International Biodeterioration & Biodegradation 133 (2018) 133–141

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

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

Requirement of ecological replication with independent parallel analysis of each replicate plot to support soil remediation

Yan Sun, Anna-Lea Rantalainen, Martin Romantschuk, Aki Sinkkonen*

Faculty of Biological and Environmental Sciences, Ecosystems and Environment Research Programme, University of Helsinki, Niemenkatu 73, FI-15140, Lahti, Finland

ARTICLE INFO

Keywords:
Ecological replication
Pseudoreplication
Soil heterogeneity
Bioremediation
Natural attenuation
Biostimulation
Gasoline contamination
Naphthalene

ABSTRACT

Consequences of pseudoreplication or the sole use of plot averages even within one ecosystem on the reliability of laboratory remediation tests have barely been studied even though they may mask field variability. Soils from three field plots were tested separately to reveal the consequences of inadequate use of replicates. Parallel remediation experiments consisting of treatments control (non-contaminated soil), natural attenuation (gasoline-contaminated soil) and biostimulation (gasoline-contaminated and methylene urea fertilized soil) were conducted to each plot soil. Three destructive sampling days and three pseudoreplicates per treatment, per plot and per sampling day were designed. The concentrations of total and individual gasoline aromatics were followed using both within-plot pseudoreplicates and plot averages. Results showed that natural attenuation of total or individual gasoline aromatics depended on field plots and that fertilization showed enhancing, inhibiting and negligible effects, respectively, also depending on field plots. Additionally, plot averages masked within-treatment variation between field plots. Conclusively, within-plot pseudoreplication using a single field plot or interpretations based solely on plot averages cannot reveal remediation patchiness observed in practice. Due to the high soil heterogeneity, ecological between-plot replication together with the parallel analysis of each replicate is encouraged in laboratory studies aiming to design *in situ* remediation strategies.

1. Introduction

Owing to their natural ecosystem function as degraders, microorganisms have been extensively studied and utilized for the remediation of organic contaminants in soils (Leahy and Colwell, 1990; Suja et al., 2014; Adams et al., 2015; Ghosal et al., 2016; Wu et al., 2016; Marchand et al., 2017). Bioremediation takes place when microorganisms feed on the organic contaminants for carbon and energy. For some organic contaminants natural attenuation – i.e. merely monitoring the situation – shows a significant potential for *in situ* remediation, while biostimulation (e.g. fertilization, aeration, application of biosurfactant) and bioaugmentation are frequently applied in cases where significant progress does not take place by natural attenuation, or this progress is too slow (Souza et al., 2014; Adams et al., 2015; Wu et al., 2016).

Laboratory experiments investigating soil remediation are a common first step in research projects mapping possibilities for soil remediation. The current work was initiated to optimize laboratory soil remediation studies for a more successful practical application. Gasoline aromatics were used as the model contaminants. Since biochemical mechanisms or pathways of biodegradation of aromatic compounds have been widely studied (Woo and Rittmann, 2000; Hendrickx et al., 2006; Haritash and Kaushik, 2009; Fuchs et al., 2011; Ghosal et al., 2016; Aydin et al., 2017) and gasoline aromatics (monoaromatics and naphthalene homologues) are fairly simple aromatic compounds, this study was focused on the methodological aspects in studying soil remediation, i.e. experimental design and result analysis.

Some important aspects that may lead to misleading or inconclusive laboratory results when studying biodegradation have been pointed out by Gu (2016), including the lack of knowledge of the biochemical mechanisms of biodegradation and the degradative microorganisms. Furthermore, small-scale lab experiments intended to test the suitability of a remediation technique may give promising results, while large-scale field efforts still turn out to be unsuccessful or patchiness prevails in the remediation of contaminated soils. This is largely explained by huge variation in field conditions that greatly influence e.g. the degradation performance of the microbial community. After all, microbial degradation of xenobiotics is a series of enzymatic reactions and the involved enzymes can be highly species specific (Leahy and Colwell, 1990; Cao et al., 2009; Fuchs et al., 2011; Gu, 2016). For instance,

* Corresponding author.
E-mail addresses: yan.sun@helsinki.fi (Y. Sun), anna-lea.rantalainen@helsinki.fi (A.-L. Rantalainen), martin.romantschuk@helsinki.fi (M. Romantschuk), aki.sinkkonen@helsinki.fi (A. Sinkkonen).

https://doi.org/10.1016/j.ibiod.2018.06.006
Received 9 March 2018; Received in revised form 6 June 2018; Accepted 6 June 2018
0964-8305/

Proteobacteria and Actinobacteria, among all the bacterial phyla, are found to produce almost exclusively intradiol dioxygenase that is essential in ring-cleavage of aromatic compounds. Successful ring-cleavage of aromatics in lab experiments may thus mean that these types of bacteria thrive in the set conditions (pH, redox situation, temperature etc.), which might not be extrapolated to the situation in field conditions. Or practical failure can simply mean the lack of degrading genes due to soil heterogeneity. The precise reason for significant differences when comparing lab and field success can vary from case to case, or remain obscure, and therefore more efficient, and especially, more diverse and/or site relevant laboratory experiments are of general interest.

In soil remediation, it is always essential to have representative samples that generate enough information to facilitate the planning and execution of remediation of the whole site. Theoretically, the more samples are taken and tested, the better the site properties are described, provided that the sampling is well designed. However, exhaustive sampling is time-consuming and economically expensive, especially on large sites, e.g. forests. To solve these problems, pseudoreplication and composite sampling are used.

Pseudoreplication refers to experimental setups where a single source of contaminated material is divided into several units that are treated as independent replicates (within-plot pseudoreplicates) in statistical analysis (Hurlbert, 1984; Pennock, 2004; Lazic, 2010). Pseudoreplication occurs quite often in ecological or soil studies (Hurlbert, 1984; Tavares et al., 2016). A traditional way of performing pseudoreplication in the environmental tests is to first map contamination gradients and soil characteristics in the field, and thereafter semi-randomly select an average field plot where all soil is collected for laboratory experiments. As a result of pseudoreplication, between-plot replication is missing and the results characterize only a single field plot. The rationale behind the use of pseudoreplication is to avoid difficulties caused by environmental heterogeneity: it is well-known that temporal and microscale variation can be high in natural attenuation studies (Ghosh et al., 2000; Kauppi et al. 2011, 2012; Sinkkonen et al., 2013b; Dechesne et al., 2014; Yu et al., 2015), and that this can fundamentally affect the fate of contaminants in soils, i.e. whether or not being remediated (Davis et al., 2003). Similarly, it is easier to study the suitability of a remediation technique if experimental units represent the so-called typical characteristics of a contaminated site. The obvious conclusion has been that pseudoreplication ignores field patchiness and the results gained from it cannot represent the whole field (Hurlbert, 1984). Knowing that Hurlbert (1984) published his classic report more than three decades ago, it is surprising how little research attention the potential negative consequences of pseudoreplication have received in the field of bioremediation.

Another common way to overlook environmental heterogeneity is to sample several field plots, combine, and mix them (pooling) before laboratory tests. Sometimes these composite samples consist of subsamples of an inadequate size that are taken from a single field plot in order to gather enough soil for lab bioassays. While this is often necessary in order to perform any lab tests, a composite sample may also consist of separate subsamples from several field plots that are combined and mixed (i.e. pooled) before the screening of chemical and microbiological characteristics (Walton and Anderson, 1990; Walsh et al., 1997; Lancaster and Keller-McNulty, 1998; Patil, 2002; Ottesen et al., 2008; Jensen et al., 2009). A composite sample then represents average characteristics of individual samples, given that pooling was made carefully (Tan, 2005). Another way to draw conclusions based on average characteristics of a field site is to use mean values of ecologically relevant replicates, instead of using the actual values of each replicate separately, such as in a Randomized Complete Block Design (RCBD) in agricultural studies that avoid experimental error arising from soil heterogeneity (Qiu et al., 1994; Fageria, 2007; Gotelli and Ellison, 2012; Tang and Yang, 2012; Tavares et al., 2016). Even though the dangers of composite sampling and the use of plot averages are well described in the investigation of contaminated sites (Hagström and Stapleton, 2005; Gotelli and Ellison, 2012), these methods are still applied while testing bioremediation potential (Vinas et al., 2005; Garg et al., 2016). This can lead to false negatives, e.g., detectable levels are diluted below the detection limit when mixed with clean samples. This dilution effect happens frequently in the medical screening of diseases (Dorfman, 1943; Ciampa et al., 2010; Bilder and Tebbs, 2012), especially when the size of the final pooled sample is very large. A similar loss of positive samples was described in detail in a contamination case study by Correll (2001) and therefore it was advised that composite sampling should be planned so that it does not miss risky contaminated patches (Belle et al., 2001; Patil, 2011). Despite the advances by Correll (2001) and Patil (2011), there is still the risk of missing highly contaminated field plots as a result of using average values, e.g. composite sampling, even though the mean level of contamination and soil remediation are acceptable. Thus, when soil remediation is studied, the dilution effect should be taken into consideration.

Since either pseudoreplication or average based conclusions neglect soil heterogeneity, there is a good chance that practical application based on these conclusions can fail. Therefore, the current study was designed to illustrate how pseudoreplication and interpretations based solely on plot averages (ecologically relevant between-plot replication) reduce the reliability of bioassays while studying natural attenuation and biostimulation of gasoline-contaminated soils and how to avoid it. For this purpose, three visually similar field plots were randomly selected in a pine forest, the same treatments were performed to the soil collected from each field plot, and simultaneously another set of experiments were conducted where three pseudoreplicates were prepared per field plot and treatment. The hypotheses were that 1) remediation outcomes of gasoline aromatics vary between soil samples collected from different field plots, 2) the use of within-plot pseudoreplication does not allow distinguishing the variability of one field plot from another, and 3) dilution effect occurs when average values of field plots are used to test time and treatment effects. If these hypotheses are true, pseudoreplication using soil originating in a single soil source and interpretations based solely on plot averages should be avoided. Instead, upcoming soil remediation lab bioassays should always include several non-pooled field plots as soil sources and the statistical analysis should be capable of distinguishing differences between field plots.

2. Materials and methods

2.1. Soil sampling and treatment

The soils were collected from three 1.5 m^2 plots in a pristine boreal pine forest in Hollola, Finland (67°67′N 34°18′E, details in Rantalainen et al., 2006; Sinkkonen et al., 2013b) and only from the surface organic layer (depth 3–15 cm). The distance between the plots was at least 5 m. From each plot, 10 L of soil was collected and stored in 10 L buckets. The soils collected from the three plots were not pooled. The buckets were then covered by lids with two 10 mm holes filled with cotton wool to allow air exchange (Kauppi et al., 2012), and stored at + 5 °C for 3 weeks before use. The collection was performed in November 2013. Soil moisture content and organic matter content were determined (Table 1) and common in surface soils in Finland.

Three treatments were designed: non-contaminated control, contaminated soil without fertilization (natural attenuation), and

Table 1
Soil dry matter and organic matter content of the three plots of soil.

Plot	Soil dry matter content, %	Soil organic matter content, %, dw
1	59	54
2	52	67
3	44	79