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Original Research

Metabolic and Molecular Profiling of Microbial Communities Following Controlled Kerosene Pollution

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Abstract

The release of xenobiotic compounds as petroleum and its derivatives still represents a problematic and not fully manageable consequence of various anthropogenic activities. Thus, endogenous microbial communities facing such pollutants are constantly adapting to new conditions, their evaluation being an important asset for environmental preservation. This study analyzes structural and metabolic shifts in a soil microbial community following kerosene pollution. Metabolic profiles from Biolog EcoPlate, analyzed as a guild grouping, Shannon diversity index (H index) and functional divergence revealed a significant reorganization followed by specialization in communities' metabolic function, also being supported by molecular profiles from ribosomal RNA (*rRNA*) intergenic spacer analysis (RISA) fingerprinting. Highly intense metabolic activity and structural changes are suggested by the increase in *alkB* and *rRNA* genes copy number, having similar trends. Thus, *alkB* gene copy number increased in 70 days from 5.53×10^{9} to 1.67×10^{11} copies. In this study we managed to report the changes that occur in a soil microbial community facing kerosene pollution using a significant number of complementary techniques, resulting in a complex characterization that can be of great use when facing kerosene-type pollutants.

Keywords: kerosene pollution, metabolic profiling, risa, *alkB* copy number, *16S rRNA* copy number

Introduction

Modern society's demand for petroleum and its derivatives is constantly increasing, leading to anthropogenic pollution with a great variety of xenobiotic compounds, mostly due to processing, transportation and improper stocking. Thus, all types of ecosystems can be compromised, endangering human well-being as well as wildlife and plants, the last one having a main role in the living world [1]. Plants perform many ecological functions in their environment, shaping the life of all living things that become directly or indirectly dependent on plants [2, 3]. The ability of plants to fulfil their functions primarily depend on the appropriate climatic and edaphic conditions [4]. Therefore, the complex structure of soil alongside the biological and biochemical processes mediated by microorganisms represent the basis of a terrestrial ecosystem, as well as its relationship with plants [5-7].

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During the last decades, increasing pollution has become a major concern for worldwide authorities engaging scientists in finding eco-friendly alternatives and, most important, developing efficient methods for remediation of damaged ecosystems [8, 9]. The latest research has described bioremediation as being the most promising method in comparison with conventional, physicochemical methods, being both technically and economically viable [10]. Bioremediation relies on the ubiquity and versatility of microorganisms being able to adapt to new unfavorable conditions, having the capacity to utilize a great variety of pollutants as the sole source of carbon converting them to less or non-toxic compounds [11-13]. For an increased remediation efficiency besides microorganism, plants are also used in decontamination of soil, mostly in heavy metals pollution [14-16] Even if a significant number of bioremediation technologies has been elaborated upon and implemented, considering the great abilities of microorganisms, natural attenuation can still represent the first and easiest choice in environmental cleaning. Thus, numerous studies are focused on microbial communities' structural dynamics from polluted sites, as a measurement of ecosystem state [8, 17-19]. In assessing microbial community structure, the most suitable approach are ones based on metagenomics [20, 21]. Even though high-quality DNA retrieval from environmental samples is problematic [22], and the great amount of data can be difficult to analyze, the evaluation of culturable and unculturable microorganisms offers a more complex and comprehensive view on microbial community structure [23] to laboratory cultivation approach. Even if molecular assays are best suited for community structure evaluation, to get a bigger picture, one must also address the metabolic activity. Biolog EcoPlates are used as a proper method in assessing the metabolic profile, represented by the capacity to grow on 31 different carbon sources, of a community at a given time point [24-27]. However, data processing and interpretation can be problematic, and various studies are still focusing on finding the most appropriate way to cover all data [28-30].

This study follows the dynamics of an indigenous microbial community during kerosene pollution, using an outdoor microcosm system. Structural changes were represented by molecular community fingerprinting by ribosomal intergenic spacer analysis (RISA) and assessment of the total number of bacteria from soil via quantitative PCR. Metabolic arrays were realized using EcoPlates, and hydrocarbon degradation potential and rate were determined by *alkB* gene quantification.

Material and Methods

Study Site and Sampling

Experimental soil, 120 kg, retrieved from a botanical garden (Bucharest, Romania) was air-dried, ground, sieved and loaded into a 512 L glass tank. To simulate naturally occurring processes, the tank was buried outdoors at a depth of 0.4 m. Soil characteristics were as follows: pH 6.94; humic acids 60.86%; conductivity 154.3 μS/cm. Subsequently, the microcosm system was intentionally polluted with 58.8 g/kg kerosene and regularly aerated and humidified. Samples were collected before and after pollution at 7, 14, 30, 50, 70, 80 days. Each sample consisted of soil collected from 5 points of the microcosm.

Community-Level Physiological Profiling

Metabolic profiles of microbial community at given time points were obtained using EcoPlates (BIOLOG Inc., Hayward CA., USA) consisting of 96 wells, with 31 different carbon sources and a water blank, in triplicate. The EcoPlates inoculum was represented by 20 g of sampled soil mixed with deionized sterile water (v:v) incubated for 2 hours at 25ºC and 150 rpm, followed by 10 min vortexing. From obtained suspension, 10-fold serial dilutions of up to 10-6 were performed. EcoPlates were inoculated from each dilution with 150 μl/well and incubated at 28ºC. Absorbance readings were taken at 590 nm using a plate reader (BIOLOG microstation) every 24 h for 7 days.

Community Fingerprinting by Ribosomal Intergenic Spacer Analysis

Molecular essays were conducted following DNA extraction from 500 mg of soil for each sample in duplicate as previously described by Tanase et al. [31].

RISA assays were conducted with the following primers: ITSF (5-GTCGTAACAAGGTAGCCGTA-3) and ITSReub (5-GCCAAGGCATCCACC-3) on ~25ng soil total DNA according to Cardinale et al. [32]. The reactions were carried out on an Eppendorf Mastercycler gradient thermocycler. Amplicons were visualized on 10.5% polyacrylamide gel and bands pattern analysis was made using Quantity One 1-D Analysis Software (BIO-RAD).

Quantification of Total Bacteria in Soil Samples

To estimate the total number of soil bacteria, a region of *16S rRNA* genes were directly amplified from diluted metagenomic DNA using universal primers Eub338 5-ACTCCTACGGGAGGCAGCAG-3(forward) and Eub518 5-ATTACCGCGGCTGCTGG-3 (reverse) in duplicate [33]. Reactions were carried out on a fast real-time PCR System900HT, by Applied Biosystems at a final volume of 20 μL volume containing: 10 μL Power SYBR Green PCR Master Mix (Applied Biosystems), 0.2 μL (10 μM) of each primer, 1 μL bovine serum albumin (3 mg/ml), 1 μL DNA (~25 ng) and distilled sterile water. Amplification conditions were as follows: 10 mins at 95ºC followed by 40 cycles at 95ºC for 50 s and annealing at 57ºC for 50 s, and

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elongation at 72ºC for 50 s.

Quantification of Alkane Monooxygenase Gene *alkB* in Soil Samples

Quantification of *alkB* genes from soil samples was conducted in the same conditions as described for *16S rRNA* genes using degenerate primers: alkBFd 5-AACTACMTCGARCAYTACGG-3 (forward) and alkBRd 5-TGAMGATGTGGTYRCTGTTCC-3 (reverse), (where M = AC, R = AG and Y = CT) [34].

Standards were constructed using 10-fold serial dilution of 1500 bp *16S rRNA* and a 550 bp *alkB* purified amplicons from a known hydrocarbon degrader *Tsukamurella* sp. MH1 [35] known to have a single copy of *alkB* gene and two copies of *16S rRNA* in the genome. Standards ranged from 10^{-4} to 10^{-9} molecular copies, depending on each case (efficiency = 0.99, R2 = 0.999). For reproducibility confirmation, all standards were used in every reaction. Results were analyzed with the manufacturer's provided software.

Data Analysis

CLPP analysis was conducted as described by Garland [36]. A single time point data for each sample was selected according to corresponding temporal dataset, represented by color development over incubation time (0-168 h). The optimal time point represented by 72 h data was normalized according to Weber et al. [28] by the average well color development (AWCD). Normalized absorbance for well k was calculated as follows:

$$\overline{A_k} = \frac{A_k - A_0}{\frac{1}{31}\sum_{i=1}^{31}(A_i - A_0)}$$

A_i – absorbance reading of well i
A_0 – absorbance reading of control well
Eq. 1 denominator is AWCD

Based on carbon source utilization patterns, Shannon diversity index (H index) was calculated as Weber and Ledge [37] described:

$$H_{CLPP} = -\sum p_i \ln(p_i)$$

p_i – OD 590 nm at 72h

Even though it is commonly used in tracking and evaluating community shifts over time and space, in this case it was used to evaluates microbial community metabolic diversity changes in time.

On the other hand, the large amount of data, represented by OD from 31 wells with different carbon sources, was organized in 5 guilds: 1) carbohydrates, carboxylic acids, 3) amino acids, 4) amines and amides, 5) polymers [38]. Thus, highly complex 31-dimensional data was compressed into 5 dimensions, and the total carbon source utilization percentage was divided in 5 guilds accordingly.

Functional community divergence measure was represented by Euclidean distance [37] between two-time points: reference point, day 0 and every given moment following pollution. Euclidean distance in this case measured for all 31 dimensions (31 carbon sources) dissimilarities of any given time point and day 0.

Results and Discussion

Microbial Community Metabolic Dynamics

CLPP profile analysis managed to capture a series of important events regarding functional diversity and metabolic dynamics of indigenous microbial community from initially pristine soil before and after kerosene pollution event. During the 80-day period of observation, the development of Shannon diversity index (Fig. 1) revealed a rapid increase until day 7, most probably due to the high metabolic activity of medium chain n-alkane degraders, compounds known to be more easily metabolized [39] by many microbial taxa. The following abrupt decrease, from day 7 to day 14, and the slow but consistent increase (until day 70) in diversity values could be associated with the microbial community adaptation, specialization and metabolic shifts from easily degraded n-alkanes to other kerosene compounds. Our Shannon diversity index data revealed comparable values with previous similar studies [40, 41].

Guild Grouping

EcoPlate carbon source utilization percentage for each guild (Fig. 2) continuously changed during our study without seeming to follow any set pattern. Noting the fact that patterns recorded on day 80 were highly different compared to all samples, especially

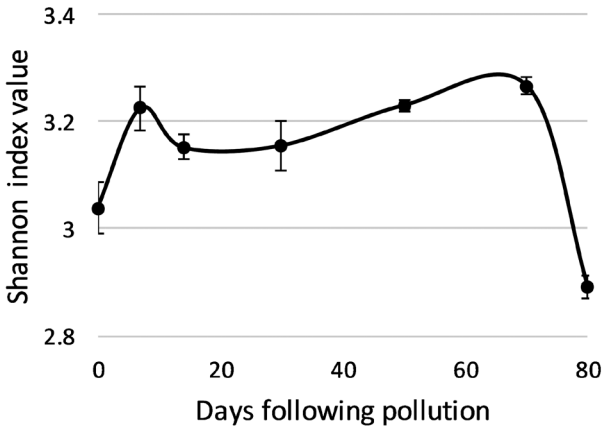

Fig. 1. Metabolic diversity represented by differences in substrate utilization following kerosene pollution (day 0) for 80 days, measured as Shannon diversity index value.

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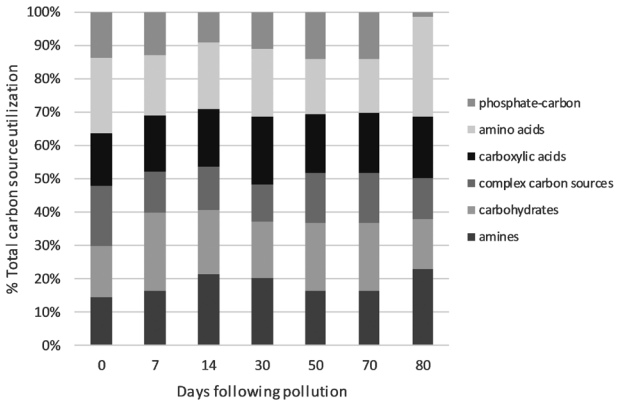

Fig. 2. Total carbon source utilization percentage divided in 6 different guilds, tracked over 80 days, following kerosene pollution.

day 0, it can be assumed that, considering the studied time frame, the analyzed microbial community did not tend to return to its initial state. Carboxylic acids were well metabolized, being noted that these compounds are metabolic intermediates in *n*-alkane degradation pathway. All shifts recorded represent a highly dynamic response to kerosene pollution, considered a disturbing factor.

Divergence

Using Euclidean distance between CSUPs (community substrate utilization patterns) with reference to day 0, we managed to capture divergence trends in bacterial communities following a kerosene pollution event. As seen in Fig. 3, indigenous communities diverged rapidly and drastically during the first 7 days of observation due to low survival rates to highly toxic pollutants [42], but mostly counted as a metabolic specialization for *n*-alkane degradation. After 7 days, community divergence reached a much steadier state, obtained values being similar when compared to day 0. In concordance with the observed results from guild grouping analysis, divergence trends also showed that the indigenous microbial community adapted rapidly to the harsh new environmental condition, subsequently specializing on using the given carbon sources. As mentioned before, data from day 80 highlighted the fact that, after restructuration events, the indigenous community diverged even more.

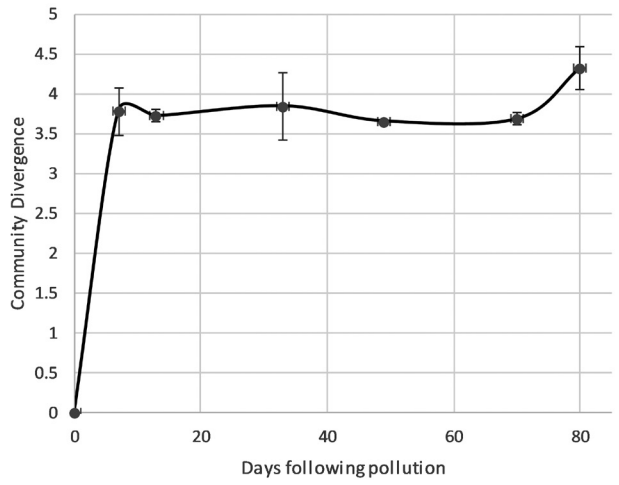

Fig. 3. Community divergence measured as Euclidean distance for 80 days, following kerosene pollution.

Assessing Microbial Community Structure and *alkB* Genes

RISA

ITS (internal transcribed spacer) phylotype revealed a total number of 38 OTUs, the maximum number of 31 being observed on day 0, accounting for the indigenous microbial community's great taxonomic variation. A rapid decrease in OUTs number to 21 from day 0 to day 50 suggested in this case a taxonomic restructuration related to kerosene toxicity, but mostly to the availability of new complex carbon sources in the environment. Samples from days 70 and 80 revealed a second taxonomic restructuration, with OTU numbers reaching 10, suggesting a highly specialized community comprising specific taxa with great tolerance and degradation abilities.

Based on a pairwise comparison of OTU presence/absence, 43-90% of shared OTUs was observed through the entire analyzed period. A major bacterial community shift appeared up to day 70, with a loss of between 33-41% for the first 50 days, followed by a significant decrease of 56% between samples from days 50 and 70. A stable state was reached between days 70 to 80, with a shared OTU of 90.9%. It is generally assumed that regarding available carbon sources specific taxa will be selected, certain bacterial community individuals being able to adapt and multiply, highlighting once again the versatility and dynamics of indigenous communities [43].

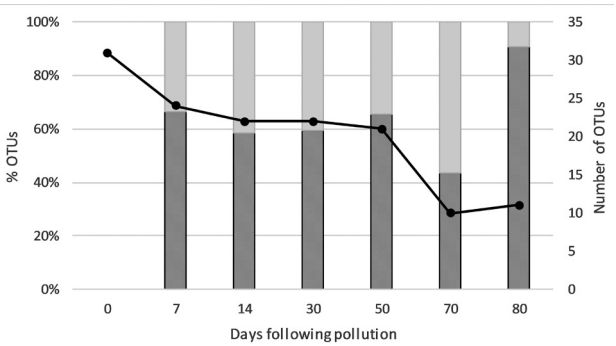

Fig. 4. OTU turnover for 80 days; the line represents the number of OTUs for every sample, and bars show the percentage of OTUs shared (striped)/not shared (not striped) between a sample and the previous one.

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qPCR 16S

The impact of kerosene pollution on the bacterial community was also analyzed by quantitative evaluation of *16S rRNA* gene copy number and resulting data was normalized with DNA extraction yields. As seen in Fig. 5, the amount of *16S rRNA* gene copies varied from 1.5×10^9 to 6.67×10^9, with the lowest value being determined after 7 days of pollutant exposure and the highest value reached after 70 days. After the decrease from day 0 to day 7, as a response to given stress factor, kerosene exposure led to the selection of bacterial populations able to use it as a carbon source and energy, favoring the growth of certain individuals adapted to the contaminated environment. After day 7 the number of gene copies increased continuously, reaching a maximum level on day 70, followed by a slight decrease from 6.67×10^9 to 6.01×10^9 copies for day 80, justified by the decrease in kerosene concentration due to biodegradation events. Thus, the association of these opposite effects of pollutant exposure, a decrease in abundance and diversity because of kerosene toxicity, followed by an increase in abundance of specialized bacteria, determined the changes in structure and abundance of indigenous bacterial communities.

qPCR alkB

Accounted as an evaluation for metabolic activity correlated to *n*-alkanes degradation, the *alkB* genes copy number was estimated (Fig. 5). The dynamic trend regarding *alkB* genes copy number is similar to the one from *16S rRNA*, excepting the fact that the copies amount was much higher, starting from 5.53×10^9 on day 0 and reaching 1.67×10^{11} on day 70. The significantly higher number compared to *16S rRNA* genes can suggest that the studied indigenous soil microbial community has a great biodegradation potential, comprising individuals with the enzymatic equipment suited for *n*-alkane catabolism. At the same time, it can be noted that some bacterial strains can have more than one *alkB* gene. These aspects can explain the initial great number of *alkB* genes, but also the maximum value reached. As also observed for *16S rRNA* genes, at day 80 we noticed a decrease in gene numbers to 1.36×10^{11}, which can be directly correlated to the decrease of bacterial individuals.

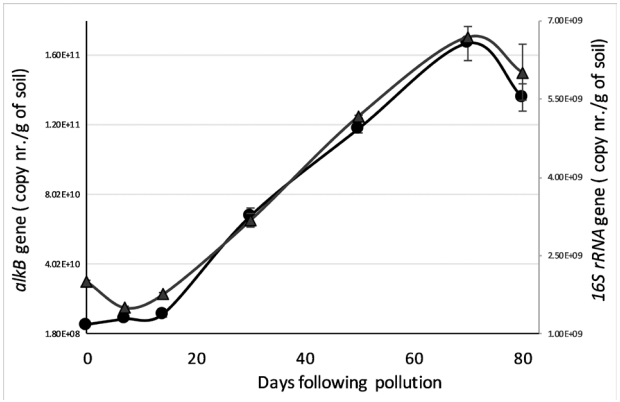

Fig. 5. Changes in *16S rRNA* (▲) and *alkB* (●) gene copy numbers per gram of soil for 80 days, following kerosene pollution.

Conclusions

This research brings valuable information in the field of microbial ecology, providing a detailed analysis regarding structural and functional changes in a soil microbial community during kerosene pollution. Novelty and originality of our result lies in the significant number of techniques, comprising metabolic profiles, molecular fingerprinting and gene quantification (*16S rRNA* and alkane hydroxylase – *alkB*) used for characterizing an indigenous microbial community from a pristine soil polluted with kerosene in controlled conditions.

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Conflict of Interest

The authors declare no conflict of interest.

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