Addition of Rubber to soil damages the functional diversity of soil

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Abstract Rubber is a polymer of isoprene, consisting mainly of cis-1,4-polyisoprene units. The unmanageable production and its irresponsible disposal pose severe threats to environmental ecology. Therefore, the current study focuses extensively on the ill-effects of Rubber disposal on soil microbial functional diversity as it reflects the health of ecosystem by acting as a key component in ecosystem productivity. To investigate the effect of Rubber on soil microbial functional diversity, soil samples were collected from landfill sites and three different soil microcosms (Rubber treated, untreated, and sterile soil) were prepared. The soil enzymatic activity was determined by fluorescein diacetate hydrolysis followed by the determination of the microbial metabolic potential and functional diversity by average well color development and Shannon–Weaver index (*H*), respectively. BiOLOG ECO plates were used for determining the microbial functional diversity of the soil microcosms. Higher heterotrophic microbial count as well as higher soil microbial activity was observed in Rubber untreated soil than Rubber treated

soil microcosm. The result indicated that the addition of Rubber to soil reduced soil heterotrophic microbial count and soil microbial activity considerably. Similarly, soil microbial metabolic potential as well as microbial functional diversity of soil had been decreased by the addition of Rubber gloves in it. Variation in soil microbial metabolic spectrum between Rubber treated and untreated microcosm was confirmed by multivariate analysis. Collectively, all the results demonstrated that the addition of Rubber to soil reduced the soil microbial functional diversity considerably. Therefore, it is necessary for the commission of serious steps regarding Rubber disposal and protection of the environment from serious environmental issues.

Keywords Rubber · Soil microbial community · Microbial functional diversity · Ecosystem stability

Introduction

Natural Rubber latex is produced by over 2000 plant species, and its main constituent is poly isoprene (C_2H_5), a highly unsaturated hydrocarbon having double bond in cis configuration (Rose and Steinbüchel 2005). Although more than 2000 plant species produce this natural Rubber latex, its main source originates from the Brazilian Rubber tree *Hevea brasiliensis* (Rose and Steinbüchel 2005). In addition to the isoprene units present in the Rubber structure, natural Rubber latex also contains non-Rubber constituents such as protein, carbohydrates, neutral lipids, polar lipids, inorganic components, amino acids, amides, etc. (Subramaniam 1995). For commercial purposes, this natural Rubber normally undergoes the process of vulcanization. Vulcanization is a process of altering its molecular

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structure through the cross-linking of isoprene chains. Vulcanization is achieved either by heating in the presence of sulphur (Bode et al. 2000) or by per oxidation (Matherell 1992) and irradiation (Subramaniam 1995). In vulcanized Rubber, sulphur bridges are introduced either at their reactive allylic sites or at the site of double bond. The presence of double bond in the Rubber molecule makes it highly reactive as it provides allylic hydrogen that permits the formation of cross links between different chains. The presence of these cross links increases the toughness, strength, and hardness of Rubber. Due to the presence of sulphur bridges, individual chains can no longer slip over one another but are locked together in a giant size molecule. The process of vulcanization is mostly used in making commercial Rubber which has diverse applications including tire production, playground equipment, shoes, mats, flooring, healthcare supplies, household supplies, balls, toys, and thousands of other Rubber products. The country wise consumption of Rubber across the Globe has been presented in Table 1. Although there has been a drastic rise in use of Rubber material, there is a lack of safe disposal practices. Thus, Rubber waste is getting accumulated considerably and this poses a serious threat to environment mostly due to contamination of the soil microbial ecosystem.

Functional diversity plays an important role in soil microbial ecology stabilization, because it is capable of influencing several aspects of ecosystem functioning like ecosystem dynamics, stability, nutrient availability, etc. (Tilman 2001). Functional diversity is a component of biodiversity that generally covers the range of functional traits of microorganisms prevailing in an ecosystem (Schleuter et al. 2010). Thus, it could be assumed that an ecosystem having higher functional diversity has higher metabolic potentiality and ecosystem having lower functional diversity has lower metabolic potentiality (Sarkar et al. 2017). Therefore, in the current study, efforts had been given to examine the effect of Rubber addition on soil

microbial functional diversity. BiOLOG-based carbon source utilization technique is considered as the most commonly used technique for analyzing the microbial metabolic activity and functional diversity of the microbial communities (Al-Mutairi 2007; Tribedi and Sil 2013a, b). The extensive use of the BiOLOG plates for assessing the functional diversity of the microbial community is mainly due to limitation of the conventional microbiological methods that can be used for measuring the functional diversity of microbial communities (Sarkar et al. 2017). Thus, in the current study, efforts had been given to investigate the change in functional diversity of soil microbial communities between Rubber gloves treated and untreated soil using BiOLOG ECO plates.

Materials and methods

Chemicals

Luria agar (LA) and Luria broth (LB) were procured from SRL. At the same time, few of the chemicals like sodium chloride (NaCl), phenol, and chloroform were purchased from Merck, India. Fluorescein di acetate (FDA) procured from Sigma-Aldrich was a kind gift from Dr. Alok Kumar Sil, Department of Microbiology, University of Calcutta to us. The Rubber gloves that were used in the study were purchased from the local market of Kolkata, India.

Soil microcosm preparation and Rubber addition

Soil samples (~10 cm depth) were collected from Kolkata municipal solid-waste landfill ground and placed in sterile polyethylene bags. The bags in sealed condition were then transported from the collection site to the Laboratory aseptically. Thereafter, the isolated soil sample was homogenized and sieved through a 2-mm pore size sieve. After that, the soil samples were air-dried.

Table 1 Country wise usage of Rubber (in '000 tonnes)

Country	2014	2015	% growth	Reference
China	4760	4680	−1.7	Rubber Statistical News, May, 2016, Rubber Board, Ministry of Commerce and Industry, Govt of India. (http://rubberboard.org.in/monstatsdisplay.asp)
India	1014.8	993.3	−2.1	
U.S.A	932.1	936.5	0.5	
Japan	709	691	−2.5	
Malaysia	447.3	474.7	6.1	
Indonesia	539.6	579.4	7.4	
Thailand	541	600.6	11.0	
Republic of Korea	402.1	387.7	−3.6	
Others	2791.1	2823.8	1.2	
World total	12,137	12,167	0.3	

Table 2 Physicochemical properties of soil collected from landfill

Organic carbon (%)	Total nitrogen (%)	Total phosphorous (%)	pH
3.42 ± 0.37	0.18 ± 0.02	0.79 ± 0.05	6.27 ± 0.03

Physicochemical properties of soil (soil organic carbon, total nitrogen, total phosphorous, and pH) were determined and presented in Table 2. Three types of soil microcosms (soil treated with Rubber, soil not treated with Rubber, and sterile soil microcosm) were prepared for the current study. For each soil microcosm preparation, 450 g of air-dried soil was separately taken in different sterile glass beaker. Sterile soil microcosm was prepared by autoclaving the soil at 121 °C for 15 min at 15 psi. Soil microcosm sterility was confirmed by the absence of microbial growth on Luria agar (LA) plates. To prepare soil microcosm treated by Rubber, Rubber gloves were cut into small pieces (2 × 2 cm). Before the addition of Rubber gloves to the soil, all the gloves were made microorganism free by washing them with 70% ethanol three times. Thereafter, equal weights (100 mg) of the air-dried Rubber gloves pieces were added to each soil microcosm aseptically. Each microcosm was covered with aluminum foil and then incubated at 30 °C for 28 days. During the course of incubation, moisture content of each microcosm was maintained by adjustment with sterile Milli-Q water. After the incubation, all the Rubber gloves pieces were taken out separately from each soil microcosm. Thereafter, a series of experiments were performed on Rubber gloves and soil samples of each microcosm to examine the effect of Rubber on the functional diversity of soil.

Heterotrophic microbial count analysis from soil

To investigate the abundance of heterotrophic microbial population in soil after Rubber addition, three different soil microcosms (soil treated with Rubber, soil not treated with Rubber, and sterile soil microcosm) were prepared and incubated at 30 °C for 28 days. After 28 days of incubation of Rubber in soil microcosms, 1 g of soil was taken from each soil microcosm and subsequently added to 9 ml of 0.85% NaCl in sterile test tubes. Thereafter, a series of dilution from 10^{-2} to 10^{-5} were prepared in sterile 0.85% NaCl. An aliquot (0.1 ml) of the appropriate dilution from each microcosm was spread onto Luria agar (LA) plates for enumeration of heterotrophic microbial organisms. LA plates were incubated at 30 °C for 2 days. After the incubation, the colonies developed on LA plate were counted.

Soil enzyme activity determination

To investigate the microbial activity among different microcosms under different treatment, fluorescein di acetate (FDA) hydrolysis assay was carried out. Viable microbial cells produce a large array of hydrolytic enzymes, which can cleave FDA to produce fluorescein that can be detected spectrophotometrically, and this assay has widely been used to measure cell metabolic activity (Tribedi and Sil 2014). FDA hydrolysis of soil was determined according to the modified method of Adam and Duncan (2001). In brief, 5 g soil sample was taken from each microcosm and mixed properly with 15 ml phosphate buffer (pH 7.6) in a 50 ml Erlenmeyer flask. Thereafter, 0.2 ml of a solution of FDA (1 mg ml⁻¹) in acetone was added to each flask. Flasks were then incubated at 30 °C for 20 min on a rotary shaker. After incubation, fluorescein was extracted with chloroform–methanol (2:1) solution. Thereafter, fluorescein concentration was measured spectrophotometrically at 490 nm. Results were expressed as micrograms of fluorescein produced per gram of soil.

Functional diversity measurement of soil microbial community

To measure the microbial metabolic functional diversity of soil, community-level physiological profiles (CLPP) were assessed for soil samples isolated from both Rubber treated and untreated microcosm. For this purpose, BiOLOG ECO plate system containing triplicates of different environmentally relevant carbon sources was used (Choi and Dobbs 1999). To measure the physiological profiles of soil microbial community, 1 g soil was taken from each microcosm and then was added into 10 ml sterile double distilled water in a test tube and shaken for 10 min. 150 µl of 10^{-3} dilution of soil suspension containing $\sim 10^3$ cfu from each microcosm was separately added to each well of the BiOLOG ECO plates. All plates were incubated at 30 °C for 60 h and absorbance was recorded at 590 nm. The Shannon–Weaver index (H), an indicator of metabolic functional diversity, was calculated by the following equation: $H = -\sum p_i \ln p_i$, where p_i is the ratio of the activity on each substrate (OD_i) to the sum of activities of all substrates ($\sum \text{OD}_i$) (Garland 1997; Tribedi and Sil 2013a, b). The corresponding Lorenz curve was also plotted to get the idea of microbial metabolic pattern prevailing in each microcosm (Tribedi and Sil 2013a, b). This curve was used to get the Gini coefficient (G), which is a measure of inequality, using the formula:

$$G = 1 - 2 \int_0^1 L df,$$

where L is the Lorenz curve and f is the standardized cumulative distribution of the standardized population.

Multivariate analysis

For principal component analysis (PCA), data from the richness tests using BiOLOG ECO Plates were collected and the correlation matrix was generated to construct the loading plot for the first two components. PCA was performed using the software Minitab 16.

Statistical analysis

Experimental results were subjected to statistical analysis of one-way analysis of variance (ANOVA). Mean values were compared at the 5% level using the software Minitab 16.

Results and discussion

Variation in microbial population among different microcosms under different treatment

Maximum number of heterotrophic microorganisms was found in Rubber untreated soil microcosm and lowest microbial count was observed in sterile soil microcosm (Fig. 1a). The microbial count in Rubber treated soil was found to be significantly lower than the Rubber untreated soil (Fig. 1a). The result indicated that the microbial count in soil had been reduced in the presence of Rubber. The result obtained from the above study indicated that addition of Rubber to soil created a stress to the soil microorganisms resulting in reduced heterotrophic microbial population.

Variation in microbial activity among different microcosms under different treatment

Consistent with the heterotrophic microbial count, the highest level of fluorescein di acetate hydrolysis was observed in soil sample taken from the microcosm free from Rubber and moderate level of hydrolysis was observed in soil sample taken from the microcosm that was treated with Rubber (Fig. 1b). However, the lowest level of fluorescein di acetate hydrolysis activity was observed in sterile soil microcosm (Fig. 1b). It was observed that the exposure of Rubber to soil significantly reduced the microbial activity in soil compared to soil which was free from Rubber (Fig. 1b). It was also observed that the variation in microbial activity among different microcosms followed the same pattern with the variation in heterotrophic microbial population among different microcosms

(Compare Fig. 1a, b). The reduction in microbial activity in Rubber treated soil could be attributed to the reduction in heterotrophic microbial load, since only viable microbial cells produce the enzyme like esterase and hydrolase. Thus, with the reduction in heterotrophic microbial load, the enzyme pool also reduces that resulted in the reduction in microbial activity in Rubber treated soil.

Variation in microbial functional diversity in different microcosms under different treatment

To investigate the effect of Rubber exposure on the soil microbial functional diversity, 1 g soil was collected from each soil microcosm and an effort was made to establish a comparative analysis between the two soil microcosms regarding their ability to utilize different carbon sources present in BiOLOG ECO plate. BiOLOG ECO plates have widely been used in applied ecological research to detect spatial and temporal changes in soil microbial communities (Sarkar et al. 2017). BiOLOG ECO plates contain pre-dried 31 different types of carbon sources and tetrazolium violet redox dye that turns purple upon the utilization of carbon source by the microorganisms. Since the average well color development (AWCD) is directly related to the total biological metabolic potential of a microcosm, AWCD of each soil microcosm was examined to investigate the metabolic potential of each microcosm under different treatment. It was observed that Rubber treated soil microcosm showed reduced AWCD level than Rubber untreated soil microcosm (Fig. 2). Thus, the result obtained from the above study indicated that Rubber addition and subsequent incubation in soil significantly reduced the overall microbial metabolic potential. To further validate the findings, the functional diversity of each soil microcosm was examined by measuring the Shannon diversity index (Fig. 3). In a given ecological niche, metabolic functional diversity influences the metabolic richness of an ecosystem by targeting nutrient utilization ability. Functional diversity indicates the reliability of an ecosystem by addressing their different metabolic potential (Goswami et al. 2017). Thus, it was documented that with the increase in functional diversity, the metabolic diversity increases that results in the formation of stable ecosystem (Tribedi et al. 2015). Consistent with the AWCD, Shannon diversity also showed the similar result where it was observed that Rubber addition significantly reduced the functional diversity of soil microcosm (Fig. 3). The result indicated that the addition of a xenobiotic component like Rubber caused stress to the preexisting microbial community of soil which resulted in lowering of metabolic potential and functional diversity of soil microbial communities. For gaining further confidence, we added different amount of Rubber to soil microcosm to examine the change in microbial functional

Microbial abundance and metabolic activity

Fig. 1 Heterotrophic microbial abundance and microbial activity profile. **a** Heterotrophic microbial population of soil microcosms under different treatment. Heterotrophic microorganisms were collected from soil and counted on Luria Agar plate. Three replicates had been used for each type of microcosm, and the result represented the average of these three replicates. *Error bars* indicated standard deviation ($\pm$ SD). Statistical significance among the experimentally used samples was evaluated by ANOVA at 5% level. Mean values with different letters represent that there is significant difference among the experimentally used samples. **b** Analysis of microbial

activity in soil microcosms under different treatment. The microbial activity of the soil samples were determined by fluorescein di acetate hydrolysis assay as described in “Materials and methods”. Three replicates had been used for each type of microcosm, and the result represented the average of these three replicates. *Error bars* indicated standard deviation ($\pm$ SD). Statistical significance among the experimentally used samples was evaluated by ANOVA at 5% level. Mean values with *different letters* represent that there is significant difference among the experimentally used samples

Metabolic potentialities of soil microcosm

Fig. 2 Analysis of metabolic potentialities of soil microcosms under different treatment. The average well color development (AWCD) assay was performed to examine the metabolic potentiality of different microcosms as metabolic potentiality is directly related to AWCD. Three replicates had been used for each type of microcosm, and the result represented the average of these three replicates. *Error bars* in the diagram indicated the standard deviation ($\pm$ SD). Statistical significance among the experimentally used samples was evaluated by ANOVA at 5% level. Mean values with *different letters* represent that there is significant difference among the experimentally used samples

Soil microbial functional diversity measurement

Fig. 3 Analysis of Shannon-Weaver Index (*H*) of soil microcosms under different treatment. Shannon-Weaver index of each soil microcosm under different treatment was derived from the well color of BiOLOG ECO plates. Three replicates had been used for each type of microcosm, and the result represented the average of these three replicates. *Error bars* indicated standard deviation ($\pm$ SD). Statistical significance among the experimentally used samples was evaluated by ANOVA at 5% level. Mean values with *different letters* represent that there is significant difference among the experimentally used samples

diversity. The result showed that with the increase in the amount of Rubber addition to the soil, there is a significant reduction in microbial function diversity suggesting a direct effect of Rubber on microbial functional diversity (Supplementary Fig. 1). In general, the anthropogenic stress reduces the microbial stability of an ecosystem by mostly affecting the microbial community structure of a given habitat (Tribedi and Sil 2013a). To address the ecosystem stability, univariate statistical analysis cannot be recommended, because it is often affected by the sample size. To resolve the limitation, multivariate cluster analysis was carried out to examine the metabolic fingerprinting pattern by both soil microcosms under different treatment. In multivariate cluster analysis, PCA was conducted. PCA is a multivariate technique that analyzes a data table in which observations are described by several inter-correlated quantitative-dependent variables. Its goal is to take out the important information as a set of new orthogonal variables called principal components, and to display the pattern of similarity of the observations and of the variables. In the current study, the effect of Rubber on soil microbial ecology was addressed using BiOLOG ECO plates. Since BiOLOG ECO plate contains 31 different environment sensitive carbon sources, each carbon source is considered as one variable in analysis. To extract the information from 31 variables in an effective way, PCA had been applied to reduce variables and reflect the considerable difference in carbon sources utilization between Rubber treated and untreated soil microcosms. Existing literature also documented that PCA can be performed to differentiate microbial communities with respect to their carbon sources utilization profiles (Sarkar et al. 2017). In the current study, in PCA, loading plot was made for

Fig. 4 Principal component analysis of soil microcosms. Loading plot was constructed to differentiate the soil microbial community between Rubber treated and untreated soil microcosms using carbon source utilization spectrum of BiOLOG ECO plates. Three replicates had been used for each type of microcosm and the result represented the average of these three replicates

interpreting relations among variables. The result of loading plot clearly showed that microbial community in Rubber treated and untreated microcosms were distinctly different (Fig. 4). It is likely that if the soil microbial community is altered then the changed microbial community of soil would show a different metabolic pattern compared to its native form and that has been reflected in the result of loading plot of PCA. To lend support, Gini coefficients of Rubber treated and untreated soil microcosms were measured. Gini coefficient is a measure of inequality or evenness in a community, ranging from zero, when all individuals are equal and most evenly distributed, to a maximum of one, where the population exhibits maximum inequality and unevenness (Tribedi and Sil 2013b). The result of Gini coefficient was found to be higher for Rubber treated microcosm in comparison with that of Rubber untreated soil microcosm (Fig. 5). The result so obtained indicated that the addition of Rubber built a kind of stress to the soil microbial community that resulted in an alteration in the natural distribution pattern

Soil microbial metabolic potentialities distribution

Fig. 5 Analysis of Gini coefficient of soil microcosms under different treatment. Gini coefficient was calculated from the Lorenz curve as mentioned in “Materials and methods”. Three replicates had been used for each type of microcosm, and the result represented the average of these three replicates. The error bars in the diagram indicated the standard deviation ($\pm$ SD). Statistical significance among the experimentally used samples was evaluated by ANOVA at 5% level. Mean values with different letters represent that there is significant difference among the experimentally used samples

of microbial community. Thus, the result revealed that microorganisms were more evenly distributed in soil where the soil was not treated with Rubber, whereas in Rubber treated condition, microorganisms were less evenly distributed. Taken together, the overall results indicated that the addition of Rubber to soil reduced the microbial count, microbial activity, metabolic potential, as well as microbial metabolic functional diversity extensively.

Conclusion

Thus, in conclusion, indiscriminate littering of Rubber wastes in soil can damage the soil microbial ecology by targeting soil microbial metabolic functional diversity. Therefore, efficient solid-waste management practice needs to be developed to get rid of this pollutant globally.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

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