids seemed to be lower compared compared to IncP-1γ and IncP-δ. Similarly fewer sequences were obtained for the latter plasmid groups by amplicon pyrosequencing. However, as different primers were used to target the IncP-1γ and IncP-1δ plasmids, differences in the intensity of hybridizations signals produced by variations in primer-amplification efficiency might not be excluded. Comparisons should only be made between the IncP-1α, IncP-β and IncP-ε group which were amplified by the same pair of primers. The diversity of different IncP-1 groups in BPS bacterial communities was remarkable and similar to the findings of Bahl *et al.* (2009) for sewage. Hybridizations of *trfA* amplicons revealed an increased abundance of IncP-1β plasmids over the agricultural season while IncP-ε seemed high and stable. Basic clustering of the *trfA* sequences obtained by pyrosequencing into the different IncP-1 subgroups (Fig. 1a) gave a much more detailed picture of IncP-1 plasmid diversity and also provided insights into the dynamics of bacterial

populations hosting these plasmids which likely reflected their exposure to different concentrations of various pesticide compounds. Significant increases in IncP-1β-1 and IncP-1β-2 abundance from March to July were found, and a decrease in IncP-1ε abundance in the same period. Interestingly, the Southern blot hybridization of *trfA* amplicon from TC-DNA revealed an equally high abundance of IncP-1ε plasmids over the season likely indicating a limitation of this method when target sequences are highly abundant. A more detailed clustering of the large subgroups (Fig. 1b) gave no further details on changes in subgroup abundance, but showed that almost all sequences had high similarity to the well known IncP-1 plasmids used for reference. Moreover, results obtained by sequencing cloned IncP-1 *trfA* amplicons also indicated that different pesticide concentrations might influence the diversity and distribution of IncP-1 plasmids (Fig. S3).

The high abundance of class 1 and class 2 integrons (*intI1, intI2*) and sulfonamide resistance genes (*sul1, sul2*) in March and July indicated that BPS are hot-spots of antibiotic resistance genes and integrons likely introduced via manure (Table 4). This could explain why the abundance of *sul1* and *sul2* genes seemed to decrease over the agricultural season (Jechalke *et al.*, 2013b). Contrary to the *intI* and *sul* genes, the bacteria with aminoglycoside adenylating gene cassette (*aadA*) were stably maintained in the BPS at all sampling times indicating their occurrence also outside of the integron classes investigated here. The *aadA* genes are frequently found on class 1 integrons of environmental isolates (Binh *et al.*, 2009), and also the *sul1* genes are associated with class 1 integrons (Hall & Collis, 1998), while *sul2* are typically found on IncQ plasmids (Scholz *et al.*, 1989, Smalla *et al.*, 2000a). The increased abundance intI2 genes in BPS samples taken in July might point to a response of their hosts to pesticides. The addition of manure as nutrient source before the agricultural season might have also contributed to the high abundance of plasmid groups such as IncQ, IncW and IncP-1ε, whichare typically associated with antibiotic resistance genes and integrons. The application of manure to field soil is known to introduce not only antibiotic compounds and their metabolites but also bacteria carrying plasmids, integrons and antibiotic resistance genes (reviewed by Heuer *et al.*, 2011).

To the best of our knowledge, this is the first study on the occurrence of a range of different MGEs in an on-farm BPS over an agricultural season. Here we showed that on-farm BPS might be considered as another “hot spot” of horizontal gene transfer (Gaze *et al.*, 2013). The exposure of BPS bacteria to various pollutants might have fostered the adaptation to rapidly changing environmental conditions via horizontally acquired mobile genetic elements. In particular, the amplicon sequencing results revealed not only an impressive diversity of IncP-1 plasmids but also a dynamic of bacterial population carrying IncP-1β and IncP-1ε plasmids over the agricultural season. The present study showed that

bacterial populations carrying IncP-1β plasmids were increased in abundance (positively affected) by the exposure of the on-farm BPS to various pesticides. The results strongly indicated that the IncP-1β subgroup plays a major role in pesticide degradation in such polluted systems.

Acknowledgments

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Tables and Figures

Table 1. Pesticides and their concentrations found in the BPS material from March, July and September

		March				July				September				
Samples	LOQ	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	
Azoxystrobin	15	53	43	22	34	23	35	19	16	26	37	15	16	
Bentazon	6	27,850	39,480	70,030	85,820	40,870	40,870	58,000	75,000	41,000	51,000	47,000	*5,000*	
Diflufencian	20	576	827	601	681	1,199	1,425	1,591	750	1,711	2,393	1,420	1,063	*
Diuron	10	85	69	83	83	97	106	140	92	120	120	104	101	*
Epoxiconazol	30	1,071	1,284	641	748	759	1,102	538	831	571	749	327	800	
Ethofumesat	20	175	104	97	101	98	222	97	128	106	98	61	100	
Fenpropimorph	2	4	<LOQ	<LOQ	<LOQ	6	6	5	2	8	8	5	3	
Flouroxypyr	6	89	79	72	119	1,346	1,259	1,043	1,356	740	700	504	190	*
Flufenacet	10	154	100	208	138	4,685	5,831	4,269	5,462	4,808	5,808	4,523	4,077	*
Metamitron	10	32	38	45	41	62	72	60	61	62	45	58	18	*
Metribuzin	30	98	117	174	173	1,541	2,486	3,012	1,222	1,226	1,088	1,680	672	*
Propiconazol	30	68	74	52	46	47	66	44	27	201	243	130	100	*
S-Metalochlor	1	2,000	3,000	5,000	3,000	2,000	4,000	4,000	4,000	11,000	13,000	11,000	12,000	*
Tebuconazol	3	421	836	1,071	857	700	1,036	729	1,243	779	1,243	786	957	
Terbuthylazin	2	398	917	685	778	6,963	9,880	7,657	9,815	1,352	1,037	2,148	2,889	*
Total (µg/g)		**33.1**	**47.0**	**78.8**	**92.6**	**60.4**	**68.4**	**81.2**	**100.0**	**63.7**	**77.6**	**69.8**	**28.0**	

All numbers except the total are ng μl^{-1}. LOQ, Limit of quantification. Colors represent changes in pesticide concentrations. For pesticides with an asterisk (*) there were a statistical significant difference (one-way ANOVA, $p< 0.05$) in pesticide concentrations between high and low. Italicizes number is a runaway measurement.

Table 2. Pesticides added to the BPS. List provided by the farmers of Kortrijk, Belgium

Product	**Aramo**	**Artist**	**Aspect T**	**Asulox**	**Atlantis**	**Bacara**	**Beetix**	**Bofix**	**Butizyl**	**Centium**	**Challenge**	**Cycocel**
Active compound(s)	tepraloxydim	flufenacet metribuzin	flufenacet terbuthylazine	Asulam	iodosulfuron-methyl-sodium mefenpyr-diethyl metsulfuron-methyl	diflufenican flurtamone	metamitron	clopyralid fluroxypyr	4-(4-chloro-o-tolyloxy)butyric acid (MCPB)	clomazon	aclonifen	chlormequat

Product	**CIPC**	**Comet**	**Defi**	**Dianal**	**Diquanet**	**Dual Gold**	**Equip**	**Ethomat**	**Frontier**	**Goltix**	**Linuron**	**Matrigon**
Active compound	isopropyl (3-chlorophenyl)carbamate	pyraclostrobin	prosulfocarb	Fenmedifam	Diquat	S-Metolachlor	foramsulfuron	ethofumesate	dimethenamid-P	metamitron	linuron	clopyralid

Product	**MCPA**	**Mikado**	**Mitron**	**Opus**	**Pyramin**	**Primstar**	**Primus**	**Round-up**	**Samson**	**Starane**	**Stomp**	**Tapier**
Active compound	2-methyl-4-chlorophenoxyacetic acid (MCPA)	Sulcotrione	metamitron	fenpropimorf	Chloridazon	Florasulam	florasulam	glyphosate	nicosulfuron	fluroxypyr	pendimethalin	difenoconazol

Table 3. PCR-Southern blot hybridization detection of plasmids in TC-DNA from the BPS. Hybridization signals: (++++) very strong, with exposure time up to 2 minutes; (+++) strong, with exposure time up to 30 minutes; (++) strong, with exposure time up to 1 hour ; (+) weak, with exposure time up to 3 hours; (-) none, with exposure time above to 3 hours.

Samples	IncP-1	IncP-1 β	IncP-1 γ	IncP-1 δ	IncP-1 ε	IncP-7	IncP-9	IncQ	IncU	IncN	IncW
March 1	++	+++	++	+++	++++	+++	+++	++++	+	-	++++
March 2	+++	+++	++	++	++++	++	+++	++++	-	-	++++
March 3	-	+++	++	++	++++	++	+++	++++	++	-	++++
March 4	++	+++	++	+++	++++	++	+++	++++	++	-	++++
July 1	+++	+++	+++	+++	++++	+++	+++	++++	+	-	++++
July 2	+++	+++	+++	++	++++	+++	+++	++++	+	-	++++
July 3	+++	+++	++	++	++++	+++	+++	++++	+	-	++++
July 4	+++	+++	++	+++	++++	++	+++	++++	+	-	++++
Sep. 1	+++	++++	+++	+++	++++	+++	+++	++++	+	-	++++
Sep. 2	+++	++++	+++	++	++++	+++	+++	++++	+	-	++++
Sep. 3	+++	++++	+++	++	++++	++	+++	++++	+	-	++++
Sep. 4	+++	++++	+++	+++	++++	+++	+++	++++	+	-	++++
negative control	-	-	-	-	-	-	-	-	-	-	-
RP4 (IncP-1α)	+++										
R751 (IncP-1β)	+++	+++									
pQKH54 (IncP-1γ)	+++		++++								
pEST4011 (IncP-1δ)	++			+++							
pKJK5 (IncP-1ε)	+++				+++						
pCAR1	+++					+++					
pNF 142							+++				
RSF1010								+++			
pRA3									+++		
RN3										+++	
R388											+++

Table 4. PCR-Southern blot hybridization detection of integrase and antibiotic resistance genes in TC-DNA from the BPS. Hybridization signals: (++++) very strong, with exposure time up to 2 minutes; (+++) strong, with exposure time up to 30 minutes; (++) strong, with exposure time up to 1 hour; (+) weak, with exposure time up to 3 hours; (-) none, with exposure time above to 3 hours.

Samples	*IntI1*	*IntI2*	*aadA genes*	*sul1*	*sul2*
March 1	+++	+	+++	++	++++
March 2	++++	+	+++	+++	++++
March 3	++++	+	+++	+++	++++
March 4	+++	++	+++	-	++++
July 1	++	++	+++	+	++++
July 2	++	+++	+++	++	++++
July 3	++++	+++	+++	++	++++
July 4	++++	+++	+++	+	++++
Sep. 1	+++	++	+++	+	++
Sep. 2	+++	++	+++	+	++
Sep. 3	+++	++	+++	++	++
Sep. 4	++	+	+++	+	++
negative control	-	-	-	-	-
pKJK5	+	+	++		
R388				++	
RSF1010					++

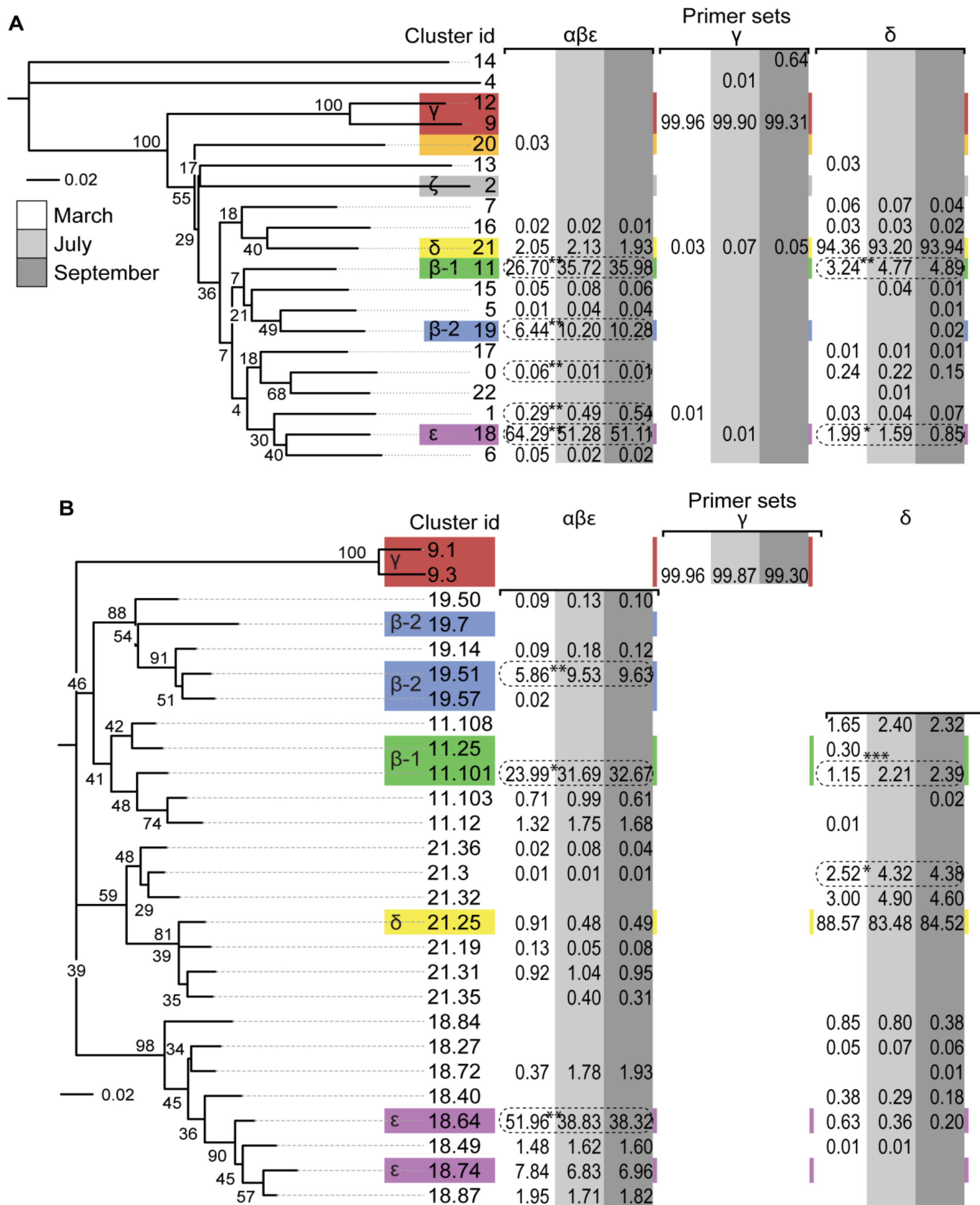


Fig.1 Rooted neighbor-joining trees of clusters of IncP-1 trfA amplicon sequences from BPS TC-DNA. Each branch represents one cluster of sequences. Sequences were first clustered at (A) 89% nucleotide sequence similarity and sequences from large clusters were then re-clustered at (B) 97% similarity with small clusters excluded. Known reference sequences were included in the clustering and their location and subgroup names are indicated by colored boxes. The tables show the mean relative abundance of sequences from each primer set. There is a small overlap in targets between primer sets αβε and δ. Dotted circles indicate significant changes in abundance between March, July and September. (One-way ANOVA, n = 3, * p<0.05, ** p<0.01, *** p<0.001). Numbers of branches are percent bootstrap support of 1,000 iterations

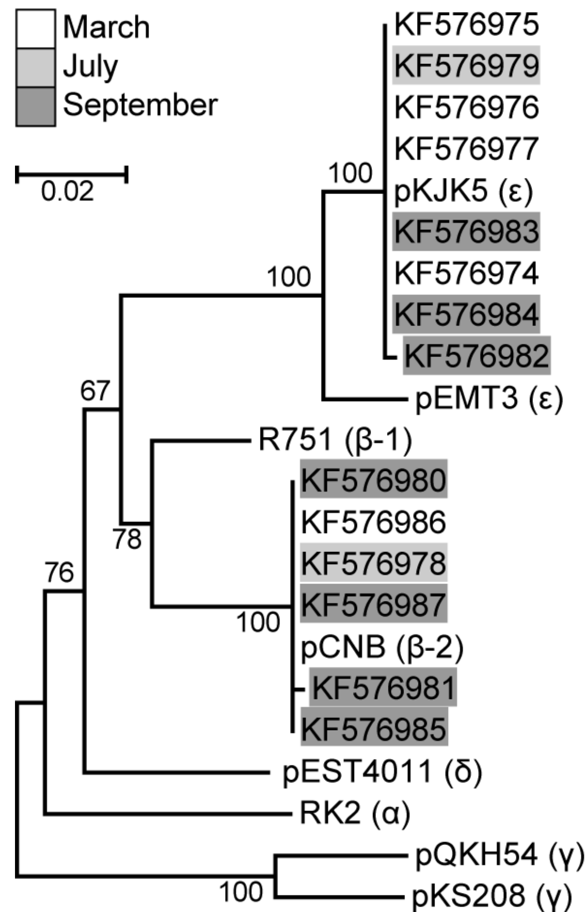
Supplement Material

S1. Primer systems and plasmids used for probe generation for Southern blot hybridizations.

MGEs	Gene fragment	Product size (bp)	Primers	Reference plasmids for probe generation
IncP-1	*trfA*	281	Bahl *et al.*, 2009	RP4; R751; pKJK5, pQKH54 and pEST4011
IncP-7	*rep*	524	Izmalkova *et al.*, 2005	pCAR-1
IncP-9	*oriV-rep*	610	Dealtry *et al.*, in prep.	pM3; PBS2; pBS265; pSN11; pMG18 ; R2; pNL60; pNL15; pSVS15; pNL22; pNF142
IncQ	*oriV*	436	Götz *et al.*, 1996 / Binh *et al.*, 2008	RSF1010
IncN	*repA*	165	Götz *et al.*, 1996 / Binh *et al.*, 2008	RN3
IncW	*oriV*	1140	Götz *et al.*, 1996 / Binh *et al.*, 2008	R388
aadA genes (aadA1, aadA2, aadA9, aadA11, aadA13)	-	-	Binh *et al.*, 2009	pKJK5
Intl1	-	280	Binh *et al.*, 2009	pKJK5
Intl2	-	233	Moura *et al.*, 2010	pKJK5
sul1	-	433	Heuer & Smalla, 2007	R388
sul2	-	293	Heuer & Smalla, 2008	RSF1010

S2. Accession number of plasmids used to design primers to detect IncU-specific amplicon plasmids in environment samples.

Plasmid	Accession number
pRA3	DQ401103.1
pMATVIM-7	NC_009739.1
pRSB105	DQ839391.1
pRms149	NC_007100.1
pKBB4037	AJ877266.1
pFBAOT6	NC_006143.1
pHH2-227	JN581942.1
Plasmid2	NC_008342.1
pAb5S9	NC_009476.1
pEIB202	NC_013509.1
Plasmid	FP340278.1



S3. Rooted neighbor-joining phylogenetic tree based on the multiple alignment of trfA gene of IncP-1 plasmids sequences from translated amino acid sequences. Value at each node is percent bootstrap support. "BPS1", "BPS2" and "BPS3" are corresponding to the sampling times in March, July and September, respectively. IncP-1 *trfA* partial sequences from clone library are submitted to GenBank with accession numbers KF576974 - KF576987.

Chapter V: Response of the bacterial community in an on-farm biopurification system to diverse pesticides introduced over an agricultural season

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Abstract

Previous studies have pointed to diverse bacterial populations as central players in the biodegradation of man-made pollutants in soils and sediment. Biopurification systems (BPS) are used on farms to clean pesticide-contaminated water, and while the degradation of specific pesticides has been intensely studied in BPS, their bacterial community has not. Due to the high pesticide load a BPS is a hot spot of mobile genetic elements (MGEs) and genes encoding enzymes involved in the biodegradation of xenobiotic compounds such as pesticides. In the present work, through DGGE and amplicon pyrosequencing of 16S rRNA gene fragments amplified from total community DNA, the diversity and abundance of the bacterial community in a BPS contaminated with different pesticides was for the first time elucidated. Increased pesticide amount in the BPS as the agricultural season progressed resulted in significant community changes with a significant fall in diversity. Additionally, a significant increase in the abundance of *Proteobacteria*, mainly the *Gammaproteobacteria*, was found and OTUs affiliated to *Pseudomonas* responded positive to increases in pesticide concentrations. Furthermore, DGGE analysis indicated that the *Betaproteobacteria* might play an important part. Interestingly, a decrease of *Firmicutes* and *Bacteriodetes* was observed, indicating their selective disadvantage in a BPS heavily polluted with pesticides.

Introduction

The overuse and misuse of pesticides in agriculture has resulted in serious pollution of the environment (Field *et al.*, 1997, Spliid & Koppen, 1998). In Europe, ground and surface water in many places are contaminated with pesticides often as a result of point contamination due to spillage and residue water after cleaning the spray apparatus (Kreuger 1998, De Wilde*, et al.*, 2007). In order to control pesticide point contamination, a simple, low cost and practical approach has been applied on farm, so-called biopurification systems (BPS) (Karanasios *et al.*, 2012). In on-farm BPS, pesticide-contaminated water is percolated over a solid matrix, the biomix, which typically is a homogenized mixture of local soil, chopped straw and peat, or composted material. BPS reduces pesticide contamination through sorption followed by biodegradation processes by microorganisms. Biodegradation has been indicated as the major process of pesticide removal in different environments (Cullington & Walker, 1999, Sørensen *et al.*, 2003). By using 16S rRNA gene cloning and sequencing, bacterial populations affiliated to *Proteobacteria, Actinomycetes and Acidobacterium* were described as dominant taxa in environments polluted with organic compounds (Paul *et al.*, 2006). Although several studies have focused on the degradation of selective pesticides in BPS, e.g. linuron (Sniegowski *et al.*, 2011a) and isoproturon (von Wiren-Lehr *et al.*, 2001), as well as the important role of mobile genetic elements (MGEs) (Dunon *et al.*, 2013, Jechalke *et al.*, 2013a, Dealtry *et al.*, chapter II and IV), the entire bacterial community in a BPS has not be thoroughly studied. A recent study demonstrated that the BPS located in Kortrijk, Belgium (the same BPS studied in the present work) has a high abundance of mobile genetic elements (MGEs), such as IncP-1 and IncP-9 catabolic plasmids (Jechalke *et al.*, 2013a, Dealtry *et al.*, chapter II) and IS*1071* (Dunon *et al.*, 2013). However, investigations of the bacterial populations hosting such MGEs are still missing. The degradative genes present on MGEs cannot be expressed or transferred when the bacterial metabolism is not active or working properly. The "health" of the bacterial community directly influences the stability, maintenance and transferability of MGEs carried by it. Therefore, it is extremely important to study the composition and structure of the bacterial population likely carrying genes facilitating pesticide degradation. Based on the hypothesis that pesticides that are toxic, i.e. unfavorable to some bacteria (DeLorenzo *et al.*, 2001) but favorable to bacteria who can utilize the pesticides as energy source (Breugelmans *et al.*, 2007), the goal of this study was to get a deeper insight into the response of the bacterial community in an on-farm BPS to different concentrations of pesticides. In the present work, a large BPS located in Kortrijk receiving different pesticides of different concentrations over a year and reflecting what the farmers used over the agricultural season was studied. Samples were collected before the pesticide spraying season, during the spraying season and after the spraying season of 2011, and total community (TC) DNA was extracted. The bacterial community was

investigated through DGGE and amplicon pyrosequencing of the 16S rRNA gene fragments amplified from TC DNA.

Materials and Methods

Biopurification system (BPS) samples

Samples were collected from a large BPS located in Kortrijk, Belgium, and the samples were previously described (Dealtry *et al.*, chapter IV). Briefly, the BPS was divided into four compartments and each was sampled before the pesticide spraying season (March), during spraying season (July) and after spraying season (September) in 2011.

The following pesticides were found by chemical analysis: azoxystrobin, bentazon, diflufencian, diuron, epoxiconazol, ethofumesat, fenpropimorph, flouroxypyr, flufenacet, metamitron, metribuzin, propiconazol, S-metalochlor, tebuconazol and terbuthylazin (Dealtry *et al.*, chapter IV).

DNA extraction

TC DNA was extracted from BPS samples as previous described (Dealtry *et al.*, in preparation) using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) and purified by using the GeneClean Spin Kit (MP Biomedicals).

DGGE analysis of 16S rRNA gene fragments

Bacterial 16S rRNA gene fragments were PCR amplified from the TC DNA using primers F984GC and R1378 (Heuer *et al.*, 1997) flanking the V6-V8 regions. A nested PCR approach was used for amplification of the 16S rRNA genes of *Actinobacteria*, *Alphaproteobacteria* and *Betaproteobacteria* (Heuer *et al.*, 1997, Gomes *et al.*, 2001). Primers are listed in Table 1. DGGE of the PCR amplicons was done as described by Ding *et al.* (Ding *et al.*, 2012) using silver staining and comparison of DGGE profiles and band intensities with the software GelCompar II 5.6 (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis based on this similarity matrix was constructed by unweighted pair-group method with arithmetic averages (UPGMA) (Smalla *et al.*, 2007). In order to evaluate slight differences in fingerprints of bacterial communities visualized by DGGE, statistical test analysis by using permutation test was applied. The permutation test was based on the pairwise similarity measures (correlation coefficients in the example), according to Kropf *et al.* (Kropf *et al.*, 2004).

Amplicon pyrosequencing

Bacterial and archeal 16S rRNA gene fragments were PCR-amplified from TC-DNA and pyrosequenced using primers 341F and 806R (Hansen *et al.*, 2012) (Table 1) flanking the V3-V4 regions as described in (Dealtry *et al.*, in preparation). Sequencing was done on a

GS FLX Titanium PicoTiterPlate using the GS FLX pyrosequencing system (Roche, Basel, Switzerland) at The Danish National High-Throughput DNA-Sequencing Centre.

Sequence analyses

16S rRNA gene amplicon sequences were trimmed, quality filtered and denoised with AmpliconNoise (Quince *et al.*, 2011). Chimeric reads were removed with uChime in uSearch 5.2.32 (Edgar *et al.*, 2011) against the GOLD database (Pagani *et al.*, 2012). Sequences were analyzed with the software package QIIME 1.5.0 (Caporaso *et al.*, 2010). Briefly, samples were rarefied to 1,903 sequences per replicate. Sequences were then clustered into operation taxonomic units (OTUs) at 97% sequences similarity and taxonomically assigned against the GreenGenes database (DeSantis *et al.*, 2006). The number of OTUs and Shannon diversity index was calculated after rarefaction as above but iterated 10 times. Statistical significances between sampling times were found by one-way analysis of variance (ANOVA, $p < 0.05$) and if significance was found Tukey Honest Significant Differences (Tukey HSD) test was subsequently used to determine means that were statistically significant from each other. Data was $\log_{10}+1$ transformed and variance homogeneity tested with Bartlett's test. The largest OTUs containing 50% of the sequences were extracted (108 of 3,947) and OTUs with significant differences in relative abundance between sampling times were identified by one-way ANOVA as described above. The distribution of genera and sequences between sampling times was done to identify genera of potential interest. Statistics were done in R 2.15.2 (R Core Team, 2012).

Nucleotide accession numbers

The 16S rRNA gene amplicon sequences have been submitted to the NCBI SRA database under accessory numbers SRA072602 in samples SRS408209, SRS408210, SRS408211, SRS408213, SRS408214, SRS408215, SRS408216, SRS408218, SRS408219, SRS408221 and SRS408222.

Results

Bacterial community composition analyzed by DGGE

The diversity of the total *Bacteria* community was analyzed by DGGE of the 16S rRNA gene along with specific DGGE fingerprinting of the *Alphaproteo-*, *Betaproteo-* and *Actinobacteria*. No clear differences between samples and sampling times were visible by eye in most of the DGGE fingerprints, except a slight difference in the intensity of few bands corresponding to *Betaproteobacteria* in the second and third sampling times (Fig. 1A). The DGGE gel pictures were subsequently submitted to computer analysis of the banding patterns and intensities. The dendrograms resulting from these analyses showed a difference in community composition between the three sampling times for the *Betaproteobacteria* (Fig. 1B). The samples from July formed a separate cluster and were more similar to the September samples than to the March samples. The UPGMA clustering of the DGGE patterns did not detect any difference between sampling times for total bacterial diversity, *Alphaproteobacteria* and *Actinobacteria* (Fig. S1-S3). Permutation test analysis of the different bacterial groups evaluated by DGGE fingerprints revealed a significant difference in the composition of *Betaproteobacteria* community in July and September samples compared to the March samples (Fig. 1C). Permutation test of total *Bacteria*, *Alphaproteo*bacteria and *Actinobacteria* DGGE fingerprints revealed no significant differences in the composition of these communities over the agriculture season sampling times (data not shown).

16S rRNA gene amplicon pyrosequencing

After quality filtering, denoising and removal of chimeric sequences between 1,903 and 5,096 sequences were obtained per sample. All samples were rarefied to 1,903 sequences before estimation of Shannon diversity and OTU clustering. Rarefaction curves did not reach a plateau (data not shown). From March to July a highly signification drop (ANOVA, $p < 0.001$) in both Shannon diversity and number of OTUs occurred (Fig. 2). From July to September the mean number of OTUs and Shannon diversity did not change, but for the diversity the standard error increased.

If possible sequences were taxonomically classified from phylum to genus level. Twenty-seven phyla including unclassified sequences were identified and 99.4% of sequences were classified to the 16 phyla in Fig. 3. Around 10% of sequences could only be taxonomically classified as *Bacteria*. Moreover, 1-2% of sequences could not be classified to anything known (Fig. 3). The in-depth characterization of BPS based on 16S rRNA gene amplicon pyrosequencing, revealed that within the *Bacteria Proteobacteria* were the most dominat phylum over the agricultural season, followed by *Firmicutes*, *Actinobacteria*, *Bacteriodetes*,

Acidobacteria, *Chloroflexi*, TM7, *Gemmatimonadetes*, *Verrucomicrobia*, *Nitrospira*, *Chlamydiae* and *Deinococcus-Thermus. Crenarchaeota* and *Euryarchaeota* were found within the *Archaea* (Fig. 3). The addition of pesticides resulted in a highly significant (ANOVA, $p < 0.01$) drop in relative abundance of these unclassified sequences along with *Bacteroidetes* between March and July along with a significant (ANOVA, $p < 0.05$) drop in *δ-Proteobacteria*. A significant decrease in the relative abundance of *Firmicutes* was observed from July to September. The positive responders to the addition of pesticides were *Proteobacteria*, more specifically *Gammaproteobacteria*, and TM7 which quickly recovered after pesticide amount started to decrease in September. Although not statistically significant, *Chloroflexi* and *Deinococcus-Thermus* also displayed a positive response, while *Crenarchaeota* appeared to have a similar relative abundance pattern as TM7 with a drop in July and quick recovery in September.

Of the 421 genera identified, those 260 with more than three sequences were used for comparison and these contained 98.81% of sequences. 212 genera were found in samples from March, July and September (Fig. 4). No unique genera were found in March samples, and only two were unique to July and three unique to September. However, the genera present at only one of two of the sampling times contained very few sequences (Fig. 4b, Table S1).

As the majority of genera contained sequences from all three sampling times, a deeper analysis was carried out to identify if any OTUs changed significantly in relative abundance between sampling times. Only the largest 108 OTUs out of the total of 3947 OTUs were used, and these 108 include 50% of the sequences. Twenty-one OTUs were found to significantly change (ANOVA, $p < 0.05$) in relative abundance between sampling times (Fig. 5). The two largest of these OTUs were classified as *Pseudomonas* and *Bacillaceae,* and while *Pseudomonas* increased in abundance from March to July, *Bacillaceae* decreased in abundance from July to September. Four additional OTUs were also classified to *Pseudomonadaceae* with some increasing in abundance in July and others in September. Two OTUs classified as *Sphingomonadales* (*Alphaproteobacteria*) gradually increased in abundance between sampling times. Two *Bacillus* OTUs had increased in abundance in July and had fallen again in September.

Discussion

Biopurification systems (BPS) are effective systems for pesticide degradation, and both aspects, which physical conditions enhance pesticide degradation and how certain pesticides get degraded have intensively been studied (reviewed by Karanasios *et al.*, 2012). However, the mechanisms involved in this degradation have not been yet elucidated. The work presented here is part of a larger study of the Kortrijk BPS using the samples collected in March, July and September 2011. From March to July a significant increase in abundance of IncP-1 plasmids happened (Jechalke *et al.*, 2013a). In addition, very high abundance of IncP-1, IncP-7, IncP-9, IncQ and IncW plasmids along with Class 1 and 2 integrons have been found (Dealtry *et al.*, chapter IV). Along with the MGE observations the concentrations of 30 different pesticides were measured and pesticide concentrations found to be highest in July (Dealtry *et al.*, chapter IV).

The decrease in the numbers of OTUs and diversity from March to July correlates with the increased amounts of pesticides added to the BPS in summer (Fig. 2, Dealtry *et al.*, chapter IV) and is thus in line with what is normally observed in polluted environments (Ding *et al.*, 2012, 2013). The DGGE targeting *Betaproteobacteria* (Fig. 1A) and subsequent analysis showed a significant difference ($p < 0.05$) (Fig. 1C) between the diversity of the *Betaproteobacteria* at the second and third sampling times. However, this was not observed in the amplicon sequence data indicating that a deeper level of sequencing is required to obtain the same level of resolution provided by the taxon-specific DGGE profiles. Hence, DGGE still has its validity and the results conducted for future studies focusing on the *Betaproteobacteria* group.

The effect of pesticides on bacterial communities has previously been investigated in soil (Paul *et al.*, 2006) and sediment (Widenfalk *et al.*, 2008) but not in a BPS. The large majority of the genera identified were present at all three sampling times (Fig. 4) and the very few that were not were only found in very low abundance (<0.2% of sequences). This indicates that the BPS does not undergo dramatic changes in the abundance of specific taxonomic groups, but rather gradual changes over the season in already established taxa. This could partly be explained by the fact that 2011 was not the first operational season of the BPS and hence the pesticide degrading bacterial community was already established.

Some large changes within the bacterial community between the sampling times were, however, observed. The increase in relative abundance of the *Gammaproteobacteria* (Fig. 3) and especially OTUs affiliated with *Pseudomonas* (Fig. 5) in July and September is in accordance with the previous findings that *Pseudomonas* has been found to be involved in pesticide degradation in aquatic environments (Lew *et al.*, 2013) as well as soil (Viegas *et al.*,

2012, Zhang *et al.*, 2013) and groundwater (Caracciolo *et al.*, 2010). In addition to the plasmids associated with *Pseudomonas* their genomes often carry genes involved in pesticide degradation; e.g. of atrazine (BoundyMills *et al.*, 1997). Moreover, *Pseudomonas* is a well-known host of many broad host range plasmids such as IncP-1, IncP-7 and IncP-9 that often carry genes associated with biodegradation (Dennis, 2005, Shintani *et al.*, 2010) which fits with the high abundance of IncP-1 and IncP-9 plasmids found in these samples (Dunon *et al.*, 2013, Jechalke *et al.*, 2013a, Dealtry *et al.*, chapters II, III, IV). All these observations pinpoint *Pseudomonas* as one of the key group involved in the degradation of pesticides in the BPS and elsewhere.

The high numbers of *Firmicutes* and *Bacteroidetes* in the samples compared to what is found in soil can be explained by the addition of manure to the BPS early in the year as the two phyla have been shown to be in high abundance in manure (Snell-Castro *et al.*, 2005, Marti *et al.*, 2011). The increase of candidate phylum TM7 in September is difficult to explain as no species from the phylum has yet been cultured. However, members of TM7 have been put in relation to toluene (Luo *et al.*, 2009, Xie *et al.*, 2011) and benzene degradation (Xie *et al.*, 2011). Moreover, the sequence of a TM7 member revealed the presence of several genes involved in sugar metabolism and a high abundance of TM7 were detected in the rhizosphere of potato (Weinert *et al.*, 2011).

Interestingly, the composition of bacterial communities in BPS exposed to pesticides was rather stable when compared to the huge shifts in the abundance and composition of MGEs (Jechalke *et al.*, 2013a, Dealtry *et al.*, chapter IV). This suggests that the adaptation process occurring in the BPS is driven by horizontal transfer of catabolic plasmids rather than change in community composition due to selection for already present pesticide degrading bacteria. Due to their wide host range IncP-1 plasmids are likely to play an important role in the adaptation of various taxonomic groups affiliated to the *Proteobacteria* to the varying types and concentrations of pesticides (Jechalke *et al.*, 2013a). Our data, however, indicate that other bacterial groups might also be important for the pesticide degradation in the BPS. Future microcosm studies spiked with specific pesticides of choice (Dejonghe *et al.*, 2003, Sørensen *et al.*, 2005a) combined with deeper sequencing of specific phyla or classes such as *Betaproteobacteria,* will likely help to better understand and predict the roles of different bacterial groups in the biodegradation of pesticides in BPS. Of specific interest could be 16S rRNA gene amplicon sequencing of microcosm material to find out where TM7 might play a role.

In conclusion, the data obtained in the present work showed that changing concentration of pesticides shaped the composition of the bacterial communities in the BPS, selecting bacterial populations able to survive by using pesticides as carbon and nitrogen

sources. However, the overall community changes were minor compared to previously seen changes in the composition of conjugative plasmids, indicating that horizontal transfer is an important factor in the development of an effective pesticide degrading community in the BPS. This is thus an important piece of the puzzle to elucidate the bacterial community composition and its changes in a BPS as a result of pesticides addition.

Acknowledgments

This work was funded by the European Commission's 7th Framework Programme (MetaExplore 222625) and the Deutsche Forschungsgemeinschaft project SM59/8-1.

Tables and Figures

Table 1. Primers targeting the 16S rRNA gene

Target	Primer	Sequence (5' – 3')	Reference
Bacteria & *Archaea*	341F	CCTAYGGGRBGCASCAG	(Hansen, *et al*., 2012)
Bacteria & *Archaea*	806R	GGACTACNNGGGTATCTAAT	(Hansen, *et al*., 2012)
Bacteria	F984GC	AACGCGAAGAACCTTAC	(Heuer, *et al*., 1997)
Bacteria	R1378	CGGTGTGTACAAGGCCCGGGAACG	(Heuer, *et al*., 1997)
Alphaproteobacteria	F203α	CCGCATACGCCCTACGGGGGAAAGATTTAT	(Gomes, *et al*., 2001)
Betaproteobacteria	F948β	CGCACAAGCGGTGGATGA	(Gomes, *et al*., 2001)
Actinobacteria	F243	GGATGAGCCCGCGGCCTA	(Heuer, *et al*., 1997)
GC-clamp [a]		CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG	(Nübel, *et al*., 1996)

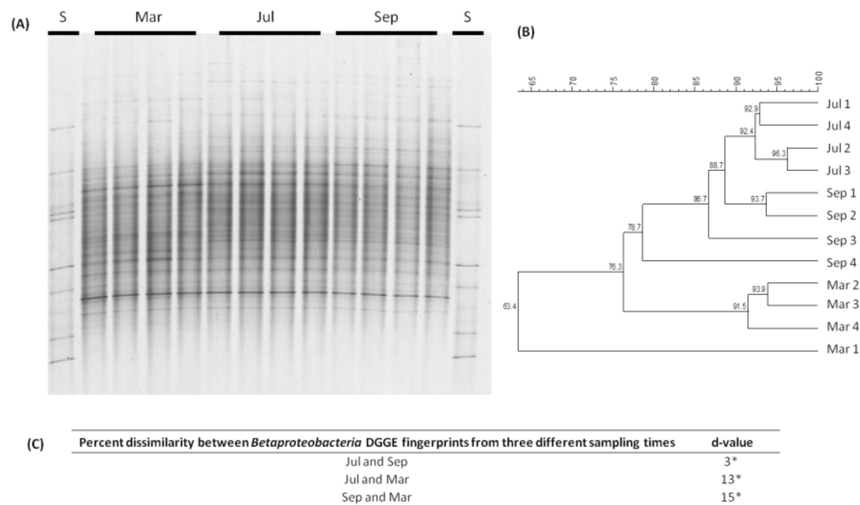

Fig. 1. (A) DGGE profile of PCR amplified 16S rRNA gene of *Betaproteobacteria* from four replicates (1-4) over three sampling times (March, July and September). S indicates bacterial DGGE standard. (B) UPGMA cluster analysis of *Betaproteobacteria* based on Pearson correlation. Bootstrap values in percent are indicated for each node. Scale is percent sequence similarity. (C) Significant (*, $p < 0.05$) difference between *Betaproteobacteria* over three sampling times. Percent dissimilarity = average within-group pairwise Pearson's correlation – average between between-group pairwise Pearson's correlation.

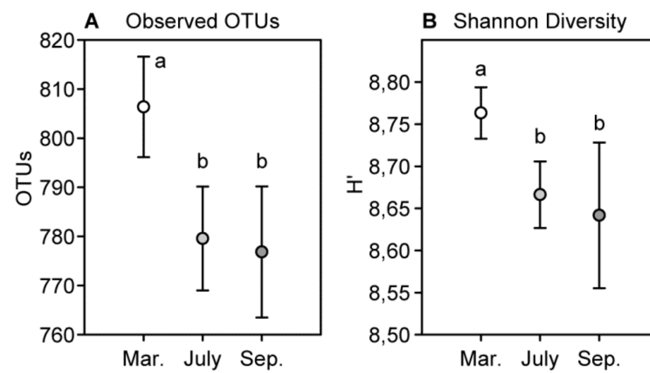

Fig. 2. The number of OTUs (a) and Shannon Diversity (b) at the three sampling times. Letters indicate significant differences between sample means (Tukey HSD). The difference between March and July was highly significant (ANOVA, $p < 0.001$). Dots are mean with S.E. ($n = 4$).

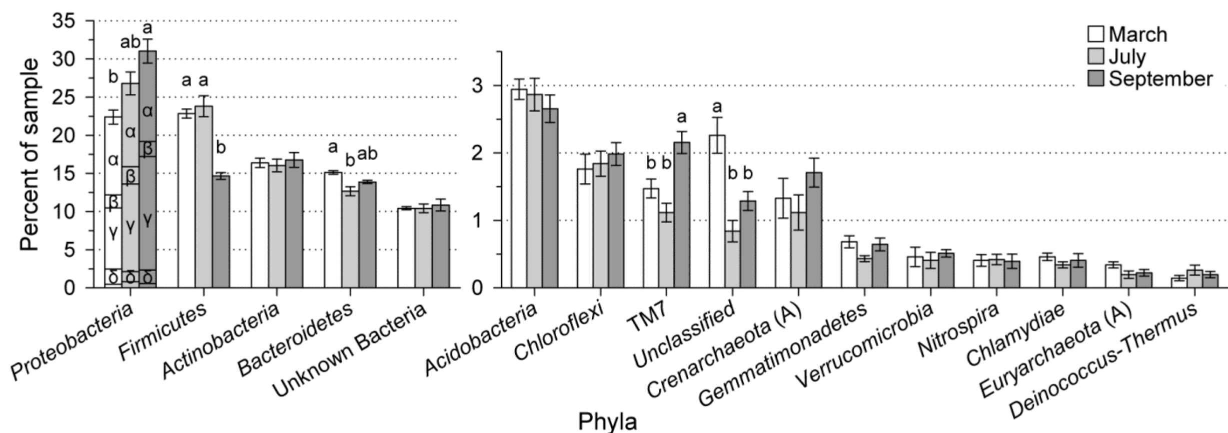

Fig. 3. Relative abundance of the 16 largest phyla present in the samples encompassing 99.4% of sequences. Classes are shown for *Proteobacteria*. Statistical significance changes in abundance within phyla was identified by one-way ANOVA and found to be highly significant ($p < 0.01$, *Firmicutes* $p < 0.001$) and differences in means within a phyla (Tukey HSD, $p < 0.05$) are shown by letters a and b above bars. From March to September a significant (ANOVA, $p < 0.05$) increase in *Gammaproteobacteria* and decrease in *Deltaproteobacteria* was also identified. *Archaea* are marked by (A). Bars are mean with S.E. ($n = 4$).

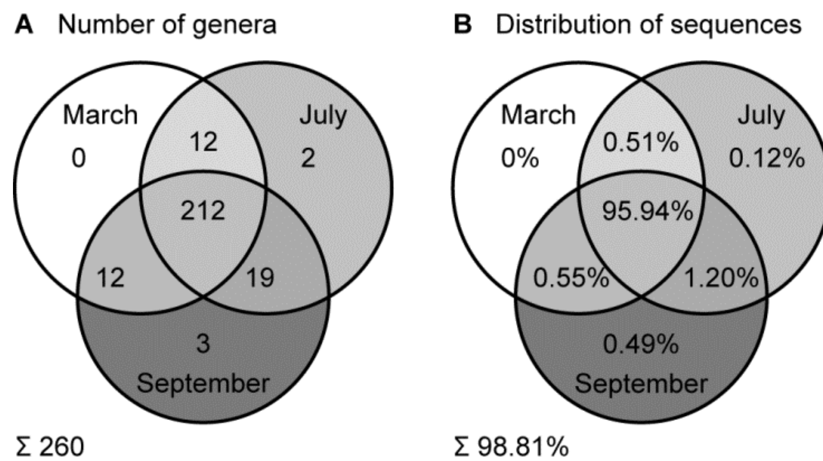

Fig. 4. Distribution of genera (A) and the sequences associated with them (B) between the three sampling times. Only the 260 genera that include more than three sequences are shown out of the total 421. The names of the genera not found at all sampling times are found in Supplemental Material Table S1 along with their relative abundances.

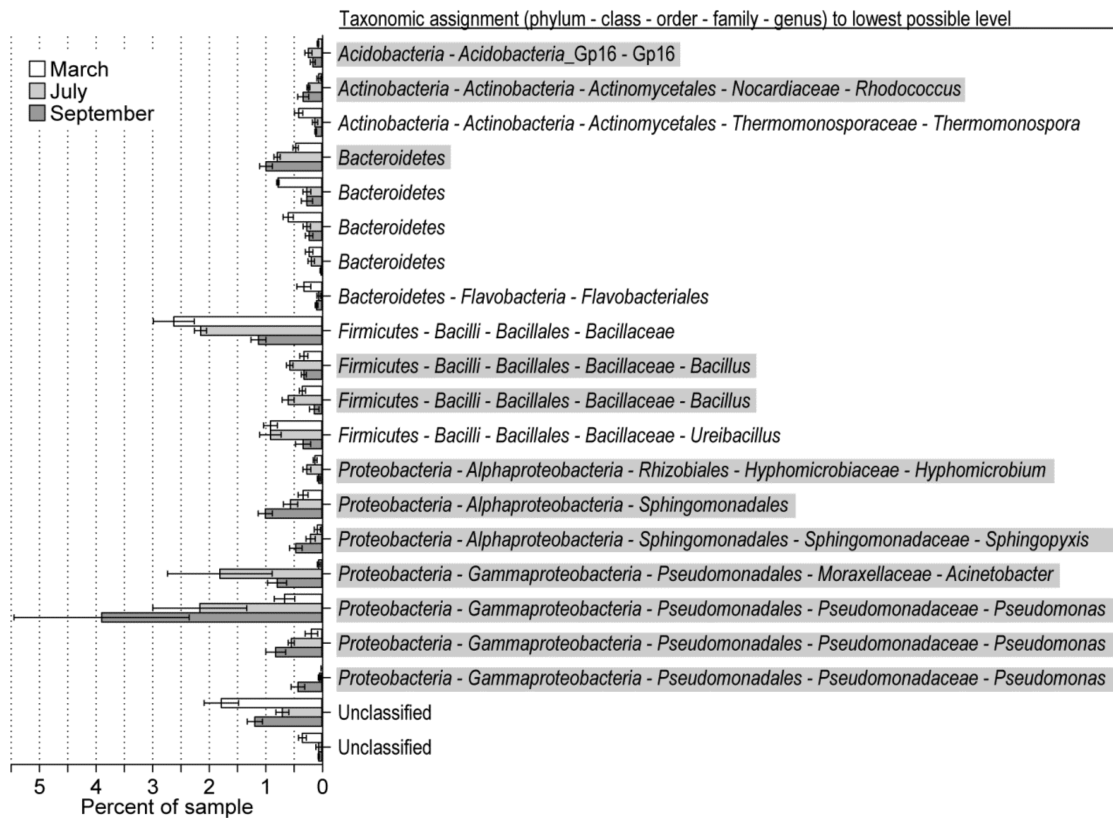

Fig. 5. OTUs found to have a statistically significant change (ANOVA, $p < 0.05$) in relative abundance between sampling times. The OTUs have been taxonomically classified to the lowest possible level against the GreenGenes database. OTUs that increased in abundance in July were pesticide concentrations where the highest are marked by gray boxes. Bars are mean with S.E. ($n = 4$).

Supplement material

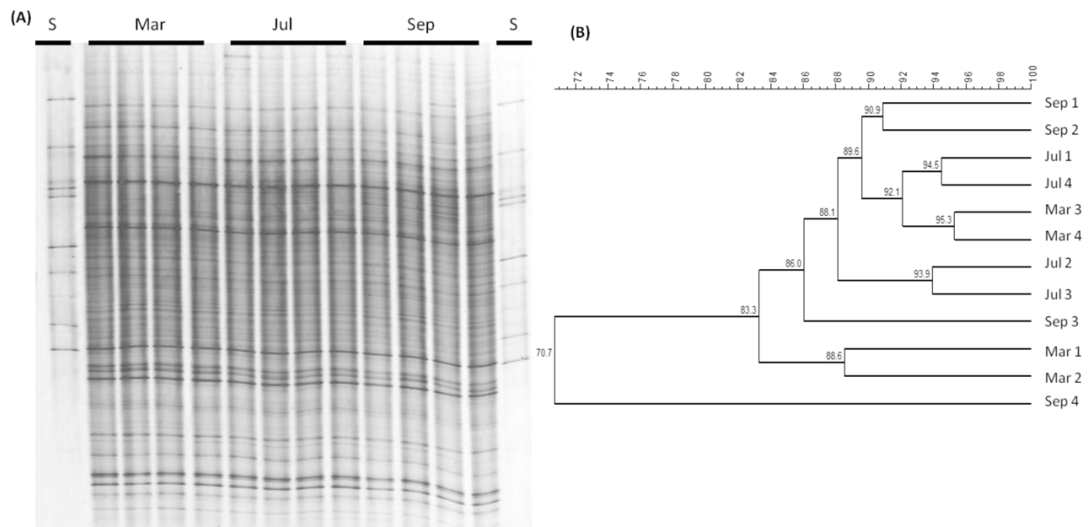

Fig. S1. (A) DGGE profile of PCR amplified 16S rRNA gene of the total bacterial community from four replicates (1-4) over three sampling times (March, July and September). S indicates bacterial DGGE standard. (B) UPGMA cluster analysis of the total bacterial community based on Pearson correlation. Bootstrap values in percent are indicated for each node. Scale is percent sequence similarity.

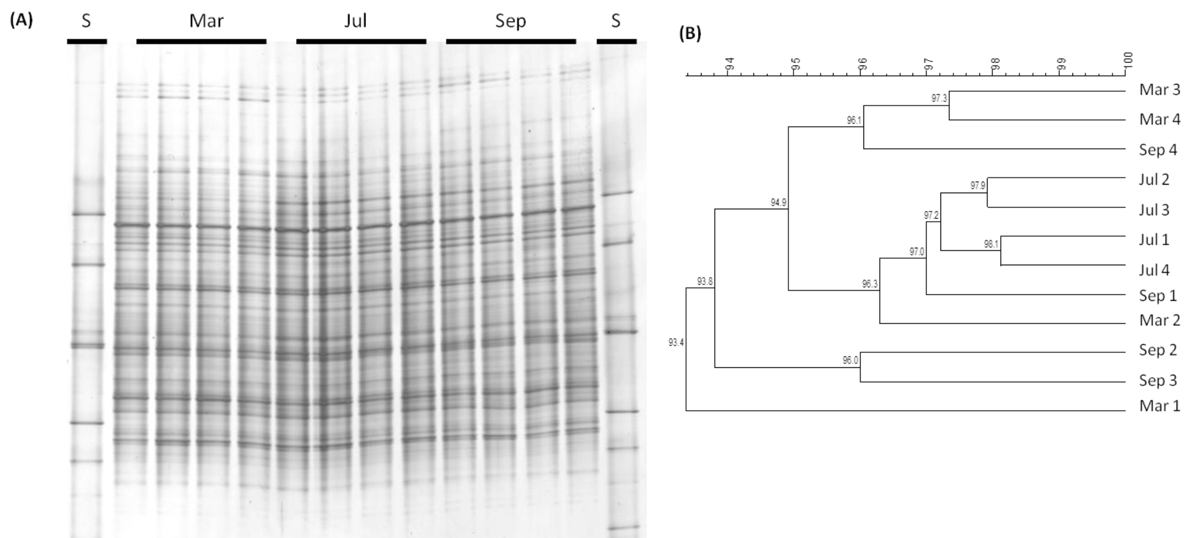

Fig. S2. (A) DGGE profile of PCR amplified 16S rRNA gene of *Alphaprotobacteria* from four replicates (1-4) over three sampling times (March, July and September). S indicates bacterial DGGE standard. (B) UPGMA cluster analysis of *Alphaprotobacteria* based on Pearson correlation. Bootstrap values in percent are indicated for each node. Scale is percent sequence similarity.

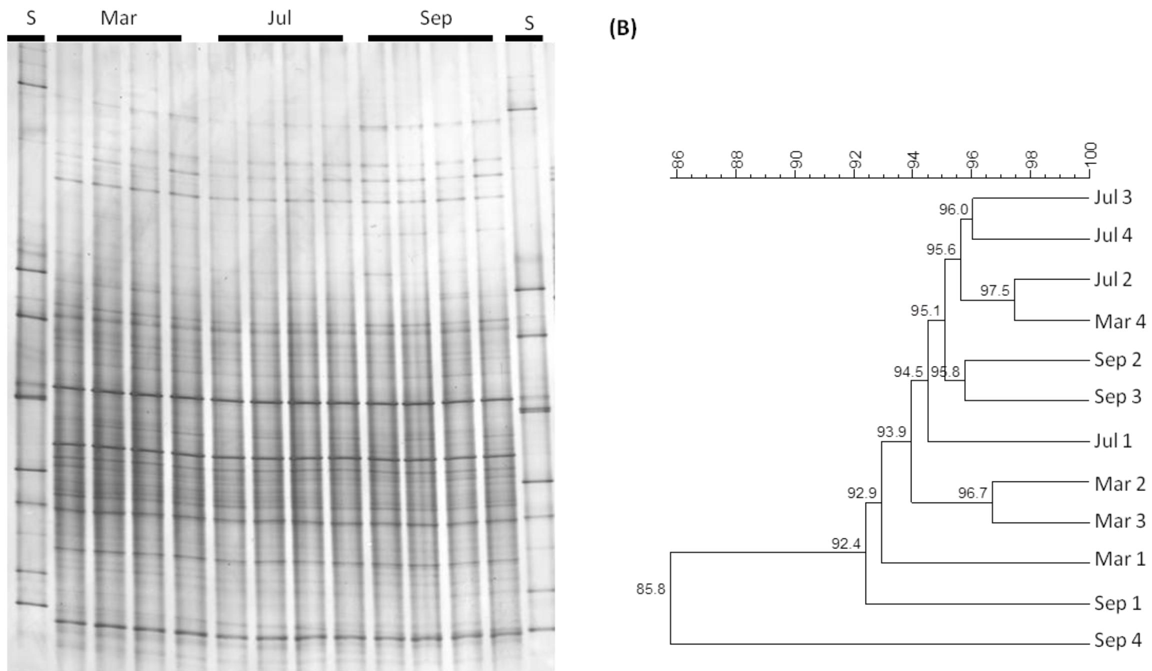

Fig. S3. (A) DGGE profile of PCR amplified 16S rRNA gene of *Actinobacteria* from four replicates (1-4) over three sampling times (March, July and September). S indicates bacterial DGGE standard. (B) UPGMA cluster analysis of *Actinobacteria* based on Pearson correlation. Bootstrap values in percent are indicated for each node. Scale is percent sequence similarity.

Table S1. Genera unique to sampling times

A total of 421 genera were found and 260 contained more than three sequences in total. Of these 212 genera (81.5%) were found at all sampling times and contained 95.94% of all sequences. The 48 genera not found at all three sampling times are listed below with taxonomic classification and mean relative abundance. *Archaea* is marked with gray. 0.0525% is equal to 1 sequence.

	Phylum	Class	Order	Family	Genus	Mar	Jul	Sep
Taxa only in Jul	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Thermomonosporaceae*	*Actinocorallia*		0.05	
	Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Myroides*		0.07	
Taxa only in Sep	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Micrococcaceae*	*Nesterenkonia*			0.08
	Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Limnobacter*			0.07
	Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	*Halomonadaceae*	*Halomonas*			0.34
Common taxa in Mar and Jul	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Flammeovirgaceae*	*Reichenbachiella*	0.05	0.01	
	Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Ruminococcus*	0.03	0.07	
	Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Rhodoplanes*	0.03	0.03	
	Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Methylocystaceae*	*Methylocystis*	0.04	0.03	
	Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Amaricoccus*	0.03	0.03	
	Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Acetobacteraceae*	*Roseomonas*	0.03	0.05	
	Proteobacteria	*Betaproteobacteria*	*Hydrogenophilales*	*Hydrogenophilaceae*	*Thiobacillus*	0.08	0.04	
	Proteobacteria	*Betaproteobacteria*	*Methylophilales*	*Methylophilaceae*	Other	0.07	0.04	
	Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacteraceae*	*Geobacter*	0.01	0.04	
	Proteobacteria	*Deltaproteobacteria*	*Myxococcales*	*Cystobacteraceae*	Other	0.07	0.03	
	Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Perlucidibaca*	0.07	0.13	
	Verrucomicrobia	Other	Other	Other	Other	0.04	0.01	
Common taxa in Mar and Sep	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*	*Methanoculleus*	0.13		0.04
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Dermabacteraceae*	*Brachybacterium*	0.01		0.04
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Streptosporangiaceae*	*Microbispora*	0.07		0.03
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Streptosporangiaceae*	*Sphaerisporangium*	0.04		0.01
	Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	*Chitinophagaceae*	*Lacibacter*	0.03		0.04
	Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	*Cytophagaceae*	*Cytophaga*	0.08		0.01
	Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*	*Pelagibius*	0.03		0.03
	Proteobacteria	*Deltaproteobacteria*	*Bdellovibrionales*	*Bacteriovoracaceae*	*Bacteriovorax*	0.04		0.03
	Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Desulfuromonadaceae*	Other	0.01		0.04
	Proteobacteria	*Deltaproteobacteria*	*Myxococcales*	*Nannocystaceae*	*Enhygromyxa*	0.09		0.08
	Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Shewanellaceae*	*Shewanella*	0.01		0.07
	Proteobacteria	*Gammaproteobacteria*	*Methylococcales*	*Methylococcaceae*	*Methylobacter*	0.11		0.04
Common taxa in Jul and Sep	*Acidobacteria*	*Acidobacteria*_Gp7	Gp7	Other	Other		0.07	0.01
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Brevibacteriaceae*	*Brevibacterium*		0.03	0.16
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	*Salinibacterium*		0.01	0.04
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Nocardiaceae*	Other		0.01	0.04
	Actinobacteria	*Actinobacteria*	*Solirubrobacterales*	*Solirubrobacteraceae*	*Solirubrobacter*		0.04	0.04
	Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Chryseobacterium*		0.04	0.03
	Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	*Cyclobacteriaceae*	Other		0.05	0.07
	Firmicutes	*Bacilli*	*Bacillales*	*Planococcaceae*	*Planococcus*		0.04	0.05
	Firmicutes	*Clostridia*	*Thermoanaerobacterales*	*Thermoanaerobacteraceae*	*Tepidanaerobacter*		0.03	0.03
	Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	Other		0.03	0.04
	Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*	*Rhizobium*		0.03	0.03
	Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Paracoccus*		0.04	0.04
	Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Herminiimonas*		0.07	0.09
	Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	Other		0.14	0.08
	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Other		0,04	0,01
	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas		0,04	0,22
	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Pseudoxanthomonas		0,12	0,12
	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas		0,18	0,18
	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Thermomonas		0,11	0,03

Chapter VI: Conclusions and main findings

- ✓ Rich reservoirs of catabolic plasmids such as IncP-1, IncP-7 and IncP-9 were indicated and suggested hot-spots of horizontal catabolic gene transfer (Chapter II);
- ✓ IncP-1 plasmids seem to be widespread in different environments, indicating their stability, broad-host-range and important role in the biodegradation (Chapter II);
- ✓ Biopurification systems (BPS) have an uncommonly high abundance of IncP-1 plasmids which we assume to be essential players in the degradation of man-made compounds such as pesticides (Chapter III);
- ✓ The data presented in this PhD thesis provided a proof of correlation between pollution and abundance of plasmids (Chapters III and IV);
- ✓ The concentration of pollutants seemed to influence the abundance of different mobile genetic elements (MGEs);
- ✓ Exogenous plasmid isolation through biparental assay using $HgCl_2$ as selective pressure can be used as a good alternative to co-capture catabolic genes carried by degradative plasmids (Chapter III);
- ✓ For the first time IncP-1 and IncP-9 plasmids could be captured into the same host, which might be possible due to the permissiveness of *Pseudomonas putida KT2442 gfp* used as recipient cell in exogenous plasmid isolation from BPS (Chapter III);
- ✓ By applying exogenous plasmid isolation through triparental matings different plasmids, likely carrying catabolic genes were captured in comparison to exogenous plasmid isolation through biparental mating;
- ✓ Indeed, as revealed by 16S rRNA gene amplicon sequencing, bacterial communities tend to be affected when exposed to pollutants such as pesticides, however, their composition was rather stable when compared to the shifts in the abundance and composition of MGEs, indicating an intense horizontal gene transfer in order to guarantee bacterial survival in polluted environments (Chapters III, IV and V).

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Supplement Material

S1. Quantification of IncP-1 Plasmid Prevalence in Environmental Samples

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To study the role of broad-host-range IncP-1 plasmids in bacterial adaptability to irregular environmental challenges, a quantitative real-time PCR assay was developed that specifically detects the korB gene, which is conserved in all IncP-1 plasmids, in environmental samples. IncP-1 plasmid dynamics in a biopurification system for pesticide wastes were analyzed.

Horizontal gene transfer by broad-host-range plasmids plays a vital role in the adaptation and robustness of bacteria to irregular or novel environmental challenges or opportunities (1, 2). Broad-host-range plasmids are also significantly contributing to the spread of antibiotic resistance (3). Plasmids of the incompatibility group IncP-1 (also called IncP in the classification scheme of *Enterobacteriaceae* plasmids) are assumed to especially foster the horizontal gene transfer because of their stable replication in a wide range of Gram-negative bacteria and their efficient conjugative transfer to an even wider range of taxa (4, 5). They consist of a conserved backbone carrying genes for plasmid persistence and conjugative transfer, and typically regions with diverse accessory genes that vary between plasmids. Accessory genes often encode antibiotic or metal resistances or degradative pathways (6). IncP-1 plasmids were first discovered in bacteria from clinical specimens (7, 8), and subsequently were found in many geographic regions and diverse environments including agricultural soil, salt marsh, manure, compost, sewage, water, and sediment (6, 9-13). However, the environmental distribution of IncP-1 plasmids and the factors promoting their frequency in bacterial communities are not well explored. High abundance of these plasmids seemed to be related to environmental disturbances like pollution (10, 11, 14). The detection in microbial community DNA was first based on PCR-amplification in combination with Southern blot hybridization of fragments of the *trfA* gene (12), which codes for the replication initiation protein. These primers were developed based on sequences of the subgroups IncP-1α and β. The discovery of IncP-1 plasmids with largely divergent backbone sequences (9, 15, 16) led to the development of new primer systems for detection of *trfA* of the IncP-1 subgroups α, β, γ, δ, and ε (17). However, also these primers do not target all known IncP-1 plasmid types such as the newly described ζ subgroup (18) or pKS208 (accession JQ432564) which is most similar to the IncP-1γ plasmid pQKH54 (9). The problem to design one primer system to detect all known IncP-1 plasmid backbones for all IncP-1 plasmids based on *trfA* is caused by its relatively high evolutionary rate (Fig. 1), which is probably a result of adaptation to interacting host proteins in diverse hosts (20). Thus, *trfA* does not provide enough conserved sites as primer targets and, moreover, as an intermediate target for a TaqMan probe to enable specific quantification of IncP-1 plasmids in environmental DNA by a real-time PCR 5' nuclease assay (qPCR). We searched the IncP-1 backbones for such a conserved region. All common backbone genes of the IncP-1

subgroups were aligned and relative evolutionary rates determined (Fig. 1). Among the most conserved regions found within *trbC*, *trbE*, *traG*, *traI*, and *korB*, the latter target gene was most suited to design a qPCR system. The *korB* product is evolutionarily constrained because it interacts with itself to form dimers, with IncC, KorA and multiple DNA binding sites of the plasmid as essential component of the partition system and the regulatory network of the plasmid (21, 22).

Two forward and three reverse primers were combined to minimize both degeneracy and mismatches to targets (Table 1). A single TaqMan probe was sufficient for qPCR with tested plasmids of all IncP-1 subgroups. An exception was the IncP-1γ plasmid pKS208, for which two mismatches to the first five 5' bases of the TaqMan probe impeded 5' nuclease activity. This problem could be solved by adding the TaqMan probe Pgz to the PCR to target IncP-1γ and ζ plasmids including also more divergent variants (Table 1). Target DNA was amplified in reactions containing 5 µl DNA solution, 1.25 U TrueStart Taq DNA polymerase and supplied buffer (Fermentas, St. Leon-Rot, Germany) in 50 µl, 0.2 mM of each deoxynucleoside triphosphate, 3.5 mM $MgCl_2$, 0.1 mg/ml Bovine Serum Albumin (Fermentas), 0.4 µM of primers F and R, 0.2 µM of primers Fz, Rge and Rd, 0.3 µM of TaqMan probes P and Pgz. Reactions were run at 5 min 95°C, and 40 cycles of 15 s 95°C, 15 s 54°C, 60 s 60°C in a real-time PCR system (CFX96, Bio-Rad, Munich, Germany). The annealing step at 54°C reduced the variance of cycle threshold values between cloned *korB* variants from six IncP-1 subgroups (data not shown), indicating an effect of sequence variation on amplification. To estimate the bias caused by this effect, the qPCR efficiencies of cloned *korB* fragments of plasmids representing the different subgroups and pKS208 were compared (Fig. 2). The amplification efficiencies of the *korB* variants did not differ significantly, as calculated from slopes of linear regression curves and test on parallelism (p=0.07, PROC GLM, statistical package SAS 9.3). Efficiency of the PCR was on average 88%, and detection limit was 10 copies. The lowest cycle thresholds with given initial concentrations were achieved with *korB* of pB10 and the highest with pKS208 and pKJK5. This variation between plasmids resulted in standard deviations of 0.4-0.5 log units at environmentally relevant concentrations (Fig. 3). The standard deviation increased with decreasing initial concentration. Around one third of this error could be explained by variation attributable to plasmid extraction and serial dilution of quantification standards, as shown by independent replicate preparations (Fig. 3).

The *korB* assay was applied to analyse the abundance and dynamics of IncP-1 plasmids in a large pesticide-degrading biofilter operated on a farm near Kortrijk, Belgium, which was previously characterized (23). It was sampled three times over a season with continuing applications of pesticide wastes, before start-up (March), during processing (July),

and after close down (September). The biofilter, composed of coco chips, straw and soil, received 37 different active compounds including 16 halogenated aromatics (23). Total DNA was extracted from 0.5 g of 2 mm-sieved biofilter samples using the FastPrep FP120 bead beating system for cell lysis and the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) and purified using the GENECLEAN Spin Kit (MP Biomedicals). The copy numbers of *korB* were determined as described here, and 16S rRNA genes were quantified using the qPCR system developed by Suzuki *et al.* (24). In addition, *trfA* copies of IncP-1ε plasmids were measured, as previously described (10), to test whether more plasmids are detected by the qPCR targeting *korB*. A dilution series of plasmid pKJK5 was used as a common quantification standard for both *korB* and *trfA*. Gene copies were related to 16S rRNA gene copies to account for differences in bacteria concentration or amplification efficiency in the samples. The *korB* assay detected 2 to 4 times more IncP-1 plasmids than the *trfA* assay, which was significant for all three samplings and which confirmed the intended broader specificity (Fig. 4). Concomitant with continued pesticide applications, the relative abundance of IncP-1 plasmids in the bacterial biofilter community increased from March till September reaching values of up to 0.2%. This might indicate an important contribution of IncP-1 plasmids to pesticide degradation, as previously suggested based on degradative pathways located on several IncP-1 plasmids (6).

Thus, our results indicate that we have successfully developed a rapid and sensitive method which allows the quantification of all known IncP-1 subgroups in environmental samples despite their diverse backbones. Its application will give further insight into the relative abundance of IncP-1 plasmids in bacterial populations, and their role in the spread of resistance genes, degradation pathways of recalcitrant compounds or other traits of the horizontal gene pool which allow a fast response of bacterial sub-populations to irregular environmental changes.

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TABLE 1. Alignment of primers and TaqMan probes (5‘ - 3‘) with corresponding sequences of *korB* genes which represent all known variants decribed so far for the known IncP-1 plasmid subgroups

Plasmid (accession)	Sg[a]	Forward primers[b]	Reverse primers	TaqMan probes (5’-FAM, 3‘-TAMRA)
		TCATCGACAACGACTACAACG (F)	TTCTTCTTGCCCTTCGCCAG (R)	TCAGYTCRTTGCGYTGCAGGTTCTCVAT (P)
		TCGTGGATAACGACTACAACG (Fz)	TTYTTCYTGCCCTTGGCCAG (Rge)	TSAGGTCGTTGCGTTGCAGGTTYTCAAT (Pgz)
			TTCTTGACTCCCTTCGCCAG (Rd)	
pB3 (AJ639924)	β1	TCATCGACAACGACTACAACG	TTCTTCTTGCCCTTCGCCAG	TCAGCTCGTTGCGTTGCAGGTTCTCGAT
pBP136 (AB237782)	β1	TCATCGACAACGACTACAACG	TTCTTCTTGCCCTTCGCCAG	TCAGCTCATTGCGTTGCAGGTTCTCGAT
pA1 (AB231906)	β2	TCATCGACAACGACTACAACG	TTCTTCTTGCCCTTCGCCAG	TCAGCTCGTTGCGTTGCAGGTTCTCGAT
pA81 (AJ515144)	β2	TCATCGACAACGACTACAACG	TTCTTCTTGCCCTTCGCCAG	TCAGTTCGTTGCGTTGCAGGTTCTCGAT
pAKD4 (GQ983559)	δ	TCATCGACAACGACTACAACG	TTCTTGACTCCCTTCGCCAG	TCAGTTCATTGCGCTGCAGGTTCTCCAC
pIJB1 (JX847411)	δ	TCATCGACAACGACTACAACG	TTCTTGACTCCCTTCGCCAG	TCAGTTCATTGCGCTGCAGGTTCTCCAC
pBS228 (AM261760)	α	TCATCGACAACGACTACAACG	TTCTTCTTGCCCTTCGCCAG	TCAGCTCGTTGCGTTGCAGGTTCTCGAT
pTB11 (AJ744860)	α	TCATCGACAACGACTACAACG	TTCTTCTTGCCCTTCGCCAG	TCAGCTCGTTGCGTTGCAGGTTCTCGAT
pQKH54 (AM157767)	γ	TCATCGACAACGACTACAACG	TTTTTCTTGCCCTTGGCCAG	TCAGGTCGTTGCGTTGCAGGTTCTCAAT
pKS208 (JQ432564)	γ	GGATCGACAACGACTACAACG	TTCTTCTTGCCCTTGGCCAG	TGAGGTCGTTGCGTTGCAGGTTTTCAAT
pMCBF1 (AY950444)	ζ	TCGTGGATAACGACTACAACG	TTCTTCTTACCCTTCGCCAG	TCAGCTCGTTGCGTTGCAGGTTCTCAAT
pMCBF6 (EF107516)	ζ	TCGTGGATAACGACTACAACG	TTCTTCTTACCCTTCGCCAG	TCAGCTCGTTGCGTTGCAGGTTCTCAAT
pKJK5 (AM261282)	ε	TCATCGACAACGACTACAACG	TTTTTCCTGCCCTTGGCCAG	TCAGCTCGTTGCGTTGCAGGTTCTCGAT
pAKD16 (JN106167)	ε	TCATCGACAACAACTACAACG	TTCTTCTTGCCCTTGGCCAG	TCAGCTCGTTGCGTTGCAGGTTCTCGAT

[a] Sg: IncP-1 subgroup; [b] Mismatches of primers or TaqMan probes to target are underlined

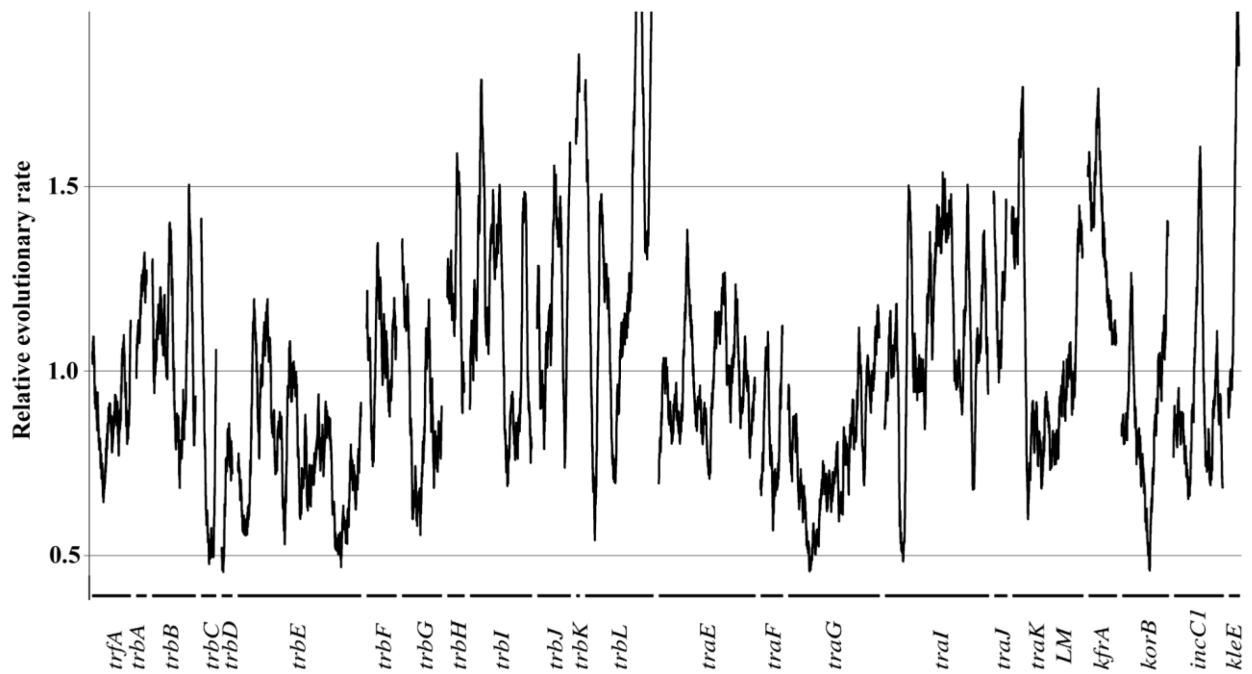

FIG. 1. Relative evolutionary rates of genes common to the backbones of all known IncP-1 subgroups (α, β1, β2, γ, δ, ε, ζ). Genes of two representative plasmids of each subgroup (see Table 1) were aligned and position-by-position evolutionary rates determined using Mega5 (19). The scaled rates (average evolutionary rate across all sites is 1) are displayed as moving averages with a window width of 100 bp.

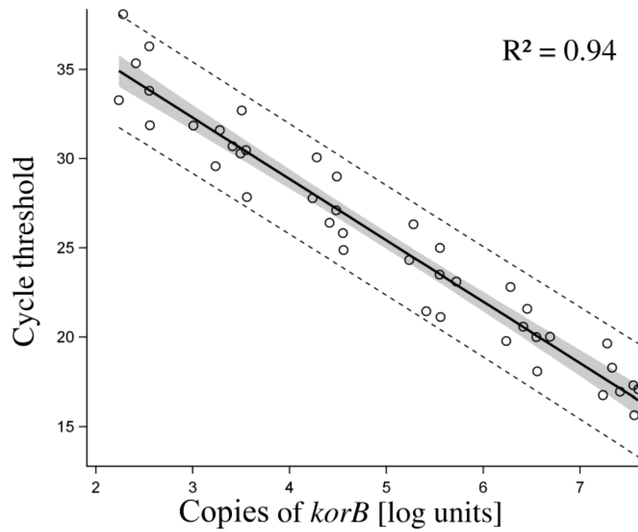

FIG. 2. Cycle threshold values related to initial gene copy numbers of cloned *korB* genes from six plasmids (pB10, pEST4011, pKJK5, pMCBF1, pQKH54, RP4) representing the different IncP-1 subgroups and pKS208. Solutions of *korB* fragments ligated into pGEM-T (Promega) with similar concentration (OD_{260} 0.5, $OD_{260}/OD_{280} > 1.8$) were serially diluted and applied to the developed real-time PCR 5' nuclease assay. Grey area and dashed lines indicate 95% confidence limits and prediction limits of linear regression analysis, respectively.

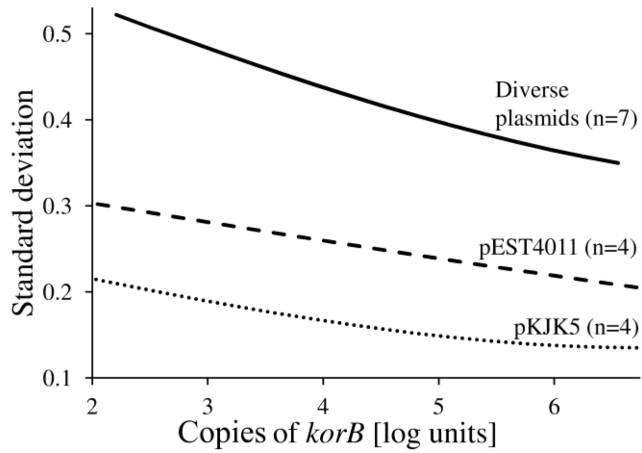

FIG. 3. Standard deviations of *korB* gene quantification in dependence of the initial concentration, calculated from regression curves, for seven *korB* variants representing the IncP-1 subgroups and pKS208 (solid line), and for four independent preparations of the quantification standards for plasmids pEST4011 and pKJK5, respectively.

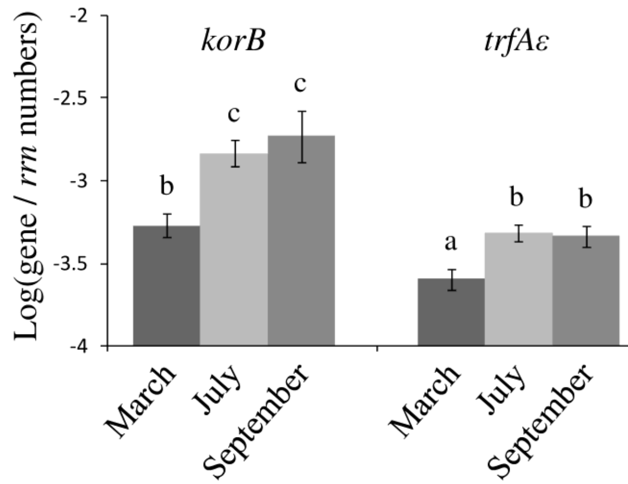

FIG. 4. Abundance of *korB* of IncP-1 plasmids and *trfA* of IncP-1ε plasmids in a biofilter that degraded various pesticides during the growing season. Plasmid copies were related to bacterial 16S rRNA gene copies (*rrn*) quantified from the same sample. Different letters indicate significant differences (Tukey test; spatially separated compartments within the 20 m x 1.2 m biofilter were used as replicates: n=3 for March, n=4 for July and September.

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