SOIL METHANOTROPHS COMPOSITION AND MICROBIAL BIOMASS LEVELS FROM LINDANE CONTAMINATED SITES

SUMMARY

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Summary

In present study, two study regions namely Lucknow (having IPL Chinhat, Chakhar village and Maati village) and Renukoot (having Adjacent to ABCIL, Sheo Park Bazar and Donki Nala) located in Uttar Pradesh state of India, were considered with following objectives:

1. To analyse the lindane residues in collected soil samples from lindane contaminated sites/area.
2. To study the soil methanotrophs compositions and microbial biomass-C, -N and -P from lindane contaminated sites.
3. To monitor the methanotrophic strains for lindane degradation tolerance level.
4. To assess the lindane degradation potential of identified methanotrophic strains at different environmental conditions.
5. To examine the end products during lindane degradation by methanotrophs in laboratory conditions.

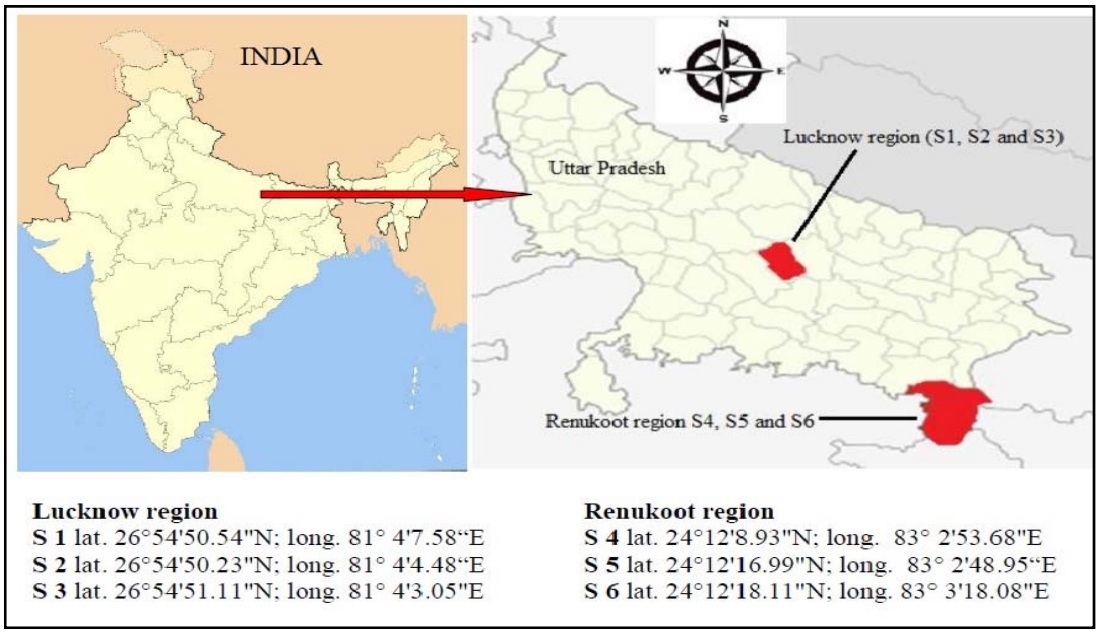

Figure Location of selected study sites (S1, S2, S3, S4, S5 and S6) distributed in Lucknow and Renukoot region of Uttar Pradesh, Northern India.

Soil physico-chemical properties

The results revealed that soil samples collected from Lucknow and Renukoot regions are slightly acidic in nature with pH values ranged from 6.1 to 6.9. The moisture content (%) for both the study region varied from 2.77 to 6.90 %. The electrical conductivity (EC) was found between 305.2 to 676.6 μS cm^{-1}across the study sites. Across the study sites, the total-C was found to be maximum (11, 250.5 μg g^{-1} soil) in soil samples of Renukoot region and minimum (6,105.2 μg g^{-1} soil) in soils of Lucknow region. Similarly, the total-N was measured maximum (705.6 μg g^{-1} soil) at S5 (Renukoot site) and minimum (500.5 μg g^{-1} soil) at S1 (Lucknow site), respectively. Across study sites the total-P was found minimum (567.5 μg g^{-1}) at S1 and maximum (766.2 μg g^{-1}) at S5 respectively, Lucknow and Renukoot sites. The soil NH_4-N content was found maximum in S6 (17.2 ± 1.9 μg g^{-1}) site of Renukoot region and minimum at S1 (6.8 ± 0.83 μg g^{-1}) site of Lucknow region. The higher total–C, –N and –P in the soils of Renukoot region compared to Lucknow region could be because soils of Renukoot region receives more plant litters via forest vegetation compared to Lucknow urban area.

Lindane residues

The GC and GC-MS/MS analysis of collected soil samples showed that the pesticides residues of HCH were found in collected soils of both the Lucknow and Renukoot region. The results confirm the presence of HCH isomers and OCPs such as α-HCH, β-HCH, γ-HCH, Aldrin, PP-DDE, β- Endo, PP-DDD, OP- DDT and PP-DDT in soil both the regions. The HCH residues in soil samples in Lucknow region

were found comparatively higher concentration as compared to Renukoot region and varied significantly due to sites. Among the HCH isomers, the concentration of α-HCH and γ-HCH were found higher in soil samples of Lucknow region and varied from 309.97 and 182.12 μg g^{-1} soil, respectively. The β-HCH was highest (169.15 μg g^{-1} soil) at S4 while lowest (5.93 ± 1.05 μg g^{-1} soil) at S5 site. The Aldrin was highest (0.02 μg g^{-1} soil) at S6 and lowest (0.006 μg g^{-1} soil) in soil of S4 site. The low concentration of pesticide residues in soils of Renukoot region compared to Lucknow region could be low anthropogenic interference because of lesser dense human population and agriculture activities. The results also showed that Folpet (fungicide) was also detected in soil of Lucknow region. The Captan compound was also detected in soils of Renukoot region. In Lucknow region on RT- 21.81, α-lindane and on RT 28.18 Captan, on RT 28.35 Folpet was confirmed by GC-MS/MS and at Renukoot region on RT- 28.18 Captan was detected. All these results shows that the soil samples of sites S1 and S2, S3 were also contaminated with the persistent organic pollutants.

Methanotrophs community composition

The cream and pink colour colonies on NMS (nitrate mineral salt media) specific media for the isolation of methanotrophs confirmed the growth of aerobic methanotrophs. After several rounds of streaking from plate to plate, pure colonies were obtained on the plates. The colonies were observed to be cream or pink in colour, round and measuring about 1 mm in diameter. The cells were found to be motile and Gram-stain negative. Different morphology of methanotrophs was observed during cultivation period. After incubation period (2-3 weeks), the methanotrophic bacterial growth on Petri plates was observed which indicates the presence of methanotrophs in lindane contaminated site of Lucknow and Renukoot

region. The result demonstrated that methanotrophic population in Renukoot region was significantly higher than Lucknow region due to large forest area and soil having higher amount of organic contents which contributes growth of methanotrophs in well aerated soil. Out of these 15 methanotrophic isolates only three (SBRJS01, SBIJS02 and SBJS03) potential isolates were selected for further study.

Metagenomics is used for the detection, identification and relative quantification of environmental microorganisms. The growing accessibility of next generation DNA sequencing (NGS) methods has greatly advanced our understanding of microbial diversity in medical and environmental science. In this study, high-throughput metagenomic sequencing provided a powerful strategy to investigate the methanotrophs community structure and functional potential associated in lindane contaminated soil. Alpha diversity is a measurement of richness and relative abundance of bacteria within the sample.

The contaminated soil contained 44,946 reads having 42,981 classified and 1,965 non-classified reads of bacterial population. The metagenomics of soil sample showed that the total number of methanotrophs present in selected site was 676. Further, the methanotrophic community composition in lindane contaminated Renukoot soil. Significant differences in methanotrophs community composition were found among soils exposed to distinct land utilization patterns and lindane contaminated sites of Lucknow and Renukoot region. The lindane concentration and soil organic matter concentrations seem to have major effects on methanotrophs community composition and abundance. The lower number of methanotrophs community in soil of Lucknow region compared to Renukoot region soil could be due to higher concentration of lindane residues at the soil of former site. Given the

fact that the dense vegetation covers at Renukoot site could be a critical factor for the greater abundance and community composition of soil methanotrophs compared to Lucknow site. Similar to this study the investigation of Chen et al. (2008) also demonstrated that the abundance and community composition size of methanotroph was decreased because of removal of the standing vegetation cover. The high proportion of physiologically distinct methanotrophs abundance in forest uplands (Renukoot) versus disturbed urban (Lucknow) soils clearly indicated that land use changes and anthropogenic disturbances could particularly select for a certain community of methanotrophs, possibly by altering ecological niches such as nutrient concentration and chemical (lindane) status of targeted bacteria.

Soil microbial biomass

The data on SMB-C, -N, -P (μg g^{-1} soil) across different study sites of Lucknow and Renukoot region are presented in Table 5.9. ANOVA revealed significant variation ($P < 0.001$) in SMB-C, -N and –P values due to sites and being highest in soils of Renukoot region. The SMB-C quantity across the sites ranged from 102.7 (S1) to 389.3 (S5) μg g^{-1} soil. Similarly the SMB-N ranged from 15.8 (S3) to 69.1 (S5) μg g^{-1} soil. The values of SMB-P ranged between 12.2 (S3) to 32.9 (S5) μg g^{-1} soil (Table 5.9). A significant variation in values of SMB-C, -N, -P in both the study region may be because the organic contents and quality due to the variation in vegetation compositions and anthropogenic activities (Singh et al., 2010). Since the six selected sites and regions of present study varied in terms of soil physico-chemical properties, HCH and OCPs residues concentrations and vegetation composition therefore, significant variations in SMB–C, –N and –P contents among sites may be also expected. It possible that across Lucknow and Renukoot region that high SMB-C, -N and -P at S4, S5 and S6 sites of Renukoot

region may be because of more suitable soil conditions, sufficient soil moisture contents, organic matters via availability of sufficient quantity of plant litters. The unfavourable soil moisture condition and low organic matter contents at S1, S2 and S3 sites of Lucknow region could suppress the SMB quantity too.

The selected sites (S1 to S6) distributed in Lucknow and Renukoot region varied significantly in terms of soil physico-chemical properties (total-C, -N, -P, moisture contents, pH, etc.), pesticides (HCH and OCPs) residues and SMB-C -N and -P values. The soils of Lucknow region sites, having higher amount of pesticides residues, may reduces the SMB-C -N and -P quantity compared to sites of Renukoot region. A higher litters and plant residues available at sites of Renukoot region due to dense natural forest soil as compared to Lucknow urban region sites, may considerably enhances the favourable soil conditions and therefore, a higher SMB-C, -N and -P values at sites of Renukoot region might be expected. A negative correlation between pesticides (lindane residues) concentrations and SMB-C, -N and -P suggests that higher HCH and OCPs present in soil may adversely influence the growth and multiplication of microbial diversity/biomass. The results of this study strongly confirms that variations in soil physico-chemical properties and HCH and OCPs residues may significantly affects the SMB-C, -N and -P compositions. Therefore, removal of lethal HCH and OCPs residues from soils via bioremediation/ phytoremediation can be a viable and eco-friendly soil management practice to improve the beneficial species of soil microbial communities and biomass which may consequently support the soil, agriculture and environmental sustainability.

Tolerance level and degradation potential of methanotrophs at different environmental conditions

After 20 day of incubation the three methanotrophic isolates (SBRJS01, SBIJS02, and SBJS03) were monitored for their potential to withstand at different lindane concentrations (10 mgL^{-1}, 20 mg L^{-1}, 30 mg L^{-1}, 40 mg L^{-1}, 50 mg L^{-1} and 60 mg L^{-1}) by inoculating into NMS broth media. Across different doses of lindane compared to control (*Methylosinus trichosporium* URRH3) the SBRJS01, SBIJS02 and SBJS03 showed maximum growth in terms of turbidity at 40 mg L^{-1} lindane concentration. The growth of all methanotrophs isolates was found to be diminished at higher lindane concentrations (50 and 60 mg L^{-1}).

The methanotrophic isolates (SBRJS01, SBIJS02 and SBJS03) showing lindane (40 mg L^{-1}) degrading potential was subjected to 16S rDNA sequence analyses. The National centre of biotechnology information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used as basic local alignment search tool BLASTn to identify the most similar sequences. The comparative analysis of SBRJS01, SBIJS02 and SBJS03 DNA sequences showed close relationship to the known methanotrophs (already available NCBI database) *Methylobacterium radiotolerans, Methylobacterium radiotolerans* and *Methylosinus trichosporium.* The 16S rRNA sequences of SBRJS01, SBIJS02 and SBJS03 have been deposited in GenBank indicated SBRJS01 is a strain of *Methylobacterium* sp. (accession no. MK481040), SBIJS02 is a strain of *Methylobacterium radiotolerans* (accession no. MK481064) and SBJS03 is a strain of *Methylosinus trichosporium* (accession no. MK503991).

Dechlorination (removal of Cl^- atom from organic halogenic compounds) is the main reaction in microbial mediated degradation of lindane. In dechlorination,

Cl^- atoms are substituted by hydrogen or hydroxyl group to the lindane (Camacho-Perez *et al*., 2012). In present study during lindane degradation, a decrease in pH was observed due to dechlorination of lindane. The coloured change in reaction mixture red to yellow was confirmed the release of CL during lindane degradation process by the methanotrophic isolates (Chapter 6; Figure 6.4). The quantification of chloride ion concentration showed its gradual increase from 0 day to 20^{th} day of incubation in experimental setup and no significant increase were observed in control. The maximum chlorine release and optical density (600 nm) was followed a trend as SBJS03 > SBRJS01 > SBIJS02 > Control URRH3 at 20 days of incubation (Chapter 6; Figure 6.5 and 6.6). These results depict that SBRJS01, SBIJS02 and SBJS03 isolated from lindane contaminated soil have potential to utilize lindane as source of carbon in place of CH_4 because during incubation no methane was added. Further, it was confirmed that the concentration of Cl ions in the NMS medium was measured highest at 20^{th} days of incubation. The maximum growth of methanotrophic isolates and lindane degradation potential on 20^{th} day of incubation at 40 mg L^{-1} lindane concentration exhibited a linear relationship between lindane degradation and chlorine release (Chapter 6; Figure 6.7).

The pH and temperature has been found as important environmental parameters affecting the microbial growth, activity and degradation potential of toxins in laboratory and natural conditions. Effect of different environmental parameters like temperature (20–40°C) and pH (5–9) were studied to find out their effects on lindane degradation by methanotrophic isolates. The *Methylosinus trichosporium* strain URRH3 was used as reference control in all the experiments. The maximum degradation of lindane was obtained at neutral (pH 7.0) after 20 days of incubation time (Chapter 6; Figure 6.8**)**. But when the pH was increased the

growth of methanotrophic isolates was decreased. When temperature was considered at environmental driver at 30 ºC maximum degradation of lindane was observed on 20th days of incubation time. However, when the temperature was increased the growth of different methanotrophic isolates was decreased significantly. Similar results were observed by Benemeli et al. (2007) showing maximum lindane removal by *Streptomyces* sp. at pH 7 and temperature 30 ºC. This suggested that as the pH and temperature increases from lower to higher values, the growth and lindane degradation potential of methanotrophic isolates were also decreases.

In order to investigate the end product during lindane degradation (after 20 days of incubation) by most tolerant methanotrophic isolate SBJS03, the resulted samples after degradation process were analyzed by GC-MS/MS technique. The results showed the presence of lindane isomers at different RT peaks as shown in Chapter 6 (Table 6.2). The *Methylosinus trichosporium* SBJS03 showed the lindane degradation potential which may be seen with clear peaks in GC-MS/MS chromatogram of cell extract as shown in Chapter 6 (Figure 6.10). The GC-MS/MS spectra showed that the lindane might be completely degraded therefore; final end product could not identified using the available library database. The presence of end product was observed in between 6.17 to 48.13 RT minutes. No any intermediate product was found as reported by in previous study. So, *Methylosinus trichosporium* SBJS03 was able to degrade lindane completely without forming any end product as per data available in The National Institute of Standards and Technology (NIST) library of high-quality database.

Conclusions

The selected sites (S1 to S6) distributed in Lucknow and Renukoot region varied significantly in terms of soil physico-chemical properties (total-C, -N, -P, moisture contents, pH, etc.), pesticides (HCH and OCPs) residues and SMB-C -N and -P values. The identified methanotrophic isolates SBRJS01, SBIJS02 and SBJS03 were tolerant to different lindane concentrations. Lindane concentration (60 mg L^{-1}) was toxic to bacteria because a significant decrease in growth rates was noted in almost all the methanotrophs isolates of present study. Among the methanotrophs isolates the maximum specific growth rate was noted in *Methylosinus trichosprium* SBJS03 strain at different environmental conditions and was positively correlated with maximum release of chlorine. So, *Methylosinus trichosprium* SBJS03 strain could be a good bioremediation tool for lindane residues and other related pesticides in soils. The methanotrophs strains investigated in the present study are highly recommended for bioremediation of soil systems contaminated by lindane and other chlorinated pesticides. The results from present investigation showed that across different study environmental parameters the pH (7.0) and temperature (30 ºC) was main factors affecting methanotrophs growth at 40 mg L^{-1} lindane concentration. Present study provided an example of a methanotrophs isolates that degrade lindane, thus extending the bioremediation potential of methanotrophs isolated from lindane contaminated soils.