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PHYTOREMEDIATION OF ABANDONED CRUDE OIL CONTAMINATED DRILL SITES OF ASSAM WITH THE AID OF A HYDROCARBON-DEGRADING BACTERIAL FORMULATION

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Environmental deterioration due to crude oil contamination and abandoned drill sites is an ecological concern in Assam. To revive such contaminated sites, a field study was conducted to phytoremediate four crude oil abandoned drill sites of Assam (Gelakey, Amguri, Lakwa, and Borholla) with the aid of two hydrocarbon-degrading Pseudomonas strains designated N3 and N4. All the drill sites were contaminated with 15.1 to 32.8% crude oil, and the soil was alkaline in nature (pH8.0–8.7) with low moisture content, low soil conductivity and low activities of the soil enzymes phosphatase, dehydrogenase and urease. In addition, N, P, K, and C contents were below threshold limits, and the soil contained high levels of heavy metals. Bio-augmentation was achieved by applying Pseudomonas aeruginosa strains N3 and N4 followed by the introduction of screened plant species Tectona grandis, Gmelina arborea, Azadirachta indica, and Michelia champaca. The findings established the feasibility of the phytoremediation of abandoned crude oil-contaminated drill sites in Assam using microbes and native plants.

KEY WORDS: environment, crude oil, hydrocarbon degrading, mixed inoculation

INTRODUCTION

Assam is one of the leading states in India for crude oil production and has a stock of over 1.3 billion tons, of which an estimated 58% is yet to be explored. Therefore, degradation of the environment due to various activities of crude oil exploration is inevitable and alarming. Crude oil contamination has occurred in Assam due to storage tank failure, flooding, accidental spillage and abandonment of drill sites (Mashreghi and Marialigeti 2005; Gogoi *et al*. 2003). Crude oil is a complex mixture of hundreds of hydrocarbons including straight-chain alkanes from C_1 to C_{40}, C_6–C_8 branched-chain compounds, cyclohexanes, aromatics and compounds containing sulphur, nitrogen and oxygen (Stafford

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et al. 1982). Crude oil contamination is also recognised as persistent organic pollutants and hazardous waste (Bossert and Bartha 1984). In addition to organic components, crude oil also contains heavy metals such as lead, cadmium, and so on (Essiett *et al*. 2012). Therefore, remediation of crude oil-contaminated soil by eco-friendly means is essential to manage the environment.

At present, the remediation of crude oil-contaminated soil by biological means is recognised as one of the most viable and eco-friendly technologies. Physical and chemical processes have been employed to remediate crude oil contaminated soil; however, these methods are costly, not eco-friendly and require site restoration (Aldrett *et al*. 1997; Salt *et al*. 1995; Cunningham and Ow 1996). In bioremediation, the application of indigenous hydrocarbon-degrading bacteria and planting tolerant plants are useful to recover crude oil-contaminated sites. Several reports highlight the integrated use of microbes and plants to remediate crude oil-contaminated soil (Greg *et al*. 2003; Elena and John 2004; Kuyukina *et al*. 2003; Okoh 2006; Saikia *et al*. 2009; Weyens *et al*. 2009). The plant species suitable for growth in different types of polluted soil have been reported previously (Prasad 2004; Dowarah *et al*. 2009). However, the success of phytoremediation in crude oil-contaminated soil depends on the nature of the contamination and on the ability of the plants and microbes to tolerate and survive in the soil (Paul *et al*. 2005). The aim of the present study was to phytoremediate the crude oil-contaminated abandoned drill sites of Assam by integrated biotechnological means using native hydrocarbon-degrading bacteria and plant species. Emphasis was given to validate the results of a controlled study utilising *in situ* reclamation of four abandoned drill sites: namely, Gelaky, Amguri, Lakowa, and Borholla of upper Assam, India.

MATERIALS AND METHODS

Site Description and Climatic Condition

Four crude oil-contaminated, abandoned drill sites (Gelaky, Amguri, Lakowa, and Borholla of upper Assam) were selected for the present study (Figure 1). The Gelaky, Amguri and Lakowa sites are in the Sivasagar district, and the Borholla drill site is in the Jorhat district. Sivasagar is situated between 94.25° and 95.25°E longitude and 25.45° and 27.15°N latitude, at an elevation of 86.6 meters; Jorhat is situated between 26.75°Nand 94.22°E at an elevation of 116 metres above sea level. The annual temperature ranges from 8°C in winter to a maximum of 35°C during summer, the humidity is high and the average annual rainfall is 94″ in both districts.

Soil Sampling

Soil samples from the surface were collected by inserting a borer to a depth of 100 cm at five distinct locations in each site. Bulk samples for each point were prepared, and a representative of five samples from the bulk samples were taken in polythene zipper bags and stored immediately at 4°C. The soil was taken to the laboratory for biological characterisation including most probable number (MPN) and was analysed within seven days of sampling.

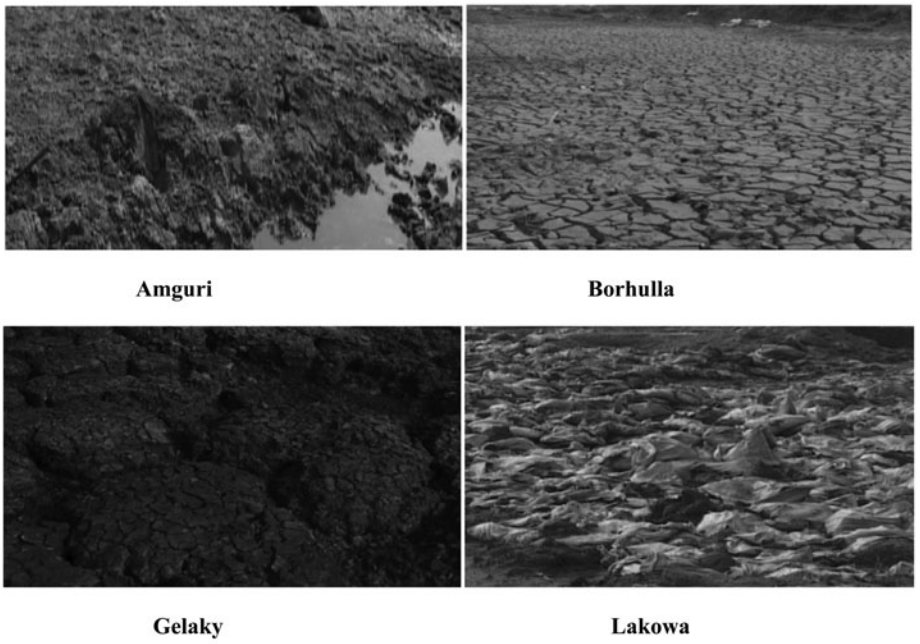

Figure 1 Crude oil contaminated drill sites of Assam before reclamation.

Plant Material and Bacterial Strains

The native plant species, including *Aegle marmelos, Albizia lebbek, Anthocephalus indicus, Azadirachta indica, Bambosa sp., Cassia fistula, Dalbergia sisso, Delonix regia, Dipterocarpus, Ficus bengalensis, Gmelina arborea, Michelia champaca, Mussaenda erythrophylla, Nerium indicum, Saraca indica, Shorea robusta, Tectona grandis, Termemenellia arjuna, Terminalia chabula,* belonging to twenty different families, were screened. The bacterial strains *Pseudomonas aeruginosa* N3 and *P. aeruginosa* N4 were isolated from crude oil-contaminated soil from Assam. Both strains degrade crude oil and possess plant growth-promoting abilities (data not shown).

Chemical and Biological Characteristics of Contaminated Soil

The pH of the soil samples was determined in 1:2.5 soil-water suspensions. Total petroleum hydrocarbon (TPH) content in the soil was estimated according to Saikia *et al*. (2009). In brief, 5.0 g of dried, crushed and sieved soil sample was placed in a cellulose extraction thimble. This sample was then subjected to Soxhlet extraction using chloroform as the solvent for 8 h at 70°C. The extracted oil was concentrated, and the total petroleum hydrocarbon content was determined.

Determination of Individual Crude Oil Fraction

Asphalt. The individual crude oil fraction was determined according to the method described by Hubbered and Stanfield (1948). In brief, 150 ml of n-heptane was added to

5.0 g of crude oil and heated under reflux conditions at 100°C for 1 h in a round-bottom flask. The flask was fitted with a ground glass stopper and allowed to cool in the dark for 2 h. The content was then filtered through Whatman No. 42 filter paper without agitation. The residue was dried and weighed, and the filtrate was used for estimations of resin and wax.

Resin. After the removal of asphalt, the filtrate was absorbed in 200 g of silica gel. The material was heated in a water bath for 1 h with continuous stirring. The material was then stored overnight undisturbed and filtered. The residue was collected and washed with distilled water, then re-dissolved in a toluene-methanol (90:10) mixture and dried over a boiling water bath; finally, the percentage of resin was calculated.

Wax. The filtrate was heated briefly in an oven and treated with 5 ml of H_2SO_4 and the mixture was cooled down to room temperature. The residual asphalt was separated from the liquid. The wax solution was decanted and washed with warm water and ammonium hydroxide solution several times to remove the acid. Then, the crude wax was dissolved in ethylene chloride ($CHCl_2$), cooled to 32°C, filtered through a cold filter funnel and the wax was collected. The filter funnel was washed with hot n-heptanes and collected in a weighed flask. This material was evaporated and dried. The percentage of wax content in the crude oil was determined from the weight after drying.

Aliphatic and aromatic hydrocarbon. After the separation of asphalt, the extracted oil was fractionated further to aliphatic and aromatic hydrocarbons by column chromatography using an activated alumina column (heated at 100°C for 1 h). The aliphatic fraction was eluted with petroleum ether. Subsequently, the aromatic hydrocarbon fraction was eluted with benzene-MeOH (85:15), and the total aliphatic and aromatic contents were determined after removing the eluent.

Determination of essential and heavy metals in soil and plant parts. The total organic carbon in the soil samples was determined according to Jackson (1973). The total nitrogen content in the soil samples was determined by Kjeldahl digestion, and the available phosphorus was determined by the phosphor molybdic acid method (Bray and Curtz 1945; Bremner 1965). The wet digestion methods described in AOAC (1990) were adopted for the preparation of soil and plant samples to determine essential, trace and heavy metals in crude oil-contaminated soil and in control soil and plant samples. For wet digestion of soil samples, 5 ml of nitric acid, 3 ml of perchloric acid and 3 ml of hydrofluoric acid were added to 0.5 g of soil and heated in platinum crucibles for 4 h at 100°C. Nitric acid-perchloric acid digestion was performed to prepare plant samples for determination of the essential, trace and heavy metal content in leaves, shoots and root parts of each plant. For this procedure, 1 g of plant material was digested with 5ml of HNO_3 at 100°C until a grey-white ash was obtained. The sample was allowed to cool for 30 min and then 5ml of perchloric acid was added. The solution was evaporated to dryness. The residue obtained after digestion was dissolved in 25 ml de-ionised water and filtered. The volume was brought up to 25 ml, and the filtered sample was analysed for Mg, Ca, Mn, Fe, Cd Cr, Cu, and Pb using an atomic absorption spectrometer (A Analyst -100 Perkin Elmer, Inc., USA).

Biological Properties of Soil

MPN. Comparison of MPN determinations of organisms in crude oil contaminated soil and in uncontaminated soil was performed by utilizing the soil dilution technique. These determinations could be performed in specialized media for estimations of general types

of soil bacteria (Alexander 1965), sulphur oxidisers (Skinner 1971), cellulolytic bacteria, nitrogen fixers and chemolithotrophic sulphur metabolizers (Bezbaruah *et al*. 1995).

Soil enzyme activity. The activities of the soil enzymes phosphatase, dehydrogenase and urease were determined following the standard methods of Bremner and Tabatabai (1969), Camina *et al*. (1998) and Smith and Chalk (1980), respectively.

Control and *In Situ* Phytoremediation

Preparation of bacterial inocula. Bacterial inocula were prepared by culturing the crude oil degrading bacteria N3 and N4 in standard Kings B media for a period of 48 h. This incubation period produced 5×10^7 cfu ml^{-1}. The 48 h-old bacterial inocula were then mixed thoroughly with cow dung: coal: bacterial broth at a ratio of 5:2:3 (w/w/v). This mixture was used in the field.

Screening of plant species for use in field trials. For a lab-scale study, earthen pots were filled with either crude oil-contaminated soil, crude oil-contaminated soil treated with bacterial formulation prepared at the rate of 5 g kg^{-1} soil or uncontaminated soil. Twenty different varieties of tree seedlings and saplings belonging to twenty different families were planted in these earthen pots, including the plant species *Aegle marmelos, Albizia lebbek, Anthocephalus indicus, Azadirachta indica, Bambosa, Cassia fistula, Dalbergia sisso, Delonix regia, Dipterocarpus, Ficus bengalensis, Gmelina arborea, Michelia champaca, Mussaenda erythrophylla, Nerium indicum, Saraca indica, Shorea robusta, Tectona grandis, Termemenellia arjuna*, and *Terminalia chabula*. The survival rate of each plant species was recorded (data not shown), and seven species (*A. indicus, A. indica, G. arborea, M. champaca, N. indicum, S. robusta, T. grandis*) were found to grow well. Of these species, *T. grandis* belongs family lamiaceae; *G. arborea* belongs to family verbenaceae*; A. indica* belongs to family malieaceae *and M. champaca* belongs to the family magnoliaceae were chosen for *in situ phytoremediation* due to their tolerance to growing in soil treated with the bacterial formulation.

Field preparation and application of bacterial inocula for the in situ experiment. Before initiating phytoremediation, unwanted materials such as plastic bags, boulders, etc., were removed, and each site was levelled. After levelling, the field was treated with the bacterial formulation at a dose of 20 kg h^{-1} of land. The site was then allowed to acclimatise for 15 days, after which plant saplings were transplanted.

Transplantation. Three-month-old seedlings of *A. indica* and *M. champaca* and stumps (saplings) of *T. grandis* and *G. arborea* were transplanted to the field that had been treated with the bacterial formulation. Beginning one month after transplantation, the bacterial formulation was applied to the collar region and to the soil by broadcasting at one-month intervals as described above.

Data recording. Plant height was measured at one-month intervals for a period of 24 months using a ruler from the base of the soil to the highest point on the tree crown. The overall survival rate of the introduced plants was measured and recorded at 12 and 24 months. The accumulation patterns of different essential, trace and heavy metals were determined in the 24-month-old plants.

STATISTICS

Student's t test was performed to evaluate the effects of the reclamation process on the crude oil contaminated soil. Analysis of variance (ANOVA) was performed to evaluate

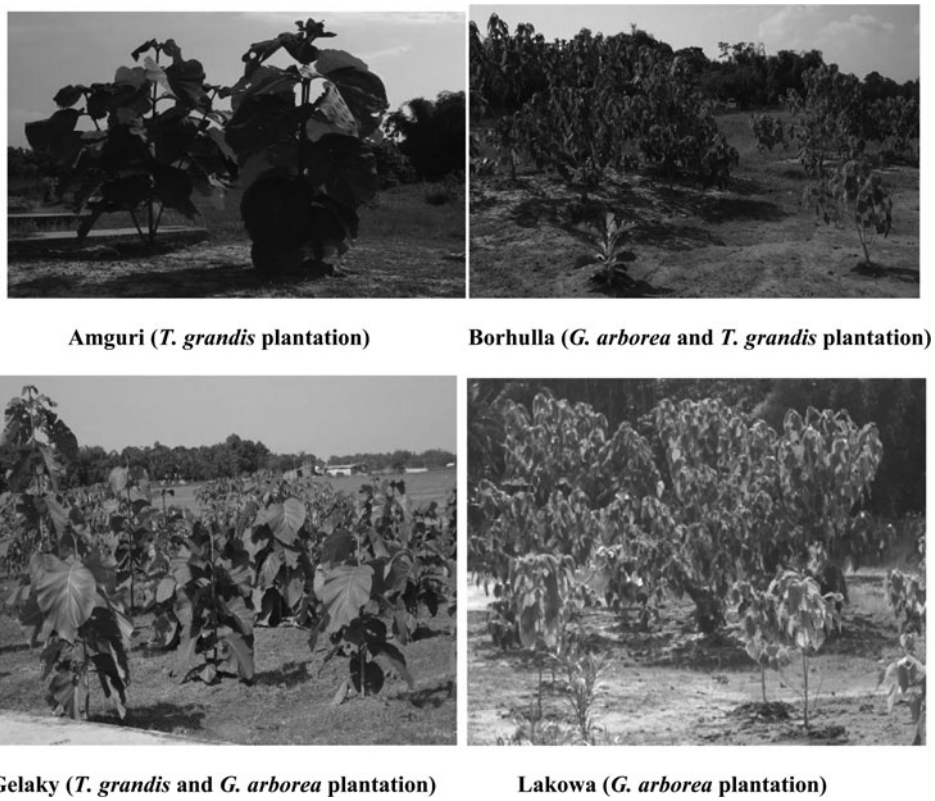

Figure 2 Crude oil contaminated drill sites of Assam after reclamation.

the significant differences and post test Duncan's multiple range test (DMRT) was used to compare the differences at $p < 0.05$.Statistical analyses were performed using SPSS software.

RESULTS AND DISCUSSION

The Gelaky, Amguri, Lakwa, and Borholla drill sites of upper Assam are contaminated with spilled crude oil, sludge, drilling mud, chemicals, polyethenes, as well as stones and concrete (Figure 1). Detailed characterisations of crude oil-contaminated soil before reclamation and after reclamation and of uncontaminated soil are shown in Table 1. Compared to uncontaminated soil, the crude oil-contaminated soil of Gelaky, Amguri, Lakwa, and Borholla drill sites were alkaline in nature (pH 8.4–8.7) with low moisture-holding capacity and low conductivity (1.0–3.6 mMhos). The low moisture content and conductivity of the crude oil-contaminated soil of Assam were due to the non-polar nature of the crude oil, which prevents ionic movement and water retention. Previous studies on crude oil-contaminated soil reported alkalinity, low conductivity and low moisture retention (Njoku *et al*. 2009; Andrade *et al*. 2004). Like moisture and conductivity, the soil quality indicators microbial population size (Figure 3), including soil enzyme activities phosphatase, urease and dehydrogenase were significantly low in crude oil contaminated soil then uncontaminated soil (Table 1). Earlier studies reported that the monitoring of

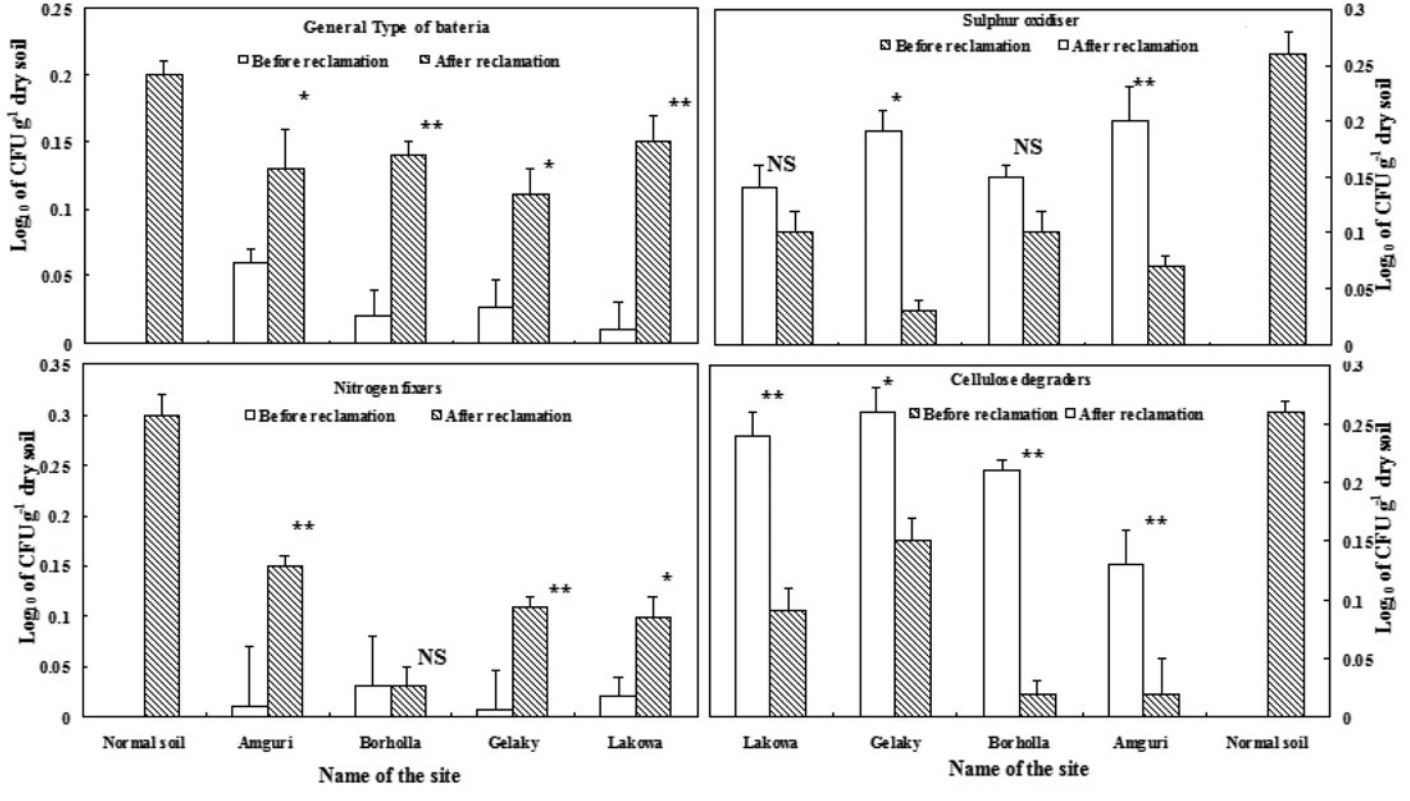

Figure 3 Microbial population in crude oil contaminated abandoned drill site soil. Data's are means of five observed values; Error bars = standard error means of observed values; * = Significant at $p < 0.05$ level; ** = Significant at $p < 0.01$ according to paired comparison.

Table 1 Comparison of physicochemical and soil biological characters of crude oil contaminated soil before and after reclamation.

Physicochemical character	Treatments	Name of the sites				LSD
		Amguri	Borhulla	Gelaky	Lakowa	
pH	Un-contaminated soil	6.8 ± 0.7b	6.8 ± 0.7b	6.8 ± 0.7c	6.8 ± 0.7b	
	Before reclamation	8.2 ± 0.7a	8.1 ± 0.7a	8.7 ± 0.7a	8.0 ± 0.9a	0.628
	After reclamation	7.3 ± 0.3b	7.0 ± 0.9b	7.5 ± 0.5b	7.0 ± 0.8b	1.02
Moisture (%)	Un-contaminated soil	20.6 ± 2.7a	20.6 ± 2.7a	20.6 ± 2.7a	20.6 ± 2.7a	
	Before reclamation	12.6 ± 1.0c	9.0 ± 1.0c	9.2 ± 1.0c	7.3 ± 1.0c	0.84
	After reclamation	14.8 ± 1.8b	15.5 ± 2.0b	14.7 ± 3.0b	10.8 ± 1.0b	1.83
Conductivity (mMhos)	Un-contaminated soil	10.7 ± 2.2a	10.7 ± 2.2a	10.7 ± 2.2a	10.7 ± 2.2a	
	Before reclamation	3.6 ± 0.8a	1.9 ± 0.3a	2.1 ± 0.6a	1.0 ± 0.5c	0.82
	After reclamation	9.3 ± 0.8a	7.0 ± 0.6a	6.2 ± 0.8a	5.8 ± 1.0b	2.16
TPH (%)	Un-contaminated soil	0	0	0	0	
	Before reclamation	15.1 ± 0.7a	15.1 ± 0.7	26 ± 3.0	30.2 ± 2.0a	3.82
	After reclamation	3.0 ± 1.0b	3.0 ± 1.0	6.3 ± 0.9	10.5 ± 2.0b	2.51
Wax (%)	Un-contaminated soil	0	0	0	0	
	Before reclamation	22.7 ± 0.7a	22.7 ± 0.7	7.7 ± 1.0	20.5 ± 2.0a	4.56
	After reclamation	20.7 ± 0.9a	20.7 ± 0.9	6.3 ± 0.9	18.5 ± 2.0a	5.85
Resin	Un-contaminated soil	0	0	0	0	
	Before reclamation	39.5 ± 2.0a	39.5 ± 2.0	5.5 ± 0.6	44.7 ± 2.0a	3.75
	After reclamation	29.9 ± 1.0b	29.9 ± 1.0	4.9 ± 0.1	42.1 ± 1.0a	7.38
Asphalt	Un-contaminated soil	0	0	0	0	
	Before reclamation	5.3 ± 0.8a	5.3 ± 0.8	16.7 ± 2.0	7.3 ± 1.0a	1.65
	After reclamation	5.0 ± 0.1a	5.0 ± 0.1	11.6 ± 1.0	6.6 ± 0.5a	4.83
Aliphatic	Un-contaminated soil	0	0	0	0	
	Before reclamation	14.0 ± 1.0a	4.4 ± 0.5	11.0 ± 0.2	8.8 ± 0.2a	3.67
	After reclamation	12.5 ± 0.7a	3.7 ± 0.4	8.7 ± 1.0	4.5 ± 0.6a	1.85
Aromatic	Un-contaminated soil	0	0	0	0	
	Before reclamation	9.6 ± 0.6a	36.8 ± 3.0a	12.6 ± 3.0a	14.8 ± 1.0a	4.63
	After reclamation	9.0 ± 0.7a	31.2 ± 3.0b	12.0 ± 0.1a	12.0 ± 1.0a	2.76
		Soil enzyme s (μg g^{-1} h^{-1})				
Dehydrogenase	Un-contaminate soil	126.5 ± 2.1a	126.5 ± 2.1a	126.5 ± 2.1a	126.5 ± 2.1a	
	Before reclamation	56.0 ± 3.0a	26.0 ± 1.0c	90.0 ± 3.0c	60.9 ± 1.0c	4.85
	After reclamation	99.3 ± 2.0a	80.0 ± 1.0b	100.0 ± 5.0b	101.4 ± 1.0b	10.12
Phosphatase	Un-contaminate soil	49.6 ± 1.6a	49.6 ± 1.6a	49.6 ± 1.6a	49.6 ± 1.6a	
	Before reclamation	27.3 ± 1.6c	2.4 ± 0.2c	17.6 ± 2.0c	10.9 ± 2.0c	7.81
	After reclamation	41.5 ± 6.0b	12.8 ± 3.0b	34.4 ± 0.7b	13.5 ± 2.0b	6.58
Urease	Un-contaminate soil	138.3 ± 3.0a	138.3 ± 3.0a	138.3 ± 3.0a	138.3 ± 3.0a	
	Before reclamation	14.4 ± 3.0c	38.8 ± 7.0c	34.2 ± 5.0c	108.6 ± 6.0b	12.83
	After reclamation	17.9 ± 1.0b	59.7 ± 1.0b	47.3 ± 7.0b	109.3 ± 14.0b	8.85

Data were mean of five individual values, ±1.0 = SE of observed values; SE followed by similar letter are not significantly different between three treatments un-contaminated soil, before reclamation and after reclamation within the column at $p < 0.05$ as per student's t test; LSD values as per Duncan's multiple range test ($p < 0.05$) for different sites within the rows.

microbial population size and soil enzyme activity as indicators of soil health and decontamination (Alef 1995; Anderson and Domsch 1990; Colwell *et al*. 1977; Reak *et al*. 2007; Insam and Domsch 1988; Devinny and Chang 2000). The phosphatase activity (2.4 μg g^{-1}) and urease activity (14.4 μg g^{-1}) were significantly lower in Borholla and Amguri drill sites compared to the other three sites. Saikia *et al*. (2002) reported

Table 2 Comparison of essential, trace and heavy metal content in crude oil contaminated soil before and after reclamation.

Name of the metals	Treatments	Name of the sites				LSD
		Amguri	Borhulla	Gelaky	Lakowa	
N (%)	Un-contaminated soil	6.4 ± 1.2a	6.4 ± 1.2a	6.4 ± 1.2a	6.4 ± 1.2a	
	Before reclamation	0.9 ± 0.4b	0.8 ± 0.2c	0.7 ± 0.2c	0.5 ± 0.3c	0.25
	After reclamation	7.0 ± 0.2a	5.8 ± 1.0b	5.0 ± 0.6b	4.7 ± 0.5b	1.81
P (%)	Un-contaminated soil	3.1 ± 0.9b	3.1 ± 0.9a	3.1 ± 0.9b	3.1 ± 0.9b	
	Before reclamation	1.9 ± 0.2c	2.0 ± 0.3c	1.5 ± 0.2c	1.6 ± 0.6c	1.11
	After reclamation	8.5 ± 0.7a	8.5 ± 0.8a	5.6 ± 1.0a	6.5 ± 0.9a	1.23
K (%)	Un-contaminated soil	7.6 ± 1.7a	7.6 ± 1.7a	7.6 ± 1.7a	7.6 ± 1.7a	
	Before reclamation	0.7 ± 0.4c	6.3 ± 0.2a	2.8 ± 0.4c	1.8 ± 0.3c	0.75
	After reclamation	1.5 ± 0.6b	7.5 ± 0.6a	3.5 ± 1.0b	5.0 ± 0.7b	0.87
OC (%)	Un-contaminated soil	10.8 ± .3b	10.8 ± .3a	10.8 ± .3b	10.8 ± .3a	
	Before reclamation	4.8 ± 0.1c	1.4 ± 0.1c	4.1 ± 0.1c	2.8 ± 1.0c	2.10
	After reclamation	17.5 ± 1.0a	3.9 ± 0.6b	16.4 ± 1.0a	5.0 ± 0.9b	5.63
Ca (mg kg^{-1})	Un-contaminated soil	340.0 ± 0.7a	340.0 ± 0.7a	340.0 ± 0.7b	340.0 ± 0.7b	
	Before reclamation	140.0 ± 2.0c	194.0 ± 3.8c	112.0 ± 0.6c	183.0 ± 5.0c	7.56
	After reclamation	270.0 ± 1.0b	202.0 ± 11.1b	476.0 ± 2.8a	370.0 ± 11a	13.71
Mg (mg Kg^{-1})	Un-contaminated soil	298.0 ± 0.4b	298.0 ± 0.4b	298.0 ± 0.4b	298.0 ± 0.4b	
	Before reclamation	159.0 ± 1.0c	133.0 ± 4.0c	149.0 ± 9.1a	237.0 ± 22.0c	5.63
	After reclamation	398.0 ± 1.0a	506.0 ± 4.0a	714.0 ± 3.0a	458.0 ± 5.0a	12.37
		Trace and heavy metals ($\mu g\ g^{-1}$)				
Fe	Un-contaminated soil	132.5 ± 9.0a	132.5 ± 9.0a	132.5 ± 9.0a	132.5 ± 9.0a	
	Before reclamation	126.2 ± 1.0b	74.5 ± 8.0b	99.2 ± 1.0c	93.9 ± 1.0c	10.21
	After reclamation	132.9 ± 1.0aa	67.6 ± 1.5c	108.7 ± 1.2b	101.8 ± 7.7b	6.75
Mn	Un-contaminated soil	0.19 ± 0.01	0.19 ± 0.01c	0.19 ± 0.01c	0.19 ± 0.01b	
	Before reclamation	1.7 ± 0.6a	1.8 ± 0.3a	1.2 ± 0.8a	1.3 ± 1.0a	0.21
	After reclamation	1.3 ± 0.4a	1.2 ± 0.3b	1.7 ± 0.7a	1.9 ± 0.4a	1.11
Cu	Un-contaminated soil	0.1 ± 0.01	0.1 ± 0.01b	0.1 ± 0.01b	0.1 ± 0.01b	
	Before reclamation	2.7 ± 0.8a	1.5 ± 0.9a	1.7 ± 0.6a	1.8 ± 1.0a	0.76
	After reclamation	0.1 ± 0.02b	0.09 ± 0.05c	0.09 ± 0.01c	0.1 ± 0.06b	0.21
Cr	Un-contaminated soil	ND	ND	ND	ND	
	Before reclamation	2.0 ± 0.2a	0.6 ± 0.1a	0.7 ± 0.2a	1.9 ± 0.1a	0.75
	After reclamation	0.3 ± 0.02b	0.3 ± 0.07a	0.1 ± 0.01b	0.1 ± 0.06b	0.23
Ni	Un-contaminated soil	0.02 ± 0.0	0.02 ± 0.0	0.02 ± 0.0	0.02 ± 0.0	
	Before reclamation	0.5 ± 0.01a	0.4 ± 1.0a	1.3 ± 0.3a	0.03 ± 0.0b	0.53
	After reclamation	0.2 ± 0.02a	0.3 ± 0.01a	0.5 ± 0.01b	1.0 ± 0.02a	0.47
Cd	Un-contaminated soil	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	
	Before reclamation	1.2 ± 0.1a	0.9 ± 01a	1.0 ± .002a	1.9 ± 0.1a	0.63
	After reclamation	0.2 ± 0.01b	0.04 ± 0.03b	0.5 ± 0.2a	0.8 ± 0.02b	0.27
Pb	Un-contaminated soil	0.1 ± 0.01	0.1 ± 0.01b	0.1 ± 0.01c	0.1 ± 0.01c	
	Before reclamation	3.4 ± 0.2a	0.3 ± 0.01a	5.4 ± 0.4a	7.8 ± 0.4a	0.63
	After reclamation	0.2 ± 0.07b	0.8 ± 0.09a	0.8 ± 0.2b	0.8 ± 0.09b	0.65

Data were mean of five individual values, ±1.0 = SE of observed values; SE followed by similar letter are not significantly different from each other within the column as per student t test; LSD = least significant difference among the different observation within rows according to Duncan's multiple range test.

that crude oil affects the biochemical nature of soil. Among all four drill sites, the TPH content was highest at the Gelaky drill site (32.8%) before remediation, followed by Lakwa (30.2%) > Borholla (26%) > Amguri (15.1%). Like other soil physical and biological characters, the levels of N, P, K, and C, were below the threshold limit in the crude oil-contaminated soil with above threshold limit of trace and heavy metals (Table 2). Kpoveta,

et al. (2011) found low N and P contents in crude oil-contaminated soil. Compared to uncontaminated soil, the crude oil contaminated soil also contain significantly higher amount of trace and heavy metals. In crude oil contaminated soil, the trace metals Fe (74.5–126.2 mg Kg^{-1}), were recorded lowest followed by Ca and Mg, at all the contaminated sites. Similarly, heavy metals Pb, Cd and Ni concentrations were also considerably higher in the contaminated soil, and the highest concentration of lead (5.4 $\mu g\ g^{-1}$) was detected at Gelaky, Amguri and Lakwa. In Borholla soil, the concentration of Mn (1.8 $\mu g\ g^{-1}$) was estimated to be the highest, followed by Cu > Cd > Cr > Ni and Pb. High concentration of trace and heavy metals in crude oil contaminated soil including Ni and V in the asphaltene fraction were earlier reported (McGrath and Zhao 2005).

It was found that the crude oil-contaminated drill soil of Assam is not a good medium for growing plant species. Consequently, bio-augmentation was performed utilising two hydrocarbon-degrading bacterial strains N3 and N4. Hydrocarbon degrading bacteria bearing plant beneficial characters facilitate the improvement of quality contaminated soil and plant survival under stress (Glick and Pasternak 2003). It was found that soil inocula containing bacterial strains N_3 and N_4 enhanced the plant survival rate and growth in 40% of crude oil contaminated soil due to the degradation crude oil and also effect on plant survival (data not shown). The nature of growth and survival of four species (*T. grandis*, *G. arborea, M. champaca,* and *A. indica*) grown in crude oil contaminated soil after the soil was treated with the bacterial formulation is described in Table 3. The bacterial treatment significantly improved the survival rate of the introduced plants twenty four months after planting in Gelaky, Amguri and Borholla, while the plant survival rate during this same period of time was not significantly different at the Lakwa drill site. *T. grandis* showed 93.5%, 99%, 73.5%, and 81.5% survival rates after 24 months in Gelaky, Amguri, Lakwa and Borholla, respectively. *M. champaca*, *G. arborea*, and *A. indica*, had survival rates of 87.5%, 89%, and 95% in Amguri. At the Borholla drill site, the survival rates of *M. champaca* and *G. arborea* were 82.5% and 77.5%. However, all of the tree species showed lower survival rates at the Lakwa drill site over the 24-month study-period due to the high content of crude oil, low nutrient content and relatively high levels of heavy metals.

Comparison of the growth of *T. grandis, G. arborea, A. indica,* and *M. champaca* was recorded at one-month intervals for 24 months (Figure 4). It was found that the application of bacterial strains N3 and N4 led to an increase in the introduced plant height. *T. grandis* grown at Gelaky, Amguri, Lakwa and Borhola drill sites attained heights of 343, 269, 195, and 200 cm, respectively. Overall, the smallest plant height was measured for all species grown at the Lakwa drill site, with *M. champaca* height being the lowest (95 cm).

Since crude oil contaminated soil contains above threshold limits of available trace and heavy metal contents; therefore introduced plants accumulate trace and heavy metals to a different level. Several studies have reported bioaccumulation of trace and heavy metals by different plant parts (McIntyre 2003; Yang *et al*. 2002a; 2004; He *et al*. 2002; Tong *et al*. 2004; Dickinson *et al*. 2009; Rai 2008). The concentrations of the heavy metals Cd, Cr, Cu, Pb, and Ni in various parts of plants grown at the four drill sites are presented in Table 4. Significantly higher accumulations of trace and heavy metals were found in roots, followed by stems and leaves. In the plants grown at the Gelaky drill site, the highest accumulation of Pb (700 $\mu g\ Kg^{-1}$) was found in roots of *T. grandis*, followed by Cd > Ni > Cr > Cu. In the root part of *G. arborea,* Ni concentration (1000 $\mu g\ Kg^{-1}$) was found to be the highest, followed by Cr (800 $\mu g\ Kg^{-1}$) > Cu (500 $\mu g\ Kg^{-}1$) > Cd (140 $\mu g\ Kg^{-1}$) > Pb (100 $\mu g\ Kg^{-1}$). The concentration of Cr (800 $\mu g\ g^{-1}$) was highest in roots of *A. indica*, followed by Pb (250 $\mu g\ g^{-1}$) > Cd (240 $\mu g\ Kg^{-1}$) > Ni (200 $\mu g\ Kg^{-}1$) > Cu (100 $\mu g\ Kg^{-1}$).

Table 3 Comparison of plant survival rate over the period of observation grown in crude oil contaminated soil with or without treatment.

	Plant survival rate (%)							
	Tectona grandis		*Gmelina arborea*		*Azadirachta indica*		*Michelia champaca*	
Name of the site	After 12 month	After 24 month	After 12 month	After 24 month	After 12 month	After 24 month	After 12 month	After 24 month
Amguri	77.5 ± 3.0	99.0 ± 1.0**	65.0 ± 7.0	89.0 ± 1.0**	52.5 ± 2.0	95.0 ± 1.0**	63.5 ± 4.0	87.5 ± 3.0**
Borholla	53.4 ± 4.0	81.5 ± 4.0**	58.5 ± 9.0	77.5 ± 3.0**	48.5 ± 2.0	85.7 ± 7.0**	48.0 ± 2.0	82.5 ± 10.0**
Gelaky	67.5 ± 3.0	93.5 ± 2.0**	52.5 ± 3.0	73.4 ± 4.0**	62.5 ± 3.0	83.5 ± 2.0**	52.5 ± 3.0	68.5 ± 2.0**
Lakowa	57.5 ± 3.0	73.5 ± 2.0**	65.0 ± 3.0	62.5 ± 6.0**	43.5 ± 4.0	51.0 ± 2.0**	64.0 ± 8.0	67.5 ± 3.0**
LSD	5.8	10.0	5.9	7.0	6.8	12.3	8.8	5.6

Data were mean of five individual values, ±1.0 = SE of observed values; ** = statistically significant at $p < 0.01$ by paired comparison between 12 and 24 months, LSD = Comparison of mean data within the column as per DMRT test at $p < 0.05$.

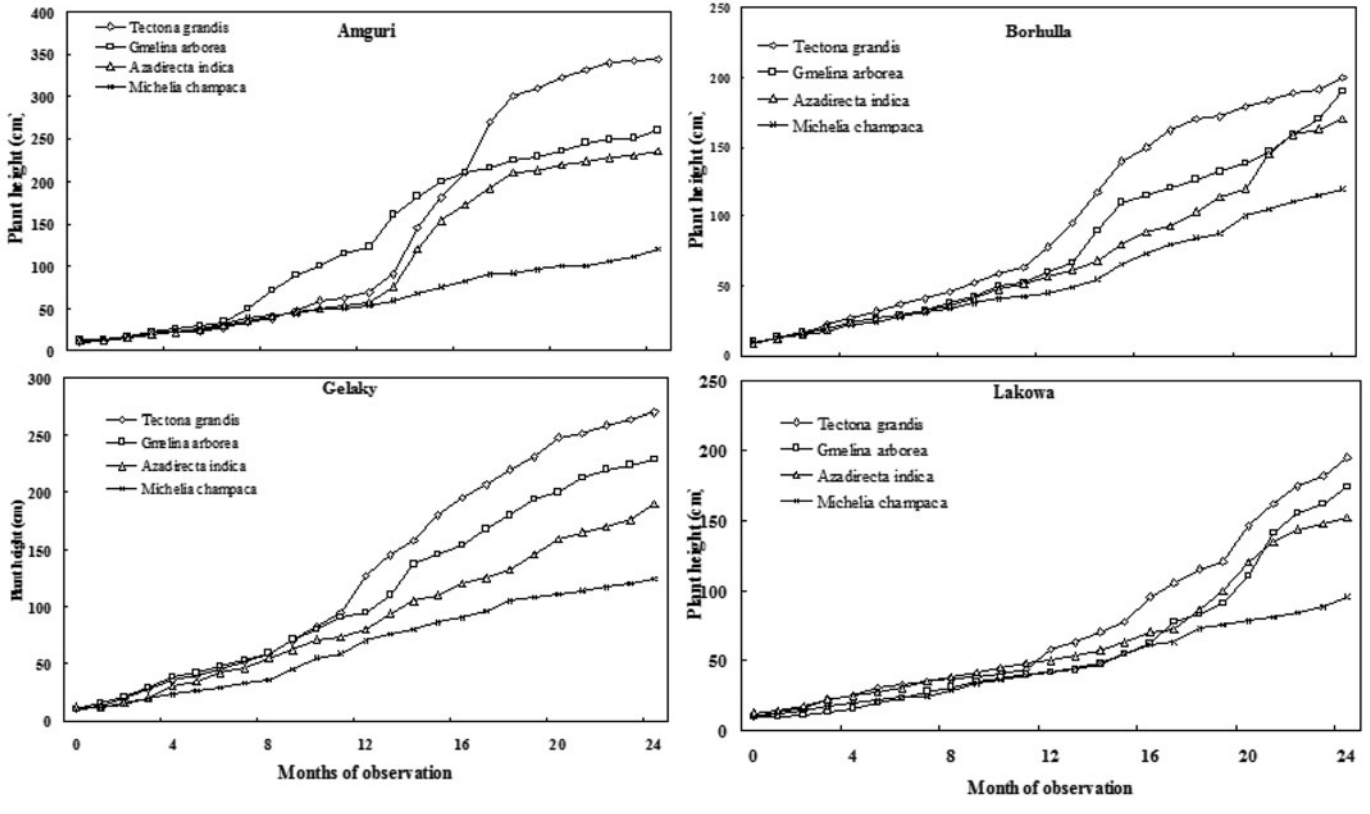

Figure 4 Plant height of four different plant species used to remediate crude oil contaminated drill sites of Assam.

Table 4 Comparison of accumulation pattern of different metal by different parts of the plant used for reclamation of crude oil contaminated abandoned drill sites.

Trace and heavy metal content (μg kg^{-1} dry plant materials)	Name of the plant and plant parts											
	Tectona grandis			*Gmelina arborea*			*Azadirachta indica*			*Michelia champaca*		
	Leaf	Shoot	Root	Leaf	Shoot	Root	Leaf	Shoot	Root	Leaf	Shoot	Root
						Amguri						
Cr	50 ± 3d	130 ± 2b	100 ± 1c	50 ± 1b	800 ± 4a	900 ± 4a	50 ± 1c	90 ± 6c	120 ± 14c	60 ± 1a	120 ± 3d	570 ± 2a
Cd	80 ± 2a	680 ± 3a	900 ± 8a	40 ± 2c	580 ± 2b	800 ± 15a	70 ± 3b	480 ± 7a	350 ± 3b	30 ± 3c	200 ± 0.1b	120 ± 1c
Cu	30 ± 2e	80 ± 0.5d	100 ± 1c	80 ± 5a	100 ± 7d	800 ± 4a	50 ± 5c	70 ± 2c	120 ± 14c	20 ± 2d	400 ± 2a	590 ± 2a
Ni	70 ± 8b	90 ± 0.7c	74 ± 4d	50 ± 2b	80 ± 3e	200 ± 2c	70 ± 1b	90 ± 1c	120 ± 2c	60 ± 1a	80 ± 2e	90 ± 9d
Pb	60 ± 3c	80 ± 8d	150 ± 2b	50 ± 3b	240 ± 4c	280 ± 1b	90 ± 2a	460 ± 2b	600 ± 1a	50 ± 1b	150 ± 2c	400 ± 3b
						Borhulla						
Cr	60 ± 2b	90 ± 2a	100 ± 4a	90 ± 1a	100 ± 6a	150 ± 0.4a	400 ± 2a	80 ± 0.3b	120 ± 8b	50 ± 7c	50 ± 0.3d	90 ± 3c
Cd	60 ± 4b	80 ± 2d	90 ± 1b	60 ± 3c	90 ± 2b	100 ± 2b	20 ± 7d	40 ± 2e	60 ± 6d	20 ± 0.8e	40 ± 2e	70 ± 5d
Cu	20 ± 2c	50 ± 0.9b	80 ± 2c	30 ± 6e	50 ± 6c	70 ± 0.9d	30 ± 2c	70 ± 3c	90 ± 7c	40 ± 9d	80 ± 0.7c	100 ± 2b
Ni	20 ± 6c	50 ± 3b	80 ± 0.8c	50 ± 6d	50 ± 2c	90 ± 6c	80 ± 1b	50 ± 2d	90 ± 6c	90 ± 0.7b	100 ± 2b	100 ± 3b
Pb	70 ± 5a	90 ± 6a	120 ± 0.4a	70 ± 4b	100 ± 2a	150 ± 2a	80 ± 3b	100 ± 1a	160 ± 1a	100 ± 2a	130 ± 2a	200 ± 4a
						Gelaky						
Cr	50 ± 5b	130 ± 2a	110 ± 5d	60 ± 2a	80 ± 4	800 ± 4b	170 ± 2b	500 ± 6a	800 ± 1a	20 ± 5d	100 ± 3b	500 ± 4a
Cd	40 ± 6c	60 ± 3d	400 ± 5b	30 ± 1b	50 ± 2d	140 ± 2d	10 ± 4d	40 ± 1d	240 ± 2b	30 ± 1c	200 ± 2a	280 ± 2b
Cu	40 ± 9c	60 ± 3d	100 ± 0.5d	60 ± 3a	300 ± 6a	500 ± 0.9c	90 ± 4c	90 ± 3c	100 ± 0.5d	50 ± 3b	70 ± 4c	90 ± 4c
Ni	40 ± 2c	70 ± 4c	600 ± 2c	60 ± 2a	90 ± 1b	1000 ± 1a	100 ± 3c	100 ± 5b	200 ± 11c	100 ± 4a	100 ± 7b	400 ± 1a
Pb	80 ± 4a	100 ± 7b	700 ± 5a	60 ± 4a	80 ± 2c	100 ± 2d	1000 ± 4a	100 ± 9b	250 ± 9b	30 ± 1c	30 ± 2d	50 ± 7d
						Lakowa						
Cr	30 ± 2d	50 ± 1c	90 ± 7d	60 ± 2b	100 ± 4a	250 ± 7a	30 ± 4c	40 ± 7d	60 ± 5d	90 ± 1b	160 ± 3b	410 ± 3b
Cd	50 ± 1b	40 ± 3d	200 ± 2b	30 ± 3d	50 ± 2c	90 ± 1c	50 ± 4a	80 ± 4a	100 ± 1c	50 ± 4d	90 ± 0.7c	100 ± 2c
Cu	50 ± 3b	70 ± 6b	90 ± 0.6c	20 ± 6e	25 ± 8e	80 ± 5d	40 ± 2b	50 ± 2c	50 ± 7e	30 ± 2e	40 ± 0.4d	50 ± 7d
Ni	60 ± 1a	50 ± 3c	300 ± 1a	40 ± 4c	70 ± 2b	100 ± 4b	20 ± 4d	60 ± 1b	200 ± 9b	70 ± 3c	200 ± 2a	500 ± 4a
Pb	40 ± 5c	80 ± 8a	200 ± 7b	70 ± 7a	40 ± 4d	80 ± 2d	40 ± 6b	80 ± 4a	300 ± 2a	100 ± 2a	200 ± 7a	500 ± 6a

Data were mean of five individual values, ±1.0 = SE of observed values; SE followed by similar letter within the column are not significantly different from each other according to DMRT for different metals at $p < 0.05$.

In roots of *M. champaca*, Cr concentration (500 μg Kg^{-1}) was found to be the highest, and Cu concentration (50 μg Kg^{-1}) was observed to be the lowest. The nature of trace and heavy metals accumulation were highest in root followed by stem and leaves for all the plants. Like gelaky plants, the plants grown in Amguri crude oil-contaminated soil also showed highest accumulation of different metals in roots followed by stem and leaves except Ni in *T grandis*. Overall highest accumulation in root was found for the metal Cd (900 μg Kg^{-1}) in *T. grandis;* Cr (900 μg Kg^{-1}) in *G. arborea*; Pb (600 μg Kg^{-1}) in *A. indica;* and Cu (590 μg Kg^{-1}) in *M. champaca*. In Lakowa plants, except *T. grandis* for Ni (50 mg kg^{-1} dry plant), *G. arborea* for Pb (40 mg kg^{-1} dry plant) higher accumulation was found in root and lowest in leaves. Similar trend of higher accumulation of trace and heavy metals were found for the Borhulla crude oil contaminated plants except *A. indica* for Ni. The accumulation of Pb, Zn, Cd, Ni, Co, Cd by hyperaccumulator species, such as *Thlaspi caerulescens* and fast-growing plants, such as *Salix* and *Populus* spp. were reported in the review of Dickinson (2009). Further, higher accumulation of metals in roots accomplished by lower concentrations in stems and leaves was reported earlier by Baker *et al*. (2000). The restriction of trace and heavy metals in roots are the constitutive and adaptive mechanisms of a plant (Tripathi *et al*. 2008). The higher accumulation of metals in roots than in stems and leaves saves the metabolically active part of the plant from deleterious effects of access trace and heavy metals.

To draw the efficacy of phytoremediation in improvement of soil quality, removal of trace and heavy metals from the restored site; the chemical and biological characters of remediated sites were re-evaluated (Tables 1 and 2). There were improvements in moisture-holding capacity, conductivity and pH in the reclaimed crude oil-contaminated soil. These improvements were due to the degradation of available crude oil by 65.23% to 88.5% at the remediated drill sites. Enhanced soil biological characters like enzyme activities dehydrogenase, phosphatase and urease from 6–520% and total microbial population in the reclaimed crude oil-contaminated soil. The enhanced soil biological characteristics of present findings are supported by the earlier reported work on integrated biological means of overburdened reclaimed wasteland of mines (Gogoi *et al*. 2012; Finkenbein *et al*. 2012). It was also found that there was a subsequent increase in essential elements such as N, P, K, C, Mg, Mn, Ca, and Fe in the reclaimed crude oil-contaminated drill soil, along with enhanced MPN (Figure 3). This change may be due to the external application of microbial formulations and the activity of the introduced plants along with the plant litter produced by the latter. The improvement of soil biological and chemical characters in the reclaimed crude oil contaminated soil showed the efficacy of present integrated phytoremediation strategy.

CONCLUSION

Alkaline pH, low concentrations of macro- and micronutrients and low biological activity are unique characteristics of abandoned crude oil-contaminated soil in upper Assam. No vegetation grows in such crude oil contaminated soil for a more than two to three decades. Using the crude oil-degrading bacteria *P. aeruginosa* N3 and *P. aeruginosa* N4 led to decreases in the concentration of TPH and increases in the soil biological activity and nutrient content. Treatment of crude oil contaminated-soil with bacteria also led to enhanced survival rates of the native plant species *T. grandis, G. arborea, A. indica,* and

M. champaca from 51–99%. These findings establish the feasibility of the phytoremediation of abandoned crude oil-contaminated drill sites in Assam using microbes and native plants.

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