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Assessment of bacterial diversity associated with crude oil-contaminated soil samples from Assam

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Abstract   In this study, we are reporting bacterial diversity in crude oil-contaminated soil of Assam, India. Integration of physiological community profiling, culture-dependent and culture-independent (metagenome) approaches, was employed to obtain a complete picture of the total bacterial diversity. Samples collected from 10 sites contaminated with crude oil ranging from 0.22 to 89.36% were analysed, and altogether 160 culturable bacteria were isolated (117 Gram-positive and 43 Gram-negative bacteria). Molecular identification showed the predominance of genera *Lysinibacillus, Alcaligenes*, *Bacillus*, *Clostridium, Enterobacter* and *Pseudomonas*. Conversely, denaturing gradient gel electrophoresis (DGGE) profiles of 16S rDNA phylotypes showed the predominance of *Sphingomonas*, *Ralstonia*, *Sphingobium*, *Massilia*, *Acinetobacter* and *Pseudomonas*. Both culture-dependent and culture-independent approaches resulted in 11 genera of which *Bacillus* and *Pseudomonas* were the key inhabitants creating most favourable bacterial milieu in the presence of crude oil contaminants. Metabolic fingerprints data depicted in PCA plot demonstrated that sites CTF-D-1 and Core-10 were most diverged. It was further confirmed that variations of bacterial species dominance in different sites were due to origin of hydrocarbon contamination. We here claim that the present findings is a first-hand report on combined physiological community profiling, culture-based and culture-independent approaches in assessing total bacterial diversity in crude oil-contaminated soil of Assam.

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Introduction

The inherent problem associated with hydrocarbon contamination caused by anthropogenic activities of crude oil drilling, exploration, refining, transport and storage has led to making these compounds recalcitrant environmental pollutants (Allen et al. 2007; Paisse et al. 2008; Mnif et al. 2009; Lu et al. 2012). Studies have reported that hydrocarbon-contaminated environments are inhabited by microorganisms which selectively grow and utilize petroleum hydrocarbons as carbon source for growth and development (Liu and Liu 2013; Beazley et al. 2012; Bargiela et al. 2015). Earlier studies also established that the presence of hydrocarbons determines the dominance of specific microbes (Vila et al. 2010; Hazen et al. 2010). Various reports on variations of bacterial diversity in the presence of hydrocarbons in an environment have shown that the presence of hydrocarbon contaminants often leads to selective enrichment of hydrocarbon-utilizing bacteria (Abed et al. 2002; Saul et al. 2005; Hamamura et al. 2006).

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Thus, determination of community structure of hydrocarbon-contaminated sites is required for getting first-hand information on microbial diversity (Albokari et al. 2015).

Conventionally, characterization of microbial community in contaminated soil was limited to culturing microorganisms from environmental samples termed as culture-dependent approach. Hydrocarbon-contaminated soils appear to be more favourable to conventional culture-dependent sampling than other soils; the reason being hydrocarbon contamination often leads to a shrink in microbial diversity (Bell et al. 2013; Saul et al. 2005; Kim et al. 2015; Abbasian et al. 2016). Consequently, a minimal sampling effort is required to isolate a representative fraction of the active community inhabiting those sites. In culture-dependent methods, only a small part approximately >3% of the microorganisms that inhabit soil contaminated with hydrocarbons could be cultured in the laboratory, and the rest >97% remain unreported (Macnaughton et al. 1999; Keller and Zengler 2004; Achtman and Wagner 2008). Hydrocarbon contaminants have been reported to restrain growth of certain responsive microbial groups (Santos dos et al. 2011) and selectively trigger subgroups like that of the *Actinobacteria* and *Proteobacteria* (Bell et al. 2013, 2014; Jung et al. 2016). Selectivity of growth media, lack of knowledge of the actual conditions under which most bacteria grow in soil, problems with soil sampling, recognition of small cell size bacteria and bacterial viability are critical limitations when trying to gain an understanding of bacterial diversity and community structure in soil. The understanding of microbial diversity has been extended by culture-independent studies, which involve comparative analysis of gene sequences directly from DNA extracted from the environmental samples. It seems likely that the uncultured majority provides a vast, unexploited reservoir of useful enzymes and biological probes. Therefore, integration of culture-dependent and culture-independent (metagenome) approaches is essential to get a complete picture of total bacterial diversity (Vaz-Moreira et al. 2011; Beazley et al. 2012; Mason et al. 2012; Liu and Liu 2013; Kimes et al. 2013; Stefani et al. 2015). Approaches for predicting total bacterial diversity include PCR amplification of 16S rRNA genes from cultured isolates (Aislabie et al. 2004; Saul et al. 2005; Roy et al. 2014); 16S rRNA V3 region amplified directly from soil DNA, followed by denaturing gradient gel electrophoresis (DGGE) profiling and sequencing specific amplicon (Muyzer et al. 1998; MacNaughton et al. 1999; Vinas et al. 2005; Greer et al. 2010), phylogenetic analysis and community-level physiological profiling through carbon utilization pattern (Mishra and Nautiyal 2009; Nautiyal 2009). Integration of both culture-dependent and culture-independent methods is crucial in getting a complete representation of total bacterial diversity.

Crude oil-contaminated areas in Assam are stretched to a large extent, have persisted for long duration and are mostly untapped (Gogoi et al. 2003; Yenn et al. 2014; Roy et al. 2014). Thus, these sites have a high likelihood of harbouring large diversity of unidentified bacteria that may have the ability to grow under hydrocarbon stressed and their utilization as bio-formulations may restore the environment. Recently, Li et al. (2015) and Howard and Howard (2016) had shared a vision to collaborate all environmental researchers towards a common goal, the "New Silk Road" for both beneficially and sustainably minimizing environmental degradation. In the present study, we attempt to determine the soil physicochemical profiles, soil enzymes and bacterial diversity of 10 hydrocarbon-contaminated sites of Duliajan and Jorajan districts in Assam state. To the best of our knowledge, this is the first report where we demonstrate the bacterial diversity associated with hydrocarbon-contaminated soil sites of Duliajan and Jorajan districts in Assam for its further exploitation as a source for bioremediation purposes.

Materials and methods

Site description, soil physical and chemical analysis

Crude oil-contaminated samples were collected from different locations operated under Oil India limited, Duliajan of Tinsukia District. Sampling was done randomly at ten oil drilling-contaminated sites of Duliajan (27°21′42″ N, 95°19′ 6″ E) and Jorajan (95°11′–95°30′ E; 27° 06′–27° 22′ N) of Dibrugarh and Tinsukia, Assam (Fig. 1). Soil, sludge and mud samples from different locations of crude oil-contaminated sites were collected at random to a depth of 0–10 cm. To represent uniformity, bulk samples were prepared by mixing minimum five to ten samples collected from each location and mixed well. From these homogenous bulk samples, minimum three representative samples were collected and immediately stored at 4 °C and taken to the laboratory for analysis. Samples were analysed for their total hydrocarbon, pH, moisture content, soil organic carbon (SOC), soil enzyme activities and total bacterial count. For total hydrocarbon content, 5 g of contaminated sample was taken in a glass beaker and extracted with diethyl ether. The solvent was then filtered out into another fresh glass beaker. The process was repeated minimum three times or till the soil was found to be free of the oil content. The solvent obtained from all the extraction steps was pooled together. This filtrate was then allowed to dry under room temperature and weighed. The amount of dried filtrate is equal to the total hydrocarbon content in the contaminated sample and expressed as percentage (%). The process was repeated minimum three times. The filtrate
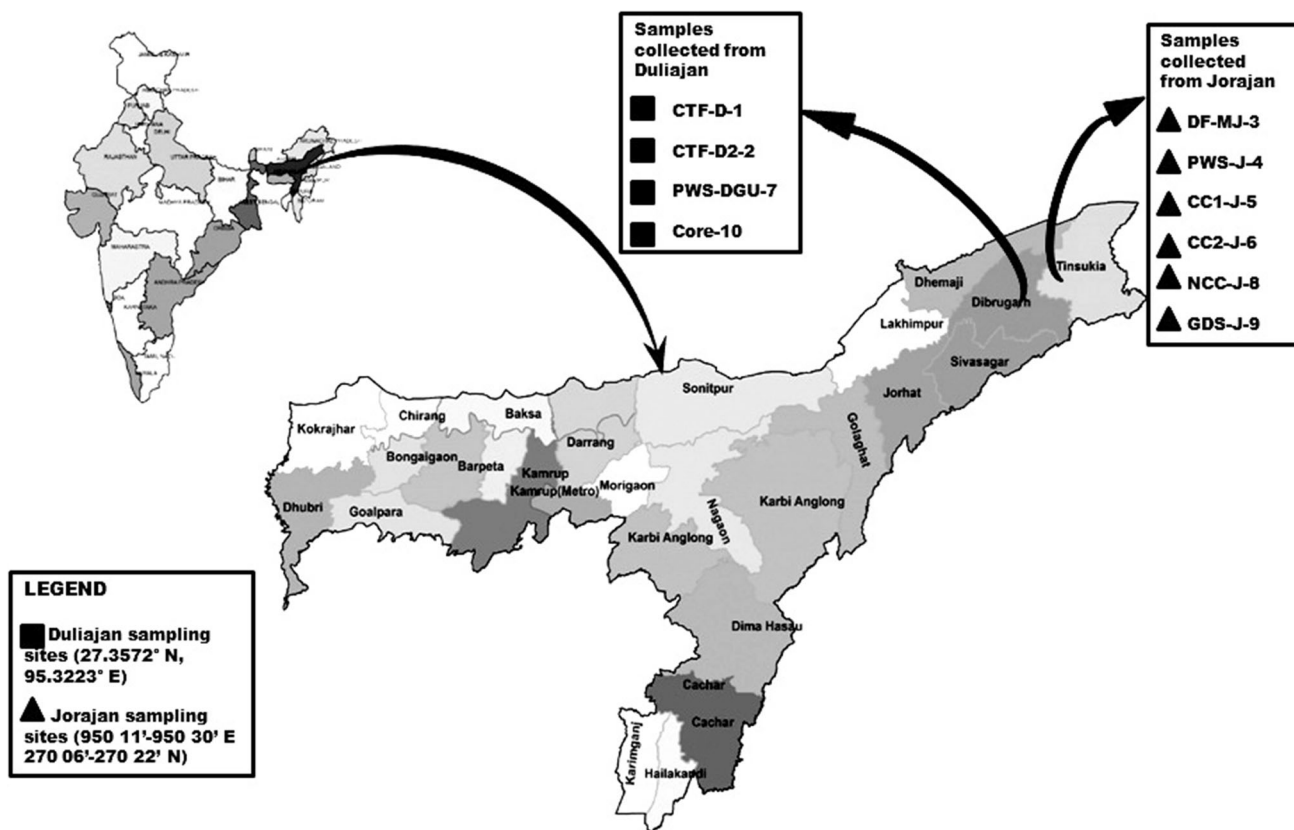
Fig. 1 Schematic representation of study sites in Assam

obtained was dried and weighed. pH of the contaminated sample was estimated in sample/water suspension (1:2.5) using pH meter (Eutech, Malaysia); moisture content was determined gravimetrically by drying the samples at 70 °C until constant weight. Total SOC was determined according to Walkley and Black (1934) Total organic carbon of the samples was determined according to Walkley and Black (1934). Briefly, to 1 g sample 10 ml of 1 N potassium dichromate and 20 ml of concentrated H_2SO_4 were added, agitated for about a minute and allowed to stand for 30 min. Following this, 200 ml distilled water, 10 ml orthophosphoric acid and 1 ml diphenylamine indicator were added. The solution was than titrated against standard ferrous ammonium sulphate (FAS) till bluish green colour. Organic carbon is calculated in percentage. Dehydrogenase activity was estimated by using 2,3,5-triphenyl tetrazolium chloride (TTC) red technique (Casid et al. 1964). To 1 g fresh sample, 0.1 gm of $CaCO_3$ and 1 ml of TTC solution were added in 3 replicates each and incubated at 30 °C for 24 h. The resulting slurry was passed through Whatman No. 1 filter paper and extracted with successive aliquots of methanol, adjusted to 50 ml and read at 485 nm using Specord 210 UV–Vis spectrophotometer (Analytikjena). Dehydrogenase activity of the samples per gm was expressed in terms of µg TPF per g dry sample per 24 h.

Urease activity of the samples was measured following the method of McGarity and Mayers, (1967) for which to 1 g of sample, 1 ml of toluene was poured and allowed to stand for 15 min. Subsequently, 10 ml buffer (pH 7) and 5 ml of 10% urea solution were added and incubated for 3 h at 37 °C, adjusted to 100 ml with distilled water, mixed thoroughly and filtered through Whatman No. 5 filter paper. From this filtrate, 0.5 ml was taken and made to 30 ml with distilled water and treated with 2 ml phenolate solution and 1.5 ml sodium hypochlorite solution. Absorbance was read at 630 nm. The amount of NH_4^+-N released was calculated by a reference calibrated curve and expressed as NH^+–N mg per gm dry sample per 3 h. Phosphatase activity was determined as per by Tabatabami and Bremner (1969). To 1 g sample, 4 ml of universal buffer, 0.25 ml of toluene and 1 ml of 0.115 ml *p*-nitro phenyl phosphate solution were added and incubated at 37 °C for 1 h. One milligram of 0.5 M $CaCl_2$ and 0.5 M NaOH solution were introduced to the mixture. The suspension was filtered through Whatman No. 1 filter paper, and optical density was measured at 430 nm. The phosphatase activity was calculated in terms of concentration of PNP in each sample from a standard curve of PNP in water and expressed as mole of PNP released per g dry sample per hour.

Crude oil fractionation, i.e. asphaltene, resin, wax, aliphatic/aromatic fraction, was separated according to Yenn et al. (2014). To 5 g of crude oil, 150 ml of n-heptane was added and heated under reflux conditions at 100 °C for 1 h in a round-bottom flask. The flask was with stopper and allowed to cool under dark condition for 2 h. The content was then filtered through Whatman No. 42 filter paper without agitation. The residue obtained was asphaltene and was dried and weighed for further experiments. The filtrate then was absorbed in 120 g of silica gel and heated in a water bath for 1 h with continuous stirring. The resulting material was then allowed to stand undisturbed overnight and then filtered. The residue obtained after filtration was collected and washed with distilled water, dissolved in a toluene–methanol (90:10) mixture and dried in a boiling water bath, and the dried fraction was weighed to estimate the percentage of resin. The filtrate was heated in an oven briefly, treated with 5 ml of H_2SO_4 and then allowed to cool down to room temperature. The wax solution was decanted and washed with warm water and ammonium hydroxide solution several times till the acid in it got removed. Then, the crude wax thus obtained was dissolved in ethylene chloride ($CHCl_2$), cooled to around 32 °C and filtered through a cold filter funnel, and the wax was collected. The filter funnel was washed with hot n-heptane solvent and collected in a weighed flask. The collected material was evaporated and dried to obtain the residual aliphatic and aromatic components.

Enumeration and isolation of bacterial strain

Enumeration of bacterial population was done as described by Roy et al. (2014) and expressed as log_{10}. Briefly, serial dilution technique followed by plating on nutrient agar (NA), Reasoneŕs 2A agar (R2A), Pikovskaya, N_2-Free media and mineral media M1 with crude oil as carbon source was done for isolating colonies. The incubation temperature was maintained at 30 °C for 24 h. Morphologically distinct colonies were picked and purified in fresh NA plates. The purity was further confirmed microscopically. Morphological and biochemical properties of each isolate were documented as described in Cappuccino and Sherman laboratory manual (1998) and Bergey's Manual of determinative bacteriology (Weeks and Breed 1957).

Identification of bacterial strains by 16S rDNA sequencing analysis

Bacterial genomic DNA was extracted from the isolates obtained using GenElute bacterial genomic DNA kit (Sigma Aldrich). PCR amplification of the genomic DNA was carried out using the universal 16S rDNA primers fD1 AGAGTTTGATCCTGGCTCAG and rP2 ACGGCTACCT TGTTACGACTT (Weisburg et al. 1991) with reaction conditions set at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 45 s and 72 °C for 1.30 min and then final extension at 72 °C for 10 min. 16S rDNA amplicon from each strain was sequenced, and the data were searched using NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast) search tool for identification of the strain type.

Soil DNA extraction and 16S rRNA gene PCR amplification

DNA was extracted from approximately 0.5 g of soil using UltraClean^{TM} soil DNA isolation kit (Mo Bio, CA, USA) following the manufacturer's instructions. Further, the V3 region of 16S rRNA gene from these metagenomic DNA samples was obtained by the partial amplification of the respective gene using specific primers 341f and 518r (Muyzer et al. 1998). Briefly, PCR mixture was carried out in 50 μl volume consisting of 1.0 U Taq polymerase, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM of each dNTPs, 10 μM of each primer (forward and reverse) and 20–30 ng of soil DNA. PCR-grade water was used as a negative control in PCR. The resultant amplicons were electrophoresed to check their integrity against 100-bp DNA ladder (Thermo Scientific, USA) using image acquisition software (Universal Hood III, Bio-Rad, USA). Thermal cycling was done in an GENEI-TC3000 thermocycler (GENEI, India) with the conditions given hereunder: 3 min initial denaturation at 94 °C; 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 58 °C and 60 s elongation at 72 °C; and a 10-min final elongation. PCR amplicons were further subjected for DGGE-based fingerprinting analysis and then sequencing.

DGGE Profiling and 16S rRNA gene sequencing

Gels comprising of 40% (w/v) polyacrylamide (37.5:1 acrylamide/bisacrylamide) were cast using a Universal Mutation Detection System (Dcode^{TM}, Bio-Rad, USA) for denaturing gradient gel electrophoresis (DGGE) analysis. The gels had a linear gradient from 40 to 60% denaturant, where 100% denaturing acrylamide contained 7 M urea and 40% (v/v) formamide (Muyzer et al. 1998). Electrophoresis was conducted for 16 h at 60 V at a constant temperature of 60 °C in 7 L of 1 × TAE. The gels were stained in ddH_2O containing 0.5 mg l^{-1} ethidium bromide for 10 min, and the gel images were digitally captured using image acquisition software (UVITEC, GENEI, India). Any bands migrating to the same position in the gel were assumed to be identical amplicons. The DGGE bands were excised from the gel using a sterile scalpel and incubated for 24 h at 4 °C. Further, PCR was done with the

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same primers devoid of GC-clamp, and the resultant amplicons were outsourced for sequencing (3130xl Applied Biosystems, ABI, USA).

Phylogenetic analysis

To determine evolutionary relationship among the bacterial isolates as well as the uncultured 16S rRNA V3 sequences, the gene sequence were compared against those obtained from GenBank database using NCBI-Blast as mentioned above and Seqmatch tool (Cole et al. 2007). Multiple sequence alignment was carried out using ClustalW (http://www.ebi.ac.uk/clustalw/) sequence alignment tool. Phylogenetic tree was then constructed using the neighbour-joining method of MEGA6 program (Tamura et al. 2013). Branching orders of the trees were ascertained and compared using distance-based neighbour-joining algorithms with Kimura two-parameter corrections to improve the reliability of internal branches (Kimura 1980).

Carbon source utilization pattern-based community-level physiological profiling

Metabolic fingerprint of microbial community were obtained from Biolog Ecoplates (Biolog, Inc, Hayward, CA, USA). Utilization of 31 different carbon sources in triplicate was analysed by inoculating the plates with aqueous preparation of samples. Data were recorded at every 24-h interval till day 7 at 590 nm. The results obtained on 7th day were used for comparison. Microbial activity in each microplate was expressed as average well colour development (AWCD) (Garland 1996). Shannon diversity and evenness indices were calculated following the method as described by Staddon et al. (1997) and Nautiyal (2009).

Statistical analyses

All the data were mean of three independent experiments each. All the data were subjected to analysis of variance (ANOVA) after normalization, and significant differences among the study sites were compared at $p < 0.01$ according to Tukey's test. Data related to CFU were converted into logarithmic values, before statistical analysis. Further, to establish the relationship on physiological community profiling of each site based on carbon source utilization pattern, principal component analysis (PCA) was performed. For this, dimension reduction in the Biolog data of 10 soil samples was normalized and transformed as described by Weber et al. (2007). Statistical analysis was performed using IBM SPSS Statistics 20 software (version 16.0).

Results and discussion

Physicochemical properties

A schematic map of the study site is depicted in Fig. 1. Site description and characteristics of samples are summarized in Table 1. The total hydrocarbon content of the contaminated soil was obtained in a range of 0.40–89.36% as revealed by gravimetric assessment. Of the total samples, 90% were of basic pH (7.8–9.1). The sample collected from Core-10 site was found to have significantly different properties as compared to the other study sites. Organic carbon content was found to be high in all the soil samples, ranging from 1.38 (in CTF-D-1) to 6.9 (in CTF-D2-2 and Core-10). Exceptionally low moisture content was found in the core sample compared to the others. Overall, a significantly different moisture content for Core-10 (1.25%), peat water samples PWS-J-4 (52.5%), PWS-DGU-7 (38.8%) and drilling fluid site DF-MJ-3 (30.6%) was observed. The variations in physical and chemical properties of hydrocarbon-contaminated soils were mainly due to the source of hydrocarbon contamination as well as the extent of crude oil contamination.

Soil enzyme activity

Considerable differences in soil enzyme activity were found among the samples (Fig. 1). Highest amount of dehydrogenase (68.6 ± 0.01 μg/g of fresh soil/h), alkaline phosphatase (8.2 ± 0.01 μg/g of fresh soil/hr) and neutral phosphatase (2.9 ± 0.03 μg/g of fresh soil/h) activity was found for the samples collected from CC1-J-5 site. Nevertheless, urease enzyme activity was found to be higher in site NCC-J-8 (0.002 ± 0.004 μg/g of fresh soil/h). From the observation, it was noticed that compared to dehydrogenase and phosphatase activities, very low urease activities were observed in the hydrocarbon-contaminated soil. In addition, Pearson correlation analysis revealed a significant positive correlation between the bacterial colony counts on crude oil supplemented media ($r = 0.6, 0.7$) and the total hydrocarbon and organic carbon content (as shown in supplementary table S1). The positive correlation may be due to the selective preference of bacterial growth.

Cultivable bacterial diversity

Using culture-dependent approach, a total of 160 morphologically distinct culturable strains were isolated from the 10 different crude oil-contaminated sites using different culture media (Table 1). Based on Gram staining, biochemical and morphological observation 117 strains were found to be Gram-positive bacteria and the rest 43 strains

Table 1 Physical, chemical and biological characteristics of oil-contaminated soil of Assam used for isolation of hydrocarbonoclastic bacteria

Sample ID	Description	Total hydrocarbon (%)	pH	Moisture content (%)	Organic carbon (%)	Soil enzymes (µg/g fresh soil/hour)				Total no. of morphologically distinct isolates picked
						Dehydrogenase	Neutral phosphatase	Alkaline phosphatase	Urease	
CTF-D-1	Oil sludge pit central tank farm (CTF), Duliajan	15.8 ± 0.07^c	9.1 ± 0.3^a	66.6 ± 0.3^a	1.4 ± 0.07^a	14.5 ± 0.1^c	0.2 ± 0.02^c	1.1 ± 0.003^c	0.001 ± 0.005^a	52
CTF-D2-2	Sludge having mostly crude oil, CTF, Duliajan	89.4 ± 0.02^a	8.8 ± 0.3^a	10.1 ± 0.3^f	6.9 ± 0.2^a	22.7 ± 0.1^b	0.1 ± 0.04^c	0.5 ± 0.001^d	0.001 ± 0.005^a	13
DF-MJ-3	Mud sample (drilling fluid) DGU drilling site, Jorajan	1.1 ± 0.02^e	8.6 ± 0.1^a	30.6 ± 0.4^d	6.9 ± 0.3^a	10.4 ± 0.2^d	0.5 ± 0.1^c	0.4 ± 0.004^d	0.001 ± 0.001^a	21
PWS-J-4	Mud like peat water sample DGU drilling site, Jorajan	0.04 ± 0.02^e	8.9 ± 0.2^a	52.5 ± 0.3^b	5.4 ± 0.2^a	11.8 ± 0.1^d	0.3 ± 0.1^c	0.5 ± 0.001^d	0.001 ± 0.081^a	14
CC1-J-5	Crude oil-contaminated soil sample (1) near Jorajan drilling site	14.6 ± 0.02^c	8.0 ± 0.2^a	11.1 ± 0.3^e	5.4 ± 0.3^a	68.6 ± 0.2^a	2.9 ± 0.2^a	8.1 ± 0.01^a	0.001 ± 0.003^a	12
CC2-J-6	Crude oil-contaminated soil sample (2) near Jorajan drilling site	38.5 ± 0.05^b	7.9 ± 0.2^a	10.8 ± 0.3^f	5.6 ± 0.2^a	11.2 ± 0.1^d	0.64 ± 0.5^{bc}	2.9 ± 0.01^b	0.001 ± 0.001^a	13
PWS-DGU-7	Peat water sample DGU	0.2 ± 0.02^e	8.7 ± 0.2^a	38.8 ± 0.4^c	3.7 ± 0.05^a	3.7 ± 0.2^e	0.2 ± 0.1^c	0.9 ± 0.001^{cd}	0.002 ± 0.001^a	11
NCC-J-8	Soil taken from areas near contaminated sites in Jorajan	1.9 ± 0.01^{de}	8.6 ± 0.3^a	11.1 ± 0.3^{ef}	2.2 ± 0.3^a	11.8 ± 0.8^{cd}	0.5 ± 0.1^c	0.9 ± 0.001^d	0.002 ± 0.004^a	11
GDS-J-9	Contaminated soil sample abandoned gas drilling site, Jorajan	3.0 ± 0.02^d	8.8 ± 0.2^a	67.1 ± 0.3^a	4.7 ± 0.2^a	25.7 ± 0.5^b	1.1 ± 0.6^b	2.3 ± 0.001^b	0.001 ± 0.000^a	8
Core-10	Core sample depth ∼3600 m (Eocene formation)	0.4 ± 0.04^e	3.0 ± 0.2^a	1.25 ± 0.2^g	6.9 ± 0.2^a	11.4 ± 0.6^d	0.1 ± 0.1^c	0.3 ± 0.001^d	0.002 ± 0.007^a	5

Data shown are mean of three individual observations. SD value followed by similar letter denotes no significant difference

The samples were collected on March, 1, 2013 from Duliajan and Jorajan of Dibrugarh and Tinsukia districts, respectively. The photograph of the sites is shown in Fig. 1. The outdoor temperature during collection was 26 °C and an average sunny day. The area was contaminated more than a decade with crude oil. The Core-10 sample collected is distinctively different from the others having a pH of 3. This was collected from a depth of around 3600 m (Eocene formation) that is characterized by large sequestration of carbon dioxide in the form of methane clathrate, coal and crude oil that reduced the atmospheric carbon dioxide

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were identified as Gram-negative bacteria. Highest number of bacterial colonies ($n = 52$) were identified from the CTF-D-1 site, whereas only 5 isolates could be collected from Core-10 site. The order of bacterial population was highest in site CTF-D-1 followed by DF-MJ-3 > PWS-J-4 > CTF-D2-2, CC2-J-6 > CC1-J-5 > PWS-DGU-7, NCC-J-8 > GDS-J-9 > Core-10, respectively. The variation in the number of isolates was mainly due to the differences in the TPH levels in these sites.

Based on the sequence similarity of 136 strains that were obtained from 160 isolates, we found that the majority of the strains belonged to the phylum Proteobacteria (Betaproteobacteria-2, Gammaproteobacteria-17) and Fermicutes (Bacilli-115, Clostridia-2), under the genus *Enterobacter*, *Pseudomonas*, *Alkaligenes*, *Bacillus*, *Lysinibacillus* and *Clostridium*. List of bacterial strains identified in the present study along with their closest relatives from NCBI database is shown in table S2. From the observation, it was found that, in site CTF-D-1 the presence of genera *Enterobacter*, *Pseudomonas*, *Bacillus*, *Lysinibacillus* and *Clostridium*, in site CTF-D2-2 *Enterobacter*, *Bacillus* and *Alkaligenes,* in site DF-MJ-3 *Clostridium*, *Bacillus*, *Lysinibacillus* and *Paenibacillus,* in sites PWS-J-4, CC2-J-6 and PWS-DGU-7 *Pseudomonas, Bacillus* and *Lysinibacillus,* in site CC1-J-5 *Pseudomonas, Bacillus, Exiguobacterium* and *Lysinibacillus,* in site NCC-J-8 *Bacillus* and *Lysinibacillus,* in site GDS-J-9 *Bacillus, Lysinibacillus* and *Alkaligenes* and in site Core-10 *Pseudomonas and Bacillus* were identified (Table 2). The dominance of genus *Enterobacter*, *Pseudomonas*, *Bacillus*, *Lysinibacillus*, *Alkaligenes* and *Clostridium* could be due to their adaptability to the presence of crude oil and their ability to use petroleum hydrocarbons as carbon source for growth processes.

Culture-independent bacterial community profiling

In order to obtain the uncultured bacterial diversity associated with crude oil-contaminated sites, soil metagenome isolation followed by DGGE of PCR-amplified 16S rDNA fragments was carried out (Fig. 2). All the 10 sites showed prominent DGGE banding profiles, having a total of 33 bands, of which 9 were unique, while the others were present in two or more sites. Highest number of bands ($n = 11$) were observed in PWS-J-4 site, showing maximum species richness. Dendrogram obtained from clustering of all the sites based on DGGE banding patterns of the 10 sites is shown in Fig. 3. From this analysis, 6 distinct clades were identified with sites CC1-J-5 and CC2-J-6 as two separate branches and CTF-D-1 and CTF-D2-2; PWS-DGU-7 and GDS-J-9; NCC-J-8 and Core-10; and DF-MJ-3 and PWS-J-4 comprising the four clades. From the DGGE profile, 17 distinct bands, referred as operational taxonomic

unit (OTU) were successfully sequenced (Fig. 4, Table S3). From this analysis, it was confirmed that the bacterial community mainly originated from the genera *Sphingomonas*, *Ralstonia*, *Sphingobium*, *Massilia*, *Acinetobacter* and *Pseudomonas*. Further, DGGE fingerprints of each site were analysed to identify the species richness and diversity of the crude oil-contaminated sites (Fig. S2). Among the sites, PWS-J-4 was found to have highest bacterial richness (~ 11) and bacterial diversity (~ 2.3), whereas CC1-J-5 site was found to have least bacterial diversity as well as bacterial richness. Form the observation, we found that the lowest degree of crude oil contamination showed the maximum species richness and hence the highest diversity.

Carbon source utilization pattern-based community-level physiological profiling

Evaluation of community-level physiological profiling based on carbon utilization pattern, a significantly different Shannon diversity ($p < 0.05$), Simpson diversity index and McIntosh evenness was observed in CTF-D-1 and Core-10 than in the other study site (Table 3). Principal component analysis (PCA) also showed no distinct association (Fig. 5). It was found that Core-10 (V10) was most diverse, indicating the presence of significantly different microbial community. Similar to culture-dependent and culture-independent approach, in physiological community profile we observed that source and crude oil contamination affected the overall microbial community-level physiological activities.

Among other ecological niche crude oil-contaminated environment is one of the ecologically degraded environments which harbour numerous bacteria (Aislabie et al. 2004; Saul et al. 2005). The microbes inhabited in crude oil-contaminated environments determine the fate of crude oil in nature. Therefore, assessment of diversity and species richness of microbial population in crude oil-degraded environment is being given utmost important (Hazen et al. 2010; Beazley et al. 2012; Liu and Liu 2013). Assam houses many crude oil-contaminated environments which have emerged as major matter of concern. In our previous study, we have compared bacterial diversity in different locations of Sivasagar and Jorhat districts and maximum of oil-contaminated soil environment was found to contain 15.0–56.0% (Roy et al. 2014; Yenn et al. 2014; Das et al. 2015), whereas in the present study, the origin and nature of contamination were quite different (Table 1). Overall hydrocarbon content in the present study was fairly high and ranged from 0.04 to 89.36% which was due to bottom water tank sludge, drilling fluid, drilling sites, etc. The highest soil organic carbon (6.99%) was found in mud sample, whereas lowest (1.38%) was seen for oil sludge

Table 2 Comparative analysis of bacterial isolates from 10 crude oil-contaminated sites under study

Strain	Sites under study									
	CTF-D-1	CTF-D2-2	DF-MJ-3	PWS-J-4	CC1-J-5	CC2-J-6	PWS-DGU-7	NCC-J-8	GDS-J-9	Core-10
Enterobacter hormaechei	KF843695 (R1) KF843696 (R2) KF843700 (R11)									
Enterobacter sp.	KM459022 (R3) KF843698 (R8) KM459025 R15	KJ499800 (RC4)								
Pseudomonas putida	KF843697 (R5) KU752806 (R39)			KU752847 (R89)						
Clostridium sp.	KM459023 (R6)		KU752884 (RP1)							
Lysinibacillus sphaericus	KM459024 (R7)			KU752845 (R87)	KU752854 (R96)					
Enterobacter cloacae	KF843699 (R9)									
Pseudomonas stutzeri	KJ499784 (R16)									KJ499802 (RC11)
Bacillus sp.	KJ499785 (R17) KJ499789 (R21) KJ499792 (R24) KJ499794 (R26) KJ499795 (R27) KJ499797 (R29)		KU752828 (R61)	KU752839 (R80)		KU752865 (R107)	KU752869 (R111) KU752871 (R114) KU752872 (R116) KU752873 (R117)		KU752892 (RR3)	
Lysinibacillus sp.	KJ499786 (R18) KJ499796 (R28)		KU752886 (RP3)	KU752844 (R86) KU752848 (R90)	KU752850 (R92) KU752852 (R94)	KU752861 (R103) KU752891 (RR2)	KU752895 (RR7)	KU752877 (R121) KU752887 (RP4) KU752888 (RP5) KU752893 (RR4)	KU752881 (R127)	
Bacillus megaterium	KJ499787 (R19) KU752818 (R51)	KU752820 (R53)								

Table 2 continued

Strain	Sites under study									
	CTF-D-1	CTF-D2-2	DF-MJ-3	PWS-J-4	CC1-J-5	CC2-J-6	PWS-DGU-7	NCC-J-8	GDS-J-9	Core-10
Bacillus cereus	KJ499788 (R20) KJ499790 (R22) KU752811 (R44) KU752812 (R45) KU752813 (R46)		KU752832 (R71) KU752835 (R76)	KU752836 (R77) KU752838 (R79)	KU752856 (R98)	KU752860 (R102) KU752867 (R109)	KU752874 (R118) KU752894 (RR5)			
Pseudomonas plecoglossicida	KJ499791 (R23)									
Bacillus aryabhattai	KJ499793 (R25)	KU752825 (R58)							KM459019 (RC6)	
Bacillus anthracis	KJ499798 (R30)									
Bacillus methylotrophicus	KU752799 (R31) KU752803 (R35) KU752804 (R37) KU752808 (R41) KU752810 (R43) KU752814 (R47) KU752817 (R50)				KU752855 (R97) KU752858 (R100)	KU752862 (R104)		KU752876 (R120)		
Bacillus subtilis	KU752800 (R32) KU752802 (R34) KU752805 (R38) KU752807 (R40) KU752809 (R42) KU752816 (R49)	KU752819 (R52) KU752822 (R55) KU752827 (R60) KF953985 (RC2)	KU752829 (R64) KU752834 (R74) KU752885 (RP2) KJ499804 (RC4)	KU752843 (R85)		KU752863 (R105) KU752866 (R108)		KU752878 (R122) KU752879 (R123)		KM459020 (RC10)
Bacillus pumilus	KU752801 (R33)	KU752823 (R56) KU752824 (R57) KU752826 (R59)	KM459021 (RC13)				KU752875 (R119)			

Table 2 continued

Strain	Sites under study									
	CTF-D-1	CTF-D2-2	DF-MJ-3	PWS-J-4	CC1-J-5	CC2-J-6	PWS-DGU-7	NCC-J-8	GDS-J-9	Core-10
Bacillus amyloliquefaciens	KU752815 (R48)									
Bacillus altitudinis		KU752821 (R54)								
Bacillus tequilensis			KU752830 (R65)							
Bacillus safensis			KU752831 (R66)							
Paenibacillus sp.			KU752833 (R73)							
Bacillus thuringiensis				KU752837 (R78) KU752840 (R81)		KU752890 (RR1)				
Lysinibacillus fusiformis				KU752842 (R84) KU752846 (R88)	KU752857 (R99)	KU752868 (R110)	KU752870 (R112)	KU752889 (RP6)		
Pseudomonas fluorescens					KU752849 (R91)					
Exiguobacterium sp.					KU752851 (R93)					
Pseudomonas koreensis					KU752853 (R95)					
Bacillus flexus						KU752859 (R101) KM505011 (RC8)				
Pseudomonas sp.						KU752864 (R106)				
Bacillus aerophilus									KU752880 (R126)	
Bacillus badius										KU752882 (R130) KU752883 (R131)
Alcaligenes faecalis		KF843701 (RC1)								

Table 2 continued

Strain	Sites under study									
	CTF-D-1	CTF-D2-2	DF-MJ-3	PWS-J-4	CC1-J-5	CC2-J-6	PWS-DGU-7	NCC-J-8	GDS-J-9	Core-10
Enterobacter cancerogenus		KJ499799 (RC3)								
Alcaligenes sp.									KF953984 (RC5)	
Bacillus stratosphericus	KM505010 (RC7)									
Bacillus licheniformis					KJ499803 (RC12)					

collected from pit, Duliajan. The contaminated samples were alkaline in nature except for the Core-10 sample collected from a depth of 3600 m. Biological activities of the present soils were very low. This wide variation in physical, chemical and biological properties of crude oil-contaminated soil of present study was due to source of hydrocarbon contamination and hydrocarbon load. Soil biological activity, including soil enzymatic activity, has been reported to be influenced by a range of environmental stresses and contaminants (Alrumman et al. 2015).

The natures of contamination of crude oil in different soil environment were due to nature of the operational contamination. Overall hydrocarbon content in the present study was also quite high and ranges from 0.04 to 89.36% which was due to bottom water tank sludge, drilling fluid, drilling sites, etc. Studies on special environmental conditions of crude oil contamination soils reported that there were overall less microbial diversity and species richness in oil-contaminated soil (Aislabie et al. 2004; Saul et al. 2005) which is in agreement with the present findings. Hydrocarbon contaminants have also been reported to restrain growth of certain responsive microbial groups (Santos dos et al., 2011) and selectively trigger subgroups like that of the Actinobacteria and Proteobacteria in contaminated soils (Bell et al. 2013, 2014), hence affecting the microbial diversity. In the present study, we were able to isolate 160 morphologically distinct bacteria and 16S rDNA analysis confirmed that the isolates belonged to the genus *Bacillus, Alkaligenes, Enterobacter, Clostridium, Exoguibacterium* and *Pseudomonas*. The highest number of population isolated was from CTF-D-1 followed by TFD2-2 > DF-MJ-3 > PWS-J-4 > CC1-J-5 > CC2-J-6 > PWS-DGU-7 and NCC-J-8 > GDS-J-9 > Core-10, respectively. We are also reporting for the first time the presence of *Exiguobacterium* sp. from CCJ2-J-6 which might be due to drilling sludge and other stress. To forecast any relationship between microbial population and hydrocarbon, we found that except TPH with microbial population in M1 media, no other parameters showed significant positive correlation (Table S1). The positive correlation between TPH and M1 media is due to crude oil-specific growth. Interestingly, previous study conducted by Margesin et al. (2003) also showed no correlation between concentration of TPH in soil environment and microbial population.

From the DGGE banding profile, 17 distinct bands were successfully sequenced. BLAST-N analyses revealed prevalence of genus *Sphingomonas*, *Ralstonia*, *Sphingobium*, *Massilia*, *Acinetobacter* and *Pseudomonas* in the samples. *Proteobacteria* was reported to typically dominate microbial assemblage in hydrocarbon-contaminated soils (Greer et al. 2010). Several previous studies reported *Pseudomonas* and *Acenitobacter* in various hydrocarbon-

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Fig. 2 DGGE band profiles of PCR-amplified 16S rDNA fragments obtained from 10 crude oil-contaminated soils. Bands marked with *arrow* (*n* = 25) showed prominent bands that were excised for sequencing
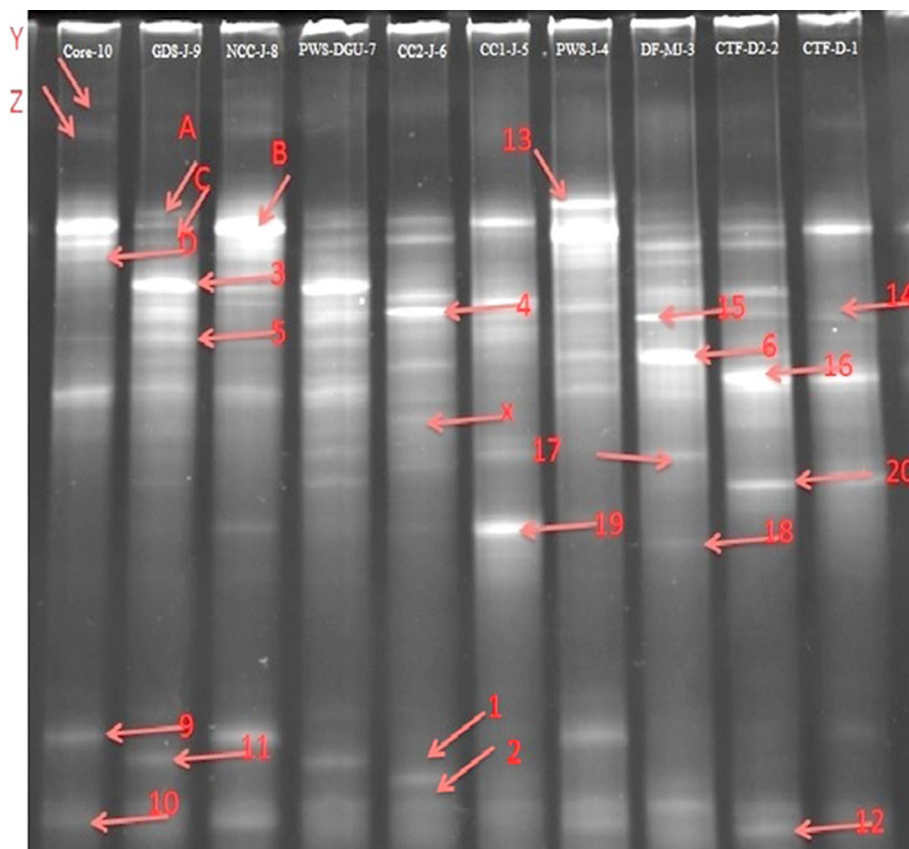
Fig. 3 Clustering based on DGGE banding pattern of crude oil-contaminated sites under study. **a** DGGE gel showing banding profiles of 16S rRNA-V3 gene region isolated from the each soil DNA; **b** dendrogram obtained from clustering of DGGE bands of the 10 soil samples. A total of six distinct clades with sites CC1-J-5 and CC2-J-6 as two separate branches; CTF-D-1 and CTF-D2-2; PWS-DGU-7 and GDS-J-9; NCC-J-8 and Core-10; and DF-MJ-3 and PWS-J-4 comprising the four clades were identified
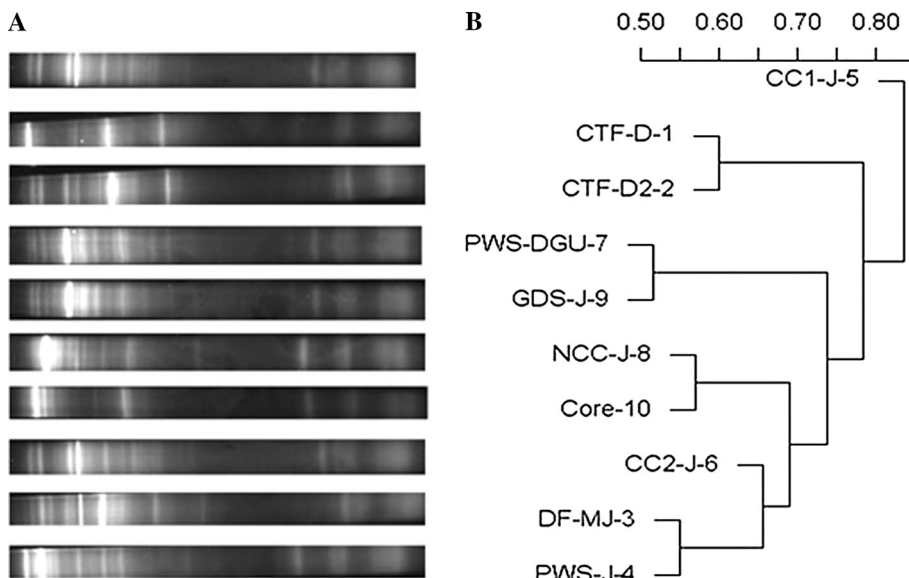
contaminated environments using culture-dependent (Belhaj et al. 2002; Bhattacharya et al. 2003) and culture-independent (Kaplan and Kitts 2004; Margesin et al. 2003; Roling et al. 2004) approaches. The presence of *Spingomonas* and *Raslstonia* in contaminated sites was also

Fig. 4 Phylogenetic tree showing the relationships among the 16S ▶ rRNA-V3 gene sequences of DGGE bands obtained from the 10 crude oil-contaminated soils. Single OTUs identified from the sequenced data of amplified 16S rRNA-V3 gene region revealed the occurrence of genera *Sphingomonas*, *Ralstonia*, *Sphingobium*, *Massilia*, *Acinetobacter* and *Pseudomonas* in the contaminated sites

◆ KT186601 Uncultured Pseudomonas mendocina clone 6.9
gi|817250498|emb|LN835456.1|:249-442 Pseudomonas mendocina partial 16S rRNA gene strain HWS1044
gi|817250498|emb|LN835456.1|:249-442 Pseudomonas mendocina strain HWS1044
gi|820948535|gb|KP282812.1|:271-464 Pseudomonas mendocina strain ZJY-503
gi|820948547|gb|KP282824.1|:267-460 Pseudomonas mendocina strain ZJY-262
◆ KT186606 Uncultured Pseudomonas mendocina clone 10.D
◆ KT186600 Uncultured Pseudomonas mendocina clone 8.B
◆ KT186598 Uncultured Pseudomonas mendocina clone 6.2
gi|596301869|gb|KC901552.1|:328-521 Pseudomonas sp. 69ene04
gi|664802716|gb|KJ573547.1|:231-424 Pseudomonas sp. TSASRA007
gi|827529026|gb|KP175321.1|:281-474 Pseudomonas alcaliphila strain 2F
◆ KT186599 Uncultured Pseudomonas alcaliphila clone 6.4
gi|822590896|gb|KP319030.1|:280-473 Pseudomonas alcaliphila strain Cd-t1
gi|752480603|gb|KP410048.1|:289-482 Uncultured prokaryote clone seq G07 Z117731 Z066856a X-S43 16SF
◆ KT186597 Uncultured Pseudomonas clone 6.1
gi|436429271|gb|KC140599.1|:289-482 Pseudomonas sp. WS-7
gi|192758014|gb|EU781490.1|:261-454 Pseudomonas sp. USTB-04
◆ KT186604 Uncultured Pseudomonas clone 9.A
gi|42557834|emb|AJ544240.1|:315-508 Pseudomonas stutzeri CLN100
gi|342314709|gb|JN221506.1|:285-478 Pseudomonas sp. 1.5-03
gi|227002202|gb|FJ866731.1|:240-433 Pseudomonas sp. WH364
gi|55831299|gb|AY789545.1|:269-462 Pseudomonas pseudoalcaligenes strain IH8-15
◆ KT186596 Uncultured Acinetobacter clone 5.19
gi|238312567|gb|GQ076390.1|:309-503 Uncultured bacterium clone nbw364e02c1
gi|723219625|gb|KM243756.1|:329-523 Acinetobacter sp. HA 16S
◆ KT186602 Uncultured bacterium Duliajan oil clone 9.5
gi|460185328|gb|KC521859.1|:289-482 Uncultured bacterium clone 2-61
gi|460185298|gb|KC521829.1|:286-479 Uncultured bacterium clone 2-30
gi|460185294|gb|KC521825.1|:288-481 Uncultured bacterium clone 2-26
◆ KT186603 Uncultured bacterium Duliajan oil clone 9.11
gi|760236177|gb|KP183053.1|:338-531 Uncultured Ralstonia sp. clone 12L 38
gi|171703178|dbj|AB366318.1|:310-504 Ralstonia sp. SBI-24
gi|395144683|gb|JQ433928.1|:316-509 Ralstonia sp. LT3
◆ KT186592 Uncultured Ralstonia clone 2.16
gi|821345914|dbj|LC052930.1|:2-193 Uncultured bacterium clone: E7LCFH403FO8RX
◆ KT186595 Uncultured Massilia aurea clone 3.18
gi|746874407|gb|KM877032.1|:326-519 Uncultured bacterium clone XZ6-24
gi|674640769|emb|LM994750.1|:243-436 Massilia aurea strain PomC5.2
gi|823651820|gb|KP400444.1|:282-450 Uncultured bacterium clone SS31 partial sequence
gi|762069466|gb|KP257600.2|:302-470 Sphingobium sp. SC 3
gi|751935397|gb|KM978212.1|:258-426 Sphingobium scionense strain SC-5 partial sequence
◆ KT186605 Uncultured Sphingomonas clone 10.9
gi|516545666|gb|KC711591.1|:257-425 Uncultured bacterium clone CON 8F e09
◆ KT186591 Uncultured Sphingomonas clone 2.12
gi|778565764|gb|KP177380.1|:214-382 Bacterium C0144
gi|699448602|gb|KM221303.1|:303-471 Uncultured bacterium clone 12-2B-130
gi|685505229|gb|KJ958399.1|:1-167 Uncultured bacterium isolate DGGE gel band 14
◆ KT186590 Uncultured bacterium Duliajan oil 2.20
gi|498922521|gb|KC884580.1|:41-209 Uncultured bacterium clone Dagang oil-16
◆ KT186593 Uncultured Sphingobium clone 3.6
gi|498922523|gb|KC884582.1|:41-206 Uncultured bacterium clone Dagang oil-18
◆ KT186594 Uncultured bacterium Duliajan oil clone 3.15
gi|498922511|gb|KC884570.1|:41-207 Uncultured bacterium clone Dagang oil-6

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Table 3 Diversity indices for contaminated sites of Assam derived from carbon sources utilization pattern

Sample ID	Shannon diversity	Shannon evenness	Simpson	McIntosh	McEvn
CTF-D-1	1.97 ± 0.10[e]	0.65 ± 0.13[b]	0.86 ± 0.02[b]	0.72 ± 0.02[c]	0.76 ± 0.08[b]
CTF-D2-2	3.30 ± 0.03[abc]	0.96 ± 0.01[a]	0.99 ± 0.00[a]	0.98 ± 0.00[ab]	0.98 ± 0.00[a]
DF-MJ-3	3.32 ± 0.01[ab]	0.97 ± 0.01[a]	0.99 ± 0.00[a]	0.98 ± 0.00[ab]	0.98 ± 0.01[a]
PWS-J-4	3.16 ± 0.10[c]	0.92 ± 0.01[a]	0.98 ± 0.00[a]	0.95 ± 0.01[b]	0.95 ± 0.01[a]
CC1-J-5	3.33 ± 0.03[a]	0.97 ± 0.00[a]	0.99 ± 0.00[a]	0.98 ± 0.00[ab]	0.98 ± 0.00[a]
CC2-J-6	3.35 ± 0.03[a]	0.98 ± 0.01[a]	1.00 ± 0.00[a]	0.98 ± 0.00[a]	0.98 ± 0.00[a]
PWS-DGU-7	3.15 ± 0.05[bc]	0.93 ± 0.01[a]	0.98 ± 0.00[a]	0.95 ± 0.01[ab]	0.95 ± 0.01[a]
NCC-J-8	3.22 ± 0.03[abc]	0.94 ± 0.02[a]	0.99 ± 0.00[a]	0.97 ± 0.00[ab]	0.97 ± 0.01[a]
GDS-J-9	3.32 ± 0.09[abc]	0.98 ± 0.01[a]	0.99 ± 0.00[a]	0.98 ± 0.01[ab]	0.98 ± 0.01[a]
Core-10	2.25 ± 0.11[d]	0.68 ± 0.04[b]	0.82 ± 0.03[c]	0.67 ± 0.03[c]	0.68 ± 0.04[c]

Diversity indices for the contaminated sites under study were calculated from 168-h data in triplicate obtained from Biolog Ecoplates. The average values followed by ±0.1 = SD of observed values. SD followed by superscript similar letter is not significantly different from each other within the column. All the contaminated sites except CTF-D-1 and Core-10 have a Shannon diversity index above 3 and McIntosh index close to 1, showing that the structure of these habitats is relatively stable and balanced
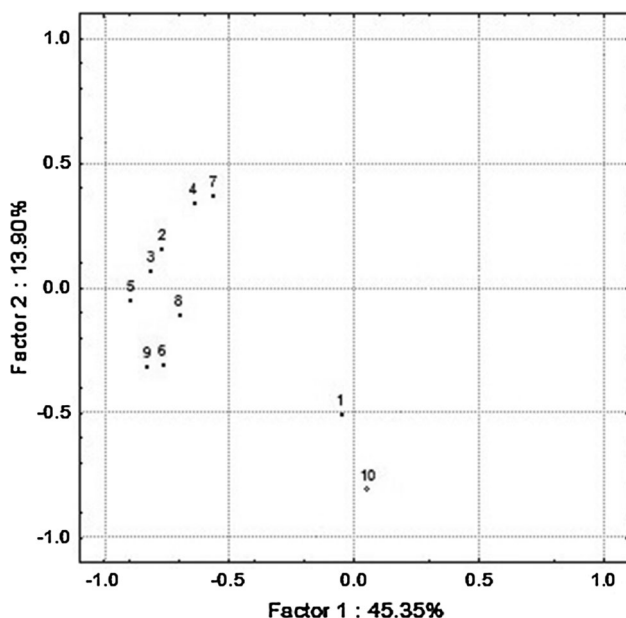
Fig. 5 Principal component analysis (PCA) based on metabolic fingerprint obtained from 10 crude oil-contaminated sites. From the PCA plot, it was seen that site Core-10 was most diverse at 45.35 and 13.9% on first 2 principle components, indicating that this had significantly different bacterial phyla. Site CTF-D-1 also had a diverse pattern as depicted by their physiological profile. Again sites CC2-J-6 and GDS-J-9, CC1-J-5 and NCC-J-8, PWS-J-4 and PWS-DGU-7 were seen to have metabolic fingerprint pattern similar to each other but different from the rest. Sites CTF-D2-2 and DF-MJ-3 were seen to be different from each other in bacterial community structure and also to the others

reported by various researchers (Juhasz and Naidu 2000; Zocca et al. 2004). We also found many of the unidentified clones having >99% sequence similarity to the bacterial clones (Accession nos. KC884580.1, KC884570.1, KC521869.1 and EU491331.1) (Table S3 and Fig. 4) that were reported by researchers from other oil-contaminated sites including Dagang oil fields of China (Li et al. 2006). Interestingly, we observed a disparity between culture-based techniques to that of uncultured diversity assessment which was in agreement with the earlier reports (Lagier et al. 2012; Shade et al. 2012) and contrary to many reports (Lagier et al. 2012; Carraro et al. 2011; Vaz-Moreira et al. 2011). Hamamura et al. (2006) in their study also reported cultivation of numerous HCB from oil-contaminated soil which did not show relationship with the results obtained using culture-independent molecular techniques. Here the difference in culture-based techniques to that of uncultured diversity assessment is mainly due to the lack of ability of some of the bacteria to grow under laboratory condition. This lack of ability may be as a result of inability of providing optimum conditions for the indigenous to grow in laboratory media plates.

The PCA plot of carbon source utilization pattern by microbial community of soil samples: sites CTF-D-1 and Core-10 was seen to be most diverse and was also found to have the highest pH of 9.11 and lowest pH of 3, respectively, as compared to the rest and hence different microbial community inhabiting these sites. Previous studies

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have also reported bacterial community composition to be associated with the quality of soil organic matter, geographical region and environmental factors such as temperature, nutrient availability, soil pH (Wallenstein et al. 2007; Coolen et al. 2011; Lauber et al. 2009; Shen et al. 2013; Bartram et al. 2014). High moisture content of CTF-D-1 (66.6%) and significantly low moisture content of Core-10 (1.25%) as compared to the other sample were another factor that can be responsible for variation in community structures of these sites. Soil water content determines microbial community structure, and Zhou et al. (2002) suggested that free water connecting soil particles may influence diversity patterns, by controlling nutrient availability and cell movement. Low soil moisture was reported to decrease microbial activity by reducing diffusion of soluble substrates, microbial mobility and intracellular water potential (Zhou et al. 2002).

The current study determined the diversity of indigenous bacteria in the crude oil-contaminated sites of Duliajan and Jorajan, Assam. Bacterial isolates obtained from culture-based approach showed predominance of *Lysinibacillus, Alcaligenes*, *Bacillus*, *Clostridium*, *Enterobacter* and *Pseudomonas* in these study sites. Culture-independent DGGE technique confirmed the presence of genera *Sphingomonas*, *Ralstonia*, *Sphingobium*, *Massilia*, *Acinetobacter* and *Pseudomonas*. For the first time, we are reporting the presence of genus *Exoguibacterium* in crude oil-contaminated soil of Assam. PCA analysis revealed CTF-D-1 and Core-10 to be most diverged, indicating significant bacterial phyla, while sites CC2-J-6 and GDS-J-9 were found to have closely similar bacterial phyla. Both culture-dependent and culture-independent approach resulted in a total of 11 genera. The present findings is the first-hand report on total bacterial diversity in crude oil-contaminated soil of Assam, which can be assisted in further exploitation of these for bioremediation purposes.

Conclusion

Pollution by hydrocarbons may stimulate the growth of hydrocarbon-utilizing/hydrocarbon-degrading bacteria and alter the structure of microbial communities in the contaminated area. Thus, classification of the organisms that participate in hydrocarbon biodegradation is important for assessing and developing in situ bioremediation strategies. Assessment of diversity and species richness of microbial population in crude oil-degraded environment is being given utmost importance. The present study demonstrated

the assessment of bacterial population of crude oil-contaminated samples collected from different locations operated under Oil India limited, Duliajan (27°21′42″ N, 95°19′ 6″ E) and Jorajan (95°11′ - 95°30′E; 27° 06′ - 27° 22′ N) of Dibrugarh and Tinsukia, Assam. Here, we report 160 morphologically distinct bacteria which could be isolated using conventional culturing techniques of which 117 strains were found to be Gram-positive bacteria and the rest 43 strains were identified as Gram-negative bacteria; majority of the strains were seen to belong to the phylum Proteobacteria (Betaproteobacteria, Gammaproteobacteria and Fermicutes (Bacilli, Clostridia), under the genus *Enterobacter, Pseudomonas, Alkaligenes, Bacillus, Lysinibacillus* and *Clostridium*. Moreover, an alternative approach, the denaturing gradient gel electrophoresis (DGGE) profiles of 16S rDNA phylotypes showed the predominance of *Sphingomonas*, *Ralstonia*, *Sphingobium*, *Massilia*, *Acinetobacter* and *Pseudomonas.* Both culture-dependent and culture-independent approaches resulted in 11 genera of which *Bacillus* and *Pseudomonas* were the key inhabitants creating most favourable bacterial milieu in the presence of crude oil contaminants. Previous studies have reported that there was an overall less microbial diversity and species richness in crude oil-contaminated environments which is in agreement with the present findings. The highest bacterial population was obtained from CTF-D-1 followed by CTF-D2-2 > DF-MJ-3 > PWS-J-4 > CC1-J-5 > CC2-J-6 > PWS-DGU-7 and NCC-J-8 > GDS-J-9 > Core-10, respectively. This study also reports for the first time *Exiguobacterium sp.* from site CCJ2-J-6 to inhabit crude oil-contaminated areas of Assam. Currently, hydrocarbonoclastic bacteria are being given much importance by researchers and have been found to be efficient alternatives in controlling the fate of natural and anthropogenic crude oil seepage. Hence, increased interest is being paid in studying the diversity of indigenous bacteria capable of degrading hydrocarbon contaminants. This study thus helped in the identification of the principal bacterial species inhabiting the contaminated sites that might be potential in degradation of the contaminants, and these results will prove to be significant for chalking-out ways of most favourable in situ bioremediation strategies.

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