Environmental Pollution xxx (2017) 1–9

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Response of the bacterial community in an on-farm biopurification system, to which diverse pesticides are introduced over an agricultural season☆

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ARTICLE INFO

Article history:
Received 7 March 2017
Received in revised form
6 July 2017
Accepted 8 July 2017
Available online xxx

Keywords:
Bacterial communities
Shannon diversity
16S rRNA gene sequencing
DGGE profiling
Biopurification system
Pesticide degradation

ABSTRACT

A biopurification system (BPS) is used on-farm to clean pesticide-contaminated wastewater. Due to high pesticide loads, a BPS represents a hot spot for the proliferation and selection as well as the genetic adaptation of discrete pesticide degrading microorganisms. However, while considerable knowledge exists on the biodegradation of specific pesticides in BPSs, the bacterial community composition of these systems has hardly been explored. In this work, the Shannon diversity, the richness and the composition of the bacterial community within an operational BPS receiving wastewater contaminated with various pesticides was, for the first time, elucidated over the course of an agricultural season, using DGGE profiling and pyrosequencing of 16S rRNA gene fragments amplified from total community DNA. During the agricultural season, an increase in the concentration of pesticides in the BPS was observed along with the detection of significant community changes including a decrease in microbial diversity. Additionally, a significant increase in the relative abundance of *Proteobacteria*, mainly the *Gammaproteobacteria*, was found, and OTUs (operational taxonomic units) affiliated to *Pseudomonas* responded positively during the course of the season. Furthermore, a banding-pattern analysis of 16S rRNA gene-based DGGE fingerprinting, targeting the *Alpha*- and *Betaproteobacteria* as well as the *Actinobacteria*, indicated that the *Betaproteobacteria* might play an important role. Interestingly, a decrease of *Firmicutes* and *Bacteroidetes* was observed, indicating their selective disadvantage in a BPS, to which pesticides have been introduced.
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1. Introduction

The use and/or misuse of pesticides in agriculture have resulted in serious pollution of the environment, eventually leading to adverse effects on human health (Field et al., 1997; Spliid and Koppen, 1998). In Europe, pesticide contamination has in many places been observed in ground and surface water, and is often a result of point source contamination due to pesticide handling involving spillages from the filling of sprayers, leakages of pesticide containers, spray leftover, and rinsing water from the internal and external cleaning of the spray apparatus (Kreuger, 1998; De Wilde et al., 2007). In order to control pesticide point source contamination, a simple, low cost and practical on-farm bioremediation approach has been proposed, referred to as biopurification systems (BPSs) (Karanasios et al., 2012). In on-farm BPSs, pesticide-contaminated wastewater is percolated over a solid, biologically active matrix, the biomix, which typically is composed of a homogenized mixture of straw, peat, manure, or composted material, as well as local agricultural soil, the latter being the main source of pesticide-degrading microorganisms. In the BPS, the pesticides are

☆ This paper has been recommended for acceptance by Klaus Kummerer.

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http://dx.doi.org/10.1016/j.envpol.2017.07.026
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removed from the wastewater by sorption and/or biodegradation processes. Biodegradation has previously been indicated as a major process of pesticide removal in different environments (Cullington and Walker, 1999; Verma et al., 2014). By using 16S rRNA gene cloning and sequencing for studying changes in community structure during bioremediation, bacterial populations affiliated to *Proteobacteria, Actinobacteria and Acidobacteria* were previously described as the dominant taxa in environments polluted with organic compounds and pesticides (Paul et al., 2006). In other studies focusing on BPSs exposed to mixtures of different fungicides commonly applied in vineyards, denaturing gradient gel electrophoresis (DGGE) analysis revealed that either fungal strains in general (Marinozzi et al., 2013), or yeast flora and ascomycete filamentous fungi in particular (Coppola et al., 2011), seemed to be involved in the biodegradation process. Although several studies have focused on the degradation of selected pesticides in on-farm BPSs, e.g. linuron (Sniegowski et al., 2011), atrazine (Tortella et al., 2013a), carbendazim (Tortella et al., 2013b) and isoproturon (von Wiren-Lehr et al., 2001), as well as on the important role of mobile genetic elements (MGEs) (Dunon et al., 2013; Jechalke et al., 2013; Dealtry et al., 2014), the bacterial community composition in an operational BPS has not yet been thoroughly studied. The efficiency of the BPS is largely based on the capacity of the biomixture to degrade pesticide loads being discharged on the biomix during the season. The microbial communities inhabiting the biomix, therefore, are key in controlling the depuration efficiency of the BPS, and accordingly the understanding of the microbial community dynamics within these systems is of use to further optimize BPS performance. Recent studies demonstrated that a large operative BPS located on a farm in Kortrijk, Belgium (the same BPS as studied in the present work), showed a high prevalence of MGEs including IncP-1 and IncP-9 catabolic plasmids (Dealtry et al., 2014) and IS*1071* insertion sequences (Dunon et al., 2013). These genetic elements are key factors in controlling horizontal gene transfer (HGT) in xenobiotic degrading bacteria. However, investigations of the bacterial populations hosting such MGEs are still inadequate. As the metabolic activity of the bacterial community is expected to directly influence the stability, maintenance, expression level and transferability of the MGEs carried by it, it becomes crucial to study the diversity, richness and composition of the bacterial populations of the BPS likely to carry MGEs encoding genes facilitating pesticide degradation.

Based on the hypothesis that pesticides are toxic to some bacteria (DeLorenzo et al., 2001), but favorable to bacteria, which can utilize the pesticides as energy source (Breugelmans et al., 2007), the aim of this study was to gain a deeper insight into the response of the bacterial community inhabiting an operational on-farm BPS in Kortrijk, to which different concentrations of various pesticides had been introduced during a year, which reflects the farmers' consumption over the course of the agricultural season. Samples were collected at three time points of the agricultural season 2011, i.e., in March before the pesticide spraying season started, in July during the spraying season and in September after the spraying season, and total community DNA (TC-DNA) was extracted. The bacterial community composition, representing each sampling point, was subsequently investigated through pyrosequencing of the 16S rRNA gene fragments amplified from TC-DNA, and through DGGE profiling targeting only the specific taxa, *Alpha-* and *Betaproteobacteria* as well as *Actinobacteria*.

2. Materials and methods

2.1. Biopurification system (BPS) sampling

Samples were collected from a large BPS (20 m long, 1.2 m wide) located in Kortrijk, Belgium, operational since 2008, and containing a biomix composed of agricultural soil (25 vol%), composted material (25 vol%), and straw mixed with stable manure originating from a nearby horse manége (50 vol%). Sampling was performed as previously described (Dealtry et al., 2014). Briefly, the BPS was divided into four compartments, representing four replicates, and each compartment was sampled three times in the agricultural season of 2011, i.e., before (in March), during (in July) and after (in September) pesticide application.

The following pesticides from spillage and residue water collected during cleaning of spraying equipment were found by chemical analysis of the BPS samples, performed in a previous study (Dealtry et al., 2014): azoxystrobin, bentazone, diflufenican, diuron, epoxiconazole, ethofumesate, fenpropimorph, fluroxypyr, flufenacete, metamitron, metribuzine, propiconazole, S-metolachlor, tebuconazole and terbuthylazine. The measurements of these detected pesticides are listed in Table 1 along with additional pesticides that were included in the chemical analysis of the BPS material, but not detected. In addition to the pesticides detected in the BPS material (Table 1), a previous study reports the application of several other active pesticide compounds to the BPS (Dealtry et al., 2014). These are: aclonifen, asulam, chloridazon, chlormequat, chlorpropham, clomazon, clopyralid, dimethenamid-P, diquat, epoxiconazole, fenmedifam, florasulam, flurtamone, foramsulfuron, glyphosate, iodosulfuron-methyl-sodium, linuron, MCPA (2-methyl-4-chlorphenoxyacetic acid), MCPB (4-(4-chloro-o-tolyloxy)butyric acid), mefenpyr-diethyl, metsulfuron-methyl, nicosulfuron, pendimethalin, prosulfocarb, pyraclostrobin, sulcotrione and tepraloxydim. Of these, only MCPA was included in the chemical analysis of the BPS material, but not detected (Table 1).

2.2. Total community DNA extraction

Aliquots of 12 BPS samples (four replicates, each from three sampling times) were used for extraction of TC-DNA as previously described (Dealtry et al., 2014), using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). The extracted DNA samples were subsequently purified using the GeneClean Spin Kit (MP Biomedicals, Santa Ana, CA, USA).

2.3. DGGE analysis of 16S rRNA gene fragments

A nested PCR approach was performed on TC-DNA for amplification of the 16S rRNA gene fragments of *Actinobacteria*, *Alphaproteobacteria* and *Betaproteobacteria*, since these specific bacterial groups are known to include prominent pesticide degrading taxa (Paul et al., 2006). The nested PCR reactions were conducted as previously described (Heuer et al., 1997; Gomes et al., 2001), using the group-specific forward primers F243 (Heuer et al., 1997), F203α (Gomes et al., 2001), and F948β (Gomes et al., 2001), respectively, together with a general bacterial reverse primer, R1378 (Heuer et al., 1997), flanking the V3-V8, V2-V8, and V6-V8 variable regions of the 16S rRNA gene of *Actinobacteria*, *Alphaproteobacteria* and *Betaproteobacteria,* respectively. Primers are listed in Table 2. DGGE of the PCR amplicons was done as described by Ding et al. (2012) using silver staining, and comparison of DGGE profiles and band intensities was performed with the software GelCompar II 5.6 (Applied Maths, Sint-Martens-Latem, Belgium). A cluster analysis based on this similarity matrix was constructed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Smalla et al., 2007). In order to evaluate slight differences in fingerprints of bacterial communities visualized by DGGE, a statistical test analysis was applied by using permutation test. The permutation test was based on the pairwise similarity measures (correlation coefficients in the example), according to Kropf et al. (2004).

Please cite this article in press as: Holmsgaard, P.N., et al., Response of the bacterial community in an on-farm biopurification system, to which diverse pesticides are introduced over an agricultural season, Environmental Pollution (2017), http://dx.doi.org/10.1016/j.envpol.2017.07.026

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