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Assessment of Microbial Community in Fluoride Contaminated Soils using Phospholipid Fatty Acid Analysis

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Abstract

A biochemical method that does not rely on culturing of microorganisms is phospholipid fatty acid (PLFA) analysis. The PLFA patterns give an indication of the composition of the microbial community and can be obtained relatively quickly and easily. The effects of fluoride (F^-) pollution on soil microbial communities have always been a captivating area that has received little attention in the unique tropical Kuttanad agro-ecosystem. No study has utilized PLFA profiles as a means to detect changes in the soil microbial community structure caused by F^-. In the present study, the microbial community in F^- contaminated soils of Kuttanad was assessed using PLFA. PLFA analysis showed that different F^- levels in the soil have an impact on the community structure of specific microbial groups. General bacteria and actinomycete PLFA a15 16:0 18:0 showed the highest abundance in low F^- soil whereas most gram-negative bacteria, cyanobacteria and fungal PLFA a12 16:1ω4c increased in medium F^- soil and most PLFA decreased or disappeared in high F^- soil suggesting that the status of F^- can change the structure of microbial communities in Kuttanad soils.
Keywords: PLFA, microbial community, fluoride contamination, agro-ecosystem, principal component analysis, Kuttanad

Резюме

Анализът на фосфолипидните мастни киселини (PLFA) е биохимичен метод, който не разчита на култивиране на микроорганизмите. Стойностите на PLFA дават индикация за състава на микробните съобщества и могат да бъдат получени сравнително бързо и лесно. Ефектът от замърсяването на почвите с флуориди (F^-) върху почвените микробни съобщества винаги е бил интересна област, която е получавала малко внимание в уникалната тропическа агро-екосистема Kuttanad. Липсват съобщения за използването на PLFA профилите като средство за откриване на промени в структурата на почвеното микробно съобщество, причинени от F^-. В настоящото проучване с помощта на PLFA е извършена оценка на микробното съобщество в замърсени с F^- почви на Kuttanad. Анализът на PLFA показва, че различните нива на F^- в почвата оказват влияние върху структурата на специфичните микробни групи. Резултатите от PLFA a15 16:0 18:0 показват най-голямо количество на бактерии и актиномицети в почва с ниско съдържание на F^-, докато количеството на Грам-отрицателните бактерии, цианобактериите и гъбите се увеличава в почви със средно съдържание на F^-. В почви с високо съдържание на F^- се отчита намаляване или дори изчезване на тези микроорганизми. Резултатите предполагат, че съдържанието на F^- може да промени структурата на микробните съобщества в почвите Kuttanad.

Introduction

Soil microbial communities are in charge of 80-90% of biotic soil processes, biogeochemical cycling and mediating soil organic matter decomposition, and assume exceptionally essential roles in the survival of plants. Soil microbial ecology has consistently been hindered by the difficulty in observing the activities of microorganisms in their regular habitat. A few techniques have been utilized to gauge the amount of microbial biomass in soil, yet few differentiate between various groups of microorganisms. Microscopic techniques can be utilized for this, however, they are tedious. They have additionally been scrutinized both for challenges in recognizing living and dead organisms and for underestimating biomass, since life forms covered

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up inside or behind soil particles are not counted (Schallenberg *et al*., 1989). In addition, conventional cultivation techniques may find limitations in characterizing most of the soil microorganisms.

Be that as it may, for a long time soil microbial biomass stayed notably a black box until the appearance of molecular methods started to uncover the extensive diversity of the microbial community. Therefore, a culture-independent approach is utilized to determine soil microbial community composition by phospholipid fatty acids analysis of microbial membranes, which offer the most amazing way to determine microbial biomass, shift in microbial community structure and activities (Zhao *et al*., 2016; Kuppusamy *et al*., 2018).

Meanwhile, less complex strategies dependent on specific biomarkers were developed which can defeat the issue of selective culturing while giving a fair perspective of the structure of complex microbial communities (Zelles, 1999; Insam, 2001). Among these was the utilization of phospholipid fatty acid (PLFA), first applied to soil by Frostegård (1991). Polar lipids, which are principally phospholipids in microbes, are present in all unscathed cells. More than 200 distinctive fatty acids have been characterized from different prokaryotic and eukaryotic organisms. They fluctuate essentially among various organisms, making them integral assets in taxonomic studies.

Phospholipid fatty acid (PLFA) analysis has been proved to be highly successful as an indicator in environmental studies for determining microbial community structures (Kaur *et al*., 2005). Changes in PLFA profile are indicative of changes in the overall structure of microbial communities (Frostegård *et al*., 1996) and "signature" PLFA can provide information on specific groups of microorganisms present in a community. Soil contamination can result in shifts in PLFA profiles for microbial communities (Pennanen *et al*., 1996; Griffith *et al*., 1997). PLFA profile analysis has therefore been made use of in monitoring the changes in microbial communities due to soil contamination caused by copper-based fungicides (Zelles *et al*., 1994), heavy metals (Kelly *et al*., 2003), 2.4.6 trinitrotoluene (TNT) (Wilke *et al*., 2004), and crude oil contaminated soil (Erdoğan *et al*., 2013).

PLFAs have a few highlights that strengthen their utilization as a marker of environmental stress, which enables them to react both in intracellular and extracellular environmental conditions, and henceforth can be utilized as an indicator in environmental monitoring and assessment. Taking the above facts into consideration, the aim of the study was to assess the microbial community in F^- contaminated soils of Kuttanad, using PLFA. Besides, this method provides results which can be visualized by multivariate statistical methods.

Materials and Methods

Study area

The study area selected was the agricultural lands of Kuttanad, the "rice-bowl" of Kerala, India. This unique wetland lies at the very heart of Alappuzha district, and is a part of Vembanad-Kole wetland, the Ramasar site in Kerala. The rice fields in this region are known as "Puncha vayals".They are comprised of 55 000 ha below mean sea level where sowing takes place in November and where harvesting takes place between March – April. Kuttanad has a warm and humid climate with slight seasonal fluctuation in temperature (21-38°C) and average rainfall of 300 cm, 83% of which is received during two monsoon periods from June to October.

Sampling scheme

Soil samples were collected from 15 locations Viz Veeyapuram, Thakazhi, Ramankari, Edathwa, Thalavady, Muttar, Nedumudy, Monkompu, Veliyanad, Pulinkunnu, Kainakary , Kavalam, Pandy, Kunnumma and Ponga of Kuttanad rice fields during the fallow period (January) of 2016 from a depth of 20 cm below the surface layer. Soil-subsamples for phospholipid fatty acid (PLFA) analysis were frozen within 24 hrs of sampling. All the soil samples taken were analyzed for F^- concentration. Whereas, samples for PLFA analysis, soils from three locations which had a low (from Thalavady soil), medium (from Monkompu) and high F^- concentration (from Muttar soil) (Roshni and Harikumar, 2021) were chosen retaining the same number of replications.

Phospholipid Fatty Acid (PLFA) analyses

Lipid extraction and PLFA analysis were performed using a modification of Bligh and Dyer (1959) method as described by Bossio *et al*. (1998). Briefly, 2 g of lyophilized soil samples were used to extract the PLFAs with a single phase mixture of chloroform/methanol/citrate buffer (15.2 ml at a 1:2:0.8 volume ratio). The extracted fatty acids in the chloroform were fractioned into neutral lipids, glycolipids and polar lipids using a silica-bonded phase column (SPE-Si, Supelco, UK) with chloroform, acetone and methanol, respectively. The recovered polar lipids were transesterified to the fat-

ty acid methyl esters (FAMES) by a mild alkaline methanolysis. Fatty acids and methyl esters were quantified by gas chromatograph (6890N, Agilent Technologies, Santa Clara, CA) and identified with MIDI SHERLOCKS microbial identification system (Version 4.5, MIDI, Inc., Newark, DE). Nonadecanoic acid methyl ester (19.0) was added as the internal standard before methylation and fatty acid methyl esters were identified automatically by the MIDI peak identification software (Wu *et al*., 2009).

Fatty acids were designated as A:BωC, where 'A' is the number of carbon atoms in the chain, 'B' is the number of double bonds, and 'C' is the number of carbon atoms from the methyl end of the molecule to the first unsaturated bond (Frostegard *et al*., 1993; Zelles, 1999). The prefixes *i, a,* and *cy* refer to iso-, anteiso- and cyclopropyl ring structure, respectively. The suffixes *c* and *t* refer to cis- and trans- configurations, respectively. 10Me indicates a methyl group on the tenth carbon atom from the carboxyl end of the molecule (Arao, 1999; Bååth and Anderson, 2003). Fatty acid nomenclature was used as described by Frostegård *et al*. (1993). The PLFAs i14:0, i15:0, a15:0, i16:0, a16:0, i17:0, and a17:0 were considered to represent gram-positive bacteria; 16:1ω9c, cy17:0, 18:1ω6c, 18:1ω7c, and cy19:0 to represent gram-negative bacteria (Frostegård and Bååth, 1996); 18:1ω9c, 18:3ω6c and 16:1ω5c for fungi; 10Me16:0, 10Me17:0 and 10Me18:0 for indicating actinomycetes (Zelles, 1997). Total microbial biomass was estimated using the total concentration of PLFAs (nmol g^{-1}). The abundance of individual PLFAs was indicated by their relative abundance (% mol) in each sample.

Results

Phospholipid fatty acids (PLFAs) in F⁻ contaminated soil.

The soil under different F^- concentrations contained various PLFAs composed of straight, branched, monounsaturated fatty acids, polysaturated fatty acids, dimethyl acetal and methyl branched fatty acids, and branched chain fatty acids (Fig. 1).

Eleven PLFAs with chain lengths ranging from C12 to C24 were identified. The PLFA patterns varied in response to different F^- levels as revealed by their relative abundance. For example, the proportion, expressed as mol% of the branched a12:0 was more in soils of medium F^-, whereas the proportion of 12:0 was higher in low F^- soil compared to medium and high F^- soil. The mol% of a15:0 and 16:0 were more in low F^- soil compared to medium

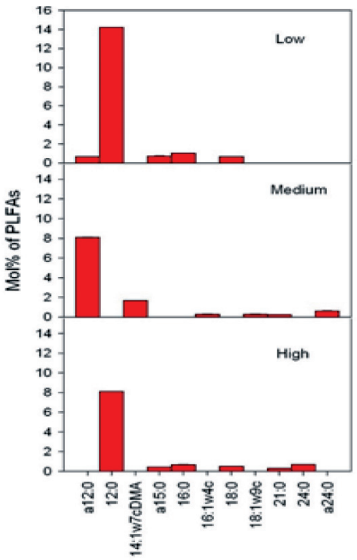

Fig. 1. Mol% of different PLFA under different F^- levels

and high F^- soil. PLFA 16:1ω4c increased in medium F^- soil and fatty acid 18:0 showed an increase in low F^- soil. PLFA 21:0 and 24:0 increased in high F^- soil while a24:0 showed an increase only in medium F^- soil. The soil with low F^- concentration had high percentage of gram-negative bacteria followed by medium F^- concentration. The PLFA 18:1ω9c, which is a signature fatty acid of cyanobacteria, green algae and fungi, was relatively higher under medium level of soil F^- concentration. Other PLFA proportions showed an increase in both medium and high level of soil F^- concentration.

Principal component analysis

The coordinates plot in Fig. 2 illustrates the difference in the PLFA composition under three levels of F^- concentration, where PC1 and PC2 account for 75.4% and 24.3% of the variation, respectively. The medium F^- level data formed a cluster and had positive scores for PC2, whereas data points for

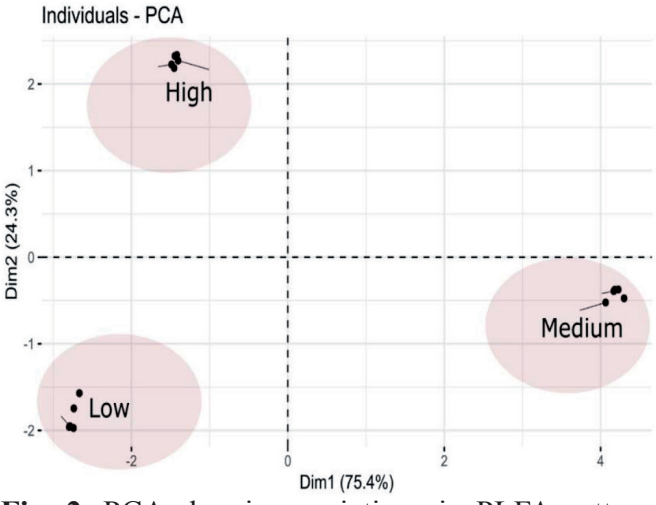

Fig. 2. PCA showing variations in PLFA pattern under different F^- levels

low and high F^- clusters were away from the other level and had negative scores for PC1 and PC2. It was fitting to separate the three F^- levels into three kinds of cluster analysis.

Principal component analysis also identified fatty acids that were important in explaining the variability in PLFA profiles (Fig. 3).

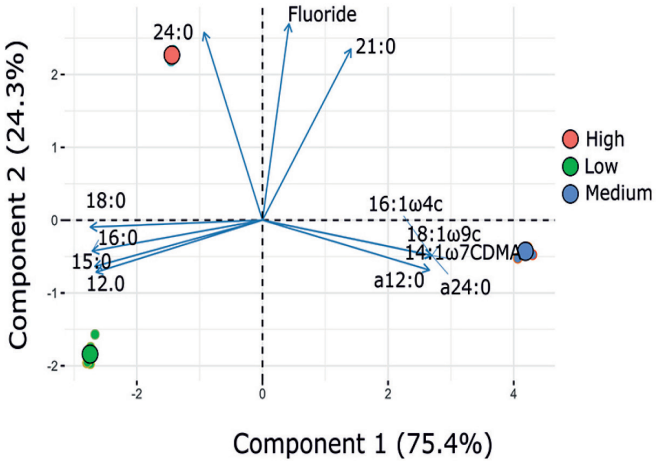

Fig. 3. PCA showing loading values for individual PLFAs

Certain specific PLFAs that were identified, including a12:0, 14:1ω7CDMA, 16:1ω4c, 18:1ω9c, 21:0 and a24:0, were found in the right-hand side in the plot. It was evident from the plot that medium level of F^- in the soil did not affect certain gram-negative bacteria as indicator fatty acids a12:0, 14:1ω7CDMA, 16:1ω4c are characteristic of gram-negative bacteria (Zelles, 1999; Buckeridge *et al.*, 2013). Similarly, the indicator fatty acids 18:1ω9c for cyanobacteria, green algae and fungi (Bühring *et al.*, 2014) were not affected by medium level of F^- concentration in the soil. However, the fatty acids for certain gram-negative bacteria (12:0, 18:0), general bacteria (15:0, 16:0) were negatively affected by F^- concentration even at a lower level. The proportion of fatty acids was the highest 109.43% under low F^- soil, whereas the lowest was 91.94% in high F^- soil (Fig. 4).

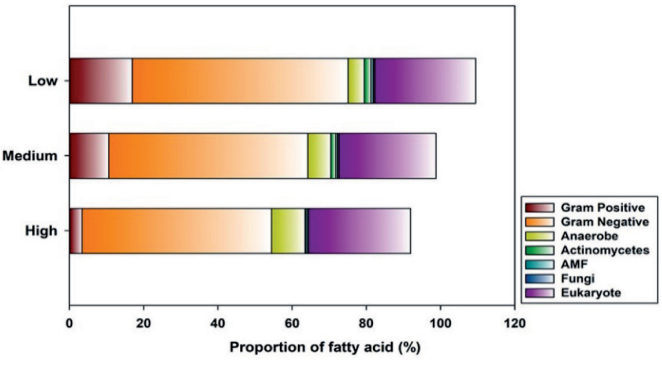

Fig. 4. Proportion of fatty acids corresponding to microbial groups

The total PLFA in the soil was found to be adversely affected by increasing F^- concentration as was evident from the exponential fit (Fig. 5)

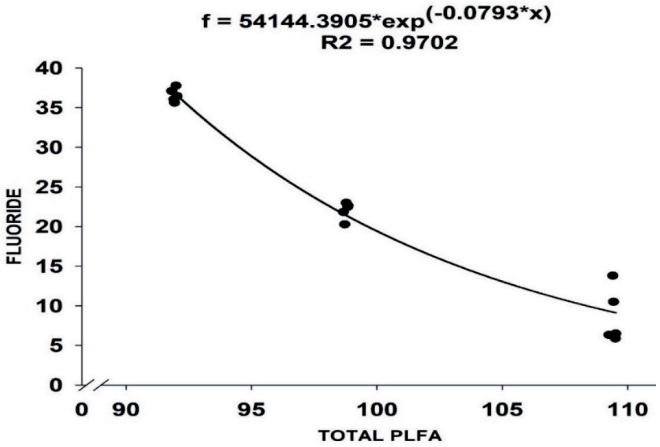

Fig. 5. Relationship between total PLFA and F^- in soil

Discussion

PLFA dynamics in F^- contaminated soils

Phospholipids are essential membrane components of living cells and are not found in storage products or dead cells. Under the conditions expected in naturally occurring communities, phospholipids make up a relatively constant proportion of the biomass of the organisms (Lechevalier, 1989). PLFA is a commonly used analytical procedure for the evaluation of soil biological characteristics. In environmental studies, PLFA analyses have mostly been used to describe microbial communities in seawater and lake water (Tulind and White, 1992). Only a few studies have used this approach to detect changes in the community structure due to different environmental disturbances, especially in soil. Smith *et al.* (1986) found a shift towards a more gram-negative bacterial community in subsurface sediment contaminated with creosote wastes. An increase in gram-negative bacteria as well as actinomycetes, due to liming, ash fertilization and alkaline deposition was indicated by altered PLFA patterns of different coniferous forest soils (Bååth *et al.*, 1992; Frostegård *et al.*, 1993). No study has utilized PLFA profiles as a means to detect changes in the soil microbial community structure caused by F^-. In the present study, the microbial community in F^- contaminated soils of Kuttanad was assessed using PLFA. The study revealed that different F^- levels in the soil have an impact on the community structure of specific microbial groups. General bac-

teria and actinomycete PLFA a15 16:0 18:0 showed the highest abundance in low F^- soils, whereas most gram-negative bacteria, cyanobacteria and fungal PLFA a12 16:1ω4c increased in medium F^- soil and most PLFA decreased or vanished in high F^- soil. A predominance of gram-negative over gram-positive bacteria is often found in metal contaminated soils (Doelman 1985; Duxbury, 1985). In this study, some evidence of similar shift was indicated by a decrease in PLFA, suggesting that the status of F^- can change the structure of microbial communities in Kuttanad soils.

Conclusion

This method can be used to detect changes in soil microbial community structure and can be used for a wide range of soil types. Though some of the changes found in PLFA patterns were restricted mainly to one of a few signature fatty acids, these were interpreted as changes in the proportions of some major groups of organisms in soil samples. PLFA analysis can thus be considered a fast and reliable method for the limited detection of overall changes in the microbial community structure strengthening their use as a bioindicator of environmental stress.

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