BACTERIAL DIVERSITY AND ACTIVITY OF SEMIARID SOILS OF MAHI RIVER BASIN, WESTERN INDIA

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DECLARATION

STATEMENT UNDER O. Ph.D. 8/(iii) OF
THE M. S. UNIVERSITY OF BARODA, VADODARA

The work presented in this thesis has been carried out by me under the guidance of Dr. G. Archana, Department of Microbiology and Biotechnology Centre, Faculty of Science, The M. S. University of Baroda, Vadodara, Gujarat, India. The data reported herein is original and has been derived from studies undertaken by me.

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Date: — Candidate

This is to certify that the above declaration is true.

Place: Vadodara — **Dr. G. Archana**

Date: — Research Supervisor

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List of Abbreviations

AAS	Atomic absorption spectrophotometer
APA	Phosphatase activity
As	Arsenic
ACC	1-Aminocyclopropane-1-carboxylate
ANOVA	Analysis of variance
ARDRA	Amplified rDNA restriction analysis
BLAST	Basic local alignment search tool
Bp	Base pair
Cfu	Colony forming unit
Cm	Centimetre
Cr	Chromium
CTAB	Cetyl trimethylammonium bromide
DB	Devenze and Bruni medium
DGGE	Denaturant gradient gel electrophoresis
DHA	Dehydrogenase activity
D/W	Distilled water
E	Evenness index
EDTA	Ethylene diamine tetra acetic acid
GSA	ß-Glucosidase activity
H	Shannon-Weaver index
Ka	Kilo year
Kb	kilo-basepair
Km	Kilo meter

M	Meter
Mm	Millimeter
MPN	Most probable number
Mol	Mole
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
Pb	Lead
PCR	Polymerase chain reaction
PGPB	Plant growth promoting bacteria
PSA	Protease activity
q-PCR	Quantitative real-time PCR
Rr	Range weighted richness index
Rrna	Ribosomal ribonucleic acid
R2A agar	Reasoner and Geldreich agar
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TAE	Tris Acetate EDTA
TE	Tris EDTA
Tris –base	Tris (hydroxymethyl) aminomethane
UPGMA	Unweighted pair-group method using arithmetic average
XRD	x-ray Diffraction
XRF	x-ray Fluorescence

Note: The full forms of several rarely used abbreviations have been described within the text

List of Symbols

α	Alpha
β	Beta
γ	Gamma
μ	Micro
3^1	3 prime
5^1	5 prime
d	Days
g	g-force
k	Kilo
^{O}C	degree Celsius
l	Litre
m	Milli (10^{-3})
n	Nano (10^{-9})
p	Pico (10^{-12})
%	Percentage
g	Gram
min	Minutes
h	Hours
s	Second
M	Molar
U	Unit
V	Voltage

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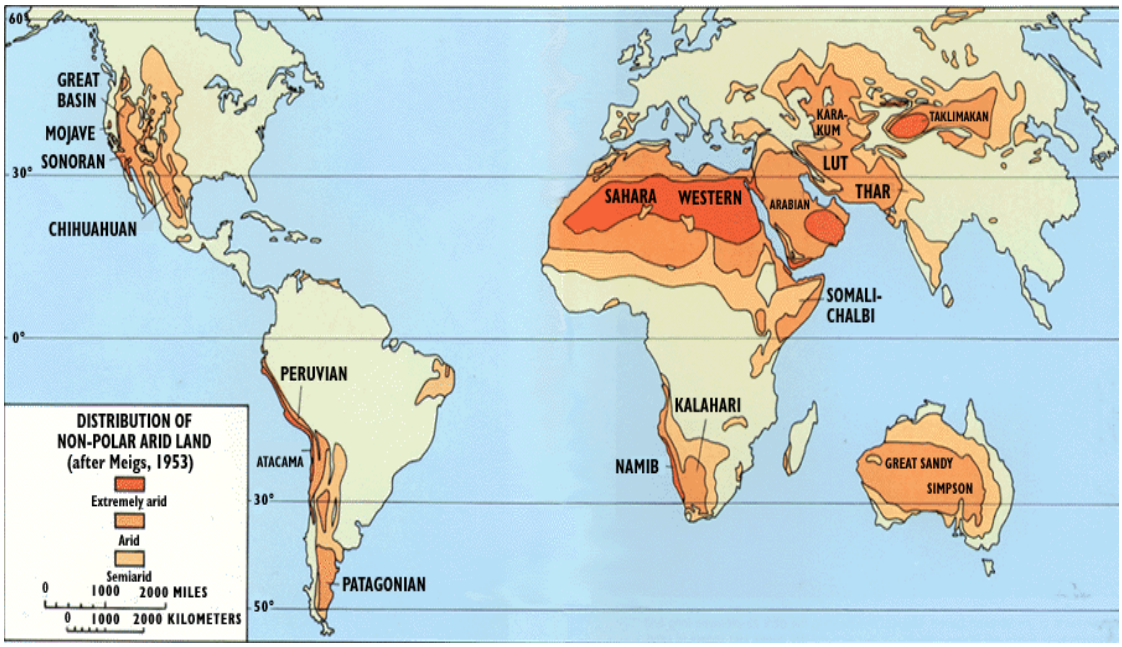
Chapter 1

Review of Literature

1.1. Semiarid landscapes

Semiarid tropics (arid index 0.20-0.50) are characterized by relatively low annual rainfall where annual precipitation varies from 300-600 to 700-800 mm, with summer rains, and from 200-250 to 450-500 millimeters with winter rains (http://www.fao.org/docrep/T0122E/t0122e03.htm). Arid and semi-arid lands occupy (Fig. 1.1) about one-third of the global terrestrial surface area (Dregne, 1991). Nearly 1.4 billion people are living in these particular tropics (Thurston, 1997).

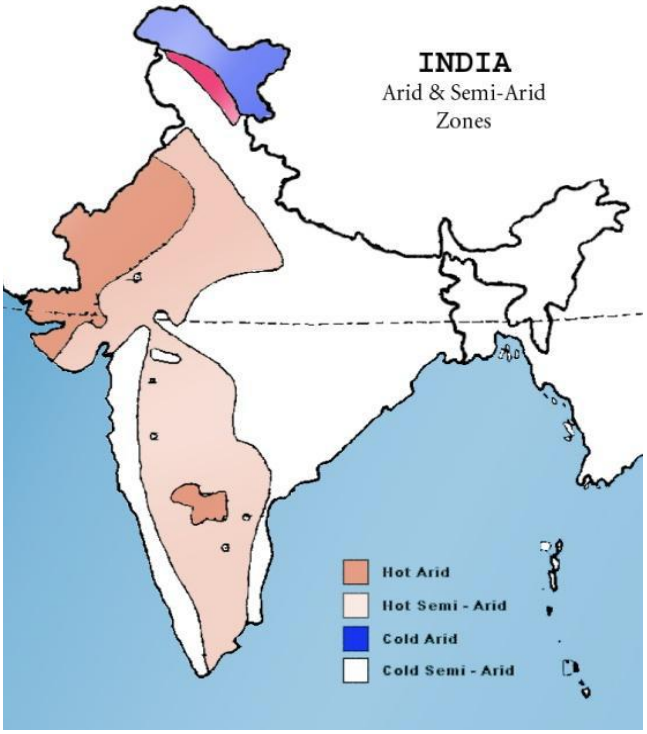
Fig. 1.1: *Global distribution of arid and semiarid landscapes. (adapted from pubs.usgs.gov/gip/deserts/what/world.gif)*



According to Ministry of Environment and Forests, Government of India, about 69% of India is dry land distributed in arid, semi-arid and dry sub-humid climates. Among these, the semi-arid region extends to a larger portion in India (Fig. 1.2). Most of the area across 14 states and over 75% of the cropped area (i.e. 131 million ha out of 174 million ha) are in this particular semiarid climate (http://www.ced.org.in/docs/inecc/arid_booklet/Arid-3-Arids.pdf). Water availability is scarce in these regions as the annual precipitation is low. These areas are heavily populated and considered as population hot spots. Soil erosion and land degradation have severe implications for livelihood and food security for millions of people living

in these areas. Environmental constraints of these particular landscapes include low and unpredictable seasonal rainfall, high mean annual temperatures and high evaporative demand, which severely limit water supplies for agricultural use. Poor soil fertility of semiarid soil may also limit agricultural productivity.

Fig. 1.2: *Distribution of arid and semiarid regions in India. (adapted from http://www.ced.org.in/docs/inecc/arid_booklet/Arid-3-Arids.pdf)*



Studies of soil environments have received lot of attention because of their importance in human health, ecosystem functions, agriculture, and environmental management. The extent of the diversity of microorganisms in soil is seen to be critical to the maintenance of soil health and quality, as a wide range of microorganisms are involved in vital soil functions (Pereira e Silva et al., 2013). Although information is available on microbial diversity and activity with response to magnitude of spatial *and temporal* changes, such information is sparse in arid/ semiarid regions which provide essential land area for cultivation and other applications. Therefore, it is of fundamental concern to understand the response of land use changes on microbial activity in arid/semi-arid landscapes. Moreover,

microbial activity, diversity and their critical determinants in the semiarid alluvial plains are inadequately understood in Indian subcontinent. Since the findings emerging from reported studies cannot be directly extrapolated to soils that are geographically distant and geochemically distinct, it is imperative that systematic studies on factors influencing bacteriological activities and communities to be carried out on Indian soils. In the present thesis, such studies were undertaken in semiarid alluvial soils around Mahi River basin, western India. By characterizing microbial diversity and activity, we may able to better understand and manipulate ecosystem functions for sustainability of agriculture or for biogeochemical cycling. Bacterial communities in natural systems are great resource for biotechnological exploration of novel organisms, products and processes which is largely unexploited because of paucity of biodiversity indexing of microorganisms in diverse environments.

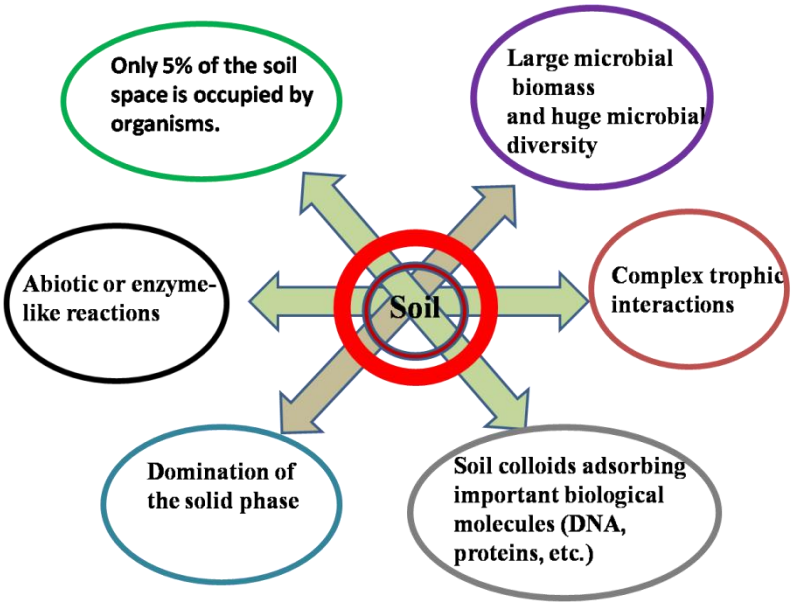
The present review of literature comprises of information about soil habitat, microbial activity and diversity, important methods for studying soil microbial functions, soil quality indicators and scope of the thesis.

1.2. Soil as highly heterogeneous and complex microhabitat

Soil, the upper most layer of Earth's crust is fundamental and irreplaceable living medium. As an interface between the Earth's crust, atmosphere and hydrosphere, the soil is a non-renewable resource that fulfils vital functions and is a major contributor to global nutrient cycling processes. Soil is considered to be complex, dynamic and highly heterogeneous ecosystem (Fig. 1.3).

Soil is said to be structured, discontinuous and poor in nutrients. Soil is a major reservoir of organic carbon on earth. Soils undertake a range of functions in the environment which include provision of a suitable medium for plant growth, immobilisation of contaminants derived from anthropogenic activities, exchange of gases with the global atmosphere and the creation of a suitable environment for the maintenance of biodiversity**.**

Fig. 1.3: *Schematic representation of the main characteristics of soil as a microhabitat. (adapted and redrawn from Nannipieri et al., 2003)*



Soil microbes play an important role in soil functions and by identifying the activities and communities of microbes in the soils it may be possible to identify process-based quality indicators. Soil microbes living in discrete microhabitats and perform vital soil functions such as degradation of organic matter and pollutants, recycling of nutrients, plant health, soil formation and it is believed that 80 to 90% of the relevant soil functions may be mediated by microbes (Nannipieri et al., 2003; Pereira e Silva et al ., 2013). The capacity of soils to sustain such functions is a key feature of what has been denominated "soil health/quality". The physiochemical and biological characteristics of soil microhabitats change in both time and space. The sequential occurrence of maxima and minima in relevant parameters of different soil processes are considered to be the 'natural' limits of variation in soil functioning, on the basis of which a normal operating range (NOR) has been defined and external disturbances can be visualised to bring about changes from NOR for sensitive parameters (Pereira e Silva et al., 2013)

1.3. Soil microbial activities

Microorganisms make up the largest part of the total biomass in the soil and are key drivers in processes that contribute to the provision of essential ecosystem services,

such as respiration, decomposition of organic matter and nitrification and other N-related processes (Winding et al., 2005).

1.3.1. Estimation of soil microbial activity

Soil microbial activity is gauged by various biological indicators such as soil enzymes, respiration and microbial biomass etc. Some important methods are described in the Table 1.1.

Table 1.1: *General methods to assess the biomass and activity of soil organisms (modified from Bunemann et al., 2006).*

Name	Method description	Reference
Microbial biomass C, N, and P	C, N and P in microbial biomass by fumigation extraction method	Vance et al., 1987; Kouno et al., 1995
Adenosine triphosphate (ATP)	Extraction of ATP indicate size of microbial biomass	Contin et al., 2001
CFU	Colony forming units; plate counting techniques	Sarathchandra et al., 1993
Soil respiration	CO_2 release from incubated soil	Alef, 1995
Metabolic quotient	Ratio of soil respiration to microbial C; higher values can indicate physiological stress	Anderson and Domsch, 1990
Soil enzyme activities	Dehydrogenase, phosphtase, glucosidase and urease etc.	Tabatabai, 1994
FDA hydrolysis	Fluorescein diacetate (FDA) hydrolysis as a measure of total microbial activity	Adam and Duncan, 2001
Adenylate energy change, leucine and thymidine incorporation	Microbial incoroporation of leucine and thymidine indicates active biomass	Nannipieri et al., 1990

1.3.1.1. Soil enzymes

Soil enzymology is considered to be one of the oldest fields of research in soil biochemistry (Burns et al., 2013) with an extensive literature available. Some of the important review articles about the basic and applied aspects of soil enzymology are shown in the Table 1.2.

Soil enzyme activities play vital biochemical functions in the overall process of organic matter decomposition or transformation in the soil system (Sinsabaugh et al., 1991). These enzymes are said to be constantly being synthesized, accumulated, inactivated and decomposed in the soil. Extracellular enzyme activities play an

important role in the microbial acquisition of C, N, P and are believed to be the rate-limiting steps in microbially mediated decomposition of organic matter (Caldwell et al., 2005). Some of the important soil enzyme activities which play important role in nutrient cycling are summarized in Table 1.3.

Table 1.2: *Table showing important review articles (from 1982 to 2013) about basic and applied aspects of soil enzyme activities*

Si no	Review on	Reference
1	Enzyme activity and their possible role in microbial ecology	Burns, 1982
2	Importance of ureolytic enzymes in soil fertility	Hasan, 2000
3	Important developments in soil microbiology since the mid 1960s	Insam, 2001
4	Overall review about soil enzyme research	Zahir et al., 2001
5	Functional aspects of soil enzymes	Emmerling et al., 2002
6	Enzyme activities as a component of soil biodiversity	Caldwell, 2005
7	Different approaches to evaluating soil quality using biochemical properties	Gil-Sotres et al., 2005
8	Potential role enzymes in the ecosystems with good examples	Makoi and Ndakidemi, 2008
9	Stoichiometry of soil enzyme activity at global scale	Sinsabaugh et al., 2008
10	Emerging tools for measuring and modeling the in situ activity of soil extracellular enzymes	Wallenstein and Weintraub, 2008
11	Classical and molecular approaches of soil enzymology	Nannipieri et al., 2012
12	Soil enzymes in a changing environment: Current knowledge and future directions	Burns et al., 2013

Soil enzyme activity measurements are the direct expression of the soil community to metabolic requirements and available nutrients (Caldwell, 2005). Soil enzyme acitivity assays are accurate, simple, cheap and generally based on short-term incubations (Nannipieri et al., 2012). A better understanding of the role of this soil enzymes activity in the ecosystem will potentially provide a unique opportunity for an integrated biological assessment of soils.

Table 1.3: *Certain important soil extracellular hydrolases participating in C, N and P cycling*.

Nutrient cycle	Enzyme	Enzyme function	Enzyme assay reference
Carbon	Cellulase	Degradation of cellulose	Deng and Tabatabai, 1994
	ß-Glucosidase	Rate limiting enzyme in the microbial degradation of cellulose to glucose. The enzyme catalysis the hydrolysis of glucosides	Eivazi and Tabatabai, 1988
	Amylase	Catalyses the hydrolysis of starch	Pancholy and Rice, 1973
	Xylanase	Catalyses the hydrolysis hemicelluloses	Speir et al., 1984
	Chitinase	Catalyses the hydrolysis of chitin	Rodriguez-Kabana et al., 1983
	Phenoloxidase	Catalyses the hydrolysis of aromatic compound lignin	Sinsabaugh et al., 1992
Nitrogen	Endo-protease	Proteins	Ladd and Butler, 1972
	Aminopeptidase	Peptides	Ladd and Butler, 1972
	Urease	Primary amines	Sinsabaugh et al., 2000
Phosphorous	Phosphomonoesterase	Catalyses the hydrolysis monophosphate esters	Eivazi and Tabatabai , 1977
	Phosphodiesterase	Catalysis the hydrolysis of diphosphate esters	Sparling et al., 1986

Soil enzyme activities have been correlated well with soil physiochemical characters, vegetation metal pollution succession and microbial community structure (Kourtev et al., 2002; Sinsabaugh et al., 2002; Tscherko et al., 2003; Khan et al., 2007). Soil enzymes are found to be highly responsive and exceptionally sensitive to anthropogenic and environmental parameters (Nannipieri et al., 2002). Some of the important extracellular enzyme (hydrolases) activities involving in soil C, P, and N cycles are ß-glucosidase, alkaline phosphatase, and protease respectively. The effect of various land use changes including pollution on soil hydrolases are comprehensively summarized in Table 1.4. One more important soil enzyme is dehydrogenase (DHA). Active dehydrogenases are considered to exist in soils as integral parts of intact cells. They do not accumulate extracellularly in the soil. This enzyme is associated with living cells and provides an assessment of respiration, and has been widely used to estimate microbial activity in a range of soils. As like as hydrolases, DHA is also sensitive to various perturbations (Mora et al., 2005). DHA is found to be sensitive to amendment of agricultural inputs on soil organisms

(Bunemann et al., 2006). Measurements of dehydrogenase activity represent immediate metabolic activities of the soil microorganisms at the time of the test.

Table 1.4: *Effect of various anthropogenic activities on soil dehydrogenase, alkaline phosphatase, ß-glucosidase and protease. Values in the related articles are expressed as following units: dehydrogenase: µg TPF dwt g-1 soil 24 h^{-1}; alkaline phosphatase : µg p-NP dwt g-1 soil h^{-1}; ß-glucosidase: µg p-NP dwt g-1 soil h^{-1}; protease: µg p-NP dwt g-1 soil $2h^{-1}$*

Enzyme	Effect	Enzyme response	Range of activity (approximate)	Reference
Dehydrogenase (EC 1.1.1.)	Heavy metals (Cd and Pb)	Decreased	45-75	Khan et al., 2007
	Long term metal contaminated soils	Decreased	4-50	Oliveira and Pampulha, 2006
	Municipal waste compost and biosolid waste compost	Increased	60-70	Mora et al., 2005
	Organic fertilization with conventional tillage	Increased	20-35	Mijangos et al., 2005
	Pesticide (fonofos)	Decreased	20-100	Stêpniewska et al., 2007
	Organic forming (green manure and farmyard manure)	Increased	200-250	Okur et al., 2009
Alkaline Phosphatase (EC 3.1.3.1)	Heavy metals (Cd and Pb)	Decreased	450-750 270-350 4808-6135	Khan et al., 2007 and 2010 ; Renella et al., 2006.
	Mixture of soil stabilizers beringite and zerovalent	Increased	700 -800	Ascher et al., 2009
	Organic fertilization with conventional tillage	Increased	650-900	Mijangos et al., 2005
	Addition of Organic acids (citrate, glutamate and oxalate)	Increased in both clay and sandy soil	3800-9500	Renella et al., 2007
	No tillage practice	Increased	139 to 207	Balota et al., 2004
	Organic forming (green manure and farmyard manure)	Increased	450-550	Okur et al., 2009
ß-Glucosidase (EC 3.2.1.21)	Heavy metals		400-1000	Renella et al., 2006.
	Mixture of soil stabilizers Beringite (B) and zerovalent	Increased	100-230	Ascher et al., 2009
	Municipal waste compost and biosolid waste compost	Increased	400-800	Mora et al., 2005
	Organic fertilization with conventional tillage	No significant change	200-250	Mijangos et al., 2005
	Dry residues amendment(for soil moisture content (%)	Increased	120-450	Geisseler et al., 2012

	adjustment)			
Protease (EC *3.4.21.19*)	Addition of dry residues for soil moisture content (%) adjustment	Increased (after 5% moisture content)	50 – 170	Geisseler et al., 2012
	Organic forming (green manure and farmyard manure)	Increased	150-180	Okur et al., 2009
	Fungicides (propiconazole and chlorothalonil, 10 kg h^{-1})	Decreased	540-682	Ramudu et al., 2012
	Cattle manure	Increased	8000-11000	Cenciani et al., 2008

1.3.1.2. Soil respiration

Soil respiration is a routinely used technique for quantifying microbial activity in soils. Active living cells of aerobic heterotrophic microorganisms derive energy degradation or oxidation of organic matter consists of cellulose proteins, nucleotides and humified compounds where oxygen functions as the end electron acceptor. The end product of this reaction is CO_2 production or O_2 consumption (Alef, 1995).

The basal respiration is defined as the respiration without the addition of organic substrate to the soil. Substrate induced respiration (SIR) is the soil respiration measured in the presence of an added substrate such as glucose, amino acid, etc. Respiration is influenced by soil moisture, temperature, the availability of nutrients and soil structure. Soil respiration decreases with depth of the soil and correlates significantly with soil organic matter (C_{org}) and most microbial parameters.

Soil respiration has been most frequently used for the assessment of the side effects of chemicals such as pesticides and heavy metals, etc. Some of the land use changes including pollution affecting soil respiration are summarized in Table 1.5.

1.3.1.3. Soil microbial biomass

Microbial biomass has been defined as the part of the organic matter in soil that constitutes living microorganisms small than 5 to 10 µm (Joergensen, 1995). One hectare of agricultural soil may contain up to 3000 kg of bacteria in the upper 25 cm. Typically, biomass is measured in terms of biomass carbon (C_{mic}) and the C_{mic} value ranges from 1 to 5% of soil organic matter (Smith and Paul, 1990). The interest in estimating soil microbial biomass is related to its function as a pool for subsequent

delivery of nutrients and its role in structural formation and stabilization of soil and an ecological marker.

Several methods have been used to estimate microbial biomass in soil. Vance et al. (1987) proposed the chloroform fumigation for indirect microbial biomass determination which is currently used. Estimation of soil microbial biomass C including biomass N certainly offers a mean of assessing the response of total microbial population to the magnitude of changes in soil management practices (Table 1.6). Studies indicate that microbial biomass carbon decreases in soils which are contaminated by elevated levels of heavy metals. Microbial biomass and activities are regulated by many soil and environmental factors including soil organic matter quality (Moore et al., 2000), physiochemical soil characteristics and amendment of agricultural inputs (Bünemann et al., 2006).

Table 1.5: *Effect of certain land use changes and pollution on soil respiration.*

Soil ecosystem Type	Treatment/Effect of	Respiration response	References
Salt affected soils	Salinity	Decreased	Iwai et al., 2012
Clay-loam soil	Conventional tillage	Decreased	Mijangos et al., 2006
Vertic Aquic Ustorthent soil	Compost amendment Herbicides	Increased Decreased	Perucci et al., 2000
Agricultural soils	Heavy metals (Cd, Cr and Pb)	Decreased	Verma et al., 2010
Polluted soils	Heavy metals (Fe, Al, Mn, Zn, Cr, Ni, V, Cu, Cd and Pb)	Decreased	Bech et al., 2000
Agricultural soil	Organic amendment	Increased	Treonis et al., 2010
Silt-loam Luvisol	Fungicides (Benomyl)	Decreased	Chen et al., 2001

Table 1.6: *Effect of some perturbations on soil microbial biomass C (C_{mic})*

Soil ecosystem type	Effect of	Microbial biomas C response	Range (approximate) mg kg^{-1} soil	References
Oxisol	Soil degradation	Decreased	20 to 120	Nunes et al., 2012
Rhizosphere	Organic amendment	Increased	300-500	Das and Dkhar, 2012

	(farmyard manure)			
Alpine meadow soils	Grazing	Decreased	270-650	Fu et al., 2012
Agriculturasl soil	Tillage	Decreased	160-209	Balota et al., 2004
Semiarid soil	Cadmium (Cd) pollution	Decreased	390-440	Ros et al., 2009
Vertic Aquic Ustorthent soil	Herbicide (Rimsulfuron)	Decreased	350-370	Perucci et al., 2000

1.4. Soil microbial biodiversity

As for the International Union for Conservation of Nature and Natural Resources (IUCNNR), biodiversity includes all life forms, ecosystems and ecological processes and acknowledges the hierarchy at genetic, taxon and ecosystem levels. Bacterial diversity in soils is particularly large, with thousands to millions of genomes (a surrogate for species) in just a few grams of soil (Torsvik et al., 1990; Gans et al., 2005). Similarly, Curtis et al. (2002) estimated the number of bacterial taxa in a gram of soil between 6400 and 38000 based on a log-normal species abundance curve. Equations used to calculate diversity indices including species richness and evenness have been discussed by Kennedy and Smith (1998). The richness and evenness of bacterial communities reflect selective pressures that shape diversity within soil communities. Measuring these parameters is most useful when assessing treatment effects (e.g., physical disturbances, land use changes, pollution, climate change, etc.) on community diversity.

1.4.1. Studying soil microbial diversity

The immense phenotypic and genetic diversity found in soil microbial communities makes it one of the most difficult communities to investigate. It has been suggested that at least 99% of bacteria observed under a microscope are not cultured by common laboratory techniques (Pace et al., 1997). Given the high genetic and physiological diversity of bacteria, it is impossible to investigate all of them by using one single method. However, different types of techniques can be applied to explore

soil microbial diversity. Problems associated with the study of microbial community are not only due to methodological problems, but also to the lack of taxonomic knowledge. According to Kirck et al. (2004), certain general limitations in exploring microbial diversity are as follows

- Spatial heterogeneity of soil microbial communities
- Inability to culture soil microorganisms
- Limitations of molecular-based methods
- Taxonomic ambiguity of microbes

A number of technological advances have been made in the recent past leading to the development of new and more powerful methods for resolving microbial community structure and studying community dynamics vis-à-vis biotic and abiotic factors. Methods to measure microbial diversity in soil can be categorized into two groups: biochemical-based techniques and molecular-based techniques however each method has its own advantages and disadvantages. Fig. 1.4 summarizes the general methodology for opening the black box of soil microbial community. Recently, several fingerprinting techniques have been developed and used in microbial ecology studies (Van Elsas and Boersma, 2011).

1.4.2. Culture dependent vs culture independent soil microbial diversity studies

Microbial diversity studies can be divided into two broad categories, culture dependent and independent ones (Nocker et al., 2007; Van Elsas and Boersma, 2011). Culture dependent methods are based on isolation of bacteria from environmental samples by suitable culture medium. However, the biggest challenge in exploring bacterial biodiversity is the issue of viable but non-cultivable organisms (VBNC). In culture dependent diversities, bacterial communities are normally estimated by phenotypic characterization of isolated bacteria, but the problem is that phenotypic methods can be used for only those bacteria which can be grown and cultured. Therefore, traditional characterization of microbial strains has been subjected to debate, as it is dependent on the ability of the strains to grow under specific environmental conditions. Moreover, such methods have been frequently met with strong limitations as only a limited portion of the soil microbiota can be accessed on the basis of cultivation. This phenomenon has been defined as the Great Plate Count

Anomaly (Van Elsas and Boersma, 2011). The isolated bacteria may be represent only a minor proportion of the total bacterial diversity in a given soil ecosystem. Although culturing has been indispensable for increasing our understanding of specific organisms, problems with using culturing for community analysis arise from the fact that an artificial homogenous medium virtually does not represent natural environment and typically allows growth of only a small fraction of the organisms. This has apparently led to the uncertainty regarding the true extent of soil bacterial diversity. The disparity between culturable and in situ diversity has increased the importance of culture-independent molecular approaches (Nocker et al., 2007)

Culture independent methods involve direct extraction of community nucleic acids from soil samples. It often involves the amplification of nucleic acid (extracted directly from environmental samples) by selective PCR and the subsequent analysis of the diversity of the amplified molecules (community fingerprinting). Alternatively, the amplified products may be cloned and sequenced to identify and enumerate bacterial species present in the sample. It is thought that the direct extraction of nucleic acids from environmental samples accounts for the very large proportion of microorganisms that are not readily cultured in the laboratory, but that may be responsible for the majority of the ecosystem activity of the interest. Although culture independent methods are more advanced than cultivation dependent ones, no single tool allows perfect assessment of the soil bacterial diversity. Therefore, the use of a polyphasic approach involving a combination of cultural, biochemical and molecular biology techniques is necessary to obtain a better understanding of the interaction between the microorganisms, their natural environment and ecosystem functions.

1.4.3. Studying soil microbial diversity by DGGE*:*

Genetic fingerprinting tools provide a pattern of the genetic diversity in a microbial community. Denaturing gradient gel electrophoresis (DGGE) is one of the routinely using molecular fingerprinting tool for studying microbial diversity including functional gene diversity in a range of environments (Table 1.7). Muyzer et al. (1993) first time applied DGGE technique to study microbial communities in soil microbial ecology. For this technique, community DNA is extracted from soil samples and amplified using PCR with universal primers targeting part of the 16S or

18S rRNA gene sequences or any other gene of interest. A 40 base pair GC clamp is attached to the 5'-end of the forward primer. The general principle is "On denaturation, DNA melts in domains, which are sequence specific and will migrate differentially through the polyacrylamide gel with a gradient of the denaturant (in general 40 to 60%, Muyzer, 1998). In other words separation of DNA fragments depends on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of a urea and formamide. Theoretically, DGGE can separate DNA with one base-pair difference (Muyzer, 1999). DGGE has the advantages of being reliable, reproducible, rapid and somewhat inexpensive. Most importantly, multiple samples can be analyzed simultaneously, making it suitable to detect changes in microbial populations. Nevertheless, it has also some limitations such as PCR biases (Wintzingerode et al., 1997) and variable DNA extraction efficiency (Theron and Cloete, 2000).

1.4.4. Quantification of microbial abundance and diversity by quantitative PCR

Most of the molecular techniques currently being used in microbial ecology lack a quantitative constituent. Nevertheless, quantification of microbial abundances is of great relevance for the understanding of microbial community structure and dynamics in natural environments. In recent years, quantitative PCR (q-PCR, also referred to as real-time quantitative PCR) has emerged as a promising technique for studying soil microbial communities from different environments (Walker, 2002; Smith and Osborn, 2009). q-PCR is based on the real-time detection of a reporter molecule whose fluorescence increases as PCR product accumulates during each amplification cycle (Smith and Osborn, 2009). The q-PCR approach is somewhat unique among methods of community analysis in that it allows for a relatively rapid yet quantitative assessment of the abundances of specific phylogenetic groups of microorganisms in soil (Table 1.8). Recently, applications of real-time PCR have also been demonstrated with marine samples to quantify numbers of cells of *Synechococcus* spp., *Prochlorococcus* spp. and *Pseudomonas stutzeri* ; with soil samples to quantify cells of *Rhodococcus* spp. , methanotrophs , and fungi ; with sediments to quantify cells of *Geobacter* spp. Real-time quantitative -PCR is sensitive and highly specific, allowing one to detect and quantify low levels of transcripts, which makes it highly suited for

the analysis of microbial gene expression (function) in soil (Table 1.8; Novinscak and Filion, 2011; Chi et al., 2013).

Fig 1.4: General methodology for exploration of soil microbial diversity.

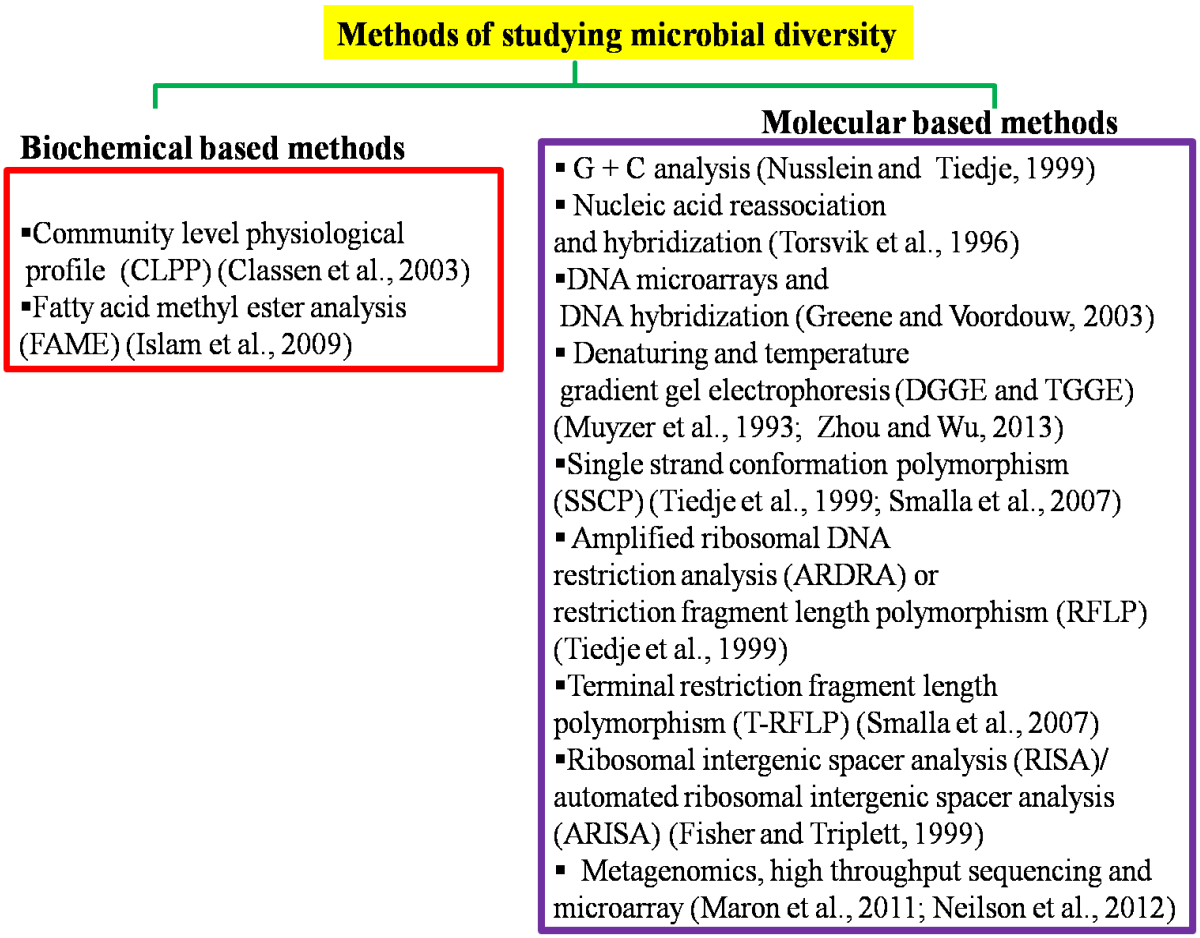


Table 1.7: *Recent applications of DGGE tool (particularly) in soil ecosystems for exploration of general and functional soil microbial diversity.*

Ecosystem type	Targeted gene for DGGE	References
Heavy metal contaminated scrubland soil	16S rRNA, 18S rRNA, α-proteobacteria, β-proteobacteria and acidobacterial 16S rRNA	Ros et al., 2009
Alkaline sandy loam and acidic soils	Bacterial *amoA* gene and Archaeal *amoA gene*	Zhang et al., 2012; Chen et al., 2010; Shen et al., 2008
Rhizosphere and Bulk soil samples	16S rRNA	Shi et al., 2011
Polycyclic aromatic hydrocarbon-contaminated soil	16S rRNA	Mao et al., 2012
Grassland soils	*nrxA* gene	Wertz et al., 2008
Agricultural soil	*nirS* and *nirK* genes	Zhou et al., 2011
Tropical ferralitic soil	*frc gene*	Khammar et al., 2009
Pristine black soil	*28S rRNA*	Yu et al., 2010

Table 1.8: *Recent application of q-PCR tool in various soil ecosystems for quantification different general and functional genes of distinct soil ecosystems.*

Ecosystem	Targeted gene	Targeted microbial group	Reference
Acidic luvisols	AOA-*amoA*, AOB-*amoA genes*	Ammonia oxidizing archaea (AOA) and bacteria (AOB)	Xu et al., 2012
Acidic soils	AOA-*amoA*, AOB-*amoA genes*	Ammonia oxidizing archaea (AOA) and bacteria (AOB)	Zhang et al., 2012
Forest soils	fungal ITS, bacterial 16S-rRNA archaeal 16S-rRNA genes	Fungi, eubacteria and archaea	Yarwood et al., 2010
Rhizosphere of rice	*nirK* gene	Denitrifiers	Hussain et al., 2011
Agricultural soils	*hcnC* gene	*Pseudomonas* sp.	Novinscak and Filion, 2011
Nitrophenol spiked soil	*pnpA, mnpA and onpA, 16S rRNA* genes	Nitrophenol degrading bacteria	Chi et al., 2013

1.5. Soil health and soil quality

Despite great progress in overall agricultural productivity in recent decades, land degradation has reduced the productive capacity of soils on nearly 40% of the world's agricultural land. These soils suffer physical degradation, such as erosion and compaction; chemical degradation due to acidification, nutrient depletion, pollution from industrial wastes etc. and biological degradation due to organic matter depletion and loss of biodiversity. In light of these threats, there is growing interest in the factors governing soil health, biodiversity, and resilience, as well as in the fundamental relationships between them. Soil health or soil quality has been defined as "the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation". It is often postulated that many healthy soils show a certain degree of resilience, *i.e*. they possess the capacity to return to their original status following a disturbance or stress (Allison and Martiny, 2008; Shade et al ., 2012). In addition to their importance to the functioning of soil ecosystems, microorganisms confer resistance or resilience to soil in the event of an external disturbance. Studies demonstrate that changes in microbial composition are often associated with changes in ecosystem process rates (Allison and Martiny, 2008). Thus, changes in microbial communities due to disturbance may directly affect soil ecosystem processes. Microbial resistance, resilience and functional redundancy are the three prime features for predicting soil ecosystem process rates under disturbances (Allison and Martiny, 2008). The potential impacts of a disturbance on microbial composition and ecosystem processes are illustrated in Fig. 1.5. Microbial composition might be resistant to the disturbance, and may not change. Otherwise, the community may be sensitive and does change, but it could be resilient and quickly recover to its original composition. Finally, a community whose composition is sensitive and not resilient might produce ecosystem process rates similar to the original community, only if the members of the community are functionally redundant.

Important mechanisms responsible for microbial resistance, and resilience in distrusted environments are recently reviewed by Shade et al. (2012) and are shown in Fig. 1.6.

Fig 1.5: *A schematic representation of how disturbance can change microbial community composition and thereby affect ecosystem processes versus when disturbance would not have this effect (when the microbial community is resistant, resilient, or functionally redundant)* ***(adApted from Allison and Martiny, 2008).***

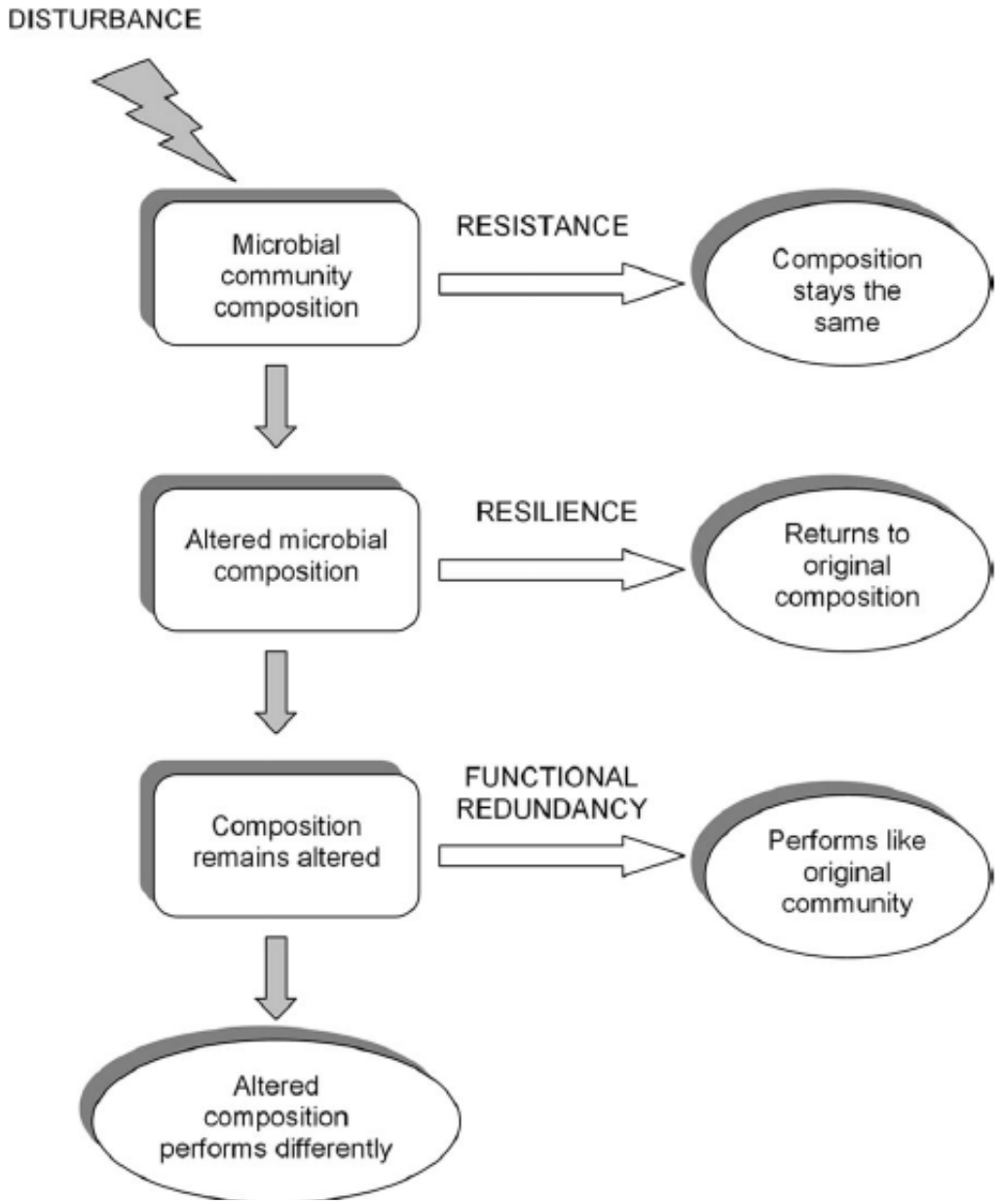
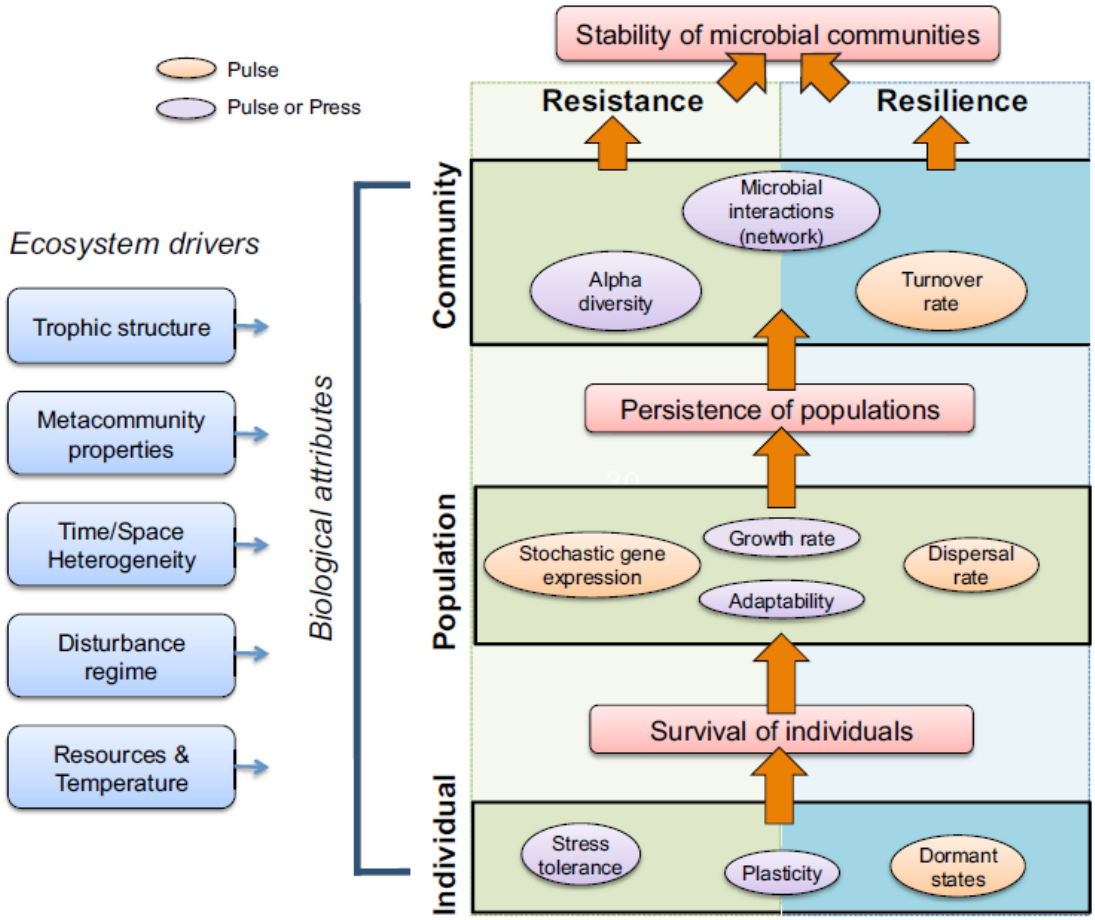


Fig 1.6: *Model of ecosystem properties governing microbial community resistance to and resilience after disturbance. Ecosystem stability is influenced by individual-, population-, and community-level biological attributes that contribute to community resistance (left, green background) and/or resilience (right, blue background), or both (center). Ecosystem drivers are indicated in leftmost blue boxes, biological attributes will be differently advantageous given a pulse (orange or purple) or press (purple) disturbance (adapted from Shade et al., 2012)*

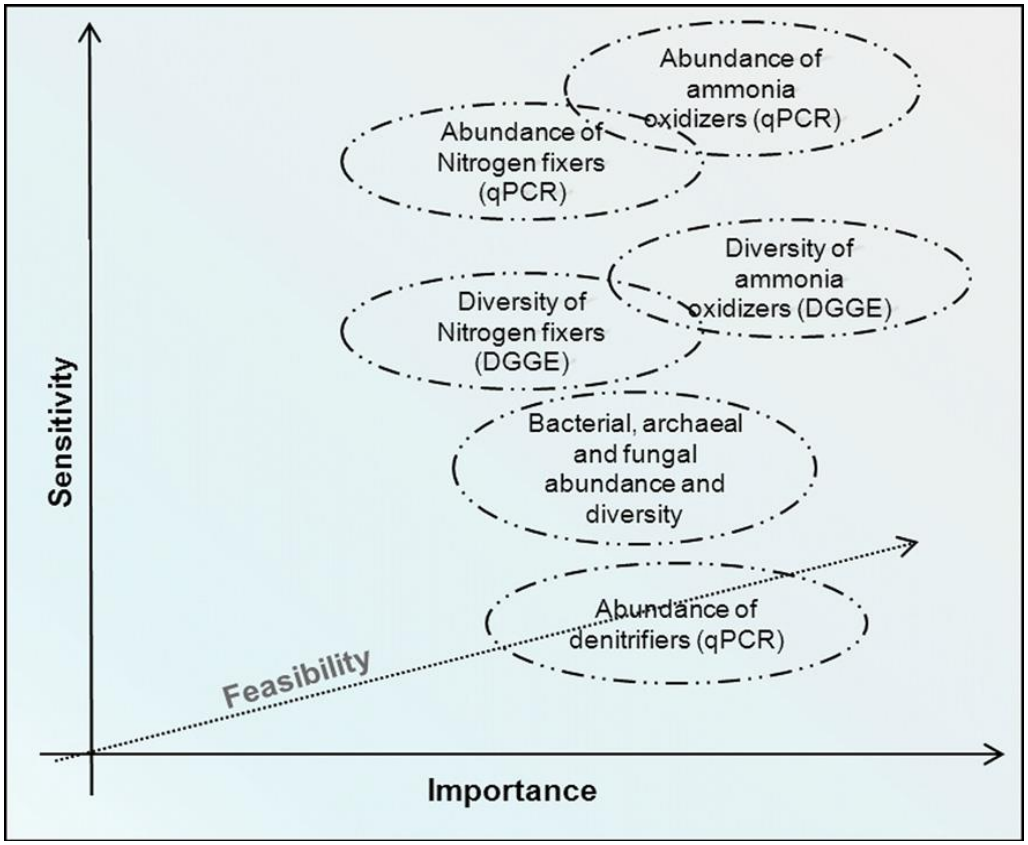


If community composition is sensitive to a disturbance, not resilient, and not functionally redundant and does change, then only it matters for predicting soil ecosystem process rates. For instance, even if microbial composition is highly sensitive and not resilient to a disturbance, but they are nearly functionally redundant, then predictions of ecosystem process rates will not be directly dependent on information about microbial composition alone. Allison and Martiny (2008) reviewed a total of recent 110 studies and assessed whether microbial community composition is resistant, resilient, or functionally redundant in response to four different common soil disturbances such as elevated CO_2, mineral fertilization, temperature changes,

and C amendments. They found that in 80% of studies microbial composition is sensitive and could be used as sensitive indicators for soil disturbances.

These soil quality parameters should include sensitive processes, which mean those processes that are poorly redundant and easily deviate following a stress situation (Pereira e Silva et al., 2013). Therefore the first step in the development of a framework of soil quality and may form the basis of a soil normal operating range (NOR) is thus the selection of an appropriate set of indicators, and their threshold values, at different points in time. For example, as depicted in Fig. 1.7 among the different biological parameters, top ranked parameter is nitrification-related measurement in relation to other less sensitive measurements.

Fig 1.7: *Classification of potential biological parameters of soil health and the tradeoff between their importance and sensitivity (adapted from Pereira e Silva et al., 2013) .*



Similarly, attempts have been made to distinguish the concept of soil quality from that of soil health. It is currently accepted that the term quality refers to the capacity of the soil for carrying out a specific function, while health refers to its

overall condition (Doran and Safley, 1997; Doran, 2002,; Gil-Sotres et al., 2005). Maintenance of good soil quality is of prime importance for sustainable agriculture. Soil quality can be assessed by various parameters among which microbial based parameters gained lot of attention (Gil-Sotres et al., 2005).

1.5.1. Microbiological soil quality indicators

According to Gil-Sotres et al. (2005) for estimation of soil quality, 40% of the consulted published papers used a general biochemical parameter such as microbial biomass C, dehydrogenase activity, soil respiration, nitrogen mineralization capacity, FDA hydrolysing capacity or ATP content, whilst the remaining 60% considered a specific biochemical parameter such as phosphatase, urease and glucosidase activities. Gil-Sotres et al. (2005) stated that amongst the soil quality parameters, the microbial biomass C is considered to be the most reliable (41% of authors) followed by dehydrogenase activity (28%) and N mineralization capacity (16%). Phosphatase (28%), ß-glucosidase (16%) and urease (11%) activities are the most frequently used among the specific biochemical parameters and, appropriately, represent the P, C and N cycles (Gil-Sotres et al., 2005). Microbial biomass C and N mineralization capacity have primarily been used to estimate changes in soil quality due to land use changes at the same time, dehydrogenase activity (as a general measure of viable microorganisms) has also been employed in soils affected by heavy metals and pesticides, as well as for the diagnosis of the degree of recovery of degraded soils.

1.5.1.2. Soil enzymes as soil quality indicators

Following enzymes are considered to be good soil quality indicators and the detailed description of these enzymes is reviewed by Makoi et al., 2008.

1.5.1.2.1. ß-glucosidase

ß-glucosidase is characteristically useful as a soil quality indicator, and may give a reflection of past biological activity, the capacity of soil to stabilise the soil organic matter, and can be used to detect management effect on soils (Ndiaye et al., 2000). ß-glucosidase enzyme is very sensitive to changes in pH, and soil management practices (Acosta-Martinez and Tabatabai, 2000).

1.5.1.2.2. Dehydrogenase

The dehydrogenase enzyme activity is commonly used as an indicator of biological activity in soils. Dehydrogenase activity processes are part of respiration pathways of soil micro-organisms and are closely related to the type of soil and soil air-water conditions. Since these processes are part of respiration pathways of soil microorganisms, studies on the activities of dehydrogenase enzyme in the soil is very important as it may give indications of the potential of the soil to support biochemical processes which are essential for maintaining soil fertility. Dehydrogenase activity was found to be a sensitive assay for determining the effect of heavy metals on physiologically active soil microbial biomass and sustains the high applicability of this parameter for soil ecotoxicological testing (Oliveira and Pampulha, 2006).

1.5.1.2.3. Phosphatases

Among the hydrolases, acid phosphomonoesterase activity has been the most frequently used for estimating changes in soil quality due to either management or the presence of pollutants. It is a good index of the quality and quantity of organic matter in the soil (Nannipieri et al., 2011). In soil ecosystems, these enzymes are believed to play critical roles in P cycles as evidence shows that they are correlated to P stress and plant growth. This enzyme can be very high in arable soils as long as the levels of organic matter in the soil are maintained (Dick et al., 1994). Apart from being good indicators of soil fertility, phosphatase enzymes play key roles in the soil system.

1.5.1.2.4. Proteases

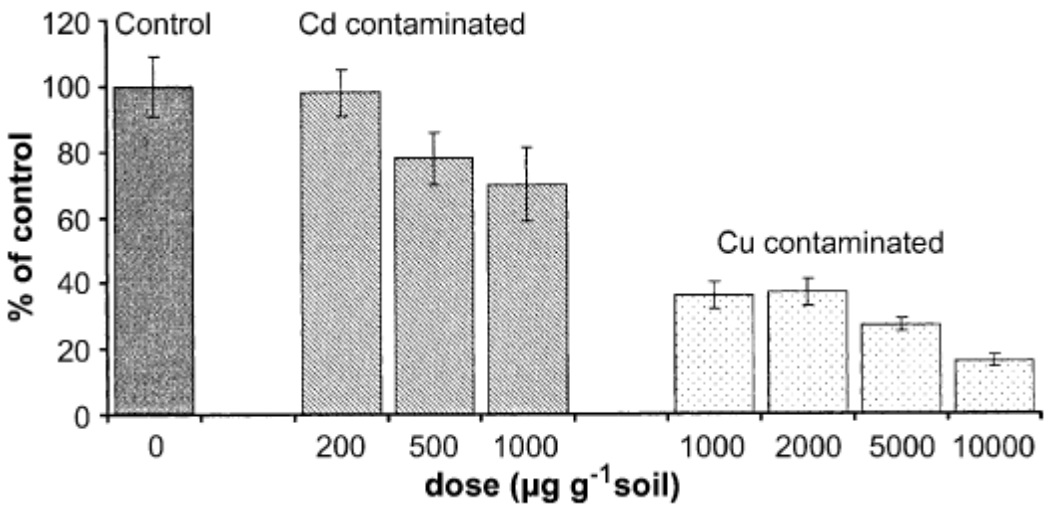
Proteases in soil play a significant role in N mineralization, an important process regulating the amount of plant available N and plant growth. This enzyme in the soil is generally associated with inorganic and organic colloids (Nannipieri et al., 1996). The amount of this extracellular enzyme activity may be indicative not only of the biological capacity of soil for the enzymatic conversion of the substrate, which is independent of the extent of microbial activity, but might also have an important role in the ecology of micro-organisms in the ecosystem (Burns et al., 2013).

All these above said enzymes are highly sensitive to both biotic and abiotic factors. Certain important land use factors and perturbations on these enzymes are selectively summarized in Table 1.4.

1.5.1.3. Microbial biomass, soil respiration and simple indexes as soil quality indicators

Microbial biomass pool sizes have been proven to be consistent indicators of soil quality, and contribute to the understanding of nutrient dynamics, both on the long term and short term seasons. Soil microbial biomass responds rapidly to land management strategy changes and is an indicator of soil health in the carbon cycle (Nielsen et al., 2002). It allows comparisons of different soils at a time, and reflects soil management changes, or pollution impact (Insam, 2001) or environmental effects. Soil microbial biomass is highly sensitive to heavy metals (Fig. 1.8).

Fig. 1.8: *Microbial biomass C in soils contaminated with different doses of cadmium and copper as a percentage of the value of the microbial biomass C in uncontaminated soil used as a control (Gil-Sotres et al., 2005).*



The microbial biomass C is increased with the intensity of grazing in meadow soils and with the cereal-pasture rotation (Dalal, 1998), decreased when the soil is cultivated (Caldwell et al., 1999) or metal polluted (Khan et al., 2007 and 2010). Furthermore, microbial biomass C increased in soils supplied with organic fertilizers (Dalal, 1998), showed an unpredictable behavior for inorganic N fertilization (Ladd et al., 1994). The effect of different agricultural inputs including fungicides on soil microbial biomass C, N and P was comprehensively summarized by Bünemann et al. (2006). Effect of some land use changes on C_{mic} is summarized in Table 1.6.

Soil respiration, depends on many biotic and abiotic factors, which prevents comparison of the degree of soil microflora functioning on its respiration level. Soil respiration is also used as biological indicator for soil quality. For example soil respiration decreased with increasing heavy metal concentrations (Verma et al., 2010). The sensitivity of soil respiration aganist various land use changes are given in Table 1.5.

Most widely used simple indices for measuring soil quality are the metabolic quotient (q-CO_2), the death rate quotient (qD), the ratio of organic C to biomass (C_{org}: C_{mic}) and the ratio between biochemical properties (Landi et al., 2000). q-CO_2 is the microbial respiration rate per unit biomass and this is one of the most popular and reliable of other simple indexes. It represents the quantity of substrate that is mineralized per unit of microbial biomass carbon and per unit of time. In general, relatively, in a imprecise ecosystem the q-CO_2 value increases in relation to a stable ecosystem (Dalal, 1998). In relation with soil quality evaluation, q-CO_2 has been considered a good index of heavy-metal induced stress in soil (Filip, 2002) as high q-CO_2 values were found in long-term contaminated soils (Brookes et al., 1986).

Next to q-CO_2, specific enzymatic activities (SPA) have been used to compare and discuss values of enzymatic activities in soils with different organic matter contents and also been considered as simple indexes of soil quality. Ecological information on specific nutrient cycling aspects of microbial activity can be obtained by calculating the ratio between soil enzyme activity to microbial biomass (Gil-Sotres et al., 2005).

1.6. Microbial studies in River basins

Although microbial activity and diversity in general terrestrial soils are established, river basins are a poorly characterized ecosystem from a microbiological point of view. Most of the available river basin microbiological studies are pointed on river sediment or river waters (Winter et al., 2007; Ghai et al., 2011), while the alluvial soils around the river basins have got less attention. Artificial neural network- modal for microbial richness in Danube River (Europe) waters indicate that 90% of the bacterial community richness could be explained with N-NO_2 and P-PO_4 (Winter et al., 2007). The riverine bacterial community was majorly influenced by intrinsic factors (Winter et al., 2007). Metagenomic studies of Amozon River water column

revealed the dominance of *Actinobacteria* among the microbial taxa and the study also pointed that river microbes appeared to be specialized in taking up and mineralizing allochthonous carbon derived from plant material (Ghai et al., 2011). The prokaryotic diversity associated with highly metal-contaminated river (Coeur d'Alene) sediment samples revealed that 75.8% of bacteria associated with Beta-proteobacteria while the remaining 24.2% were with Gamma-proteobacteria (Rastogi et al., 2009). Recent microbial diversity studies of freshwater, intertidal wetland, and marine sediments (along Pearl River, China) indicated that bacterial richness and evenness were the highest in freshwater sediment, medium in intertidal sediment, and lowest in marine sediment (Wang et al., 2012). Freshwater sediment was enriched with *Acidobacteria, Nitrospira, Verrucomicrobia, Alphaproteobacteria, and Betaproteobacteria*. The bacterial genera in intertidal sediment had a unique community with diverse primary producers such as Ch*loroflexi, Bacillariophyta, Gammaproteobacteria, and Epsilonproteobacteria)* as well as saprophytic microbes such as *Actinomycetales, Bacteroidetes, and Firmicutes*). Flood-induced disturbance causes major shifts in the diversity and function of microbial communities. Microbial activity in floodplain soils were strongly influenced by the hydrological gradient in elevation, type of soil, flooding duration, and time since the soils were last flooded (Rinklebe and Langer, 2006). Such type of studies are very much important in Indian subcontinent where there is much potential to develop or design models for practical applications such as soil ecosystem restoration or eco-engineering for sustainability of agriculture. However, unfortunately, no single study yet explored microbial aspects of Indian river basins or flood plains in a spatio-temporal context.

1.7. Microbial activity in soil genesis: Biological weathering

The dissolution of minerals by microbes is a critical process in biological weathering for soil formation (Fig. 1.9). One of the principal mechanisms that influence elemental recycling on the Earth's surface is "weathering", which plays an important role in the release of nutrients into the biosphere as a result of rock dissolution (Hamdan and Burnham, 1996) or the regulation of long-term climate by the consumption of atmospheric CO_2 during the alteration of silicate materials (Berner and Kothavala, 2001). Many weathering processes can be affected, or even driven, by the presence of microbial communities (Fig. 1.9; Banfield et al., 1999; Gorbushina

and Krumbein, 2005). Microbial weathering action is due to formation of metabolic products such as organic acids or protons or the production of metal-chelating siderophores, especially when the microbes live in a film on the surface of a rock or mineral (Ehrlich., 1998; Brantley et al., 2001). It has been reported that organic acids enhance the dissolution of aluminosilicate minerals or quartz both in field observations and from laboratory experiments (Fig. 1.9). Welch et al. (1999) reported that a variety of extracellular polysaccharides significantly enhanced the dissolution of plagioclase at pH 4. Similarly Maurice et al., (2001) found that the dissolution of well and poorly ordered kaolinites by siderophore, iron chelating compounds. It is well established that bacteria appeared to have a strong influence on granite weathering by forming pits (Song et al., 2007, Fig 1.10). It is also been reported that during heterotrophic bacterial metabolism, pH was decreased and can greatly accelerate Ca release from trace phases in granite, such as calcite, fluorapatite, and fluorite (Wu et al., 2007).

Fig. 1.9: *Schematic representation showing some general mechanisms involved in soil genesis. The puple colour circle represents the biological weathering mechanisms by soil bacteria (modified from http://www.seafriends.org.nz/enviro/soil/geosoil.htm)*

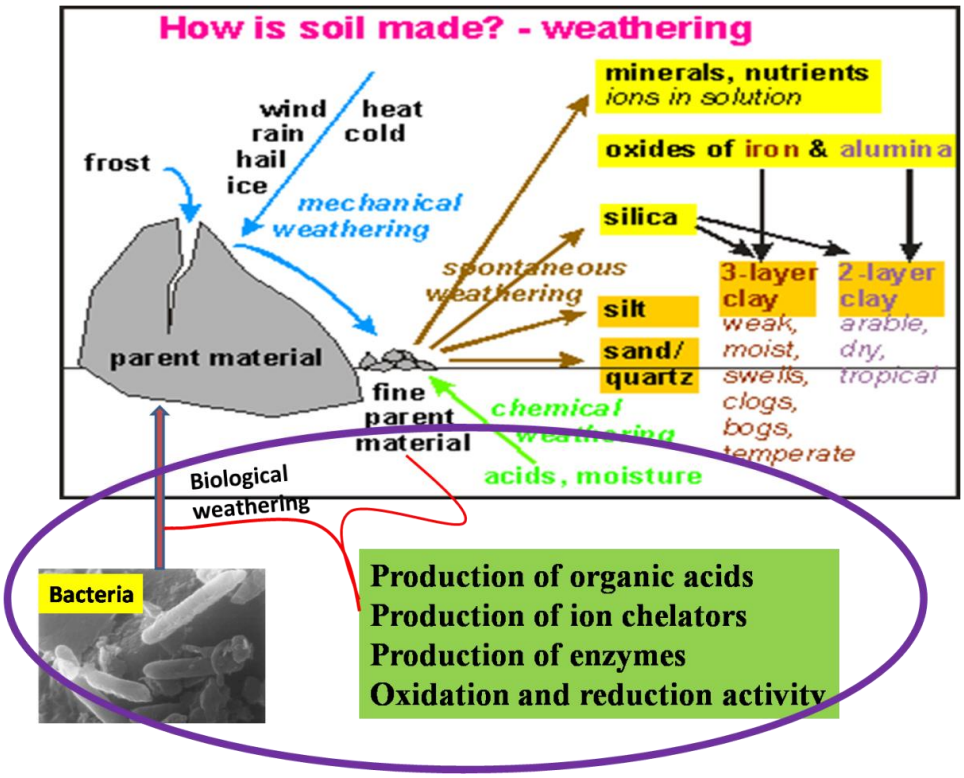
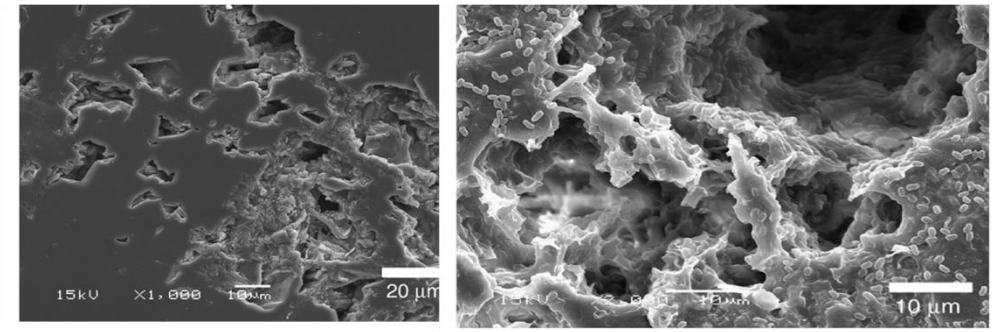


Fig. 1.10: *Effect of bacteria on weathering of granite. The amount of new pits and widened micropores on the bacteria-bearing sample was 2.4 times higher and reached an area 2.3 times wider than bacteria-free specimen (adapted from* Song et al., 2007)



Recently investigations on the microbial diversity and weathering of silica-rich rhyolitic glass (obsidian) from a lava flow in Iceland are reported (Herrera and Cockell, 2008). In addition to bacteria, fungal activities on mineral transformations by biochemical and biophysical mechanisms also have been reported (Gadd, 2007; Boswell et al., 2002). Apart from organic materials, fungi are also involved in attack of inorganic materials. They are well suited to bioweathering of rocks and minerals because of their morphological and physiological attributes and also through their symbiotic relationships (Burford et al., 2003; Gadd, 2007). Fungi have also been suggested as being instrumental in the formation of clay minerals in soils and weathered rocks (Barker and Banfield, 1996 and 1998). Some of the minerals, such as biotite and hornblende are more susceptible to biological weathering than harder or more nutrient-poor minerals such as feldspars and quartz (Barker and Banfield, 1996; Banfield et al., 1999; Roux et al., 2006). Laboratory investigation of role of bacteria on weathering of basalt revealed that chemoorganotrophic bacteria are involved in the cycling of Fe and Mn and the weathering of basalt near deep see hydrothermal wents (Daughney et al., 2004). Bacterial population includes *Actinobacteria, Proteobacteria, Bacteroidetes Acidobacteria, Gemmatimonadetes and Verrucomicrobia* are notived in Weathered Basaltic Glass (Charles et al., 2009). Geomicrobiology of pyrite weathering at Iron Mountain, CA, was investigated by (Edwards et al., 1999). Substantial literature also has been accumulated on silicate and feldspar weathering (Bennett et al., 2001). Little is known about the bacterial mediated lime stone weathering.

1.5.1. Weathering of carbonated rocks

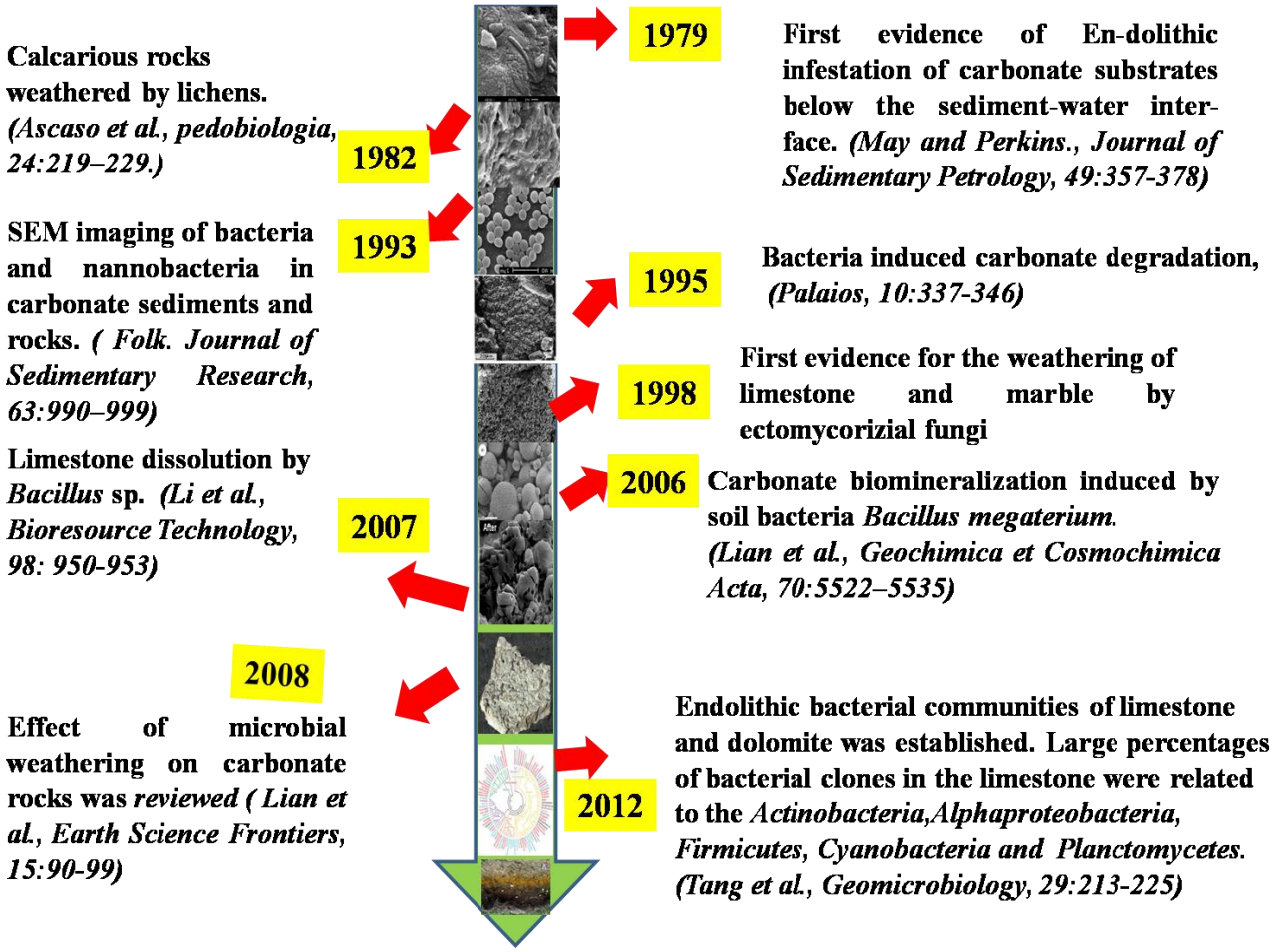
Weathering of carbonate rocks which is an important link within the exogenic biogeochemical cycle of elements in the karst environment (Zhu and Li., 2004). Microbial weathering processes of carbonated rocks include biological eluviation (or bio-leaching), mineral dissolution, sand or soil formation and substance transmission. The microbial damaging effects on carbonate rocks include biological corrosion promoting the inorganic dissolution of carbonate rocks in the land environment with effective surface, and biological abrasion making the surface of carbonate rocks become loose and speed up rock corrosion. Furthermore, the textures of carbonate rocks can be damaged because of boring by the organism, creeping, and splitting. Fig. 1.11 summarizes the important developments in carbonate rocks geomicrobiology during 1979-2012.

The mechanisms of microbial weathering refer to the corrosion of carbonate rocks by metabolism products and secreted chemical substances, or the erosion of carbonate rocks because of diagenetic changes of minerals or the oxidation and reduction of dissolved rock constituents. The main actions include acidolysis, alkaline hydrolysis, complexation, oxidation or reduction of iron and manganese (Ehrlich., 1998). Acidolysis not only includes the actions of inorganic acids, such as nitric acid, sulphuric acid, and carbonic acid but also includes the actions of organic acids, such as citric acid, oxalic acid, and gluconic acid produced by fungi, and formic acid, acetic acid, lactic acid, succinic acid, and pyruvic acid produced by bacteria (Krumbein, 1983). Alkaline hydrolysis is a process, in which organic base is formed in the growing process of heterotrophic microorganisms. The organic base can increase the pH value of mineral surface and promote the dissolution of silicon dioxide. Complexation is the process, in which chelating agents or complexants of soluble mineral elements (Fe, Al, Cu, Mg, Zn, Mn, Ca) are formed by microbial actions. The chelates include organic acids, such as citric acid, oxalic acid, tartaric acid, ethanone-gluconic acid, and phenols, such as salicylic acid and 2-3dihydroxy-benzoic acid (Krumbein, 1983). Oxidizing or reducing substances of Fe and Mn are mostly produced by bacteria, including Fe^{3+} produced by *Thiobacillus ferrooxidans* and sulfide produced by sulfate-reducing bacteria. Fe^{3+} in acidic solution can react with sulfide minerals, such as CuS, whereas sulfide can reduce MnO_2. The studies of

Bennett et al., 2001 showed that microbial weathering of carbonate rocks was not a simple process of metabolism, instead, it was a process, in which microorganisms need to extract limited nutrition they need from special minerals (Wu et al., 2007) Therefore, the microbial weathering rate of carbonate rocks depends on trace nutrition components and microbial ecologic factors.

Limestone is one of the most abundant rock types in karst areas, and is predominantly calcium carbonate. Carbonate minerals play important roles in global carbon cycling, alkalinity generation, cycling of major and trace elements, and transfer of matter among the oceans, the continents, and the atmosphere (Warren et al., 2001). Understanding limestone weathering, therefore, has a wide range of implications, from the interpretation of biogeochemical cycles to the potential impact of atmospheric CO_2 sink and the mysterious missing sink in carbon cycling.

Fig. 1.11: *Birds eye view of the geomicrobiology of the carbonates. Important developments of bacterial weathering of carbonates from 1979 to 2012 are shown with references.*



1.8. Aims and scope of the thesis

Microorganisms are key to the living soil as 80 to 90 % of the relevant soil functions may be mediated by them (Nannipieri et al., 2003). Thus, soil microbial communities play central roles in the maintenance of soil fertility and ecosystem functions. Understanding soil microbial activity and diversity would be useful to develop effective soil conservation measures and sustain food production in semiarid tropics. The overall aim of the present thesis is to add to the sparse knowledge about mechanisms driving bacterial community structure and activity in soils around river basin and to assess potential microbiological indicators of soil quality within these landscapes. Influence of anthropogenic and environmental factors on soil microbial activity and diversity has been investigated by polyphasic approach. Soil quality indicators for imposed perturbations and environmental stress have been assessed.

The Mahi River basin, western India was the site of study and has the following features that made it an interesting site to study from microbiological point of view.

- The Mahi River basin falls under semiarid climatic zone, a major climatic zone of India.
- Geomorphological, geochemical and sedimentological aspects of the Mahi River basin have been thoroughly investigated by earlier workers.
- Distinct soil and sediment sequences are exposed in open sections along the river channel and are easily accessible. They provided samples of unique soil types situated in the same climatic region
- We can find soils of same sedimentary origin but under different land use managements (e.g. agriculture and pristine soils)
- Several sites located along the river basin offer the study of unique ecosystems (e.g. Buried palaeosols, flood affected agricultural fields and industrial effluent contaminated soils).

Further these studies were extended to costal soils where potential involvement of specific bacterial groups in dissolution of Quaternary carbonates has been evaluated.

Accordingly the work was aimed at the following:

- **Establishing a link between soil characteristics and microbial processes using a polyphasic approach**
- **Microbial activity and diversity profiles of agricultural, pristine, flood affected and unaffected soils of common sedimentary origin: Case studies from Rayka and Mujpur, Gujarat, western India.**
- **Microbial activity and diversity profiling of long-term industrial waste effluent contaminated semiarid landscapes**
- **Exploring multimetal resistant plant growth promoting bacteria (PGPB) for enhancing plant growth in heavy metal polluted soils**
- **Bacteriology of miliolite, a bioclastic lime stone: Bacterial diversity, activity and potential role of bacteria in weathering of miliolite**

CHAPTER 2

Common methodology

Details for the common methodology used for several chapters are described in this section. Description of the specific methodology used only in a particular chapter is mentioned in the "Materials and Methods" section of individual chapters.

2.1. Soil physicochemical analysis

Soil pH was determined (with a soil to water ratio of 1:2.5) potentiometrically in 0.01M $CaCl_2$ solution using glass electrode. Soil moisture was determined by 1.0 g oven drying the soil samples at 105^{o}C for 24 h. Soil water holding capacity (WHC) was measured by standard gravimetric method. In brief the principle is an excess amount of water is percolated through a known amount of field moist soil; the volume of the percolate is determined and the volume of the water stored in the soil is calculated (Forster, 1995a). Soil organic matter (SOM) was determined by heated dichromate oxidation method as described by Walkley and Black (1934). In brief, 0.5 g of dried soil was mixed with 5 ml potassium dichromate and 7.5 ml concentrated sulfuric acid in a block digester tube. The tube was allowed to heat at 100^{o}C for 30 min in oven. After cooling the solution, 0.3 ml N-phenylanthranillic acid (indicator solution) and titrated with 0.2 M ferrous ammonium sulfate solution. Total organic carbon ($C_{org)}$ was measured by wet $K_2Cr_2O_7$ digestion method (Froster, 1995b; Nelson and Somers, 1975; 1996). Soil texture and particle size distribution was measured using the rapid sieving procedure (Kettler et al., 2001). Soil micro and macro elements such as total carbon (TC), total nitrogen (TN) and total sulfur (TS) were determined using the Element Analyser (Vario EL III, Elementar, Hanau, Germany). Total available phosphorous (TAP), total available potassium (TAK), Zn, Fe, Mn, and Cu were measured in the laboratory of Gujarat State Fertilizers Corporation (GSFC), Vadodara, Gujarat. All determinations were performed in triplicates and values are reported as means with standard deviation.

2.2. Soil biochemical analysis

2.2.1. Soil respiration measurement

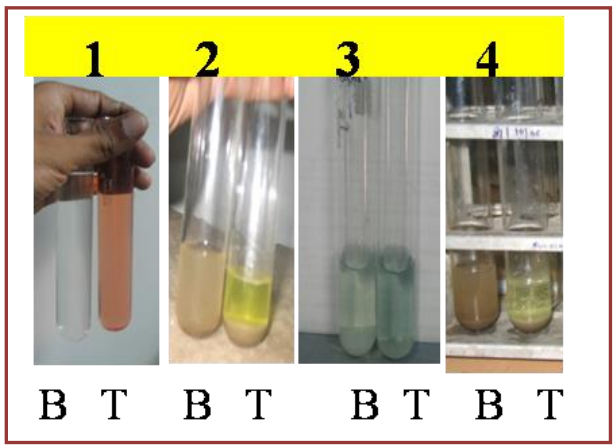
Basal soil respiration (BSR) was determined by measuring CO_2 evolution (Anderson, 1982). In brief, field-moisture soil samples (equivalent to 20 g oven-dry soil) were placed in gauze and incubated in 500 ml air-tight glass vessels at 30^{0}C for

24 h. The CO_2 evolved from the soil was absorbed in 15 ml 0.1 M NaOH and the unconsumed base titrated with 0.1 M HCl following additions of $BaCl_2$. Glucose at final concentration of 10 mg/g of soil sample (Vinther et al., 2008) was used in substrate induced respiration (SIR) and the procedure followed as above. All determinations were performed in triplicates and values are reported as means with standard deviation.

2.2.2. Soil enzyme activity determination

Dehydrogenase, protease, phosphatase and glucosidase activities were estimated from soil solutions (Fig. 2.1). All the determinations were performed in triplicates and the values are reported as means with standard deviation.

Fig 2.1: *Soil enzyme activities. 1: dehydrogenase; 2: phosphomonoesterase; 3: protease 4: glucosidase activities. B: Blank; T: test.*



2.2.2.1. Dehydrogenase activity (DHA)

Soil dehydrogenase (EC 1.1.1.) activity was measured by reduction of soluble tetrazolium salt 2, 3, 5-triphenylotetrazolium chloride (TTC), as an artificial electron acceptor, to red coloured formazans, which were extracted and estimated colorimetrically at 546 nm (Alef, 1995) by a UV-visible spectrophotometer (UV-1601, Shimadzu Corporation, Japan). Because of light sensitivity of TTC, all the experiments were performed under diffused light. In brief, field-moist soil (5 g) was mixed with 5 ml of TTC dissolved in 100 mM Tris–HCl buffer. The tubes were sealed with air tight stoppers and incubated in dark at 30ºC for 24 h. The control sample was

incubated with 5 ml of Tris-HCl buffer without TTC. After the incubation, 40 ml of acetone was added to each tube and further incubated for 2 h in dark. The triphenyl formazan (TPF) extracted was calculated by reference to a calibration graph prepared from TPF standards. The DHA is expressed as µg of triphenyl tetrazolium formazan (TPF) g^{-1} 24 h^{-1} oven dried soil. Glucose at a final concentration of 10 mg g^{-1} of sample was used in substrate induced dehydrogenase (SI-DHA) and same procedure was followed.

2.2.2.2. Protease activity (PSA)

Soil protease (EC 3.4.2.21-24) activity was determined by using the method given by Ladd and Butler (1972) with sodium caseinate as a substrate. The amount of tyrosine released was calculated by reference to a calibration graph prepared from tyrosine standards. Field moist sieved soil (1 g) was incubated with 5 ml of 50 mM Tris (hydrox methyl amino methane) buffer (pH 8.1) and 5 ml of 2 % Na-caseinate at 50°C for 2 h on a shaking water bath; enzyme action was then stopped by the addition of 5 ml trichloroacetic acid (15%). Centrifuged the resulting soil suspension at 6000 x *g*. The aromatic amino acids released in the supernatant were measured colorimetrically using Folin-Ciocalteu reagent (33%) at 700 nm (UV-1601, Shimadzu Corporation, Japan), with tyrosine as standard. Absorbance was measured several times until the measured value become constant. Protease activity is expressed as µg of tyrosine g^{-1} $2h^{-1}$ oven dried soil (Alef and Nannipieri, 1995a).

2.2.2.3. β-D-glucosidase activity (GSA)

The method was based on the estimation of released *p*-nitrophenol after the incubation of soil with *p*-nitrophenyl glucoside for 1 h at 37^0C (Eivazi and Tabatabi. 1988; Alef and Nannipieri, 1995b). Soil *β*-D-glucosidase (EC 3.2.1.21) activities were determined by incubating 1g of moist sieved of fresh soil with 2 ml of substrate *p*-nitrophenyl *β*-D-glucopyranoside, 0.25 ml toluene and 4 ml of 0.1M maleate buffer (pH 6) at 37^0C. After 1 h, 0.5 ml of 0.5 M $CaCl_2$, 2 ml of 0.5 M NaOH and 5 ml of distilled water (D/W) were added, samples were filtered (Whatman, 42), and *p*-nitrophenol in the filtrate was measured spectrophotometrically at 398 nm (UV-1601, Shimadzu Corporation, Japan). Units for GSA are expressed as µg of *p*-nitrophenol g^{-1} h^{-1} oven dried soil.

2.2.2.4. Phosphomonoesterase activity (APA)

The method is based on the estimation of *p*-nitrophenol released after incubation of soil with substrate *p*-nitrophenyl-phosphate (PNPP) (Tabatabai and Bremner. 1969; Alef et al., 1995). In the thesis, the term phosphatase has been used as an alternative for phospmonoestearse. Alkaline/Acidic phosphatase (EC 3.1.3.1) activity was determined by incubating 1 g of moist sieved fresh soil with 1 ml PNPP (15 mM), 0.25 ml toluene and 4 ml of modified universal maleate buffer (pH 11 for assay of alkaline phosphatase and pH 6 for assay of acidic phosphatase activity for 1 h at 37^0C). After the incubation, 1 ml of 0.5 M $CaCl_2$, 4 ml of 0.5 M NaOH and 5 ml of D/W were added, samples were filtered (Whatman, 42), and *p*-nitrophenol in the filtrate was measured spectrophotometrically at 398 nm (UV-1601, Shimadzu Corporation, Japan). Units for APA are expressed as µg p-nitrophenol g^{-1} oven dried soil h^{-1}.

2.3. Soil microbiological analysis

2.3.1. Soil microbial biomass carbon estimation

Soil microbial biomass C (C_{mic}) was determined by the chloroform fumigation extraction combined with dichromate digestion procedure (Vance et al., 1987; Tate et al., 1988). Fresh soil sample of 10 g was exposed to alcohol-free chloroform ($CHCl_3$) vapor in a vacuum desiccator containing soda-lime at 25^0C for 48 h. The fumigated soil was transferred in to an empty desiccator and residual $CHCl_3$ was removed from the fumigated soils by repeated evacuations. The fumigated soil was extracted immediately following $CHCl_3$ removal by shaking for 30 min with 50 ml 0.5 M K_2SO_4. The un-fumigated 10 g soil (oven dry weight) was extracted at the time of fumigation commencement. C_{mic} was estimated with $K_2Cr_2O_7$ oxidation method by following formula.

$$C_{mic} = (C_{fum}-C_{nf})/k_{ec}$$

where C_{fum} is the C extracted from the fumigated sample, C_{nf} the C extracted from the non-fumigated sample and k_{ec} is the conversion factor. The competence of C_{mic} extraction is generally accommodated by the k_{ec} factor. The k_{ec} value for soils collected from depths greater than 40 cm is 0.2 (Dictor et al., 1998), and for

agricultural soils is 0.45 (Wu et al., 1990) and for all other soils, the value is 0.38 (Vance et al., 1987). All determinations were performed in triplicates and values are reported as means with standard deviation.

2.3.2. Isolation and enumeration of heterotrophic bacteria

Total viable heterotrophic bacteria of soils were enumerated on R_2A agar (Edenborn and Sexstone, 2007). The composition (g L^{-1}) for R2A agar is as follows Proteose peptone, 0.5; Casamino acids, 0.5; Yeast extract, 0.5; Dextrose, 0.5; Soluble starch 0.5; Dipotassium phosphate, 0.3; Magnesium sulfate $7H_2O$, 0.05; Sodium pyruvate, 0.3; Agar, 15, pH 7.2±0.2. In brief, 10 g of moist sieved (4 mm) soil was homogenized in 90 ml of N-saline (0.85% NaCl [wt/vol]) and serially diluted (10 folds) in the same. Aliquots 100 µl were spread on triplicate plates of R_2A agar (HiMedia, HiMedia Laboratories, Mumbai), incubated at 30^0C for 2 d and colonies were counted.

2.3.3. Ecophysiological indices and specific enzymatic quotients (SEQ)

In order to evaluate the soil microbial ecophysiology, the ratios between microbiological parameters have been used in the present investigation (Table 2.1). Ecophysiological indices such as microbial quotient (C_{mic}/C_{org}) and metabolic quotient (qCO_2) were measured (Moscatelli et al., 2005). Microbial quotient (C_{mic}/C_{org}) was calculated by expressing C_{mic} as a percentage of total soil C_{org} (Anderson and Domsch, 1989). The microbial q-CO_2 (Wang et al., 2010) was determined by dividing basal respiration (mg CO_2–C g^{-1} dry soil h^{-1}) by microbial C (g C_{mic} g^{-1} dry soil). For evaluating the significance of microbial populations and microbial activity in the cycling of elements, specific enzymatic quotients (SEQ) were determined in the present study. SEQ for dehydrogenase, alkaline phosphatase, protease and β-glucosidase were calculated by dividing enzymatic activities per unit time by C_{mic} (enzyme activity g^{-1} soil per unit time µg C_{mic} g^{-1} dry soil). All determinations of enzymatic activities were performed in triplicate and values are reported as means with standard deviation

Table 2.1: *Different ecophysiological indices and their respective units expressed in the present study.*

Ecophysiological indices	Units for measurement
Microbial quotient (C_{mic}/C_{org}))	%
metabolic quotient (q-CO_2)	µg CO_2-C mg C_{mic}^{-1} $24h^{-1}$
SEQ for dehydrogenase	µg TPF $24h^{-1}$ µg C_{mic} g^{-1} dwt soil
SEQ for phosphatase	µg PNP h^{-1} µg C_{mic} g^{-1} dwt soil
SEQ for protease	µg Tyrosine $2h^{-1}$ µg C_{mic} g^{-1} dwt soil
SEQ for *β*-glucosidase	µg PNP h^{-1} µg C_{mic} g^{-1} dwt soil

2.4. Molecular analysis

2.4.1. Molecular phylogeny of bacterial isolates by 16S rRNA gene analysis

2.4.1.1. Genomic DNA extraction

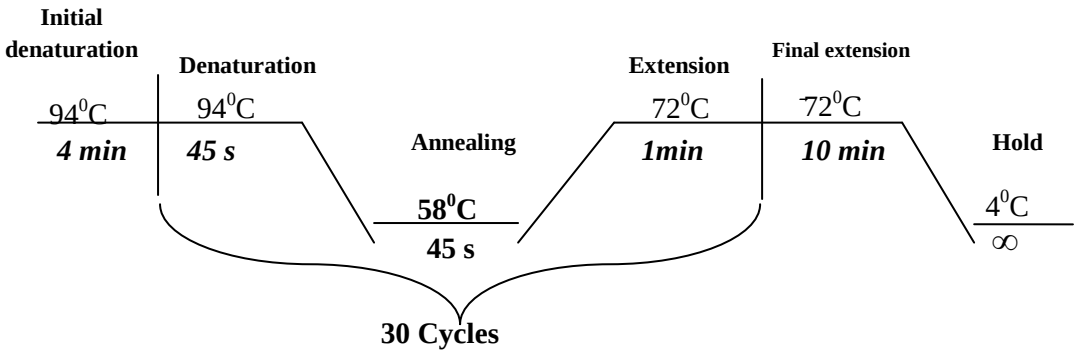
Genomic DNA was extracted from bacterial isolates by CTAB-method (Sambrook and Russel, 2001). In brief, 1 ml of the overnight grown culture, 30 µl of 10 % SDS, 3 µl of Proteinase K (20 mg/ml) was added, mixed, incubated at 37 ^{0}C. After 1h 100 µl 5M NaCl was added followed by 80 µl 2X CTAB, mixed well and incubated at 65^{0}C for 20 min. After incubation supernatant was collected by centrifugation at 13,000 *x g* for 5 min and supernatant was extracted once with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) mixture and once with chloroform: isoamyl alcohol (24:1). Two volumes of ice cold absolute alcohol was added to supernatant, DNA pellet was collected by centrifugation at 13,000 x *g* for 5 min, washed with 70 % alcohol, air dried, dissolved in double D/W. Extracted DNA samples were electrophoresed to check the quality and approximate quantity of the DNA by using 0.8% agarose gels stained with ethidium bromide (0.5 µg/ml). The gels were cast and run in TAE buffer (Sambrook and Russel, 2001)

2.4.1.2. 16S rRNA PCR amplification

PCR amplifications of the variable regions of the 16S rRNA genes using universal eubacterial primers (Rajendran et al., 2008) was carried out with forward primers 27F corresponding primer to positions 9-27 (5^{l}-CGAATTCAGAGTTTGATCCTGGCT CAG-3^{l}) and reverse primer 1107R corresponded to position 1107 (5^{l}- GCT CGT TGC

GGG ACT TAA CC-3[l]). 16S rRNA PCR products were evaluated for their product size of 1080 bp on 1% agarose gels impregnated with ethidium bromide (0.5 µg/ml).

Conditions for PCR amplifications are as follows



Ingredients for PCR reaction mixture is shown in the following Table 2.2.

Table 2.2: *Components for 16S rRNA PCR reaction mixture*

Ingredient	**Quantity in µl**
10X Taq DNA polymerase buffer (with 2.5 mM $MgCl_2$)	5
dNTPs mix (10 mM)	1
Forward primer (27F, 2 mM)	1
Reverse primer (1107R, 2mM)	1
Template DNA (approx. 50 ng/ µl)	1
Taq polymerase (3U/µl)	0.5
Millipore Sterilized Water	40.5
Total volume	**50µl**

2.4.1.3. Culturable bacterial diversity by Amplified rDNA Restriction Analysis (ARDRA)

Cultural bacterial diversity of soils was established by ARDRA analysis (Shukla et al., 2007; Patel et al., 2010). Amplified 16S rDNA products were directly subjected to restriction digestion for comparative ARDRA. Restriction enzyme digestion (RE) was carried out using a tetra cutter *Hinf* I restriction endonuclease. Reaction mixture for RE was summarized in the Table 2.3. The RE digests were electrophoresed on 2.5% (w/v) agarose gel to obtain well-resolved bands at 60 V for 2.5 h. Gels were stained

with ethidium bromide and visualized under UV transilluminator and photographed with gel documentation system (Alpha Innotech, CA). Data were computed with NTSYS-pc 2.02 version (Exeter Software) by unweighted paired group mean average (UPGMA) clustering using SAHN subroutine of NTSYS pc 2.02 programme. A binary scoring system (1 for the presence of the band and 0 for the absence) was used to generate an input matrix, which was analyzed using the UPGMA clustering and subsequently dendograms were generated.

Table 2.3: *Reaction mixture of RE for ARDRA*

Ingredient	**Quantity in µl**
10x restriction enzyme buffer-C	3
10X BSA	3
16S rDNA amplicons 500-700ng equivalent	15-20
Restriction endonuclease (*Hinf* I)	0.5
Total volume made by using sterile D/W	30

2.4.1.4. 16S rRNA sequencing and phylogenetic analysis

Amplified products of 16S rRNA gene were sequenced by using the commercial sequencing service provided by Bangalore Genei (Genei^{TM}, Merck India Pvt. Ltd., Bangalore, India). The best read from the chromatogram was converted to FASTA format and NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast/) analysis as well as RDP-II project database SeqMatch (http://rdp.cme.msu.edu/seqmatch/) were performed. Multiple sequences alignment was carried out in RDP-II project by taking appropriate database sequences and a phylogenetic tree was constructed for the isolates using Neighbor joining method in MEGA 5.0 programme (Tamura et al. 2011). The evolutionary history was inferred using unweighted paired group mean average (UPGMA) method. 16S rRNA sequences obtained from the present study were submitted to GenBank and the details of the accession numbers are given in the respective Chapters.

2.5. Denaturant gradient gel electrophoresis analysis (DGGE)

2.5.1. Soil community DNA extraction

Community DNA was extracted directly from soil samples by a modified protocol of Zhou et al. (1996). The reagents and chemicals used in the procedure as follows

Reagents and chemicals used:

A: Extraction buffer

100 mM Tris-Cl (pH 8.0)

100 mM sodium EDTA (pH 8.0)

100 mM sodium phosphate (pH 8.0)

1.5 M NaCl

B: Polyethylene glycol (PEG) solution

29% PEG-6000 IN 1.5M NaCl

C: 2X CTAB solution

2% CTAB

1.4 M NaCl

0.1M EDTA

Soil 0.5 g was mixed with 1.3 ml extraction buffer, 10 µl protinase-K (10 mg ml^{-1}) and 10 ul lysozyme (500 µg/ml) in 2 ml sterilized microcentrifuge tubes. The tubes were vortexed for 10 s and allowed to incubate horizontally on shaker at 200 rpm for 30 min at 37^0C. After incubation, 150 µl SDS (20% w/v) was added and the contents were mixed by vortexing. Bacterial cells were lysed for 2 h by incubating tubes at 65^0C with occasional tapping at every 20 min. The lysed cell content was obtained by centrifuge the tubes at 5000 x *g* for 10 min (Eppendorf 5415R, Chennai). DNA from remaining soil pellet was extracted twice by adding 0.45 ml extraction buffer and 50 µl SDS solution. Supernatants were collected in fresh tubes and cooled by keeping tubes at -20^0C for 20 min. The tubes were again centrifuged at 12,500 x *g* for 10 min at 4^0C. The supernatants were collected in fresh tubes and 10 µl potassium acetate (5 M) , pH 5.2 and 600 µl of PEG were added, the content were mixed and tubes were incubated at -20^0C for 30 min after that, the tubes were centrifuged at 12,500 x *g* for 15 min at 4^0C. The supernatants were discarded and 900 µl of 2X

CTAB was added with gentle tapping of tubes and incubated for 10 min at 68^0C. After that, 900 µl of chloroform was added and the tubes were centrifuged at 12,000 x *g* for 10 min. The supernatant was taken in a fresh tube and 1ml of isopropyl alcohol was added and then the tubes were kept at -20^0C for 20 min. After the end of the incubation, tubes were again centrifuged for 12000 x *g* for 10 min. The pellet was washed with 450 µl ammonium acetate (2.5M) and chilled absolute alcohol and then centrifuged 12000 x *g* for 10 min. The supernatant was discarded and the obtained pellet was washed twice with 70% alcohol by centrifugation. The pellet was air dried and resuspended in 50 µl autoclaved D.W and the DNA was dissolved at 65^0C for 10 min. The extracted DNA was evaluated on a 1% agarose gel containing 0.5 µg/ ml ethidium bromide EtBr. The gels were visualized under Uv light in trans-illuminator and photographed.

2.5.2. 16S rRNA PCR amplification for DGGE analysis

Universal eubacterial primers 341F (5^1-CCTACG GGAGGCAGCAG-3^1) and 534R (5^1-ATTACCGCGGCTGCT GG-3^1) targeted for V3 region of 16S rRNA corresponds to the positions 341 and 534 in *E. coli* (Muyzer et al., 1993) were chosen for DGGE analysis (Muyzer and Smalla, 1998). A 40 bases GC-clamp (Muyzer, 1999; Kowalchuk et al., 2002; Dar et al., 2005) was added to forwarded primer (GC-341F: 5^1CGCCCGCCGCGGCGGCGGGCGGGGCGGGGGCACGGGGGCCTACGGGA GGCAGCAG3^1). Components for PCR reaction are shown in the Table 2.4.

Table 2.4: *Components of 16S rRNA PCR reaction mixture for DGGE analysis*

Reactants	**Quantity in µl**
10x Taq DNA polymerase buffer (with 2.5 mM $MgCl_2$)	5
dNTPSs mix (10 mM)	1
Forward primer (GC-341-F, 2 mM)	1
Reverse primer (534R, 2 mM)	1
Template DNA (10-40 ng/µl)	1
Taq polymerase (3U/µl)	0.5
1x BSA (10 mg/ml stock)	1
Millipore Sterilized Water	39.5
Total volume	**50 µl**

High concentrations of primers are used here to prevent heteroduplex formation during PCR, which can result in false bands in DGGE analysis (Crump et al., 2003). Touchdown PCR cycling conditions for amplifying 16S rRNA were 95^0C for 15 min, followed by 20 cycles of 94^0C for 1 min, 65^0C (anneal temperature reduced by 0.5^0C each cycle) for 45 sec and 72^0C for 1 min; 15 cycles of 94^0C for 1 min, 55^0C for 45 s and 72^0C for 1 min followed by final extension 72^0C for 10 min.

2.5.3. DGGE

The D-code universal mutation detection system is used for DGGE (Fig. 2.2, Bio-Rad laboratories Ltd, UK) analysis. A 0% stacking gel was poured over each denaturant gel to ensure comb wells contained no denaturant material. Gels were allowed to polymerize for at least 4 h. 16S rRNA PCR amplified samples (about 200 ng) were loaded on to 8% polyacrylamide-bisacrylamide gels (37.5:1) with denaturing gradient from 40 to 60% where 100% denaturant was 7 M urea and 40% (vol/vol) deionized formamide with 1x TAE as electrophoresis buffer. Electrophoresis conditions were 60 V for 16 h at constant 60^0C. Gels were stained with 0.5 mg/ml silver nitrate and visualized (Radojkovic and Kusic, 2000).

Fig. 2.2: *DGGE Apparatus*

2.5.3.1. Silver staining protocol

DGGE gels were removed carefully from glass plates and rinsed with D/W for two times. Gels were kept in 10% methanol for 12 min. Just enough 0.7% nitric acid was added and incubated for 6 min under shaking. Repeated the water wash steps 2 times and then added just enough 0.2% silver nitrate and allowed to keep for 30 min under shaking. This step was performed under dark condition. After 30 min gels were washed with D/W for 2 times and then added 150 ml developer (2.29 g sodium carbonate + 150 µl formaldehyde + 2 ml of 0.2% sodium thiosulfate and made up to 100 ml with D/W) and shaked till the bands appear clear. In order to prevent appearance of high back ground, 50 ml of acetic acid was added to stop the developing reaction at the end.

2.5.4. Analysis of DGGE band patterns

DGGE gels were scanned by Alpha imager® HP gel documentation system (Alpha Innotech, CA). Band patterns were analyzed with AlphaEase FC™ software (version 4.0, Alpha Innotech, CA). Diversity indices such as Shannon-Weaver index (*H*) and evenness (*E*) were used to calculate 16S rRNA band pattern diversity on the following equations as described by Shen et al. (2008)

$$H = -\sum_{i=1}^{S} p_i \ln p_i = -\sum_{i=1}^{S} (N_i/N) \ln (N_i/N)$$

$$E = H/H_{max} = H/\ln S$$

where N_i is the abundance of the i^{th} ribotype (height of the i^{th} band peak), N is the total abundance of all ribotypes in the sample (sum of total heights of all band peaks in a lane of DGGE gel) and S is the number of ribotype (total number of bands in DGGE gel lane).

The species richness and border carrying capacity of the soils for bacterial species were determined by range weighted richness index (*Rr*) following by the equation as described by Marzorati et al. (2008).

$$Rr = (N^2 \times Dg)$$

Where, N represents the total number of bands in the DGGE pattern, where as Dg represents the denaturing gradient comprised between the first and the last band of the pattern.

CHAPTER 3

Establishing a link between soil characteristics and microbial activity processes in semiarid Mahi River basin by polyphasic approach

3.1. Introduction

Microbial activity in floodplains can control the ecosystem and metabolism of rivers (Fischer and Pusch, 2001; Fischer et al., 2003) and is significantly influenced by variability in hydrologic regimes, flow dynamics, sediment movement (Baldwin and Mitcheel, 2000; Fischer et al., 2003) and chemical pollutants (Onweremadu and Nwufo, 2009). Microbiology of the floodplains has received a lot of attention (Baldwin and Mitcheel, 2000; Gergel et al., 2002; Kang and Stanly, 2005; Klebercz et al., 2012). Investigations on the sediments of river channels have shown that the microbial activity is controlled by sediment structure, hydraulic conditions and availability of organic carbon and nitrogen (Fischer et al., 2005). Bacteria were found to be dominant components of flood plain soils. In general, microorganisms were more abundant and diverse, and distributed more evenly in the oxic layers than the anoxic layers of flood plains (Song et al., 2008). Gram-positive bacteria consisted >16% of the community in food plain soils and their abundance increased with increasing soil depth (Song et al., 2008). Results indicate that the soil microbial activity-changes in flood plains were attributed to modified hydrology regimes, (which caused differences in water availability), soil chemistry, and in substrate quality (Kang and Stanly, 2005). Carbon, nitrogen, and fine grain sediment contents are positively correlated with sediment microbial community in polluted riverine sediments (Bushaw-Newton et al., 2011)..

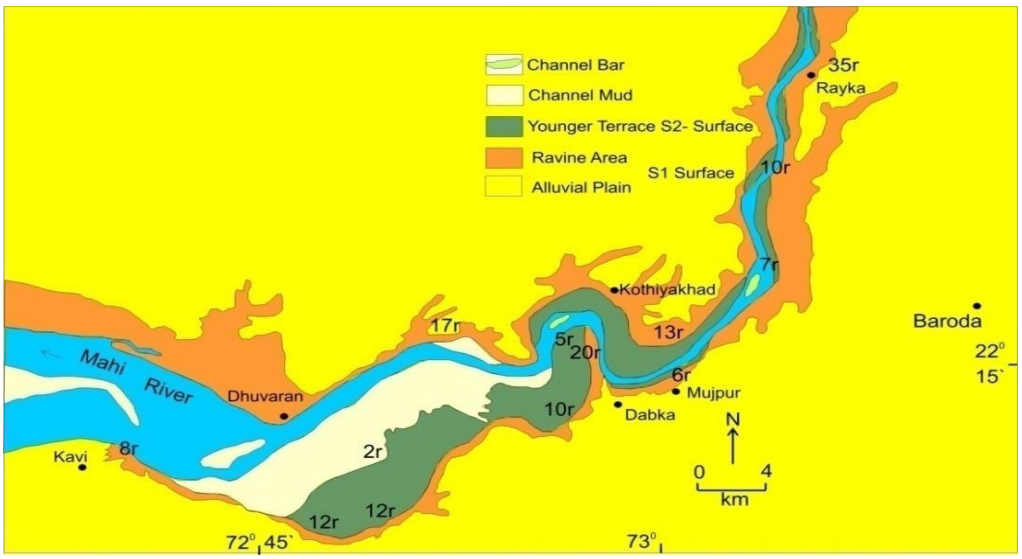
Soil enzymes catalyze biochemical reactions and are an integral part of nutrient cycling in the soil (Nannipieri et al., 2002). Soil enzymes are believed to be derived primarily from soil microbes and are considered to be good indicative measures for soil fertility and quality (Zahir et al., 2001, Carina et al., 2012; Han et al., 2012; Vinhal-Freitas, 2013). Soil enzymes participate in elemental cycling and decomposition of organic residues (Caldwell, 2005). In general, the activities of enzymes can be used to assess the abundance and metabolic activity of soil microbes (Tabatabai, 1994, Caldwell, 2005; Chodak and Niklińska, 2012).

Palaeosols (fossil soils) are preserved throughout the geologic record and provide signatures of past climatic and environmental changes (Sheldon and Tabor, 2009) as well as landscape evolution including allogenic processes (Kraus, 199; Zech et al., 2000; Buynevich and Bitinas, 2007). Several studies pertaining to palaeosols from

various parts of the Indian sub-continent have been carried out (Khadkikar et al., 2000; Achyuthan 2003 and 2004). However, studies on microbial activity and biogeochemical cycling which control ecosystem functions and shapes the landscape are rare, especially from the Indian subcontinent, though elsewhere such studies have been dealt with in great detail (Kieft et al., 1996; Brockman et al., 1998; Khomutova et al., 2006; Demkin et al., 2008; Demkina et al., 2000, 2007and 2008). It has been shown that the microbial species active at the time of the formation of palaeosols have survived till date (Demkina et al., 2008). The microbial DNA extracted from the palaeosol correlates strongly (r^2 = 0.97) with the total microbial biomass (Blagodatskaya et al., 2003). The microbial communities have also been used to infer paleoclimate for the last 5 ka (Demkina et al., 2000; Khomutova et al., 2006). Most of the palaeosol microbial studies have been restricted to total microbial biomass and active microbial biomass measurements (Demkina et al., 2000; 2007, 2008; Khomutova et al., 2006) however microbial ecology in relation to paleoenvironments is not yet fully understood. Very little is known about microbial diversity and the biogeochemical cycling of elements of the palaeosols.

Although microbial activity of river channels, streams, sediments and hyporheic zone are well studied, microbial activity and their critical determinants in the alluvial plains is inadequately understood. Studies aimed at overall understanding of alluvial soil quality will enable us to manage soil resources to ensure sustainable agriculture. Realizing this, the present study was initiated to investigate microbial parameters and their critical decisive determinants in the semiarid soils and sediments of alluvial plains of Mahi River basin in western India. Mahi River originates near Moripara in Dhar district of Madhya Pradesh and flows through the Precambrian Aravalli mountain range (in Rajasthan) and the vast Gujarat alluvial plains and merges in the Gulf of Cambay. The Mahi River basin falls under semiarid climatic zone, a major climatic zone of India comprising 37% of the total geographical area of the country and the alluvial soils are under intense weathering as well as erosion which are significant processes in landscape evolution. Generalized geomorphic view of the Mahi River is shown with Fig. 3.1.

Fig: 3.1. *Gemorphological setting of Mahi River, Gujarat, western India*



In earlier studies, the Mahi River basin has been investigated for understanding Quaternary environmental and tectonic changes and their implications on the fluvial systems and landscape of the dry lands of Gujarat (Merh and Chamyal, 1997; Chamyal et al., 2003). Nevertheless, microbial activity of these dry lands and fluvial sediments are not yet characterized. The significance of the exposed sedimentary records in the cliffy banks of the Mahi River has been and used for reconstruction of Quaternary continental stratigraphy and understanding the geomorphic evolution of the Mahi River basin (Juyal et al., 2000; Chamyal et al., 2003; Sridhar, 2007). At estuarine area, this river receives both metal and organic contaminants from industrial effluent. Since microbial activity is linked with several ecosystem processes including soil formation, organic matter transformation, soil fertility and nutrient cycling, it is important to understand the factors that control microbial distribution and activities in river alluvial soils and sediments. In addition to this, stratified palaeosol (fossil soils) sequences are noticed in Mahi river basin.

The present study was initiated to address following aspects

- **Soil microbial activity in alluvial estuarine soils of Mahi River basin**
- **Microbial activity and diversity in the late Pleistocene palaeosols**
- **Microbial activity and diversity in the exposed shallow subsurface soils and sediments: Correlations between soil microbial and physiochemical variations**

3.2. Soil microbial activity in alluvial estuarine soils of Mahi River basin

3.2.1. Materials and methods

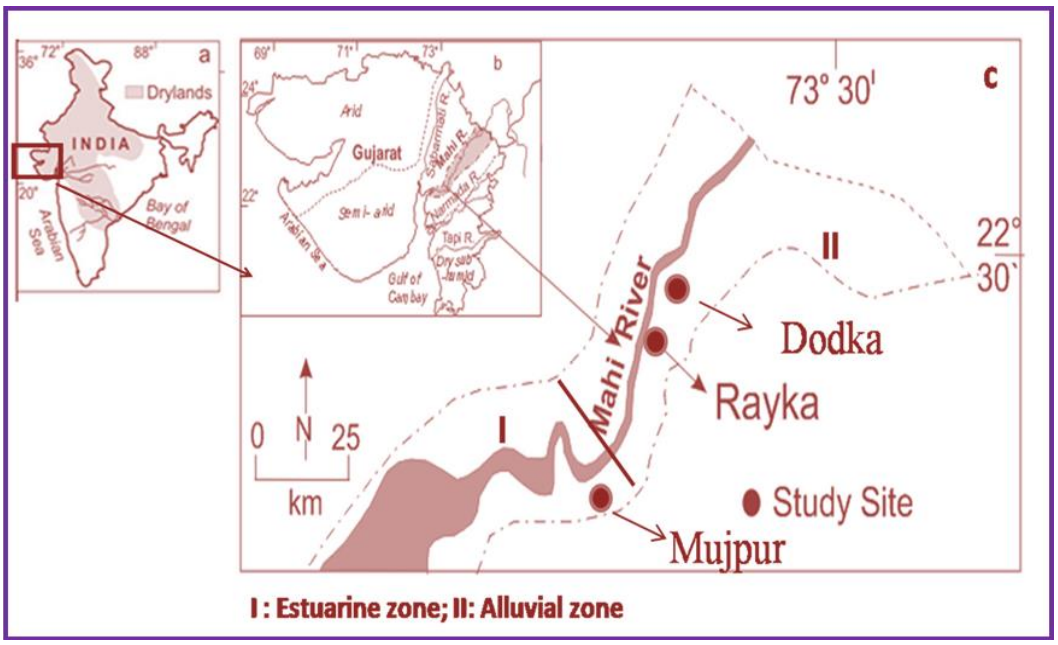
3.2.1.1. Study sites description and sample collection

The sites studied are located in the semiarid Mahi River basin, Western India (Fig. 3.2), with mean annual rainfall about 600 to 650 mm spanning three locations Rayka, Dodka and Mujpur. The alluvial zone of the Mahi River basin at Rayka (22° 26' 56.59"N, 73° 05' 16.95"E) comprises distinct geomorphic units and alluvial deposits of Pleistocene and Holocene age. River channel on the convex banks is bordered by cliffs rising about 30–40 m from the river level. The sediment and soil sequences along these cliffs have been dated back to 125 ka (Juyal et al., 2000). At Rayka, the sediments comprising these cliffs are deeply incised inland forming ravines exposing sediment sequences. The alluvial zone of the Mahi River basin at Dodka (22° 28' 54.0"N, 73° 06' 5.67"E) lying upstream of Rayka. The sediment sequence at Dodka is capped by aeolian deposits which show intense weathering. Significant soil erosion is also observed in Dodka section. In both Dodka and Rayka, landscape is covered with *Calotrophis procera, Zyzyphus nummulariea, Acasia,* and grass like *Heterophogan , Setaria* sp, *Cymbopogon* sp, *Digitaria* sp*, and Brachiaria* sp. Mujpur section lies at estuarine zone located downstream of Rayka and is made up of sediments of mid to late Holocene forming a younger terraced surface.

A total of 33 triplicate composite samples at an interval of 25 cm from the three exposed sections at Dodka, Rayka and Mujpur were collected for the present study. Due to lot of heterogeneity at alluvial sites and varied stratigraphy, more number of samples (twenty seven) were collected from alluvial zone. However, only 6 samples were collected from the estuarine zone of Mahi River basin because of low heterogeneity. All the sites selected were unexposed to anthropogenic activities. Prior to the collection of soil samples, the sites were cleaned by scraping the surface layer. Due to difference in geomorphic settings, at Rayka 5 different sites (each harboring distinct soil sequences) such as Rayka ravine soil (RR), Rayka ravine red soil (RRR), Rayka aeolian soil (RW), Rayka surface soil (RS) and Rayka surface red soil (RD) were selected and a total of 22 samples were collected. These 5 sites represent the overall soil sequences developed over river born sediments along the bank of Mahi

River at Rayka. Eleven samples were collected from 3 sites (RR, RRR, and RW) at the Rayka section exposed along the Mahi River bank and remaining eleven samples were collected from other 2 sites (RS and RD) at a section exposed landward in a deeply incised ravine. Five samples were collected from Dodka section (D) and six samples from Mujpur (M) located in the estuarine zone of Mahi River.

Fig. 3.2: *Location map of the study area showing the study sites and generalized geomorphic and climatic zones. (a) Distribution of dry lands in India (b) Major climatic zones and rivers of Gujarat and (c) Geomorphic zones in the Mahi River basin (after Rachana et al., 1998). Sites sampled are marked with filled circles.*



Due to distinct stratified soil successions that occurred in the exposed sites, the different thickness of the soil profiles and the material was available at different depths; therefore the gradient in depth was not same for all the samples. For example, at RW site which is a soil profile developed over wind-borne material of above 75cm thickness, the samples were collected up to a depth of 75 cm. Same case was observed in RRR but here the profile has been developed over a river-borne sediment layer measuring 125cm. No distinct pedon was noticed below the 125cm (D5) in the Dodka site which is comprised of loose fluvial sediments. Autoclaved polyethylene bags were used for sampling which were immediately kept on ice packs before being

transported to the lab. All the samples were sieved (< 4 mm), cleaned of visible roots and plant residues, and stored at 4^0C.

3.2.1.2. Determination of soil parameters

Soil samples were analyzed for soil moisture content (SMC), pH, soil texture and soil organic matter (SOM) according to the protocols described in Chapter 2. All the determinations were performed in triplicate, and values reported are mean with standard deviation.

3.2.1.3. Determination of soil enzymes

Soil dehydrogenase and protease activities were estimated as decribed in Chapter 2. All determinations of enzymatic activity were performed in triplicate and values are reported as means with standard deviation. Specific enzyme activities were determined by dividing enzyme activities with unit total organic carbon (TOC) and the results were expressed as TPF (µg) dwt g^{-1} C $24h^{-1}$ for specific dehydrogenase activity, Tyrosine (µg) dwt g^{-1} C $2h^{-1}$ for specific protease activity.

3.2.1.4. Statistical analyses

The results obtained were examined statistically for linear regression, and Pearson Product Moment Correlation. For identification of meaningful variables in the studied samples, Principal component analysis (PCA) was carried out by using XLSTAT version 2009.6.01 (trade mark of Adinosoft)

3.2.2. Results

3.2.2.1. Soil parameters

Characteristics of soil samples taken from 3 locations are summarized in Table 3.1. All the samples were neutral to slight alkaline in nature with a pH ranging from 6 to 7.6. Relatively, Mujpur soils were characterized by high amount of SOM (1.7 to 3.7 %), SMC (5 to 22 %) and clay (36.4 to 40.8 %) in comparison to the other two sites (Table 3.1). This may be due to geographical location of Mujpur section which is near the estuarine zone of Gulf of Cambay where frequent back waters inundate during high tides. All the Rayka samples are loam in texture, where as Dodka and Mujapur soils were silty loam and clay loam respectively.

Table 3.1: *Properties of soils used in this study. RR, RRR, RW, RS and RD are samples series collected at Rayka section. D and M are soils taken at Dodka and Mujpur respectively. Rayka and Dodka samples are obtained from.*

Sample	Soil Depth (cm)	pH	SOM (%)	SMC (%)	Sand (%)	Silt (%)	Clay (%)	Texture
Rayka								
RR1	0-25	7.4	1.9	5.0	33.3	46.6	20.8	loam
RR2	25-50	7.1	1.7	5.0	31.1	47.1	21.8	loam
RR3	50-75	7.0	1.4	5.0	46.6	40.0	13.4	loam
RR4	75-100	6.9	1.25	5.0	42.6	45.3	12.1	loam
RR5	100-125	7.0	1.2	4.0	46.6	40.6	12.8	loam
RRR1	0-25	7.0	3.6	6.0	45.3	41.3	13.4	loam
RRR2	25-50	7.5	2.2	5.0	47.3	41.3	11.4	loam
RRR3	50-75	7.3	2.3	5.0	45.0	42.6	11.4	loam
RW1	0-25	6.0	2.5	5.0	42.6	43.3	14.1	loam
RW2	25-50	6.8	2.9	6.0	45.2	41.3	13.7	loam
RW3	50-75	6.9	1.8	5.0	44.6	45.3	10.1	loam
RS1	0-25	6.9	1.43	4.0	42.0	45.3	12.1	loam
RS2	25-50	7.1	1.4	4.0	44.6	41.3	14.1	Loam
RS3	50-75	7.3	1.2	4.0	44.6	42.6	12.8	Loam
RS4	75-100	6.9	1.4	4.0	46.0	42.0	12.0	Loam
RS5	100-125	7.2	0.93	5.0	46.0	42.6	11.4	Loam
RS6	125-150	7.2	0.4	4.0	47.3	41.3	11.4	Loam
RD1	0-25	7.4	2.0	6.0	41.3	45.3	13.4	Loam
RD2	25-50	7.5	2.0	6.0	42.4	42.0	15.4	loam
RD3	50-75	7.3	2.63	7.0	41.3	40.6	18.1	loam
RD4	75-100	7.2	2.9	6.0	45.3	40.6	14.1	loam
RD5	100-125	7.2	2.66	6.0	41.3	41.3	17.4	loam
Dodka								
D1	0-25	7.4	1.8	6	18.4	68.0	14.6	Silty loam
D2	25-50	7.4	1.2	5	20.0	73.3	12.5	Silty loam
D3	50-75	7.2	1.4	5	18.6	67.2	14.2	Silty loam
D4	75-100	7.5	1.5	5	19.5	67.3	14.2	Silty loam
D5	100-125	7.6	1.7	5	20.0	61.3	18.7	Silty loam
Mujpur								
M1	0-25	7.2	1.7	5.0	22.6	40.0	36.4	Clay loam
M2	25-50	7.2	2.3	6.0	21.3	42.6	36.1	Clay loam
M3	50-75	7.2	2.3	8.0	19.3	44.0	36.7	Clay loam
M4	75-100	7.2	2.5	11.0	20.0	42.0	38.0	Clay loam
M5	100-125	7.2	3.0	20.0	20.0	40.0	40.0	Clay loam
M6	125-150	7.3	3.7	22.0	18.2	40.0	40.8	Clay loam

3.2.2.2. Soil enzymes and their correlation to soil parameters

The activities of soil enzymes (dehydrogenase and protease) and their relation to soil characters were investigated in semiarid alluvial and estuarine soils around Mahi River basin. Both the enzyme activities were relatively high in estuarine soil samples.

Dehydrogenase and protease activities of the 3 locations (Fig. 3.3A and B) were varied with the sampling site and soil properties. Both the enzyme activities are comparatively high in Mujpur site. Significant positive correlation was noticed between dehydrogenase activity (cluster of 27 samples from both Rayka and Dodka) and SOM (Fig. 3.4, r = 0.786, P <0.001, n = 27) in alluvial soils and it is very well correlated in estuarine zone (Fig. 3.4 inset, r = 0.955, P < 0.001, n = 6). Protease activity does not show any significant relation with SOM (Fig. 3.4, r = - 0.312, P > 0.11, n = 27). However, in estuarine soil samples, a positive correlation with SOM (Fig. 3.4, inset) was obtained and was significant (r = 0.98, P < 0.001, n = 6).

Positive correlation was noticed between dehydrogenase and SMC (r = 0.56, P <0.001 n = 27) in alluvial soils. No statistical relationship is seen between overall protease activity and SMC (P > 0.05) in alluvial soils, however, the positive correlation found in estuarine soils (Fig. 3.5, r = 0.94, P < 0.001, n = 6) and this is in agreement with other results indicating that the Mujpur section is microbiologically active than other two sites.

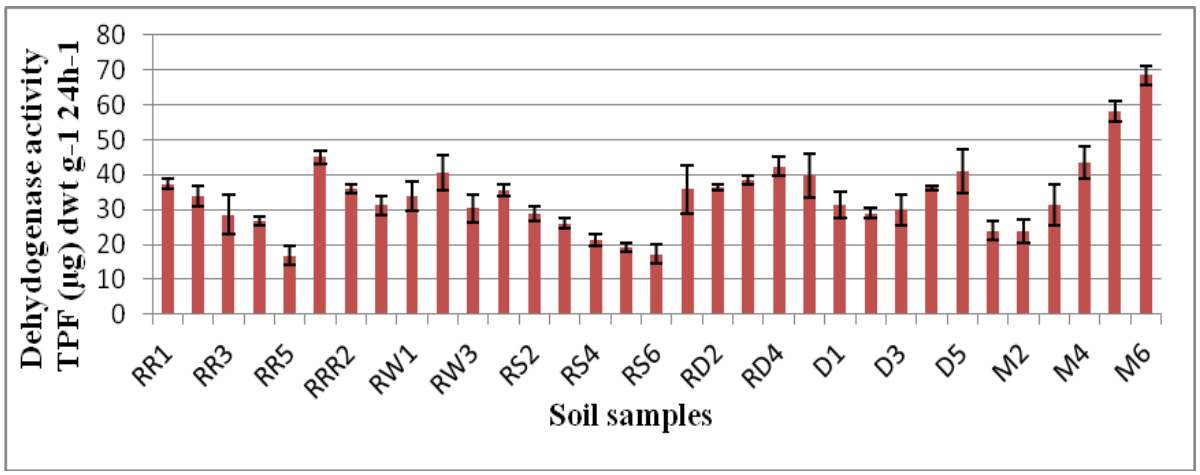
The other variables considered in this study are the sand, silt and clay content and soil depth. Since soils were collected at different depth gradients, correlations have been made for individual sites. Soil depth showed a negative correlation with dehydrogenase and protease activities (Table 3.2). The dehydrogenase activity shows a moderate positive correlation with clay content of the soils (Fig. 3.6, r = 0.52, P < 0.004, n = 27). No significant correlation was, however noticed with protease activity (r = -0.45, P < 0.177, n = 27) barring a positive correlation in Mujpur site (Fig. 3.6, r = 0.92, P < 0.001). No apparent relation was found for both enzymes with pH, sand and silt content of the soils at P level 0.05, however, a negative correlation of dehydrogenase activity and protease activity with sand proportion (r = -0.85, P < 0.02, n = 6, r = -0.94, P < 0.004, n = 6 respectively) was noticed in Mujpur section and it is site specific. No significant correlation was observed between the two enzymes (Fig. 3.7, r = -0.32, P > 0.1, n = 27) in alluvial soils. On the contrary significant positive correlation was observed between dehydrogenase and protease in estuarine zone of Mahi River (Fig. 3.7, r = 0.94, P < 0.001, n = 6).

Since the studied soils possess variable amounts of SOM, specific enzyme activity indices were calculated per unit of total C. These activity indices were correlated with

soil parameters (Table 3.3) and the results show that dehydrogenase specific activity is moderately correlated with clay content and significantly correlated with SMC and SOM in both alluvial and estuarine soils (Table 3.3). Protease specific activity in the alluvial soils does not show any correlation with soil indigenous properties however, a strong correlation was observed with SOM and clay percentage in estuarine soils.

Fig. 3.3: *Dehydrogenase and protease activities of soil samples. A: Dehydrogenase activities of alluvial (from RR1-D5) and estuarine samples (M1-M6). B: Protease activities of alluvial (from RR1-D5) and estuarine (M1-M6) samples.*

A:



B:

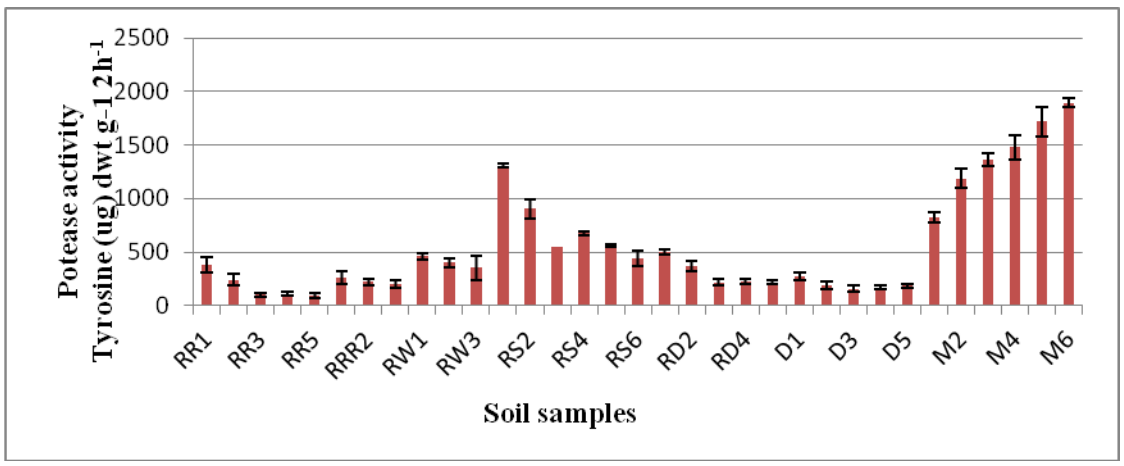
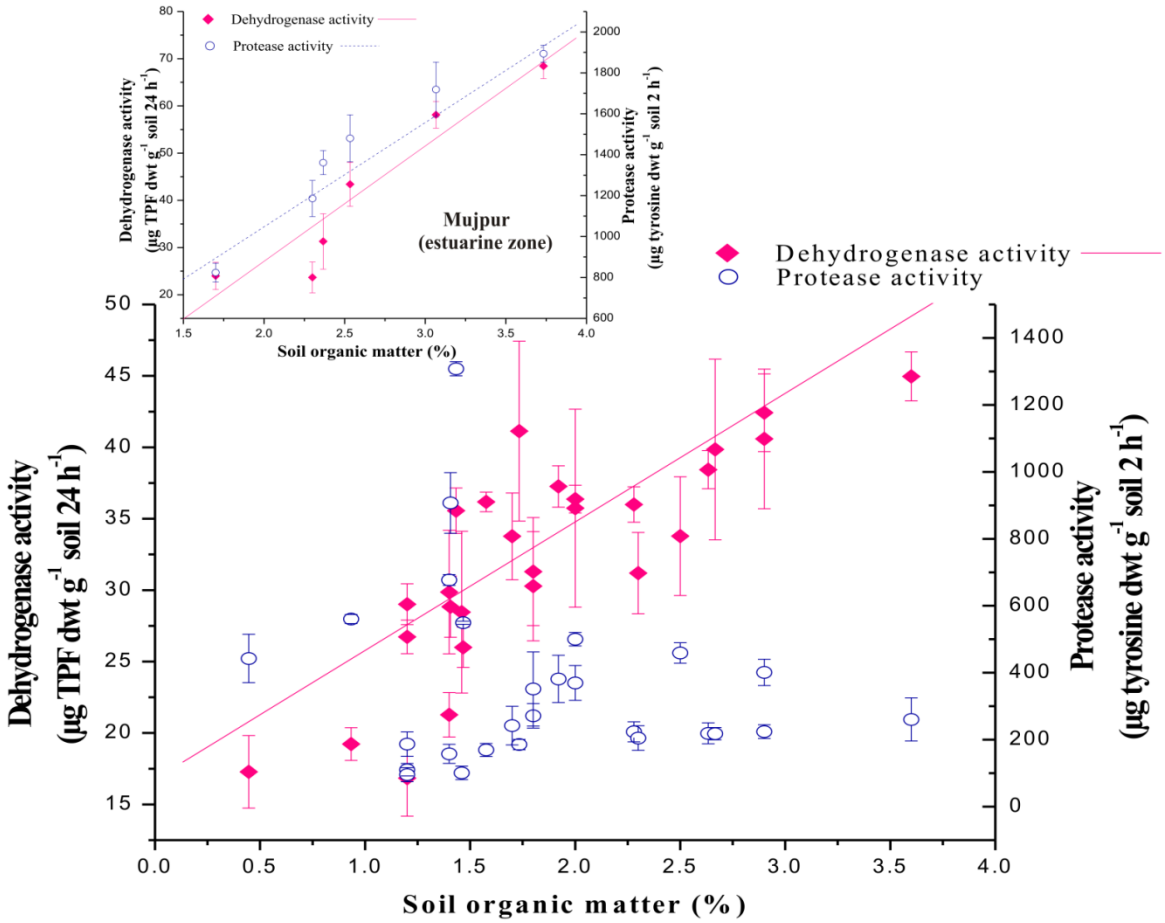


Fig. 3.4: *Correlation of dehydrogenase and protease activities with SOM in alluvial soils. Regression line shown for significant positive correlation in case of dehydrogenase activity (r = 0.786, P <0.001), whereas no correlation was noticed with protease activity. Inset shows data for estuarine (Mujpur soil) samples, where both dehydrogenase and protease activities showed significant positive correlation (r = 0.95, and r = 0.98 respectively) with SOM at P = <0.001.*



Principal component analysis (PCA) is a variable reduction method which can be used to identify groups of observed variables that tend to cluster together. Present data were subjected to PCA analysis and principal components were extracted by Scree test from both alluvial and estuarine soils (Fig. 3.8A and 7B). In both the cases, the first two components displayed Eigen values greater than 1.5. This suggested that the first two components were meaningful. Therefore, only the first two components were retained for rotation (varimax orthogonal). Combined, components 1 and 2 of alluvial soil variables accounted for 67.19 % of total variance (Fig. 3.8A) whereas combined components 1 and 2 of estuarine soil variables accounted for 90.63 % of total variance (Fig. 3.8B). PCA plots (Fig. 3.8A and 3.8B) indicates that SMC, SOM, clay, dehydrogenase of both alluvial and estuarine zone form a closer cluster and hence are correlated with each other whereas sand, silt and pH showed different

patterns and are scattered from the cluster. Strong correlation among both dehydrogenase and protease was observed in estuarine soils (Fig. 3.8B).

Table 3.2: *Pearson product moment correlation between soil enzymes and soil depth.*

Sample site	Protease activity		Dehydrogenase activity		Protease activity/C		Dehydrogenase Activity/C	
	r	***P***	**r**	***P***	**r**	***P***	**r**	***P***
RR	-0.89	0.04	-0.97	0.001	-0.61	0.266	-0.95	0.01
RRR	- 0.98	0.01	- 0.98	0.012	0.28	0.817	-0.83	0.37
RW	-0.99	0.002	- 0.34	0.07	0.93	0.239	-0.62	0.56
RS	-0.87	0.002	-0.97	0.001	0.46	0.358	-0.83	0.03
RD	-0.88	0.041	- 0.83	0.07	-0.80	0.100	0.84	0.071
D	-0.66	0.21	0.83	0.07	0.64	0.24	0.15	0.80
M	0.98	0.001	0.96	0.001	0.80	0.051	0.95	0.002

Protease activity: Tyrosine (µg) dwt g^{-1} $2h^{-1}$., dehydrogenase activity: TPF (µg) dwt g^{-1} $24h^{-1}$.,
Protease activity/C: Tyrosine (µg) dwt $g^{-1}C$ $2h^{-1}$., Dehydrogenase activity/C: TPF (µg) dwt g^{-1} C $24h^{-1}$

3.2.3. Discussion

Relatively both soil dehydrogenase and protease activities are high in Mujpur samples, indicating high microbial activity as well as high soil quality (Zahir et al., 2001) in estuarine zone. The enzyme dehydrogenase being one of the principal agent in the degradation of SOM (Tabatabai, 1994), it is inferred that SOM transformation in estuarine zone (Mujpur soils) is more than other two sites (Rayka and Dodka). Generally, protease activity depends on the distribution of proteolytic bacteria and the amount of proteinaceous substrate availability in the SOM. The results of protease activity indicate that proteolytic bacteria and the amount of proteinaceous substrates are comparatively high at Mujpur which lies in estuarine zone.

Present findings demonstrate that dehydrogenase activity is sensitive to SOM, SMC, clay and soil depth. The SOM is one of the critical determinative parameter to soil dehydrogenase because organic matter is substrate for microbial dehydrogenase activity. Gajananda (2007) demonstrated similar relationship between organic carbon and dehydrogenase ($r = 0.85$, $P < 0.001$) and stated that organic C is an important factor in controlling the development of dehydrogenase in Arctic soils. Shang et al. (2012) also found a positive relationship between dehydrogenase and organic C ($r = 0.99$, $P < 0.01$). Similar kind of observation was found by Padre et al. (2007) and Niemi et al. (2005). Also immobilization of organo-mineral complexes by clay minerals allows retention of substrate to dehydrogenase. In contrast the protease

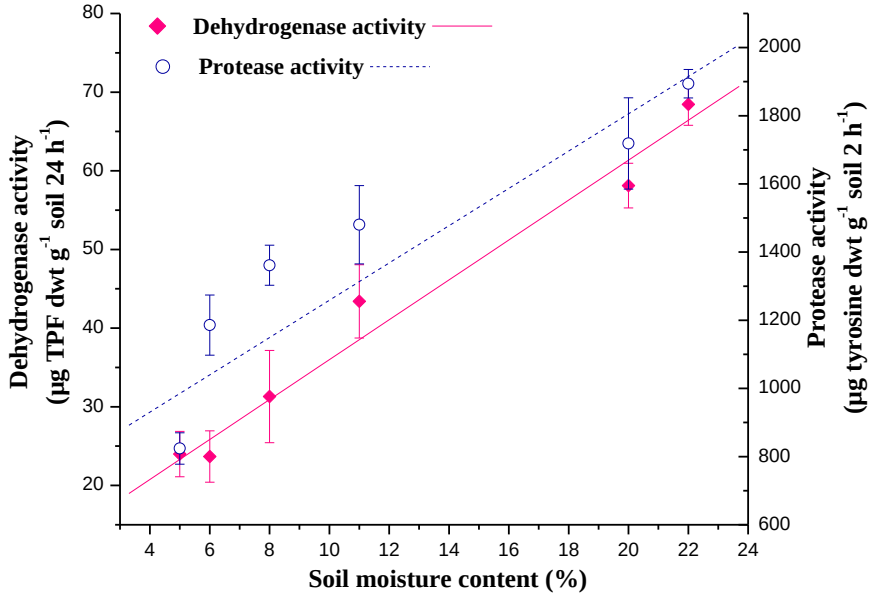
activity is poorly correlated with SOM in alluvial zone. This may be due to either low amount of proteinaceous matter in SOM or less distribution of proteolytic bacterial population in studied semiarid alluvial soils.

Table 3.3: *Correlations between soil characteristics and specific enzyme activity indices in semiarid alluvial and estuarine soils.*

Soil parameters	**Alluvial soils**				**Estuarine soils**			
	Dehydrogenase		**Protease**		**Dehydrogenase**		**Protease**	
	r value	*P* Value	r value	*P* value	r value	*P* value	r value	*P* value
SOM	0.998	0.0001	-0.705	0.001	0.999	<0.001	0.683	0.135
SMC	0.706	0.001	-0.426	0.02	0.933	0.006	0.857	0.029
Sand	0.171	0.393	-0.233	0.241	-0.793	0.059	-0.534	0.276
Silt	-0.257	0.195	0.273	0.169	-0.417	0.411	-0.715	0.111
Clay	0.234	0.241	-0.065	0.746	0.897	0.015	0.921	0.009
pH	0.0665	0.742	0.159	0.430	0.429	0.396	0.797	0.057
Dehydrogenase activity	_	_	-0.371	0.056	_	_	0.667	0.148
Protease activity	-0.193	0.335	_	_	0.667	0.148	_	_

Mujpur is situated in the estuarine zone of Mahi River; it gets inundated frequently during annual floods, high tides and subsequently high availability of substrate enhancing the protease activity. Multiproxy study on Mujpur section may provide meaningful information in respect of enzyme dynamics vis-a-vis temporal changes. Theoretically, a general consideration is that soils with high SMC content should possess high dehydrogenase activities because SMC enhances the microbial activities and dehydrogenase exists in soils as integral parts of intact cells. This was seen in Mujpur soils where SMC is positively correlated with its corresponding dehydrogenase activity (Fig. 3.5). Brzezinska et al. (2001) determined the relationship between the enzyme activity and soil aeration parameters in a pot experiment with barley vegetation and envisaged a positive relation between SMC and dehydrogenase activity (r = 0.59). Similar kind of observation was found in the present study (Fig. 3.5).

Fig. 3.5: *Correlation of dehydrogenase and protease activities with SMC in estuarine soils (r = 0.99, and r = 0.94 respectively) at P <0.001.*



Niemi et al (2005) demonstrated a negative correlation between soil depth and enzyme activities and a positive correlation between SOM and enzyme activities. Similar type of correlations was found by Sardans and Penuelas (2005) on hydrolytic enzyme activities in relation to soil depth. The present study and obtained data are supportive to their observations (Table 3.2). However, an increase in dehydrogenase with depth in the Mujpur section is suggestive of the increased enzymatic activity of anaerobic and facultative bacteria. Increase in protease activity with depth at Mujpur may be due to increased substrate availability and microbial activity. Wright and Reddy (2001) reported that a decreased protease and glucosidase activity with depth is due to decrease in substrate quality along with depth.

Fig. 3.6: *Correlation of dehydrogenase and protease activities with clay content in alluvial soils. Regression line shown for positive correlation in case of dehydrogenase activity (r = 0.52, P < 0.004) where both dehydrogenase and protease activities showed significant positive correlation (r = 0.99, and r = 0.92 respectively) with clay at P = <0.001.*

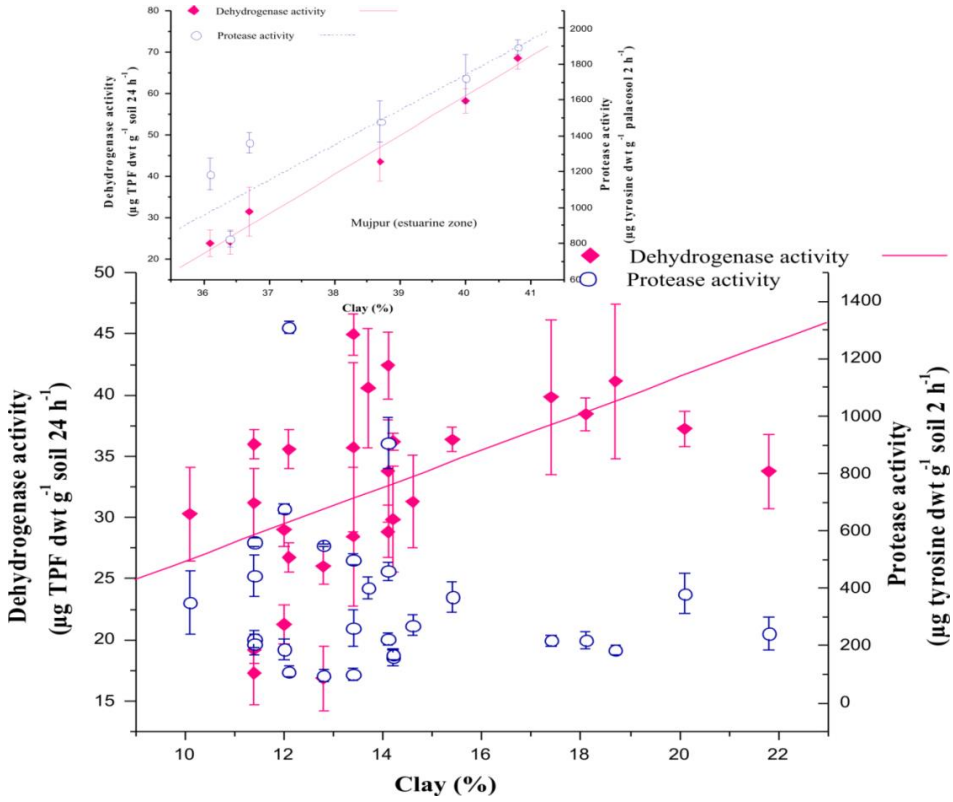


Fig. 3.7: *Correlation between dehydrogenase and protease activities was not found in alluvial soils however inset data shown for estuarine samples and a linear strong correlation (r = 0.94) was observed among both the enzymes at P = <0.001.*

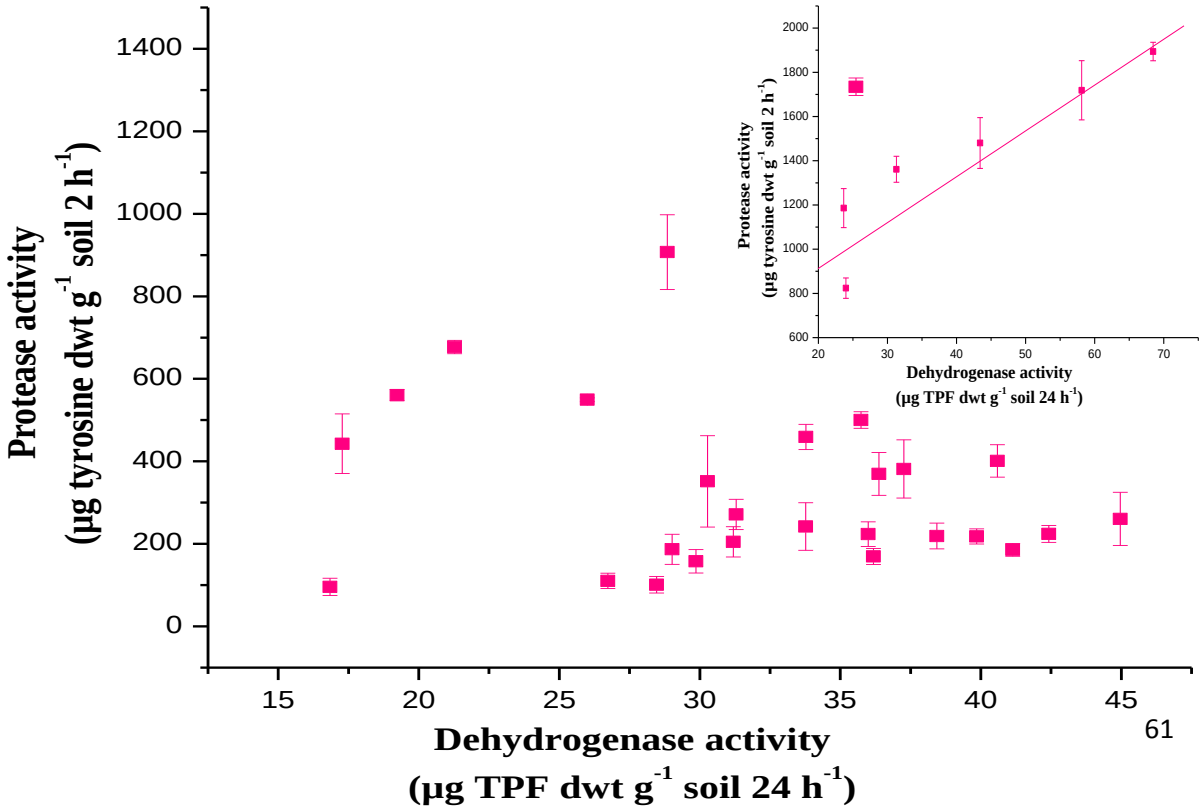
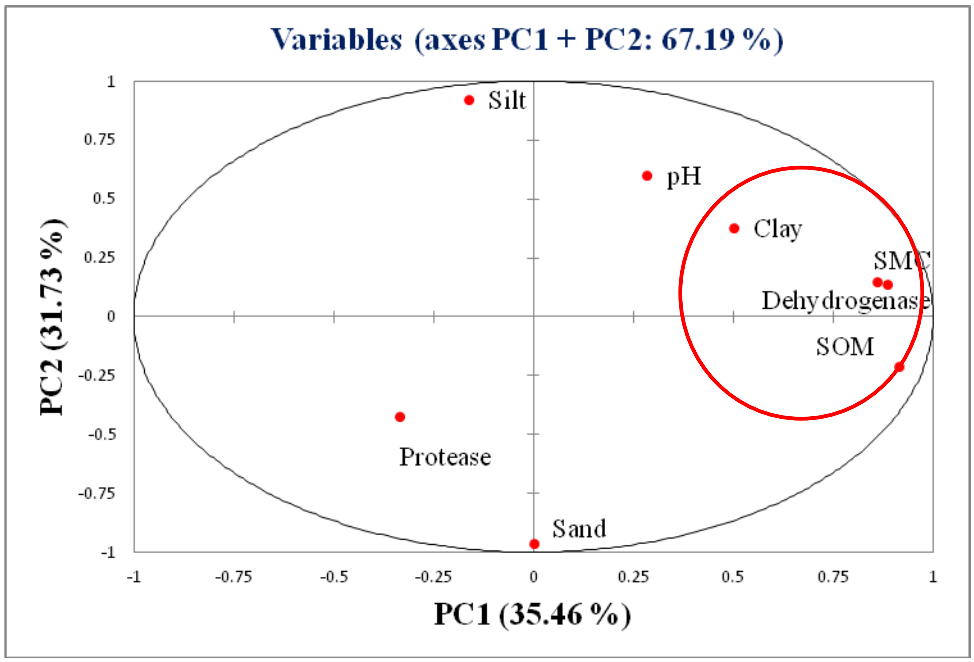
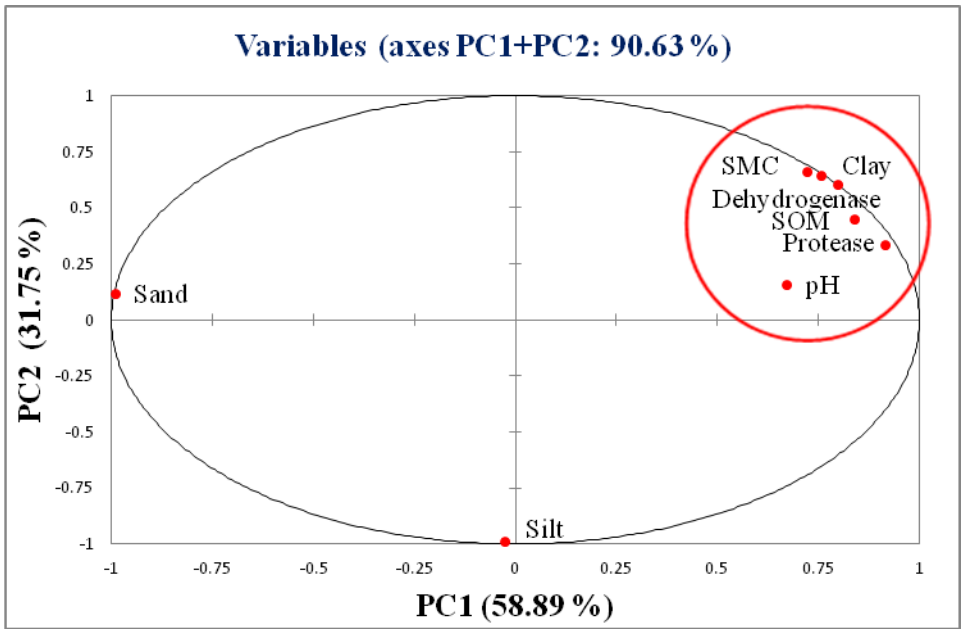


Fig. 3.8: *Principal component analysis (PCA) for soil properties and enzymes. A: PCA loading plot of alluvial zone variables. B: PCA loading plot of estuarine zone variables.*

A:



B:



Both the enzymes in alluvial soils are independent of each other (Fig. 3.7), indicating soil organic matter transformation and initial breakdown of proteins are self regulated processes in alluvial systems. However, a trinity between higher microbial activity and higher protease activity was observed in Mujpur soils (Fig. 3.3B and Fig. 3.7) indicating high metabolic state of the estuarine soils. Initial organic matter

transformation by dehydrogenase (during microbial respiration) made substrate available to protease and subsequently higher protease activity achieved in estuarine zone. In contrast, the alluvial site is lacking a high amount of proteinaceous substrate in its integral part of SOM.

We presume that lower soil enzyme activities in alluvial soils were associated with progressive soil erosion. It has been demonstrated that dehydrogenase activity can be used as a sensitive marker of soil degradation and soil microbial activity in semiarid Mediterranean region of Spain (Garcia et al, 1997). In the same study it was found that no correlation between the dehydrogenase activity of the soils and organic matter content ($r = 0.32$, $P = 0.18$) and it is contradictory to present findings which are showing positive correlation between dehydrogenase and SOM ($r = 0.52$, $P < 0.004$, $n = 27$) in the semiarid alluvial zone which is under intense weathering. In another investigation which is on soil quality degradation processes along a deforestation chronosequence in the Ziwuling area, China, it was found that the soil erosion was the primary process responsible for the degradation of soil physical, chemical, and microbiological properties (An et al., 2008). However, they have studied alkaline phosphatase activity and invertase activity. In another report from northern China (Zhong et al., 2009), found decreased soil enzyme activities in a degraded sandy grass land however their studies were mainly focusing on influences of continuous grazing and livestock exclusion on sandy grass land soil erosion. Present findings are in general agreement with their results. Although restricted to a limited number of soils, results seem to be encouraging and the obtained data suggests that, dehydrogenase and protease could be considered as indicators for intense erosion of semiarid alluvial soils. In the alluvial soils, the decrease in the activity of soil enzymes which involves in recycling of nitrogen and carbon may effect in long term on soil nutrient availability and reducing the nutrient supply to plants. Moreover in both alluvial and estuarine ecosystems, oxidation of organic content and subsequent transformation could be depending on SMC, clay and soil depth.

3.2.4. Conclusions

Two soil enzymes dehydrogenase and protease showed different relationships with soil properties depending upon the alluvial and estuarine zones. SOM, SMC, clay content and soil depth are the main determinants for soil dehydrogenase activity

and subsequent organic matter transformation in both zones. Soil protease activity and its critical determinates were found to be site specific. Relatively estuarine zone showed high microbial activity as compared to alluvial zone in the semiarid Mahi River region.

3.3. Microbial activity and diversity in the late Pleistocene palaeosols of Mahi River basin

3.3.1. Materials and methods

3.3.1.1. Study site description and sample collection

The site Rayka under study is about 22 km NE of Vadodara located on the left bank of the semi arid Mahi River basin, Gujarat, Western India (Fig. 3.9A). The mean annual rainfall in the area is about 600 to 650 mm. The site lies within the alluvial zone of the Mahi River basin. About 38 m exposed section on the left bank of the Mahi River shows alluvial deposits and intercalated palaeosols (Fig. 3.9B and C) of late Pleistocene age (Maurya et al., 1997; Raj et al., 1998; Khadkikar et al., 2000). A summary of the chronology (Figure 3.9B) and inferred palaeo-monsoon variability based on the fluvial and aeolian record from the Mahi basin has been well documented by Juyal et al. (2006). Palaeosol P1 has been dated to ~45 ka whereas P2 to ~30 ka. Information on paleoenvironments in which these palaeosols have formed is well documented (Juyal et al., 2000). The base of the sediment sequences along these cliffs is dated back to 125 ka (Juyal et al., 2000 and 2006).

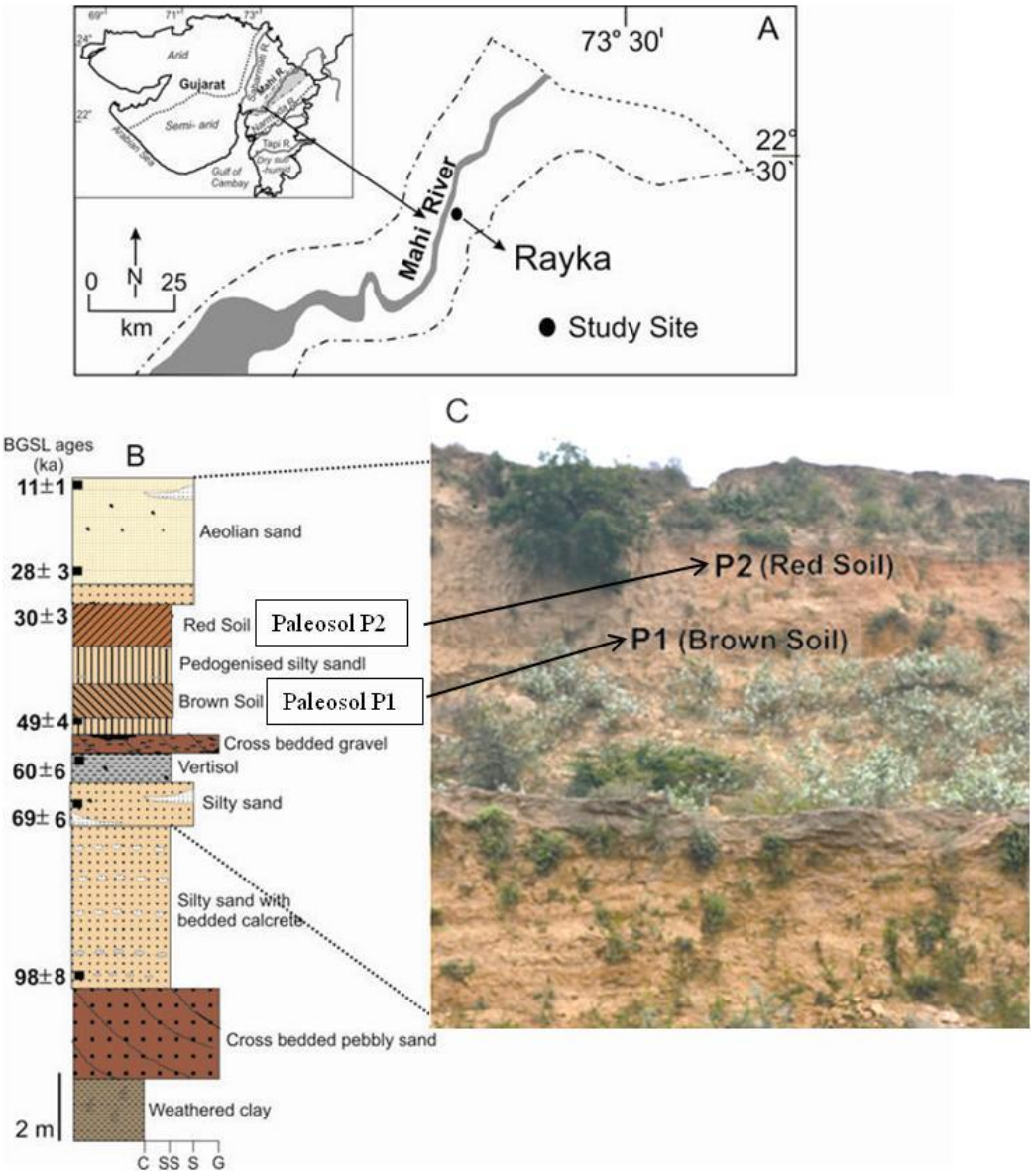
Triplicate composite samples of two different buried palaeosols P1 and P2 were collected from the exposed section at Rayka (Fig. 3.9C). Prior to the collection of soil samples, the exposed surface of the palaeosol was scraped clean. Autoclaved polyethene bags were used for collecting the samples which were immediately kept in a box containing ice packs before being transported to the laboratory. All the samples were sieved (< 4 mm), cleaned of visible roots and plant residues, and stored at 4 ^{0}C.

3.3.1.2. Palaeosol physiochemical analysis

Palaeosol samples were analysed for soil moisture content, pH, soil texture and water holding capacity following the standard protocols. Palaeosol macro and micro elements including soil organic C (C_{org}) were analysed at the laboratory of Gujarat

State Fertilizers Corporation (GSFC), Vadodara, Gujarat. All the determinations were performed in triplicate, and values reported are mean with standard deviation.

Fig. 3.9: *Overview of the sampling site. A. Map showing location of Mahi River basin and study site, (B) Stratigraphic log at Rayka (after Juyal et al., 2006). (C) Exposed section at Rayka showing locations of P1 and P2.*



3.3.1.3. Soil enzyme activity assays

Soil enzymes dehydrogenase (DHA), β-glucosidase (GSA), alkaline phosphatase (APA) and protease (PSA) involved in C, P and N cycling respectively,

were estimated (Chapter 2). All determinations of enzyme activities were performed in triplicates and values are reported as means with standard deviation.

3.3.1.4. Palaeosol microbiological analysis

Palaeosol microbial biomass C (C_{mic}), basal soil respiration (BSR) and substrate induced respiration (SIR) were determined (Chapter 2). In order to evaluate the palaeosol microbial ecophysiology, the ratios between microbiological parameters have been used in the present study. Ecophysiological indices such as microbial quotient (C_{mic}/C_{org}) and metabolic quotient (q-CO_2) were measured (Moscatelli et al., 2005). For evaluating the significance of microbial populations and microbial activity in the cycling of elements, specific enzymatic quotients (SEQ) were determined. All determinations of enzymatic activities were performed in triplicate and values are reported as means with standard deviation. Total viable heterotrophic bacteria of palaeosols were enumerated on R2A agar (Edenborn and Sexstone, 2007).

3.3.1.5. Microbial diversity studies of palaeosols by DGGE

Heterotrophic bacterial diversity was established in palaeosols of Mahi River by PCR-16S rRNA based denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1998; Chapter 2). In brief; 10 gm of palaeosol was taken in 90 ml of R_2A broth and allowed to incubate for 2 d at room temperature. After which community DNA of heterotrophic bacteria was extracted and checked for the purity and molecular size using conventional agarose gel electrophoresis. Universal eubacterial primers GC-341F and 534R) (Muyzer et al., 1993; Chapter 2) were used for the amplification of 16S rRNA gene fragments suitable for DGGE. The DCode system for DGGE (Bio-Rad laboratories Ltd, UK) was used. PCR amplified samples were loaded on to 8% polyacrylamide-bisacrylamide gels with denaturing gradient from 40 to 60%. Band patterns were analyzed with AlphaEase FC software (Alpha Innotech, CA). The diversity of the microbial community was expressed as the Shannon index of general diversity (*H*), eveness (E) and range-weighted richness (*Rr*) (Marzorati et al., 2008, Chapter 2).

3.3.2. Results

3.3.2.1. Physicochemical analysis palaeosols

Physicochemical characteristics of palaeosols are important to understand the soil productivity. Tables 3.4A and 3.4B show the physicochemical properties of P1 and P2. The lower palaeosol (P1) dating back to ~ 45 ka has high soil organic C (Corg), clay content and soil water holding capacity as compared to the upper palaeosol (P2). The Fe content is found more in the P2 rendering red color to the palaeosol (Juyal et al., 2006).

Table 3.4: *Physical (A) and chemical parameters (B) of Rayka palaeosols P1 and P2.*

A:

Palaeosols	Soil moisture content (%)	Soil dry-mass (%)	Soil water holding capacity (%)	Soil texture		
				Sand (%)	Silt (%)	Clay (%)
P1	12	88	12	40.5	51	8.5
P2	6	94	13	44	50.2	5.8

B:

Palaeosols	Organic Carbon (%)	pH	EC	S (ppm)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)
P1	0.61	8.1	0.41	26.40	1.10	4.8	3.82	3.66
P2	0.47	7.6	0.44	16.9	0.58	6.2	2.32	0.46

3.3.2.2. Microbial analysis palaeosols

Soil microbial biomass carbon is the living component of soil organic matter and it generally constitutes about 1-5% of the total organic matter (Nsabimana et al., 2004). Relatively high microbial biomass C (Fig. 3.10A) was found in P1 than P2 which indicates higher bacterial distribution per g of P1 palaeosol. These results are corroborated by total viable bacterial counts (Table 3.5). Slightly higher microbial and metabolic quotients were found in P1 (Table 3.5). Results show that P1 had a greater BSR and SIR activities than P2 (3.10B and C).

Table 3.5: *Total viable count, empirical indices such as microbial quotient (Cmic/Corg), metabolic quotient (q-CO2) and diversity indices like species richness (H) and evenness (E) and range weighted richness (Rr) of Rayka palaeosols.*

Palaeosols	Total viable count (10^5 cells / g^{-1} of soil)	Microbial quotient (C_{mic}/C_{org}) (%)	Metabolic quotient (qCO2) (µg C mg C_{mic}^{-1} h^{-1})
P1	6.6 ± 1.52	4.43 ± 0.15	11.54 ± 0.484
P2	4.2 ± 2.08	4.30 ± 0.21	7.49 ± 0.81

3.3.2.3. Palaeosol enzyme activities

To evaluate the fluxes of elements in palaeosols, hydrolytic enzymes such as GSA, PSA and APA involved in C, N, P cycles, respectively (Masciandaro et al., 2004) were determined. Relatively, higher GSA, PSA and APA were noticed with P1 palaeosol than P2 ((Fig. 3.11B, C and D) and the results are statistically significant ($p< 0.05$). Particularly, 2.44 folds higher GSA was found in P1. Higher DHA activity was observed in P1 than P2. In addition, higher specific enzymatic quotients (SEQ, per unit microbial biomass carbon) as well as higher specific enzymatic indices (SPI, per unit soil organic Carbon [C_{org}]) were also obtained in P1 (Table 3.6).

3.3.2.4. Molecular diversity fingerprinting of palaeosols by DGGE

In the present investigation DGGE of the 16S rRNA gene was used to compare the heterotrophic bacterial communities of palaeosols (Fig. 3.12). Relatively more number of bands were observed in P1 than in P2 indicating high heterotrophic bacterial diversity in P1. Diversity indices *H* and *E* were relatively high in P1 than P2 (Table 3.7).

Range weighted richness (Rr) value for P1 was found to be 20.22 where as Rr value for P2 was found to be 10.12, allowing the inference that the P1 palaeosol has a broad carrying capacity that the system supports the growth diverse group of bacteria about two times greater than P2. Bands numbered 3, 4, 6, 7, 8 and 9 were common in both the palaeosols (Fig. 3.12) indicating distribution of these ribotypes in both the palaeosols. Bands numbered 1, 2 and 5 (Fig. 3.12) are highly specific to P2 and these

bands were virtually absent in P1. Bands denoted as a, b, c, d, e, f and h are specific to P1 (Fig. 3.12) and are absent in P2 indicating development and maintenance of distinct species in the two soils during the time course.

Fig. 3.10: *Soil microbial activities in the Rayka palaeosols P1 and P2. (A) Microbial biomass C, (B) Basal soil respiration and (C) Substrate induced respiration.*

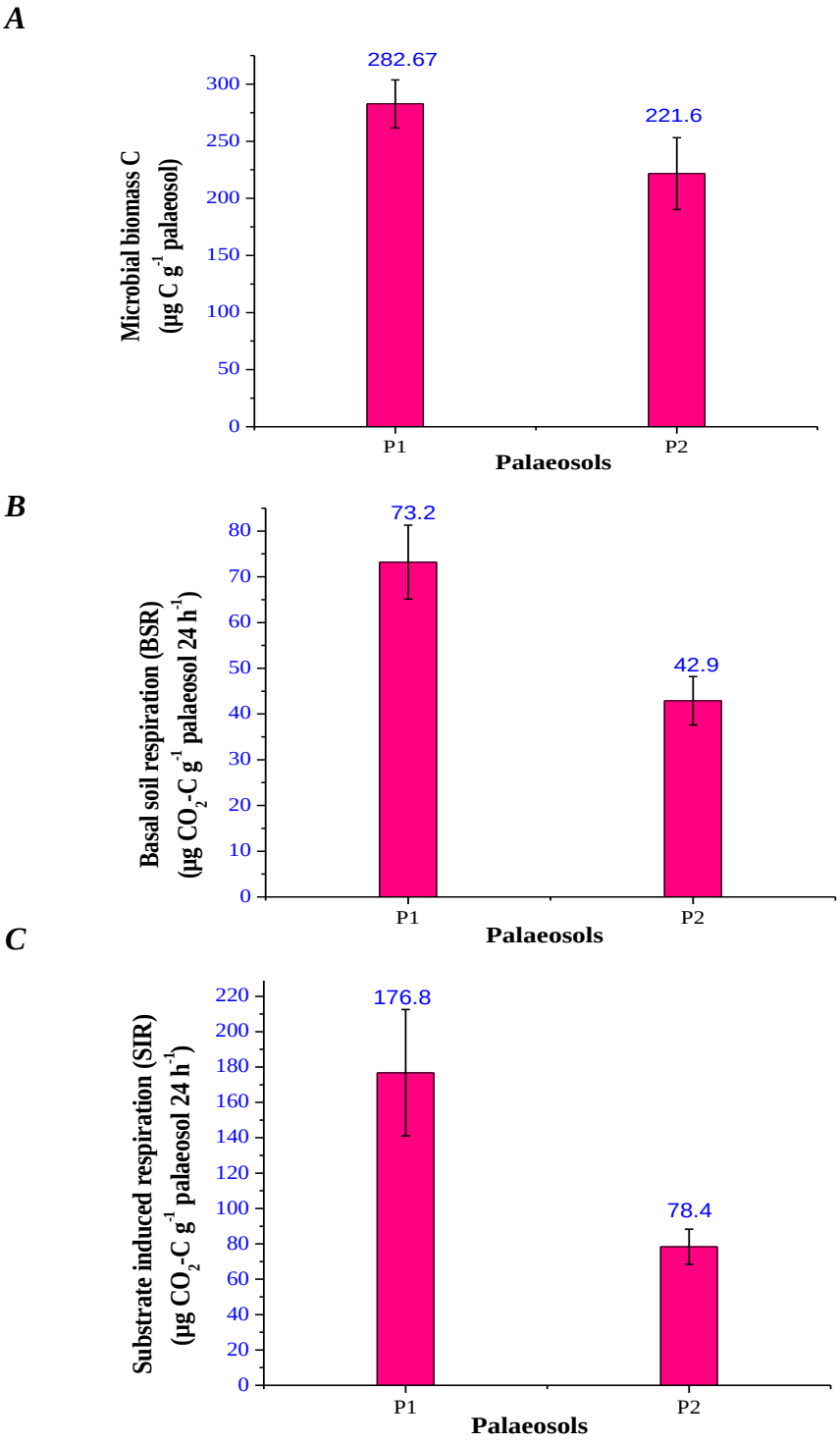
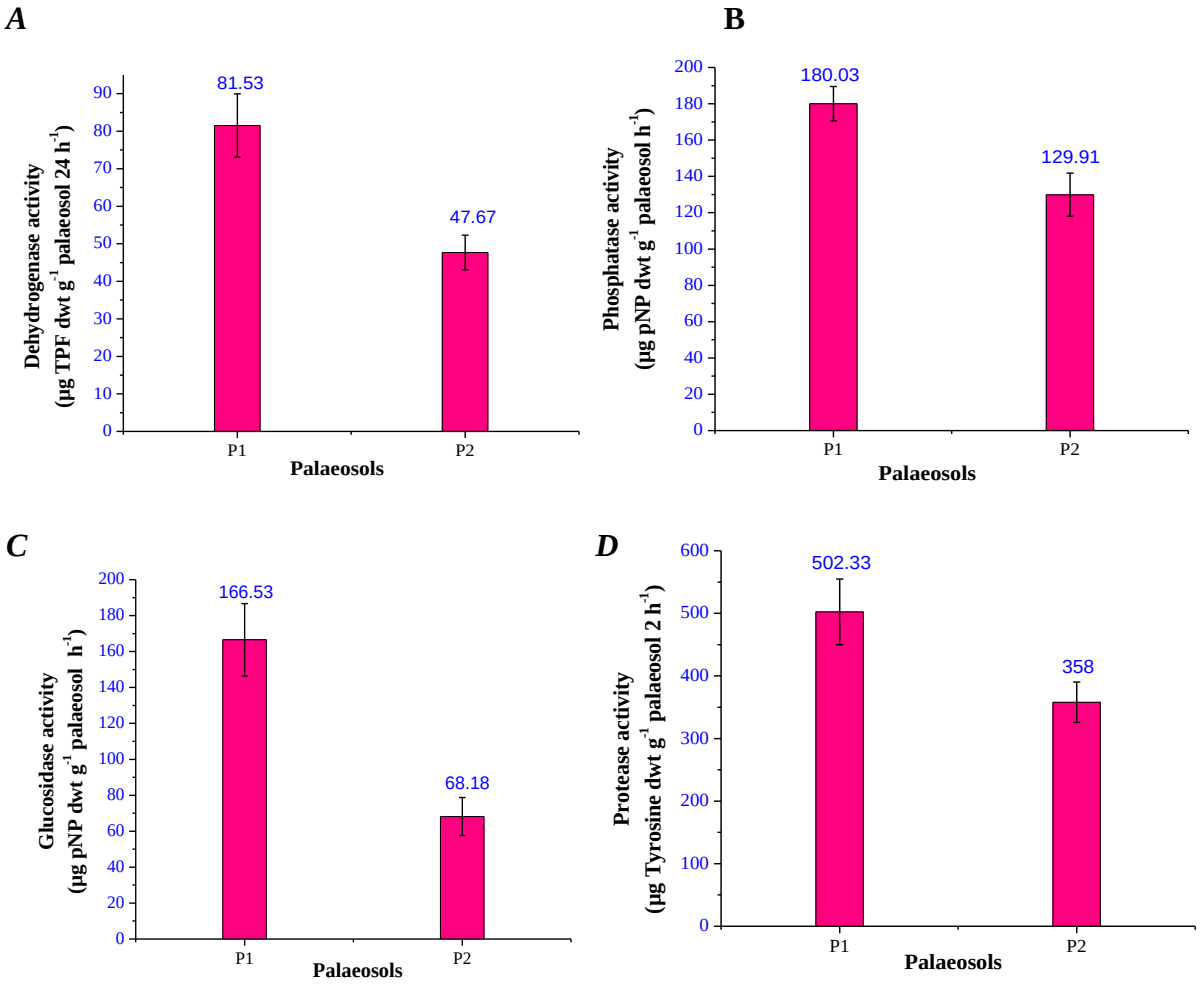


Fig. 3.11: Soil microbial enzymes activities in the Rayka palaeosols P1 and P2. A: Dehydrogenase; B: Phosphatase; C: Glucosidase and D: Protease activities.



3.3.3. Discussion

Juyal et al. (2006) suggested that during the last 125 ka, the fluvial sedimentation pattern in the Mahi basin changed frequently. The variation in the nature of sediments provide a clue to understand changes in the precipitation regime and fluctuating water budget in the basin that receives the southwest monsoon. The palaeosols P1 and P2 are estimated to have an age difference of about 15,000 years. Palaeosol P1 is the older, well pedogenised silty brown soil whereas P2 is a red alfisol.

Table 3.6: *Specific enzymatic quotients (SEQ) and specific enzymatic indices (SPI) for Rayka palaeosols. SEQ were calculated per unit microbial biomass (C_{mic}) where as SPI were calculated per unit soil organic Carbon (C_{org}).*

Palaeosols	SEQ-DHA	SEQ-APA	SEQ-GSA	SEQ-PSA	SPI-DHA (%)	SPI-APA (%)	SPI-GSA (%)	SPI-PSA (%)
P1	0.288 ±0.02	0.635 ±0.03	0.59 ±0.07	0.885 ±0.08	1.33 ±0.13	2.94 ±0.15	2.72 ±0.32	3.74 ±0.34
P2	0.211 ±0.02	0.583 ±0.05	0.308 ±0.04	0.80 ±0.06	0.89 ±1.01	2.76 ±0.25	1.44 ±0.22	3.80 ±0.30

SEQ-DHA: µg TPF $24h^{-1}$ µg C_{mic} g^{-1} dwt palaeosol; SEQ-APA: µg PNP h^{-1} µg C_{mic} g^{-1} dwt palaeosol; SEQ-GSA: µg PNP h^{-1} µg C_{mic} g^{-1} dwt palaeosol; SEQ-PSA: µg Tyrosine $2h^{-1}$ µg C_{mic} g^{-1} dwt palaeosol; SP-DHA: µg TPF $24h^{-1}$g-1 C_{org} g-1 dwt palaeosol; SPI-DHA: µg TPF $24h^{-1}$g-1 C_{org} g-1 dwt palaeosol; SPI-APA: µg PNP h^{-1} g-1 C_{org} g^{-1} dwt palaeosol; SPI-GSA: µg PNP h^{-1} g-$^{1}C_{org}$ g^{-1} dwt a; SPI-PSA: µg Tyrosine $2h^{-1}$ g^{-1} C_{org} g^{-1} dwt palaeosol.

Fig. 3.12: *16S rRNA molecular microbial diversity profiling of Rayka palaeosols P1 and P2 by DGGE***.**

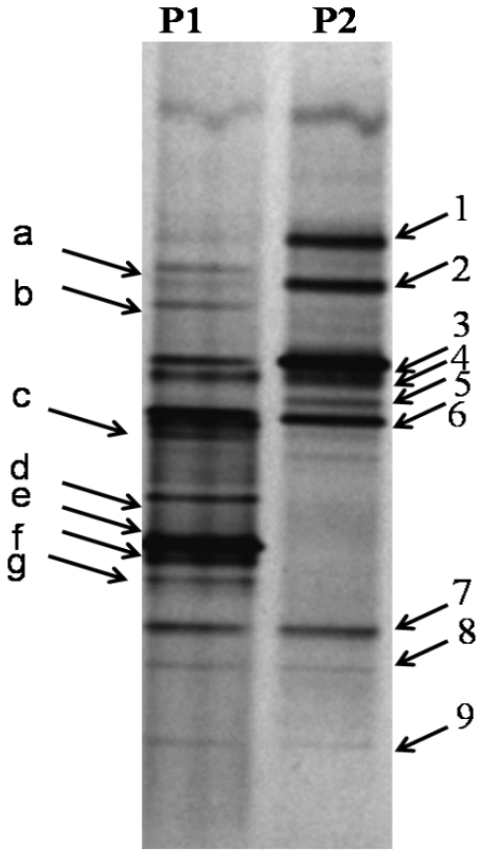


Table 3.7: *Microbial diversity indices of palaeosols*

Palaeosol	Species richness (*H*)	Species evenness (*E*)	Range weighted richness (*Rr*)
P1	2.66	2.20	20.2
P1	2.28	2.13	10.12

In the present study, palaeosol organic matter decomposition rates were understood as a function of three parameters: microbial biomass, microbial respiration and microbial enzymes (Bekku et al., 2004). All these parameters were relatively high in P1 than P2 indicating enhanced organic matter transformation in P1. Microbial activity was measured by respiration, metabolic CO_2 and general evaluation of soil enzymes involved in transformations of elements. It is known that the soil CO_2 emission rate is determined by microbial activity and is considered as the basic portion of the total carbon flow to the atmosphere (Schlesinger and Andrews, 2000). The microbial activity related to the mineralization of palaeosols organic matter was estimated on the basis of CO_2 evolution rate (Fig. 3.10B and C). Results show that P1 had a greater basal soil respiratory (BSR) activity than P2 suggesting that the soil microorganisms in P1 have a higher availability of SOM for respiration and also the capability of its breakdown as compared to those in P2.

In general, soils receive exogenous substrates at regular intervals during the course of landscape evolution. The organic substrates introduced into soil may enhance or reduce the microbial mineralization of soil organic matter that is defined by positive or negative priming effect (PE). Positive PE is considered as one of the factors escalating the atmospheric CO_2 concentration (Kuzyakov, 2006). In order to evaluate the microbial response and their concurrent PE as a result of addition of exogenous substance, substrate induced respiration (SIR) was monitored with readily utilizable substrate, glucose. Although carbon mineralisation rates increased in both soils, and the increase was relatively higher (by 141.53%) in P1 as compared to that in P2 (by 82.8%). Overall P1 had high SIR activity (Fig. 3.10C) than P2. Based on the values of both BSR and SIR, it can be inferred that the microbes in P1 have greater potential in SOM decomposition and carbon flow to the atmosphere (Schlesinger and Andrews, 2000). The composition of the microbial communities and in particular the

competition between K-strategists and r-strategists is used for determining the priming effects (Schlesinger and Andrews, 2000). The input of an easily metabolized substrate may favour the growth of r-strategists in comparison to K-strategists, since the r-strategists start to grow within hours and utilize the major portion of such substrates. Hence, the greater SIR activity associated with P1 is a likely response of r-strategists. Activity of r-strategists can reflect the nutritional status of the environment; such activity was lower in P2 due to oligotrophic nature. The microbial potential in decomposition of organic matter was elucidated by metabolic quotient (q-CO_2, Table 3.5). Based on the respiration rates as well as q-CO_2, we infer that the soil organic matter decomposition is relatively higher in P1 than P2.

Soil microbial enzymes play a key role in decomposition of organic residues and are considered fundamentally good indicators for soil quality (Caldwell, 2005). Several enzymes are known to be involved in the cycling of nutrients and can be used as potential indicators of nutrient cycling processes (McLatchey and Reddy, 1998). DHA is associated with living cells, linked with microbial oxidoreduction processes and is principal agent in the degradation of SOM (Tabatabai, 1994; Alef and Nannipieri, 1995). Remarkably high amount of DHA activity was found in P1 (Fig. 3.11A) and is in general agreement with high microbial respiration activity indicating that organic matter decomposition was much active in P1.

To evaluate the fluxes of elements in palaeosols, extracellular hydrolytic enzymes such as GSA, PSA and APA involved in C, N and P cycles, respectively (Masciandaro et al., 2004) were determined for the palaeosols (Fig. 3.11B, C and D). Hydrolase-enzyme activities indicate the soil potential to carry out specific biochemical reactions and also maintain soil fertility (Nannipieri et al., 2002; Masciandaro et al., 2004). It was found that all the three enzymes were relatively high in P1 (Fig. 3.11B, C and D) indicating that the microbially mediated C, N and P cycle fluxes are higher in P1 dated to ~45ka. Specific enzymatic quotients (SEQ) per unit microbial biomass carbon give an idea of the microbial role in biogeochemical cycles. The order of biogeochemical cycling in P1 is N >P ≥ C whereas the order for P2 is N > P >C. Same trend was observed with specific enzymatic indices (SPI) calculated by unit C_{org} (Table 3.6). Both SEQ and SPI were relatively high in P1 indicating good soil quality and health.

Microbial activities in relation with soil physical and chemical properties in the present day (surface) soils of the Mahi River basin have been studied. Dehydrogenase activities of the surface soils of Rayka showed strong correlation with soil organic matter and soil moisture content, however protease activity did not correlate with any of the soil parameters studied. Studies reported here show similar trend in the buried palaeosols of Rayka, where higher organic matter and moisture content (in P1 palaeosol) was concurrent with higher enzyme activities. The results indicate that physicochemical properties of the soils remain the main determinants of microbial activity regardless of the age of the soil.

3.3.4. Conclusions

Organic matter decomposition and carbon flow to the atmosphere are much higher in the P1 brown palaeosol dated to ~45 ka. Soil in situ enzymes, SEQ and SPI reveal greater productivity and quality of P1 (~45 ka) than P2 (~30 ka). The order of microbial activity in biogeochemical cycling in P1 was found to be $N > P \geq C$, whereas in P2 it was $N > P > C$. P1 showed high heterotrophic bacterial diversity and broader carrying capacity than P2. Contrasting microbial activity and diversity in these palaeosols indicate two different environments and are in agreement with climatic inferences drawn by earlier workers (Juyal et al., 2000 and 2006).

3.4. Multivariate statistical analysis of soil/sediment microbial and physiochemical variations in a exposed vadosezone at Rayka, Mahi River basin

3.4.1. Materials and methods

3.4.1.1. Sample collection

Soils and sediments investigated in this study were collected from an exposed shallow subsurface (22°26'48.50"N, 73° 5'12.22"E) measuring about 28 m length near Rayka in the vicinity of Mahi River basin, Vadodara window. Generalized overview of the section is showed in Fig. 3.13. Lithological and stratigraphic details of the sequences are given in Fig. 3.14. The exposed section consisted of various sediment sequences and distinct soil profiles (Fig. 3.14) such as surface sandy soil, silty soil (red in color), loam soil (brown in color) and organic rich basal vertisol. Rayka geomorphic setting has been discussed in previous section (Section 3.2). A total of 51

triplicate composite samples were collected at an interval of 25 cm from exposed soils and sediments. To the best of our knowledge the samples were unexposed to anthropogenic activities. Prior to the collection of soil and sediment samples, the sites were cleaned by scraping the surface layer. Autoclaved polyethylene bags were used for sampling. Samples were immediately kept in a transport box containing ice packs before being transported to the lab. All the samples were sieved (< 4 mm), cleaned of visible roots and plant residues, and stored at 4^{o}C.

3.4.1.2. Physicochemical analysis

Samples were analyzed for soil moisture content (SMC), pH, soil texture according to the standard protocols (Alef and Nannipieri, 1995; Chapter 2). Soil organic matter was estimated by dichromate digestion method (Walkley and Black, 1934). Detailed geochemical data of the soils (micro and macro elements) has been obtained with collaboration from Dr. Anupam Sharma, BSIP, Lucknow, India. Different phosphorus fractions such as organic (OP), inorganic (IP), inorganic-apatite (AP) and inorganic-non apatite (NAP) fractions have been analyzed (Ruban et al., 2001). All the determinations were performed in triplicate, and values reported are mean (n = 3).

3.4.1.3. Soil microbial activity studies

Soil microbial activities were estimated as an index of enzyme activities for dehydrogenase and phosphatase and respiration studies (BSR and SIR). All the determinations were performed in triplicate, and values reported are mean with standard deviation (n = 3).

Fig. 3.13: *Generalized overview of the sampling site at Rayka. Satellite image (Source- www.googleearth.com) showing the sampling site (indicated with orange mark) of exposed shallow subsurface section near Rayka (A). Longitudinal (B) and vertical (C) views of the exposed section are also shown.*

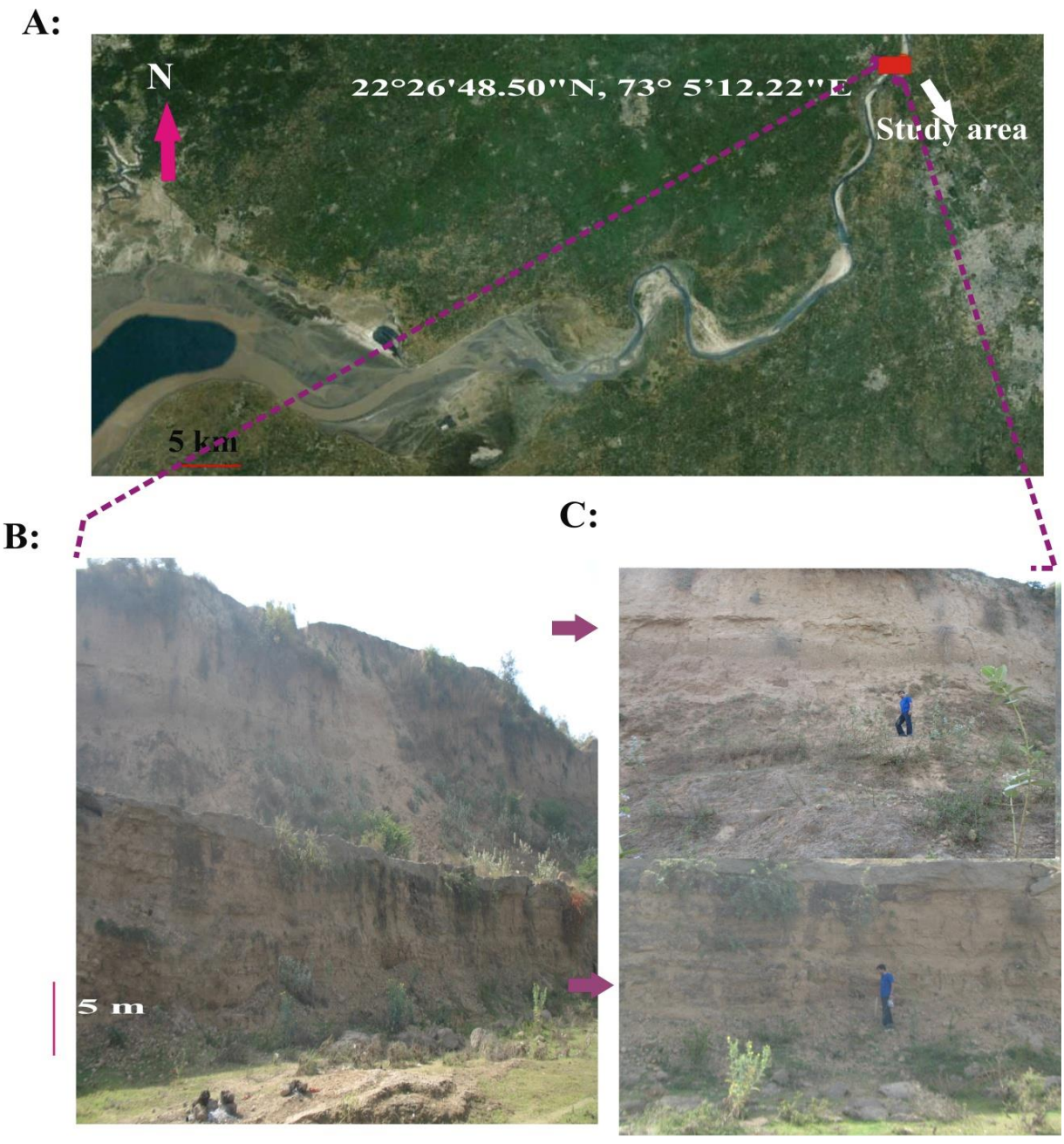
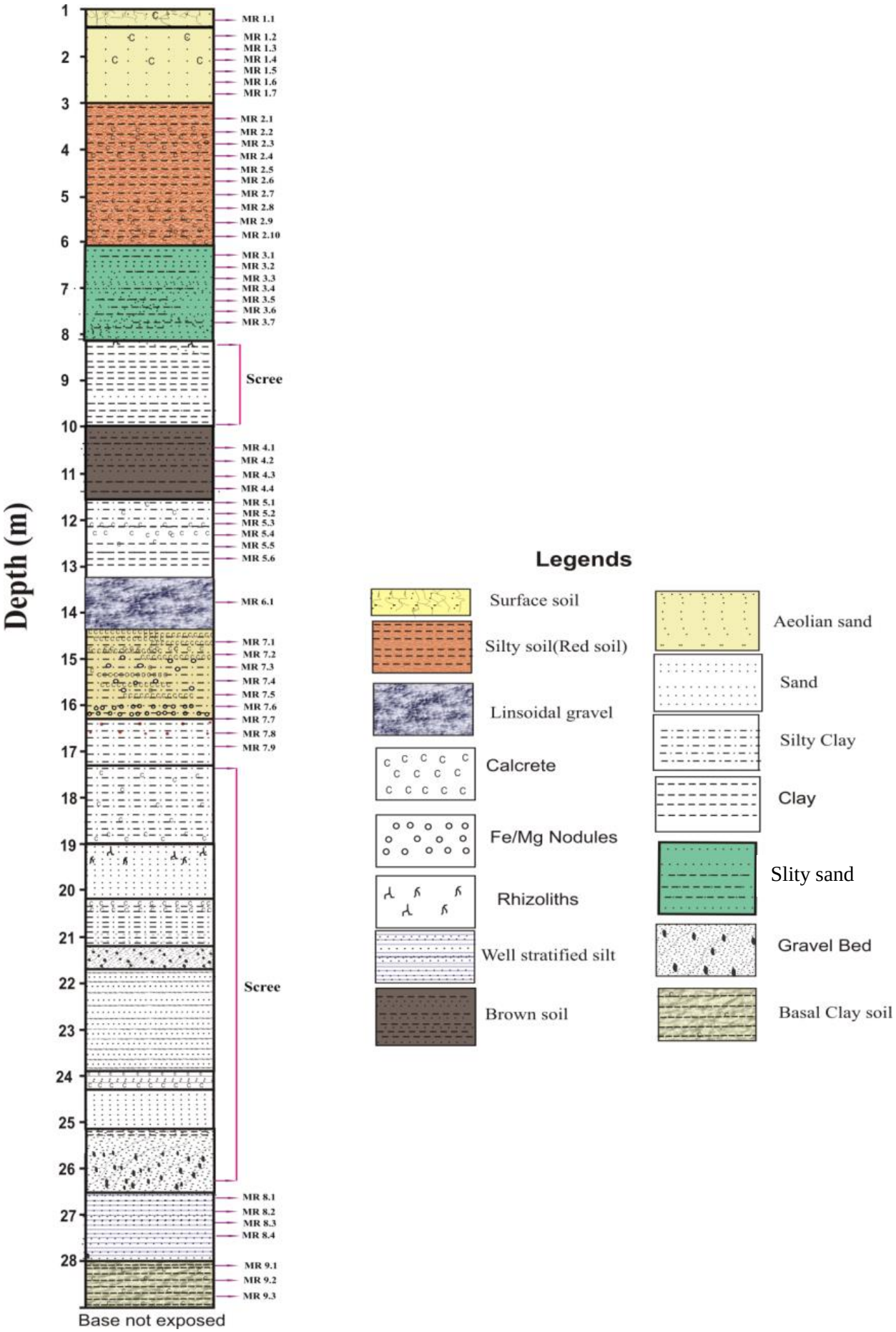


Fig. 3.14: *Stratified litholog of the exposed shallow subsurface section near Rayka, Mahi basin. Sample names and their locations are mentioned with arrows. A total of 51 triplicate composite samples (MR 1.1 to MR 9.3) were collected along the 28 m depth profile. Portions under "Scree" is contaminated or mixed with surface sediments and thus excluded from the present study.*


Depth (m)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
MR 1.1
MR 1.2
MR 1.3
MR 1.4
MR 1.5
MR 1.6
MR 1.7
MR 2.1
MR 2.2
MR 2.3
MR 2.4
MR 2.5
MR 2.6
MR 2.7
MR 2.8
MR 2.9
MR 2.10
MR 3.1
MR 3.2
MR 3.3
MR 3.4
MR 3.5
MR 3.6
MR 3.7
Scree
MR 4.1
MR 4.2
MR 4.3
MR 4.4
MR 5.1
MR 5.2
MR 5.3
MR 5.4
MR 5.5
MR 5.6
MR 6.1
MR 7.1
MR 7.2
MR 7.3
MR 7.4
MR 7.5
MR 7.6
MR 7.7
MR 7.8
MR 7.9
Scree
MR 8.1
MR 8.2
MR 8.3
MR 8.4
MR 9.1
MR 9.2
MR 9.3
Base not exposed
Legends
Surface soil
Silty soil(Red soil)
Linsoidal gravel
Calcrete
Fe/Mg Nodules
Rhizoliths
Well stratified silt
Brown soil
Aeolian sand
Sand
Silty Clay
Clay
Slity sand
Gravel Bed
Basal Clay soil

3.4.1.4. Soil microbial diversity analysis

Molecular fingerprinting of the microbial diversity of the Rayka exposed section was established by denaturing gradient gel electrophoreses (DGGE) (Muyzer and Smalla, 1998; Chapter 2). Universal eubacterial primers GC-341F and 534R) (Muyzer et al., 1993) were used for the amplification of 16S rRNA gene fragments suitable for DGGE. DNA extraction and conditions for PCR and procedure for DGGE are described (Chapter-2). Group specific primers i.e. GC-517f (5^1CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCGTGCCAGC AGCCGCGG-3^1) and Alf968r (5^1-GGTAAGGTTCTGCGCGTT-3^1) targeted for α-proteobacteria were used to determine α-proteobacterial community dynamics (Gich et al., 2005). Diversity indices such as H^I, E^I and *Rr* were estimated (Shen et al., 2008; Marzorati et al., 2008).

3.4.1.5. Statistical analysis

In this study relation between soil/sediment microbial activity and their physicochemical characters has been explored by undertaking multivariate statistical approach. All the physical and chemical variables are broadly categorized in two distinct groups for ease of analysis.

- Group-1: 12 different soil physical, organic and inorganic C based variables **:** pH, Sand, slit, clay, soil moisture content (SMC), Organic C (OC), structured C (SC), Brown C (BC), Charcoal, carbonate (CO_3^-), Loss of ignition carbon (LOI) and fungal spores (FS).

- Group-2: 22 different soil micro and macro elements : V, Co, FeO, MnO, MgO, CaO, Na_2O, K_2O, LOI, Ba ,Ni ,Cr, Sr, Rb, Sc, Y, Zr, Zn, CIA, SiO_2, TiO_2 and Al_2O_3.

Principal component analysis (PCA) was performed to reduce data dimensions and also to determine the critical (principal) factors that relate to the microbial activity in exposed vadosezone. Linear regression models including correlation coefficients were determined between enzymes and soil properties. Canonical correspondence (CCA) analysis was performed to simulate exposed shallow subsurface microbial activity and

their relation to physical and chemical factors. Canoco 4.5 and sigma stat 4.0 were used for the statistical analysis.

3.4.2. Results

3.4.2.1. Microbial activity studies of samples

Soil microbial enzymes i.e. dehydrogenase and phosphatase activities (Fig. 3.15A and B) were estimated. Enzyme activities were varied particularly with the sampling depth and nature of the soil. Both the enzyme activities are comparatively high in the samples collected at a depth of 2 to 2.5 m, 10.25-11m, 11.5 to 12.5 m and 26.75 to 28 (Fig. 3.15A and B). It is indicative of high microbial activity in these regions.

In the present study, the microbial activity related to the mineralization of subsurface organic matter was estimated on the basis of CO_2 evolution rate. Basal respiration (BSR) and substrate induced respiration (SIR) of exposed shallow subsurface samples are shown in Fig 3.16A and B. High basal respiration activity was noticed in samples collected at a depth of 10.25 to 11 m, 11.5 to 12 m, 16.5 m and 26.25 to 28 m (Fig. 3.16A). High amount of SIR was noticed in upper portion of Rayka section distributing at a depth of 2 to 2.5 m, 4.75 m, 6.5 m and 7.5 m (Fig. 3.16B).

Soil samples containing high enzyme activities as well as BSR activity are considered to be microbial activity hotspots of Rayka and were shown in the litholog (Fig. 3.17). There are 4 alleged hot-spots which are distributed along the 28 m Rayka section. The details of the hot-spots are mentioned below.

- Hot-spot 1: located in between 2 to 2.5 m depth which is the portion of upper portion of silt soil.
- Hot-spot 2: located in between 10.25 to 11 m depth which is the portion of loam soil (brown soil)
- Hot-spot 3: located in between 11.5 to 12.5 m depth which is the portion of fluvial silt
- Hot-spot 4: located in between 26 to 27 m depth which is the portion of stratified vertisol.
- Hot-spot 5: located in between 27 .5 to 28 m which is the portion of basal clay soil

Fig. 3.15: *Dehydrogenase (A) and phosphatase (B) activities of soils and sediments in exposed Rayka section measuring about 28 m in the vicinity of Mahi river basin. Values are mean of three replicate samples (n = 3) and the error bar represents standard deviation.*

A: ***B:***

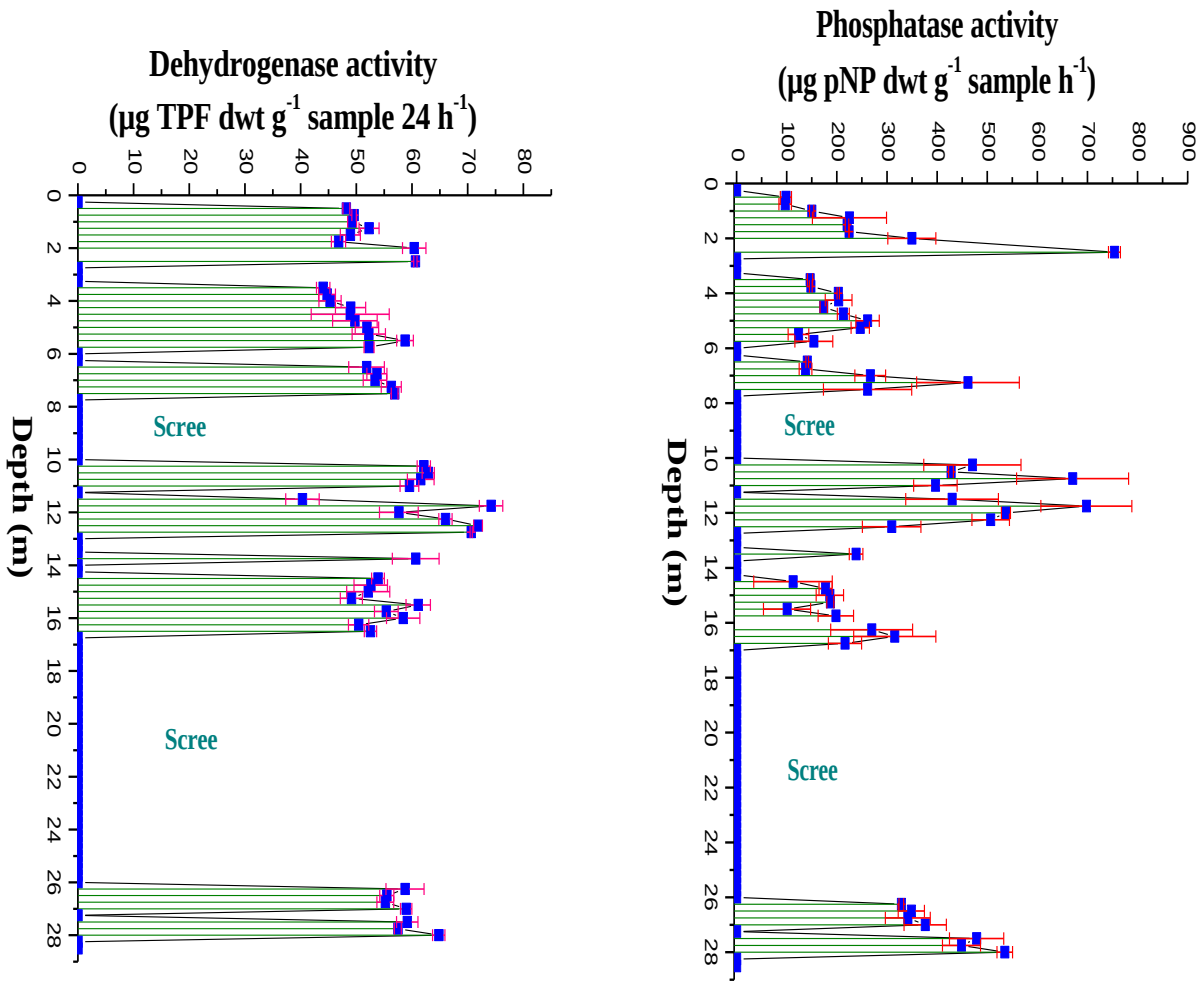
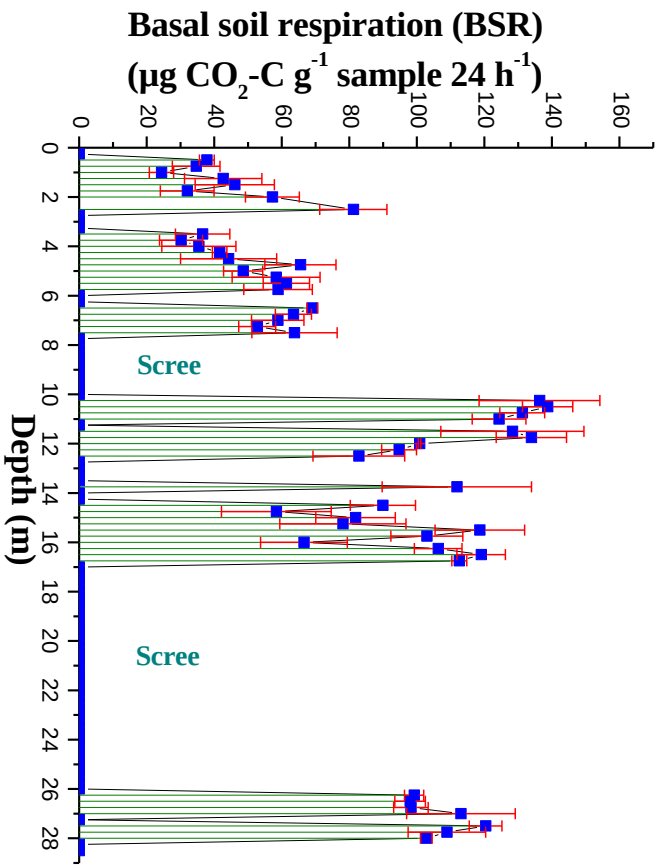
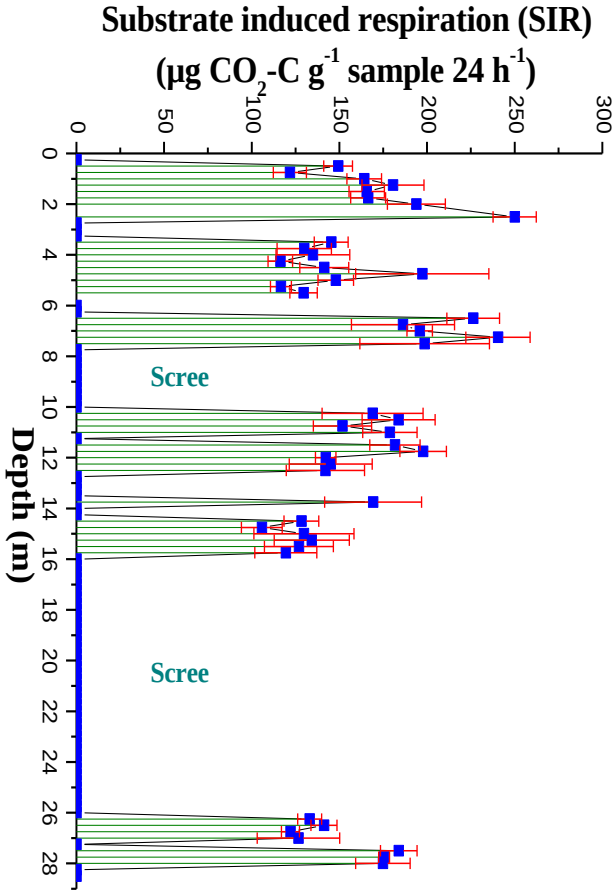


Fig. 3.16: *Basal (A) and substrate induced respiration (B) activities of soils and sediments in exposed Rayka section measuring about 28 m in the vicinity of Mahi river basin. Values are mean of three replicate samples (n = 3) and the error bar represents standard deviation.*

A:



B:



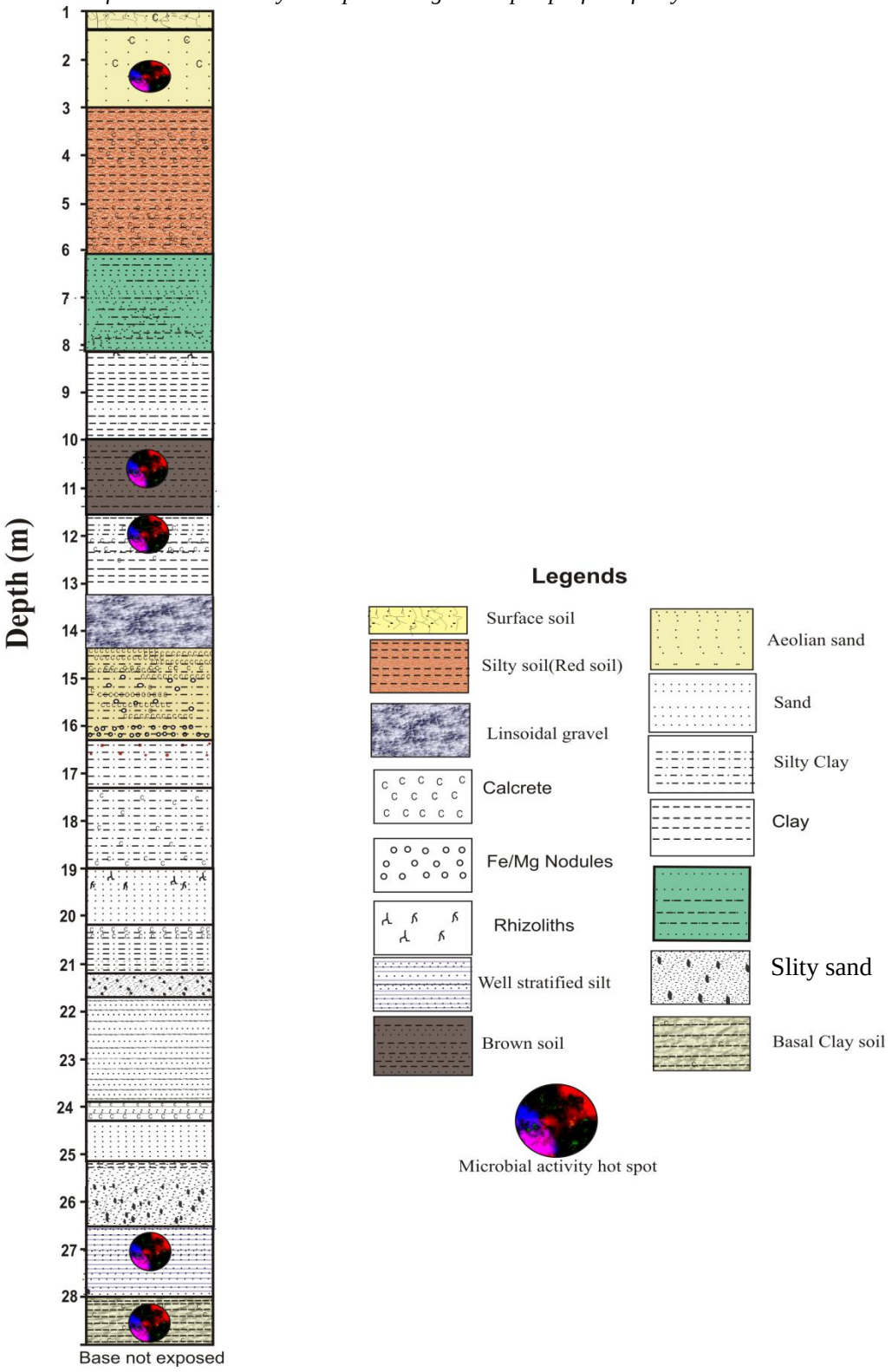
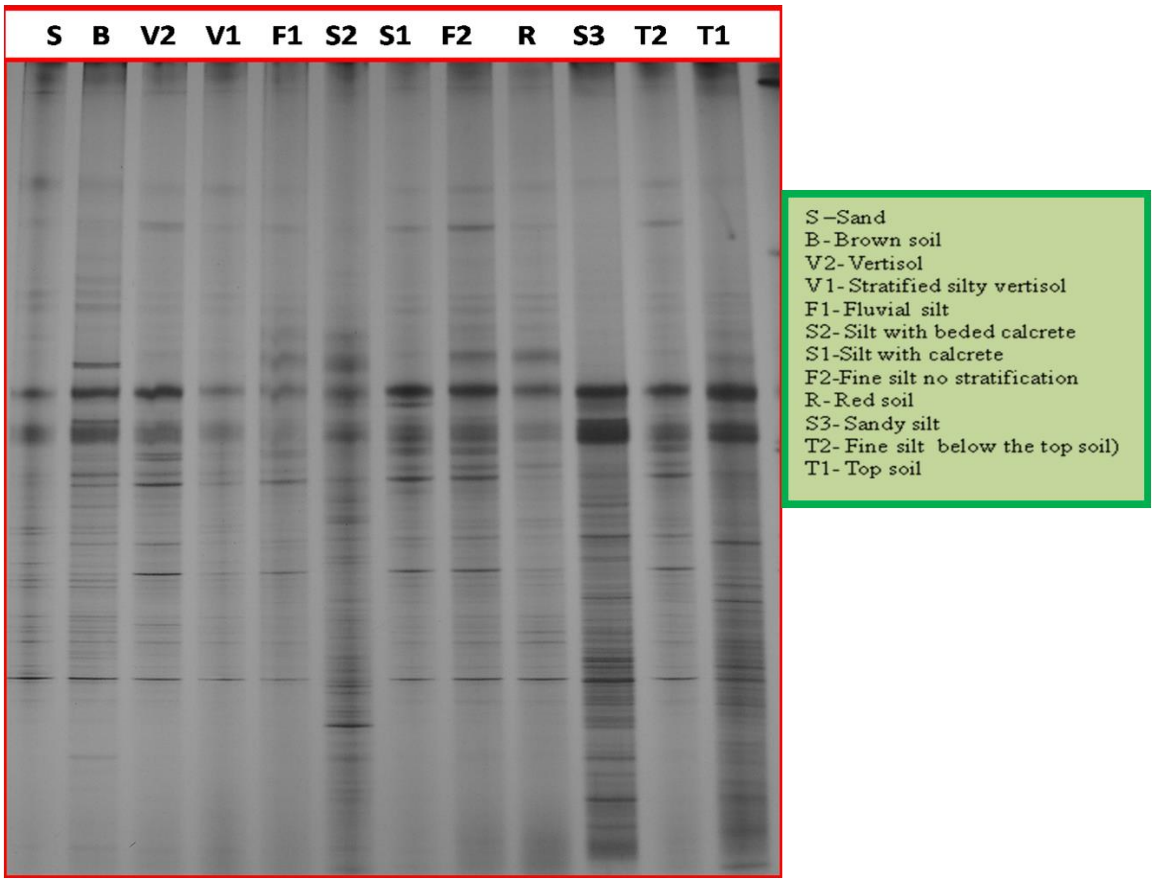


Fig. 3.17: *Distribution of microbial activity hot-spots along the depth profile of Rayka section. Hot spots were determined based on the soil enzymes and BSR activity studies.*

3.4.2.2. Microbial diversity analysis by DGGE

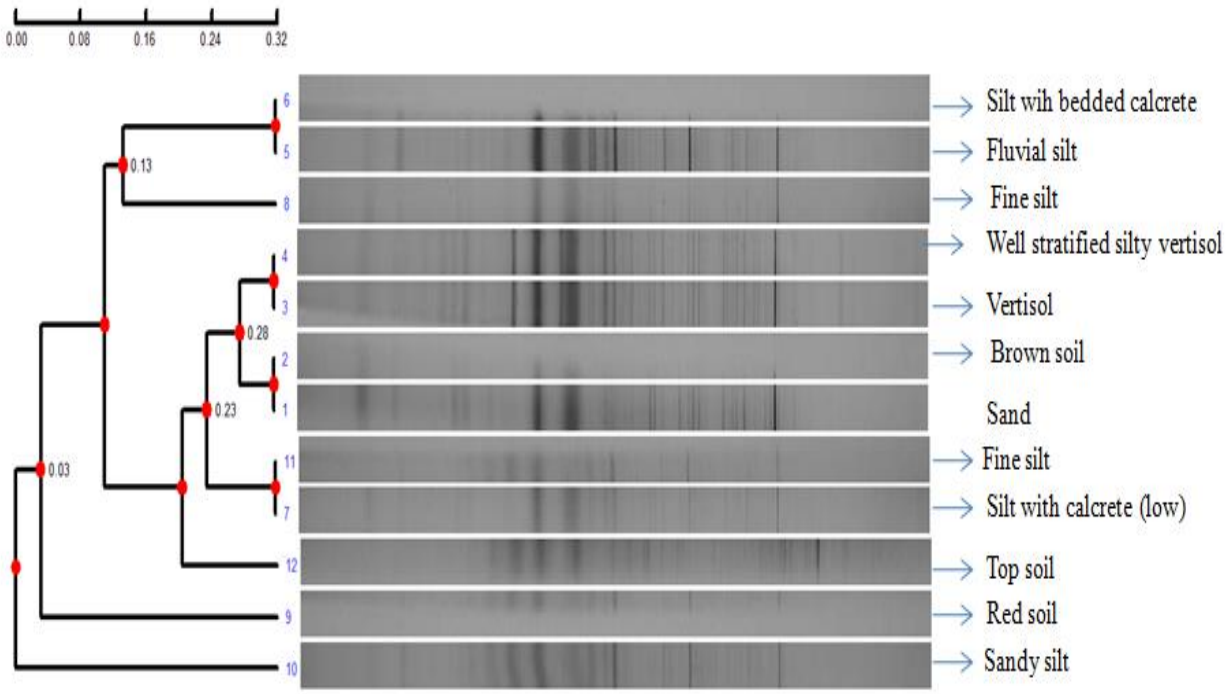
Community DNA was directly extracted from samples of each soil and sediment type. 16S rRNA gene fragments suitable for analysis of denaturing gradient gel electrophoresis (DGGE) were obtained. Molecular fingerprinting of bacterial community of Rayka section by DGGE is depicted in Fig. 3.18.

Fig. 3.18: *16S rRNA based DGGE fingerprintipriting of bacterial community of Rayka subsurface soils and sediments.*



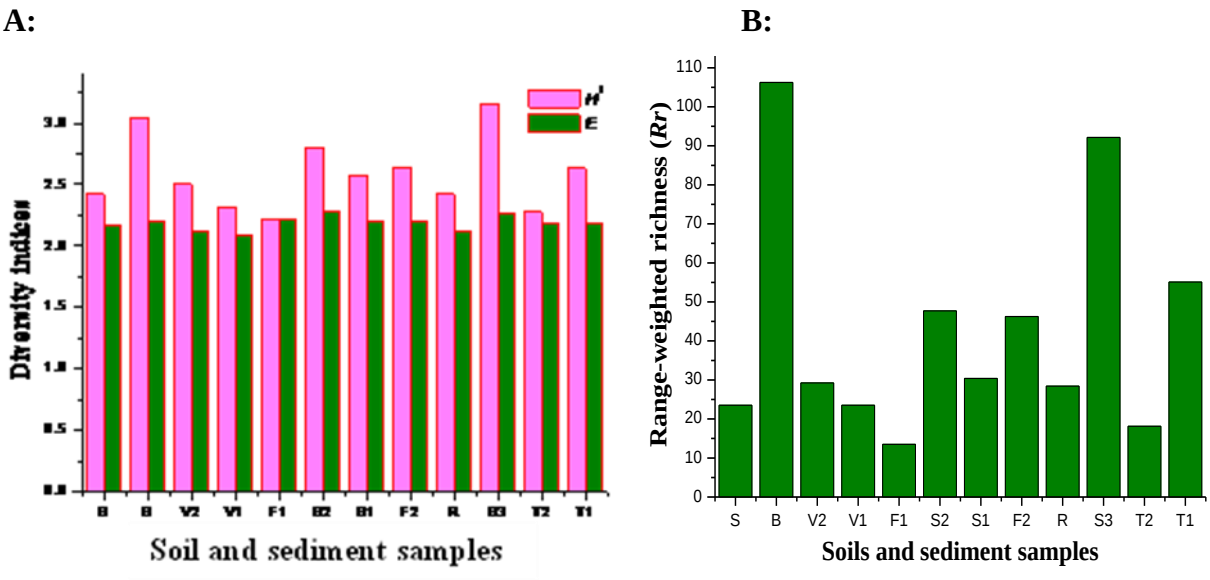
High microbial diversity was noticed in sandy silt (S3), silt with bedded calcrete (S2), brown soil (B) and surface top soil (T1). Most of the bacteria were evenly distributed along the entire profile; however, certain bacterial species were specific to corresponding samples. The subsurface soil samples were grouped based on similarity in bacterial diversity (Fig. 3.19). Vertisol and stratified vertisols had similar kind of bacterial diversity, similarly brown soil and sandy soil had related bacterial diversity.

Fig. 3.19. *Phylogenetic relation and sample relatedness of of Rayka samples based on 16S rRNA DGGE analysis.*



Bacterial diversity of red soil, brown soil and vertisols were distinct from each other. Relatively, red soil had lower bacterial diversity than brown soil. The similarity dendrogram generated by DGGE data cluster analysis (Dice similarity coefficient, UPGMA clustering method) indicated that the samples from each site produced distinct clusters, which could be well discriminated, but not in all cases (Fig. 3.19). In particular, at a 0.32 similarity level, 8 main molecular fingerprinting clusters were identified. *Shannon diversity indices (H^I) ranged from 3 to 2.21. Highest H^I values are noticed with B, S2, S3, F2 and T1, where as lower H^I values are associated with F1, V1 and T2 (Fig. 3.20A).* Pielou's evenness indices (E^I) ranged from 2 (in V1) to 2.27 (in S2). Range weighted richness values (Fig. 3.20B) which indicate broader species carrying capacity ranged from 13.5 (in F1) to 106.2 (in B).

Fig 3.20: *Diversity indices (A) such as Shannon- Wiener species diversity index (H^I) and Pielou's evenness index (E). Range weighted richness indices of the exposed shallow surface are also been shown (B).*

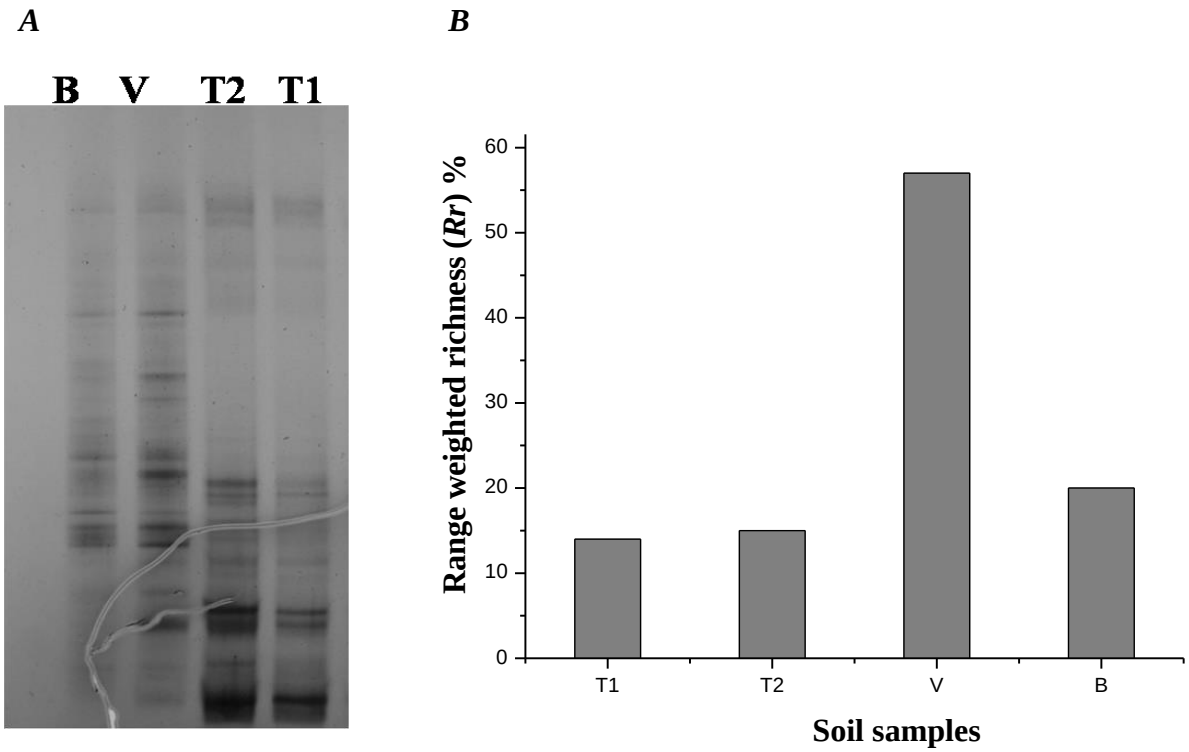


DGGE based α-proteobacterial diversity studies revealed that among the soil hot spots, vertisol (V2) had alpha proteobacteria diversity (Fig. 3.21A) and is an indicative of good soil health than other 3 soil samples distributed in the profile. The H^I diversity and E^I indices for the soil samples were as follows V2> T2>V3> T1. Relatively vertisol (V3) had higher Rr values (Fig. 3.21B) than other microbial hot spots.

3.4.2.3. Relation between soil/sediment physiochemical variables and microbial activity

In order to explore critical determinants that confer higher microbial activity in the exposed subsurface soils and sediments, multivariate statistical analysis was undertaken. Principal component analyses (PCA), a data reduction statistical tool was performed with group-1 variables (12 different Soil physical, organic and inorganic C based variables) of subsurface soils and sediments. PCA loading factors (known as eigenvectors or weights) as well as two dimensional plot for group-1 variables have been shown in the Table 3.8 and Fig. 3.22 respectively. Values for loading factors > 0.55 are shown in bold/italic characters.

Fig. 3.21: *Moleculer fingerprinting of Alpha proteobactreial diversity in vertisol (V), brown soil (B) and top soils (T1 and T2) collected at Rayka. Rr (range weighed richness) values for diversity measurements were also shown (B)*



PCA models were found to be congruent with group-1 data. Principal component 1 (PC1, 58.45%) and principal component 2 (PC2, 16.08%) together explained (eigenvalue > 1.0) 74.54% of the total variance among group-1 data (Table 3.9 and Fig. 3.22). Loading factors indicated that, charcoal, BC, FS, SC, OC, have been represented more correlation along PC1, where as PC2 was associated mainly with sand, clay, CO_3 and LOI.

Table 3.8: Principal component analysis of Group-1 based Soil physical, organic and inorganic C based variables. *PC1 and PC2 are principle component 1 and 2 respectively.*

Variables	**PC1**	**PC2**
pH	0.263	0.064
Sand	-0.511	***-0.570***
Silt	0.079	0.045
Clay	0.574	***0.662***
Charcoal	***-0.908***	-0.151
Brown C	***0.886***	0.099

FS	***0.897***	0.034
SC	***0.771***	0.475
CO_3	-0.127	***0.933***
Loi	0.467	***0.816***
OC	***0.599***	-0.023
SMC	***0.830***	0.377

FS: fungal spores; SC: structured carbon. CO_3: carbonate; OC: Organic carbon; SMC: soil moisture content

Fig. 3.22: *Two-dimensional plot of the principal component analysis (PCA) for the group-1 dataset (12 different soil physical, organic and inorganic-C based variables). Positive relation among the group-1 based physical, organic and inorganic C based variables are also shown with ellipsis 1 and 2.PC1 and PC2 are principle component 1 and 2 respectively.*

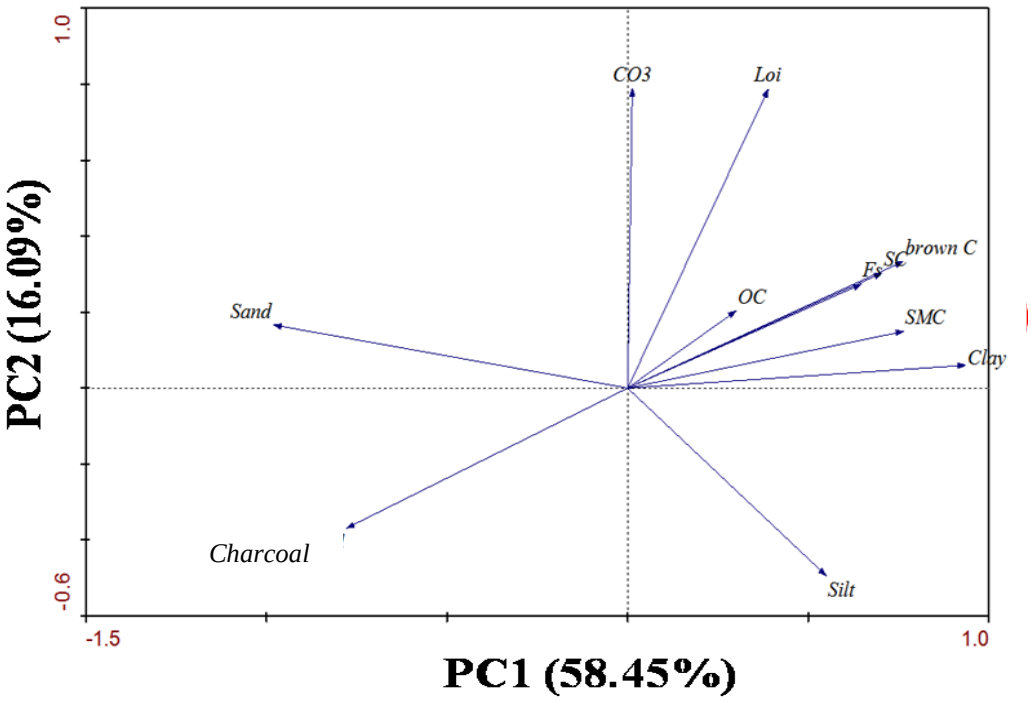


Table 3.9: *Properties of principal components extracted from group-1 data.*

Principal component	Eigen value	Variance among data explained (%)	Cumulative variance (%)
1	**7.01**	**58.45**	**58.45**
2	**1.93**	**16.08**	**74.54**

Extracted PC1 and PC2 components of group-1 variables are further reduced and analyzed for predicting critical determinants for microbial activity in exposed shallow

vadosezone of Mahi basin. Linear regression model between microbial activity and extracted PC components indicated that about 55% of DHA, 71% of APA and 86% of BSR could be explained by extracted PC1 variables (Table 3.10) and the results were highly significant (P <0.001). Group-1 variables of PC2 components are not significant enough to address microbial activity of the section. Both PC1 and PC2 are failed to explain or predict SIR in Rayka soils.

Table 3.10: *Linear regression model for predicting microbial activity with extracted principal components (PC1 and PC2) of group-1. Significance of the model was evaluated by ANOVA*

Microbial	**PC 1**			**PC2**		
activity	**R**	**F**	***P***	**R**	**F**	***P***
DHA	0.554	16.4	0.0001	0.023	0.020	0.887
APA	0.714	38.5	< 0.0001	0.123	0.574	0.453
BSR	0.867	112.8	< 0.0001	0.010	0.007	0.930
SIR	0.056	0.118	0.732	0.23	2.199	0.146

Since, PC1 is found to be critical for the microbial activity, Pearson linear correlation (PLC) analysis (Table 3.11) was performed between PC1 retained variables (with high loading scores) and microbial activity parameters. PLC reveled that among group-1 variables, OC, SMC, FS, BC and SC are the critical determinants for Rayka soil and sediment microbial activity (as an index of DHA, APA and BSR). Charcoal was negatively correlated with microbial activity. Neither positive nor negative correlations were observed in case of SIR.

Similarly, the relation between soil macro and micro elements and microbial activity of shallow subsurface zone was addressed with multivariate statistics. Group-2 data set (22 different soil micro and macro elements of 49 soil and sediment samples from differing horizons) was reduced to identify principal chemical elements for determining microbial activity of exposed section.

PCA loading variables for group-2 was given in the Table 3.12. Values for loading factors > 0.55 are shown in bold/italic characters. It was found that first 6 PCs (eigenvalue > 1.0) together explained 81.5% of total variance in the group 2 chemical data (Table 3.13). Two-dimensional loading plot for PC1 and PC2 of group-2 data

was shown with Fig. 3.23, where the positive relationship among the group-2 data would be seen (3 ellipses). Linear regression model between microbial activity and extracted PC components indicated that the loading variables of PC3 and PC4 can explain 64% of DHA, 55% of APA and about 60% of BSR in Rayka section, nevertheless SIR could not be explain by any mean of PC variables (Table 3.14).

Significant variables of PC3 and PC4 were further analyzed for linear correlation with microbial activity data. Pearson linear correlation analysis (Table 3.15) revealed that Al, Fe, Mg, Mn, Zr are found to be significant positive detriments for Rayka subsurface microbial activity (DHA, APA and BSR). Moreover, microbial activity as an account of BSR is positively correlated with weathering indices "chemical index of alteration" (CIA). Si is found to be negatively correlated with microbial activity in Rayka section. These results are further confirmed with a canonical correlation analysis (CCA) where all the above said variables had significant positive correlation (P <0.05) with microbial activity (Fig. 3.24A and B).

Canonical correlation ordination diagram between the first chemical canonical variate (C-CV1) and the first microbial canonical variate (M-CV1) was significant with a goodness of fit of P = 0.001. Figure 3.24 shows biplot ordinations with soil physiochemical variables represented by points and microbial activity represented by arrows, pointing in the direction of maximum variation, with their length proportional to the rate of change. Each arrow determines an axis on which the variable points can be projected. Variables which are distributed along the direction of arrow indicate positive relationship with microbial activity. The Length of the arrow indicates strengthens of the relationship. The angles between the arrows indicate correlation among individual microbial parameters. It was found that OC, SMC, FS, SC, MgO, FeO, Zr, MnO and Al are critical determinants for microbial activity in Rayka exposed section.

Further, microbial activity (as an index of enzymes and respiration) and their relation with various phosphorus fractions are deciphered by Pearson linear correlation analysis (Table 3.16). Significant positive relation r = 0.8; p<0.001) between APA and OP was observed. A slight negative correlation (r = -3.9) between IP and APA was also noticed at p<0.05. Clay content also showed a positive correlation with APA (r = 0.3, P = 0.05). BSR is positively correlated with both IP (r

= 0.55, P< 0.05) and OP (r = 7.8, p<0.01). Though there is a significant (p<0.05) relation between OP and DHA, the strength of the relation is (r = 3.9) weak. A negative correlation was observed between AIP% and BSR (r = -58) and the relation is significant (p<0.05).

3.4.3. Discussion

Soil microbial activity and diversity are critical for element cycling, organic matter turnover, and maintenance of soil health and function (Nannipieri et al., 2003). Understanding the soil microbial activity and community structure is considered to be important for the sustainability of terrestrial ecosystems. Microbial properties have been proved to be powerful indicators of soil/sediment quality (Kennedy and Papendick, 1995; Bastida et al., 2006; Stark et al., 2008). Previous studies indicated the changes of microbial parameters with respective to erosion, devegetation (Garcia et al., 1997a), pesticides (Lobo et al., 2000), heavy metals (Moreno et al., 2003), fertilizers (Sun et al., 2003; Stark et al., 2007), salinization (Garcia et al., 2000) and land use patterns such as abandonment (Garcia et al., 1997c) and agricultural practices (Bossio et al., 2005). Nevertheless, this information pertained in the semiarid regions of western India is obscure. Moreover, critical determinants to the indigenous microbial activity of semiarid soils and sediments are not yet characterized in Indian subcontinent. In the present study, microbial activity and diversity were linked to changing physicochemical properties as due to differing pedogenic processes along vertical gradients and across contrasting soil and sediment types. In view of this, we have investigated distinct soils and sediments sequences of a typical model section measuring 28 m length under the semiarid climatic zone in the vicinity of Mahi River basin.

In the present study, distribution of microbial activity along the profile was not depth dependent (Fig. 3.15 and 3.16) phenomenon. Previous studies indicated that enzyme activities and microbial diversity often decreased along the depth of surface and subsurface horizons (Niemi et al., 2005; Goberna et al., 2005; Holden and Fierer, 2005; Goberna et al., 2006; Gelsomino and Azzellino, 2011), nevertheless, our results are not comparable to those as the present study is restricted to a unique exposed shallow-vadose zone comprised of distinct soil and sediment sequences. Soil enzyme activity is an important indicator of the potential of the soil microbial activity to support biochemical processes which are essential for the maintenance of soil fertility

(Nannipieri et al., 1990; Garcia et al., 1997b, Obbard, 2001). In the present study, enzyme activities were affected by contrasting soil or sediment type (Gelsomino and Azzellino, 2011). Enzyme dehydrogenase provides correlative information on the microbial composition in the soil. Dehydrogenase activity can reflect the strength of biochemical processes and metabolic status in soil (Garcia et al., 2000; Liu et al., 2008). The enzyme dehydrogenase being one of the principal agent in the degradation of SOM (Tabatabai, 1994; Alef and Nannipieri, 1995), we could infer that the SOM transformation and metabolic state at these samples was relatively high than other samples collected at different depths along the Rayka profile (Fig. 3.15A).

Table 3.11: *Pearson linear correlation analysis of microbial activity and principal soil and sediment variables of group 1data*

Microbial activity	Correlation	DHA	PSA	BSR	SIR	Clay	Charcoal	BrownC	FS	SC	OC	SMC
DHA	Pearson Correlation	1	0.484 (**)	0.456 (**)	0.305 (*)	0.107	-0.407 (**)	0.408 (**)	0.392 (**)	0.307 (*)	0.591 (**)	0.54 (**)
	significance	-	.000	0.001	0.033	0.462	0.004	0.004	0.005	0.032	0.000	0.000
	N	49	49	49	49	49	49	49	49	49	45	43
PSA	Pearson Correlation	0.484 (**)	1	0.617 (**)	0.344 (*)	0.30 2(*)	-0.614 (**)	0.577 (**)	0.690 (**)	0.605 (**)	0.550 (**)	0.61 (**)
	significance	0.000		0.000	0.016	0.035	0.000	0.000	0.000	0.000	0.000	0.00
	N	49	49	49	49	49	49	49	49	49	45	43
BSR	Pearson Correlation	0.456 (**)	0.617 (**)	1	0.013	0.446 (**)	-0.835 (**)	0.819 (**)	0.842 (**)	0.6000 (**)	0.693 (**)	0.81 (**)
	significance	0.001	0.000		0.927	0.001	0.000	0.000	0.000	0.000	0.000	0.000
	N	49	49	49	49	49	49	49	49	49	45	43
SIR	Pearson Correlation	0.305(*)	0.344 (*)	0.013	1	-0.350 (*)	-0.039	0.064	-0.055	-0.065	0.03	-0.16
	significance	0.033	0.016	0.927		0.014	0.792	0.662	0.707	0.659	0.843	0.285
	N	49	49	49	49	49	49	49	49	49	45	43

**** Correlation is significant (*p*) at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed.)**

Table 3.12: *Principal component analysis of group 2 based micro and macro elements. Loadings of variables on six principal components (PC1 to PC6) extracted for Group 2 chemical data. Significant loading variable on principal components are shown with bold numbers.*

Variables	PC1	PC2	PC3	PC4	PC5	PC6
V	-0.111	***0.906***	-0.028	0.112	0.198	-0.160
Co	***0.846***	-0.156	0.090	-0.013	0.139	-0.006
FeO	0.352	-0.081	***0.551***	***0.499***	0.176	0.297
CaO	-0.155	0.075	***-0.893***	0.231	-0.017	-0.13
Na_2O	-0.173	0.078	-0.06	-0.049	-0.111	***-0.934***
K_2O	0.062	-0.72	0.291	-0.314	0.052	-0.049
LOI	-0.163	***0.598***	0.281	***0.554***	0.003	0.186
Ba	-0.230	-0.093	0.350	-0.093	***-0.817***	0.063
Ni	***0.756***	0.275	0.376	0.029	0.261	0.117
Cr	***0.733***	-0.055	0.225	-0.111	0.115	0.306
Sr	0.219	-0.259	-0.258	-0.387	-0.65	-0.058
Rb	***0.708***	***-0.590***	0.184	-0.043	0.062	0.057
Sc	0.344	-0.129	0.076	-0.079	***0.573***	0.459
Y	***0.764***	-0.332	0.108	0.066	-0.187	0.138
Zr	0.130	0.338	***0.502***	0.421	0.445	0.280
Zn	-0.091	***0.960***	0.046	-0.013	0.044	0.045
SiO_2	0.079	-0.345	0.065	***-0.877***	0.011	-0.036
TiO_2	0.411	0.082	***0.736***	0.244	-0.092	0.058
MgO	0.032	-0.101	0.089	***0.804***	0.354	0.074
MnO	0.085	0.116	0.138	0.416	-0.023	0.146
Al_2O_3	0.27	-0.285	***0.691***	0.248	-0.197	0.082
CIA	0.199	0.179	0.436	0.395	-0.160	***0.710***

Table 3.13: *Properties of principal components (PC1 to PC6) extracted from group-2 data.*

Principal component (PC)	Eigen values	% of Variance explained	Cumulative %
1	6.59	29.9	29.9
2	5.06	23.0	53.0
3	2.14	9.7	62.7
4	1.74	7.9	70.6
5	1.22	5.56	76.2
6	1.17	5.32	81.5

Fig. 3.23: *Two-dimensional plot of the principal component analysis (PCA) for the group-2 dataset (22 different soil micro and macro elements). Positive relation among the group-2 based chemical variables are shown with ellipsis 1, 2 and 3.*

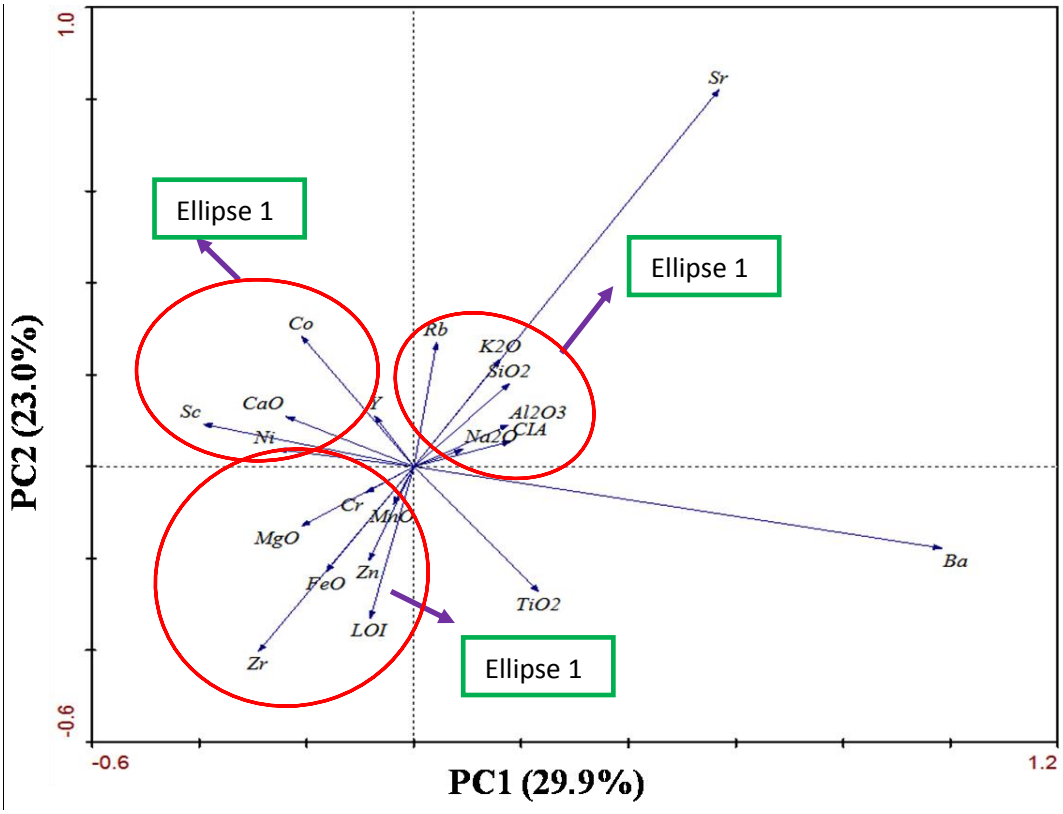


Table 3.14: *Linear regression model for predicting microbial activity with extracted principal components of group 2. Significance of the model was evaluated by ANOVA.*

Component	DHA	PSA	BSR	SIR
PC1	**R=0.135**	**R=0.080**	**R=0.144**	**R=0.230**
	F=0.581	**F=0.200**	**F=0.665**	**F=1.731**
	***P*=0.451**	***P*=0.657**	***P*=0.420**	***P*=0.197**
PC2	**R=0.118**	**R=0.071**	**R=0.062**	**R=0.359**
	F=0.438	**F=0.159**	**F=0.122**	**F=4.606**
	***P*=0.512**	***P*=0.692**	***P*=0.728**	***P*=0.039**
PC3	**R=0.315**	**R=0.188**	**R=0.578**	**R=0.232**
	F=3.433	**F=1.143**	**F=15.61**	**F=1.770**
	***P*=0.073**	***P*=0.293**	***P*=0.0004**	***P*=0.193**
PC4	**R=0.640**	**R=0.550**	**R= 0.551**	**R=0.384**
	F=21.53	**F=13.45**	**F=13.54**	**F=5.368**
	***P*=0.0000**	***P*=0.0009**	***P*=0.0008**	***P*=0.027**
PC5	**R=0.184**	**R=0.408**	**R=0.075**	**R=0.304**
	F=1.095	**F=6.194**	**F=0.180**	**F=3.167**
	***P*=0.303**	***P*=0.018**	***P*=0.674**	***P*=0.084**
PC6	**R=0.124**	**R=0.075**	**R=0.056**	**R=0.149**
	F=0.489	**F=0.176**	**F=0.099**	**F=0.707**
	***P*=0.489**	***P*=0.677**	***P*=0.753**	***P*=0.406**

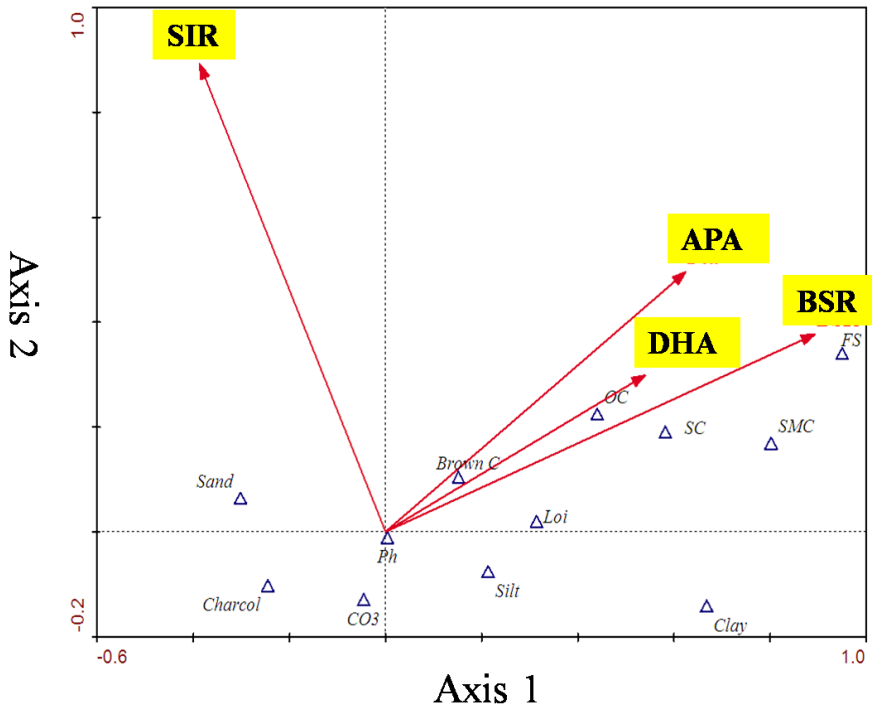
Table 3.15: *Pearson linear correlation analysis of microbial activity and principal sediment variables (group 2).*

Activity	Correlation	DHA	PSA	BSR	SIR	FeO	CaO	Loi	Zr	SiO2	TiO2	MgO	MnO	Al2O3	CIA
DHA	Pearson Correlation	1	0.49 (**)	0.44 (**)	0.36 (*)	0.50 (**)	0.12	0.40 (**)	0.41 (**)	-0.46 (**)	0.21	0.61 (**)	0.45 (**)	0.53 (**)	0.10
	Sig. (2-tailed)		0.001	0.004	0.021	0.001	0.427	0.009	0.008	0.003	0.181	0.000	0.004	0.001	0.548
	N	40	40	40	40	40	40	40	40	40	40	37	38	37	38
PSA	Pearson Correlation	0.49 (**)	1	0.57 (**)	0.43 (**)	0.43 (**)	-0.08	0.09	0.43 (**)	-0.17	0.28	0.59 (**)	0.11	0.13	0.16
	Sig. (2-tailed)	0.001		0.000	0.008	0.005	0.594	0.561	0.006	0.279	0.074	0.000	0.483	0.441	0.312
	N	40	40	40	40	40	40	40	40	40	40	37	38	37	38
BSR	Pearson Correlation	0.44 (**)	0.57 (**)	1	-0.00	0.65 (**)	-0.16	0.35 (*)	0.51 (**)	-0.34 (*)	0.42 (**)	0.59 (**)	0.33 (*)	0.41 (*)	0.50 (**)
	Sig. (2-tailed)	0.004	0.000		0.974	0.000	0.307	0.025	0.001	0.029	0.006	0.000	0.040	0.010	0.001
	N	40	40	40	40	40	40	40	40	40	40	37	38	37	38
SIR	Pearson Correlation	0.36 (*)	0.41 (**)	-0.00	1	0.14	0.15	-0.09	0.02	-0.08	-0.08	0.24	0.15	-0.10	-0.16
	Sig. (2-tailed)	0.02	0.001	0.974	0.40	0.372	0.341	0.577	0.875	0.619	0.602	0.141	0.345	0.540	0.311
	N	40	40	40	40	40	40	40	40	40	40	40	40	40	40

**** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed)**

Fig. 3.24: *Modelling of microbial activity in exposed shallow surface section at Rayka by Canonical correspondence simulation analysis. Microbial activity is predicted with soil and sediment physicochemical characters. A: CCA with Group 1 variables; B: CCA with group 2 variables.*

A:



B:

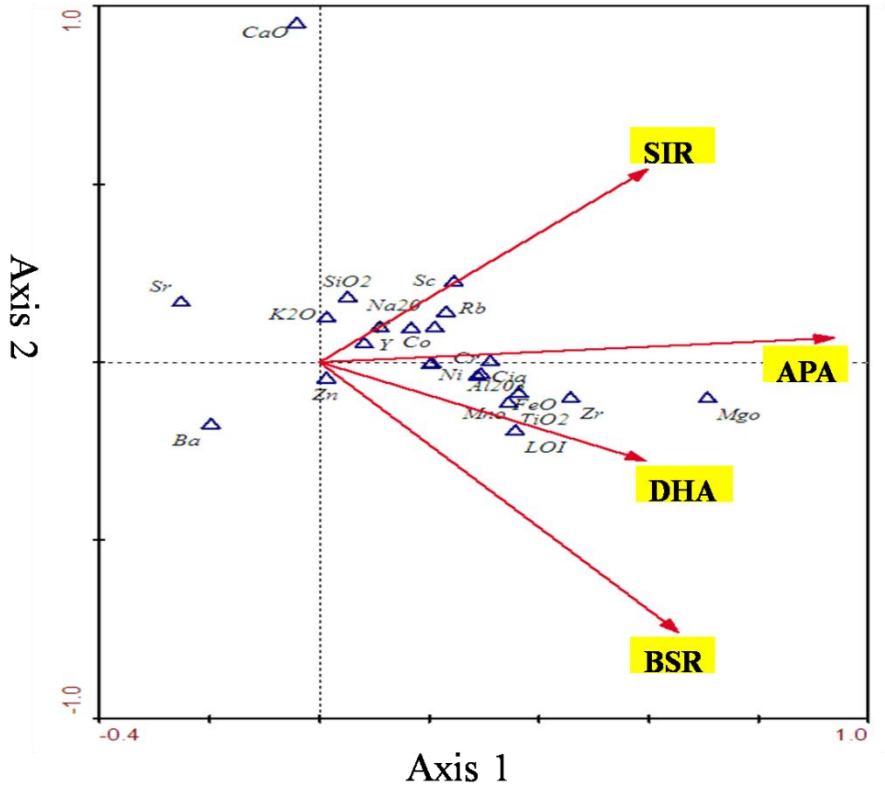


Table 3.16: *Pearson-linear correlation between microbial activity and different phosphorous fractions obtained from Rayka section.*

Microbial activity	Correlation	APA	SIR	DHA	BSR	TP	IP	OP	NAIP	AIP	OP%	NAI %	AIP%
APA	Pearson Correlation	1	0.40	0.499	0.90 (**)	-0.08	-0.39 (*)	0.85 (**)	-0.18	-0.16	0.66 (**)	-0.23	-0.52 (*)
	Sig.		0.133	0.059	0.000	0.765	0.14	0.00	0.517	0.554	0.007	0.40	0.04
	N	15	15	15	15	15	15	15	15	15	15	15	15
SIR	Pearson Correlation	.406	1	0.45	0.37	0.24	-0.04	0.49	-0.05	0.09	0.23	-0.22	-0.11
	Sig.	0.133		0.086	0.167	0.386	0.862	0.063	0.835	0.741	0.400	0.431	0.683
	N	15	15	15	15	15	15	15	15	15	15	15	15
DHA	Pearson Correlation	0.49	0.458	1	0.42	0.23	0.11	0.39 (*)	-0.30	0.08	0.27	-0.36	-0.07
	Sig.	0.059	0.086		0.116	0.409	0.680	0.150	0.277	0.761	0.330	0.181	0.798
	N	15	15	15	15	15	15	15	15	15	15	15	15
BSR	Pearson Correlation	.904 (**)	.36	.42	1	-.21	.55 (*)	.78 (**)	-.23	-.30	.71 (**)	-.19	-.58 (*)
	Sig.	.000	0.167	0.116		0.453	0.031	0.001	0.404	0.278	0.003	0.480	0.021
	N	15	15	15	15	15	15	15	15	15	15	15	15

TP: total phosphorous; IP: inorganic phosphorous; OP: organic phosphorous; NAIP: non-apatite inorganic phosphorous, AIP: apatite inorganic phosphorous.

*** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).*

Phosphatases are group of enzymes that are capable of catalysing hydrolysis of esters and anhydrides of phosphoric acid (Alef et al., 1995). In soil ecosystems, these enzyme activities are believed to play pivotal roles in P cycles (Speir and Ross, 1978) as evidence shows that they are correlated to P stress and plant growth. Phosphatase activity depends on the distribution of phosphatase producing bacteria and the amount of organo-phosphorous substrate availability in the SOM. Obtained results of phosphatases indicate that such bacteria and their substrates are comparatively high at depths of 2 to 2.5 m, 10.25-11m, 11.5 to 12.5 m and 26.75 to 28 (Fig. 3.15B). This is further confirmed with correlation between different phosphorus fractions and phosphates activity. Significant correlation ($r = 0.85$, $p<0.01$) between organic phosphorous (OP) and APA was noticed (Table 3.16). Similar kind of positive relation between APA and OP was obtained in dry-land agricultural soils (Melero et al., 2007). We also found a negative correlation between IP and APA, this may be due to feedback inhibition of inorganic phosphate as suggested by Nannipieri et al. (1979). Similar results were obtained by Madejon et al. (2001) where available IP reduced alkaline phosphatase activity. In our study, the positive effect of organic matter could be more important than the inhibitor effect of the high available IP content. Similar to previous results, a positive correlation between clay content and APA was observed (Gelsomino and Azzellino, 2011). Since, phosphatase activity plays an essential role in the mineralization of organic P in soil (dick et al., 2000), we speculate that portions of 2 to 2.5 m, 10.25-11m, 11.5 to 12.5 m and 26.75 to 28 m in Rayka section would be having important role in biogeochemistry of phosphorous.

The measurement of soil respiration provides an indication of overall biological activity in the soil without having to determine the exact types of organisms present. This method could yield information regarding soil fertility, which is valuable information for agricultural industry. It is known that the soil CO_2 emission rate is determined by microbial activity and is considered as the basic portion of the total carbon flow to the atmosphere (Schlesinger and Andrews, 2000). BSR results were both an indicative of high microbial activity that are existing at the depths of 10.25 to 11 m, 11.5 to 12 m, 16.5 m and 26.25 to 28 and further agreement with enzyme results. Results of SIR were indicative of high distribution of copiotrophic bacteria. Based on the ratios of SIR to BSR, we could infer that along the depth oligotrophic microbes are abundantly distributed.

In the present study, highest microbial activity (as an index of both enzymes and BSR) was considered to be microbial hot spots. The environmental conditions for soil microbes in the hot spots seemed to be improved as reflected by enzyme and BSR. Most of the hotspots were distributed towards the bottom of the profile and not in the surface layer. This could be explained by competition for nutritional resources between root biomass and microbes within the top surface layers, subsequently lower stress may exist towards the bottom of the profile (Agnelli et al., 2001, Villar et al., 2004). Though we have distinguished distinct microbial activity hot-spots of entire 30 m Rayka section (Fig. 3.17), further studies such as microbial biomass, metabolic potential and other soil enzyme activities should be carried out to confirm these hot-spots and their prominent role in various biogeochemical cycles.

In order to distinguish critical determinants that confer higher microbial activity within the hotspots, multivariate statistical approach based on large data set (12 physical, organic and inorganic based variables and 22 chemical variables of 49 samples) was carried out. Multivariate statistical analysis indicates that among the group-1 data, OC, SMC, FS, BC and SC, are the critical determinants for microbial activity in these samples. These results are in congruence with earlier reports; clearly indicate that OC and SMC are the key factors for enhancing microbial activity in a range of soils (Gajananda, 2007; Padre et al., 2007). The higher correlation between TOC contents, enzymes and BSR are noticed by earlier workers (Masciandaro et al. 1997; Madejon et al., 2001; Melero et al., 2007, Goberna et al., 2006). Furthermore, organic matter plays an important role in the protection of enzymes in soil humic-clay complex (Tabatabai, 1994). Among the group-2 macro and micro elements, Al, Fe, Mg, and Mn are found to be critical for microbial activity of Mahi soils and sediments as these elements are the integral part of many proteins and also act as cofactors for various enzymes. Research also suggests that Fe (III) distributed in sediments is an important potential source of electron acceptors that could support microbial activity under favorable conditions (Bishop et al., 2010).

Microbial community plays an important role in element cycling and organic matter turnover in terrestrial ecosystems (Zornoza et al., 2007; Benintende et al., 2008). Bacteria belong to the *α-proteobacteria* are critical for soil fertility/productivity as most of the symbiotic nitrogen fixers affiliated to *α-*

proteobacteria (Young and Haukka, 1998). Diversity indices of α-*proteobacteria* suggest the higher broader carrying capability and corresponding soil quality of vertisol (Marzorati et al., 2008) than other soils. The DGGE fingerprints of 16S rRNA gene fragments revealed a marked similarity among samples and also with distinct patterns being observed for certain sample depth. 16S rRNA-DGGE based fingerprint revealed complex profiles, attributable to the large genotypic diversity of bacteria present in these samples (*Torsvik* et al., 2002). It is also worth noting that an increased diversity (*i.e.*, diversity indices) was observed in DGGE profiles of samples from subsurface layers when compared to those from uppermost horizons, respectively (Fig. 3.18). Compositional shifts in bacterial-community structure and the great number of prominent bands occurring even in deep horizons along the profiles may suggest that bacterial-community structure changed with soil depth and each horizon was conducive to the dominance of particular ribotypes. Several factors have been identified as major determinants of microbial life in soil. Plants (vegetation) are generally being considered as key factors influencing soil microbial communities and their activities through C flow and competition for nutrients (*O'Donnell* et al., 2001; *Dilly* et al., 2004). However, in the present study vegetation is restricted to surface layer (25 cm). We presume that total bacterial- community composition was primarily determined by the underlying soil/sediment chemistry and texture rather than by the different vegetation or cropping regimes *(Wieland* et al., 2001; *Garbeva* et al., 2004; Gelsomino and Azzellino, 2011). These observations are consistent with the significant relationships between bacterial DNA fingerprinting and soil properties (including soil texture, inorganic C, total N, and EC) from a range of soils found by Johnson et al. (2003) and Gelsomino and Azzellino (2011).

3.4.4. Conclusions

Overall, the results show that microbial activity and diversity have been affected by contrasting soil or sediment physico-chemical gradient in semiarid region. Multivariate statistical analysis indicated that OC, SMC, FS, BC, SC, Al, Fe, Mg, Mn and Zr are the critical determinants for microbial activity in exposed shallow subsurface soils and sediments of Mahi River basin. Microbial activity hotspots of shallow subsurface soils and sediments were determined at Ryaka, Mahi River basin.

Chapter 4

Microbial activity and diversity profiles of agricultural, pristine, flood effected and unaffected soils of common sedimentary origin: Case studies from Rayka and Mujpur, Gujarat, western India

4.1. Introduction

The extent of the diversity of microorganisms in soil is seen to be critical for the maintenance of soil health and quality, as a wide range of microorganisms is involved in important soil functions. The impact of human activities on soil quality has increased over the past decades due to population growth and extensive exploitation of natural resources, including soils. Environmental and anthropogenic activities like floods, agriculture practices, industrialization and subsequent pollution can profoundly affect the soil bacterial diversity as well as activity. However, knowledge pertained in these aspects is limited particularly in semiarid landscapes, Gujarat, western India. It is of fundamental concern to understand the response of land use changes on microbial activity in arid/semi-arid landscapes, which occupy about 40% of the planet's surface (Dick-Peddie, 1991). It is documented that the microbes in agricultural soils have profound impact on the soil's fertility, particularly with respect to the availability of plant nutrients (Kennedy and Smith 1995) and suppression of soil-borne plant diseases (van Elsas et al., 2002). Thus, it is primordial to obtain a scrupulous understanding of how, and to what extent, the microbial diversity in soils is affected by agricultural practices (Abawi and Widmer 2000; Nüsslein and Tiedje 1999). For addressing the same, it is essential to compare microbial parameters of agricultural and non-agricultural (pristine) soils. In addition to this, response of agriculture practice on the key microbial functional groups such as diazotrophs (nitrogen fixers) which are crucial for soil fertility need to be addressed.

Biological nitrogen fixation occurs in species of more than 100 genera distributed among various phylogenetic divisions of prokaryotes (Eubacteria and Archaea) (Young, 1992). Over the past century, diazotrophic genera are successfully cultivated from groups as diverse as *Proteobacteria, Green sulfur Bacteria, Cyanobacteria, Firmicutes, Spirochaetes,* and *Archaea* (Izquierdo, 2006; 2007). Many different physiological types of diazotrophs have been isolated from the rhizoplane and rhizosphere (Bagwell et al., 2000a and 2000b), but the true extent of the diversity of these organisms has not been determined. However, it is clear that the soil diazotroph assemblage is quite diverse, highly active under most conditions, and that diazotrophy by these organisms can be responsive to both host primary production and several key edaphic environmental variables. It is also clear that the

diversity of this assemblage is poorly characterized at present, as is the case for most microbial groups (Lovell, 2000).

Soil is said to be heterogeneous medium, both physically and chemically (Ladd et al., 1996) and diazotrophs can be distributed very differently from one cubic centimetre to the next (Vogel et al., 2003; Izquierdo and Nüsslien, 2006). It has been reported that distribution of soil diazotrophs might be influenced by soil texture (Riffkin et al., 1999), nitrogen levels in the soil (Limmer and Drake, 1998) and the vegetation of the environment (Bardgett et al., 1999). However knowledge about the response of agriculture on diazotrophs is not yet understood. Since changes in the activity of soil organisms may reflect changes in soil quality, indicators of microbial activity and function could be used to describe the status and trends in soil conditions due to management practices (Doran and Parkin, 1994; Bucher and Lanyon, 2005).

By characterizing microbial diversity and activity, we may be able to better understand and manipulate soil ecosystem functions because the ability of an ecosystem to withstand serious disturbances may depend in part on the microbial component of the system (Nannipieri et al., 2003).

The objectives for the chapter are as follows

- Comparison of microbiological profiles of agricultural and nonagricultural soils (pristine soils) of common sedimentary origin situated adjacently
- Effect of annual floods on soil microbial activity and diversity

4.2. Comparison of microbiological profiles of agricultural and nonagricultural soils

4.2.1. Materials and Methods

4.2.1.1. Sample collection

Agricultural (Agri) and pristine (Pri) soils studied in the present investigation were collected from two different fields situated near Rayka which is in the semi arid Mahi River basin, Gujarat, western India (Fig. 4.1) with mean annual rainfall about 600 to 650 mm. Pristine soil (22°26'49.02"N, 73° 5'16.48"E) lay about 1.5 km from agricultural soil (22°26'40.49"N and 73° 5'20.92"E). Geologically both the sites have

evolved simultaneously and fall under the same sedimentary origin. No anthropogenic activities were noticed in pristine soil (undisturbed natural habitat); where as in agricultural soil *"Capsicum annuum***"** was cultivated at the time of sampling. No pesticides, herbicides and fungicides have been used in the agricultural soil for the past 3 years. However, organic manure (organic fertilizers/compost) and also conventional fertilizers (little amount) were being applied as a part of sustainable agriculture. Two months before the sampling, the agricultural soil had received synthetic fertilizer at a rate of about 50 kg N ha^{-1}. Prior to the collection of soil samples, the sites were cleaned by scraping the surface layer. Triplicate composite samples were collected from both the fields. Autoclaved polyethylene bags were used for sampling. Samples were immediately kept in a transport box containing ice packs before being transported to the lab. All the samples were sieved (< 4 mm), cleaned of visible roots and plant residues, and stored at 4^{o}C.

4.2.1.2. Soil physicochemical analysis

Soil physicochemical characters such as pH, soil texture, soil organic content, moisture content and elemental analysis were determined with standard protocols (Section 2.1).

4.2.1.3. Soil microbial activity measurements

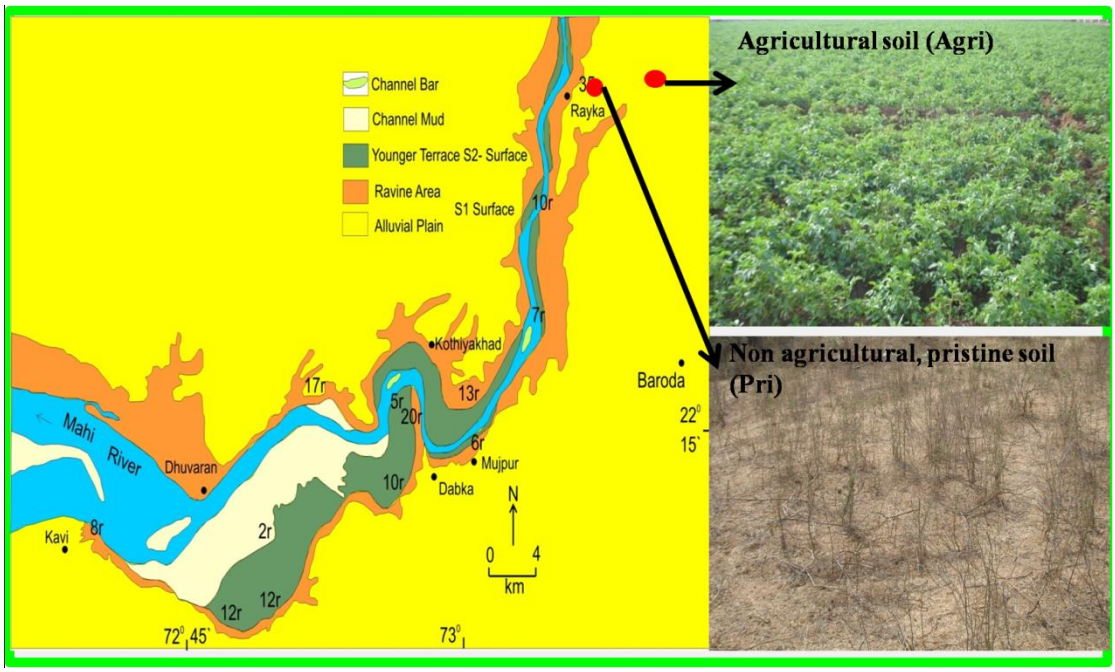
Soil microbial biomass C (C_{mic}), basal soil respiration (BSR) and substrate induced respiration (SIR) have been measured according to the standard protocols (Section 2.2 and 2.3). The microbial q-CO_2 (µg CO_2–C g^{-1} dry soil h^{-1} $24h^{-1}$) was determined. Soil microbial enzyme activities such as dehydrogenase (DHA), protease (PSA), ß-D-glucosidase (GSA) and phosphatase (APA) were determined with standard protocols mentioned earlier (Section 2.3). All the experiments were conducted in three independent replicates (n = 3) and the values are reported with standard deviation.

4.2.1.4. Soil microbial diversity studies

Molecular fingerprinting of the microbial diversity of Agri and Pri samples was established by denaturing gradient gel electrophoreses (DGGE) (Muyzer and Smalla, 1998). Methodology for soil community DNA extraction and 16S rRNA PCR

amplification for DGGE with eubacterial primers GC-341F and 534R are described in Section 2.5.2. The diversity of the microbial community was expressed as the Shannon index of general diversity (H). The range-weighted richness (*Rr*), reflecting the broader carrying capacity of the Agri and Pri samples was determined (Section 2.5.4)

Fig. 4.1: *Geomorphological map showing location of sampling sites at Mahi River basin. Agricultural and pristine soil soils of same sedimentary origin studied in the present investigation were collected at two different alluvial fields situated near Rayka in the vicinity of the semiarid Mahi River basin. Location of the agricultural and pristine landscapes are shown with red and blue circles respectively.*



4.2.1.5. Isolation and enumeration of diazotrophic bacteria

Agricultural and pristine soils were analysed for the total viable N_2 fixers count. A nitrogen free Jenson's agar medium (HiMedia Laboratories, Mumbai, India) was used for the enumeration of culturable diazotrophs (Rao, 1977; Chaintrevil et al., 2000). Ingredients for Jenson's agar as follows (g/l): Sucrose 20; Dipotassium phosphate 1; Magnesium sulphate 0.5; Sodium chloride 0.5; Ferrous sulphate 0.1; Sodium molybdate 0.005; Calcium carbonate 2.0; Agar 15.0.

In brief, 10 g of moist sieved (4 mm) soil was taken and homogenized in 90ml of 0.85% (wt/vol) saline and serially diluted (10 fold) in the same. Aliquots 100µl were spread on triplicate plates of Jenson's agar for enumeration of total viable N_2 fixers

count. Plates were incubated at 30^{o}C for 48 h and colony forming units (CFU) were counted.

Based on the abundance and colony characters, abundant isolates were taken for further analysis. The bacterial isolates were purified on Jenson's agar medium by several rounds of plating preserved in 40% glycerol at -20^{o}C .

4.2.1.6. Molecular diversity of the isolates by Amplified rDNA Restriction Analysis (ARDRA)

Phylogenetic relationship among the Agri and Pri sample bacterial isolates were ascertained by ARDRA. Genomic DNA was extracted from the bacterial isolates and checked. 27F (5l GAG AGT TTG ATC CTG GCT CAG 3l) and 1107R (5l GCT CGT TGC GGG ACT TAA CC 3l) were used for the amplification of 16S rRNA gene fragments suitable for ARDRA. Conditions for PCR and ARDRA procedures were described in Section 2.4.1.3.

4.2.1.7. 16S rRNA gene sequence analysis

As per the ARDRA-dendrogram, from each group representative isolates were subjected to PCR amplification and sequence obtained through the commercial sequencing service by Bangalore Genei (Genei^{TM}, Merck India Pvt. Ltd., Bangalore, India). PCR products were sequenced and the best read from the chromatogram was converted to FASTA format. NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast/) analysis as well as RDP-II project database SeqMatch (http://rdp.cme.msu.edu/seqmatch/) was performed. Multiple sequences alignment was carried out in RDP-II project by taking appropriate standard database sequences and a phylogenetic tree was constructed for the isolates using neighbour joining method in MEGA 5.0 programme (Tamura et al., 2011).

4.2.1.8. Activity and diversity of cultivable diazotrophic bacteria from agricultural and pristine soils

Diazotrophic bacteria of agricultural and pristine soils were enriched by using Jenson's broth (nitrogen free medium) for 10 d in a 500 ml conical flask. In brief, 10 g of each agricultural and pristine soil was individually directly added to 100 ml of Jenson's broth and incubated for 10 d at 30^{o}C. Activity of the diazotrophic bacteria was measured with modified basal respiration method. In brief 1 ml of the

enriched diazotrophic bacteria culture was taken in a closed jar and proceeded for BSR as mentioned in section 2.2.1.

Community DNA was directly extracted from enriched diazotrophs (Sambrook and Russell, 2001). 16S rRNA based diazotrophic community was established with DGGE. Detailed description of the PCR conditions, DGGE methodology was mentioned earlier section 2.5.2.

4.2.1.9. Statistical analysis

All statistical analyses were performed using SPSS version 15.0 and Canoco version 4.0. Pairwise comparisons and quantitative differences between means of microbial activity were analyzed by t-test. In this study, $p<0.05$ was considered to be statistically significant.

4.2.2. Results

4.2.2.1. Physiochemical analysis of agricultural and pristine soils

Physiochemical properties of selected soils are shown in Table 4.1. No significant difference in soil pH was found and both the soils were found to be neutral in nature. Soil chemical parameters were found to be significantly varying between these two soils of the same sedimentary origin. Available P, available K, S content, Zn content, and some other metal ions like Fe^{+2}, Mn^{+2} and Cu^{+2} were comparatively high in agricultural soils (Table 4.1). High levels of available phosphate and potassium in the agricultural soil is the indicative of anthopogenic activity i.e addition of chemical fertilizers. The ratio of % Nitrogen to organic-C was found to be less in non-agricultural soil as compared to the agricultural soil.

There was no significant difference found in soil texture of both the soils and texturally both the soils were loam in nature. This might be as a result of the common origin of from same sediment sequences and geochronologically both soils are of same age. There was no apparent difference in soil moisture content noticed.

4.2.2.2. Soil microbial activity and diversity in agricultural and pristine soils

A significant increase of (P <0.05, LSD) of both C_{mic} and BSR was noticed in agricultural soils (Fig. 4.2A and B). The values for C_{mic} were ranged from 484.53

(±35.6) mg kg^{-1} (Agri soil) to 343.8 (±22.1) mg kg^{-1}soil (in Pri soil). Similarly, significant difference between SIR and q-CO_2 was found (Fig. 4.2C and D).

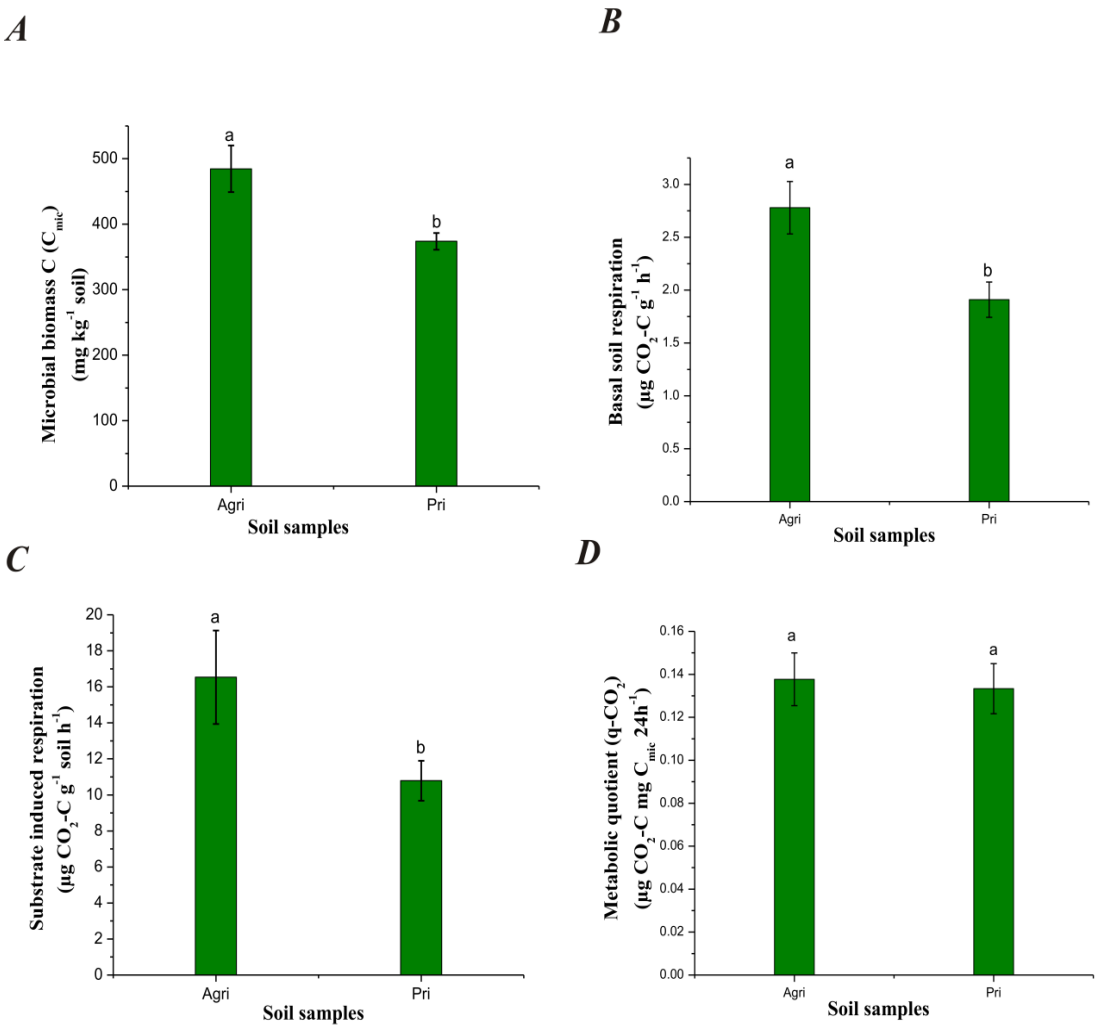
Table 4.1: *Physicochemical characteristics of the agricultural and pristine soils around Mahi River region.*

Soil characteristics	Agricultural soil	Pristine soil
TN /TC (%)	0.13	0.27
Available P (mg kg^{-1})	49	31
Available K (mg kg^{-1})	300	155
pH	7.2	6.92
EC	0.38	0.28
S (mg kg^{-1})	4.6	27.30
Zn (mg kg^{-1})	1.20	4.28
Fe (mg kg^{-1})	6.70	20.28
Mn (mg kg^{-1})	12.2	56.4
Cu (mg kg^{-1})	3.06	2.36
TOC (%)	0.82%	0.45%
Sand (%)	39.33	46.60
Slit (%)	40	33.33
Clay (%)	21.77	20.01
Soil type	Loam	Loam
SMC (%)	21	9

TN: total N; TC: Total C; EC: Electric conductivity; TOC: total organic C; SMC; soil moisture content.

A higher level of soil *in-situ* enzyme activities was noticed with agricultural soils (Fig. 4.3A, B, C and D) and the results were significant (P <0.05, LSD, n = 3). Enzymes DHA, APA, GSA, and PSA were lower by 29.1%, 43.4%, 18.9% and 25.78% respectively in pristine soils. Among all the enzyme activities estimated, APA activity was found to be significantly high in agricultural soil as compared to pristine soil.

Fig. 4.2: *Soil microbial activities of agricultural (Agri) and pristine (Pri) soils. A: Microbial biomass C (C_{mic}); B: Basal soil respiration; C: Substrate induced respiration and D: Metabolic quotient (q-CO_2). Significant differences (P <0.05, t-test) between the soils are indicated by different alphabets. Error bars represent standard deviation of mean (n =3)*



Bacterial diversity of agricultural and pristine soils were investigated with 16S rRNA based DGGE (Fig. 4.4) molecular fingerprinting. Very complex and little contrasting microbial fingerprints were observed in both agricultural and pristine soils. Relatively, high amount of total 16S rRNA based microbial diversity was noticed in agricultural soils than in pristine soils. Shannon-Weaver indices (*H*) for agricultural and pristine soils are 2.68 and 2.21 respectively; whereas evenness (*E*) values are found to be 2.6 and 2.1 respectively. Range weighted richness (*Rr*) for agricultural and pristine soil were found to be about 112.5 and 82.1 respectively.

Fig. 4.3: *Soil enzyme activities of agricultural (Agri) and pristine (Pri) soils. A: DHA; B: PSA; C: APA and D: GSA. Significant differences (P <0.05, t-Test) between the soils are indicated by different alphabets. Error bars represent standard deviation of mean (n = 3).*

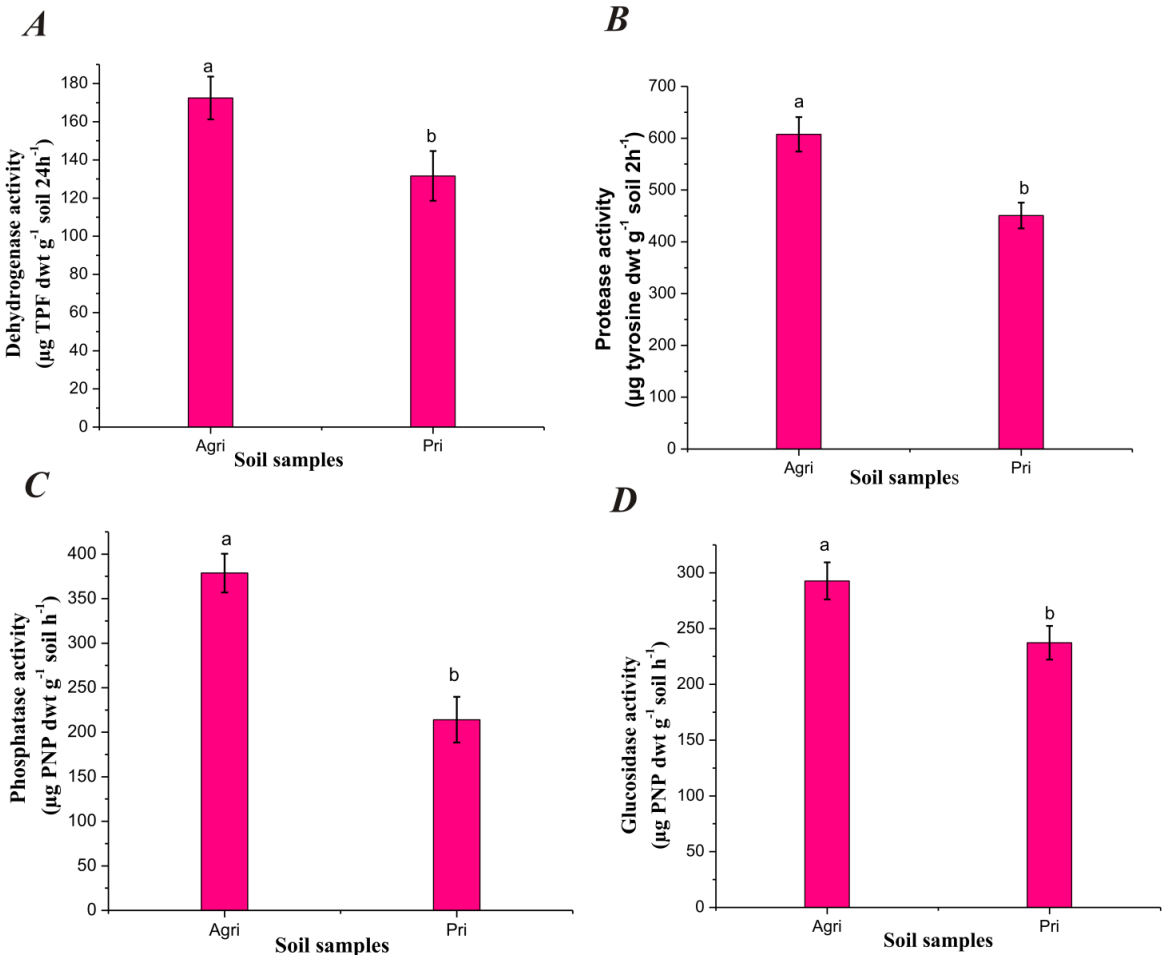
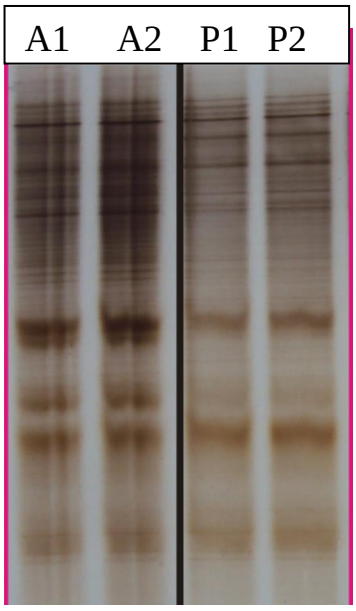


Fig. 4.4: *Soil bacterial community structure of agricultural and pristine soils by 16S rRNA based DGGE analysis. A1, A2, P1 and P2 are (replicate) samples of agricultural and pristine soils respectively.*



4.2.2.3. Isolation and enumeration of diazotrophic bacteria from Agricultural and Pristine soil samples

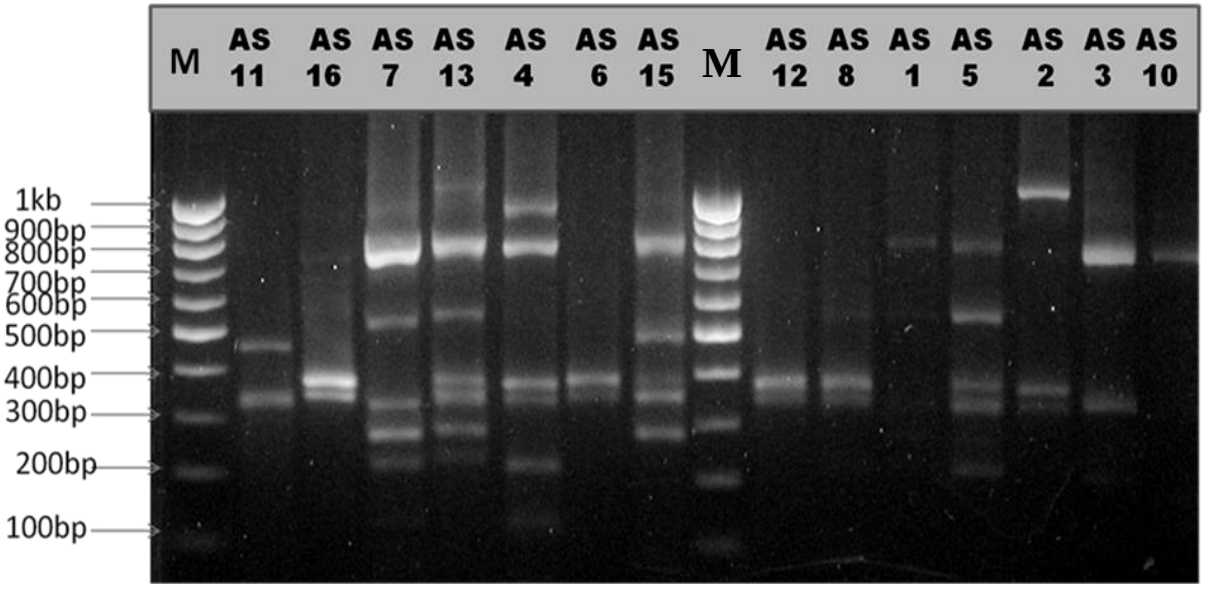
After an incubation period of 48 h, colonies developed on Jenson's agar plates were enumerated. A large discrepancy in total viable count of diazotrophs was noticed. The counts were ranged from 2.5 x 10^6 (in agri soil) to 4.5 x 10^4 (in pri soil). This was further confirmed by most probable number (MPN) method using Jenson's broth. Results obtained from colony characters of agricultural isolates showed a great degree of morphological variation with respect to their colony size, shape, margin, elevation, opacity and pigmentation. It was found that most of the colonies were producing high amounts of exopolysaccharide indicating one of the traits of N_2 fixers to protect their O_2 labile nitrogenase enzyme complex. Many of the colonies were submerged in the Jenson's agar surface. Significant differences in colony characters of non-agricultural soils were found but it was not as much as in case of agricultural soil isolates. Very few colonies were showing pigmentation like as agricultural isolates. These results were shown that comparatively high degree of phenotypic cultivable nitrogen fixer's diversity existed in agricultural soil than in non-agricultural soils.

4.2.2.4. Molecular diversity of the isolates ARDRA

On the basis of the banding pattern of ARDRA with all the bacterial isolates and one standard strain *Azotobacter*, the isolates were grouped (Fig. 4.5.A and B; Fig. 4.6A and B). It can be inferred from both the dendrograms that agricultural soil isolates fall in 12 different phylogenetic groups at a similarity coefficient of 0.90 (Fig. 4.5.B), while at the same similarity coefficient, pristine soil isolates fall in 9 different groups (Fig. 4.6B). These results tentatively indicate that there was relatively higher diversity in culturable diazotrophs of agricultural soil as compared to that in non-agricultural soil. It can be inferred from the Fig. 4.7, that many isolates of agricultural soil and non-agricultural group together indicating their comparatively close phylogenetic relatedness.

Fig. 4.5: *ARDRA pattern of diazotrophic isolates from agricultural soil. A: ARDRA pattern; B: ARDRA based dendrogram represents the phylogenetic relation among diazotrophs by UPGMA method. Isolates phylogeny was compared with standared Azotobacter sp. (AZT). "M" means 100 bp moleculer ladder.*

A



B

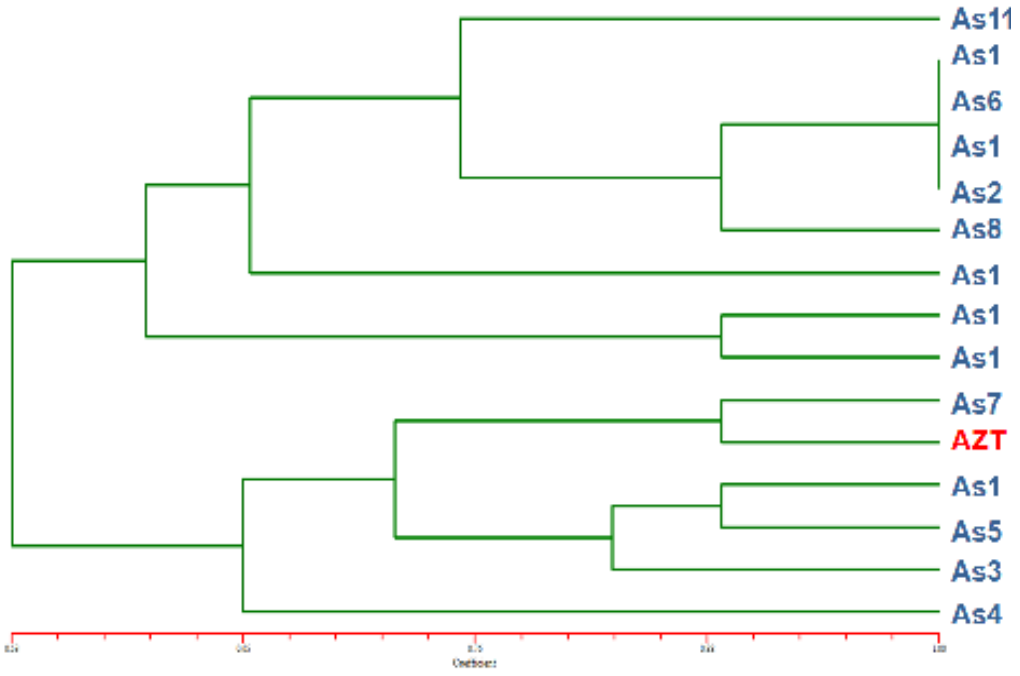
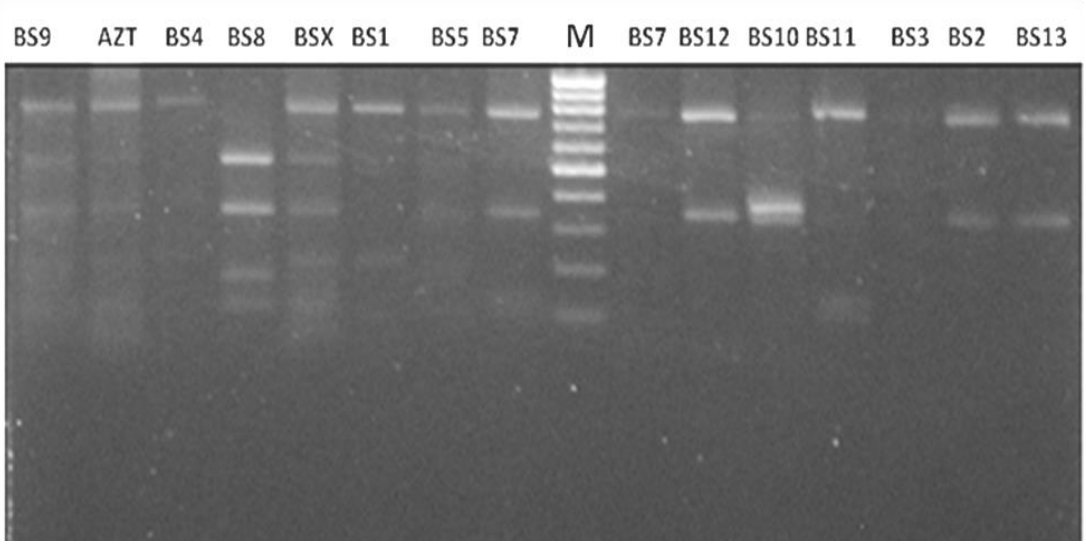


Fig. 4.6: *ARDRA pattern of diazotrophic isolates from pristine soil. A: ARDRA pattern; B: ARDRA based dendrogram represents the phylogenetic relation among Diazotrophs by UPGMA method. Isolates phylogeny was compared with standared Azotobacter sp. (*AZT*). "M" means 100 bp moleculer ladder.*

A


BS9
AZT
BS4
BS8
BSX
BS1
BS5
BS7
M
BS7
BS12
BS10
BS11
BS3
BS2
BS13

B

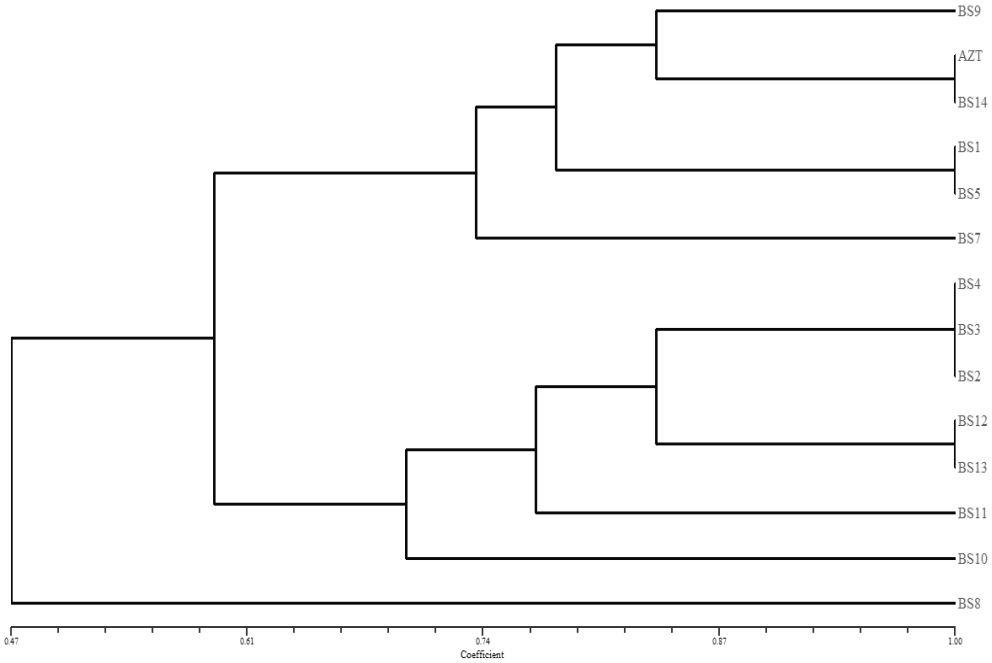
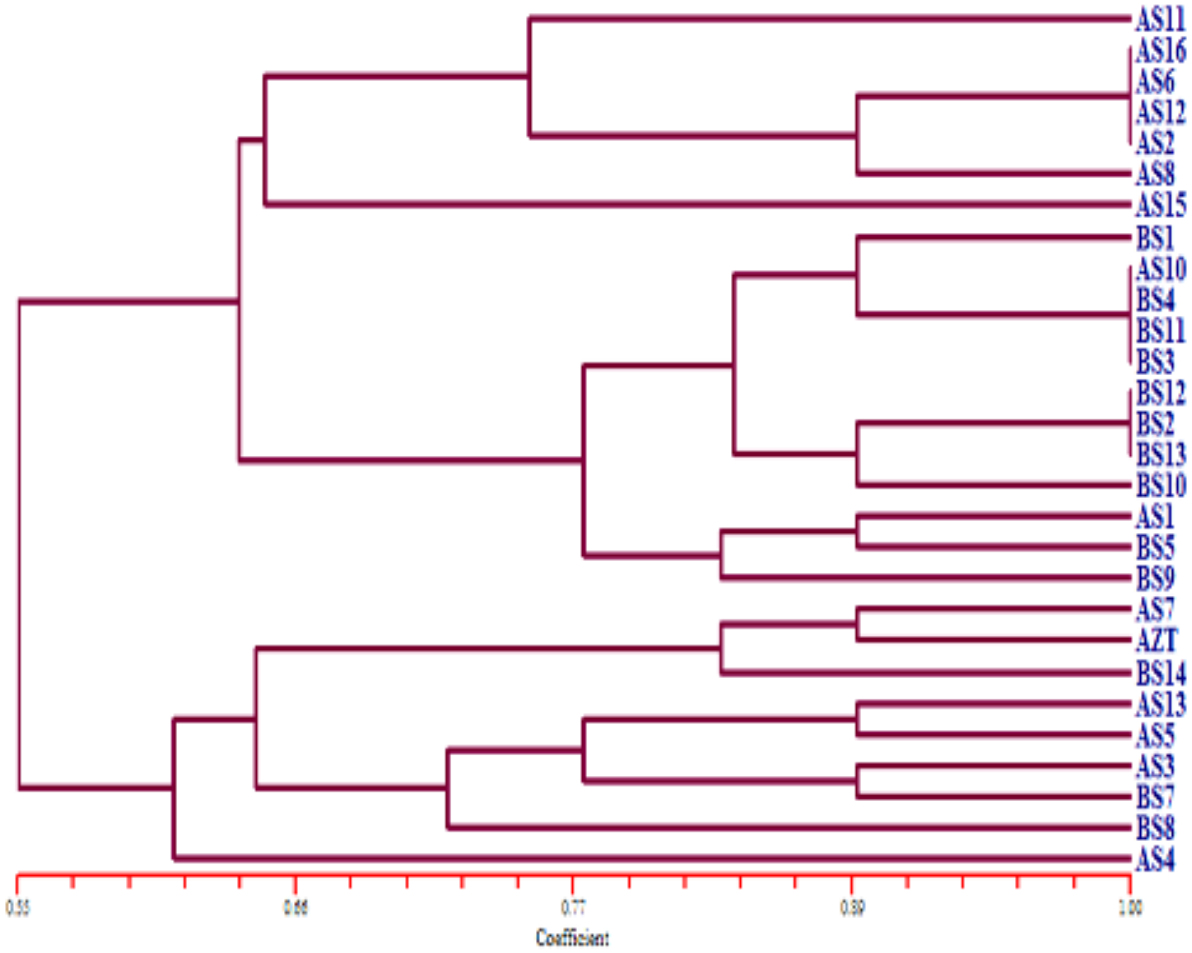

BS9
AZT
BS14
BS1
BS5
BS7
BS4
BS3
BS2
BS12
BS13
BS11
BS10
BS8
0.47
0.61
0.74
0.87
1.00
Coefficient

Fig. 4.7: *Grouping of diazotrophs obtained from both agricultural and pristine soils. Dendrogram was generated based on ARDRA pattern by UPGMA method.*



4.2.2.5. Identification of the diazotrophic bacterial isolates

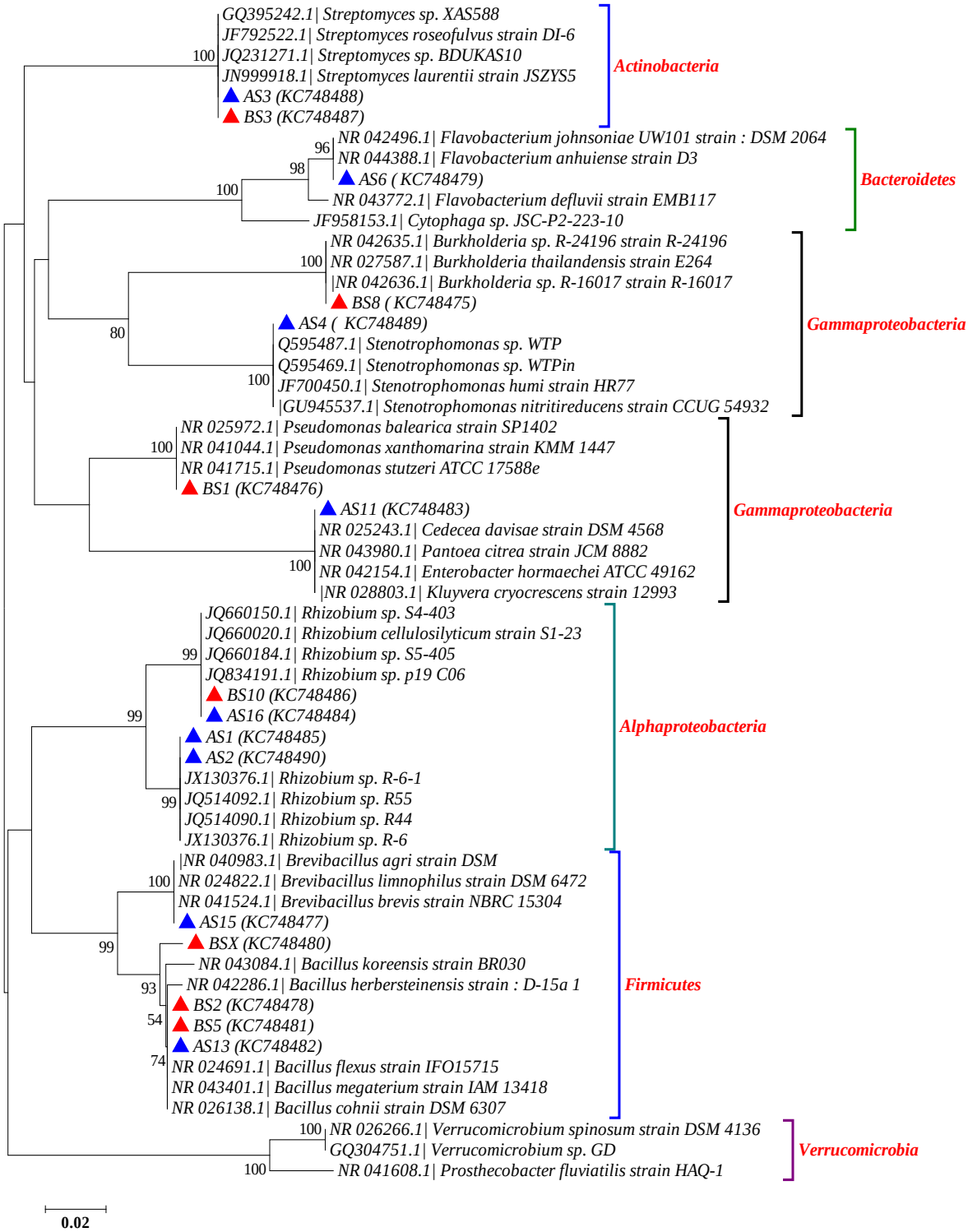
Identification of the bacteria on the basis of partial 16S rRNA gene sequence is summarized in Table 4.2 and the sequence based phylogenetic tree of the diazotrophs is shown with Fig. 4.8. Most of the agricultural soil isolates are represented under phyla *Proteobacteria, Actinobacteria* and group *Firmicutes* whereas in pristine soil, most of the isolates were affiliated to *Firmicutes*. Obtained results noticed that distribution of *Proteobacteria* is low in pristine soil when compared to agricultural soil however more sequencing studies should be undertaken. Comparatively high diversity was noticed in agricultural soil isolates. All the soil diazotrophs identified here are common to recently reported studies on cultured diaztorps obtained from nitrogen free media (Hara et al., 2009; Mirza et al., 2012).

Although, Rhizobium species are symbionts to plants, they can also grow in nitrogen free media (Mirza et al., 2012).

Table 4.2: *Diazotrophic bacterial identity based on 16S rRNA gene sequencing showing closest match.*

Isolate name	Closest match from the GeneBank	GeneBank accession number of the closest match	% similarity
BS1	*Pseudomonas stutzeri*	NR 025972.1	100
BS2	*Bacillus flexus*	NR 024691.1	100
BS3	*Streptomyces roseofulvus*	NR 041120.1	99
BS5	*Bacillus flexus*	NR 024691.1	99
BS8	*Burkholderia* sp.	NR 042636.1	99
BS10	*Rhizobium cellulosilyticam*	NR043985.1	98
BSX	*Bacillus flexus*	NR 024691.1	99
AS1	*Rhizobium* selenitreducens	NR 044216.1	97
AS2	*Rhizobium selenitreducens*	NR 044216.1	98
AS3	*Streptomyces roseofulvus*	NR 041120.1	99
AS4	*Stenotrophomonas* sp.	NR 042569.1	99
AS6	*Flavobacterium anhuiense*	NR044388.1	100
AS11	*Cedecea* sp.	NR 025243.1	99
AS13	*Bacillus flexus*	NR 024691.1	**99**
AS15	*Brevibacillus* sp.	NR041524.1	98
AS16	*Rhizobium cellulosilyticam*	NR043985.1	98

Fig 4.8: *16S rRNA based phylogenetic tree of the diazotrophic bacteria isolated from agriculture and pristine soils. The evolutionary history was inferred using the UPGMA method. Bootstrap values more than >50 are shown next to the branches. The scale bar represents 5% estimated nucleotide sequence divergence. Diazotrophs isolates reported in this study are shown with triangles with different colours (blue for Agri and Red for Pristine soil isolates). Isolates GenBank accession numbers are shown in parenthesis.*



4.2.2.6. Sequence accession numbers

Nucleotide sequences (of bacterial 16S rRNA gene fragments) retrieved from this study have been deposited in the GenBank nucleotide sequence database and the details are mentioned in Appendix 1.

4.2.2.7. Activity and diversity of cultivable diazotrophic bacteria

16S rRNA based diazotrophic community was established with culturable-DGGE (C-DGGE) and activity was determined based on their *in situ* respiration activity. Relatively high amount of BSR respiration activity (88.24± 9.3 µg CO_2-C ml broth^{-1}) was noticed with agriculture soil diazotrophic consortium than in pristine soil diazotrophic consortium (52.84± 9.3 µg CO_2-C ml broth-1). High diazotroph diversity was noticed with agricultural soil (Fig. 4.9) than with pristine soils. These results correlate with previous total soil community DGGE analysis which suggested improved diversity in agricultural land. Diversity indices such as *H* (3.1), *E* (2.64) and *Rr* (40.5) were found to be relatively high in agricultural soil based consortium than in pristine soil *H* (2.8), *E* (2.28) and *Rr* (35.2).

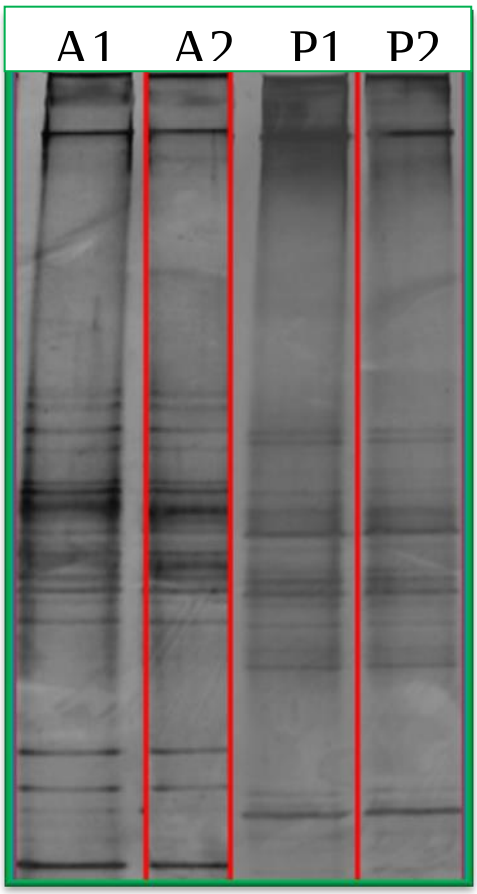
4.2.3. Discussion

Soil management practices such as crop production, crop rotations and agricultural inputs affect soil microbial communities in semiarid landscapes (Bucher and Lanyon, 2005). In the present study, impact of soil management on the quality of semiarid soils is investigated by polyphasic approach. We have chosen two different sites as pristine soils (marked by wild vegetation, situated near Rayka ravines) and adjacent agricultural plot of the same sedimentary origin.

Soil organic matter is primarily plant residues, in different stages of decomposition. The accumulation of soil organic matter within soil is a balance between the return or addition of plant residues and their subsequent loss due to the decay of these residues by microbes. Soil organic content was found to be nearly twice in agricultural soil as compared to non-agricultural soil (Table 4.1). Similar results were obtained by Santos et al. (2012) in Northeast Brazil where organic farming soils had higher organic content than in natural vegetative soils. This variation might be attributed to the residues of crops, increased use of manure and other organic amendments during cultivation in agricultural soil. Weset and Post

(2002) and Leite et al. (2010) found that changes in agricultural management can potentially increase the accumulation rate of soil organic C (SOC), thereby sequestering CO_2 from the atmosphere. Liu et al. (2003) stated that soil organic content may increase in some agricultural management systems. Banerjee et al. (2006) demonstrated that use of organic amendments can increase the soil organic content of alluvial soils and has significant influence on aeration, nutrient availability, moisture holding capacity etc. All these discrepancies may lead to change in the soil bacterial community in agricultural soils.

Fig. 4.9: *Community fingerprints of enriched diazotrophic populations of agricultural and pristine soils by 16S rRNA based DGGE analysis. A1, A2 are agricultural samples, while P1 and P2 are pristine soils.*



Microbial biomass carbon (C_{mic}) and soil respiration are considered as important soil biological activities frequently influenced by various perturbations (Chodak and Niklińska, 2012; Santos et al., 2012). Understanding the environmental as well as anthropogenic influences on C_{mic} and soil respiration is the key to envisaging changes in soil nutrient cycles (Gregorich et al., 2000; Haney et al., 2001; Marinari et al., 2006; Mathew et al., 2012; An et al., 2012). In the past, C_{mic} has been used as meaningful and sensitive indexes of soil quality evaluation (Xu et al. 2008; Khan et al., 2010; Zhang et al., 2010; An et al., 2012; Santos et al., 2012). In the present study, relatively agricultural soil had 29.65% more C_{mic} and 69% more BSR than in pristine soils. These results are in accordance with previous reports indicated that agriculture management can potentially affect both the soil C_{mic} and respiration (Garcia-Orenes et al., 2010; Mathew et al., 2012). Santos et al. (2012) also found relatively higher C_{mic} in agricultural plot (organic forming) than in natural vegetative plot (pristine soils). With the values of both BSR and SIR, it can be inferred that the microbial activity of agricultural soil have greater potential in SOM decomposition and carbon flow to the atmosphere.

Extracellular hydrolytic enzyme activities (EHE) in soils have been investigated for more than a century with a goal to understand the biogeochemistry of nutrient cycles. They have also been studied in relation to ecosystem responses to global change and other disturbances (e.g. Lipson et al., 2005; Finzi et al., 2006; Hu et al., 2013; Zhang et al., 2013). Several enzyme activities are known to be involved in the cycling of nutrients and can be used as potential indicators of nutrient cycling processes (Dick, 2000). In order to evaluate the fluxes of elements in agricultural soils, extracellular microbial hydrolases such as GSA, PSA and APA involved in C, N, P cycles respectively (Masciandaro and Ceccanti, 1999) were determined. Agricultural soil had relatively higher microbial enzyme activities than pristine soils. Similar trend was observed by Daniel et al. (2013) where significant higher enzyme activities (β-glucosidase, dehydrogenases and acid phosphatase) are noticed in agro-ecosystem than in undisturbed natural habitat. Therefore, it would infer that the microbial mediated soil C, N and P cycles considerably increases in soils as a result of agriculture practice (mainly organic forming).

It is been reported that DHA is associated with living cells, linked with microbial oxidoreduction processes and is the principal agent in the degradation of

SOM (Tabatabai,, 1994; Alef and Nannipieri, 1995). High amount of DHA was associated with agriculture soil and is in general agreement with high microbial respiration activity (Fig. 4.2B), indicating that organic matter decomposition is much active in agricultural soils.

The most valuable indicators of soil microbial activity are microbial biomass C and soil respiration, while specific indicators are related to the activity of extracellular hydrolytic enzymes such as phosphatase and glucosidase, involved in C and P nutrient cycling (Gil-Sotres et al., 2005; Daniel et al., 2103). The evaluation of biological and biochemical soil properties and glucosidase activity has been suggested because of their relationship to the soil C cycle and the sensitivity of these indicators to detect changes resulting from agricultural management practices (Nannipieri et al., 1995; Gil-Sotres et al., 2005; Lagomarsino et al., 2009; Daniel et al., 2013). The activity of phosphomonoesterase enzymes, such as acid and alkaline phosphatases, has been widely studied because of its importance in organic P mineralization, releasing orthophosphates that are readily assimilated by plants and soil microorganisms (Sylvia et al., 1999).

Relatively, high amount microbial diversity was noticed with agricultural soils than pristine soils. Nevertheless, it is virtually a complex subject and not yet known which management regime can confer high microbial diversity as diverse management practices such as irrigation, amendment of fertilizer/ compost, tillage and organic residue incorporation taking place at these soils. These results are in congruence with previous investigations pointed that the agricultural practice or soil management regime cans potential effect soil bacterial diversity (Garbeva et al., 2004). Earlier it has been reviewed that soil type, plant type and agriculture management regimes are the critical determinants for microbial diversity in a range of semiarid soils (Garbeva et al., 2004). Since the studied soils are the same type, the observed high diversity in agricultural soils could be due to either agronomic management regime and/or crop type. Furthermore, ARDRA results depict comparatively higher diversity of culturable diazotrophs in case of agricultural soil. This could be due to high organic content of agricultural soils. Mireles et al. (2007) also found a positive correlation between soil organic content and diazotrophs diversity. The semiarid pristine soils possessing lower bacterial diversity might be having higher relative abundances of genes associated with osmoregulation and

dormancy, but lower relative abundances of genes associated with nutrient cycling as suggested earlier metagenomic studies (Fierer et al., 2012).

Free-living diazotrophs are diverse and ubiquitous in soil. In the present study, we have obtained phyla *Alphaproteobacteria, Gammaproteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes* by nitrogen free Jensens medium. Similar type of cultivable free living soil diazotrophs was isolated with nitrogen free media (Hara et al., 2009; Mirza et al., 2012). Obtained results indicate that relatively distribution of proteobacteria is low in pristine soil as compared to agricultural soil (however more sequencing studies should be undertaken). It is hypothesized that the observed *diversity* resulted from the adaptation to fluctuating conditions ((Poly et al., 2001). In their study remarkable proteobacterial diversity in diazotrophic community was noticed; nevertheless their studies were mainly aimed at studying total diazotrophic diversity in rhizosphere of agricultural soil.

With these results of microbial activity and diversity studies, it can be inferred that the pristine soil quality might be improved by agricultural practice in semiarid tropics of this particulate Mahi River region. Previously, rapid improvement of soil quality has been observed in semiarid Mediterranean agro-ecosystems due to carrying out sustainable agriculture (Garcıa-Orenes et al., 2010). The results indicate that the spatial variation of the soil characteristics, and in particular the organic C content, had a major influence on the variability of the soil microbial parameters as suggested earlier (Vinther et al., 2012)

4.2.4. Conclusions

DGGE results indicated the higher diversity of eubacteria in agricultural soil as compared to pristine soil. Agriculture practice enhanced semiarid soil quality in terms of several microbial parameters including the diazotrophic bacterial population size and diversity. Phyla *Proteobacteria* was diverse in agricultural soil while in natural soils majority of the nitrogen fixers are representative of *Firmicutes* however more sequencing studies should be undertaken. Based on ARDRA pattern and sequence analysis, it can be inferred that comparatively high diversity of nitrogen fixers were noticed in agricultural soil. Semiarid soil management practices influenced soil physical and chemical characteristics and brought about changes in the soil microbial community structure and function.

4.3. Microbial activity and diversity in flood inundated agricultural field, near Sarod, Mahi River basin, Gujarat, western India

4.3.1. Materials and methods

4.3.1.1. Sample collection

The sampling sites are located about 2 km from Mujpur, Mahi River basin. Sampling method was randomized block design with triplicate composite sampling. Three soil samples named as S1 (0 -10 cm), S2 (10-20 cm) and S3 (20-30 cm) were collected with an interval of 10 cm from an agriculture field which is inundated with fluvial sediment (Fig. 4.10). The field had been effected by 3 times with flooding events during the last decade (Table 4.3). In this area flooding occurred due to Mahi River overflow and surface runoff in monsoon season (Monsoon downpour). During flooding event, piles of fluvial sediment have been accumulating in the effected field for more than 5 years. Control reference soil named S4 was collected from nearby unaffected agricultural field from Pilludra. In flood affected field, soil is mixed-up with fluvial sediment that consisted of higher amount of sand and silt (Fig. 4.10D). On January 21st, 2007, triplicate composite samples are collected in sterile polyethylene bags and immediately kept in ice box before being transported in to the lab. The soils were stored at 4^{o}C and -20^{o}C for microbial and molecular analysis respectively.

4.3.1.2. Soil physiochemical analysis

Soil physiochemical characters are determined as mentioned in chapter2

4.3.1.3. Soil biochemical analysis

Microbial biomass carbon, BSR, SIR, q-CO_2 were determined. Soil enzymes such as dehydrogenase (DHA), protease (PSA), glucosidase (GSA) and phosphatase (APA) were estimated by standard protocols discussed (Section 2.2 and 2.3).

Table 4.3: *History of flooding events (major) at the Mujpur sampling site during last decade (flood level and elevations are with respect to sea level).*

Flood dates	Flood duration	Flood elevation	Flood level	Flood depth
14-8-04	2 d	30 m	76 m	40 m
7-9-06	3 d	15 m	38 m	20 m
12-8-06	3 d	15 m	32 m	18 m

4.3.1.4. Soil microbial analysis

Total viable heterotrophic counts were enumerated with Reasoner´s 2A (R2A) agar (Section 2.3.2). Fast growing (copiotrophs or r- *strategists*) and slow growing (oligotrophs or K- *strategists*) were enumerated with modified peptone-glucose-yeast extract agar (M-PGY) supplemented with soil extract (Lorch et al., 1995; Alef, 1995). Soil extract contains growth factors, trace elements, organic substances. The composition (g L^{-1}) for M-PGY is as follows: glucose 0.25, peptone 0.25, yeast extract 0.25, dipotassium phosphate 0.10, magnesium sulfate heptahydrate 0.05, ferrous sulfate heptahydrate 0.02, soil extract 17.75 ml, agar 15.0, final pH (at 25°C) 6.8±0.2. Soil extract was prepared as follows: 500 g of soil was suspended in 1000 ml distilled water and boiled for 2 h. After cooling, the solution was filtered and then added to the medium as mentioned above. Colonies grown within 2 d were considered as fast growers (copiotrophs); while colonies that had grown within 3 to 7 d were considered as slow growers (oligotrophs).

Different physiological groups of bacteria such as cellulose degraders, nitrogen fixers, denitrifiers, sulfate reducers and iron oxidizers were enumerated with most probable number (MPN) method with statistical probability tables (Meynell et al 1965; Deman, 1983). The MPN counts of bacterial populations are expressed per gram of the soil by multiplying the tabulated results with dilution factor (Koch, 1981; Alexander, 1982). The protocols followed were based on Lorch et al. 1995 and the details are Table 4.4). The number of the phosphate solubilizing bacteria or enumeration of mineral phosphate (tricalcium phosphate) *solubilizing* bacteria was done by counting the

colony forming units (CFU) that showed zone of clearance on Pikovskaya's agar (HiMedia Laboratories, Mumbai, India) plates incubated at 30^0C for 48h. For this, 10 g of moist sieved (4 mm) soil was homogenized in 90 ml of 0.85% (w/v) saline and serially diluted (10 fold) in the same. Aliquots (100 µl) were spread on triplicate plates of Pikovskaya's agar (HiMedia Laboratories, Mumbai, India).

Fig 4.10: *General view of the sampling sites. A and B: Flood inundated agricultural field. Sample S1 is surface soil where as samples S2 and S3 collected at depth interval of 10 cm (shown in the inset). C: Unaffected control reference agricultural soil (S4). D: over view of the fluvial sediment in the S1 agricultural fled. The fluvial sediment is majorly comprised of sand and silt. Piles of fluvial sediment have been accumulating in these agricultural fields during annual floods.*

Table 4.4: *Culture media used for enumerating some functional groups of soil bacteria by MPN method.*

Target physiological group	Culture media	Reference
Denitrifiers	Casamino acid -yeast extract broth Composition: g L^{-1} : Casaminoacid 5.0, Yeast extract 5.0, Potassium nitrate 6.0, Sodium chloride 3.5, pH 7.2±0.2 **Positive reaction:** Gas in Durham vial is considered as positive	Tomlinson and Hochstein, 1972; Lorch et al., 1995.
Nitrogen fixers	Jensen's Medium. Composition: g L^{-1} : Sucrose 20.0, Dipotassium phosphate 1.0, Magnesium sulphate 0.50, Sodium chloride 0.50, Ferrous sulphate 0.10, Sodium molybdate 0.005, Calcium carbonate 2.0, **Positive reaction:** Selective media, nitrogen fixers can only grow, luxuriant growth	HiMedia Laboratories, Mumbai, India; Rao, 1977
Phosphate solubilizers*	Pikovskaya's agar. Composition: g L^{-1} : Yeast Extract 0.50, Dextrose 10.00 Calcium Phosphate 5.00 Ammonium Sulphate 0.50 Potassium Chloride 0.20 Magnesium Sulphate 0.10 Manganese Sulphate 0.0001 Ferrous Sulphate 0.0001	Himedia Laboratories, Mumbai, India; Rao, 1977.

	Agar 15.00 **<u>Positive reaction:</u>** Clear hollow zone surrounding the colony.	
Cellulose degraders	Cellulose mineral salt broth, Composition: g L^{-1} : Cellulose (filter paper strip), Yeast extract 0.02, Dipotassium hydrogen phosphate 0.5, Potassium dihydrogen phosphate 0.3, Magnesium sulphate heptahydrate 0.2, Ferrous sulphate heptahydrate 0.1, pH 7.2±0.2 **<u>Positive reaction:</u>** Degradation of the filter paper strip in the standing tubes after shaking the culture by hand at 1 week interval for 5 weeks is considered as positive	Sato et al., 1984, Lorch et al., 1995.
Sulfate reducers **(H_2S producers)**	Lactate-thioglycolate-sulphate broth. Composition: g L^{-1} : Sodium lactate 5.0, yeast extract 1.0, Dipotassium hydrogen phosphate 0.5, Ammonium chloride 1.0, Calcium chloride 0.1, Magnesium sulphate heptahydrate 3.5, Sodium thioglycolate 0.5, Ferrous ammonium sulfate 0.1, Agar 2, pH 7.2±0.2 **<u>Positive reaction:</u>** Blackening of Medium	Lorch et al., 1995.
Iron oxidizers	Iron oxidizing agar (twin pack). Composition: g L^{-1} : <u>Part A</u> Ammonium sulphate 3.000, Potassium chloride -0.100, Dipotassium phosphate 0.500, Magnesium sulphate heptahydrate 0.500, Calcium nitrate 0.010,	HiMedia laboratories, Mumbai, India; Eaton et al., 2005.

	Part B - Ferrous sulphate 44.220, Final pH (at 25°C) 3.3±0.2 **Positive reaction:** An increase of deep orange brown colour of the medium

*: Phosphatase solublizers are enumerated by plating method on the basis of zone of clearance

4.3.1.5. Identification of bacterial isolates

Dominant culturable heterotrophic bacteria from S1 and S4 samples were identified by 16S rRNA sequencing analysis. Total of 10 dominant isolates (5 each from S1 and S4) based on their colony morphology were subjected to sequencing analysis of 16S rRNA gene fragments. In brief, genomic DNA was extracted from bacterial isolates. Universal eubacterial primers 27F (5'-GAG AGT TTG ATC CTG GCT CAG-3') and 1107R (5'-GCT CGT TGC GGG ACT TAA CC-3') were used for the amplification of 16S rRNA gene fragments suitable for sequences analysis (Section 2.4.1.2**).** The best read from the chromatogram was converted to FASTA format and used for NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/) analysis as well as RDP (version 10) seqmatch tool (http://rdp.cme.msu.edu/*)*. Multiple sequence alignment was carried out in RDP (http://rdp.cme.msu.edu/myrdp) along with representative sequences from database. A phylogenetic tree was constructed using neighbour joining method in MEGA 5.0 (Tamura et al., 2011).

4.3.1.6. Soil Microbial diversity studies by DGGE

Soil community DNA was extracted from S1, S2, S3 and S4. Detailed descriptions for soil community DNA extraction, PCR conditions, DGGE methodology are as given in Section 2.5.

4.3.1.7. Statistical analysis

One-way analysis of variance (ANOVA) was used to compare soil properties among four different samples. Mean values (N = 3) of soil C_{mic}, BSR, SIR, q-CO_2 and soil enzymes were tested for differences among sites with Fischer's LSD test (LSD).

In the present study P <0.05 was considered as statistically significant. Relation between soil characteristics and microbial activity was investigated by canonical correspondence analysis (CCA) with Canoco for Windows 4.5.

4.3.2. Results

4.3.2.1. Physiochemical analysis of soils

The physicochemical characteristics of the soil samples are shown in Table 4.5. High amount of total organic carbon (TOC) % was found in S4 (Table 4.5A). Soils were texturally classified as sandy, silty sand, silt loam and clay loam based on obtained sand, silt, and clay proportions of soils used (Table 4.5B). Relatively high amount of heavy metals are detected in the flood deposited soils S1. No significant pH change was observed in case of buried (S3) and interface soil (S2). Remarkable decrease in soil moisture content (SMC) was noticed with flood effected soils (<4%); while significant higher SMC (20%) was noticed in S4 reference soil.

4.3.2.2. Biochemical analysis of soils

Inundated fluvial sediment had significantly decreased (P <0.05) biochemical properties in this particular region (Fig. 4.11). Soil C_{mic} were significantly higher in the uncontaminated S4 soil than in the flood contaminated sites (Fig. 4.11A). The lowest C_{mic} (217.16 mg kg^{-1}) was found in S1 sample. The difference in MBC was significant across 4 samples. The observed trend in C_{mic} was as follows S1<S2<S3<S4. Soil respiration rate (both BSR and SIR) was significantly high in the unaffected S4 (Fig. 4.11B and C). Lowest respiration activity was observed in S1 and S2 samples. The respiration activity of the samples were in the order of S1<S2<S3<S4. Microbial metabolic potential (q-CO_2) was significantly different among different samples (Fig. 4.11D). Higher q-CO_2 was found in flood contaminated S1and S2 samples. The trend of q-CO2 was in order of S4<S3<S2<S1.

A detrimental effect of fluvial sediment on soil enzymes was noticed (Fig. 4.12). Soil dehydrogenase activity ranged from 40 (in S1) to 94 (in S4) µg TPF dwt g^{-1} 24 h^{-1} (Fig. 4.12A). Significantly high protease activity was found in S4, while lowest was found in S1 (Fig. 4.12B). The activity of β-glucosidase showed a minimum in the flood effected soils and was maximum in the unaffected soil (Fig. 4.12D). The activity of phosphatase had a similar pattern as β -glucosidase, with a high activity in

the S4 soil and low activity in the S1 soils (Fig. 4.12C). Over all the trend for the soil enzymes has been noticed as follows S1<S2<S3<S4.

Table 4.5: *Soil chemical analysis (A) and physical analysis (B). Values in the columns followed by same alphabetical letters (a, b, c and d) are not significant at P <0.05 (LSD).*

A:

Soil sample	Sand (%)	Silt (%)	clay (%)	Soil type	SMC (%)
S1	66.6a	20a	13.3a	Sandy	4a
S2	36.6b	46.6b	16.7a	Silty sand	7b
S3	20c	56c	24b	Silty loam	12c
S4	15.3d	40b	44.7c	Loam	20d

B

Measurements	Soil samples			
	S1	**S2**	**S3**	**S4**
pH	8.20 a	8.39 a	8.50 a	8.12 a
Total OC (organic carbon) %	0.42 a	0.67a	0.65a	2.1b
Phosphorus (kg/AC)	17.00a	14.00a	15 .00 a	13.00 a
Potash (kg/AC)	175a	78 b	75b	105c
Sulphur (ppm)	-	-	-	-
Heavy metals				
Zn^{+2} (ppm)	12.80 a	0.76 b	0.44 b	0.28 c
Fe^{+3} (ppm)	22.40 a	9.38b	9.82b	4.8 c
Mn^{+2} (ppm)	29.90a	16.20b	14.30 b	13.26 b
Cu^{+2} (ppm)	18.60a	18.14 a	12.20a	2.80 b
Cr^{+2} (ppm)	23.09a	21.24a	19.20b	2.30

4.3.2.3. Microbiological analysis of flood affected and unaffected soils

Total viable count analysis revealed a large discrepancy in the number of bacteria that could be cultured from all 4 different soils. These results showed varying trends and are presented in the Table 4.6. Total viable counts ranged from 1.4 (0.33) x 10^7 (in S2) to 1.28 (0.53) x 10^8 (in S4). Significant lower total cultivable bacteria were observed in flood affected soils. It was found that the ratio of fast verses slow growers (Table 4.6) were significantly different in both flood deposited and unaffected agricultural soil but comparatively high in buried soil (S3).

Population densities of different physiological groups were measured by MPN (Table 4.7). High amount of the nitrogen fixers, denitrifiers, phosphate solubilising bacteria, cellulose degrading bacteria were observed in unaffected agricultural soil S4 where as remarkably low numbers of all these groups were observed in remaining three samples, especially in S1 and S2. High amount of H_2S producers were indentified only with S1.

4.3.2.4. Identification and phylogenetic relation of the bacterial isolates

Dominant cultivable bacteria (isolated by R2A medium) in flood affected S1 and unaffected S4 sample were deciphered with 16S rRNA sequence analysis. The phylogeny among the dominant isolates is depicted in Fig. 4.13 Relatively high cultivable diversity was noticed with S4 as compared to S1. Majority of the dominant bacterial phyla in S4 samples are *Proteobacteria*, *Actinobacteria* and *Firmicutes*; while dominant phyla in S1 samples are found to be *Firmicutes, Actinobacteria* and *Acidobacteria.* Bacterial genera *Bacillus* sp. (family *Bacillaceae*), *Naxibacter* sp. (family *Oxalobacteraceae*), *Arthrobacter* sp. (family *Micrococcaceae*) *and Beijerinckia* sp. (family *Beijerinckiaceae*) were distributed in S4 sample. Bacterial genera affiliated to *Bacillus* sp. (family Bacillaceae)*; Promicromonospora* sp. (family *Promicromonosporaceae*) and *Granulicella* sp. (family *Acidobacteriaceae*) were found in S1 sample.

4.3.2.5. Sequence accession numbers

Nucleotide sequences (of bacterial 16S rRNA gene fragments) retrieved from this study have been deposited in the GenBank nucleotide sequence database and the details are mentioned in Appendix-I.

Fig. 4.11: *Soil microbial activities of flood affected (S1, S2 and S3) and unaffected (S4) agricultural soils. A: Microbial biomass C (C_{mic}); B: Basal soil respiration (BSR); C: Substrate induced respiration (SIR) and D: Metabolic quotient (q-CO_2). Significant differences (P <0.05, LSD) between the mean values are indicated by different alphabets. Error bars represent standard deviation of mean (n =3)*

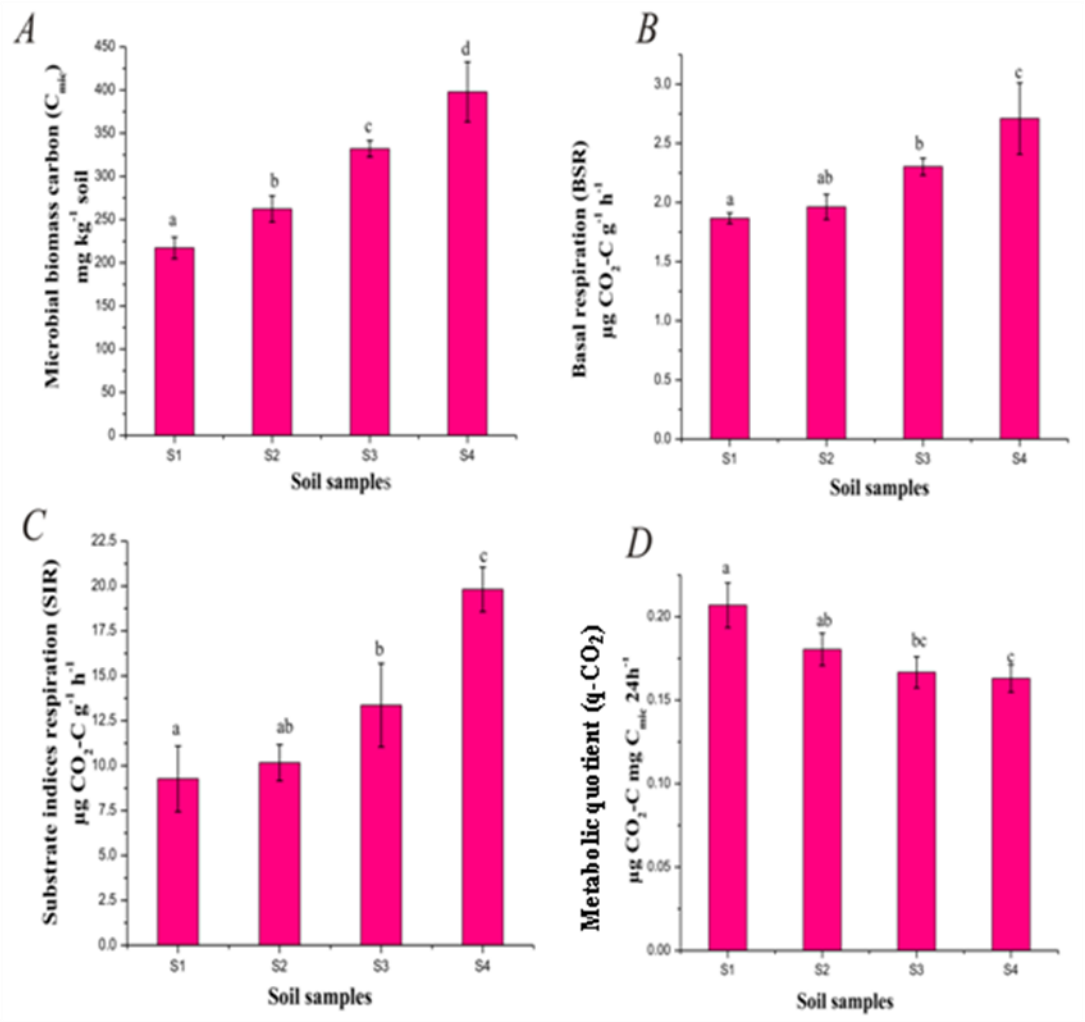
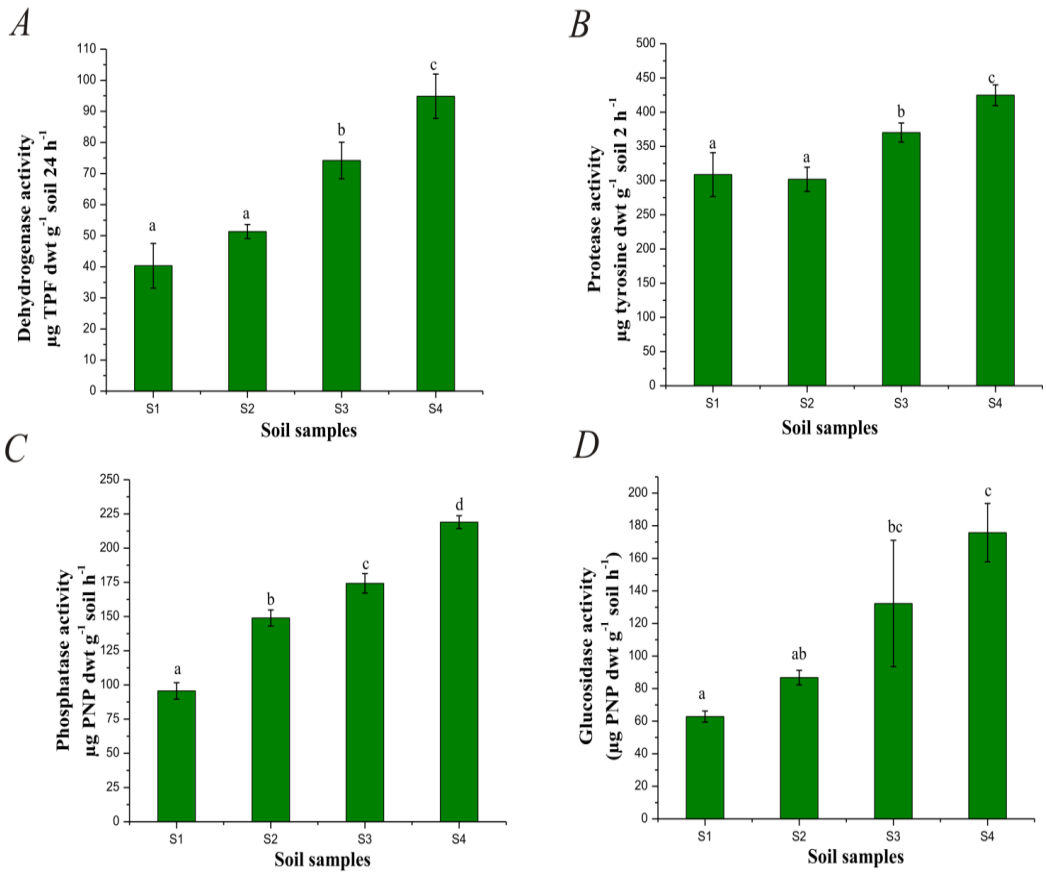


Fig. 4.12: *Soil enzyme activities of flood affected (S1, S2 and S3) and unaffected (S4) agricultural soils. A: Dehydrogenase; B: Phosphatase; C: Alkaline phosphatase and D: Glucosidase activities. Significant differences (P <0.05, LSD) between the soils are indicated by different alphabets. Error bars represent standard deviation of mean (n = 3).*



4.3.2.6 .Microbial diversity analysis by DGGE

16S rRNA-DGGE based bacterial community structure of the soils under study is shown in Fig.4.14. Complex and but discrete bacterial community structure with many faint bands was noticed in all the samples. Along the depth profile the bacterial diversity increased in flood affected field. Higher bacterial diversity is noticed in S4 as compared to flood affected samples. The magnitude of bacterial diversity was placed in this order S1≥S2<S3<S4. Relatively higher diversity indices such as Shannon (*H*) and evenness (E) were found in S4. The *H* indices for S1, S2, S3 and S4 were found to be 2.1, 2.22, 2.6 and 3.1respectively. Whereas E values for the S1, S2, S3 and S4 were 1.7, 1.9, 2.3 and 2.8 respectively. Higher carrying capability of the ecosystem for sustainability of bacterial growth as estimated by range weighted richness (*Rr*) is significantly higher in S4. These values for S1, S2, S3 and S4 were

64, 64, 72 and 112 respectively. Fluvial sediment had a negative effect on bacterial diversity.

Table 4.6: *Total culturable heterotrophic bacterial counts. Values are mean of three independent replicates (N = 3). Standard deviation is shown in parenthesis. Values in the columns followed by same letters are not significant within the group at P <0.05 (LSD).*

Soil sample	***Total viable counts (CFU g^{-1} soil)***	***Fast growers (r strategists, copiotrophs)***	***Slow growers (K strategists, oligotrophs)***	***Ratio of fast vs slow growers (r/K)***
S1	4.03 (0.17) x 10^{7a}	1.8 (0.32) x10^{6a}	2.5 ± 0.49 x10^{6a}	0.72a
S2	1.4 (0.33) x 10^{7b}	8 (0.32) x10^{6b}	13 ± 0.45 x10^{6b}	0.62b
S3	4.73 (0.49) x 10^{7c}	3.7 (0.69) x10^{6c}	4.3 ± 0.89 x$10^{6\,c}$	0.86c
S4	1.28 (0.53) x 10^{8d}	9.2 (2) x10^{6d}	13.9±0.86 x10^{6d}	0.66b

Table 4.7: *Population densities of distinct physiological groups of bacterial populations by most probable number method (MPN).*

Physiological group	**S1**	**S2**	**S3**	**S4**
Denitrifiers	4.3 x10^3	9.3 x10^2	1.50 x10^1	2.1 x10^4
Nitrogen fixers	1.5x10^2	9.2x10^1	4.3x10^2	3.8x10^5
Phosphate solublizers*	2x10^5	1x10^5	2x10^5	1.5x10^6
Cellulose degraders	2.1x10^5	4.30x10	3.6x10^1	9.2x10^3
H_2S producers	2.30 X 10^3	ND	ND	ND
Iron oxidizers	ND	ND	ND	ND

*Results obtained through plate counts. ND: done the experiment, but "not detected"

4.3.2.7. Relation between soil microbial activity and soil characteristics

Canonical correspondence analysis (CCA) is a multivariate method (Braak and Verdonschot, 1995) used to elucidate the relationships between soil microbial activity and their soil characteristics. Partial ordination biplot of CCA is showed in Fig 4.15. The first two components together explained about 97.2% variance among the data distributed. CCA ordination diagram showed an apparent negative impact of sand and heavy metals on microbial activity parameters. However q-CO_2 is positively correlated with sand and heavy metals content of the samples. Similarly, a strong positive correlation among SMC, TOC, clay and soil microbial activity parameters was noticed in this particular environment. Furthermore soil microbial parameters were grouped to each other, indicates significant positive relation existed between them. No significant relationship between silt, pH and soil microbial activity parameters was found. Significant positive correlation between the diversity indices and SMC, TOC, clay were noticed

Fig. 4.13: *16S rRNA based phylogenetic tree of dominant bacteria isolated from flood affected (S1) and uneffcted soils (S2). The evolutionary history was inferred using the UPGMA method. Bootstrap values more than >50 are shown next to the branches. The scale bar represents 5% estimated nucleotide sequence divergence. Isolates are shown with different coloured triangles. (GenBank accession numbers of the isolates are shown in parenthesis (next to isolate numbers).*

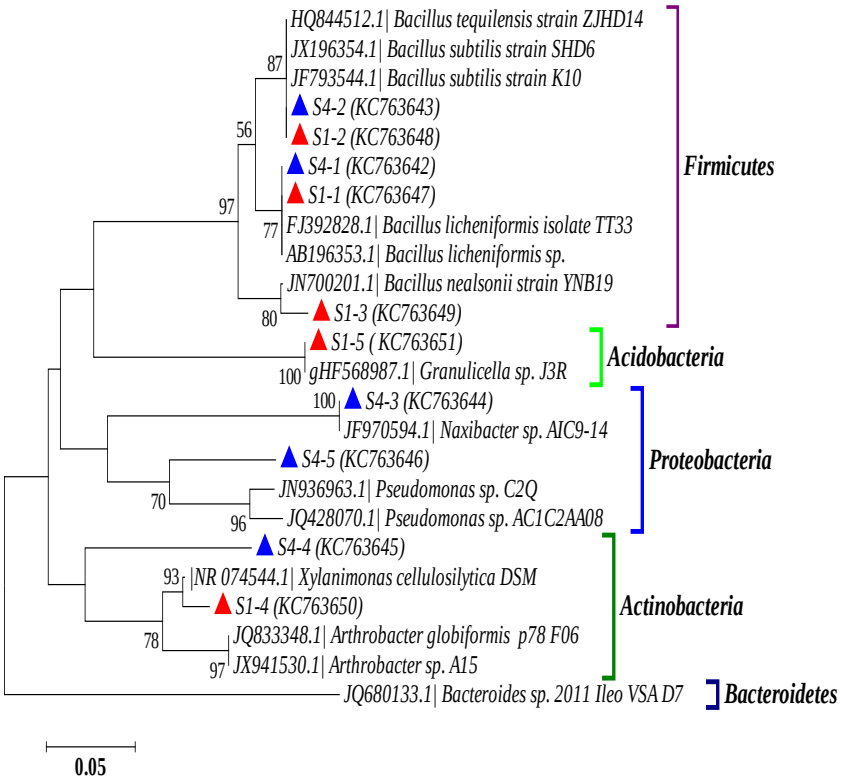
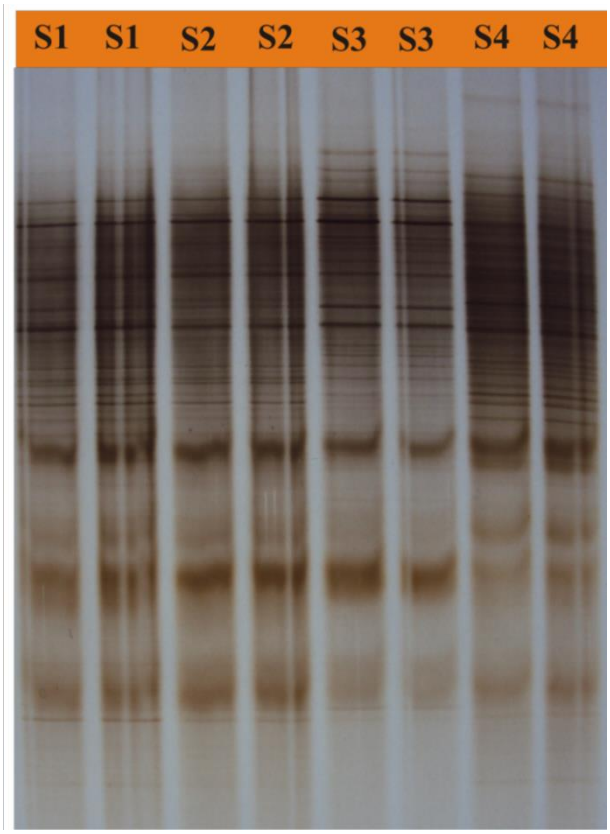
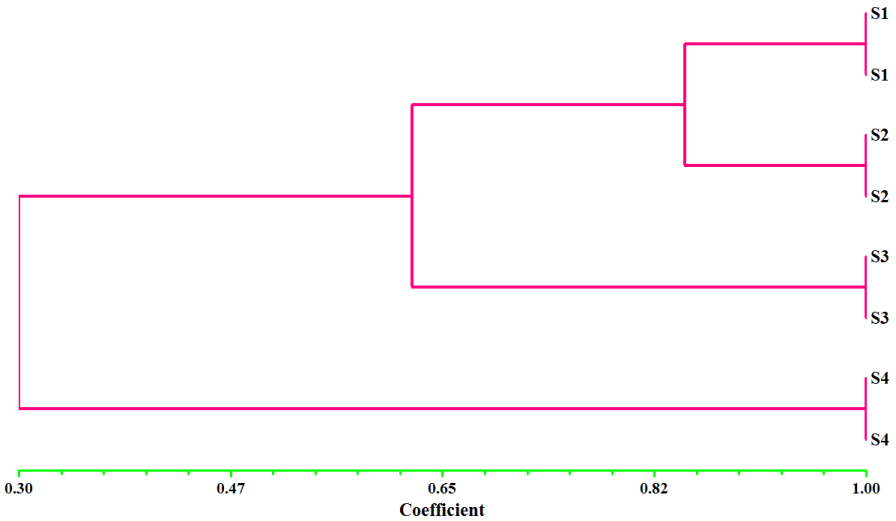


Fig 4.14: *Soil bacterial community structure of flood affected and unaffected agricultural soils by 16S rRNA based DGGE analysis. S1, S2 and S3 are flood affected soils, while S4 is unaffected soil. A: molecular profile of bacterial community structure; B: DGGE based dendrogram representing the similarity between bacterial soils by UPGMA method.*

A



B



4.3.3. Discussion

The present study aimed to extend the knowledge of functional and structural soil microbial activity and diversity as influenced by annual floods at Mahi River region, western India. Soil physiochemical characters suggest that soil texture has been considerably changed due to inundation of fluvial sediment. Relatively higher heavy metals contents are found in S1, this could be due to mixing of heavy metal contained fluvial sediment during flood. Many industries are situated on the southern bank of Mahi River. Some of these industries release their waste-effluent to Mahi stream and thus pollute Mahi sediments. Moreover, an industrial effluent channel from the industrial area heads towards the estuarine end of river and discharges the effluent near Sarod and thus pollutes the entire estuarine sediment region (Nanda et al., 2005).

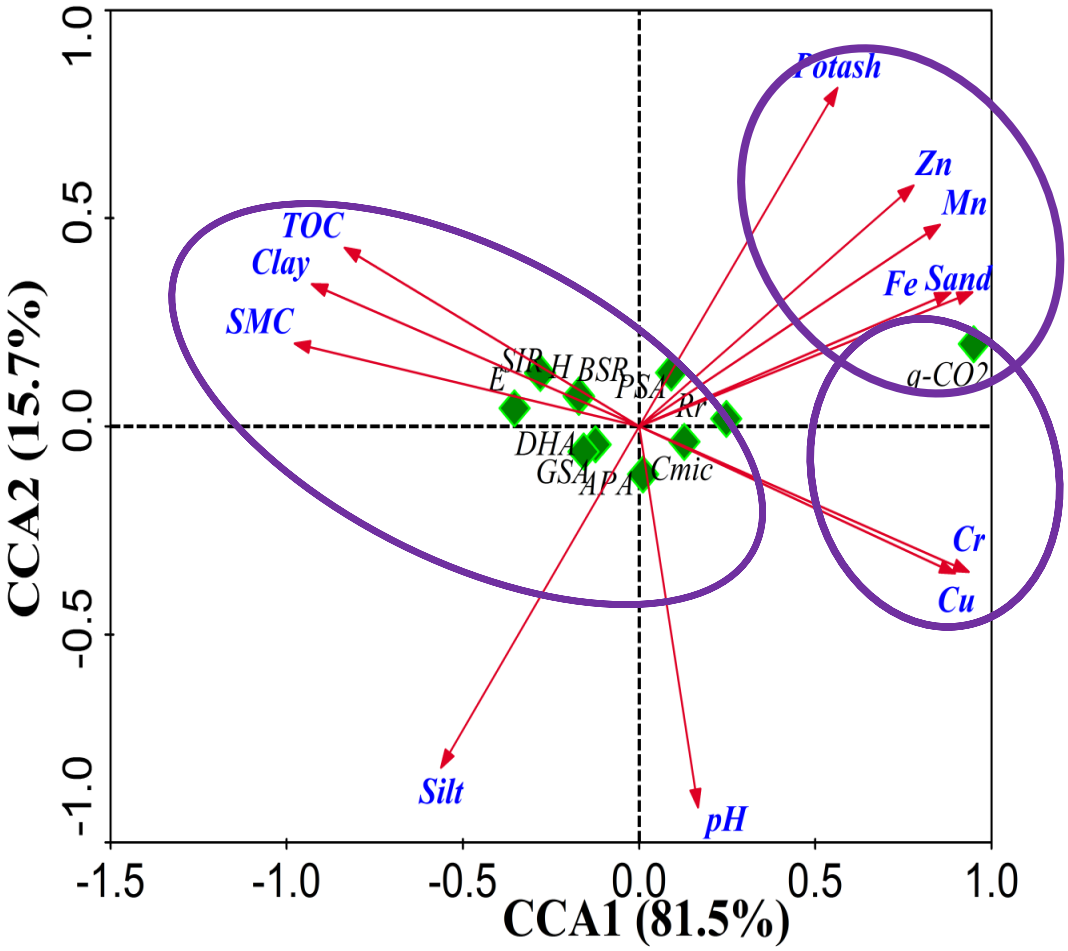
Soil enzymes involved in the C, N and P cycling enzymes and C_{mic} were significantly decreased in flood affected soils (S1, S2, and S3) and thus microbial potential in nutrient cycling might have been considerably decreased by fluvial sediment. This could be due to low content of TOC, SMC and higher heavy metals. Inhibitory effects of metals on soil phosphatases are reported (Khan et al., 2007). Soil dehydrogenase (DHA) plays imperative role in the oxidation of organic matters (Dick et al., 1996). In this study, DHA activity was significantly reduced in flood affected field indicate microbial mediated organic matter oxidation is inefficient. These enzymes are considered as good indicators in soil ecotoxicological testing caused by perturbations (Hinojosa et al., 2004; Khan et al., 2007).

The higher enzyme activities in S4 soil could be due to the more active microbial biomass or to higher substrate availability in organic content (Acosta- Martınez et al., 2004 and 2008). The multivariate CCA ordination plot indicates that TOC, SMC and clay content had a positive measurable relation with soil enzymes. Similar kind of observations were made by the earlier workers (Landgraf and Klose 2002; Taylor et al. 2002; Turner et al. 2002; Langer and Klimanek, 2006).

The marked difference q-CO_2 values between the S4 and S1sites might have resulted from a reduced water availability at S1 (SMC<4). A high q-CO_2 value may indicate a decline in substrate quality (Smith, 1993) and a decrease in microbial

metabolic efficiency (Anderson and Domsch 1990). Since the highest q-CO_2 was found in flood affected field, we assume that soil microbiota in this field must be facing multivariable stress. These stresses could be scarcity of both water and TOC and also heavy metals. In earlier higher q-CO_2 was found in perturbed ecosystems and those results indicates that when the microbial biomass becomes more efficient in the use of the ecosystem resources, less CO_2 (per unit of Cmic) is lost through respiration and a higher amount of the C is incorporated into microbial biomass, resulting on q-CO_2 decrease (Perucci et al., 2000; Hungria et al. 2009; Petrônio et al., 2011). That means that high stability is found under management systems in which the microbial community is little disturbed, thereby contributing to higher agroecosystem sustain-ability (Dilly et al., 2001). Therefore we infer that S4 field is relatively stable ecosystem than flood effected S1, S2 and S3.

Fig 4.15: *Relation between microbial activity (represented with olive colour diamonds) and soil characteristics (represented with arrows) by Canonical correspondence simulation analysis (CCA). Microbial activity is predicted with TOC, SMC and Clay.*



Heterotrophic bacteria are of paramount importance in the cycling of geoelements (Madsen, 2005; Stabili and Cavallo, 2006). Reasoner´s 2A agar (R2A) is used for the culturing of heterotrophic bacteria that do not readily grow on complex organic rich media. A low concentration of casein hydrolysate, peptone, yeast extract and glucose in this medium favors the growth of a broad range of oligotrophic bacteria which are the major portion of eubacteria in soil/sediments. R2A agar is widely used for enumeration and diversity analysis of culturable bacteria in oligotrophic environments such as soils, sediments and sea water (Cho and Giovannoni, 2004; Edenborn and Sexstone, 2007). The total culturable heterotrophic bacterial counts (TCHB) showed heterogeneity along the depth profile (S1, S2 and S3) and uncontaminated soil (S4). Relatively high bacterial numbers were found in unaffected agriculture field (S4). Further, along the depth profile the counts have been increased. This could be due to relatively high proportion of available nutrients and growth substrates along the depth profile in flood affected soils (Zelles et al., 1994).

It was found that dominant bacterial isolates belonged to phyla *Firmicutes, Actinobacteria* and *Acidobacteria* in flood affected soils. Thus, the cultural heterotrophic diversity of this environment is significantly lower than microbial diversity prevailing in normal agro-ecosystems (Øvreås and Torsvik, 1998; Patel et al., 2010). Most importantly, dominant Gram positive spore-forming *Actinobacteria, Firmicutes and Acidobacteria* represents stressful environment in flood affected soil S1. Phyla *Actinobacteria* and *Firmicutes* were found to be dominant bacteria in dry soils subjected to severe environmental conditions (Aislabie et al., 2006; Connon et al., 2007; Steven et al., 2007; Babalola et al., 2009). These results are indicating the physiological adaptability of these phyla to adverse environmental conditions such as fluctuating high and low temperatures and dryness (Wardle, 1998; Aislabie et al., 2006; Lira and Coutinho, 2007). Aislabie et al. (2006) found that *Actinobacteria* have the capability to utilize diverse range of C sources in high altitude dry soils. Borneman et al. (1996) and Hattori et al. (1997) also underlined the predominance of Gram positive bacteria in dry soils. High occurrence of Gram positive *Firmicutes* and *Actinobacteria* were also noticed in desert soils (Wang et al., 2012). However, in certain other environments such as pristine stream sediments and agricultural soils, Gram negative bacteria were the main bacterial groups (Alija and Johnston, 1999; Patel et al., 2010; Yang et al., 2012). This may reflect the variations between climate

and soil physico-chemistry. It is found that extreme environments harbour relatively lower species diversity (Smith et al., 2006). Similar to our results, phylum *Acidobacteria* was found to be dominant in hyper arid climate, dry soils, desert agricultural ecosystems and heavy metal contaminated soils *(*Barns et al., 1999 and 2007; Branco et al., 2005; Janssen et al., 2006; Kim et al, 2008; Neilson et al., 2012). The ubiquity and abundance of *Acidobacteria* in soils and their ability to withstand polluted and extreme environments suggest that they perform potential functions that are important in the environment and that are potentially quite varied as suggested by Ward et al. (2009).

The 16S rRNA-DGGE patterns showed that numerous bands were common to all treatments. There were also changes in band presence and relative intensity due to floods. DGGE molecular fingerprinting soils suggested a dominant influence of fluvial sediment on bacterial diversity. DGGE band sequencing could lead to identification of the unique members. The higher diversity indices are accompanied by higher C_{mic} and enzymes activity. Further soil bacterial diversity indices are also positively related to TOC, SMC and Clay and negatively correlated with q-CO_2, sand and heavy metals.

These studies could create an opportunity towards development of sustainable agricultural practices or improvement of soil health around flood effected soils, in this semiarid region. Based on obtained results, it can be inferred that the soil health in flood affected fields is in endangered. In order to improve the soil quality of these fields, organic farming and application of plant growth promoting bacteria might help.

4.3.4. Conclusions

Microbial mediated organic matter dynamics, nutrient cycling and decomposition processes in agriculture fields were decreased by fluvial sediment inundation. Reduced microbial diversity is noticed in flood affected soil. Overall, the soil health in the flood affected field is in endangered which suggest the importance of modifying management for sustainable agriculture. Enzymes and q-CO_2 values can be used as sensitive biological measure to assess or detect the soil disturbances lead by floods in semiarid region. Further our results indicate that phyla *Firmicutes, Actinobacteria* and *Acidobacteria* could be used as indicator organisms for detecting

environmental perturbations in semiarid soils. Floods have led to distinct alterations of the soil ecosystem functioning

Chapter 5

Microbial activity and diversity profiling of long-term industrial waste effluent contaminated semiarid landscapes, Mahi River basin

5.1. Introduction

Industrial inputs and the agronomic application of fertilizers, pesticides, metal tainted sewage and effluents continue to contribute to the accumulation of toxic metals and organic pollutants in soils (Herland et al., 2000). Unlike organic pollutants, heavy metals cannot be degraded and persist indefinitely in the environment (Baath, 1989). Soil pollution by elevated levels of heavy metals is known to adversely affect microbial activities and their functional diversity (Gremion et al., 2004; Demanou et al., 2006; Bhattacharya et al., 2008; Gleeson et al., 2008; Khan et al., 2010, Pavissich et al., 2010; Gough and Stahl, 2011; Máthé et al., 2012; Sheik et al., 2012; Thavamani et al., 2012). The diversity of soil microbial communities is exceedingly rich (Roesch et al., 2007; Sheik et al., 2011). However, knowledge about the effects of metal contamination on microbial community abundance and structure in semiarid soils/sediments has not been widely studied. Moreover, the response of heavy metals particularly on soil microbial functional groups which are important in soil biogeochemistry is poorly understood.

Microbial ammonia oxidation (MAO) is the first and rate limiting step in nitrification (Di et al., 2009), and thus plays a decisive role in the global nitrogen cycle. For more than a century, it has been believed that MAO is exclusively mediated by chemolitho-autotrophic ammonia oxidizing bacteria (AOB) from ß and γ-proteobacteria (De Boer and Kowalchuk, 2001). Recent discovery of homologs of ammonia monooxygenase genes (*amoA*) in archaea and subsequent cultivation of autotrophic ammonia oxidizing archaea (AOA) affiliates to phylum '*Thaumarchaeota*' has radically changed the general perception of MAO (Venter et al., 2004; Könneke et al., 2005). Recent molecular investigations revealed the global distribution of the putative AOA-*amoA* genes in a wide range of terrestrial and marine habitats (Leininger et al., 2006; Prosser and Nicol, 2008; Bartossek et al., 2012). Most surveys based on *amoA* genes have revealed the numerical dominance of AOA over AOB by many folds in terrestrial environments (Leininger et al., 2006; He et al., 2007; Zhang et al., 2012; He et al., 2012) which strongly supports the notion that AOA may be the keystone drivers in soil ammonia oxidation (Leininger et al., 2006; Nicol et al., 2008; Tourna et al., 2008; Zhang et al., 2012).

Acidic soils occupy about 30% of world's land area (von Uexkull and Mutert, 1995), among which alfisols are the third most important soil orders in the world, covering 13.9% of the world area. It is of paramount importance to know the effect of distinct perturbations on nutrient cycling processes of acidic soils.

Numerous studies have demonstrated the changes in abundance and diversity of AOB and AOA in response to different abiotic stressors like temperature, pH and soil type (He et al., 2007; Xia et al., 2007; Chen et al., 2008; Nicol et al., 2008; Shen et al., 2008; Nyberg et al., 2012). Nitrification is considered to be the most sensitive soil microbial processes with regards to heavy metal stress (Smolders et al., 2001). Studies have particularly emphasized the response of AOB to heavy metals such as Zn, Cu and Hg (Xia et al., 2007; Mertens et al., 2009; Liu et al., 2010; Lee et al., 2011; Vasileiadis et al., 2012), while very little is known about AOA response to Cu and As stress in acidic soils. Majority of these previous studies are confined to neutral or alkaline soils. More importantly, no single study has yet established the AOA-*amoA* gene composition in heavy metal polluted acidic/alkaline soils except DGGE or T-RFLP based community shifts (Mertens et al., 2009; Ollivier et al., 2012; Vasileiadis et al., 2012). All known terrestrial AOB to date belong to a monophyletic assemblage of *Nitrosospira* and *Nitrosomonas* in the ß-subdivision Proteobacteria (Kowalchuk et al., 2001). This assemblage originally consists of seven 16S rRNA gene sequence clusters (Kowalchuk et al., 2001). Nevertheless, it is still unclear how the composition of AOB communities varies due to different environmental and anthropogenic factors.

In the present investigation, the microbial activity and diversity has been studied with special emphasis to ammonia oxidizing microbes in semi-arid alluvial plains of Baroda window receiving concoction of pollutants consists of heavy metal and complex organic pollutants. Initial responses of ammonia oxidizing microbes (both AOA and AOB) and detailed AOA phylogeny in Cu and As amended acidic alfisols are addressed through a 10 week microcosm experiment. Accordingly, the objectives under this chapter are as follows

- Effect of industrial waste effluent (IWE) on soil microbial activity and diversity
- Effect of IWE on soil nitrification and ammonia oxidizing microbes

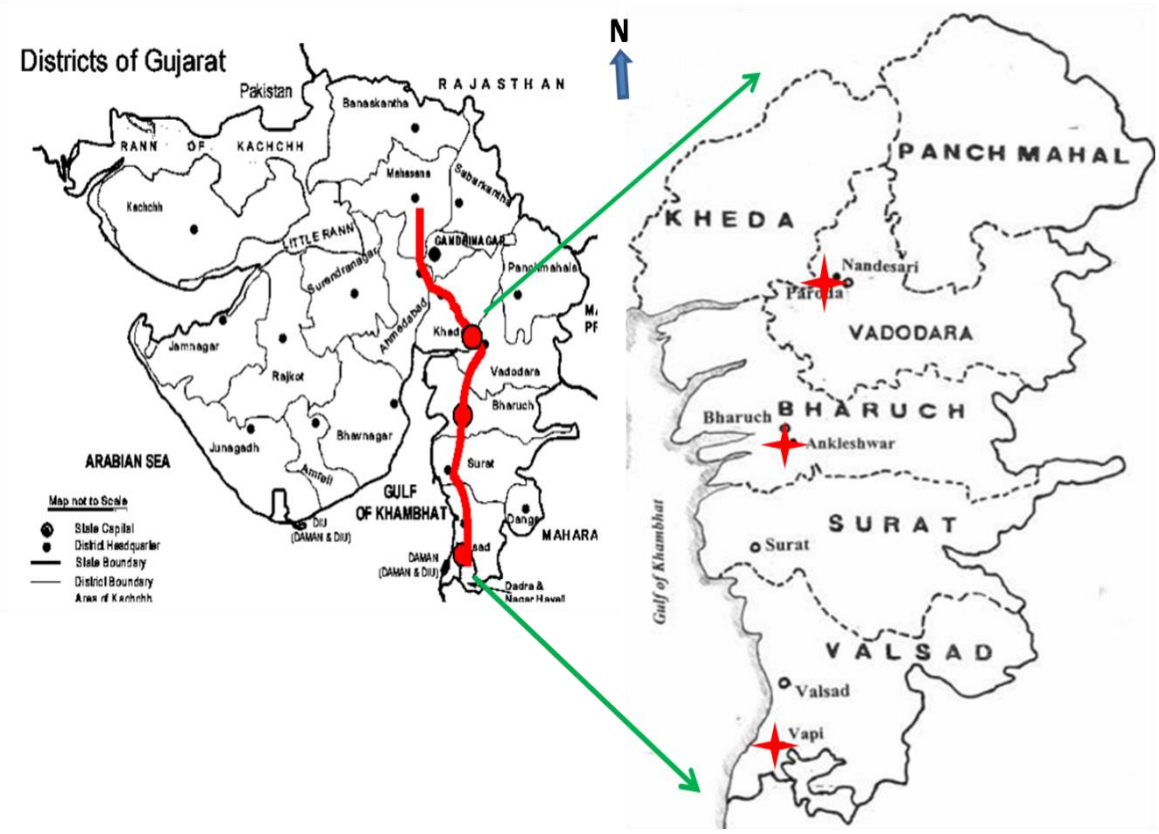
- Effect of heavy metals on soil microbial activity and ammonia oxidizing microbes: short term microcosm studies

5.2. Materials and Methods

5.2.1. Site description and soil sample collection

The "Golden Corridor" of the Indian State of Gujarat is an industrial belt that includes the large industrial estates in Nandesari, Ankleshwar, and Vapi (Fig. 5.1).

Fig 5.1: *Golden corridor of state of Gujarat (red color line). Nandesari, Ankleshwar, and Vapi industrial zones are shown with star marks.*



These industrial areas contain thousands of individual industrial units, including dye factories, textile, rubber, pesticide, fertilizers, paint manufacturers, pulp and paper producers, pharmaceutical, engineering, petrochemicals and chemical manufacturing companies (Bruno, 1995, Nagar, 1995). Nandesari industrial estate ($22^0 24' 59.83''$ N, $73^0 05' 11.89''$ E) developed in 1969, is located 15 km away from

Vadodara city ($22^0$18'26.15" N, $73^0$10'52.29" E), Gujarat, India. There are approximately 300 industrial units, of which more than 25% are manufacturing chemicals, pharmaceuticals, dyes and dye intermediates, fertilizers and plastics. These industries utilize common effluent treatment plants (CETPs) to varying degrees to treat their industrial waste effluent (IWE) and finally dispose into Gulf of Khambhat through a 55 km long, partially covered, brick lined channel. However, many industries ignores CETPs and haphazardly dispose their IWE, piles of sludge and solid bulk hazardous waste directly in to the nearby Mini River stream (a tributary of Mahi River) and also indiscriminately dumped on open ground, causing a severe threat of ground water pollution, damaging surrounding landscapes, and depriving its productive use.

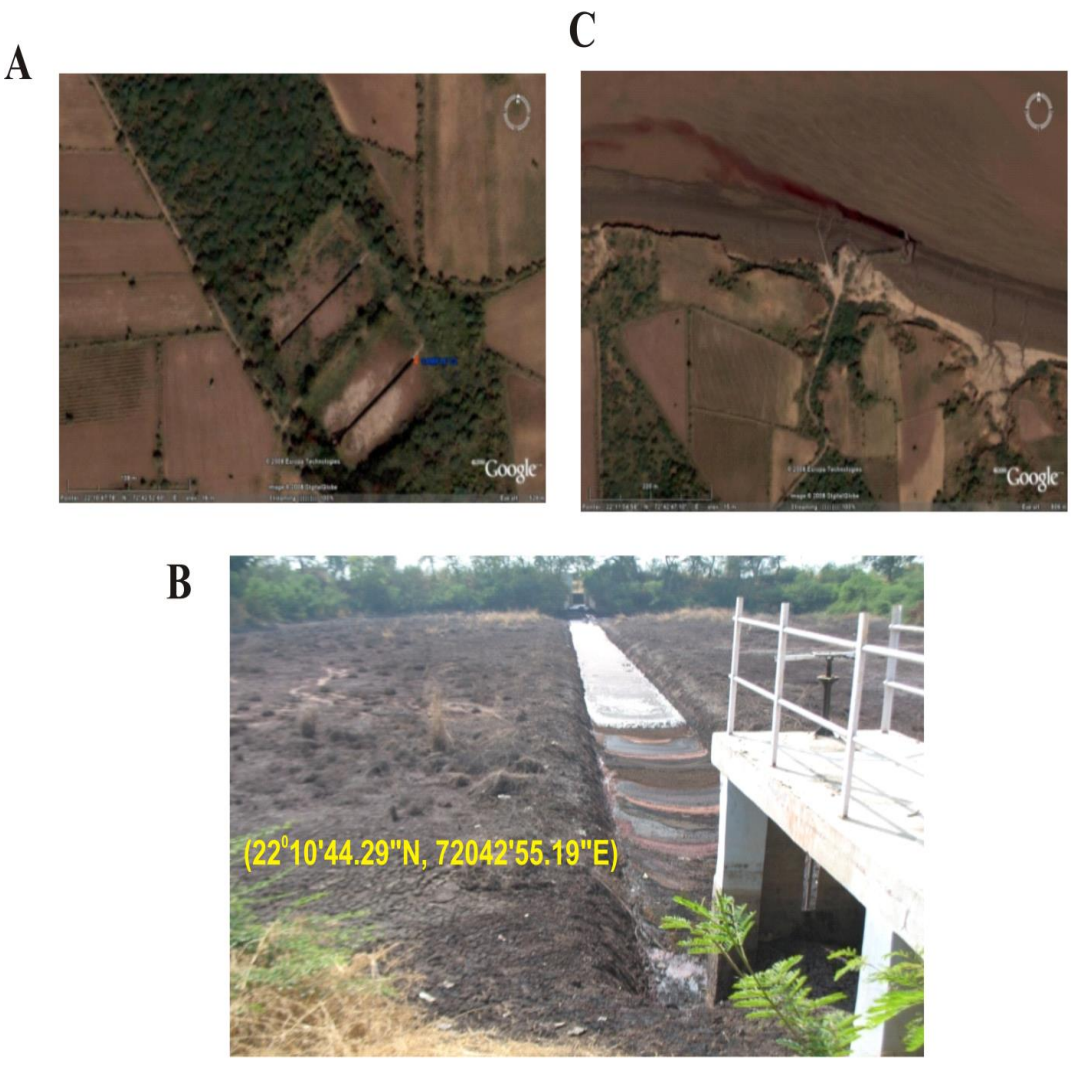
In the present study, soil samples were taken from 3 different locations under semiarid climate. Sample R1 is a natural pristine soil without any contamination, collected from a site nearby Ryaka ($22^0$26'05.28" N, $73^0$04'28.65" E). The site for R1 is located about 3.5 km upstream to Nandesari industrial estate. Samples R2 and R3 were collected from 2 different sites situated along the contaminated Mini River basin. These sites have been receiving mixed bag of contamination for more than 20 years mainly by episodic streams of Mini River consisting of industrial waste water. Sampling sites R2 ($22^0$20'50.34" N, $73^0$04'36.9'' E) and R3 ($22^0$23' 29.52" N, $73^0$0.5'45.20" E) lies about 6 and 1.5 km downstream to Nandesari industrial estate respectively. R4 is a sediment sample collected from a IEF settling lagoon (Fig. 5.2) situated near Sarod ($22^0$10'44.29"N, 72042'55.19"E). Triplicate composite surface samples (0-10 cm) including sub samples were collected and placed in sterile polyethylene bags and kept on ice, and transported to the laboratory. All samples were passed through a 2.0-mm sieve, stored at 4°C for analysis of soil characteristics and at −80°C for DNA extraction.

5.2.2. Physiochemical analysis of samples

Soil texture, pH and organic matter content were estimated by standard protocols (Section 2.1). Total C (TC), total N (TN), and total S (TS) were estimated by a combustion analyzer (Vario ELIII elemental analyzer). Heavy metal concentrations were analyzed by standard microwave oven digestion method according to the United States environmental protection agency (US-EPA, 1998). In

brief, 0.25 gm triplicate samples were digested in a microwave oven with 9 ml HNO_3 and 3 ml HCl for 5 min at 700W (to reach 180^{o}C) and10 min at 500W (to maintain at 180^{0}C). After cooling, digested extracts were centrifuged (4,000 x *g* for 5 min) and diluted to 25 ml with Milli-Q water. Heavy metals were determined by ICP-OES, Perkin Elmer Co., USA.

Fig. 5.2: *Generalized overview of the IWE settling lagoon near Sarod, Gujarat, Western India. 1: satellite image of the IWE settling lagoon (IWE-SL). 2: Close up of the IWE-SL where sediment sample R4 was collected. 3: Treated effluent is released from IWE-SL to estuarine, Gulf of Khambhat.*



5.2.3. Soil ammonium, nitrite and potential nitrification rate (PNR)

Soil ammonium and nitrate contents were extracted from fresh soil samples with 2 M KCl and determined by a Continuous Flow Analyser (SAN++, Skalar, Holland; Shen et al., 2008). PNR was measured by using chlorate inhibition method

as described previously (Kurola et al., 2005). In brief, fresh soil sample 5.0 g was added to 50 ml centrifuge tube containing 20 ml of phosphate buffer solution (g l^{-1}: Na_2HPO_4, 0.2; NaH_2PO_4, 0.2; KCl, 0.2; NaCl, 8.0; pH 7.4) with 1 mM $(NH4)_2SO_4$. Nitrite oxidation was inhibited by adding potassium chlorate with a final concentration of 10 mM. The suspension was incubated at 25°C for 24 h in dark. Nitrite was extracted with 5 ml of 2 M KCl and determined spectrophotometrically at 540 nm with N-(1-naphthyl) ethylenediamine dihydrochloride. All the analysis of soil physicochemical characters including PNR described here were performed with triplicate samples and values were represented as mean with S.D.

5.2.4. Soil microbial activity measurements

Microbial biomass C (C_{mic}), soil basal respiration (BSR) and substrate induced respiration (SIR) were determined (Chapter 2). Metabolic quotient (q-CO_2) i.e. basal respiration for unit microbial biomass (µg CO_2-C mg C_{mic} $24h^{-1}$) was calculated (Section 2.2. and 2.3). Soil extracellular microbial enzymes such as β -glucosidase (GSA), alkaline phosphatase (APA), and protease (PSA) involved in C, P and N cycling respectively, were estimated (Section 2.3). Soil dehdyrogenase (DHA), activity which is indicative of organic matter oxidation was measured (Section 2.3). All the determinations were performed in triplicates and the values were mentioned as mean with standard deviation (n = 3).

5.2.5. Soil community DNA extraction

Soil community DNA was extracted from 0.5 g subsamples of each soils using Ultra-clean^{TM} soil DNA Isolation Kit (MoBio Laboratory, Carlsbad, CA, USA) followed by the manufacturer protocol. The extracted community DNA was checked on a 1% agarose gel and the DNA extracts were evaluated by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA).

Table 5.1: *Details of the primers and probes used in the present study.*

Target group	Primer Name	Gene targeted	Sequence (5′–3′)	Amplicon length (bp)	Purpose	Reference
Bacteria	954F 1369R 954F-GC BACT1369F PROK1541R Probe TM1389F®	 Bacterial 16S rRNA	GCACAAGCGGTGGAGCATGTGG GCCCGGGAACGTATTCACCG **gc**$^{\varphi}$GCACAAGCGGTGGAGCATGTGG CGGTGAATACGTTCYCGG AAGGAGGTGATCCRGCCGCA CTTGTACACACCGCCCGTC	415 456 170	DGGE, cloning and sequencing of bands q-PCR	Yu and Morrison, 2004; Zhang et al., 2011 Suzuki *et al.*, 2000
Archaea	A364Af A934Br	Archaeal 16S rRNA	CGGGGYGCASCAGGCGC GTGCTCCCCCGCCAATTCCT	570	q-PCR	Kemnitz *et al.*, 2005
AOA	Arch-amoAF Arch-amoAR	Archaeal *amoA* gene	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	635	q-PCR and AOA-*amoA*-clone library analysis	Rotthauwe *et al.*, 1997; Liu *et al.*, 2010
AOB	amoA1F amoA2R amoA1F-GC	Bacterial *amoA* gene	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC **gc**$^{\varphi}$GGGGTTTCTACTGGTGGT	491 531	q-PCR DGGE of AOB-*amoA*, *cloning* and sequencing of bands	Francis *et al.*, 2005
AOA	CrenamoA23f CrenamoA616r	Archaeal *amoA* gene	ATGGTCTGGCTWAGACG GCCATCCATCTGTATGTCCA	593	DGGE of AOA-*amoA*, *cloning* and sequencing of bands	Tourna *et al.*, 2008; Könneke *et al.*, 2005

AOA: Ammonia oxidizing archaea; AOB: Ammonia oxidizing bacteria; **gc**$^{\varphi}$: 40 base pairs GC clamp was attached to the 5^{1} end of the primer (cgcccgccgcgccccgcgcccggcccgccgcccccgcccc), ® : Probe.

5.2.6. Quantification of genes by real-time quantitative PCR assays (q-PCR)

Quantification of bacterial 16S rRNA, archaeal16S rRNA, AOB- *amoA* and AOA-amoA genes were carried out by q-PCR with the iCycler iQ-5 thermocycler (Bio-Rad, Hercules, CA, USA). For 16S rRNA real-time PCR assays, *Taq*Man probes were labeled at the 5′ end with the reporter dye FAM (6-carboxy-fluorescein) and at the 3′ end with the quencher dye TAMRA (6-carboxy-tetramenthylrhodamine) (TaKaRa Bio, Otsu, Shiga, Japan). Amplifications were performed in 25 µl reaction mixture including 12.5 µl Premix Ex TaqTM (TaKaRa Biotechnology, Japan), 0.5 µl of each primer (10 µM), and 2 µl of tenfold diluted extracted DNA (1-10 ng) as template (Zhang et al. 2009). For each reaction 1 µl of BSA (200 ng mL^{-1}) was also added to reduce the interference of humic acid in the PCR (He et al., 2005). Quantification of bacterial 16S rRNA gene was performed using primers BACT1369F/PROK1541R and probe TM1389F (Suzuki et al. 2000). Primers A364Af and A934bR were used for quantification of archaeal 16S rRNA genes (Kemnitz et al., 2005). Primers Arch-*amoA*F/Arch-*amoA*R (Francis et al., 2005) and *amoA*1F/*amoA*2R (Rotthauwe et al., 1997) were used for quantification of AOA-*amoA* and AOB-*amoA* genes respectively with SYBR® Premix Ex Taq™ (TaKaRa Biotechnology, Japan). The parameter Ct (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected. After each run, melting curve analysis was performed to test the specificity of the qPCR. The validity of q-PCR was further confirmed by running the respective amplicons on agarose gel electrophoresis to ensure amplicons of the expected size. PCR efficiency (E) and correlation coefficients (r^2) for all the q-PCR assays ranged from 82 to 102 % and 0.994 to 0.996 respectively. Description of the primers and probes is given in Table. 5.1.

5.2.7. Soil microbial diversity studies by DGGE

Eubacterial diversity of the soils has been established by 16S rRNA-based denaturing gradient gel electrophoresis (DGGE). Universal eubacterial 16S rRNA primers, 954f-GC/1369r were used for the amplification of 16S rRNA gene fragments suitable for DGGE. Descriptions for the primers are given in the Table 5.1. Conditions for PCR were 95°C for 10 min, followed by 18 cycles of 95°C for 1 min,

59°C (reduced 0.5°C during each cycle up to 55°C) for 45 s, and 72°C for 45 s followed by 20 cycles of 95°C for 1 min, 55°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 10 min.

DGGE analysis AOA and AOB communities were performed with primer sets CrenamoA23f/CrenamoA616r and amoA1F-GC/amoA2R respectively. Descriptionof the primers are mentioned in Table 5.1. All the Amplifications were performed with TaKaRa *EX Taq DNA polymerase* (Takara Bio, Otsu, Japan). The total volume of PCR mixture was 50-µL including 10x *EX Taq* buffer, 3.0 mM $MgCl_2$, 250 µM each of dNTP, 2.5 U *EX Taq* DNA polymerase (TaKaRa), primers and BSA. Detailed thermal profiles of the primers and the percentages of denaturing gradient are comprehensively mentioned are followed in accordance to Ge et al. (2008), Shen et al. (2008) and Zhang et al. (2010). The resulting PCR products were directly subjected to DGGE.

Gels were electrophoresed in 1X TAE buffer at a constant temperature of 60^{o}C for 15 h at 100 V. After the electrophoresis, the gels were stained with 1:10000 SYBR Gold Nucleic Acid Gel Stain (Invitrogen-Molecular Probes, Eugene, OR, USA) for 30 min according to the manufacturer's instructions, scanned by a GBOX/HR-E-M (Syngene, UK) and analyzed using the software AlphaEase FC software (Alpha Innotech, CA, USA). The diversity of the microbial community was expressed as the Shannon index of general diversity *(H^I)* and evenness *(E^I).* The range-weighted richness *(Rr),* reflecting the carrying capacity of the samples were determined (Marzorati et al., 2008). Predominant bands in the DGGE profiles were selected for cloning and sequencing analysis.

5.2.8. Cloning and sequencing of selected DGGE bands and phylogenetic analysis

Bands numbered in the DGGE gel as well as bands with the equivalent mobility were excised for subsequent cloning and sequencing. Initial experiments indicated that the bands with the same mobility in a DGGE gel had highly similarity in (about > 98%) nucleotide sequences. The dominant bands in the DGGE gels were carefully excised and suspended in 40 ml of Tris-HCl buffer (pH 8.0) for 12 h (at 4^0C), and reamplified with the respective primers mentioned above without GC clamp

(Table 5.1). Reamplified PCR products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The purified PCR products were ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA) and the resulting ligation products were transformed into *Escherichia coli* JM109 competent cells following the manufacturer instructions. The positive clones were amplified using the primers T7 and SP6 and confirmed product size (in bp) genes before being sent for sequencing.

All the obtained sequences were thoroughly analyzed for their primers binding sites and checked for their correct orientation of open reading frame (ORF). Sequences were subjected to BLAST analysis in the NCBI GenBank (www.ncbi.nlm.nih.gov) database and similarity analysis was performed with DNAStar and DNAMAN version 4.0. Phylogenetic analyses were con- ducted using MEGA version 5.05 (Tamura et al., 2011). Translated aminoacid sequences (for both AOA-*amoA* and AOB-*amoA* genes) were used to construct neighbour-joining tree followed by Poisson model with 1000 replicates to produce Bootstrap values. Diversity indices such as Shannon index (*H*) and Evenness index (*E*) were measured as described previously (Shen et al., 2008).

5.2.9. Short-term microcosm studies: Response of heavy metals (As and Cu) on AOA and AOB.

5.2.9.1. Soil samples and experimental setup

Two different types of pristine alfisols (from both India and China) without any history of heavy metal exposure were collected for short-term microcosm studies. Alfisol- RA was collected from a site located in semiarid region near the Mahi River basin, Gujarat, western India. 'RA' is an oligotrophic silty-loam soil, with low organic matter (0.86%), low water content (8%) and pH of 5.1. Alfisol-HZ was collected from Hangzhou, *Zhejiang* province, eastern China. 'HZ' alfisol is a clay loam, consisting of relatively more organic matter (2.88%) with a water content of 18.9% and a pH of 5.6. Samples were air dried and ground to a size finer than 2.0 mm. Before conducting the microcosm experiments, soils were remoistened as described by Mertens et al. (2009). Two different series of microcosm setups were constructed with RA and HZ soils. Three different types of metal treatments viz. As-

spiked, Cu-spiked and combination of As and Cu-spiked (As + Cu) were set up for each microcosm series (RA and HZ series). Soil without addition of heavy metal was considered as background control treatment (CK). Aqueous solutions of $CuCl_2$ and $Na_2HAsSO_4.7H_2O$ were used as source of Cu and As, respectively and pH of the soils maintained to their natural values. In Cu-amended microcosms the final concentrations of Cu were adjusted to 0, 125, 250 and 500 mg kg^{-1} dry soil, while in As-amended microcosms the concentrations were adjusted to 0, 17.5, 35, and 70 mg kg^{-1} dry soil. Concentrations for combined Cu and As –microcosms" were as follows: As-35 + Cu-250, As-35 + Cu-500, As-70 + Cu-250 and As-70 + Cu-500 mg kg^{-1} dry soil. Microcosms were named according to their nominal As and Cu concentrations. For example, As 35 and Cu 500 means two different metal treatments received 35 mg-As kg^{-1} dry soil and 500 mg-Cu kg^{-1} dry soil respectively, similarly As 35 + Cu 500 means soil received both 35 mg-As and 500 mg-Cu kg^{-1} dry soil. The type of experimental design was completely randomized block. A total of 20 g of each sample was placed in a 200-mL-plastic jar. For each concentration of heavy metal three independent replicates were performed (n=3). The microcosms were incubated at 25°C for 10 weeks with 55% of soil water holding capacity and destructively harvested at the end of 10^{th} week. Harvested soil samples were immediately processed for soil chemical and microbial activity analysis. Parts of the samples were stored at −60°C for subsequent DNA extraction and molecular analysis. On the other hand, subsamples were processed for soil physiochemical analysis

5.2.9.2. Soil chemical analysis and potential nitrification rates

Soil pH (soil to water ratio of 1:2.5), soil organic matter and heavy metals were estimated. (Section 5.2.2) Soil ammonium, nitrate and PNR contents were estimated as described above. Particle size distribution was measured using the rapid sieving procedure. PNR was measured using the chlorate inhibition method (Kurola et al., 2005). All the analysis were performed with triplicate samples and values are represented as mean value (n = 3) with standard deviation.

5.2.9.3. Quantification of genes by real-time PCR assays

After 10^{th} week, DNA was extracted from 0.5 g of fresh soil samples using Ultra-clean^{TM} soil DNA Isolation Kits (MoBio Laboratory, Carlsbad, CA, USA). Abundance of eubacterial-16S rRNA, archaeal-16S rRNA, bacterial-*amoA* and

archaeal-*amoA* genes was determined by q-PCR with the iCycler iQ5 thermocycler (Bio-Rad, Hercules, CA, USA. Data analysis was carried out with iCycler software (version 1.0.1384.0 CR). Description for the primers, probes and the methodology is given in above section 5.2.6 and Table 5.1.

5.2.9.4. Denaturing gradient gel electrophoresis (DGGE) analysis analysis of AOA-amoA

DGGE of archaeal-*amoA* gene community was performed with a DCode Universal Mutation Detection System (Bio-Rad, Germany). Archaeal-*amoA* genes were amplified using primers Cren-*amoA*23f and Cren-*amoA*616r (Tourna et al., 2008). DGGE was performed as described by Zhang et al. (2010). Gels were electrophoresed in 1X TAE buffer at a constant temperature of 60^{o} C for 15 h at 100 V. After the electrophoresis, the gels were stained with 1:10000 SYBR Gold Nucleic Acid Gel Stain (Invitrogen-Molecular Probes, Eugene, OR, USA) for 30 min according to the manufacturer's instructions, scanned by a GBOX/HR-E-M (Syngene, UK) and analyzed using the software AlphaEase FC software (Alpha Innotech, CA, USA).

5.2.9.5. Construction of AOA-amoA clone library and sequencing studies

In order to investigate the effects of Cu, As and combination of Cu+As on soil AOA community composition, four distinct soil treatments such as CK, Cu-500, As-70, and Cu-500+ As-70 mg kg^{-1} dry soil were selected and the DNA extracted from three independent replicates samples were mixed. A total of 4 Thaumarchaeal AOA-*amoA* clone libraries (named as 1A, 2A, 3A, and 4A) were constructed. These included 1A: AOA- *amoA* gene clone library constructed from CK-soil (uncontaminated soil); 2A: AOA-*amoA* clone library constructed from As-70 (As- 70 mg kg^{-1} dry soil); 3A: AOA-*amoA* clone library constructed from Cu-500 (Cu-500 mg kg^{-1} dry soil) and 4A: AOA-*amoA* clone library constructed from As-70 + Cu-500 (As-70 mg kg^{-1} + Cu-500 mg kg^{-1} dry soil). The products of archaeal-*amoA* genes amplified with the primers Arch-*amoA*F/Arch-*amoA*R (Francis et al., 2005) were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The purified PCR products were ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA), and the resulting ligation mix transformed into *Escherichia coli* JM109 competent cells following the manufacturer's protocol.

Positive clones were chosen for PCR reamplification using the primers T7 and SP6 and confirmed with 635 bp of archaeal *amoA* gene. About 100 positive clones for each AOA-*amoA* library were sequenced. A total of 400 AOA-*amoA* sequences were obtained from 4 clone libraries and the sequences were proceeded for further phylogenic analysis. All the sequences were subjected to BLAST analysis in the NCBI GenBank database (www.ncbi.nlm.nih.gov/genbank/). Phylogenic tree was constructed by MEGA 5.05. Analysis of clone libraries was performed with Mothur v.1.27.0 (http://www.mothur.org/ v. 1.22.1; Schloss et al., 2009). Rarefaction curves were generated and operational taxonomic units (OTUs) are defined as clusters of sequences with a similarity of 97% (i.e. a distance cutoff 0.03).

5.2.10. Statistical analysis

Prior to statistical analysis, copy numbers of the genes were log transformed as needed to normalize the distributions. All statistical analyses were performed using SPSS version 15.0 and Canoco version 4.0. Pair wise comparisons and quantitative differences of all the sample means were conducted with one-way analysis of variance (ANOVA) by Fishers least significant difference test (LSD) and by t-test. In the present study, P <0.05 was considered to be statistically significantAll statistical analyses were performed using SPSS version 15.0 and Canoco version 4.0. In order to describe the median effective concentration (EC50), linear regression was performed with normalize data and fitted in to a logistic response equation {y= a/1+ $(x/x_{50})b$} as described previously (Liu et al., 2010).

5.3. Effect of industrial waste effluent (IWE) on soil microbial activity and diversity

5.3.1. Results

5.3.1.1. Soil physicochemical properties

Soil physicochemical characters are summarized in Tables 5.2A and B. Soil pH ranged from 5.3 (in R1) to 7.4 (in R3). Significantly (P <0.05) high amount of organic matter (OM, 2.92%), total carbon (TC, 21.2 g kg^{-1}), total nitrogen (3.48 g kg^{-1}) and total sulfur (TS, 4.7 g kg^{1}) were noticed in R3 (Table 5.2A). High C/N ratio was associated with R1 and was significant with R3. ICP-AES analysis revealed the elevated levels of heavy metals in the soils (R2, R3 and R4). High amount of heavy

metals (µg g^{-1}) such as Cu, As, Cr and Pb were found in R3, where as moderate concentrations of heavy metals were noticed in R2 (Table 5.2B). Soil moisture content ranged from 21% (R1) to 41%.

Highest organic matter (14.8%), TOC (7.44%), TN (17.09 g kg^{-1}) and TC (172.8 g kg^{-1}) were noticed with sediment (R4) and it is positive indication of contamination by organic rich pollutants. such as poly cyclic aromatic hydrocarbons and phenolic hydrocarbons (Labunska et al., 1999). Particularly, it was found that As, Cu, Pb and Cr concentrations exceeded the maximum permissible limits (Table 5.2B). Relatively, sediment R4 was found be most contaminated with heavy metals.

5.3.1.2. Soil microbial activity measurements

The values for C_{mic} were ranged from 298.2 mg kg^{-1} soil (in R1) to 148.4 mg kg^{-1} soil (in R4) (Fig. 5.3A). Significant *(P* <0.05, LSD) reduction in both C_{mic} and BSR values were noticed in long term IWE polluted soils and sediments R2, R3 and R4 (Fig. 5.3A and B). No significant difference between values of SIR was observed. Higher q-CO_2 values are found (*P* <0.05, LSD) in polluted soils (Fig. 5.3C and D). Relatively higher microbial enzymes were noticed with R1 soil. Significant decrease (*P* <0.05, LSD) in soil *in-situ* microbial enzymes such as DHA, APA, GSA, and PSA were found in metal polluted soils (R2, R3 and R4) (Fig. 5.4A, B, C and D). In R2 soils, enzymes DHA, APA, GSA, and PSA were reduced to 25.6%, 16.68%, 23.06% and 28.57% respectively with reference to uncontaminated control R1 soil. Similarly, these values for R3 soil were reduced to be 34.22%, 31.38%, 46.1% and 52.57% respectively. Highest enzyme reductions (*P* <0.05, LSD) were noticed with R4 sediment sample, where DHA, APA, GSA, and GSA were decreased up to 43.2%, 49.8%, 50.65% and 62.5% respectively.

5.3.1.3. Community structure of eubacteria

Contrasting eubacterial community shift along the contamination gradient was noticed by 16S rRNA based DGGE (Fig. 5.5). High degree of phylogenetic heterogeneity was associated with R1 soils, where most of the bands were equally distributed along the entire denaturant gradient. While in R2 and R3 soils, bands have been concentrated mainly in the middle of the gradient. Bands with the same mobility in the DGGE gel were marked with the same number.

Fig. 5.3: *Microbial biomass C and respiration activities of selected soil samples. A: Microbial biomass C (C_{mic}); B: Basal soil respiration; C: Substrate induced respiration; (D) metabolic quotient (q-CO_2). Values are mean of three independent replicates (n =3). Significant differences (P <0.05, LSD) between the means are indicated by different alphabets. Error bars represent standard deviation of mean (n = 3).*

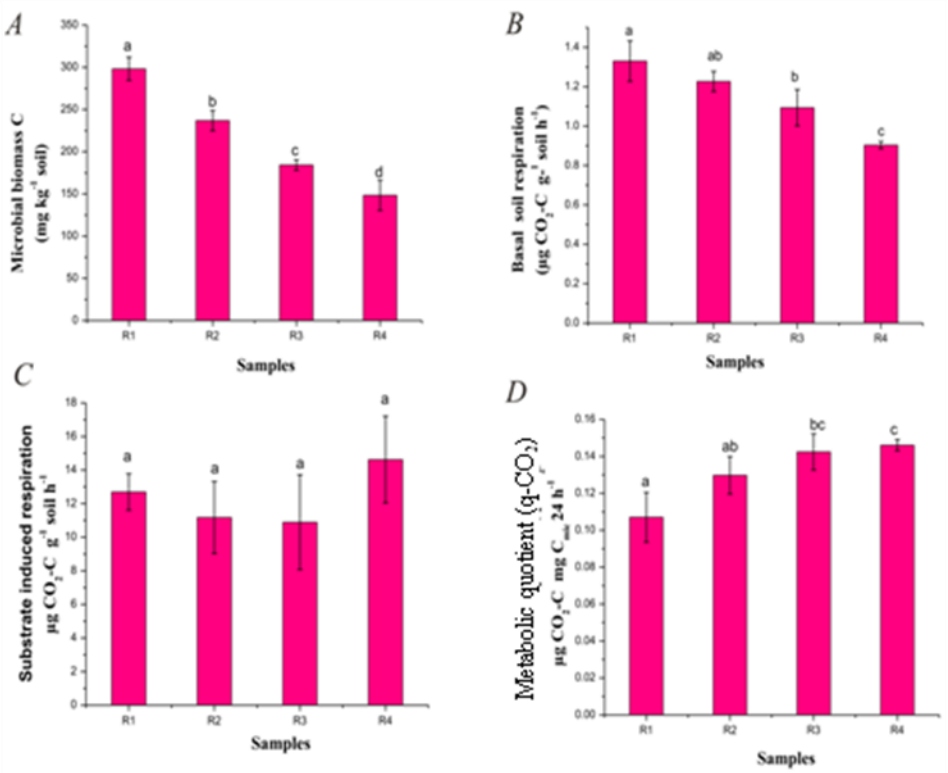
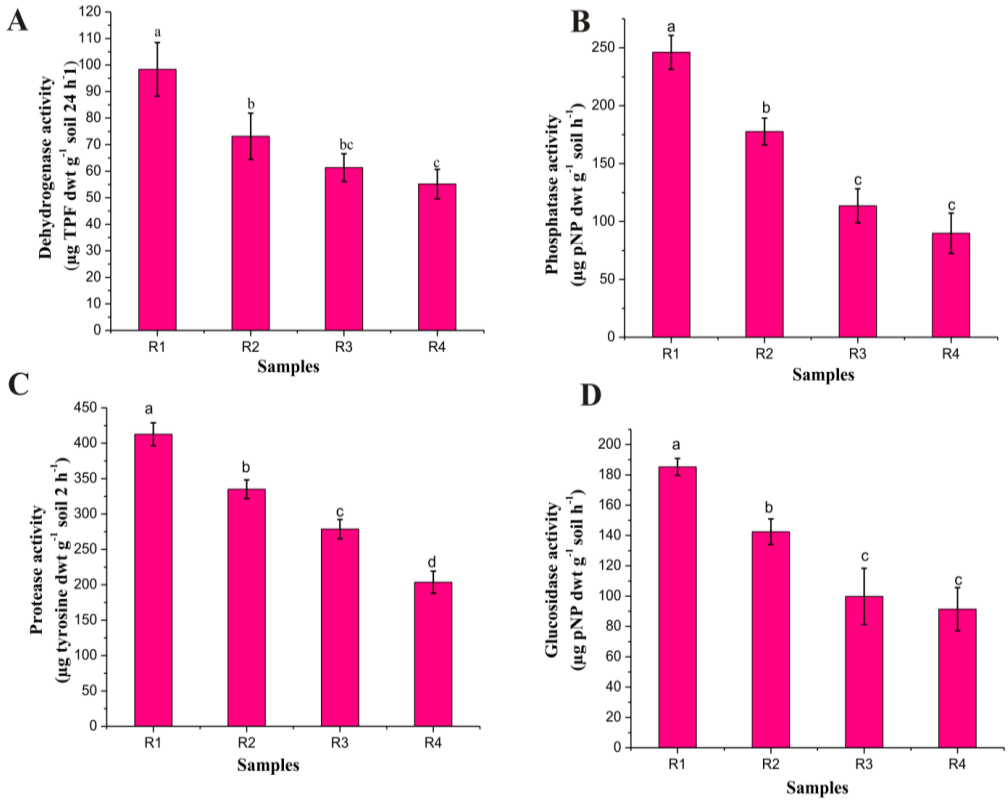


Fig. 5.4: *Soil microbial enzyme activities. A: Dehydrogenase activity B: Phosphatase activity; C: glucosidase activity; (D) Protease activity. Values are mean of three independent replicates (n =3). Significant differences (P <0.05, LSD) between the means are indicated by different alphabets. Error bars represent standard deviation of mean (n = 3).*



Bands S2, S9, S10, S11, S16 and S23 were specific to R1 soil. Similarly, bands S3, S4, S6, S8, S12, S13, S17, S21, S24 and S26 were appeared in R2 and R3 samples. Common bands (S7, S14, S15, S18, S19 and S20) in all the three soils were also noticed. However, bands S15, S18, S19 and S20 were enriched in R2 and R3 samples. No significant ($P < 0.05$) difference among the diversity indices H^I and E^I was observed. However E^I values were relatively high in R2 and R3 samples. Relatively higher *Rr* value (58.8) was obtained in R1 than R2 (43.6) and R3 (44) soils. A total of 26 bands were excised from the DGGE gel (Fig. 5.5), reamplified, cloned and sequenced for phylogenetic analysis. Three clones were randomly selected for each band being sequenced. The sequences at 2% nucleotide cut-offs were used to construct phylogenetic trees with representative sequences retrieved from the GenBank database. Evolutionary distance tree of the sequence of 16S rRNA genes amplified from excised DGGE bands are shown in Fig. 5.6 and 5.7. Major eubacterial phyla in R1 soils were affiliated to *Poteobacteria and Actinobacteria,* where as phyla *Actinobacteria, Firmicutes* and *Acidobacteria* were dominant in R2 and R3 soils (Table 5.3). Phylum *Spirochaeta* was found only in R2 soil. Classes *Alphaproteobacteria* and *Betaproteobacteria* were found to be specific to R1 where as classes *Epsilonproteobacteria* and *Gammaproteobacteria* were specific to R2 and R3 samples. Major euabcterial phyla in contaminated sediment R4 were found to be *Actinobacteria, Firmicutes and Acidobacteria.* Dominance of unclassified bacteria (bands S15, S16 and SD4; Fig. 5.5, 5.6 and 5.7) was observed in contaminated soils (R2 and R3) and sediment (R4).

5.3.1.4. Sequence accession numbers

Nucleotide sequences (bacterial 16S rRNA gene fragments) retrieved from this study have been deposited in the GenBank nucleotide sequence database and the details are mentioned in the Appendix I.

Table 5.2: *Physicochemical properties (A) and heavy metal analysis (B) of soils under investigation. Values are mean of three replicates with a deviation shown in parenthesis. Values within the same column followed by the same letter do not differ significantly at P <0.05 by Fisher's LSD.*

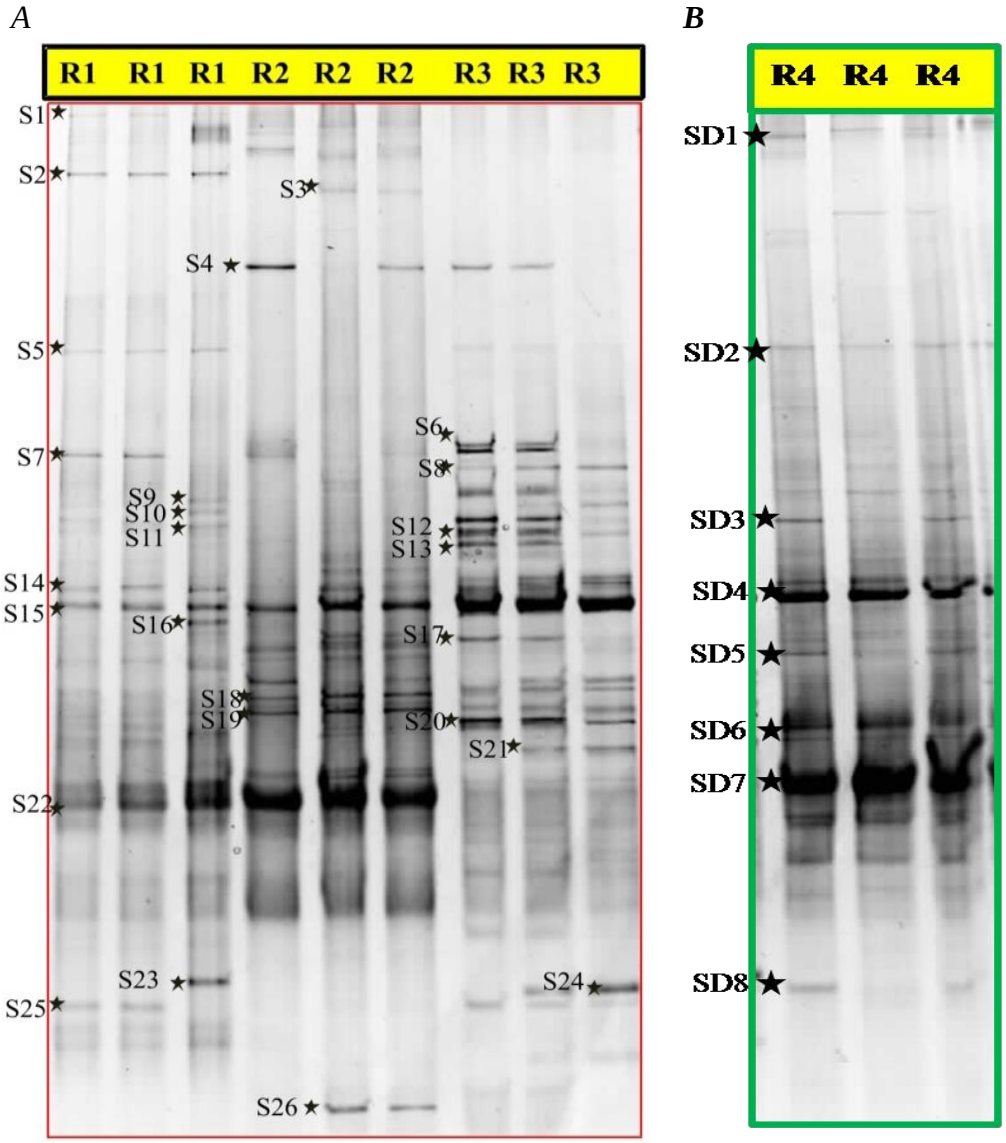
A:

Sample	Soil texture	pH	SMC (%)	OM (%)	TOC (%)	TN (g kg^{-1})	TC (g kg^{-1})	TS (g kg^{-1})	C/N
R1	Silt loam	5.4 (0.14)a	11.2 (2.3)a	0.67 (0.12)a	0.38 (0.09)a	0.5 (0.1)a	4.8 (0.82)a	0.17 (0.04)a	9.6 (1.1)a
R2	Silt loam	7.3 (0.18)b	26.4 (4.1)b	2.11 (0.38)b	1.08 (0.1)b	1.61 (0.3)b	11.4 (1.38)b	0.99 (0.13)b	7.12 (1.4)a
R3	Silt loam	7.4 (0.23)b	29.4 (3.8)b	2.92 (0.41)c	1.72 (0.14)c	3.48 (0.3)c	21.2 (3.4)c	4.7 (1.28)c	6.06 (1.4)b
R4	Clay	8.1 (0.2)c	41 (3.9)c	14.8 (1.2)d	7.44 (0.39)d	17.08 (2.4)d	172.8 (12.49)d	14.7 (5.7)d	10.12 (2.8)c

B:

Sample	Cu (µg/g)	As (µg/g)	Co (µg/g)	Mg (µg/g)	Cr (µg/g)	Mn (µg/g)	Ni (µg/g)	Zn (µg/g)	Cd (µg/g))	Pb (µg/g)	Fe (µg/g)	Al (µg/g)
R1	8.6 (2.1)a	ND	5 (0.8)a	158.6 (22.4)a	13.1 (8.4)a	103.8 (18.2)a	5.7 (0.4)a	41.4 (15.4)a	ND	32 (8.1)a	4524.9 (285)a	2518.2 (225.2)a
R2	128 (28.2)b	6.1 (1.3)a	8.3 (2.3)b	257.7 (31.6)b	28.1 (2.4)b	114.4 (61.2)a	11.6 (4.1)b	65.1 (21.1)a	ND	84 (13.1)b	6170.6 (424.3)b	5701.7 (329.74)b
R3	293.4 (18.1)c	47.6 (2.4)b	9.7 (1.6)b	343.8 (38)c	252.5 (14.8)c	277.9 (28.4)b	16.2 (6.23)b	248.4 (18.4)b	1.2 (0.48)a	198.4 (18.2)c	6012.7 (312.8)b	5298.3 (412)b
R4	562.9 (28.2)d	43.1 (2.3)c	15.6 (2.8)c	390.3 (25)d	378.8 (21.1)c	282.1 (14.4)b	35.5 (2.7)c	651.6 (32.8)c	18.5 (0.22)b	235.2 (14.6)c	8100.6 (214)c	873.7 (28.1)c

Fig. 5.5: *16S rRNA based DGGE fingerprinting of eubacterial communities in selected soil samples. Numbers indicate excised and sequenced bands. A: Soil samples R1, R2, R3; B: Sediment sample R4.*



5.3.2. Discussion

High amounts of heavy metals were found in IWE contaminated sites (R2, R3 and R4). Previous studies by Greenpeace international indicates that the industrial effluent consist of remarkable amounts of organ halogen compounds, phenolic compounds, polycyclic aromatic hydrocarbons, phthalates, aliphatics and aromatics including heavy metals (Santillo et al., 1996; Labunska et al., 1999). Microbial biomass carbon (C_{mic}) and soil respiration are considered to be important soil biological activities which are frequently influenced by heavy metal contamination. Understanding the environmental as well as anthropogenic influences on microbial

biomass and activity is the key to predict changes in nutrient cycling of semiarid soils. The soil microbial biomass is the active component of the soil organic pool, which is responsible for organic matter decomposition affecting soil nutrient content and, consequently, primary productivity in most biogeochemical processes in terrestrial ecosystems (Gregorich et al., 2000; Haney et al., 2001). In the past, C_{mic} has been used as meaningful and sensitive indexes of soil quality evaluation (Xu et al, 2008; Ros et al., 2009; Khan et al., 2010; Zhang et al., 2010). In the present study, significant reduction (P <0.05, LSD) in soil C_{mic} and BSR were noticed with heavy metal polluted semiarid tropic soils (R2, R3, R4) (Fig. 5.3A and B). These results are indicated the apparent negative effect of heavy metals on soil C_{mic} and basal respiration (Khan et al., 2010; Yan et al., 2008; Zhang et al., 2010). Based on the values of both BSR and SIR, it can be inferred that the microbes in R1 soil have greater potential in SOM decomposition and carbon flow to the atmosphere (Schlesinger and Andrews, 2000). Our finding suggests that MBC may be used a sensitive bio-indicator for assessing changes in soil environmental quality in metal polluted semiarid soils.

Soil microbial enzymes play a key role in the decomposition of organic residues and are considered fundamentally good indicators of soil quality (Zahir et al., 2001). Several enzymes are known to be involved in the cycling of nutrients and can be used as potential indicators of nutrient cycling processes. In order to evaluate the fluxes of elements in metal contaminated soils, hydrolytic enzymes such as GSA, PSA and APA involved in C, N, P cycles respectively (Masciandaro and Ceccanti, 1999; nannipieri et al., 2002) were determined. Significant (P < 0.05) heavy metal toxicity was noticed with soil *in situ* enzymes. Therefore, it could be inferred that microbial mediated soil C, N and P cycles considerably decreased as a result of IWE contamination and thus soil health may be endangered in the studied soils.

Fig. 5.6: *16S rRNA based phylogenetic tree of the eubacterial sequences from R1, R2 R3 soil samples. Phylogenetic analyses were conducted in MEGA-5.05 (Tamura et al., 2011). The evolutionary history was inferred using the UPGMA method. The percentage of replicate trees in which the allied taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. The scale bar represents 5% estimated nucleotide sequence divergence. Clades are labeled with representative bacterial phyla. DGGE bands with the corresponding GenBank accession numbers (in parenthesis) are shown with triangles.*

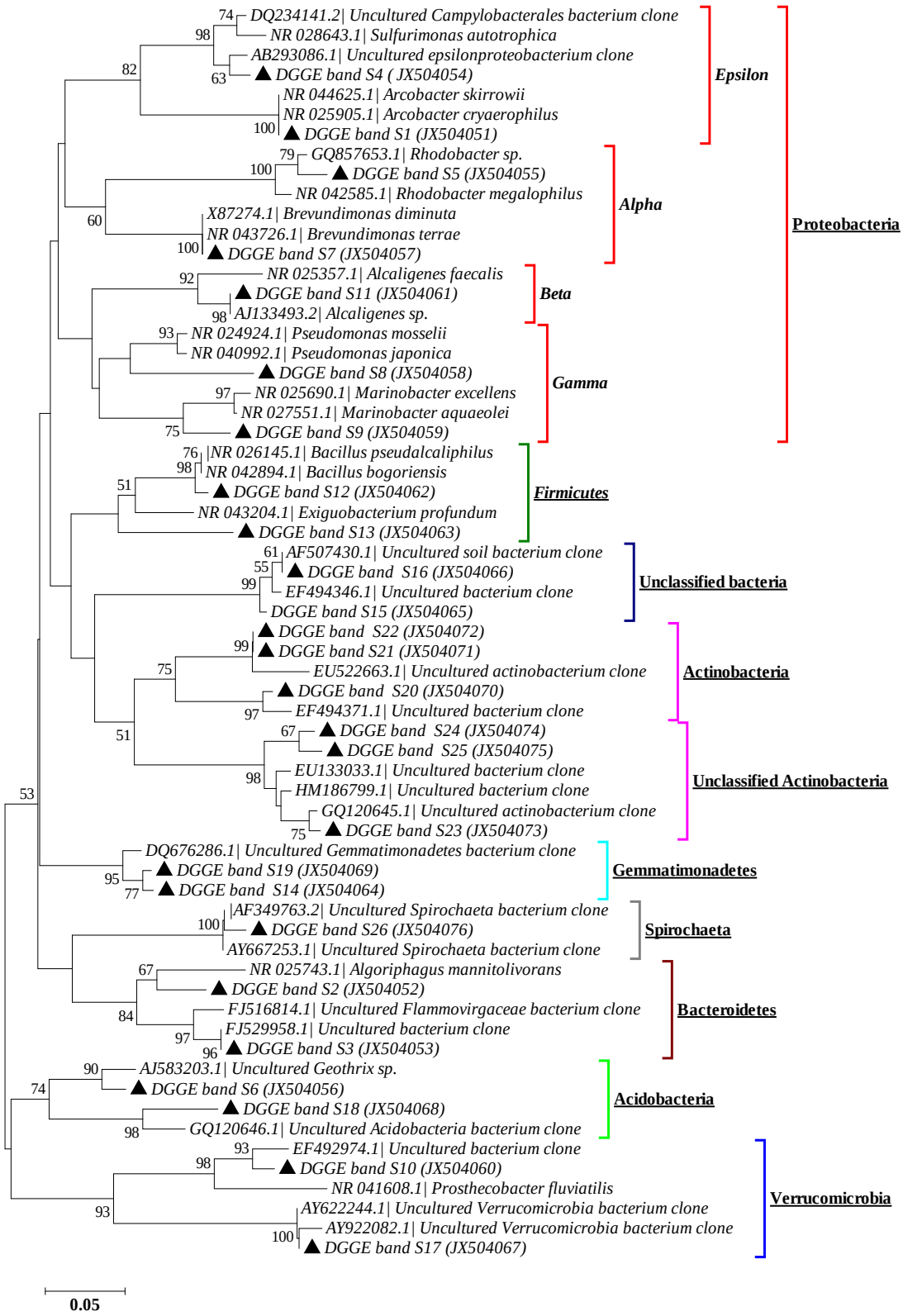

DQ234141.2| Uncultured Campylobacterales bacterium clone
NR 028643.1| Sulfurimonas autotrophica
AB293086.1| Uncultured epsilonproteobacterium clone
DGGE band S4 (JX504054)
NR 044625.1| Arcobacter skirrowii
NR 025905.1| Arcobacter cryaerophilus
DGGE band S1 (JX504051)
GQ857653.1| Rhodobacter sp.
DGGE band S5 (JX504055)
NR 042585.1| Rhodobacter megalophilus
X87274.1| Brevundimonas diminuta
NR 043726.1| Brevundimonas terrae
DGGE band S7 (JX504057)
NR 025357.1| Alcaligenes faecalis
DGGE band S11 (JX504061)
AJ133493.2| Alcaligenes sp.
NR 024924.1| Pseudomonas mosselii
NR 040992.1| Pseudomonas japonica
DGGE band S8 (JX504058)
NR 025690.1| Marinobacter excellens
NR 027551.1| Marinobacter aquaeolei
DGGE band S9 (JX504059)
NR 026145.1| Bacillus pseudalcaliphilus
NR 042894.1| Bacillus bogoriensis
DGGE band S12 (JX504062)
NR 043204.1| Exiguobacterium profundum
DGGE band S13 (JX504063)
AF507430.1| Uncultured soil bacterium clone
DGGE band S16 (JX504066)
EF494346.1| Uncultured bacterium clone
DGGE band S15 (JX504065)
DGGE band S22 (JX504072)
DGGE band S21 (JX504071)
EU522663.1| Uncultured actinobacterium clone
DGGE band S20 (JX504070)
EF494371.1| Uncultured bacterium clone
DGGE band S24 (JX504074)
DGGE band S25 (JX504075)
EU133033.1| Uncultured bacterium clone
HM186799.1| Uncultured bacterium clone
GQ120645.1| Uncultured actinobacterium clone
DGGE band S23 (JX504073)
DQ676286.1| Uncultured Gemmatimonadetes bacterium clone
DGGE band S19 (JX504069)
DGGE band S14 (JX504064)
AF349763.2| Uncultured Spirochaeta bacterium clone
DGGE band S26 (JX504076)
AY667253.1| Uncultured Spirochaeta bacterium clone
NR 025743.1| Algoriphagus mannitolivorans
DGGE band S2 (JX504052)
FJ516814.1| Uncultured Flammovirgaceae bacterium clone
FJ529958.1| Uncultured bacterium clone
DGGE band S3 (JX504053)
AJ583203.1| Uncultured Geothrix sp.
DGGE band S6 (JX504056)
DGGE band S18 (JX504068)
GQ120646.1| Uncultured Acidobacteria bacterium clone
EF492974.1| Uncultured bacterium clone
DGGE band S10 (JX504060)
NR 041608.1| Prosthecobacter fluviatilis
AY622244.1| Uncultured Verrucomicrobia bacterium clone
AY922082.1| Uncultured Verrucomicrobia bacterium clone
DGGE band S17 (JX504067)
Epsilon
Alpha
Beta
Gamma
Proteobacteria
Firmicutes
Unclassified bacteria
Actinobacteria
Unclassified Actinobacteria
Gemmatimonadetes
Spirochaeta
Bacteroidetes
Acidobacteria
Verrucomicrobia
0.05

Fig. 5.7: *16S rRNA based phylogenetic tree of the bacterial isolaes from R4 sediment sample. Phylogenetic analyses were conducted in MEGA-5.05 (Tamura et al., 2011). The evolutionary history was inferred using the UPGMA method. Bootstrap values (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. The scale bar represents 5% estimated nucleotide sequence divergence. Clades are labeled with representative bacterial phyla. DGGE bands with the corresponding GenBank accession numbers (in parenthesis) are shown with triangles.*

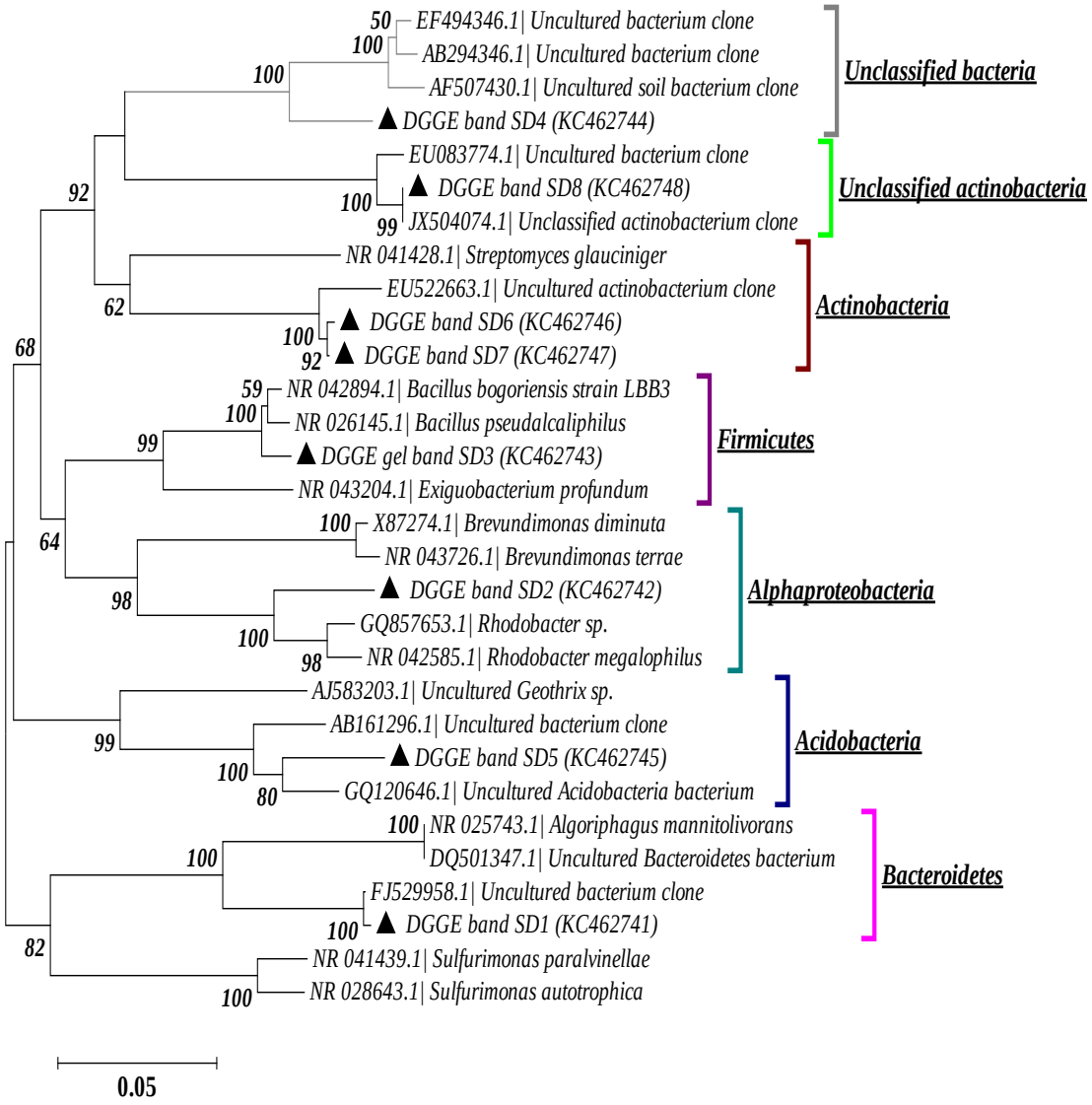


Table 5.3: *Comprehensive table consists of DGGE band numbers, their coresponing samples, GenBank accession numbers and closet 16srRNA match.*

Sample	DGGE band number	GenBank accession number for DGGE band	Closet organism from the GenBank	Accession number of closet organism	Bacterial phylum
R1	S1	JX504051	*Arcobacter* sp.	NR025905	*Epsilonproteobacteria*
R1	S2	JX504052	*Algoriphagus* sp.	NR025743	*Bacterioidetes*
R1	S5	JX504055	*Rhodobacter* sp.	GQ857653	*Alphaproteobacteria*
R1	S7	JX504057	*Brevundimonas* sp.	NR043726	*Alphaproteobacteria*
R1	S9	JX504059	*Marinobacter* sp.	NR027551	*Gammaproteobacteria*
R1	S10	JX504060	*Uncultured bacterium* clone	EF492975	*Verrucomicrobia*
R1	S11	JX504061	*Alcaligenes* sp.	NR025357	*Betaproetobacteria*
R1	S14	JX504064	*Uncultured Gemmatimonadetes* sp.	DQ676286	*Gemmatimonadetes*
R1	S15	JX504065	*Uncultured bacterium* Clone		*Unclassified bacteria*
R1	S16	JX504066	*Uncultured bacterium* clone	AF507430	*Unclassified bacteria*
R1	S22	JX504072	*Uncultured Actiobacterium* clone	EU522663	*Actinobacteria*
R1	S23	JX504073	Uncultured *Actinobacterium* clone	GQ120645	*Unclassified Actinobacteria*
R1	S25	JX504075	*Uncultured bacterium* clone	EU133033	*Unclassified Actinobacteria*
R2	S3	JX504053	*Uncultured bacterium* clone	FJ529958	*Bacterioidetes*
R2	S4	JX504054	Uncultured *Epsilonproteobacteria*	AB293086	*Epsilonproteobacteria*
R2	S18	JX504068	*Uncultured Acidobacteria* clone	GQ120646	*Acidobacteria*
R2	S19	JX504069	*Uncultured Gemmatimonadetes* sp.	DQ676286	*Gemmatimonadetes*
R2	S26	JX504076	*Uncultuerd Spirochaeta bacterium* clone	AF349763	*Spirochaeta*
R3	S6	JX504056	*Uncultured Geothrix* sp.	AJ583203	*Acidobacteria*
R3	S8	JX504058	*Pseudomonas* sp.	NR040992	*Gammaproteobacteria*
R3	S12	JX504062	*Bacillus* sp.	NR042894	*Firmicutes*
R3	S13	JX504063	*Exiguobacterium* sp.	NR043204	*Firmicutes*
R3	S17	JX504067	*Uncultured Verrucomicrobia* clone	AY922082	*Verrucomicrobia*
R3	S20	JX504070	*Uncultured bacterium* clone	EF494371	*Actinobacteria*
R3	S21	JX504071	*Uncultured Actiobacterium* clone	EU522663	*Actinobacteria*
R3	S24	JX504074	*Uncultured bacterium* clone	EU133033	*Unclassified Actinobacteria*
R4	SD1	KC462741	Uncultured bacterium clone	FJ529958	*Bacterioidetes*
R4	SD2	KC462742	*Rhodobacter* sp.	GQ857653	*Alphaproteobacteria*
R4	SD3	KC462743	*Bacillus* sp.	NR 026145	*Firmicutes*
R4	SD4	KC462744	*Uncultured soil bacterium* clone	AF507430	*Unclassified bacteria*
R4	SD5	KC462745	*Uncultured Acidobacterium* clone	AB161296	*Acidobacteria*
R4	SD6	KC462746	*Uncultured Actinobacterium* clone	EU522663	*Actinobacteria*
R4	SD7	KC462747	*Uncultured Actinobacterium* clone	EU522663	*Actinobacteria*
R4	SD8	KC462748	*Unclassified Actinobacterioum* clone	EU083774	*Unclassified Actinobacteria*

In the present study, DHA was found to be significantly *(P* <0.05, LSD) decreased in IWE contaminated soils such as R2, R3 and R4 (Fig. 5.4A). It is been reported that DHA is associated with living cells, linked with microbial oxidoreduction processes and is the principal agent in the degradation of SOM (Tabatabai, 1994; Alef and Nannipieri, 1995). Remarkably high amount of DHA activity was found in R1 (reference soil) and is in general agreement with high microbial respiration activity, indicating that organic matter decomposition is much active in uncontaminated soils. Our results indicate the pernicious effect of IWE on soil microbial functions in these landscapes. It can be inferred that soil *in-situ* enzymes particularly APA and GSA could be considered as bio-indicators to evaluate heavy metal pollution in semiarid tropic soils as enzymatic activities are reliable bio-indicators to emphasize natural and toxicological effects of various pollutants on soil microbial quality (Shen et al., 2005). It is thought that the bacterial community structure was decreased in the soils amended with different concentration of heavy metals as a result enzyme activities impaired.

In addition to the obvious effect of heavy metals on microbial activities, a change in the microbial community structure was observed by PCR–DGGE of eubacterial 16S rRNA gene fragments. These microbial responses tend to be dose-dependent, with increasing metal pollution causing a larger shift in the microbial response compared to the uncontaminated control as suggested earlier (Shi et al., 2002). The bacterial groups that were examined here are commonly found soil microbiota, which were shown to play a key role in soil functioning and nutrient cycling and also adaptable to extreme and contrasting environments (Eriksson et al. 2001; Desai et al., 2009; Ros et al., 2009; Zhang et al., 2012). In the present study, majority of bacterial phyla in contaminated soils and sediments corresponds to *Firmicutes, Actinobacteria, Acidobacteria, and γ-proteobacteria* pointing their possible potential role in functioning of contaminated systems. We have found predominance of unclassified bacteria in contaminated soils, indicating that majority of bacterial species yet to be indentified from heavy metal polluted soils. Negative impact of heavy metals on *Cytophaga-Flexibacter-Bacteroides* (CFB) bacteria was also found in contaminated sediment. Previously, Gillan et al. (2005) found the negative effect of Cu, Pb, and Zn on sediment *Bacteroidetes*. These findings reveal

that long-term IWE perturbation in semiarid soils confers soil bacterial community shifts towards a dominance of *Firmicutes* and *Actinobacteria*.

Phylogenetic analysis of 16S rRNA clearly indicated the selection in bacterial community structure with response to pollution by industrial waste water. PCR-DGGE using primers based on conserved regions of the 16S rRNA sequence yielded complex molecular profiles of soil bacteria. The profiles thus obtained reflect the composition of the dominant soil microbiota, which includes the nonculturable fractions. The DGGE patterns obtained in this study with total community DNA from soils with different levels of IWE contamination are to be interpreted as a first estimation to showing shifts in the composition of the dominant bacterial populations related to the contaminated levels. The molecular profiling data (Fig. 5.5) indicated that specific bacterial populations were selected with increasing contamination levels. In pristine R1 soil, a high percentage of bacteria belonging to the *phyla Proteobacteria* and *Actinobacteria* were found. In addition, *Verrucomicrobia, Bacteroides* and *Gemmatomonoadetes* phyla are also found. These bacterial phyla are common in semiarid pristine soils (Hendrickx et al., 2005), whereas in contaminated R2 and R3 soils, phyla *Actinobacteria, Firmicutes* and *Acidobacteria* were found to be the abundant bacterial lineages. In addition, class *Betaproteobacteria* and *Gammaproteobacteria (Pseudomonas* sp.) were found in R3. These phyla are previously observed in many studies focusing on soils those have been contaminated with persistent organic pollutants, hydrocarbons and heavy metals (Hendrickx et al., 2005, Bordenave et al., 2007; Janssen, 2006; Zhang et al., 2010; Nakatsu et al., 2005; Popp et al., 2006; Liao et al., 2010; Sheik et al., 2012). The phylogenetic identity of many of the bacteria selected under these conditions is consistent with *in situ* analyses of other metal-contaminated soils (Gremion et al., 2003) The *Actinomycetales* have been reported to be important in metal-impacted soils (Nakatsu et al., 2005). Hence, we propose here that *Actinobacteria, Firmicutes* and *Acidobacteria* could be used as biological indicative markers for sensitivity of semiarid soil contamination by mixed bag of pollutants. It is however not possible with this study to predict how microbial communities will respond when exposed to individual metals.

5.3.3. Conclusions

Contamination of IWE had significant negative impacts on bacterial abundance, activity and community structure in semiarid soil/sediments. Enzyme activity results indicate that IWE have a negative impact on microbial potentiality in nutrient cycling. Microbial parameters such as C_{mic} and extracellular enzymes are found to be good responsive measures for monitoring the toxicity of IWE deposited in the environment. We infer that *Firmicutes, Actinobacteria* and *Acidobacteria* could be used as indicative organisms for soil contamination by mixed bag of pollutants.

5.4. Effect of industrial waste effluent (IWE) on soil nitrification and ammonia oxidizing microbes

5.4.1. Results

5.4.1.1. Soil physicochemical characters and potential nitrification rate (PNR)

Soil physicochemical characters are summarized in Table 5.2A and 5.2B. High C/N ratio was associated with R1 and was significant with R3. There was a significant increasing trend in soil $NH4^+$-N (mg kg^{-1}) from R1 to R3. The values for soil NO_3^--N (mg kg^{-1}) ranging from 4.21 (in R1) to 1.62 in (R3). Potential nitrification rate was comparatively high in uncontaminated soil R1 and was significant (Table 5.4). The PNR values for R1, R2 and R3 were estimated to be 3.07, 1.26 and 0.84 mg NO_2^-- N kg^{-1} soil h^{-1} respectively.

Table 5.4: *Soil ammonium, nitrate and PNR of IWE polluted soils*

Soil	$NH4^+$-N	NO_3^--N	PNR
R1	9.45 (1.33)a	4.21 (0.27)a	3.07 (0.62)a
R2	14.28 (3.47)b	2.03 (0.14)b	1.26 (0.11)b
R3	32.3 (3.82)c	0.62 (0.1)c	0.84 (0.08)c

5.4.1.2. Abundance of 16S rRNA, archaeal 16S rRNA, AOA-amoA and AOB-amoA genes by q-PCR

Relatively higher amount of bacterial 16S rRNA, archaeal 16S rRNA, AOA-*amoA* and AOB-*amoA* gene copies (log transformed) were found in uncontaminated R1 soil than contaminated R2, and R3 soils (Fig. 5.8A and B). Both AOA and AOB-amoA gene copies significantly decreased in contaminated soils. AOA-*amoA* gene abundance ranged from $2.81x10^8$ (in R1) to $8.8x10^5$ (in R3) copies g^{-1} dry soil, whereas AOB-*amoA* gene abundance ranged from $7.63x10^6$ (in R1) to $3.09x10^6$ (in R3) copies g^{-1} dry soil (Fig. 5.8B). AOA-*amoA* genes were significantly (P <0.05) abundant than AOB-*amoA* genes in R1 and R2 soils (Fig. 5.8B). In R1 the ratio of AOA-*amoA* to AOB-*amoA* gene was found to be 36.82 ± 6.75, however the ratios significantly (P <0.05, LSD) decreased in R2 soil (6.29 ± 1.1). In R3 soil, AOB-*amoA* genes outnumbered the (P <0.05) AOA-amoA gene, and the ratio of AOB-*amoA* to AOA-*amoA* gene was found to be 3.51.

5.4.1.3. Community structure of ammonia oxidizing microbes

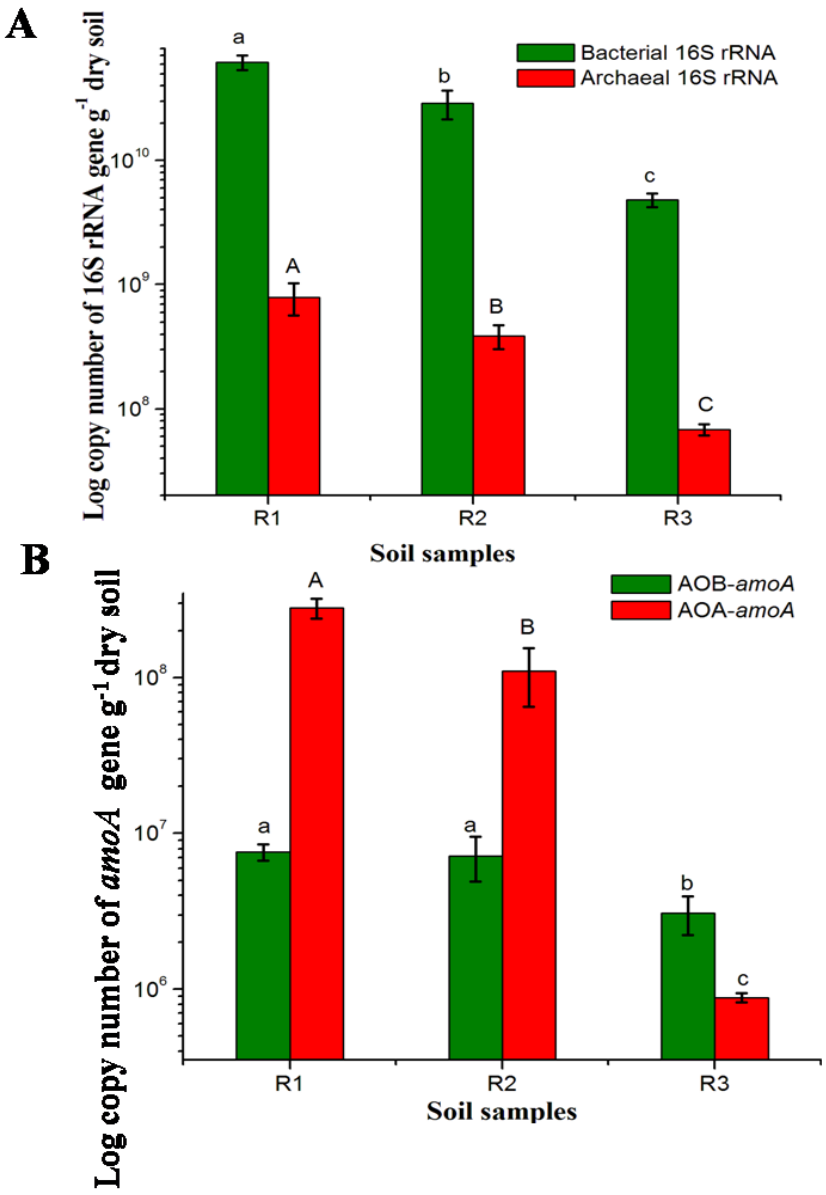
The community structure of AOB and AOA were revealed by DGGE based molecular fingerprinting. DGGE profiles of three independent replicates showed good reproducibility and are shown with Figs 5.9A and B. The sequences at 2% nucleotide cut-offs were used to construct phylogenetic trees with representative sequences retrieved from the GenBank database (Figs. 5.10A and B).

5.4.1.4. Community structure of AOA

Reproducible AOA-*amoA* gene profiles were obtained for all soils, and the numbered bands of three replicates with the same mobility in the DGGE gels were excised, reamplifieded and sequenced for phylogenetic analysis (Fig. 5.10A). Complex and contrasting AOA-*amoA* gene profiles were observed along the contamination. Relatively higher AOA-*amoA* gene diversity was noticed in pristine R1 soil than contaminated R2 and R3 soils. The diversity indices (H^I and E^I) of AOA-*amoA* gene for the samples were as follows R1 >R2 > R3 (Table 5.5). Higher *Rr* value (29.57) was found in R1 soil where as lowest *Rr* value observed with R3 soil. Phylogenetic analysis of 15 DGGE bands revealed that, Thaumarchaeal “group 1.1b” was dominant AOA in these soils (5.10A). Thaumarchaeal “group 1.1a” was found

only in contaminated soils (R2 and R3) where as Thaumarchaeal "group 1.1a associated" was found only in pristine soil R1.

Fig. 5.8: *Quantification of genes in R1, R2 and R3 soil samples. A: log transformed 16S rRNA gene copy number of both archaea and bacteria; B: log transformed amoA gene copy numbers of both AOA and AOB. Significant differences (P <0.05, Fisher's LSD) between the soils are indicated by different alphabets. Error bars represents standard deviation of mean (n = 3).*



5.4.1.5. Community structure of AOB

The DGGE profile of AOB-*amoA* gene consisted of a few DNA bands, depicting a quite simple AOB community structure than AOA (Fig. 5.10B). Greater diversity of AOA-*amoA* gene fragments (12 bands) was observed in R1 than R2 (9 bands) and R3 (7 bands) soils. Diversity indices, H^I ranged from 1.7 (in R3) to 2 (in R1). There were significant differences in the Evenness (E^I) among different soils (P >0.05; Table 5.5). Relatively higher E^1 was found in R3 (2.06) than R1 (1.84). Higher *Rr* (15.1) value was noticed with R1 soil.

Although some bands (B1, B4 and B5) were present in the profiles of all soils, intensities of these bands were different. For example, band B1 had the highest intensity in R2 and R3 soils and the lowest in R1 soil. Similarly, bands B4 and B5 had the highest intensity in R1 than in R2 and R3 soils. Bands numbered B6 and B7 were specific to R1 soil, whereas bands B2, B3 and B10 were specific to R2 and R3 soils. The classification for AOB clusters was tentatively defined as like as in previous studies (Avrahami and Conrad, 2003; He et al., 2007). All the 10 AOB-*amoA* DGGE band clones were represented to both *Nitrosospira*-like sequences and *Nitrosomona*s-like sequences (Fig. 5.10B). Nevertheless, *Nitrosospira*-like sequences were dominant ones, consisted of 70% total sequences. *Nitrosomonas nitrosa* cluster-8 was found only in contaminated soils, whereas *Nitosospira* cluster-11 was specific to pristine soil. *Nitrosospira* cluster-3a is common for all three soil samples. In addition to this, *Nitosospira* cluster-1, 2, and 4 were also noticed among soil AOB-amoA genetic profile.

5.4.1.6. Sequence accession numbers

Nucleotide sequences retrieved from this study have been deposited in the GenBank nucleotide sequence database and the details are given in appendix I.

Fig. 5.9: *Community structure of ammonia oxidizing microbes. DGGE analysis of AOA-amoA (A) and AOB-amoA (B) gene fragments obtained from R1, R2 and R3.*

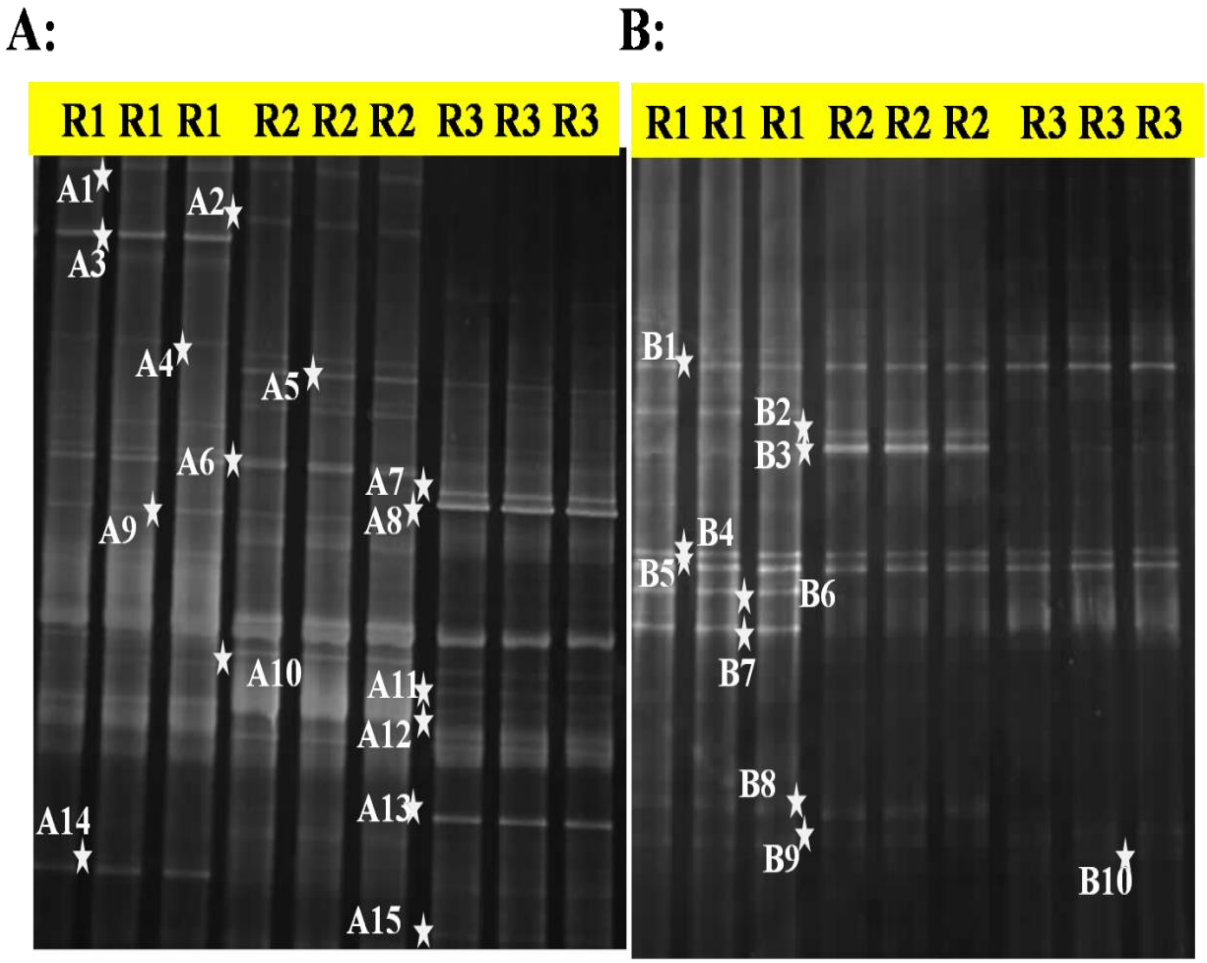
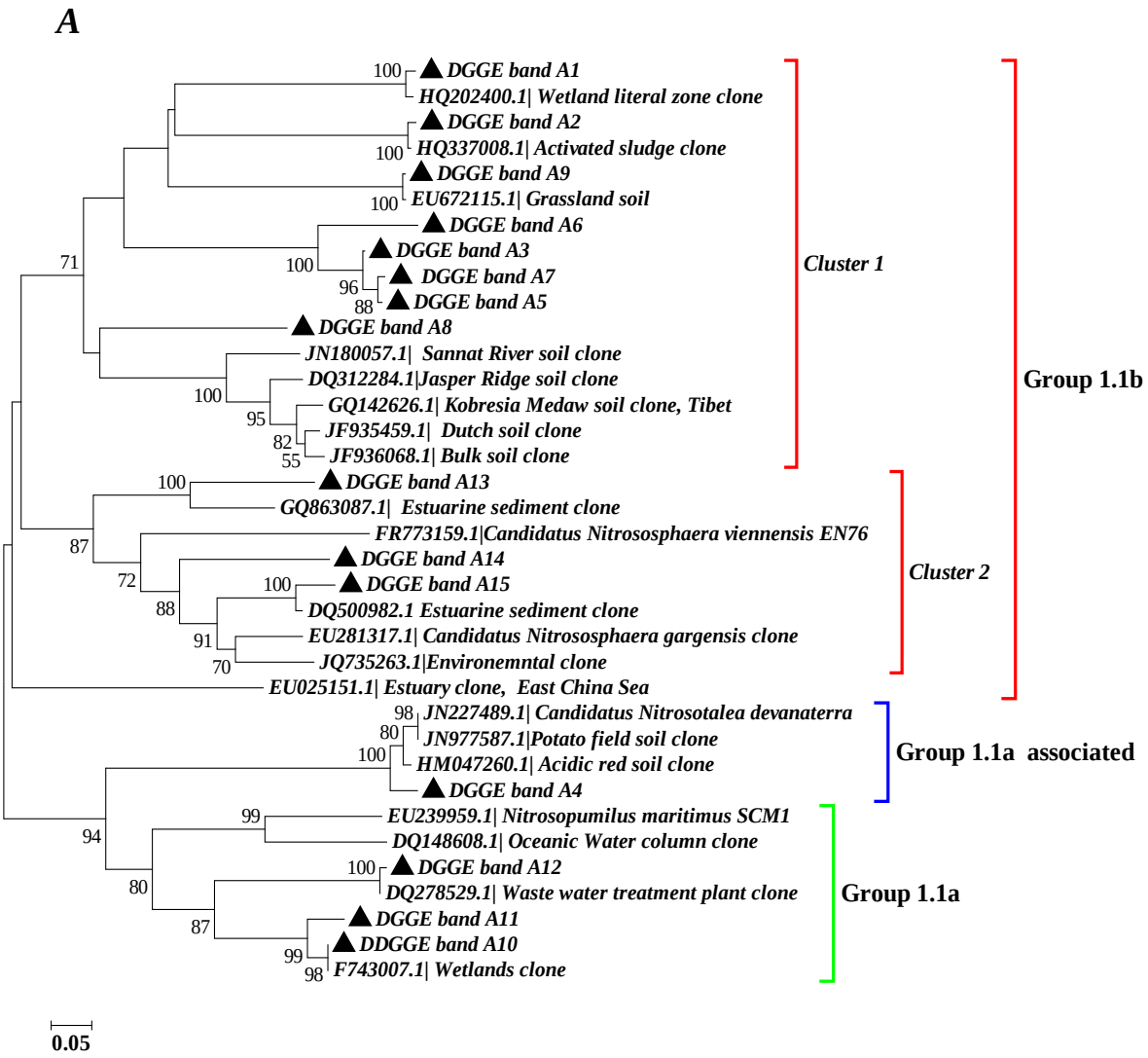


Table 5.5: *Diversity indices of ammonia oxidizing microbes in long term IWE perturbed semiarid soils. Diversity indices were estimated based on the DGGE band intensities. AlphaEase FC software (Alpha Innotech, CA, USA) was used for DGGE band analysis. Significant differences (P <0.05, Fisher's LSD) between the soils are indicated by different alphabets.*

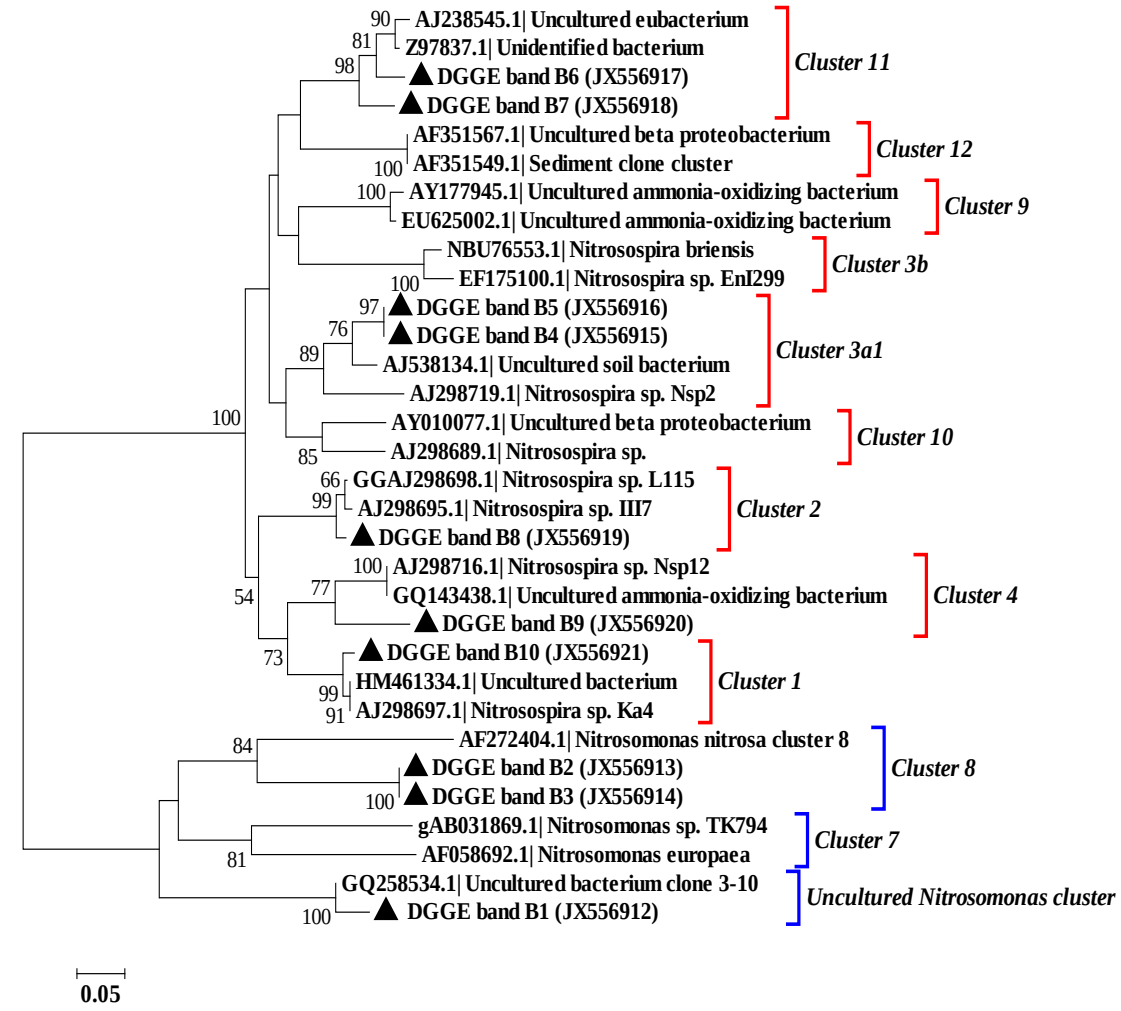
Sample	***AOB-amoA***			***AOA-amoA***		
	H^I	***E^I***	***Rr***	***H^I***	***E^I***	***Rr***
R1	*2.05a*	*1.84a*	*15.12a*	*2.12a*	*2.15a*	*29.57a*
R2	*1.99a*	*2.08b*	*9.8b*	*1.84a*	*2.01ac*	*27.04b*
R3	*1.74b*	*2.06b*	*4.5c*	*1.43b*	*1.31bc*	*12c*

H^I: Shanon diversity indices, *E^I: Evenness, Rr: Range weighted richness*

Fig. 5.10: *The phylogenetic relationship of deduced amino acid sequences of AOA-amoA and AOB-amoA genes retrieved from R1, R2 and R3 soil samples. Phylogenetic tree was constructed with Neighbor-joining method. Pairwise distances of aligned amino acid positions were calculated by using the Equal input modal with gamma distributed rates among invariable sites. Clones sequences are shown with triangle including GenBank accession numbers in parenthesis. Bootstrap Values (> 50%) are indicated at branch points. The scale bar represents 5% estimated sequence divergence.* ***A:*** *Phylogenetic relationship among deduced amino acid sequences of AOA-amoA genes. Clades are labeled with thaumarchaeal groups and their frequently represented environment. The tree was rooted with sequences affiliated to marine cluster, thaumarchaeal group 1.1a.* ***B:*** *Phylogenetic relationship among deduced amino acid sequences of AOB-amoA genes. Clades are labeled with suitable AOB clusters. The tree was rooted with sequences affiliated to Nitrosomonas clusters. Branch lengths correspond to sequence differences as indicated by the scale bar.*



B



5.4.2. Discussion

Nitrification, the rate limiting step of N cycle is carried out by autotrophic ammonia-oxidizing bacteria (AOB) and archaea (AOA) in most of the ecosystems. These microbes are responsible for the oxidation of ammonia to nitrite by the enzymes v.i.z. ammonia monooxygenase and hydroxylamine oxidoreductase (de Boer et al., 2001; Kowalchuk et al., 2001). Therefore, ammonia oxidizing microbes are of great ecological significance in global nitrogen cycle. Studies indicated the global distribution of AOA and AOB in a diverse range of environments, (Francis et al., 2005; Konneke et al., 2005; Leininger et al., 2006; He et al., 2007; Kayee et al., 2011; He et al., 2012). Molecular ecological approaches based on *amoA* gene have shed light on ecophysiology of ammonia oxidizing microbes in terrestrial ecosystems; for example abundance, distribution and community structure of ammonia-oxidizing

organisms can be significantly affected by environmental factors, including levels of ammonium, temperature (Tourna et al., 2008), moisture (Horz et al., 2004), salinity (Bernhard et al., 2010), and pH (He et al., 2007; Nicol et al., 2008). However it is not yet clear how selection pressures imposed by industrial waste effluent affect activity, diversity and dynamics of soil AOA and AOB. In the present study culture - independent experiments were conducted to gain insights into the ecophysiology of the AOA and AOB in semiarid soils which were under long-term exposure of industrial effluent with pollutants such as organic, heavy metals and elevated ammonia concentrations (Labunska et al., 1999; Santillo et al., 1996).

In the present study, soil PNR was significantly (P <0.05) inhibited by IWE. These results were in supportive of previous results indicating that PNR could be used as sensitive indicator for soil pollution (Smolders et al. 2004; Xu and Chen, 2006). The abundances of both AOA and AOB-*amoA* genes was comparable to those observed in previous studies (Leininger et al., 2006; He et al., 2007, 2012; Nicol et al., 2008; Zhang et al., 2010). AOA-*amoA* genes outnumbered AOB-*amoA* genes in uncontaminated (R1) and moderately contaminated (R2) soils and the ratios of AOA to AOB fall within those found in a wide range of soils (1.02–232; Leininger et al., 2006).

Significant reduction in AOA-*amoA* gene copies and not the AOB-*amoA* gene were noticed in highly contaminated soil (R3). Further, the reduction of AOA-*amoA* gene copies in R2 and R3 soils are corroborated to reduction in soil PNR. Therefore, the results confirm a high contribution of AOA-*amoA* genes to the nitrification of contaminated and uncontaminated semiarid soils (He et al., 2012).

Previous studies indicate the abundance of AOA over AOB in heavy metal polluted soils (Li et al., 2009; Kelly et al., 2011; Vasileiadis et al., 2012). Archaea are thought to be more tolerant to chronic stress conditions than bacteria (Schleper et al., 2005; Valentine, 2007). More recently, genome analysis of *Nitrosophaera gargensis* revealed that AOA well adapted to heavy metals containing habitats by encoding multiple metal resistance mechanisms including 21 putative metal ion efflux proteins belongs to 10 different transporter families (Spang et al., 2012). Therefore, the reduction of AOA-*amoA* gene in R2 and R3 soils may not be due to heavy metals and could be attributed to organic pollutants and higher NH_4^+ concentrations. Inhibitory

effect of organic pollutants on activity of AOA is reported in earlier studies (Martens-Habbena et al., 2009). It has been suggested that AOB growth is favored by organic rich conditions, whereas AOA have a preference for low-fertility or oligotrophic environments (Valentine, 2007; Erguder et al., 2009). AOA are highly active at 0.14 to 0.79 mM ammonium and is partially inhibited by a concentration of 3.08 mM (Hatzenpichler et al., 2008). The NH_4^+ concentrations in R1, R2, and R3 soils are 0.52, 0.9 and 2.73 mM respectively. Inhibition of AOB at such a low ammonium concentration has not been reported, to our knowledge, and some AOB strains grow at more than 200 mM ammonium at a comparable or higher pH (Suwa et al., 1997; He et al., 2 012). Additionally, soil pH is found to be critical for the abundance and activity of AOA and AOB (Nicol et al., 2008; He et al., 2012). Acidic pH favors the growth of AOA, where as alkali and neutral pH favors the growth of AOB (Nugroho et al 2007; Nicol et al.2008; Erguder et al., 2009; He et al., 2012).

Although ammonia-oxidizing microbes (AOA and AOB) play a key role in the soil nitrogen cycle, we have only a limited understanding of how the diversity and composition of soil AOB and AOA communities change across soil pollution. In the present study significant impact of IWW on AOA community structure has been observed with DGGE. Irrespective of soil pollution, thaumarchaeal "group 1.1b (*Nitrososphaera* clusters)" is found to be widely distributed in these soils. The dominance of group 1.1b in a range of soils has been noticed in earlier studies (Nicol*et* al., 2008; Gubry-Rangin *et* al., 2011; Pester et al., 2012). Thaumarchaeal "group 1.1b is shown to participate in ammonia oxidation of strongly acidic soils (Zhang et al., 2012) as well as in heavy metal contaminated thermal spring (Hatzenpichler *et al*., 2008). Studies indicate that group 1.1b-AOA adapted to its niche in a heavy metal-containing thermal spring by encoding a multitude of heavy metal resistance genes and chaperones as reveled by genome sequencing of *Candidatus Nitrososphaera gargensis* (Spang et al., 2012). In addition to this, group 1.1b-AOA also has the ability to respond to environmental changes by mannosylglycerate as compatible solute and also with signal transduction via a large number of two-component systems, by chemotaxis and flagella-mediated motility (Spang et al., 2012). Thaumarchaeal group "1.1a associated (*Nitrosoatlea* cluster)" was found only in pristine acidic R1 soil, indicates the sensitivity of this group to IWE.

Pronounced difference in AOB composition was observed among samples. Phylogenetic analysis of bacterial *amoA* gene sequences suggested that the soil AOB was dominated by *Nitrosospira*-like sequences, with *Nitrosomonas*-like sequences minimum. AOB cluster-3 is found in all the samples and this is in consistent with previous reports where AOB cluster-3 the dominant ammonia-oxidizer group in a number of arable soils (Phillips et al., 2000; He et al., 2007; Shen et al., 2008; Liu et al., 2010). The presence of *Nitrosomonas*-like sequences in this study is inconsistent with some previous studies (Webster et al., 2002; Chu et al., 2007). This could be possibly due to higher ammonium concentration in R2 and R3 soils. In general *Nitrosomonas* like species have been detected in ammonium rich environments such as manure-treated wetland (Ibekwe et al., 2003), enrichment cultures (Stephen et al., 1996), and wastewater treatment plants (Kowalchuk and Stephen, 2001). Further, Liu et al. (2010) also reported *Nitrosomoans* cluster in Chinese arable soils. In R2 and R3 samples *Nitosospira* cluster 2 and cluster 4 are present. Cluster 2 and cluster 4 were found in acidic soils and Scots pine forest soils (Laverman et al., 2001; Nugroho et al., 2005). Since R2 and R3 samples are not acidic in nature, this study suggests that pH may not the critical determinant for *Nitosospira* cluster 2 distribution in semiarid soils. This study demonstrates the ubiquitous nature of the *Nitrosospira* cluster 3a1. In polluted soils, we did not find any other *Nitrosospira* species none other than *Nitrosospira* cluster 8, 1, 2, 3a1 and 4.

5.4.3. Conclusions

Nitrification activity was found to be significantly (P <0.05) decreased by IWE in semiarid soils. Relatively, AOB-*amoA* gene diversity is more decreased than AOA-*amoA* diversity by IWE. Thaumarchaeal "group 1.1b" might be playing potential role in ammonia oxidation of pristine and contaminated soils. Important AOB in contaminated soils are found to be *Nitrosospira* cluster 8, 1, 2, 3a1 and 4 pointing their possible role in soil nitrification of IWE polluted semiarid soils.

5.5. Effect of heavy metals on soil microbial activity and ammonia oxidizing microbes: short term microcosm studies

5.5.1. Results

5.5.1.1. Soil chemical analysis

Chemical properties of the RA and HZ soil samples have been summarized in Table 5.6. Both soils were acidic in nature with pH values of 5.1 and 5.6 respectively. Relatively HZ had high OM (2.88%), TOC (16.3 g kg^{-1}) and SMC (18.9%) than RA (OM = 0.86; TOC = 5.05 g kg^{-1} and SMC (8%) and these values were found to be significant by t-Test at P <0.05. No difference in the mean values of NH_4^+N of the samples was noticed. The observed mean values of NH_4^+-N for RA and HZ soils were 9.05 (± 2.38) and 9.37 (± 1.72) mg kg-^{1} respectively. Values for NO_3^-N and PNR were relatively high with RA than HZ (Table 5.6) and the values were significant at P <0.05, t-Test. Cu-concentration in these soils were 4.2 mg kg^{-1} in HZ soil and 8.6 mg kg^{-1} in RA soil, while As was not detected in any of the soils.

Table 5.6*: Chemical characters including potential nitrification rate (PNR) of the alfisols RA and HZ. Values are mean of three replicates with a deviation shown in parenthesis. Values s followed by the same letter do not differ at P < 0.05 by t-Test.*

Soil sample	pH (H_2O)	OM (%)	TOC (g kg^{-1})	NH_4^+- N (mg kg^{-1})	NO_3^--N (mg kg-^{1})	PNR (mg $NO2^-$- N kg^{-1} soil h^{-1})	Cu (mg kg^{-1} dry soil)	As (mg kg^{-1} dry soil)
RA	5.1 (0.2)a	0.86 (0.13)a	5.05 (1.32)a	9.05 (2.38)a	23.12 (5.1)a	1.075 (0.3)a	8.6	ND*
HZ	5.6 (0.3)a	2.88 (0.32)b	16.3 (2.8)b	9.37 (1.72)a	47.35 (7.84)b	0.89 (0.18) b	4.2	ND*

OM: organic matter; TOC: total organic carbon; PNR; potential nitrification rate, * Not detected.

5.5.1.2. Quantification of genes by real-time PCR (q-PCR) assays

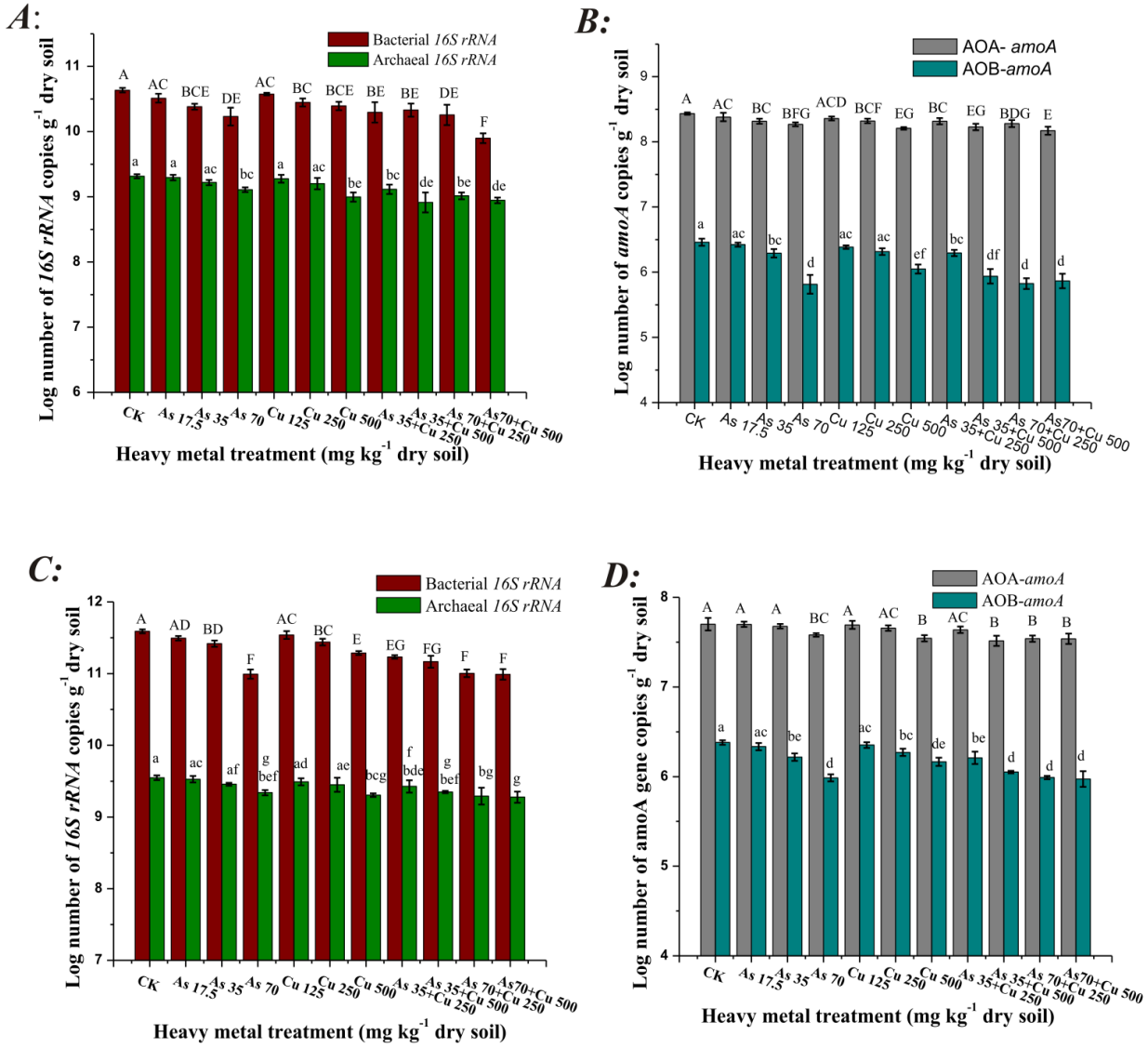
Eubacterial 16S rRNA, archaeal 16S rRNA, AOA-*amoA* and AOB-*amoA* genes were quantified by q-PCR assays.

5.5.1.2.1. Abundance of eubacterial-16S rRNA, archaeal 16S rRNA, AOA-amoA and AOB-amoA genes in As and Cu amended acidic alifisol, RA

For the RA soil, among all the genes quantified, AOB-*amoA* and eubacterial-16S rRNA genes were found to be highly sensitive to the both metals in all the treatments (Fig 5.11A and B). The eubacterial-16S rRNA gene copy number in As-treated soils was found to be relatively lower than in Cu-amended soils (Fig. 5.11A). In CK soil, copy number of eubacterial-16S rRNA gene was found to be $4.3x10^{10}$ g^{-1} dry soil, whereas in As-70 mg kg^{-1} and Cu-500 mg kg^{-1} treated soils, the copy numbers were reduced to 7% ($7.7x10^{9}$) and 5.03 % ($1.3x10^{10}$), respectively. Archaeal *16S rRNA* abundance ranged from 2.07 x 10^{9} (in CK) to 8.86 x 10^{8} (in As-70 + Cu-500) copies gm^{-1} dry soil (Fig.5.11A).

AOA-*amoA* gene abundance ranged from $2.71x10^{8}$ (in CK soil) to $1.5x10^{8}$ (in As-70+ Cu-500 soil) copies g^{-1} dry soil, whereas AOB-*amoA* gene abundance ranged from $2.9x10^{6}$ (in the CK soil) to $6.78x10^{5}$ (in the AS-70+ Cu-250 soil) copies g^{-1} dry soil (Fig. 5.11B). AOA-*amoA* gene copy numbers were significantly higher than that of AOB-*amoA* gene in the heavy metal treated as well as in the CK soils. The ratio of AOA-*amoA* to AOB-*amoA* gene in the CK soil was found to be lower than those in the soils of As-70, Cu-500 and As-70 + Cu-500 mg kg^{-1} dry soil (Table 5.7). In control soil, the ratio of AOA-*amoA* to AOB-*amoA* was found to be 94.88 ±13.75, however the ratios were significantly (P <0.05, LSD) increased at As-70 (303.09 ±122.1), Cu-500 (144.72 ±122.1) and As-70 + Cu-500 (214.7 ±72.3) mg kg^{-1} dry soil (Table 5.7). Heavy metal amendment to RA alfisols negatively impacted both AOA and AOB-*amoA* gene copies however the effect was more pronounced for AOB-*amoA* gene (Fig. 5.11B). Both archaeal and bacterial *amoA* gene copies were decreased in heavy metal impregnated alfisols however the reduction of AOB-*amoA* gene was much prominent than AOA-*amoA* gene. At a concentration of As-70mg kg^{-1} the AOA-*amoA* gene copies (log values) significantly (P <0.05, LSD) reduced to 1.94% only, while AOB-*amoA* gene copies reduced (log values) to 9.99% (Table 5.8). Similarly, AOA-*amoA* and AOB-*amoA* gene copies (log values) were reduced to 2.67% and 6.35% respectively at Cu-500mg kg^{-1} dry soil (Table 5.8).

Fig. 5.11: *Quantification of bacterial 16S rRNA, archaeal 16S rRNA, AOB-amoA and AOA-amoA genes under different As and Cu-treatments. Copy numbers (log values) of distinct genes were measured by real time quantitative PCR (q-PCR) assay. A and B: Copy numbers (log values) of various genes from RA microcosms; C and D: Copy numbers (log values) of various genes from HZ microcosms. Significant differences (P <0.05, LSD) between the microcosms at a particular metal treatment are indicated by different alphabets. Error bars represent standard deviation of mean (n = 3). Microcosms were named as per their nominal metal concentrations.*



5.5.1.2.2. Abundance of genes in As and Cu amended acidic alifisol, HZ

In the HZ soils, eubacterial-16S rRNA gene ranged from 3.89×10^{11} (in CK soil) to 9.87×10^{10} (in As-70+ Cu-500 soil) copies g^{-1} dry soil (Fig. 5.11C). When compared to CK, eubacterial-16S rRNA gene copy numbers reduced to 5.14% and 2.61% in As-70 and Cu-500 treatments respectively (Table 5.8). Archaeal *16S rRNA* abundance

ranged from 3.53 x 10^9 (in CK) to 1.91x10^9 (in As 70+Cu500) copies gm^{-1} dry soil (Fig. 5.11C).

Archaeal *amoA* gene abundance ranged from 5.08 x 10^7 (in CK) to 3.4 x 10^7 (in AS-70+ Cu-500) copies gm^{-1} dry soil, where as bacterial *amoA* gene ranged from 2.4 x 10^6 (in CK) to 9.5x10^5 (AS-70+ Cu-250) copies gm^{-1} dry soil (Fig. 5.11D). Similar to RA microcosms, AOA-*amoA* gene abundance in HZ soils was significantly higher than the AOB-*amoA* gene (Fig. 5.11D). The ratio of AOA to AOB significantly increased when exposed to elevated As and Cu (Table 5.7) concentrations. In control soil, the ratio of archaeal *amoA* to bacteria *amoA* was found to be 21.32 (± 4.5), however the ratios were apparently increased at As-70 (39.6 ± 5.1) and As-70+Cu-500 (38.1± 11.8) mg kg^{-1} dry soil (Table 5.7). Both AOA and AOB-*amoA* gene copies decreased in heavy metal amended HZ alfisols, however the reduction of AOB-*amoA* gene was much prominent than AOA-*amoA* gene. At a concentration of As-70 mg kg^{-1} dry soil, the AOA-amoA gene copies reduced to only 1.56% while AOB-*amoA* gene copies reduced to 6.18% (Table 5.8). Both AOA-*amoA* and AOB-*amoA* were reduced to be 2.04% and 3.36% respectively at Cu-500 mg kg^{-1} dry soil.

5.5.1.3. Nitrification activity and correlation with ammonia oxidizers

Potential nitrification rates (PNR) of both RA and HZ soils were found to be distinctly varied among treatments (P <0.05). An obvious negative effect of Cu and As-amendments on PNR was observed with both RA and HZ soils (Fig. 5.12). The highest PNR was noticed in CK-soils whereas the lowest PNR was observed in As-70 mg kg^{-1} and As-70mg+Cu-500mg kg^{-1} treated soils. The PNR significantly decreased with increasing Cu and As-concentrations in both soils (linear regression; P <0.05, Table 5.8). Relatively, HZ soils had the higher EC50-PNR values than RA soils. EC50-PNR of As and Cu in RA soils were determined to be 61.79 (±14.26) and 430 (± 6.9) mg kg^{-1} respectively, whereas EC50-PNR for HZ soils were determined as 85.73 (±4.26) and 557 (±119.3) mg kg^{-1} respectively (Table 5.9). The PNR was positively correlated with AOA-*amoA* and AOB-*amoA* gene copies of both RA and HZ soils; however the relationship was much stronger with AOA-*amoA* gene copies (Fig. 5.13A). Particularly, in RA microcosm's positive correlation between AOA-*amoA* copies (Fig. 5.13A) and PNR was found to be high and was very much significant (Adj. R^2 = 0.802, P <0.001, n =11) than the correlation between AOB-

amoA and PNR (Adj. R^2 = 0.64, *P* <0.001, n =11, Fig. 5.13B). In RA microcosm's, PNR could be predicted by the following equation

"y = 3.503x – 27.98, R^2 = 0.802, *P* <0.001, n =11 (Fig. 5.13A)

Where, y is the PNR (mg NO_2^--N kg^{-1} soil h^{-1}) and x is the AOA-*amoA* copies (log copy number gm^{-1} dry soil).

Similarly, correlation analysis between AOB-*amoA* copies and PNR showed following significant relation

"y = 0.961x – 5.19", Adj. R^2 = 0.74, *P* <0.01, n =11 (Fig. 5.13B)

Where y is the PNR (mg NO_2^--N kg^{-1} soil h^{-1}) and x is the AOA-*amoA* copies (log copy number gm^{-1} dry soil).

5.5.1.4. Ammonia oxidizing archaeal community shift by DGGE

Community structure of ammonia oxidizing archaea (AOA) was determined by thaumarchaeal AOA-*amoA* based DGGE. The experiment was repeated two times and confirmed the reproducibility. DGGE profile of AOA-*amoA* gene of both RA and HZ soils showed in the Figs. 5.14A and B respectively. In both RA and HZ microcosms, AOA population showed no apparent community shift in response to heavy metals amendment. No significant differences amongst AOA-*amoA* gene diversity indices were noticed in metal amended and control (CK) soils. Relatively, RA soils possessed higher diversity indices. AOA-*amoA* gene richness (*H*) and evenness (*E*) of RA soils ranged from 2.03 to 2.18 and 1.84 to 2.02, respectively; whereas, these values for HZ soils ranged from 1.26 to 1.55 and 1.17 to 1.5, respectively. Range weighted richness (*Rr*) for the samples were found to be stable and the values for RA and HZ were found to be 53.28 and 54.08 respectively

Table 5.7: *Ratios of different prokaryotic groups in RA and HZ alfisols. Ratios were calculated from results obtained by real time quantitative PCR (q-PCR). Values are mean of three independent replicates with a deviation. Values in the rows followed by the same letter do not differ significantly according to Fisher's LSD at <0.05. Treatments were named as per their nominal metal concentrations. CK stands for control treatment soil.*

RA	CK	As 17.5	As 35	As 70	Cu 125	Cu 250	Cu 500	As 35 + Cu 250	As 35 + Cu 500	As 70 + Cu 250	As70 + Cu 500
AOA/AOB	94.8 ±13.7a	92.36 ±18.72a	106.29 ±6.48ad	303.09 ±122.1bc	94.64 ±11.2a	101.05 ±5.09a	144.72 ±16.84b	105.58 ±10.5ad	197.53 ±31.4b	285.86 ±23.87bc	214.14 ±72.3b
Archaea/AOA*	7.63 ±0.57a	8.27 ±0.98a	8.02 ±0.94a	6.91 ±0.48a	8.31 ±0.81a	7.85 ±2.02a	6.26 ±1.32a	6.43 ±1.51a	5.27 ±2.34a	5.49 ±0.91a	5.94 ±0.26a
Bacteria/AOB	15016 ±1070bd	12567.6 ±2792d	12649.8 ±3045bd	12805.6 ±5441a	15588 ±1641bd	13651.6 ±1686bd	11624.6 ±3059ac	9721.4 ±334d	10608 ±2957a	12943.6 ±1467a	11151.8 ±3337bc
Bacteria/ Archaea	20.92 ±0.33a	16.76 ±3.31ad	14.63 ±1.41bd	6.11 ±0.8eg	20.02 ±2.31ac	18.49 ±6.15bdf	13.03 ±3.3bcd	14.76 ±1.62bg	11.24 ±3.38efg	8.34 ±0.78efg	9.07 ±1.65efg
HZ											
AOA/AOB®	21.32 ±4.53a	23.2 ±1.66a	28.88 ±1.34a	39.62 ±5.12b	21.96 ±1.56a	24.4 ±2.06a	23.9 ±1.07a	26.89 ±2.05b	31.63 ±0.21b	35.68 ±4.08b	38.11 ±11.8b
Archaea/AOA*	70.67 ±8.62a	67.67 ±8.99a	60.38 ±5.84a	57.49 ±3.26a	64.07 ±14.71a	64.12 ±19.01a	58.23 ±7.7a	62.25 ±11.77a	63.31 ±1.3a	59.03 ±18.2a	56.77 ±16.98a
Bacteria/AOB	162354.9 ±9607a	145620.2 ±19780a	162082.6 ±32366a	103623.6 ±21757bc	155788.8 28117a	147667.2 ±5800a	1337892± 1536ac	106935 ±19214bc	133167.4 ±23456ac	103811.1 ±9155.8bc	104103 ±6020bc
Bacteria/ Archaea	110.86 ±13.18a	93.47 ±12.19a	92.17 ±8.23ac	45.38 ±5.47b	112.41 ±15.75a	101.49 ±27.24a	95.83 ±0.42a	64.71 ±10b	66.64 ±12.58bc	52.7 ±12.06b	53.81 ±17.6b

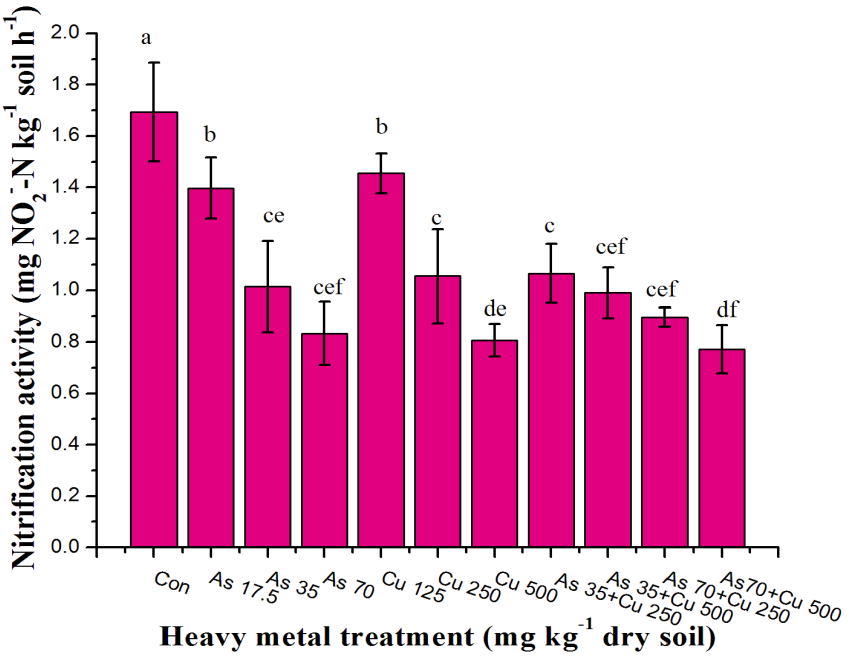
®Normality test failed, consequently significant among the means were compared with t-Test, $P < 0.05$; * Ratios are not significant at $p < 0.05$ by Fisher's LSD and/or by t-Test

Table 5.8: *Percent (%) decrease of soil nitrification and distinct gene copy numbers (log values) measured in heavy metal amended (mg kg-1 dry soil) RA and HZ soils after 10 weeks of incubation. Values of corresponding control soils (CK, 0 mg kg^{-1} dry soil) were considered to be 100%. Values are calculated as means of three independent replicates of same treatment (n=3) and represented with standard error in parentheses. Means with different letters differ significantly according to Fisher's LSD at <0.05.*

RA	As 17.5	As 35	As 70	Cu 125	Cu 250	Cu 500	As 35 + Cu 250	As 35 + Cu 500	As 70 + Cu 250	As 70 + Cu 500
Nitrification	17.51 (7.03)f	40.05 (10.4)bce	50.78 (7.21)adc	14.07 (4.58)f	37.69 (10.8)bde	52.36 (3.75)ac	37.1 (6.76)be	41.53 (5.79)ae	47.08 (2.14)ae	54.42 (5.57)a
AOA- *amoA*	0.62 (0.76)df	1.37 (0.46)def	1.94 (0.35)bc	0.88 (0.34)de	1.33 (0.41)bef	2.67 (0.23)ac	1.38 (0.56)bfe	2.42 (0.59)ac	1.81 (0.63)bce	3.09 (0.73)a
AOB- *amoA*	0.89 (0.13)d	2.61 (1)d	9.99 (2.21)a	1.15 (0.4)d	2.27 (0.77)d	6.35 (1.05)b	2.56 (0.75)c	8.08 (1.73)d	9.82 (1.26)ac	9.20 (1.71)a
Bacterial 16S rRNA	1.15 (0.63)eg	2.37 (0.41)df	7.0 (0.3)a	0.55 (0.19)g	1.75 (0.58)ef	5.03 (1.15)b	3.32 (0.44)d	6.47 (0.33)a	6.58 (0.35)a	6.94 (0.83)a
Archaeal 16S rRNA	0.50 (0.01)d	1.03 (0.43)bd	2.25 (0.38)bc	0.80 (0.3)bd	1.21 (0.92)bd	3.42 (0.76)ac	2.14 (0.77)bc	4.30 (1.63)a	3.21 (0.55)ac	3.96 (0.45)a
HZ										
Nitrification	7.67 (5.88)d	21.95 (5.08)b	42.14 (6.29)ac	5.46 (9.97)d	28.05 (7.54)b	43.8 (2.72)ac	23.07 (3.74)b	42.24 (4.89)ac	32.26 (8.08)bc	46.99 (4.81)a
AOA- *amoA*	0.25 (0.16)b	0.31 (0.32)b	1.56 (0.26)acd	0.11 (0.58)bc	0.57 (0.39)b	2.04 (0.46)ac	0.81 (0.47)bd	2.42 (0.73)a	2.1 (0.45)ac	2.12 (0.75)ac
AOB- *amoA*	0.71 (0.62)c	2.55 (0.64)bd	6.18 (0.61)a	0.76 (0.12)c	1.72 (0.63)cd	3.36 (0.72)b	2.67 (1.08)bd	5.16 (0.02)a	6.12 (0.28)a	6.38 (1.37)a
Bacterial 16S rRNA	0.82 (0.27)g	1.47 (0.37)g	5.14 (0.55)ac	0.44 (0.46)b	1.29 (0.4)b	2.61 (0.4)ef	3.08 (0.23)df	3.65 (0.71)d	5.05 (0.46)ac	5.18 (0.64)a
Archaeal 16S rRNA	0.53 (0.027)b	0.95 (0.2)c	2.18 (0.39)d	0.60 (0.51)b	1.03 (1.02)c	2.54 (0.26)a	1.27 (0.89)c	2.08 (0.06)d	2.68 (1.22)ad	2.84 (0.8)ad

Fig. 5.12: *Potential nitrification rate (PNR) of soils under different As and Cu-treatments.* ***A****: PNR of RA soils; B: PNR of HZ soils. Significant differences (P <0.05, LSD) between the microcosms at a particular metal treatment are indicated by different alphabets. Error bars represent standard deviation of mean (n = 3). Microcosms were named as per their nominal metal concentrations.*

A



B

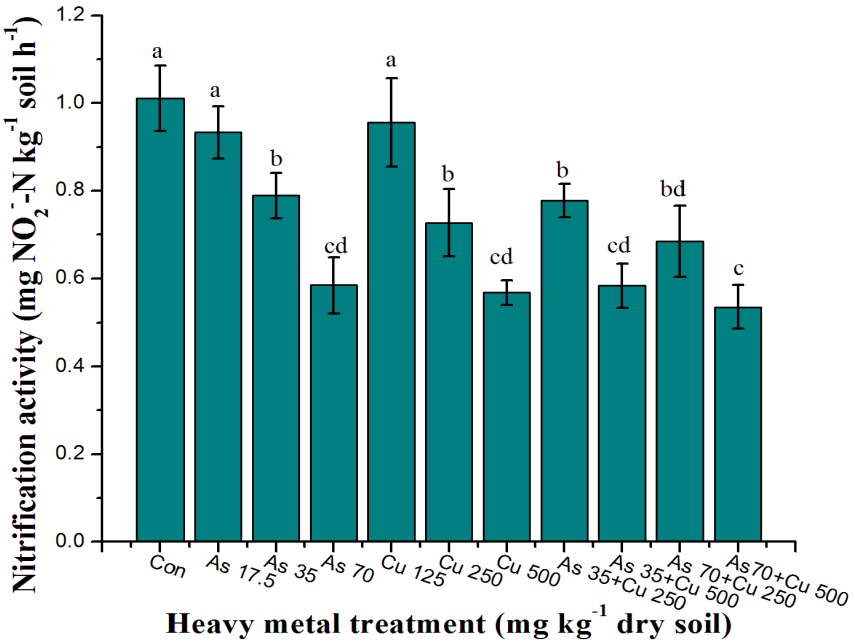


Table 5.9: *Ec-50 values of Cu and As (mg kg-1 dry soil) for nitrification and discrete genes quantified in RA and HZ soils after 10 weeks of incubation. In order to describe EC-50 values, non linear regression was performed, data was normalized (normality test passed) and fitted in to a logistic response equation {y= a/1+ (x/x0) b} described elsewhere (Liu et al., 2010). Significant values (p) are shown in italics where as standard errors are given in parenthesis. Adj. R^2 for the parameters was ranged from 0.92 to 0.99.*

	PNR	AOA-*amoA*	AOB-*amoA*	Bac-*16S rRNA*	Arch-*16S rRNA*
RA					
As	61.79 (14.26) *p* = 0.14	164.5 (56.3) *p* = 0.21	45.93 (1.17) *p* = 0.01	36.43 (5.1) *p* = 0.08	93.99 (11.67) *p* = 0.07
Cu	430 (76.99) *p* = 0.11	759.5 (121.1) *p* = 0.1	403.2 (45.4) *p* = 0.07	336.1 (9.59), *p* = 0.01	486.6 (7.68) *p* = 0.01
ZHA					
As	85.73 (4.26) *p* = 0.03	112.3 (0.1) *p* = 0.006	55.5 (1.93) *p* = 0.022	46.16 (7.84) *p* = 0.1	93.62 (9.28) *p* = 0.06
Cu	557.2 (119.3), *p* = 0.13	750.4 (9.92) *p* = 0.008	672.3 (90.03) *p* = 0.08	485.9 (28.6) *p* = 0.03	633.8 (89.65) *p* = 0.08

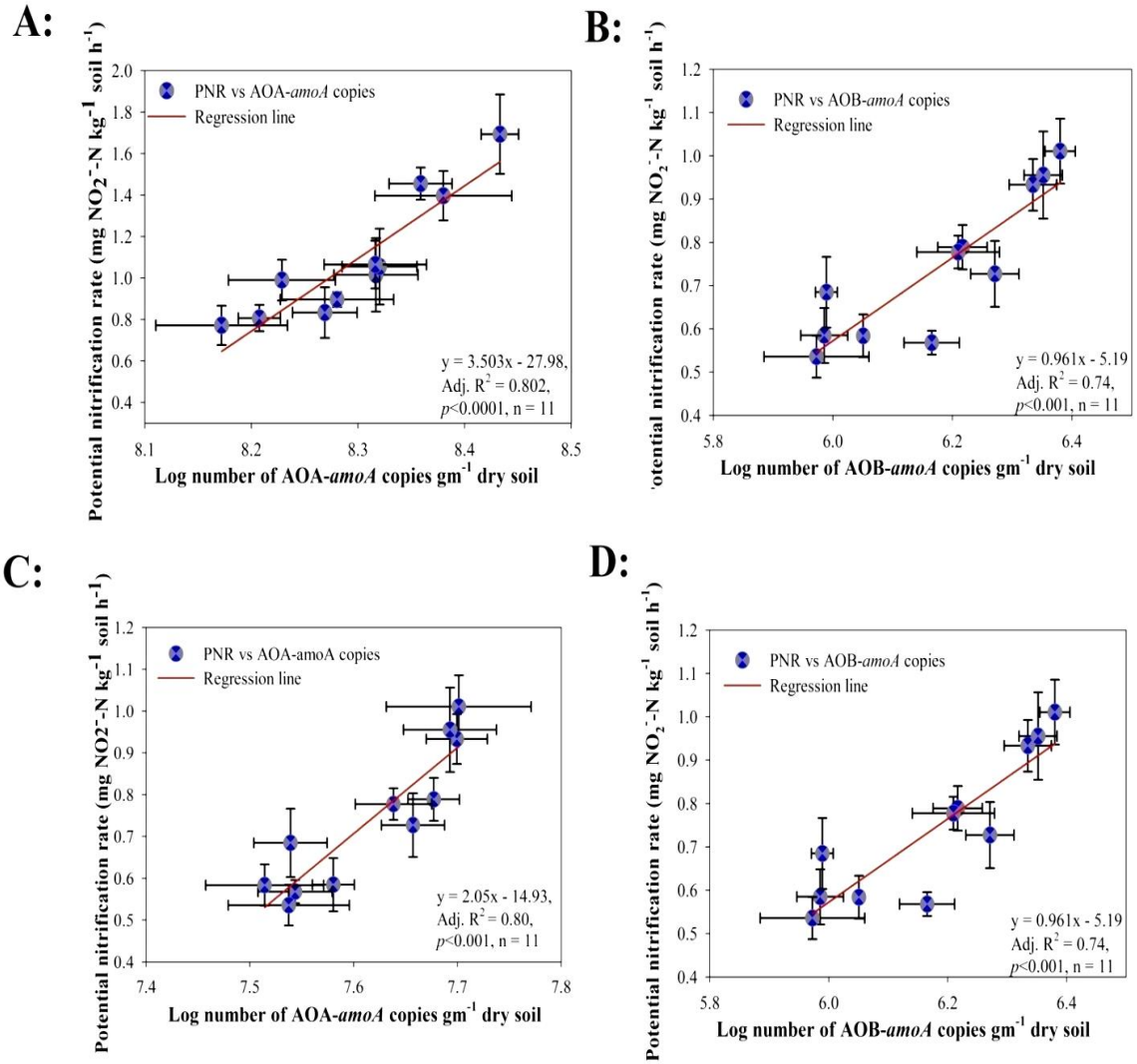
PNR (potential nitrification rate); Bac-*16S rRNA* : bacterial 16S rRNA gene; Arch-*16S rRNA* : archaeal 16S rRNA gene. Non-linear regression was performed with non logarithmic values of copy numbers. Normality test was passed, K-S statistic test also performed and the values were found to be significant.

5.5.1.5. Construction of AOA-amoA clone library and sequencing studies

Results of quantitative PCR, PNR and DGGE of AOA-*amoA* showed that AOA might be having important role in ammonia oxidation of heavy metal spiked acidic soils which impelled us to investigate the detailed composition of AOA populations using the thaumarchaeal AOA-*amoA* gene clone libraries. Rarefaction analyses of all 4 AOA-*amoA* libraries yielded asymptotic curves (Fig. 5.15). A distance of 0.03 used to define phylotypes and 18 to 22 OTUs are observed among 4 clone libraries (Table 5.10, Fig. 5.16). Shannon diversity indices for clone libraries ranged from 2.4 to 2.6, whereas Simpson diversity indices ranged from 0.083 to 0.1 9 (Table 5.10). Phylogenetic analysis of 4 archaeal-*amoA* gene clone libraries (400 sequences) showed that all the AOA-*amoA* gene sequences were placed within 3 distinct clusters likely from soil and sediment origin (Fig. 5.16). In clone libraries, cluster-III was found to be the dominant group containing 51-60 % of total sequences, whereas cluster-II was found to be less abundant and consisted of 14% to 20% of total sequences. All these sequences were found to be affiliated with only group 1.1b lineage. There was no major difference among AOA-*amoA* gene phylogeny of 4 clone libraries however, notably two changes were associated with cluster-I under group 1.1b. Firstly, one particular OTU showing absolute representative of acidic paddy soil clone (HQ215926.1) was highly distributed in metal treated soils only (Fig. 5.16).

Secondly, OTU under representation of Indian Ocean sediment clone (JN190688.1) was only distributed in CK-soil clone library.

Fig. 5.13: *Relationship between PNR and thaumarchaeal AOB-amoA and AOA-amoA genes under different As and Cu-treatments. Horizontal and vertical error bars represent standard deviation of AOA-amoA gene copies and nitrate concentrations respectively from three independent replicate samples (n=3). A and B: PNR verses AOA-amoA gene copies and PNR verses AOB-amoA gene copies respectively from RA microcosms; C and D: PNR verses AOA-amoA gene copies and PNR verses AOB-amoA gene copies respectively from HZ microcosms.*



5.5.1.6. Sequence accession numbers

All the 400 AOA-*amoA* sequences obtained in the study are deposited in GenBank nucleotide sequence database under accession numbers JX416592 to JX416690 and JX427105 to JX427403.

5.5.1.7. Relation between soil microbial activity, ammonia oxidizing microbial parameters and heavy metals Cu and As

The relation between soil ammonia oxidizing microbial parameters and heavy metals As and Cu were ascertained by principle component analysis (PCA), a multivariable statistical method. Partial ordination biplot of PCA showed apparent negative impact of As and Cu on ammonia oxidizing microbial parameters of both RA and HZ soils during 10 week of incubation (Fig. 5.17 A and B). No significant relation between thaumarchaeal AOA-diversity indices (*H, E and Rr)* and amended heavy metals was observed and therefore not included in the ordination plots. Significant positive correlation between ratios of AOA to AOB and heavy metals was noticed with both RA and HZ soils. Furthermore, contrasting negative effects of heavy metals on AOA and AOB abundance were noticed with RA and HZ soils.

5.5.2. Discussion

Acidic soils such as alfisols, aridisols, entisols and ultisols occupy about 30% of world's land area (von Uexkull and Mutert 1995) among which alfisols are the third most important soil orders in the world covering 13.9 % of the world area. These soils are abundant in semiarid tropics. It is of paramount importance to know the effect of distinct perturbations on nutrient turnover of acidic soils.

The present study aimed to determine the effect of heavy metals on microbial ammonia oxidation which plays a central role in global nitrogen cycle. Response of heavy metals such as Cu, As and combination of Cu+As on nitrification, abundance and community dynamics of ammonia oxidizing archaea (AOA) in acidic alfisols were investigated by short-term microcosm experiments. Our studies provide evidence for the important role of AOA in metal amended acidic soils and strengthen the hypothesis that AOA and AOB may occupy different niches in metal polluted low-pH environments (Schleper, 2010).

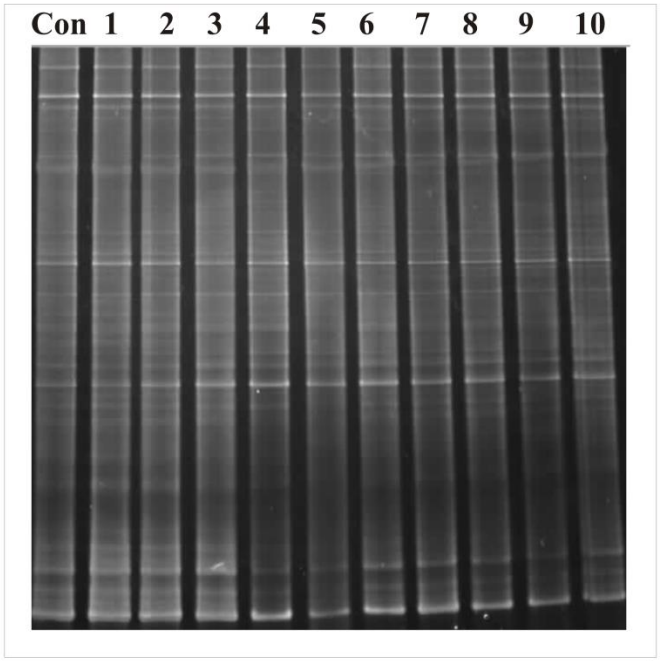
Quantification of bacterial and archaeal 16SrRNA genes showed contrasting deleterious effects of amended heavy metals. Bacterial 16SrRNA genes were found to be highly sensitive to amended-As (P <0.05) whereas archaeal copy number were greatly reduced with Cu- treatment (P <0.05). These results are congruence with quantified archaeal and bacterial amoA genes. Thus, it could be inferred that archaea and bacteria may occupy different niches in metal polluted soils as like as in acidic soils.

Gene abundances of both archaeal and bacterial *amoA* were comparable to those observed in previous studies where greater abundance of archaeal amoA genes noticed than bacterial amoA genes (Leininger et al., 2006; He et al., 2007; Nicol et al., 2008; Zhang et al., 2010; Yao et al., 2011) in most of the arable acidic soils as well as in Cu amended grassland soils (Li et al., 2009)**.** Based on the type of amended metal, ammonia oxidizers abundance clearly varied and the ratios of AOA to AOB (22 to 39, table 5.7) fall within those found in a range of soils (1.02–232; Leininger et al., 2006; He et al., 2007; Li et al., 2010). Greater abundance of archaeal *amoA* genes has been seen as evidence of a greater role for archaea in nitrification (Shen et al., 2008; Prosser and Nicol, 2008; Kelly et al., 2011). Though amoA gene abundance certainly provides information on potential ammonia oxidizing activity in soil, analysis of *amoA*-transcripts will give us more authentic panorama with respect to *amoA*-function at particular metal treatment.

The higher abundance of AOA-*amoA* over AOB-*amoA* in polluted soils indicated possible metal tolerance/resistance of AOA against amended heavy metals. Similar kind of AOA tolerance towards As and Pb was observed in soils that were impacted by mining waste however the report was restricted to root-rhizosphere complex of *Miscanthus x giganteus* grown soils (Ollivier et al., 2012). Li et al. (2009) reported similar kind of observation in Cu amended neutral and alkaline grassland soils where AOB-*amoA* was reduced to 107-232 folds while AOA-*amoA* reduction was found to be 10-89 folds.

Fig. 5.14: *Denaturant gradient gel electrophoreses (DGGE) analysis of thaumarchaeal AOA-amoA gens under different As and Cu-treatments. A: Thaumarchaeal AOA-amoA community structure under RA microcosms; B: Thaumarchaeal AOA-amoA community structure under HZ microcosms. Lane numbers indicates different As and Cu-treatments.*

A:



B:

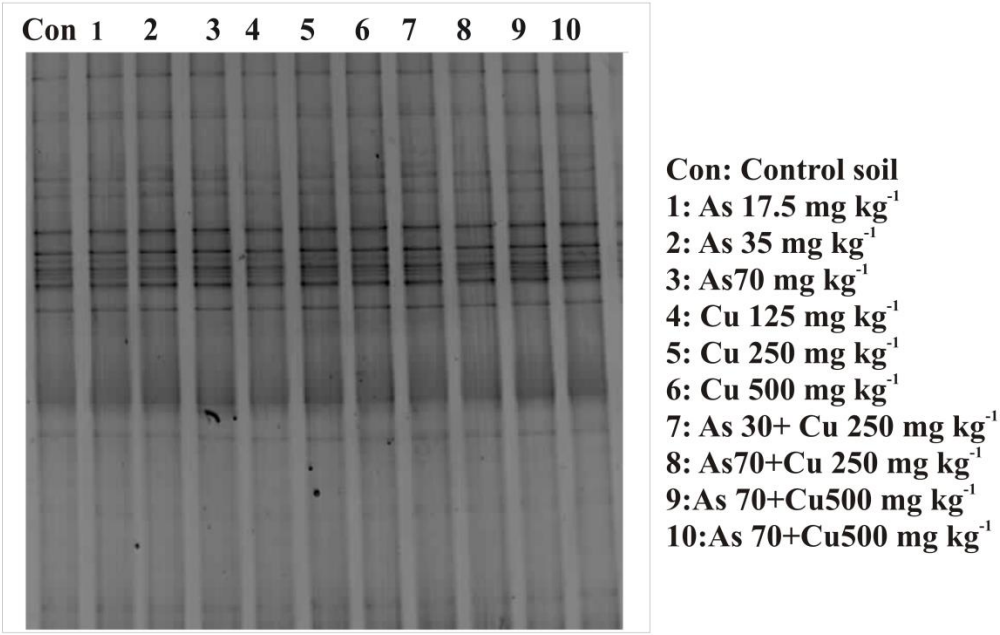


Fig. 5.15: *Rarefaction curves of the 1A, 2A, 3A and 4A-AOA-amoA gene clone libraries.* A distance cutoff value 0.03 is used for OTU determination.

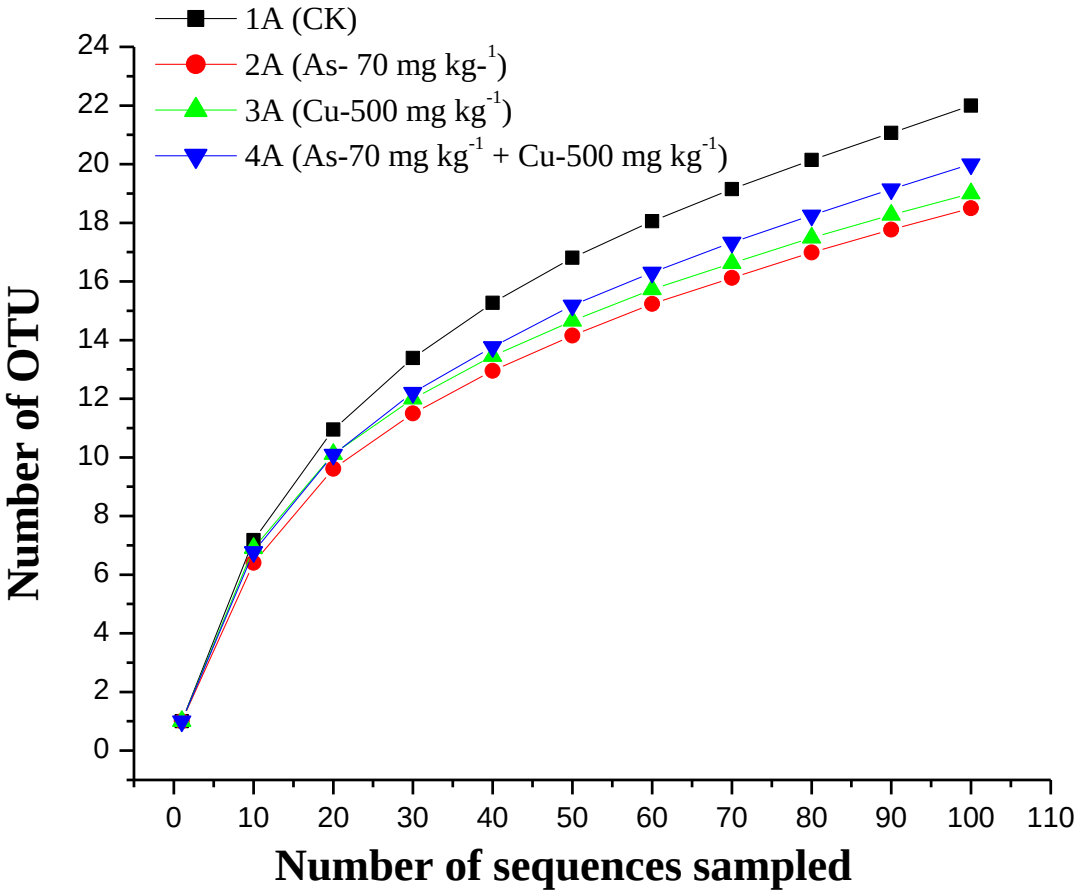


Fig. 5.16: *The phylogenetic relationship among 400 deduced amino acid sequences of AOA-amoA retrieved from 4 clone libraries of RA microcosms. Phylogenetic relation was constructed with Neighbor-joining statistical method in MEGA 5.0. Pairwise distances of 198-aligned amino acid positions were calculated by using the Equal input modal with gamma distributed rates among invariable sites. Number of clones is mentioned at subtree where as total clone numbers from representative libraries are given in parenthesis. Clone sequences are shown as triangles. Sequences named with 1A, 2A, 3A and 4A series are retrieved from 1A, 2A, 3A, and 4A clone libraries respectively. Bootstrap Values (> 50%) are indicated at branch points. The scale bar represents 5% estimated sequence divergence. Clades are labeled with thaumarchaeal groups and their frequently represented environment. The tree was rooted with sequences affiliated to marine cluster, thaumarchaeal group 1.1a.*

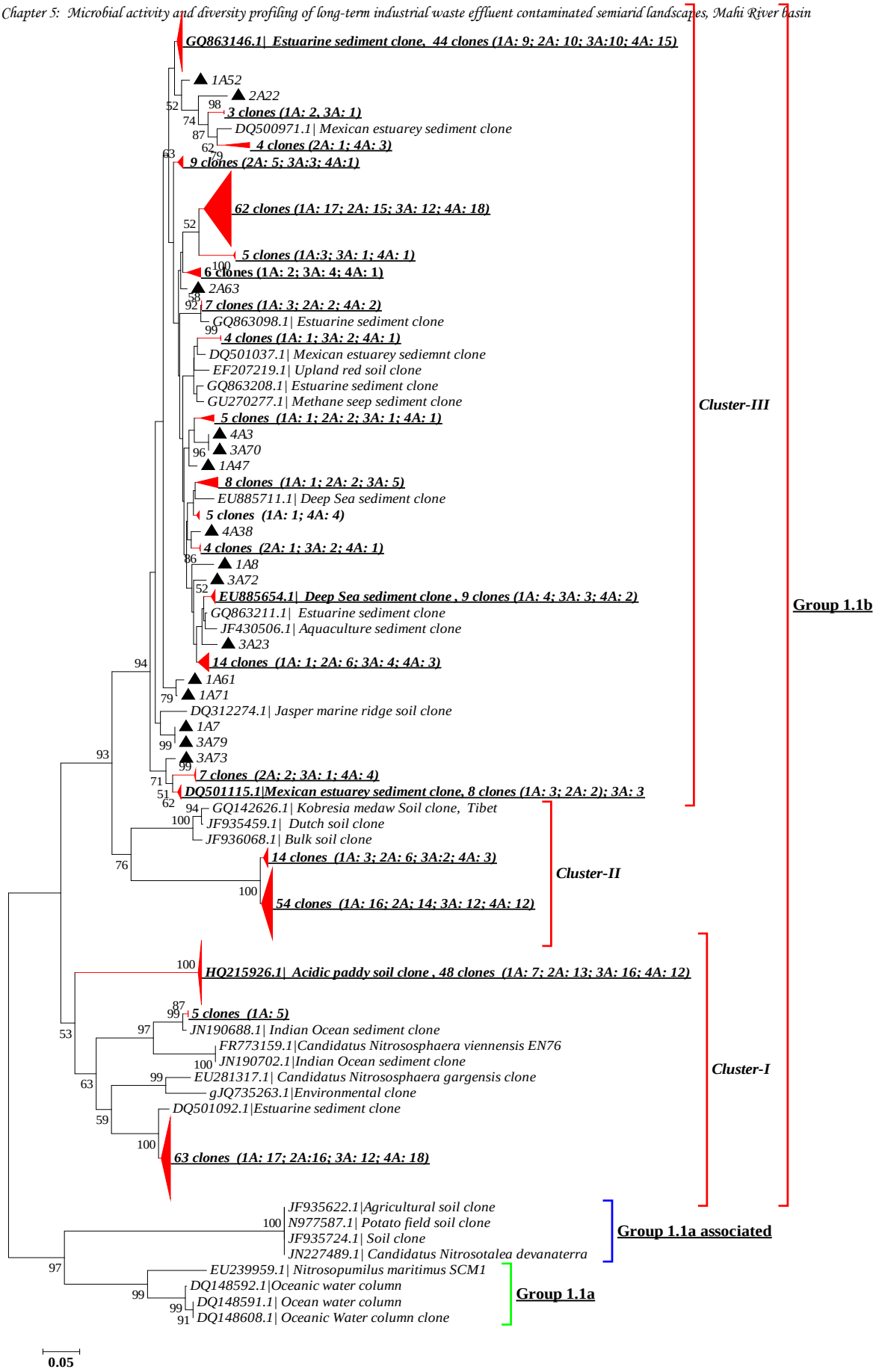

GQ863146.1| Estuarine sediment clone, 44 clones (1A: 9; 2A: 10; 3A:10; 4A: 15)
1A52
2A22
3 clones (1A: 2, 3A: 1)
DQ500971.1| Mexican estuarey sediment clone
4 clones (2A: 1; 4A: 3)
9 clones (2A: 5; 3A:3; 4A:1)
62 clones (1A: 17; 2A: 15; 3A: 12; 4A: 18)
5 clones (1A:3; 3A: 1; 4A: 1)
6 clones (1A: 2; 3A: 4; 4A: 1)
2A63
7 clones (1A: 3; 2A: 2; 4A: 2)
GQ863098.1| Estuarine sediment clone
4 clones (1A: 1; 3A: 2; 4A: 1)
DQ501037.1| Mexican estuarey sediemnt clone
EF207219.1| Upland red soil clone
GQ863208.1| Estuarine sediment clone
GU270277.1| Methane seep sediment clone
5 clones (1A: 1; 2A: 2; 3A: 1; 4A: 1)
4A3
3A70
1A47
8 clones (1A: 1; 2A: 2; 3A: 5)
EU885711.1| Deep Sea sediment clone
5 clones (1A: 1; 4A: 4)
4A38
4 clones (2A: 1; 3A: 2; 4A: 1)
1A8
3A72
EU885654.1| Deep Sea sediment clone , 9 clones (1A: 4; 3A: 3; 4A: 2)
GQ863211.1| Estuarine sediment clone
JF430506.1| Aquaculture sediment clone
3A23
14 clones (1A: 1; 2A: 6; 3A: 4; 4A: 3)
1A61
1A71
DQ312274.1| Jasper marine ridge soil clone
1A7
3A79
3A73
7 clones (2A: 2; 3A: 1; 4A: 4)
DQ501115.1|Mexican estuarey sediment clone, 8 clones (1A: 3; 2A: 2); 3A: 3
GQ142626.1| Kobresia medaw Soil clone, Tibet
JF935459.1| Dutch soil clone
JF936068.1| Bulk soil clone
14 clones (1A: 3; 2A: 6; 3A:2; 4A: 3)
54 clones (1A: 16; 2A: 14; 3A: 12; 4A: 12)
HQ215926.1| Acidic paddy soil clone , 48 clones (1A: 7; 2A: 13; 3A: 16; 4A: 12)
5 clones (1A: 5)
JN190688.1| Indian Ocean sediment clone
FR773159.1|Candidatus Nitrososphaera viennensis EN76
JN190702.1|Indian Ocean sediment clone
EU281317.1| Candidatus Nitrososphaera gargensis clone
gJQ735263.1|Environmental clone
DQ501092.1|Estuarine sediment clone
63 clones (1A: 17; 2A:16; 3A: 12; 4A: 18)
JF935622.1|Agricultural soil clone
N977587.1| Potato field soil clone
JF935724.1| Soil clone
JN227489.1| Candidatus Nitrosotalea devanaterra
EU239959.1| Nitrosopumilus maritimus SCM1
DQ148592.1|Oceanic water column
DQ148591.1| Ocean water column
DQ148608.1| Oceanic Water column clone
Cluster-III
Group 1.1b
Cluster-II
Cluster-I
Group 1.1a associated
Group 1.1a
0.05

Table 5.10: *Analysis of functional AOA-amoA gene clone libraries.*

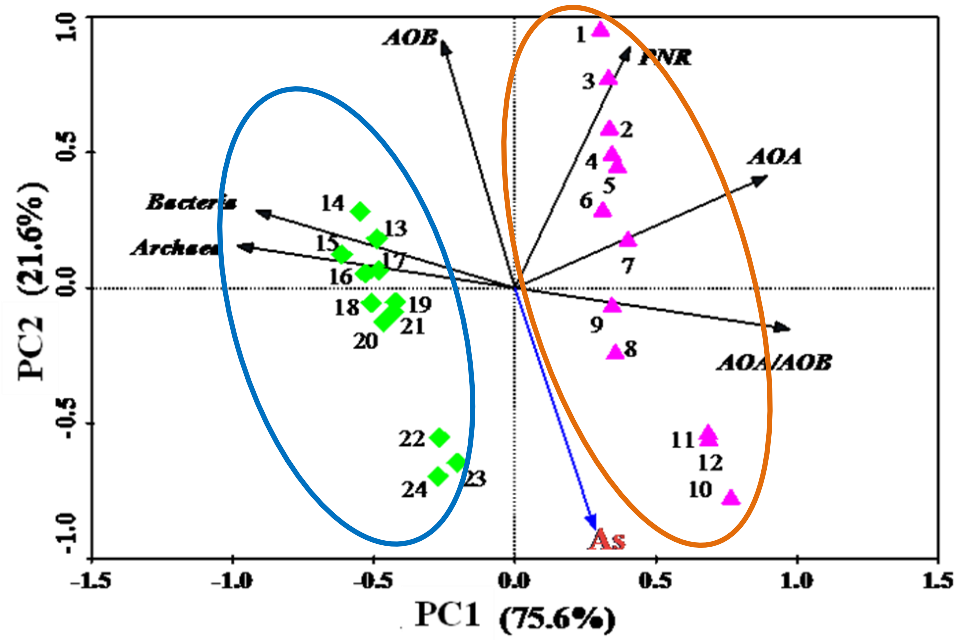
Treatment[a]	No of clones	OTUs[b]	Shannon diversity	Simpson diversity
1A	100	21	2.64	0.083
2A	100	18	2.44	0.108
3A	100	19	2.52	0.089
4A	100	20	2.51	0.09

[a] 1A : CK soil, 2A: As- 70 mg kg^{-1}, 3A: Cu-500 mg kg^{-1}, and 4A: As-70 mg kg^{-1} + Cu-500 mg $kg^{-}1$;
[b] operational taxonomic unit (DNA≥97% identity).

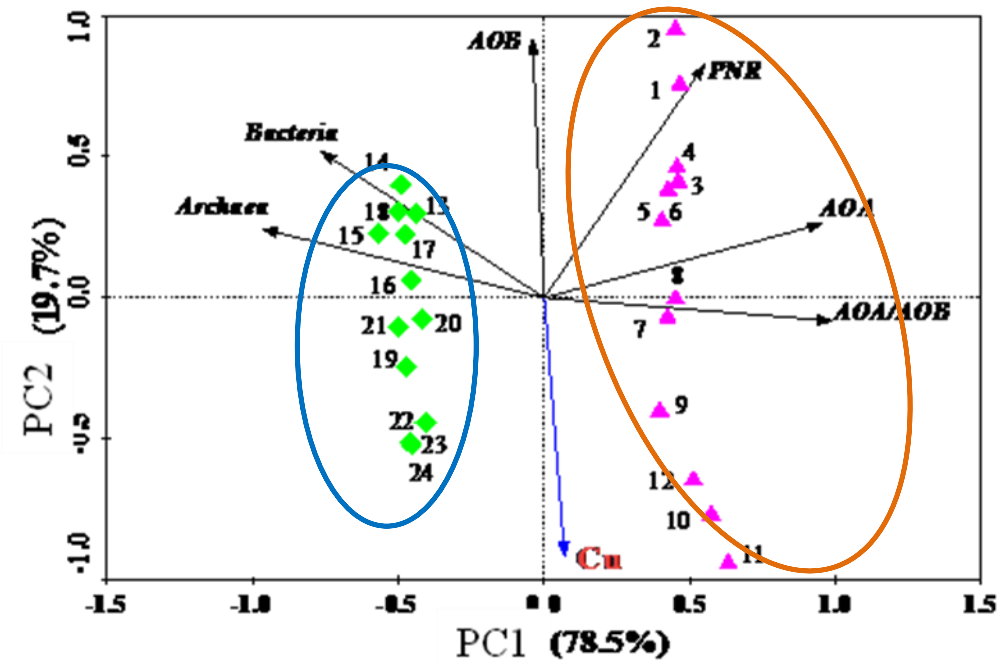
The AOA tolerance in metal polluted soils could be due to integrity and stability of archaeal membrane under chronic stresses. It was suggested that AOA might be less susceptible than AOB to inhibitor because of their remarkable different physiological and genetic characters (Schleper et al., 2005; Valentine, 2007; Schauss et al., 2009). It is reported that archaeal membranes are less permeable to ions than bacterial membranes due to exceptional chemical structure of their membrane lipids (Kandle and König, 1998). One of the unique feature of Thaumarchaeota is their cellular membrane lipid "crenarchaeol" which contains glycerol-dialkylglycerol-tetraethers (GDGT) (Damste et al., 2002; Leininger et al., 2006; de la Torre et al., 2008; Schouten et al., 2008; Pitcher et al., 2010). It has been previously suggested that cyclopentane and cyclohexane rings of GDGT confers archaeal membrane to more stable with flexible density (Gabriel and Chong, 2000) than bacterial counterparts. However, information pertaining the mechanisms that confer metal resistance in AOA remains ambiguous as limited cultivated AOA cluster (Könneke et al., 2005; de la Torre et al., 2008; Hatzenpichler et al., 2008; Lehtovirta-Morley et al., 2011) and more importantly no metal resistant AOA (MR-AOA) was so far cultivated or characterized. The credible mechanisms that confer metal-resistance to AOA could be possibly addressed by undertaking cutting edge technologies such as single cell genome sequencing (without culturing the organism) combined with powerful *in silico* genome-protein analysis and further *in situ* gene expression patterns by microarray.

Fig: 5. 17: *Partial ordination biplots of principal component analysis (PCA). Treatments from RA and HZ microcosms are shown with pink triangles and green diamond symbols respectively. Treatments numbered from 1- 12 belongs to RA alfisols whereas treatments numbered from 13-14 belongs to HZ alfisols. A: relationship between "As" and soil microbial parameters. Samples 1-3 and 13-15: Con; 4-6 and 16-18: As-17.5; 7-9 and 19-21: As-35; 10-12 and 22-24: As-70. B: relationship between "Cu" and soil microbial parameters. Samples 1-3 and 13-15: Con; 4-6 and 16-18: Cu-125; 7-9 and 19-21: Cu-250; 10-12 and 22-24: Cu-500.*

A:



B:



In the present investigation potential nitrification rates (PNR) of acidic soils wer found to be significantly (P <0.05) inhibited by amendment of As, Cu and combination of As+Cu. These results were in supportive of previous results stated that PNR could be used as sensitive indicator for metal pollution (Smolders et al., 2001; 2004; Broos et al., 2005; Xu and Chen, 2006). Similar to our results, observations were reported in earlier studies where significant reduction of PNR by heavy metals such as Hg, Zn and Cu was noticed however their reports are confined to alkaline and neutral soils (Li et al., 2009; Liu et al., 2010; Vasileiadis et al., 2012). In the present study, the soil PNR was fitted well with the logistic model, suggesting that the inhibition of metals to the oxidation of ammonia could be predicted with dose response relation in toxicology studies. Both archaeal and bacterial ammonia oxidizers were significantly correlated with PNR of heavy metal treated soils however the relation was much prominent with AOA of RA soils. Similar observations were found in Cu contained alkaline soils (Li et al., 2009) and arable acidic soils (Gubry-Rangin et al., 2010; Zhang et al., 2012). Contrasting to these results, Mertens et al. (2009) observed a significant relationship between PNR and AOB-*amoA* in Zn amended soils. This could be possibly due to the difference in the pH of the soil (i.e. 6.3), type of the heavy metal used (i.e. Zn) and the time of metal exposure (i.e. 2 years).

In addition to the soil PNR, soil microbial parameters such as C_{mic} and BSR were also significantly (P <0.05, LSD) reduced along As and Cu elevations after 10 week incubation (Table 5.11). Higher EC-50 values of Cu and As for microbial activity (Table 5.12) as well as ammonia oxidizing parameters were noticed (Table 5.9) with HZ soil and this could be due to higher clay (27%) and organic matter (2.8%) of HZ. It is known that toxicity or bioavailability of heavy metals often reduced by absorption with clay minerals and/or complexation with organic matter (He et al., 2005; Violante et al., 2010). RA soils had relatively higher (24 to 40 times, P <0.05, t-Test) AOA-*amoA* copies than HZ soil; this could be explained by oligotrophic nature of RA soil where the SOM and TOC were found to be very less (0.8% and 5.05 g kg^{-1} respectively). Previous studies have demonstrated oligotrophic life style of AOA (Martens-Habbena et al. 2009). Inhibitory effect of organic substances even at very low concentrations on AOA-activity was found (Konneke et

al. 2005). Thus, a dominating activity of AOA in AO could be found in oligotrophic environments.

Even at high concentrations of As (70 mg kg^{-1} dry soil) and Cu (500 mg kg^{-1} dry soil), the DGGE based AOA community did not change (Fig. 5.14). This is further confirmed with our 4 distinct AOA-*amoA* gene clone library sequencing analysis. With these results, it could be inferred that AOA community does not respond to heavy metals such as As (up to 70 mg kg^{-1} dry soil) and Cu (up to 500 mg kg^{-1} dry soil) in acidic soils. Though our results are confined to short term exposure (10 weeks) of metals on AOA, related observation was reported by Mertens et al. (2009) in long term Zn exposed soils where no apparent community shift found within the AOA-*amoA* gene DGGE community profile.

Earlier, AOA-*amoA* phylogenetic analysis showed that *Thaumarchaeota*-group1.1b are the major AOA in acidic soils (Nicol et al., 2008; Gubry-Rangin et al., 2011) and participate in the ammonia oxidation of strongly acidic soils (Zhang et al., 2012). Similar kind of results were obtained in the present study, where all the putative AOA-*amoA* sequences were affiliated to soil and sediment group of AOA, often referred to as group 1.1b. Therefore, we could infer that *Thaumarchaeota*-group1.1b might be having potential role in ammonia oxidation of heavy metal polluted and unpolluted acidic soils. Recent crenarchaeal-16s rRNA based phylogenetic studies of acidic soils suggested a new cluster, known as Group 1.1c – *Thaumarchaea,* however, potential involvement of group1.1c *Thaumarchaea* in ammonia oxidizing function of acidic soils still remained unclear (Lehtovirta et al., 2009; Stopnisek et al., 2010; Zhnag et al., 2012; He et al., 2012).

Correlation between microbial activity, ammonia oxidizing microbial parameters and heavy metals were interpreted with indirect gradient analysis by PCA. Ordination diagrams indicated the apparent negative impact of Cu on As on microbial parameters of both RA and HZ microcosms (Fig. 5.17 A and B). A strong correlation between ratios of AOA to AOB and heavy metals was noticed with both soils. Therefore ratios of AOA to AOB could be attributed as bio-indictor for monitoring acidic soil pollution caused by Cu and As.

Table 5.11: *Microbial activity measurements of soils amended (mg kg-1 dry soil) with As and Cu after 10 weeks of incubation. Values were calculated as means of three independent replicates of same treatment (n=3) and represented with standard error in parentheses. Means with different letters differ significantly according to LSD at <0.05 level.*

RA	**Con**	**As 17.5**	**As 35**	**As 70**	**Cu125**	**Cu 250**	**Cu 500**	**As 35+ Cu 250**	**As 35+ Cu 500**	**As 70+ Cu 250**	**As 70+ Cu 500**
C_{mic}	288.03 (14.21)a	248.07 (18.88)ac	214.07 (13.62)bc	182.56 (6.22)de	262.73 (10.40)ac	221.7 (10.63)bc	192.63 (24.64)bce	215.7 (19.38)bc	182.53 (16.92)de	164.93 (12.86)d	151.6 (2.77)d
BSR	1.603 (0.142)a	1.453 (0.095)ac	1.19 (0.108)be	0.94 (0.222)dfh	1.516 (0.028)ac	1.30 (0.072)bc	1.01 (0.144)def	1.09 (0.107)bf	0.96 (0.036)dh	0.92 (0.148)dh	0.88 (0.057)gh
SIR	5.04 (0.26)a	4.66 (0.15)a	3.81 (0.46)b	3.21 (0.09)dc	4.81 (0.16)a	4.74 (0.44)a	3.38 (0.1)bc	3.52 (0.12)bc	2.82 (0.22)d	2.76 (0.095)d	2.72 (0.41)d
HZ											
C_{mic}	477.06 (27.65)a	459.83 (17.17)ac	402.96 (11.37)be	348.1 (10.83)fg	471.5 (17.51)ac	441.2 (16.14)bc	364.53 (12.5)dg	405.96 (16.55)de	369.53 (12.47)dg	334.46 (11.17)fg	316.8 (18.17)f
BSR	3.11 (0.15)a	2.94 (0.13)a	2.72 (0.17)bc	1.99 (0.16)ef	2.98 (0.1)a	2.96 (0.21)b	2.74 (0.16)df	2.44 (0.08)dc	2.13 (0.17)efg	2.16 (0.03)eg	1.89 (0.12)g
SIR	13.17 (1.35)a	12.3 (0.31) ac	10.98 (0.22)bce	8.3 (0.61)dfg	12.45 (0.55)ac	11.16 (0.97)bc	9.48 (0.73)def	9.88 (0.67)bf	8.6 (0.33)dfg	7.62 (1.6)g	7.41 (1.05)g

Con : Control soil without heavy metals ; C_{mic} : Microbial biomass C (mg kg^{-1} soil); BSR: Basal soil respiration (µg CO_2-C gm^{-1} soil h^{-1}); SIR: Substrate induces respiration (µg CO_2-C gm^{-1} soil h^{-1}); qCO_2: Metabolic quotient i.e. basal respiration for unit microbial biomass (µg CO_2-C mg C_{mic} $24h^{-1}$,); Microbial biomass C (Cmic) was determined by the chloroform fumigation extraction method . Soil basal respiration (BSR) and substrate induces respiration were determined by measuring CO_2 evolution.

Table 5.12: *Ec-50 values of Cu and As (mg kg-1 dry soil) for microbial activity measurements of RA and HZ soils after 10 week of incubation. In order to describe EC-50 values, non linear regression was performed, data was normalized (normality test passed) and fitted in to a logistic response equation {y= a/1+ (x/x0)b} described elsewhere (Liu et al., 2010). Significant values (p) are shown in italics where as standard errors are given in parenthesis.*

	C_{mic}	BSR	SIR	qCO_2
RA				
As	127.5 (0.87) *p= 0.004***	90.1(12.41) *p=0.08*	108 (27.5) *p=0.15**	502.7 (869) *p=0.66*
Cu	1778 (460) *p=0.16*	706.9 (56.5) *p=0.05***	636.7 (60.9) *p=0.048***	1162 (272) *p=0.14**
HZ				
As	152.4 (17) *p=0.006***	99.89 (1.63) *p=0.01*	98.9 (0.39) *p=0.002***	187.7 (20.4) *p=0.06*
Cu	1160 (171) *p=0.09***	827.1 (56.8) *p=0.041***	1051(135) *p=0.08***	2289 (288) *p=0.07***

* significant at P <0.15; ** significant at P <0.05

5.2.3.4. Conclusions

The present findings demonstrated that heavy metals significantly reduced microbial ammonia oxidizing parameters in acidic alfisols. Soil nitrification rate was significantly decreased by As and Cu in acidic alfisols. AOA was found to be more abundant than AOB in As and Cu spiked acidic alfisols. AOA abundance was positively correlated with soil potential nitrification rate (PNR). All together these results may implicate the dominant role of AOA over AOB in AO observed in Cu and As amended acidic alfisols. AOA-community did not respond to As and Cu in acidic alfisols. Thaumarchaeal AOA group 1.1b might be playing potential role in ammonia oxidation of oligotrophic acidic soils. These results could support further the hypothesis that physiological differences between AOA and AOB may facilitate them to occupy distinct ecological niches.

Chapter 6

Exploring multimetal resistant plant growth promoting bacteria (PGPB) for enhancing plant growth in heavy metal polluted soils

6.1. Introduction

Industrial inputs and the agronomic management of fertilizers, pesticides, metal tainted sewage and effluent contribute to the accumulation of toxic metals in agricultural soils (Herland et al., 2000). Unlike organic pollutants, heavy metals cannot be degraded and persist indefinitely in the environment (Dacera and Babel, 2013). Contamination of agricultural soils by heavy metals has become a major environmental concern due to their toxic ecological effects (Rajkumar et al., 2012). Elevated concentrations of heavy metals in soils and their uptake by plants adversely affects the growth, symbiosis and consequently the yields of crops (Athar and Ahmad, 2002; Cheng, 2003; An et al., 2004; Karimi et al., 2013; Rajkumar et al., 2012 and 2013). It was also found that heavy metals interfere with uptake of nutrients such as Fe and P by roots and lead to plant growth retardation (Zayed and Terry, 2003). In addition to this, inundation of toxic metals in the soil inversely affects the microbial compositions and activity (Smejkalova et al., 2003; Marcin et al., 2013) and pose a major threat to plant beneficial rhizobacteria. Moreover, accumulation of heavy metals in edible parts of the crops create hazards to animal and human health

Chromium (Cr) is a widespread soil pollutant and is released from various industrial sources such as electroplating, leather tanning, alloy formation, nuclear wastes, metal cleaning and processing. Naturally occurring Cr exists in two oxidation states as trivalent (Cr (III)) and hexavalent (Cr (VI) forms. Relatively, Cr (VI) is more soluble in water and has greater mobility than Cr (III). It is well demonstrated that Cr (VI) is highly toxic to plant cellular processes than Cr (III) (Han et al., 2004; Shanker et al., 2005; Chidambaram et al., 2009). Chromium induced oxidative damage in plants includes mainly peroxidation of membrane lipids and obliteration of chlorophyll and cellular proteins (Bowler et al., 1992; Panda and Patra, 1997; Panda and Choudhury, 2005).

Lead (Pb) is one of the major contaminants of soils, sediments and water. The main sources of lead entering in soil ecosystem are atmospheric lead (primarily from automobile emissions), combustion of fossil fuels, agrochemicals (fertilisers and pesticides), lead-acid batteries, industrial processing of ores and minerals (Sengar et al., 2008). Lead deposited on the ground is transferred to the upper layers of the soil surface, where it may be retained for up to 2000 years. Uptake of lead by plants

results in impaired photosynthesis, reduced respiration and root development and pre-mature aging (http://www.lead.org.au/). In addition, lead inhibits activities of many enzymes, upsets mineral nutrition and water balance, changes the hormonal status, and affects membrane structure and permeability (Sharma and Dubey, 2005; Sengar et al., 2008). Visual, nonspecific symptoms of lead toxicity are stunted growth, chlorosis, and blackening of the root system. In most cases, lead inhibition of enzyme activities results from the interaction of the metal with enzyme -SH groups (Sharma and Dubey, 2005). Therefore, mitigating the phytotoxic effects of Cr (VI) and Pb would be beneficial for sustainable agriculture and restoration of soil health.

Metal resistant plant growth promoting bacteria (PGPB) include a diverse group of free-living soil bacteria that can improve host plant growth by alleviating toxic effects of heavy metals on the plants (Jiang et al., 2008; Rajkumar and Freitas, 2008; Li and Wong, 2012; Rajkumar et al., 2012 and 2013). In addition to this, PGPB have exceptional ability to promote the growth of the host plant by various mechanisms such as utilization of 1-aminocyclopropane-1-carboxylic acid (ACC) as a sole N source, siderophore production, phosphate solubilisation, nitrogen fixation and production of plant growth regulators (Reed et al., 2005; Rajkumar and Freitas, 2008). Metal resistant plant growth promoting bacteria suitable for escalation of phytoremediation in contaminated systems has also been established (Khan et al., 2009; Ma et al., 2010; Sheng and Xia, 2006; Li and Wong, 2012; Rajkumar et al., 2012 and 2013).

Indigenous bacteria existing in Cr contaminated areas usually acquire natural resistance against chromate and other heavy metals present in their environment (Whiting et al., 2001). Many studies on plant growth potential of metal resistant PGPB exist (Wani et al., 2007 and 2008; Chatterjee et al., 2009; Rajkumar et al., 2010, 2012 and 2013) however the study of multimetal resistant PGPB are inconspicuous. The ability metal resistant PGPB to promote plant growth in a metal-contaminated environment also makes them the preferred choice for the phytoremediation (Zaefariani et al., 2012), bioremediation and sustainable agriculture technologies. However knowledge about the effect of chromium and lead on the plant growth–promoting traits of PGPB is found to be very limited.

The present investigation was aimed at exploring novel multi-metal resistant PGPB from a highly polluted site under study for enhancing plant growth in Cr (VI) and Pb (II) amended soils. Amelioration of toxicity of Cr (VI) and Pb (II) on above and below ground biomass of *Vigna radiata* var GM4 was established for a very promising PGPB described in this Chapter. The credible mechanisms that are responsible for the plant growth promotion in Cr (VI) and Pb (II) amended soils by PGPB are elaborated.

6.2. Materials and methods

6.2.1. Site description and sample collection

Triplicate composite samples were collected from a sediment sample (R4) collected from a industrial waste effluent (IWE) settling lagoon situated near Sarod ($22^0$10'44.29"N, 72042'55.19"E), Mahi River estuarine area, Gujarat, western India. Detailed description as well as overview of the sampling site is given in Section 5.2.1 and Fig. 5.2. Autoclaved polyethylene bags were used for collecting the samples. The samples were immediately kept in an ice box before being transported to the lab. Soil samples were sieved (4 mm) and stored at 4^oC.

6.2.2. Physical and chemical analysis of the soil

The physicochemical analysis of the soil sample was done as described in Chapter 5

6.2.3. Isolation of heterotrophic bacteria.

Total heterotrophic bacteria were isolated by plating soil samples on Reasoner's 2A (R2A) agar (HiMedia Laboratories, India; as described previously (Section 2.3.1).

6.2.4. Determination of minimum inhibitory concentration (MIC) of metal ions

MIC values of the bacterial isolates against $Pb^{2+,}$ Cd^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+} and Cr^{6+} were determined by the plate dilution method (Summers and Silver, 1972; Aleem et al., 2003; Wani et al., 2007). The lowest concentration that prevented bacterial growth was considered to be MIC. The cultures were first grown in Luria Bertani (LB) medium and incubated overnight at 30^oC on a rotary shaker. These were then centrifuged at 10,835 x *g* for 20 min, followed by washing with sterile N-Saline

(0.85% NaCl). The washed pellet was resuspended in 5 ml sterile N-Saline. For determination of MIC of each isolate, for a particular metal, 1/10 th LB (1:10 dilution of LB medium) agar plates were supplemented with a particular heavy metal concentration such as 100 to 600 µg ml^{-1} for $Pb^{2+,}$ Cu^{2+}, Zn^{2+} and Cr^{6+} and 1-15 µg $ml^{-1}$for Co^{2+} and Cd^{2+}. Metal salts used were Pb $(NO_3)_2$, $CuSO_45H_2O$, $ZnCl_2$, $K_2Cr_2O_7$, $CoCl_2$ and $CdCl_2$. Culture suspension (50 µl), having O.D. = 0.2 (at 600 nm) was spotted on the above mentioned plates and growth was observed after 5 d of incubation at 30^{o}C.

6.2.5. Identification of metal resistant isolates

Genomic DNA was extracted from metal resistant bacteria. Universal eubacterial primers 27F (5'- GAG AGT TTG ATC CTG GCT CAG) and 1107R (5'-GCT CGT TGC GGG ACT TAA CC) were used for the amplification of 16S rRNA gene fragments (Rajendran et al., 2008). PCR products were sequenced using the commercial sequencing service provided by Bangalore Genei Pvt. Ltd (Merck Specialities Pvt. Ltd, Bangalore, India). The best read from the chromatogram was converted to FASTA format and NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast/) analysis as well as RDP-II project database SeqMatch (http://rdp.cme.msu.edu/seqmatch/) was performed. Multiple sequence alignment was carried out in RDP-II project by taking appropriate standard database sequences and a phylogenetic tree was constructed for the isolates using neighbour joining method in MEGA 5.0 programme (Tamura et al., 2011).

6.2.6. Plant growth promoting traits of metal resistant isolates

Plant growth promoting traits of the metal resistant PGPB were characterized as shown in Fig. 6.1. The procedures are briefly described below. All the plant growth promoting traits were measured in triplicates and the results are expressed as mean or representative values. In order to discern the credible mechanisms involved in plant growth promotion under elevated metal Cr (VI) concentrations by inoculated isolate, all the plant growth promoting traits were performed (as mentioned in Section 6.2.6) in presence of 100 ppm of Cr (VI) and Pb (II).

6.2.6.1. Mineral phosphate solubilisation (MPS)

Bacteria were grown in 5 ml LB broth overnight at 30ºC on a rotary shaker. These were then centrifuged, washed twice with N-saline and resuspended in equal volume of N-saline. A 20 µl aliquot of culture suspension (OD = 0.2 at 600nm) of each bacterial isolate was spot inoculated on Pikovskaya's (HiMedia laboratories, Mumbai, India) plates for MPS. Colonies forming a clear halo around them on Pikovskaya's agar were considered as MPS and P solubilisation index (PSI) was measured by a formula "CS+CZ/CS" where CS indicates colony size and CZ indicates Zone of clearance.

6.2.6.2. Siderophore production

Siderophore production of the isolates was checked by chrome azurol-S agar plates (Alexander and Zuberer 1991).

6.2.6.2.1. Preparation of chrome azurol-S (CAS) agar plates for siderophore detection:

The medium was prepared using the following

Dye solution (100 ml):

60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml of Fe^{+3} solution (2 mM $FeCl_3$ in 10 mM HCl). While stirring, this solution was slowly added to 72.9 mg hexadecyl trimethyl ammonium bromide (HDTMA) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved at 15 lbs pressure for 15 min.

10X MM9 salt (100 ml):

MM9 growth medium was derived from M9 by reducing concentration of phosphate salts to 0.03% KH_2PO_4. Ingredients g 100 ml^{-1}: $Na_2HPO_4.7H_2O$ 0.15, KH_2PO_4 0.15, NaCl 0.5, NH_4Cl 1.0 and D/W 100 ml. Further, 1M $MgSO_4$ and 0.1M $CaCl_2$ was prepared separately and autoclaved at 10 psi for 20 minutes. 1 ml of each was added to the above formulated medium. 10 ml 10X MM9 salts, 3.024g PIPES and 1-2g of 50% (w/w) NaOH solution were added and dissolved in 75 ml double distilled water to get pH 6.8. Autoclaved at 15 psi for 15 min. 10 ml dye solution was added lastly with agitation to prevent foaming and was poured in plates. The solution

was stored in dark in polyethylene bottles. For detection of siderophore, culture was spotted on the plate and the colonies developed were observed for yellowish orange halo around them.

6.2.6.3. pH drop and organic acid production in buffered medium (pH 8)

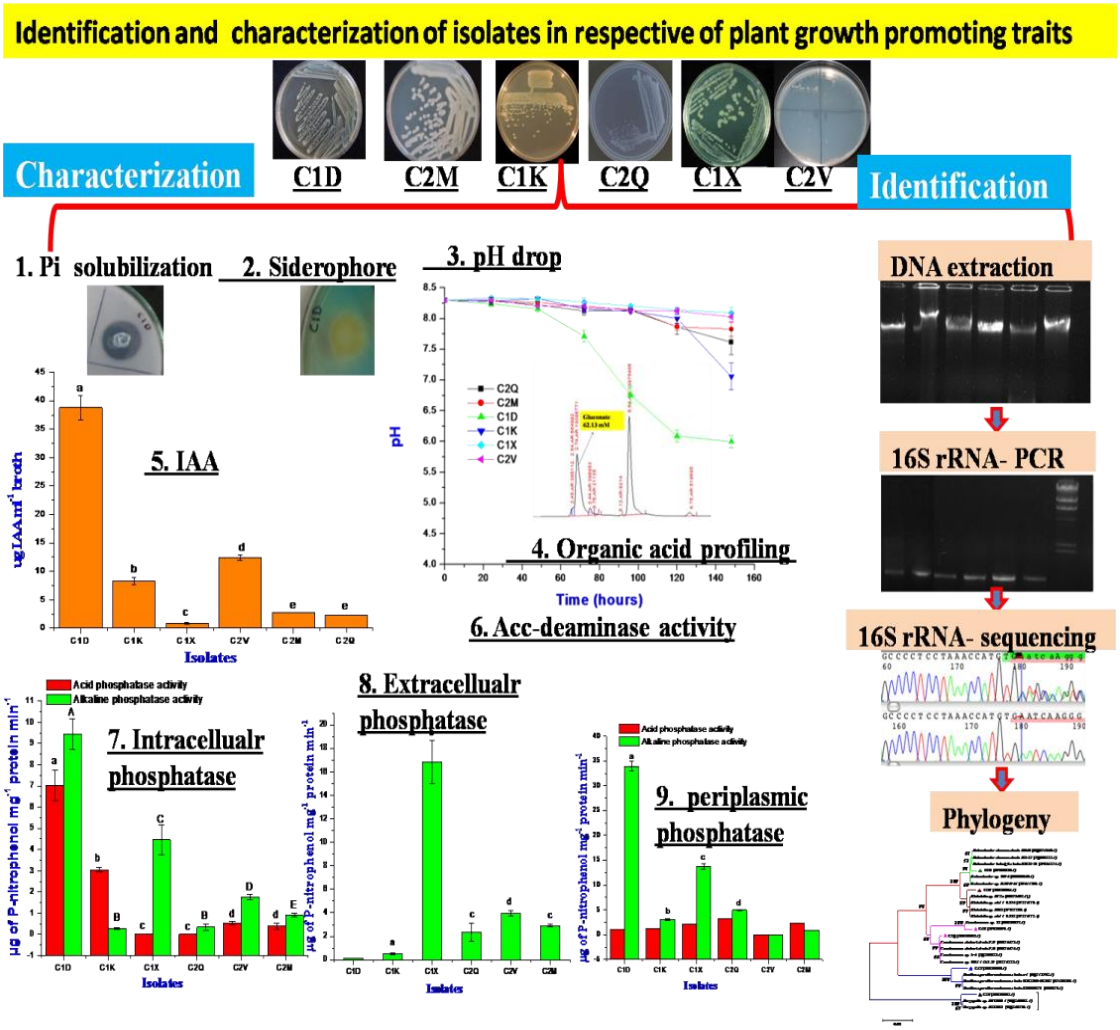
The ability of the isolates to bring about a pH drop in buffered medium (100 mM Tris-HCl, pH 8.0) was analysed in minimal medium containing Senegal rock phosphate (1 mg ml^{-1}) as the sole P source. Composition of the medium and description of the rock phosphate is given by Gyaneshwar et al. (1999 and 1998) and Sharma et al. (2005). The pH of the culture supernatant was continuously monitored over a period of 7 d. The supernatant collected when the culture showed maximum pH drop in Tris buffered rock phosphate minimal media was analyzed by high performance liquid chromatography (HPLC) to identify organic acid profile encompassing both quality and quantity of organic acids as described by Patel et al. (2010).

6.2.6.4. Phosphatase activity

Organic phosphate hydrolyzing capability of the isolates was evaluated by phosphatase assay. Both acidic and alkaline phosphatase activities of the isolates were determined by using *p*-nitro phenyl phosphate (pNPP) as a substrate. Detailed protocol for phosphatase assay is as mentioned previously (Patel et al., 2010).

In the present investigation intracellular, extra-cellular and periplasmic phosphatase activities of the isolates were evaluated. In brief, bacterial isolates were grown in 100 ml LB broth and cell pellet was obtained by centrifugation at 9000 g for 20 min followed by a wash with sterile N-Saline and then resuspended in 5 ml of sterile N-saline. The suspension was then subjected to ultrasonication for cell lysis and subsequent release of intracellular/periplasmic phosphatase. Culture supernatant was used as source of extracellular enzyme preparation. For periplasmic phosphatase activity, intact whole cells, washed and resuspended in N-saline were used. Whole cell protein of cell pellet, after 1 N NaOH treatment for 15 min, was estimated by using Lowry method (Lowry et al., 1951). Unit for enzyme activity was defined as μg p-nitrophenol mg^{-1} protein min^{-1}

Fig. 6.1: *Comprehensive picture showing characterization of multimetal resistant isolates.*



6.2.6.5. Indole acetic acid (IAA) production

IAA production of the isolates was quantified by Salkowaski's reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) as described by Brick et al. (1991) and the activity is expressed as µg IAA ml^{-1} broth.

6.2.6.6. 1-aminocyclopropane-1-carboxylate (ACC) Deaminase activity

ACC deaminase activity is of utmost importance under heavy metal stress as it involves reduction of stress ethylene in plants. Initially, ACC utilization potential of the bacterial isolates was screened by using basal minimal media agar plates comprising of ACC as the sole N-source. The composition of salt minimal media containing ACC as sole nitrogen source in g L-1 is as follows, KH_2PO_4, 1.36; Na_2HPO_4, 2.13; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.7; $FeSO_4.7H_2O$, 0.2; $CuSO_4.5H_2O$, 0.04; $MnSO_4.H_2O$, 0.02; $ZnSO_4.7H_2O$, 0.02; H_3BO_3, 0.003; $CoCl_2.6H_2O$, 0.007;

$Na_2MoO_4.2H_2O$, 0.004; Substrate ACC, 5mM; Glucose, 1.0% dissolved in 1000 mL of distilled water (Penrose et al., 2001).

The bacteria able to produce significant biomass on these plates after 48 h were considered potentially ACC deaminase positive and confirmed by ACC deaminase activity measurements with ACC as substrate (Glick et al., 1998). The product of this reaction is α-ketobutyrate, which forms phenyl hydrazone upon addition of 2, 4 dinitrophenyl hydrazine reagent. A colored complex is formed after NaOH addition. The ACC deaminase assay was performed with late log phase cells as described by Glick et al. (1998). Unit for ACC deaminase was defined as nmol α-ketobutyrate mg^{-1} protein h^{-1}.

6.2.7. Gnotobiotic plant inoculation experiments for studying effect of bacterial inoculation on plant growth in presence of metal

Metal resistant isolate (*Enterobacter* sp. C1D) which exhibited very good PGPB traits was chosen for further gnotobiotic plant studies. In order to evaluate the plant growth promoting potential of the isolate in heavy metal contaminated system, short term microcosm studies were conducted as shown below and depicted in Fig. 6.2.

Control soil without any history of heavy metal exposure was collected from *Pulse Research Station*, Anand Agricultural University, *Vadodara*- 390 003, Gujarat, India. Soil texture was found to be silty loam. Soil organic matter was found to be 0.6% and was neutral in pH (7.2 ± 0.4). The soil was sieved (2 mm) for removal of visible roots and sterilized by autoclaving at 121^{o}C, 15 psi for 1 h. After sterilization, 6 different treatments were established with soil amended with aqueous solution of $K_2Cr_2O_7$ to achieve the final concentrations of 0, 50, 100, 200, 300 and 400 mg kg^{-1} of Cr (VI). Twelve different concentrations of Pb were established with soil amended with aqueous solution of Pb $(NO3)_2$ to reach final concentrations of 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500 and 600 mg kg^{-1} of Pb (II).The experiment was completely randomized design (Leps and Smilauer, 2003).

6.2.7.1. Seed inoculation

Vigna radiata var GM4 (mung bean) seeds were obtained from *Pulse Research Station***,** Anand Agricultural University, *Vadodara*- 390 003, Gujarat, India. Healthy seeds were surface sterilized. In brief, seeds were washed thrice with sterile distilled

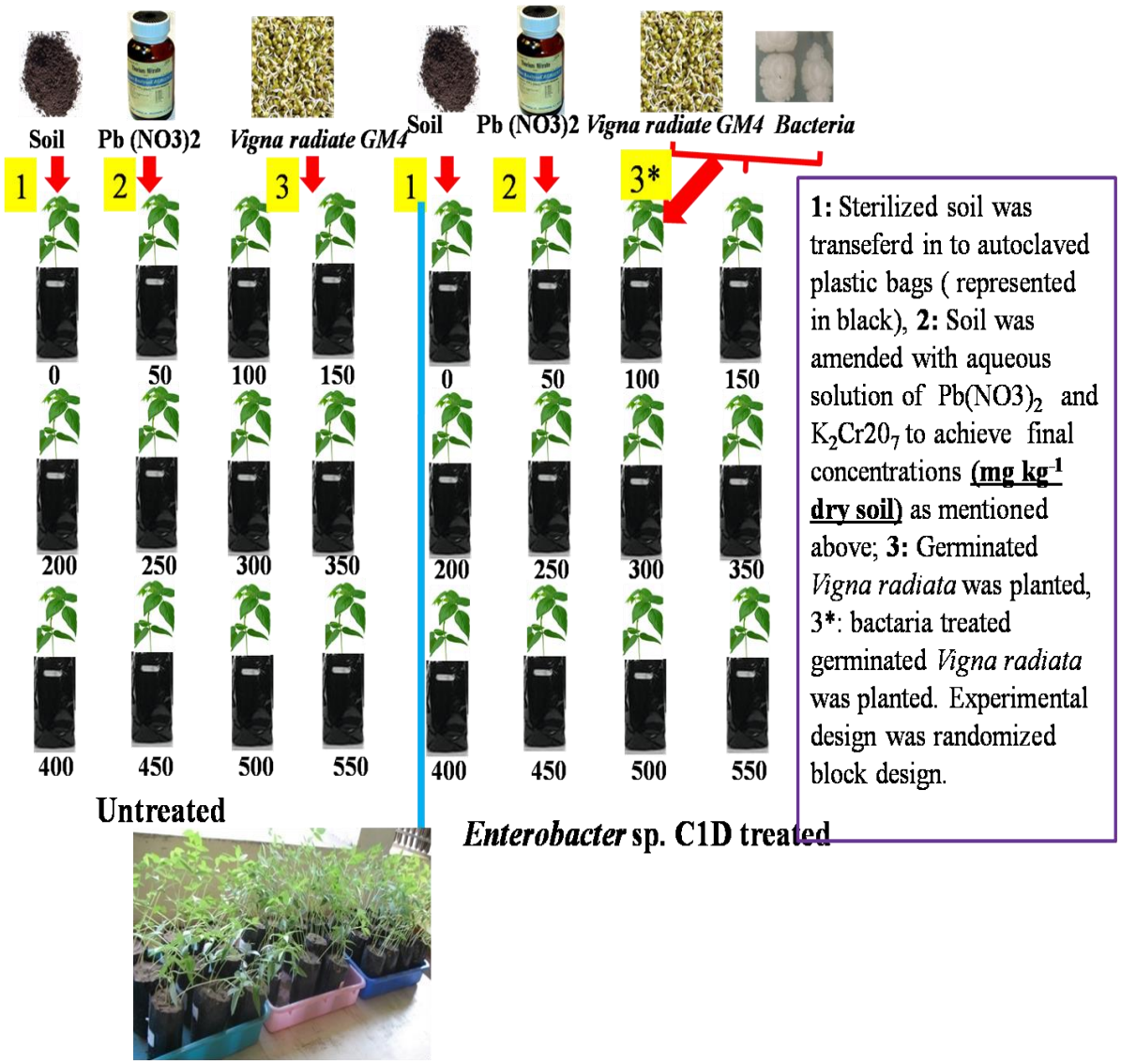
water, allowed to remain for 10 min and water was removed from it. Thereafter 0.1% $HgCl_2$ was added and seeds were allowed to soak for 5 min followed by washing for at least 6 times with sterile distilled water. To these, 70% alcohol was added and again kept for 5 min followed by 6 washes with sterile distilled water. The seeds were then transferred in sterile petri plates containing moist filter paper for germination in dark. Bacterial culture was grown for 18 h in LB broth; cells were harvested by centrifugation (9000 g, 10 min), washed twice with sterile distilled water and resuspended in N-saline. The seeds were inoculated by soaking in a bacterial suspension (O.D. = 0.6 at 600 nm) for 1 h as described by Burd et al. (2000). Seedlings soaked likewise in sterile water were used as control treatment. The inoculated and un-inoculated seeds were planted in separate sterilized plastic bags containing 500 g of autoclaved soil. The soil was moistened with water and maintained at 60% of its water holding capacity throughout the experiment. The plants were grown in a protective mesh chamber at ambient temperature and natural day-night conditions. All the experiments were performed in 3 independent replicates, with each replicate having 10 germinated seedlings. A total of 36 experimental setups were established for both inoculated and un-inoculated *Vigna radiata* GM4 seeds with both metals at concentrations indicated.

6.2.7.2. Plant growth parameter measurements

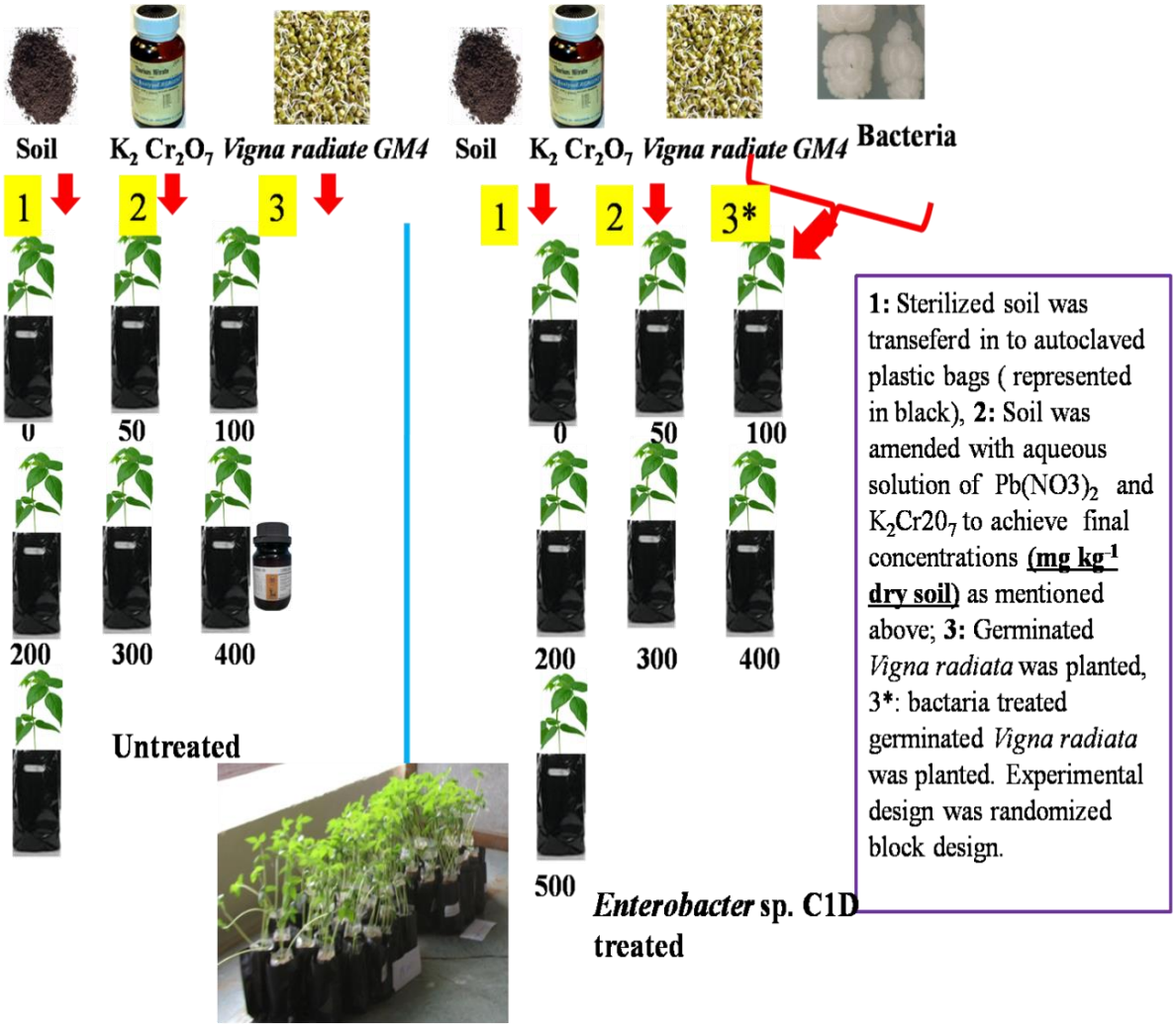
After 30 d, the plants were carefully harvested from the plastic bags and the root surface was cleaned several times with distilled water. Growth parameters such as shoot length, root length and oven dried shoot weight and root weight were measured. The chlorophyll a and chlorophyll b content of the plants were quantified following the method given by Xiong and Wang (2005). The tolerance value of Cr (VI) for every treatment was determined by effective concentration (EC50) at 30 d of incubation. In the present investigation EC50 was defined as the concentration at which the plant parameters such as shoot length, root length, shoot weight were reduced to half (50%) of the value obtained for Cr untreated control.

Fig. 6.2: *Schematic depiction of the design of microcosm studies for studying the plant growth benefit of PGPB in artificially polluted soils. a; Macrocosm studies with Pb; B; Microcosm studies with Cr*

A



B



6.2.7.3. Statistical analysis

For every treatment, data of shoot length, root length, shoot weight, root weight, chlorophyll-a and chlorophyll-b were expressed as mean value of 15 independent plants (n = 15) with standard deviation. Significant differences in means were statistically established using general analysis of variance (ANOVA) and also treatment means compared by Tukey test (P = 0.05). Effective concentration of Cr (EC50) for all the treatments were determined by non linear regression with sigmoidal, logistic 3 parameter equation described in Sigma plot version -10.0. (Systat Software, Inc., San Jose California USA, www.sigmaplot.com). Relation between different treatments and different plant growth parameters were elucidated by canonical correspondence analysis (CA). All the statistical analysis was performed by Sigma stat 4.0 (Systat Software, Inc., San Jose California

USA, www.sigmaplot.com), Sigma plot version-10.0, CANCO for Windows 4.5 version (ter-Braak, and Šmilauer, 2002; www.canoco.com) and Origin version-6.0 (http://www.originlab.com).

6.3. Results

6.3.1. Sediment chemical analysis

Physical and chemical characters of the R4 sediment sample are given in section 5.3.1.1 and Table 5.2. However, following Table 6.1 has been given for ready reference.

Table 6.1: *(A) Physical and chemical characteristics and (B) Heavy metal analysis of the Sarod sediment sample R4.*

A:	Sand (%)	Slit (%)	Clay (%)	pH	SMC (%)	OM (%)	TOC (%)	TN (g/kg)	TC (g/kg)	TS (g/kg	C/N
	390.27	30.97	29.76	8.1	41	14.8	7.44	17.08	172.8	14.7	10.12
B:	**Cu**	**Cd**	**Ni**	**Pb**	**As**	**Zn**	**Cr**	**Co**	**Mn**	**Fe**	**Al**
	562.9	18.5	35.5	235.2	43.1	651.6	378	15.6	282.1	8100.6	873.7

6.3.2. Isolation of heterotrophic bacteria and MIC determination

Total viable heterotrophic bacteria were found to be $5.2x10^5$ cfu g^{-1}. Based on the colony characters and cell morphology (by gram staining), a total of 35 different colonies were selected for MIC against selected heavy metals. Among all the isolates 6 isolates had higher MIC values (Table 6.2). Isolates C1D, C1K and C2Q were found to be multi metal resistant in nature. All the isolates showed high MIC values against Zn^{2+} while MIC values of Co^{2+} and Cd^{2+} were found to be low (Table 6.2). Isolate C1D tolerated a concentration of 400 μg ml^{-1} Cr (VI). The order of toxicity of heavy metals on the bacterial isolates was Cd > Co > Pb > Cr> Cu > Zn.

Table 6.2: *Minimum inhibitory concentrations of the heavy metals for the bacterial isolates obtained from Sarod sediment Sample R4.*

Isolate	Cu^{2+} ($\mu g\ ml^{-1}$)	Zn^{2+} ($\mu g\ ml^{-1}$)	Pb^{2+} ($\mu g\ ml^{-1}$)	Cr^{6+} ($\mu g\ ml^{-1}$)	Co^{2+} ($\mu g\ ml^{-1}$)	Cd^{2+} ($\mu g\ ml^{-1}$)
C1D	400	750	350	400	10	2
C1K	300	400	200	300	2	1
C1X	350	600	200	250	4.0	0
C2Q	400	700	200	250	6.0	4
C2V	350	500	100	200	1	0
C2M	400	550	100	200	2	0

6.3.3. Identification of metal resistant isolates by 16S rRNA sequencing

Metal resistant isolates were identified by 16S rRNA gene fragment sequence analysis. Phylogenetic identification of the isolates is shown in Fig. 6.3. Multimetal resistant isolates C1D, C1K and C2Q were found to be *Enterobacter* sp., *Pseudomonas* sp. and *Pseudomonas* sp. respectively. Isolate C2V was found to be *Bergeyella* sp. under family *Flavobacteriaceae.* Nucleotide sequences retrieved from this study have been submitted to genebank under accession numbers JN936958-JN936963.

6.3.4. Sequence accession numbers

Nucleotide sequences (of bacterial 16S rRNA gene fragments) retrieved from this study have been deposited in the GenBank nucleotide sequence database and the details are shown in Appendix I.

6.3.5. Plant growth promoting traits of metal resistant isolates

A significant variation has been noticed in plant growth promoting traits of metal resistant isolates (Table 6.3). Among the six isolates tested, four isolates C1D, C1K, C2M and C2Q were found to be to be positive for siderophore production. Isolates C1D and C1K were found to be positive for mineral phosphate solubilisation on Pikovaskya agar. Significant amount PSI index (3.5) was noticed with Isolate C1D. All the isolates were able to utilize L-tryptophan and produce IAA, however, the capability of isolates in IAA production was found to vary and followed the order

C1D > C2V > C1K > C2M > C2Q > C1X. Maximum production of IAA (24 μg ml^{-1}) was recorded with isolate C1D.

Moreover, C1D is the only isolate showed significant biomass on basal minimal media with ACC as the sole N source, while other isolates failed to grow on the medium. This was later confirmed with ACC deaminase assay (Glick et al.1998) where isolate C1D showed 237.85 nmol α-ketobutyrate mg^{-1} protein h^{-1}.

Results obtained from pH drop experiment revealed that isolate C1D had the ability to drop pH after 6 days of incubation in Tris-HCl buffered minimal media (pH = 8) containing rock phosphate as a sole P-source. No other isolate was found to be positive for pH drop. Significant amount of organic acid was noticed to be secreted by C1D in inoculated Tris-HCl buffered medium. The organic acid was found to be 62.13 mM gluconate by HPLC analysis (Table 6.3; Fig. 6.4); however, unknown major organic acid peak also noticed. Incongruity was noticed with phosphatase activity of the isolates. Relatively isolate C1X showed high extracellular alkaline phosphatase activity where as C1D had high amount of alkaline periplasmic phosphatase activity. None of the isolates showed acidic periplasmic phosphatase activity.

Overall it was noticed that isolate C1D displayed high amount of siderophore production, PSI, IAA production, ACC deaminase activity, extracellular acid phosphatase production and periplasmic alkaline phosphatase activity.

Fig. 6.3: *16S rRNA based phylogeny of the metal resistant bacterial isolates. The phylogenetic analysis was conducted with MEGA. The evolutionary history was inferred using UPGMA method. Isolates were shown with different coloured triangle. GenBank accession numbers of the isolates are shown in the parenthesis.*

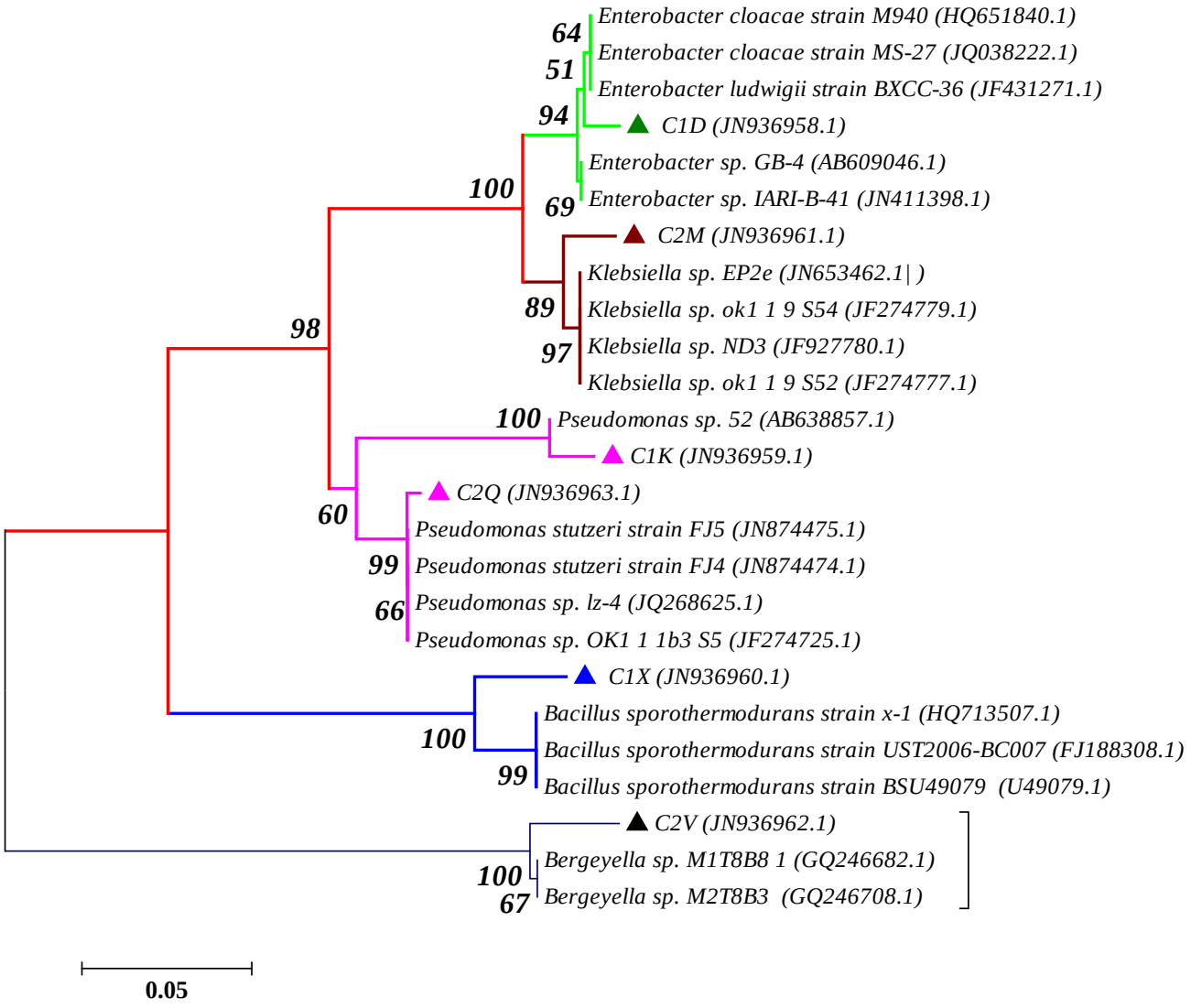


Table 6.3: *Plant growth promoting traits of metal resistant isolates from Sarod sediment Values are mean of three independent replicates (n = 3). Values in the rows followed by the same letter do not differ significantly according to Fisher's LSD at p<0.05.*

Isolate	Siderophore Production	[Δ]Mineral phosphate solubilization	[•]IAA production	[¥]ACC Deaminase activity	*pH drop	Organic acid	[ǂ]Phosphatase activity					
							E.P		I.P.		P.P	
							A	B	A	B	A	B
C1D	+++	3.5a	38.79a	237.85a	5.2 a	[Ø]Gluconate	0.13a	7.04a	9.4a	0.9a	33.9 a	UD
C1K	+++	1.25b	8.3b	85b	7.3 b	NA	0.52b	3.04b	0.3b	1.2 b	3b	UD
C1X	-	UD	0.83c	ND	8 c	NA	16.8c	0	4.4c	2 c	13.7c	ND
C2M	+	UD	2.73 d	ND	8c	NA	2.91d	0.39 c	0.8 b	2.3c	0.87d	ND
C2V	-	UD	12.38e	ND	8 c	NA	3.97e	0.5	1.7 d	0	0	ND
C2Q	++	UD	2.33d	ND	7.6 b	NA	2.34 d	0	0.8b	3.2d	4.98e	ND

[Δ] Mineral phosphate solubilisation was measure by SPI index CS+CZ/CS; [•]Unit for IAA production, µg/ml^{-1} ; [¥] Unit for ACC deminase activity, nmol α-Ketobutyrate mg^{-1} protein h^{-1}; ***** pH drop after 6days in minimal media amended with rock phosphate; [Ø]62.13 mM gluconate; [ǂ] Unit for phosphataseactivity, µg P-nitrophenol mg^{-1} min^{-1} ; E.P – Extracellular phosphataseactivity; I.P- Intracellular phosphataseactivity; P.P- Periplasmic phosphataseactivity; A-Alkaline phosphatase; B- Acidic phosphatase. UD: Undetectable; NA: Not applicable.

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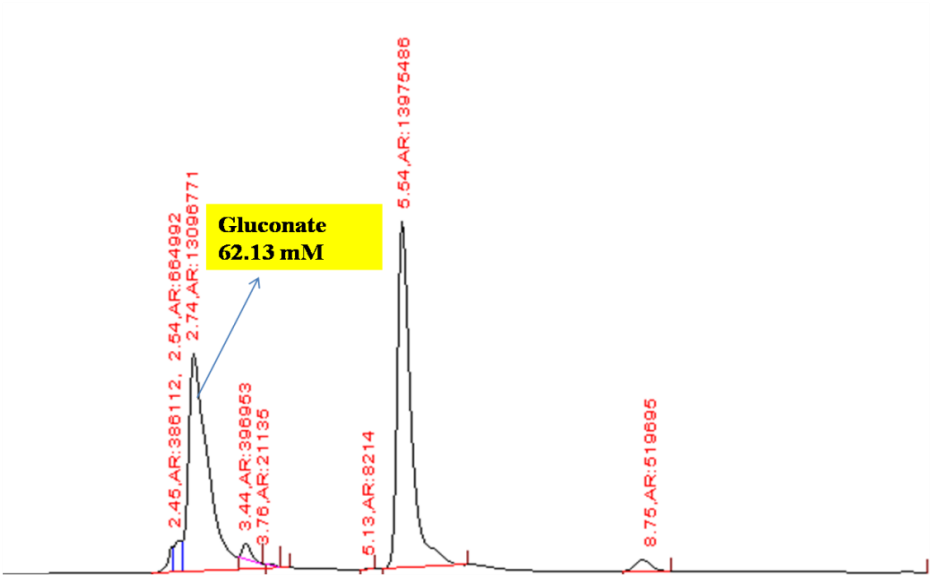
Fig. 6.4: *Organic acid profiling of Enterobacter sp. C1D by HPLC*



6.3.6. Plant growth in Cr contaminated microcosms

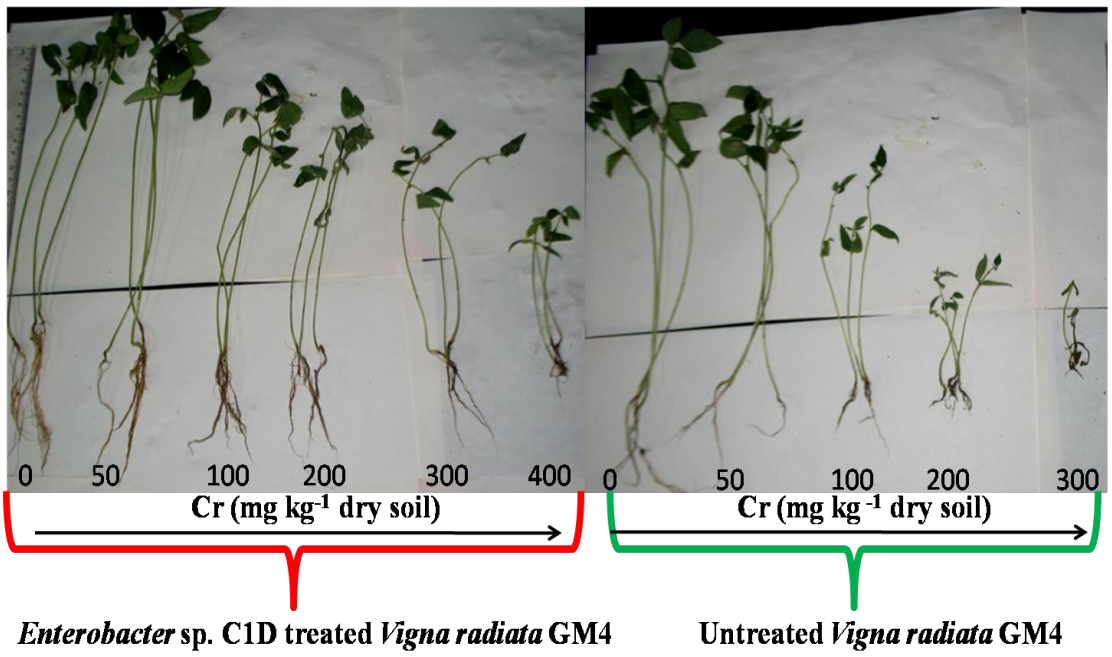
Multi metal resistant *Enterobacter* sp. C1D was chosen for gnotobiotic plant studies. The bacteria inoculated and non-inoculated plants were subjected to 6 levels of Cr (VI) concentrations (0, 50, 100, 200, 300 and 400 mg kg^{-1}) and studied for their response in terms of plant growth parameters.

The un-inoculated plants exposed to different concentrations of Cr (VI) showed a marked inhibition in the growth displaying very apparent Cr toxicity (Fig. 6.5). With the increase in concentration of Cr (VI) progressive and drastic decrease in root length, shoot length, dry shoot weight, root weight and chlorophyll content were observed (Figs. 6.5 and 6.6). Compared to control without Cr treatment, the plants grown with Cr (VI) showed reduced shoot and root parameters and the results were statistically significant at *P* level 0.05 (Fig. 6.6). The concentration of Cr (VI) amended as 300 mg/kg was found to be highly toxic to *V. radiata* plants where 80.19%, 90.69%, 77.52 and 88.3% reduction in shoot length, root length, shoot weight, and root weight respectively were observed. Similar trend was noticed with chlorophyll-a and b contents where the values for reduction were found to be 70.58% and 79.96% (Fig. 6.6), respectively. No growth was observed at Cr (VI) 400 mg/kg

treated *V. radiata* seedlings in which all the seedlings were found to be dead after a week period during the course of experiment.

Enterobacter sp. C1D considerably enhanced the plant growth of *V. radiata* in both metal containing and metal free conditions. Compared to un-inoculated plants (Figs. 6.5 and. 6.6), increased root, shoot and chlorophyll parameters were observed with *Enterobacter* sp. treated plants (Table 6.4) and the results were statistically significant (P <0.05). The growth promotion by the PGPB was found to be more prominent at elevated concentrations of Cr (VI) such as 100, 200 and 300 mg/kg (Fig. 6.6, Table 6.4). The isolate was found to increase 22.7% shoot length, 28.1% shoot weight, 24% root length, 31% root weight in the absence of Cr whereas an increase of 68.8% shoot length, 50.7% shoot length, 80.4% root length and 77.3% root weight were observed at Cr (VI) 300 mg/kg. Moreover, significant increase in both Chlorophyll-a and b contents (51.6% and 61.4% respectively) were found to be associated with isolate treated plants at Cr 300 mg/kg (Table 6.4) . Unlike un-inoculated plants, at 400 mg/kg, the PGPB treated plant showed apparent root and shoot biomass (Fig. 6.6) and this clearly indicates the improved Cr (VI) tolerance of plants upon bacterization.

Fig. 6.5: *Effect of Enterobacter sp. C1D on Vigna radiata GM4 after 30 days of incubation in Cr (VI) spiked soils.*



6.3.7. Effective concentration (EC50) of Cr (VI) at 30 d of incubation and Correspondence analysis CA analysis

Un-inoculated *Vigna radiata* plants showed significant (P = 0.05) lower values of Cr (VI) tolerance for all the plant parameters after 30 days of incubation (Fig. 6.7). The effective concentrations (EC 50) of Cr (VI) for shoot length, shoot weight, root length, root weight, chlorophyll a and b were found to be 145.4, 169.1, 96.97, 109.4, 193.7 and 137.6 mg/kg Cr (VI) respectively (Fig. 6.7) . Correspondence analysis (CA) biplot revealed that in all the 6 treatments plant parameters were closely related and clustered closer to each other whereas Cr (VI) was found to be negatively correlated to plant parameters (Fig. 6.8A). Furthermore, a strong relation between treatment 1 (Cr (VI) 0 mg) and treatment 2 (Cr (VI) 50 mg/kg) were observed where those were closely grouped to each other.

The plant tolerance towards Cr (VI) measured as effective concentration (EC50) showed these values for each and every parameter to be relatively higher with *Enterobacter* sp. C1D treated plants as compared to the un-inoculated plants at all concentrations of Cr (VI) (Fig. 6.7) and the values for shoot length, shoot weight, root length, root weight, chlorophyll a and b were found in the range of approximately 200-250 mg/kg Cr (VI) whereas in the un-inoculated plants the EC50 values ranged from 100-150 mg/kg Cr (VI)**.** CA biplot revealed that in all the 6 treatments, plant parameters were closely related and clustered closer to each other whereas Cr (VI) was still found to be negatively correlated to plant parameters (Fig. 6.8B). Furthermore, a strong relation between treatments 1 (Cr (VI) 0 mg), 2 (Cr (VI) 50 mg/kg) and 3 (Cr (VI) 100 mg/kg) were observed as those were closely grouped to each other.

6.3.8. Plant inoculation studies in Pb (II) amended microcosms

After 30 d of incubation shoot length, root length, shoot weight, root weight and chlorophyll content of *Vigna radiata* GM4 were measured. It was found that lead was very much toxic to the above and below ground biomass of *Vigna radiata* GM4 (Fig. 6.9 and Fig. 6.10). Especially the obvious reduction in below ground biomass was seen at elevated Pb concentrations (Fig 6.9 B and C). It was noticed that chlorophyll content of *Vigna radiata* GM4 was decreased by Pb^{2+} toxicity. Seeds

treated with *Enterobacter* sp. C1D were significantly protected from Pb^{2+} toxicity (Fig. 6.9 and Fig. 6.10).

Enterobacter sp. C1D had positive measurable effects on root length, shoot length, fresh shoot weight, fresh root weight and chlorophyll content of the *Vigna radiata* GM4 in Pb^{2+} contaminated soil (Fig. 6.10). The tested isolate significantly increased root biomass and shoot biomass. About 3.5 times more root biomass was achieved with *Enterobacter* sp. C1D at 600 ppm Pb^{2+} concentration. Moreover, the inoculation of *Enterobacter* sp. C1D in plants increased the chlorophyll content too (Fig. 6.10).

6.3.9. Effective concentration (EC50) of Pb (II) at 30 d of incubation and CA analysis

After 30 d of incubation un-inoculated *Vigna radiata* GM4 plants showed significantly ($P < 0.05$) lower values of Pb (II) tolerance for all the plant parameters (Table 6.5). The effective concentrations (EC 50) of Pb (II) for shoot length, shoot weight, root length, root weight, chlorophyll a and b were found to be 651.3, 519.7, 655.4, 362.4, 409.8 and 342.5 mg/kg Pb (II) respectively (Table 6.5) .

The plant tolerance towards Pb (II) measured as effective concentration (EC50) showed these values for each and every parameter to be relatively higher with *Enterobacter* sp. C1D treated plants as compared to the un-inoculated plants at all concentrations of Pb (II) (Table 6.5) and the values for shoot length, shoot weight, root length, root weight, chlorophyll a and b were found in the range of approximately 476 to 1118 mg/kg Pb (II) whereas in the un-inoculated plants the EC50 values ranged from 342 to 655 mg/kg Cr (VI)**.**

6.3.10. Characterization of plant growth promoting traits in presence of Cr (VI) and Pb (II)

Plant growth promoting traits of metal resistant isolate C1D in presence of 100 mg/kg Cr (VI) and Pb (11) are summarized in Table 6.6. Relatively decreased siderophore production and increased IAA production were noticed (VI). The concentration 100 mg/kg Cr (VI) drastically decreased both the potentiality of mineral phosphate solubilization and capability of pH drop in Tris-HCl buffered medium (pH 8). Unlike metal free environment, no organic acid was found at 100 mg/kg Cr (VI).

However, little organic acid (15.3 mM gluconate) is produced in response to 100 mg/kg Pb (II). Slightly decreased ACC deaminase activity (198.8 nmol α-Ketobutyrate mg^{-1} protein h^{-1}) was noticed. Higher ACC deaminase activity (416.93 nmol α-Ketobutyrate mg^{-1} protein h^{-1}) is noticed at 100 mg/kg Pb (II). A large discrepancy among phosphatase activities in presence of Cr (VI) AND Pb (II) were observed. Significant amount of both periplasmic acidic and alkaline phosphatase activities were noticed in presence of 100 mg/kg Cr (VI). In presence of Pb^{2+}, high amount of both acidic (55 µg pNPP mg^{-1} protein min^{-1}) and alkaline (67.43 pNPP mg $protein^{-1}$ min^{-1}) periplasmic phosphatase activities were noticed. No acidic or alkaline extracellular phosphatise activities were observed at 100 mg/kg pb (II). As compared to control (without metal), Pb^{2+} enhanced both acidic (55 times) and alkaline (2 times) periplasmic phosphatases.

6.3.11. Estimation Pb concentration in *Vigna radiate* GM4 tissues

One of the major concerns is the accumulation of heavy metals in edible parts of the crops creating hazards to animal and human health. Hence it would be worthwhile to undertake a study on the heavy metals uptake by agricultural crops. In the present study accumulation of Pb in plant tissues is noticed in both below and above ground biomass. The metal concentrations in the roots and shoots of *Vigna radiata* grown in artificially Pb amended soil are given in Fig. 6.11. In general, inoculation of *Enterobacter* sp. C1D did greatly alter the concentration of metal in plant tissues ($P < 0.05$ to $P < 0.01$). . The concentration of Pb in root tissues was decreased after inoculation with C1D. Further it can be observed that inoculation with C1D reduced the translocation of Pb from root to shoot. The highest effect was observed at elevated Pb concentrations. For example, At 100 mg/kg Pb, C1D reduced 1.26 times Pb concentration in roots and 1.32 times Pb concentration in shoots; where as at 600 mg/kg Pb, compared with the control, the inoculation of C1D decreased 1.5 times Pb accumulation in roots and 1.85 times Pb accumulation in shoots (Fig. 6.11).

Fig. 6.6: *Plant growth parameters of Enterobacter sp. C1D un-inoculated and inoculated Vigna radiata var GM4 after 30 days.* ***a:*** *Shoot length; b: Shoot weight; c: Root length; d: Root weight; Error bars represent standard deviation (n = 15). Data accompanied with different alphabets differ significantly (Tukey test, P< 0.05). Coloured star symbol denotes significance among treated and untreated plant parameters.*

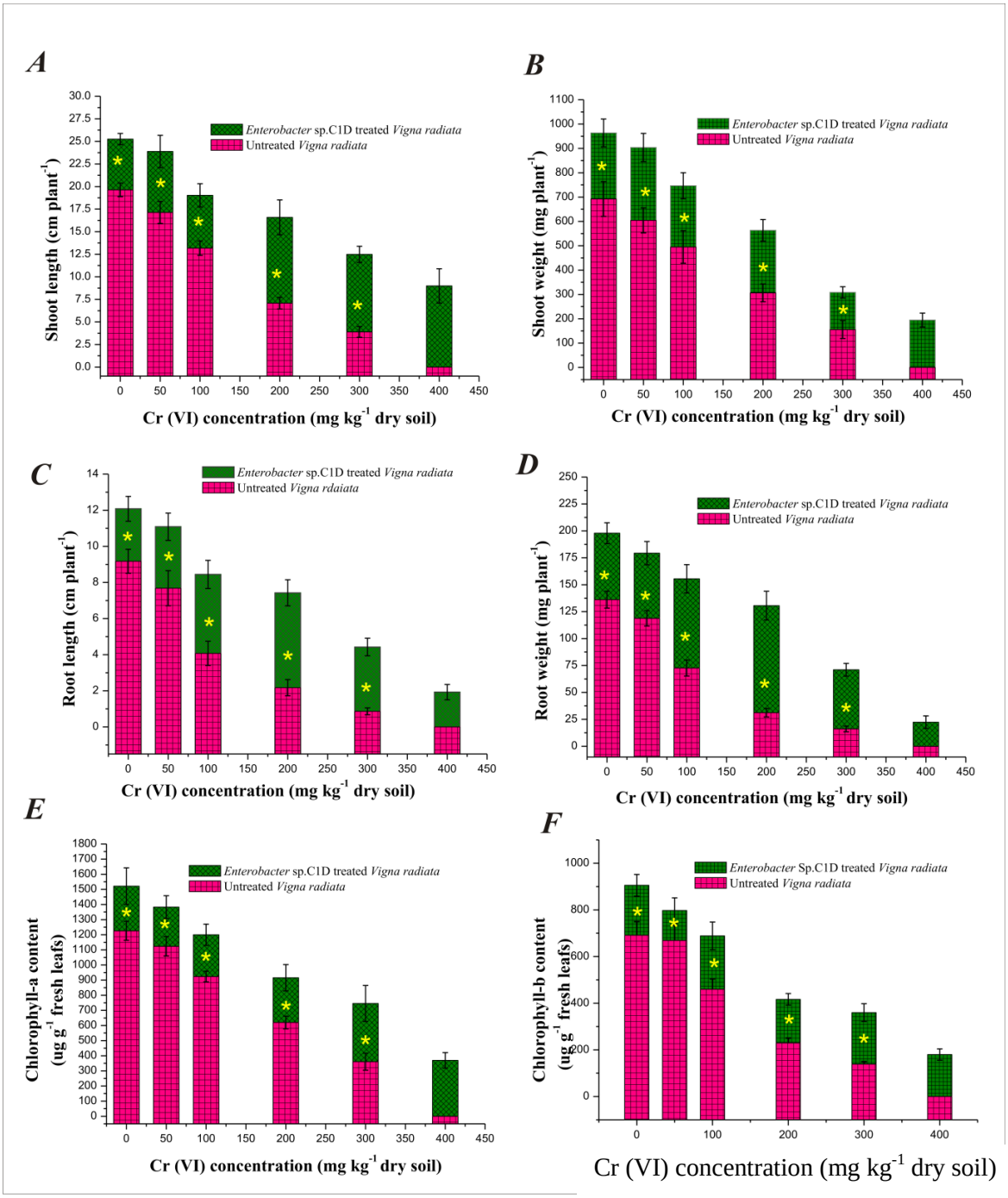


Table 6.4: *Percent (%) increase of plant growth parameters by Enterobacter sp. C1D at different Cr (VI) treatments. The differences in the mean values (n = 15) among the treatment groups are compared by pair wise multiple comparison procedure by Tukey test. An asterisk (*) denotes a value significantly greater than the corresponding control value (P < 0.05). Standard deviations are shown in the parenthesis. NA indicates not applicable.*

Growth parameters of *Vigna radiata* var GM4	Cr (VI) concentrations (mg kg^{-1} dry soil)					
	0	50	100	200	300	400
Shoot length	22.7*(±2.9)	28.2*(±5.1)	30.7*(±4.1)	57.2*(±3.9)	68.8*(±4.7)	NA
Shoot weight	28.1* (±7.4)	33.1*(±5.6)	31.8* (±9.2)	45.5*(±6.5)	50.7*(±10.4)	NA
Root length	24*(±5.4)	35.2*(±19.5)	51.7*(±7.8)	74.2*(±5.3)	80.4*(±4.3)	NA
Root weight	31.1* (±3.9)	33.7*(±4)	53.2*(±4.7)	76.1* (±3)	77.3*(±3.6)	NA
Chlorophyll-a	19.3*(±4.1)	18.7*(±4.6)	23*(±2.9)	32.3*(±4.6)	51.6*(±7.5)	NA
Chlorophyll-b	23.6*(±6.4)	16.8*(±8.3)	33.2*(±6.4)	49.7*(±5)	61.4*(±2.9)	NA

Table 6.5: *Effective concentrations (EC 50) of Pb (II) for plant growth parameters of un-inoculated and Enterobacter sp. C1D inoculated (V. radiata var GM4 after 30 days of planting. Plant growth parameters of non-inoculated and inoculated V. radiata var GM4 at Cr (VI) 0 mg kg^{-1} soil were considered as 100 %.*

Treatment	***Vigna radiata* var GM4 plant parameters**					
	Shoot length	**Shoot weight**	**Root length**	**Root weight**	**Chlorophyll-a**	**Chlorophyll-b**
Untreated	**651.3**	**519.7**	**655.4**	**362.4**	**409.8**	**342.54**
Vigna radiata	**R= 0.99**	**R = 0.94**	**R = 0.99**	**R = 0.99**	**R = 0.99**	**R = 0.99**
	***P* <0.0001**	***P* < 0.0002**	***P*< 0.0001**	***P*< 0.0001**	***P* < 0.0001**	***P* < 0.0001**
Enterobacter	**1118.7**	**4457***	**925.9**	**476.19**	**570.1**	**510.3**
C1D treated	**R = 0.95**	**R = 0.85**	**R = 0.99**	**R = 0.97**	**R = 0.99**	**R = 0.99**
Vigna radiata	***P* < 0.0001**	***P* < 0.15**	***P* < 0.0001**	***P* < 0.0001**	***P* < 0.0001**	***P* < 0.0001**

*: not significant by ANOVA

Fig. 6.7: *Effective concentrations (EC 50) of Cr (VI) for plant growth parameters of uninoculated (open symbols) and Enterobacter sp. C1D inoculated (closed symbols) V. radiata var GM4 after 30 days of planting. a: Shoot length; b: Shoot weight; c: Root length; d: Root weight; e: Chlorophyll-a and f: Chlorophyll-b. Plant growth parameters of non-inoculated and inoculated V. radiata var GM4 at Cr (VI) 0 mg kg^{-1} soil were considered as 100 % and represented as filled box. Error bars are ± standard deviation (n = 15)*

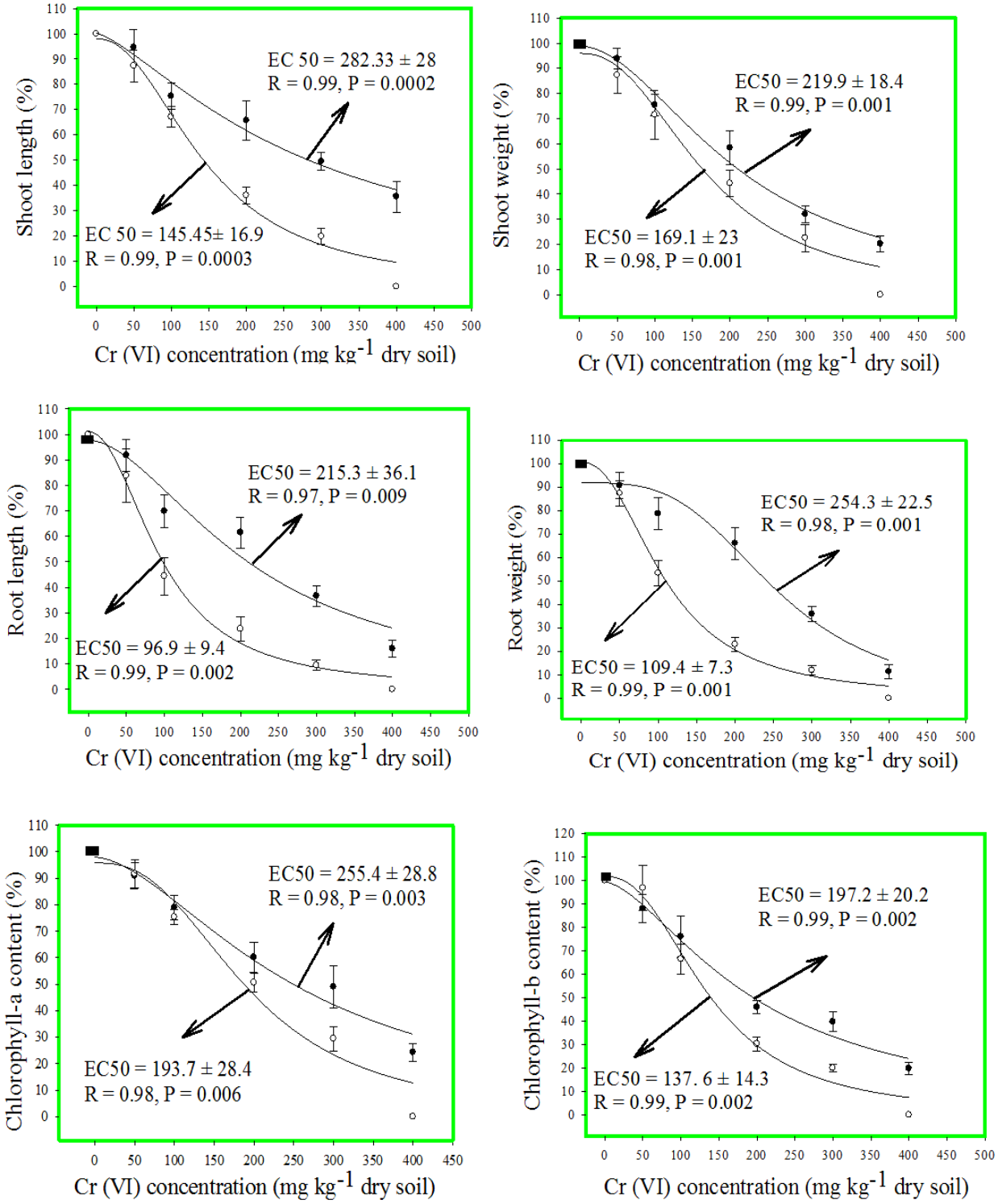


Fig. 6.8: *Correspondence analysis (CA) biplots for different treatments (filled circles) and growth parameters including Cr concentration (filled triangles) of un-inoculated* ***(a)*** *and Enterobacter sp. C1D inoculated (b) V. radiata var GM4 after 30 days of planting. Cr (VI) concentrations at particular treatment are also shown in parenthesis. SL: shoot length; SW: shoot weight; RL: root length; RW: root weight; CHA: chlorophyll-a and CHB: chlorophyll-b.*

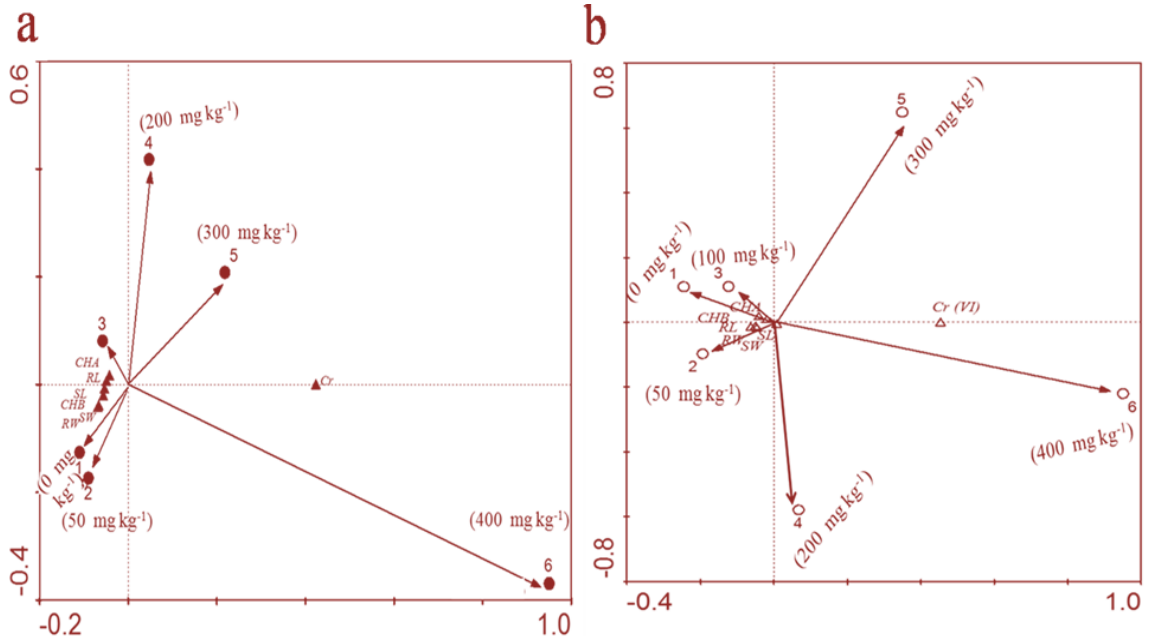
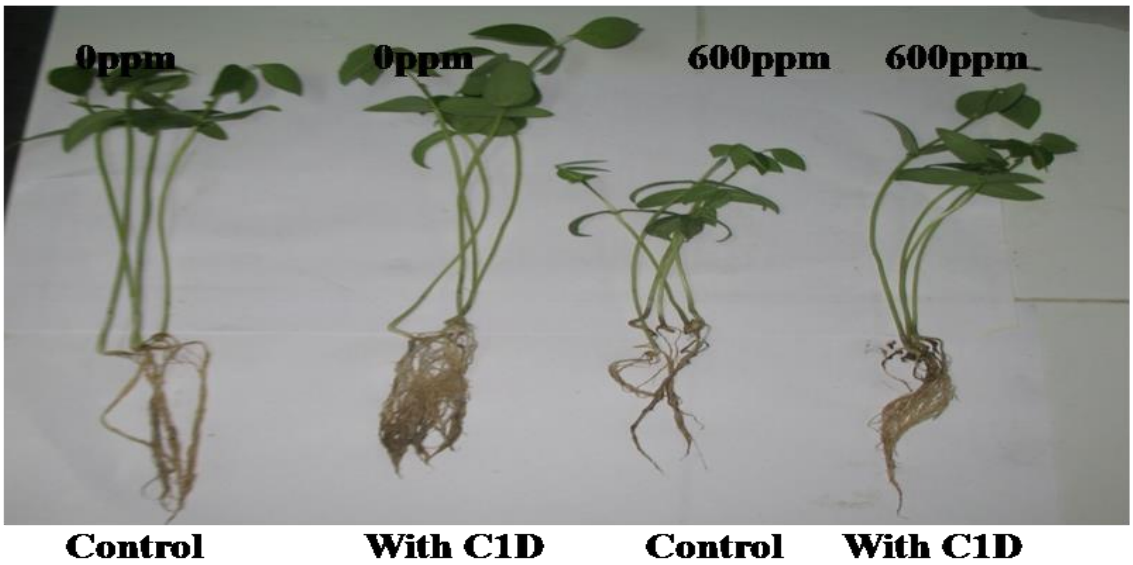


Fig. 6.9*: Effect of Enterobacter sp. C1D on above and below ground biomass of Vigna radiata GM4 in Pb (II) amended soils after 30 datys of uincubation****.*** *A: shoots B: roots; C: close up of roots (including lateral roots) at 0 mg/kg Pb(1I).*

A



B


Control
With *Enterobacter* sp. C1D
N 50 100 150 200 250
0 N 50 100 150 200 250
Control
With *Enterobacter* sp. C1D
300 350 400 450 500 600
300 350 400 450 500 600

C

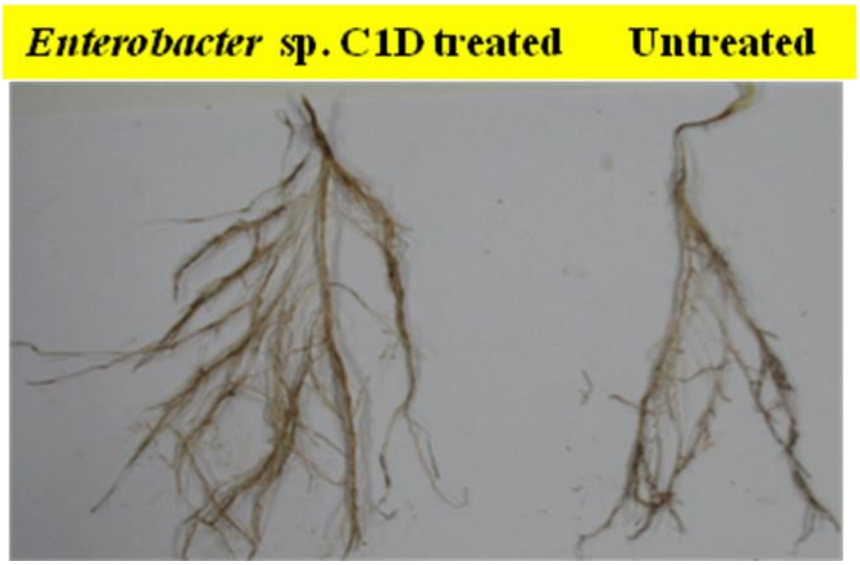

Enterobacter sp. C1D treated
Untreated

Fig 6.10: *Plant growth parameters of Enterobacter sp. C1D non-inoculated and inoculated Vigna radiata var GM4 after 30 days. a: Shoot length; b: Shoot weight; c: Root length; d: Root weight; Error bars represent standard deviation (n = 15). Data accompanied with different alphabets differ significantly (Tukey test, P< 0.05). Colored star symbol denotes significance among treated and untreated plant parameters.*

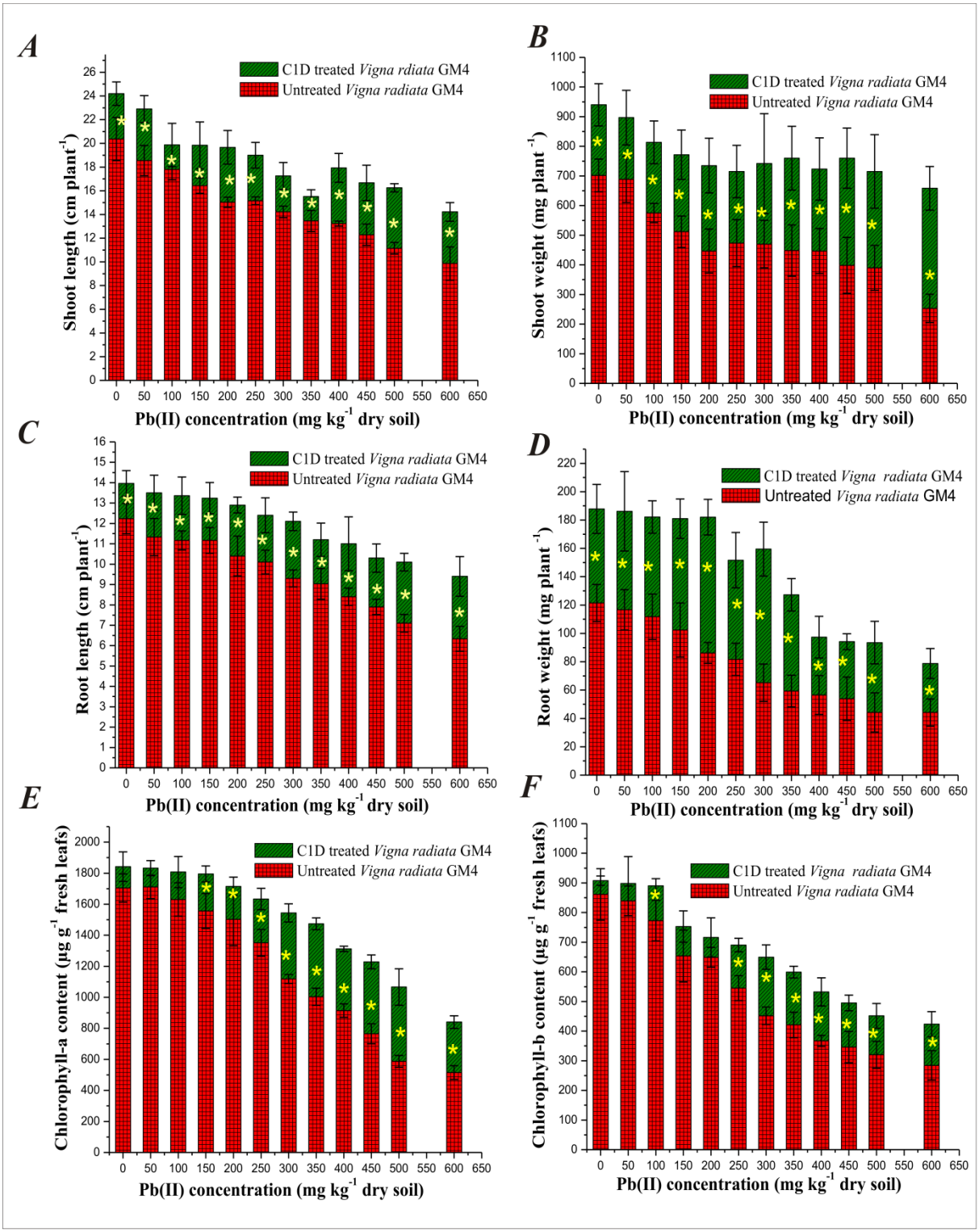
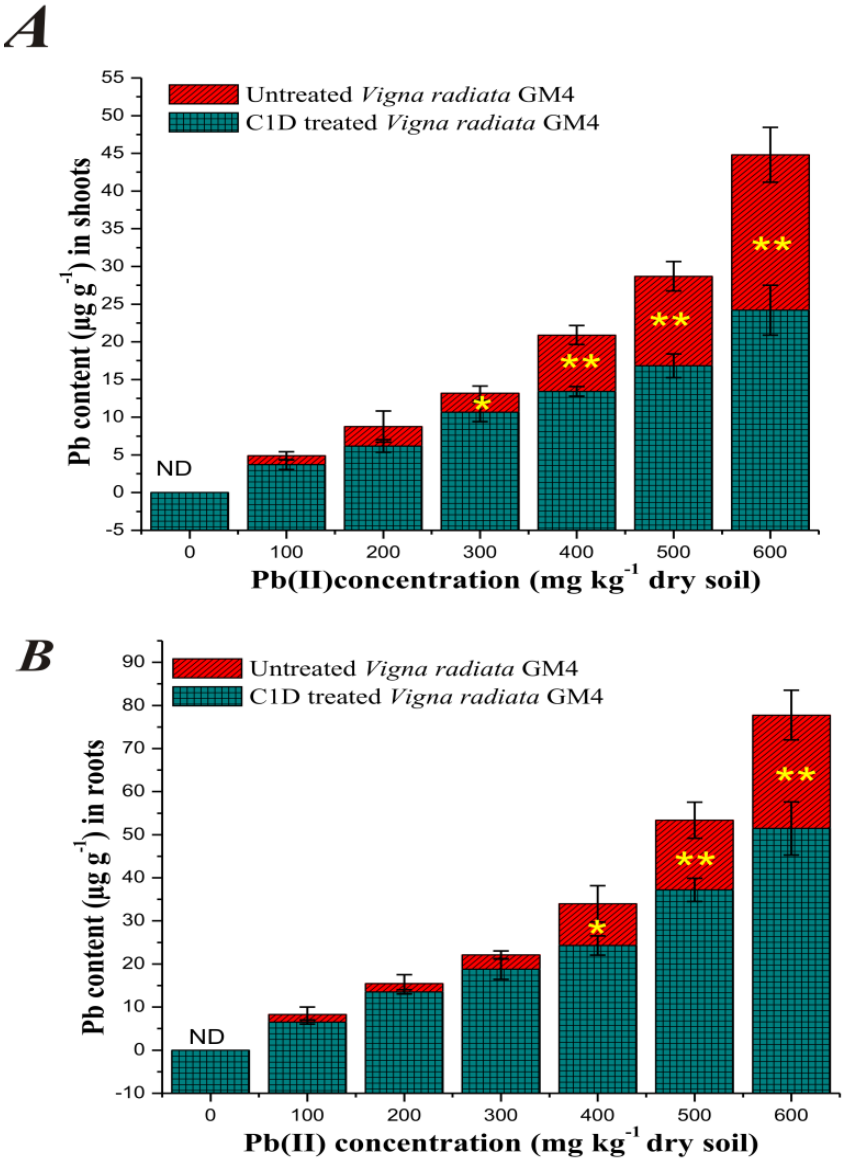


Fig. 6.11: *Lead (Pb) concentrations in Vignaradiata tissues Error bars represent standard deviation of three independent replicates (n = 3).). Coloured * denotes significance at P< 0.05(Tukey test); Coloured ** denotes significance at P< 0.001(Tukey test).*



6.4. Discussion

Chromium (Cr) and lead (Pb) are widespread soil pollutants. At elevated levels, both these metals are extremely toxic to most plants, impairing their metabolism and reducing plant growth. In the recent past, multimetal resistant plant growth promoting bacteria (PGPB) have emerged as promising candidates for both sustainable agriculture and phytoremediation in metal tainted environments (Khan et al., 2009; Kumar et al., 2009; Ma et al., 2009; Li et al., 2011; Zaefariani et al., 2012). In the

present investigation such a novel metal resistant bacterium has been isolated, characterized and established for its plant growth promoting potential. Heavy metal resistant isolates were obtained from a site that was remarkably contaminated with heavy metals such as $Pb^{2+,}$ Cd^{2+}, Cu^{2+}, Co^{2+} , Zn^{2+} and Cr^{6+}. It was thought that the bacteria and fungi isolated from polluted environments are often tolerant to higher levels of metals than those obtained from unpolluted areas and that tolerant microorganisms were reported at high frequencies in polluted habitats (Jansen et al., 1994; Huysman et al., 1994), because indigenous bacteria of contaminated areas have acquired natural resistance against heavy metals persisted in their environment (Whiting et al., 2001).

Among all the bacteria tested, isolate C1D showed high MIC values for a range of metals of which the value for Cr (VI) for isolate C1D was found to be relatively high (400 μg ml^{-1}, Table 6.2). Isolate C1D was found to be gram negative rod shaped bacterium and identified as *Enterobacter* sp., a genus which has attracted recent attention as an important group of PGPB (Jha et al., 2011; Taghavi et al., 2010). Previous reports from this laboratory have demonstrated the importance of *E. asburiae* strain PSI3 for improving the P availability to plants (Gyaneshwar et al., 1999, Sharma et al., 2006) as well as alleviation of Cd toxicity to plants (Kavita et al., 2008). However, *Enterobacter* sp. C1D is considerably more metal tolerant than the strain PSI3 and to the best of our knowledge, the first report of multimetal tolerant, multifaceted member of this group which showing a number of important plant growth promoting traits

Cr (VI) resistant PGPB, both gram negative and gram positive, have been reported (Rajkumar et al., 2005; Faisal and Hasnain, 2006; Wani et al., 2007, 2008; Chatterjee et al., 2009; Kumar et al., 2009) and the MIC value for Cr of the present bacterium is comparable to those reported. For example MIC values of Cr (VI) for *Pseudomonas* sp was found to be 450 μg ml^{-1} (Rajkumar et al., 2005) and for *Bacillus* sp. 400 μg ml^{-1} was found (Wani et al., 2007). Chromate resistance is probably based on an interaction of chromate reduction and chromate efflux (Nies, 1999; Peitzsch et al., 1998). Chromate reducing PGPB was first reported by Rajkumar et al., 2005. The present bacterium seems to also possess the ability of chromate

reduction in LB medium since after 72 h, visual observation of the colour change of the broth was observed.

Along with high metal tolerance, *Enterobacter* sp. C1D also showed remarkable plant growth promoting traits. It has been well documented that the biosynthesis of IAA along with its excretion into soil makes a major contribution to the bacterial plant growth-promoting effect (Glick, 2012). Plant exposure to heavy metals can induce the production of stress ethylene resulting in detrimental effects such as premature plant senescence (Glick et al., 1998 and 2012). In contrast, lowering of plant ethylene levels can decrease the harmful effects of many stresses. Prominent among the mechanisms used by many plant growth–promoting bacteria to facilitate plant growth is the decrease of ethylene levels by the enzyme ACC deaminase (Glick et al., 1999 and 2012). Burd et al. (2000) reported on the potential use of the ACC deaminase-producing bacterium *Kluyvera ascorbata* SUD165 to protect canola (*Brassica napus*) and tomato (*Lycopersicon esculentum*) seeds from the heavy metal toxicity induced by high concentrations of nickel (Ni), lead (Pb) and zinc (Zn). Further studies have demonstrated that when exposed to elevated cadmium (Cd) contamination in soil, the growth of Indian mustard (*Brassica juncea*) was significantly enhanced by ACC deaminase-producing PGPR from contaminated soils including mainly *Pseudomonas sp.* (Belimov et al., 2001*)*. It was also found that metal tolerant PGPB promoted plant growth in heavy metal polluted environment by production of discrete IAA and ACC deaminase activities (Rajkumar et al., 2005; Chatterjee et al., 2009; Kumar et al., 2009; Li and Ramakrishna 2011). Similarly, in the present study, *Enterobacter* sp. C1D was characterized by good amount of IAA produced and sufficient amount of ACC-deaminase activity and these values are relatively higher than previously reported Cr (VI) resistant *Enterobacter aerogenes* (Kumar et al., 2009). Although ACC-deaminase activity of the present strain is relatively lower in presence of Cr (VI) than in metal free condition, this much activity is quite sufficient to reduce ethylene levels. It is been reported that approximately $\geq$ 20 nmol α-ketobutarate mg^{-1} h^{-1} is enough to stimulate plant growth by a PGPB (Penrose and Glick, 2003).

Additionally the strain C1D has the ability to dissolve mineral phosphates and it was corroborated with a decrease in the pH of the buffered medium, due to the production of significant amount of gluconic acid (61.13 mM). This organic acid is

important in solubilization of insoluble mineral phosphates (Gyaneshwar et al., 1999) as well as in assisting the hydrolysis of phytate that is complexed with Al^{+3}, Fe^{+2}, or Fe^{+3} (Patel et al., 2010) and has also been implicated in providing metal tolerance to plants (Kavita et al., 2009). Moreover, here we are reporting first time about phosphatase activities of Cr (VI) resistant PGPB, which would be an effective feature in mobilization of organic phosphorus. In general organic phosphate (Po) is a major component of soil P, making up 20-80% of the total phosphorous (P) in the surface layers of soil. To be available to plants, Po must first hydrolyzed by phosphatase enzymes to release free phosphate (Pi). Therefore it was inferred that soil microorganisms which can mobilize phosphorous (P) are critical to the soil health by providing nutrients to plants and subsequent plant growth promotion (Gyaneshwar et al., 2002; Patel et al., 2010).

Based on the relatively higher Cr (VI) and Pb (II) tolerance and the better plant growth promoting traits *Enterobacter* sp. C1D was selected for gnotobiotic plant studies. Apparent chromium and lead toxicities have been noticed on both above and below ground biomass of non- inoculated *Vigna radiata plants.* Particularly the root system was severely affected by elevated metal concentrations (Fig. 6.6, and Fig. 6.10) and this is in agreement with previous reports (Shanker et al., 2005; Chatterjee et al., 2009). In the present study, the effective concentrations (EC 50) of Cr (VI) for root length, root weight, were found to be 96.97 and 109.4 respectively. Decrease in root growth was found to be well documented effect due to heavy metals in trees and crops (Tang et al., 2001; Shanker et al., 2005). The order of metal toxicity to roots were Cd > Cr > Pb whereas root length was more significantly affected by Cr than by other heavy metals examined (Prasad et al., 2001). General response of decreased root growth due to Cr toxicity could be due to inhibition of root cell division and /or root elongation or to the extension of cell cycle in the roots (Shanker et al., 2005).

It was found that chlorophyll content of *Vigna radiata* GM4 was decreased by Pb (II) toxicity (Fig. 6.10). Plants exposed to Pb^{2+} ions show a decline in photosynthetic rate which results from distorted chloroplast ultra structure, restrained synthesis of chlorophyll, plastoquinone and carotenoids (Sharma and Dubey, 2005). Seeds treated with *Enterobacter* sp. C1D was significantly protected from Pb (II) toxicity. About

3.5 times more root biomass was achieved with *Enterobacter* sp. C1D at 600 ppm Pb^{2+} concentration.

A strong positive correlation between plant growths parameters measured was observed on CA biplots (Fig. 6.8A). On the contrary negative effect of Cr (VI) was clearly noticed and its distance from growth parameters is significant. Moreover, *Vigna radiata* showed its natural tolerance against Cr (VI) up to 50 mg kg^{-1}. This could be clearly seen on CA biplots where the first two treatments (0 and 50 mg kg^{-1}) are highly similar. The distance (Chi-square) between the treatments in the CA biplot generally approximates the dissimilarity of their effect on plant and the first two treatments were closely placed to each other and their degree of direction also similar. On the other hand, in bacteria inoculated treatments, the plant tolerance against Cr was significantly improved as a result the first three treatments (0, 50 and 100 mg kg^{-1}) did not differ much and were clustered together (Fig. 6.8B). This was further agreement with previous EC 50 results (Fig. 6.7). Inoculation with *Enterobacter* sp. C1D resulted in greater root and shoot biomass of *Vigna radiata* GM4 and the results were much more prominent in treatments under Cr (VI) induced stress (Table 6.4). In almost all growth parameters, inoculated plants showed much better growth as compared to non-inoculated control. Moreover Cr (VI) and Pb (II) tolerances were significantly improved in inoculated plants (Fig. 6.7 and Table 6.5).

It is known that that metal resistant PGPB can affect plant growth either indirectly by ameliorating deleterious effects posed by toxic metals or directly by producing some growth promoting substances (Glick, 2003; Rajkumar et al., 2005; Wani et al., 2007 and 2008; Chatterjee et al., 2009; Khan et al., 2009). Plant growth promoting traits of the bacteria can be affected in presence of heavy metals (Wani et al., 2007; Li et al., 2011). The strain C1D reported here showed significant amount growth promoting traits in the absence of metal (Cr and Pb); however, in the presence of 100 mg kg^{-1} metal (Pb and Cr), siderophore production and mineral phosphate solubilisation were affected (Table 6.6). Wani et al. (2007) also found that phosphate solubilisation of *Bacillus* sp. decreased with an increase in Cr concentration. Li et al. (2011) showed that phosphate solubilization of *Pseudomonas* sp. was inhibited by mercury. The repression of phosphate solubilisation in presence of metals might be due to increased toxicity as a result of interaction between metal and inorganic

phosphate (Moberly et al., 2010). Alleviation of Cd toxicity to plants was attributed to chelation of the toxic metal ions by organic acids secreted by the bacterium (Kavita et al., 2008). In case of *Enterobacter* C1D, organic acid production and ability to bring about drop in pH were inhibited in the presence of Cr (VI), negating the existence of this mechanism. However, in presence of Pb, the organism was able to produce gluconic acid.

In *Enterobacter* C1D, siderophore production was adversely affected by the presence of Cr (VI) and Pb (II). Similarly, Zn and Pb have been shown to reduce the production of siderophores by *Pseudomonas* sp. (Li et al., 2011) and nickel stress caused reduction in siderophore production by other PGPB (Tank and Saraf, 2009). However, the exact mechanism of Cr, Zn and Pb in inhibition of siderophore production is not yet clear. When compared to control (no metal) Pb^{2+} enhanced both acidic (55 times) and alkaline (2 times) periplasmic phosphatases. Large amount acidic periplasmic phosphatases by *Enterobacter* sp. C1D may be attributed to Pb^{2+} resistance. Similar type ofobservations were made by Aickin and Dean, 1998. It has been previously reported that strains of *Citrobacter freundii* and of *Staphylococcus aureus* accumulated lead as Pb-phosphate when grown on media supplemented with high levels of lead salts. They postulated that the mechanism underlying Pb uptake involved hydrolysis of organic phosphate by an acid phosphatase followed by precipitation of $PbHPO_4$ on the cell surface.

Although it has been shown that IAA production can be adversely affected by metal stress (Li et al., 2011), in *Enterobacter* sp. C1D, it was not so. Therefore the plant growth promotion by present strain under chromium stress could be most likely due to the production of IAA and due to excellent ACC-deaminase activity in the presence of the metal. In addition, the possibility of directly reducing the metal toxicity, by conversion of Cr (VI) to the less toxic Cr (III), by the bacterium cannot be ruled out. The genome of plant associated *Enterobacter* sp. 638 shows the presence of genes involved in metal resistance and putative chromate reductase gene (Taghavi et al., 2010).

Based on the results obtained from present study, it is evident that the bacterial strain *Enterobacter* sp. C1D is a good candidate for plant growth promotion under elevated metal stress. Searching novel metal resistant plant growth promoting

bacteria has significance in the management of environmental and concurrent agricultural problems in polluted terrestrial systems.

6.5. Conclusions

The bacterial strain *Enterobacter* sp. C1D exerted substantial growth promoting effects on the *Vigna radiata* var GM4 plants in Cr (VI) stress, Pb (II) stress and in unstressed conditions. Plant tolerance towards both Cr (VI) and Pb (II) has been significantly increased by *Enterobacter* sp. C1D. High amount of acidic and alkaline periplasmic phosphatases, elevated IAA production and sufficient ACC deaminase activities probably enable *Enterobacter* sp. C1D to enhance plant growth in Cr (VI) and Pb (II) amended soils. Therefore, this strain could be utilized for sustainable agriculture in natural or chromium/ lead contaminated soils.

Table 6.6: *Comparison of PGPR traits of Enterobacter sp C1D in presence of metal free and metal spiked conditions. Values are mean of three independent replicates (n = 3). Values in the rows followed by the same letter do not differ significantly according to Fisher's LSD at p<0.05.*

Isolate	Siderop-hore Productio n	[Δ]Mineral phosphate solubilization	[•]IAA producti-on	[¥]ACC Deaminas e activity	*pH drop	Organic acid	[╬]Phosphatase activity					
							E.P		I.P		P.P	
							A	B	A	B	A	B
Enterobacter sp. C1D (without metal	+++	3.5a	38.79 a	237.85a	5.2 a	[Ø]Gluconate	0.13a	7.04a	9.4a	0.9a	33.9a	ND
Enterobacter sp. C1D (with 100 ppm $Cr^{6+)}$	+	0.25b	**41.6b**	**198.8b**	7.8b	UD	0.78a	2.23b	3.55b	1.55b	**32.9a**	**36.16a**
Enterobacter sp. C1D (with 100 ppm $Pb_2^{+)}$	-	1.18c	**38.94a**	**416.93c**	6.4c	[Ω]Gluconate	UD	UD	6.4c	9.54c	**55.72b**	**67.43b**

[Δ] Minral phosphate solubilisation was measure by SPI index CS+CZ/CS; [•]Unit for IAA production, µg/ml^{-1} ; [¥] Unit for ACC deminase acticity, nmol α-Ketobutyrate mg^{-1} protein h^{-1}; ***** pH drop after 6days in minimal media amended with rock phosphate; [Ø] 62.13 mM gluconate; [Ω] 15.3 mM gluconate; [╬] Unit for phosphataseactivity, µg P-nitrophenol mg^{-1} min^{-1} ; E.P – Extracellular phosphataseactivity; I.P- Intracellular phosphataseactivity; P.P- Periplasmic phosphataseactivity; A-Alkaline phosphatase; B- Acidic phosphatase. UD: Undetectable; NA: Not applicable.

CHAPTER 7

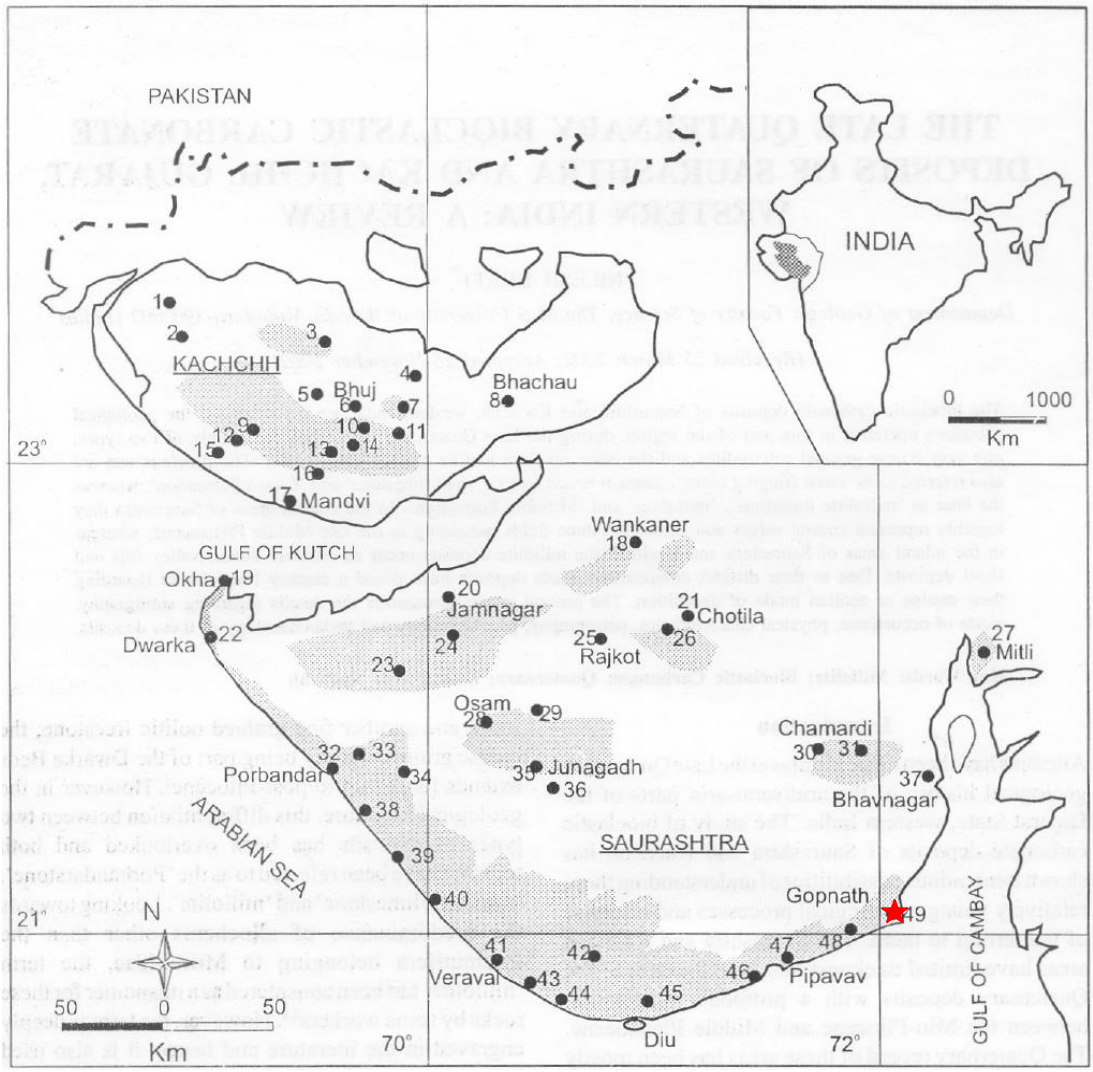
Bacteriology of miliolite, a bioclastic lime stone: Bacterial diversity, activity and potential role of bacteria in weathering of miliolite

7.1. Introduction

Biogeochemistry of carbonate minerals is considered to be vital in global carbon cycling, alkalinity generation, and the cycling of major and trace elements among the oceans, continents, and in the atmosphere (Ehrlich 1998; Warren et al., 2001). Microbially mediated weathering of carbonate rocks forms an important link within the exogenic biogeochemical cycle of elements in the karst environment (Liu et al., 2000; Viles et al., 2003; Zhu et al., 2004; Garcia-Pichel , 2006; Lian et al., 2008). The mechanisms of microbial carbonate weathering include acidolysis, alkaline hydrolysis, complexation, secretion of extracellular polysaccharides and the involvement of enzymes (Ehrlich, 1996; Huang et al., 2006; Li et al., 2007). It has been shown that bacteria are able to differentially induce the precipitation or dissolution of carbonates depending on the accessibility of nutrients for their growth (Portillo et al., 2008 and 2009). Studies show that microbial weathering of rocks is not a simple process of metabolism; instead, it is a process in which microorganisms need to extract one or more limited nutrients from special minerals (Bennett et al., 2001; Lian et al., 2008; Herrera et al., 2008). Therefore, the microbial weathering rate of carbonate rocks depends on trace nutrition components and microbial ecological factors, including their community structure. Cultivation-independent studies of the bacterial community structure of carbonated caves and crusts have been reported (Zimmermann et al., 2005; Barton et al., 2006; Tang et al., 2012). These reports indicate that cyanobacteria, proteobacteria, actinobacteria and firmicutes represent the abundant bacteria in carbonated systems; however, several questions remain to be addressed, such as the community structure of carbonate-dissolving bacterial sub-populations, which are crucial for the weathering of carbonates, and the functional role of these bacteria in carbonated environments.

Quaternary carbonate deposits known as miliolites or miliolitic limestone, also referred to as calcarenite (a type of limestone), are deposited in the Saurashtra and Kachchh provinces of Gujarat and appear as patchy outcrop inlands up to 180 km from the miliolite-bearing coast. Fig. 7.1 represents their geographical distribution and their relevant information is shown in Table. 7.1.

***Fig. 7.1**: Geographical map showing distribution of bioclastic carbonate deposits in western Gujarat (adapted from Bhatt, 2003). Sampling site for the present study Gopnath is also shown with red colour star mark.*



This limestone derived its name 'miliolite' from the foraminifer genus "*Miliolinae*", which was found abundantly in these deposits (Bhatt, 2003). Geomorphological, chronological and geochemical studies of these deposits are well established (Bhatt, 2003, Bhatt and Bhonde, 2006). The results show that miliolite deposits predominantly consist of calcite (calcium carbonate [$CaCO_3$], mean 86.8 %) with aragonite and quartz as minor components. Miliolite clays show the dominance of illite, which is principally generated by the weathering of granite and is brought by the Indus River.

Table 7.1: *Relevant details of carbonate deposits of Kachchh and Saurashtra shown in above Fig. 7.1 (M-miliolite, SL- shell limestone, OD- obstacle deposites, VF- valley fills, SH- sheets, CR- costal ridge and CD- costal dunes (adapted from Bhatt. 2003).*

Loc. No.	Location	Type of deposit/Elevation in meters/Mode of occurrence
1	Lakhapat	M/120/OD
2	Kotda	M/80-100/OD,VF
3	Jhura Hill	M/150-230/VF,SH,OD
4	Habo Hill	M/180-200/OD, SH, VF
5	Sumrasar	M/200-280/OD, SH
6	Bhuj (SE of)	M/250/OD, SH
7	Lakhand	M/180/VF, SH
8	Bhachau (SW of)	M/30/SH, CD
9	Roha	M/200-250/OD,SH
10	Jadura Mota	M/200-220/SH,OD
11	Khatrod Hill	M/280-300/OD,VF
12	Kotda (Roha)	M/150-180/OD,SH
13	Godpar	M/180-200/SH
14	Sanatorium	M/240/OD,SH
15	Boha	M/80-100/OD,VF,SH
16	Asambia nana	M/20-25/SH
17	Mandavi (E of)	M/10-15/SH,CD
18	Wankaner	M/100/SH
19	Okha	SL/0-4/SH
20	Jamnagar (S of)	M/40-60/VF,SH
21	Chotila	M/200-250/OD,SH
22	Dwarka	SL/0-6/SH,CR
23	Gop Hill	M/100-120/OD
24	Raka-Khatia	M/60-80/VF,OD,SH
25	Rajkot	M/130/SH
26	Bamanbor	M/200/VF,SH
27	Mitli	M/4-6/CD
28	Osam	M/100-150/OD,SH
29	Jetpur	M/60-80/SH
30	Sanosara	M/40-60/OD
31	Chamardi	M/40/OD,SH
32	Porbandar	SL,M/0-4/SH,CR
33	Ranavav	M/60-100/OD,SH
34	Dungarpur	M/60-80/OD
35	Junagadh	M/100-120/OD,SH
36	Gir-Gadhada	M/80-100/OD,VF
37	Bhavnagar (NW of)	M/40-50/VF,SH
38	Navibandar	SL,M/0-12/SH,CR
39	Madhavpur	SL,M/0-10/CR
40	Mangrol	SL,M/0-18/CR,SH
41	Veraval	SL,M/0-6/SH,CR
42	Ghantwad	M/40-60/VF,SH
43	Kadwar	SL,M/0-6/SH,CR
44	Sutrapada	SL,M/0-15/CR,CD
45	Una	M/12-20/CD,SH
46	Jafrabad	M/0-20/CR,CD
47	Pipavav	M/0-5/CR
48	Mahuva	M/0-10/CD
49	Gopnath	M/0-12/CD

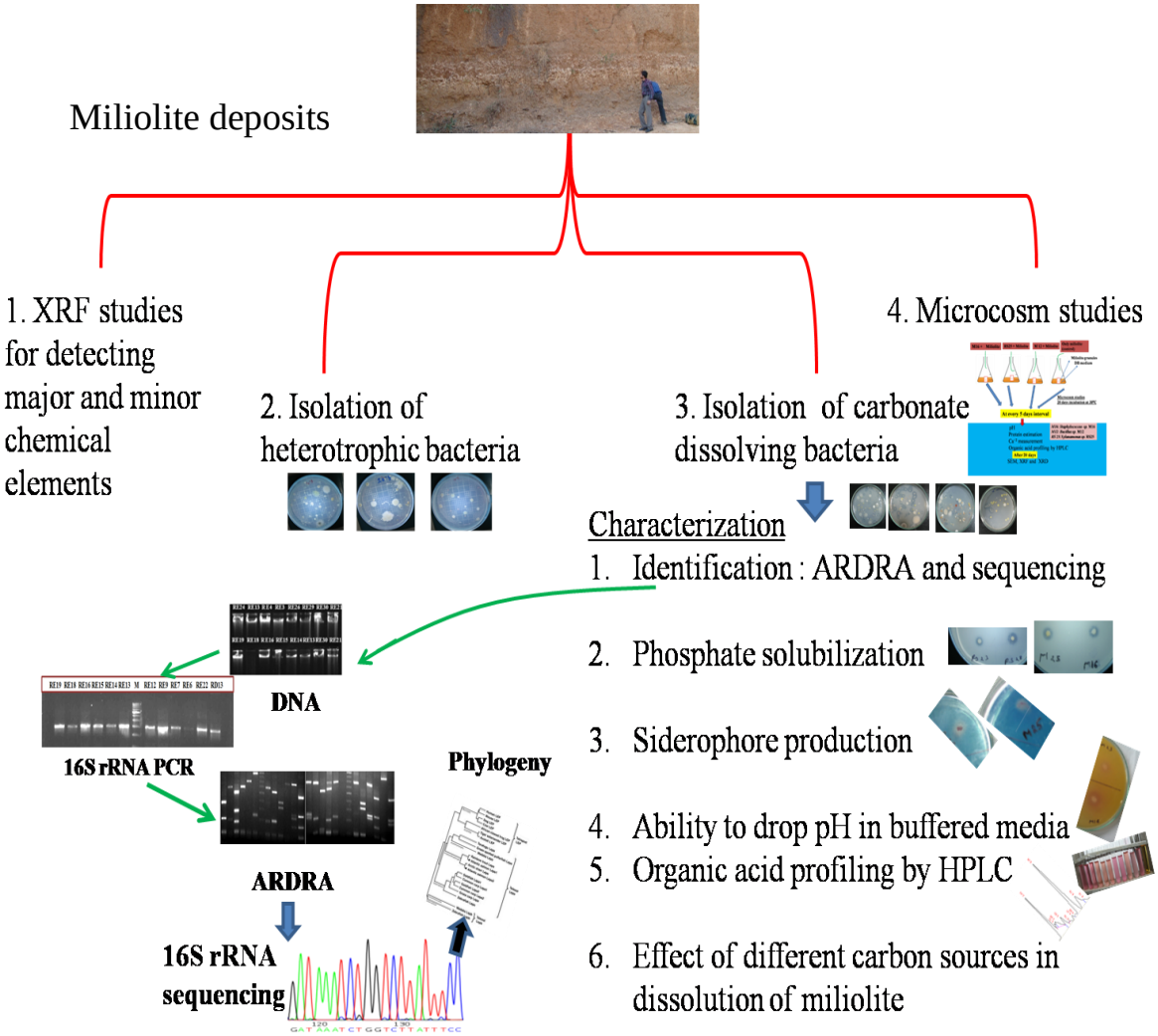
Upon weathering, miliolite forms red soil sequences, which are important landscapes in the regions of Saurashtra and Kachchh. Bacterial role in weathering of miliolite, a type of carbonated rock, is not well-studied. Therefore, the present investigation was aimed at cultural approach to study the carbonate-dissolving bacterial population from a carbonated bioclastic limestone "Miliolite". The relative abundance, distribution and carbonate-dissolving capability of the isolates are presented on the laboratory scale.

Further, the mechanisms by which the bacteria dissolve carbonated rocks is studied to understand the role of bacterial activity in miliolite weathering.

7.2. Materials and Methods

A polyphasic approach involving microbial, biochemical and molecular tools was undertaken to address the above objective (Fig. 7.2).

Fig. 7.2: *Generalized flowchart summarizing the methods employed in this study. Experiments were conducted with three independent replicates samples (n = 3).*



7.2.1. Sample collection

Miliolite and surface red soil sequences studied were collected from the miliolite-bearing natural section in Gopnath (21°12'27.21"N, 72° 6'30.08"E), Saurashtra, Gujarat, India (Fig. 7.3). The site was selectively chosen here as intensive weathering is taking place. Moreover, distinct layers of miliolite bed rock and surface weathered

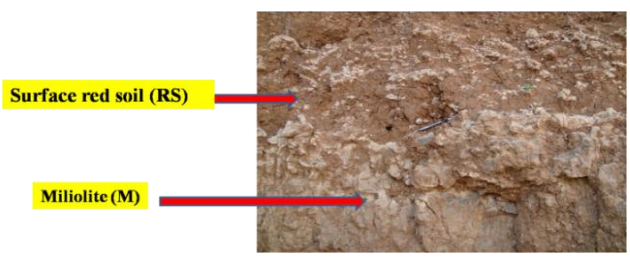
red soils are exposed at this particular site. The miliolite-bearing section is pristine in nature and no anthropogenic or animal activities have been noted. Miliolite rocks in the region are weathered and porous. The area is mostly covered with a red soil sequence; however, in many places bare limestone escarpments are present.

Triplicate composite miliolitic limestone samples up to a depth of 5 cm were collected using a sterile rock chisel and hammer and were placed in sterile plastic bags in an ice box. Prior to the collection of soil samples, the sites were cleaned by scraping the surface layer up to 1 cm. Triplicate composite red soil samples were collected in sterile plastic bags and immediately placed on ice/coolant packs for transport. Samples retrieved were denoted M for miliolite rocks and RS for red soil sequences. The soil samples were sieved (<4 mm), cleaned of visible roots and plant residues, and stored at 4°C.

Fig. 7.3: *Overview of the miliolite section near Gopnath, Saurashtra, Gujarat (A); Miliolite in the embedded section is shown with red colour arrow.Pure miliolite rock and surface red soils are shown in inset. Close-up of miliolite and its weathered surface red soils are also shown (B).*

A

B



7.2.2. Sample chemical analysis

Air dried and sieved red soil and miliolite samples (10 g each) were powdered by mortar and pestle and the fine powder was taken in a plastic beaker containing 25 ml 0.01 M calcium chloride and stirred for 1 min. The pH was measured in the supernatant after 1 h of standing with a digital pH meter. Major and minor chemical elements of the miliolite were estimated by an X-ray fluorescence (XRF) spectrometer (Tripati et al., 2010) at the Department of Geology, University of Pune, India

7.2.3. Isolation and enumeration of viable heterotrophic bacteria and carbonate dissolving bacteria

Total viable heterotrophic bacteria (THB) and total carbonate-dissolving bacteria (TCB) were enumerated using R2A medium (Edenborn and Sexstone, 2007) and Devenze and Bruni (DB) medium (Cacchio et al., 2004), respectively. In brief, miliolite rock was powdered by a sterile mortar and pestle on a clean bench. Miliolite rock powder (10 g) was then homogenized in 90 mL of N-saline and serially suspended (10 fold) in the same. Aliquots (100 µL) were spread on three independent replicate plates of R2A and DB medium, the plates were incubated aerobically at 30°C for 7 d and the colonies counted at the end of incubation. Bacterial colonies showing a halo zone of clearance around them on DB medium were considered as positive for carbonate dissolution. Composition (g L^{-1}) of DB medium was as follows: Glucose 5.0, Yeast extract 1.0, Peptone 1.0, K_2HPO_4 0.4, $MgSO_4$ 0.01, NaCl 5.0, $(NH_4)_2SO_4$ 0.05, $CaCO_3$ 5.0, Agar 15.

The colonies were further purified on DB agar medium by several rounds of plating. Based on the colony size and morphology, 21 carbonate-dissolving bacterial isolates were selected for further analysis. These purified isolates were then preserved in 40% glycerol and kept at -20º C.

7.2.4. Characterization of carbonate dissolving bacteria

7.2.4.1. Miliolite dissolution by the isolates

Miliolite dissolution efficiency of the isolates was determined by measuring the ratio of the zone of clearance (CZ) and colony size (CS) on miliolite agar as well as on DB medium. Miliolite agar is a modified DB medium in which

$CaCO_3$ is replaced by miliolite powder. The constituents (g L^{-1}) of miliolite agar are as follows: glucose, 5 ; yeast extract, 1; peptone, 1; K_2HPO_4, 0.5; $MgSO_4$, 0.01, NaCl, 5; $NH_4(SO_4)_2$, 0.05; $MgCl_2$, 0.01; sterilized miliolite rock powder, 5 g and 1.5% agar. For inoculum preparation, bacterial isolates were grown in 3 ml R2A broth for 72 h, 1 ml culture was centrifuged at 12,000 x*g* for 5 min and washed three times with 1 ml sterile 0.85% NaCl. The bacterial pellet, resuspended in 1 ml 0.85% NaCl, was used as the inoculum for experiments. A spot of 5 µl inoculum (approximately 10^6 cells ml^{-1}) was placed on miliolite agar plates and incubated aerobically at 30°C for 72 h. Plate assays were replicated three times. The opacity of miliolite agar is due to miliolite rock powder and bacteria that dissolve the miliolite can be distinguished due to the apparent halo of a clear zone around the colony. After 72 h of incubation Cz/Cs ratio was measured.

7.2.4.2. Mineral phosphate solubilisation (MPS) and Siderophore production

Carbonate dissolving isolates were grown in 5 ml miliolite broth, overnight at 30ºC on a rotary shaker. These were then centrifuged, washed twice with 0.85% NaCl and resuspended in equal volume of 0.85% NaCl. About 10 µl of culture suspension (OD = 0.2 at 600 nm) of each of these isolates was spotted on Pikovskaya's (HiMedia Laboratories, Mumbai, India) and chrome azurol-S agar plates for MPS and siderophore production, respectively (Alexander and Zuberer 1991). Colonies forming a clear halo around them on Pikovskaya's agar were considered as MPS and P solubilisation index (PSI) was measured by a formula "CS+CZ/CS" where CS indicates colony size and CZ indicates Zone of clearance. For detection of siderophore, culture was spotted on the chrome azurol-S plate and the colonies developed were observed for yellowish orange halo around them. Preparation of chrome azurol-S (CAS) agar plates for siderophore detection is described in chapter 6.

7.2.4.3. Bacterial isolates ability to lower pH of buffered and unbuffered conditions

For plate assays, the medium used was methyl red containing miliolite agar to which various concentrations of Tris –base (pH 8.0) buffer were added to give rise to following combinations.

- ✓ Methyl red containing miliolite agar (unbuffered)

- ✓ Methyl red containing miliolite agar with50 mM Tris-base buffer (buffered)
- ✓ Methyl red containing miliolite agar with100 mM Tris-base buffer (buffered)

7.2.4.3.1. Preparation of methyl red (MR) plates

Methyl red stock was prepared by taking 150 mg of methyl red powder and dissolving it in 1 ml ethyl alcohol**.** From the stock, 0.2 ml L^{-1} was added separately to autoclaved miliolite agar containing 50 mM Tris-base buffer and without buffer under sterilized conditions. Saline washed culture suspension (20 µl) for each isolate were spotted and incubated at 30°C for 4 to 5 d. Colonies were observed for clear red colour zone around the colonies. CZ/CS ratio of each colony were measured.

For broth assays, the medium used was methyl red containing miliolite broth to which various concentrations of Tris-Cl (pH 8.0) buffer were added to give rise to following combinations.

- ✓ No Tris buffer + Methyl red containing miliolite broth (unbuffered)
- ✓ 50 mM Tris buffer + Methyl red containing miliolite broth (buffered))

7.2.4.3.2. Preparation miliolite broth (MB)

The constituents for MB are same as in miliolite agar except addition of agar. Tris-base was added to the broth to obtain final concentration to 50 mM and 100 mM .

7.2.4.3.3. Monitoring pH and cell growth

At regular time interval samples were collected and its pH was measured. Cell growth was monitored by estimating protein concentration using Follin Lawry method (Lowry et al, 1951). The experiment was repeated with miliolite broth without MR for HPLC analysis.

7.2.4.3.4. HPLC analysis for identification of organic acids

HPLC analysis was carried out to identify the nature and quantity of organic acid produced by selected isolates. Sample for HPLC was prepared by inoculating 0.5% culture in DB medium and were incubated on rotary shaker at 30ºC. Cell growth and pH was monitored at regular interval. Then 1.5 ml sample was collected,

centrifuged at 5000g for 10 min to remove cells. Supernatant was transferred to fresh autoclaved microfuge tubes and stored at -20°C for HPLC analysis. At the time of HPLC, these samples were thawed and filtered through 0.2 µm nylon filters and 20 µl of filtered samples were injected in the HPLC instrument (from Shimadzu equipped with C_{18} reverse phase silica column) with the help of Hamilton syringe. In this experiment, 5 mM H_2SO_4 was used as a mobile phase with a flow rate kept constant at 1.0 ml/min and oven temperature of 40^{o}C. To identify the peaks of our interest, standard organic acids like gluconate (0 to 80 mM), citrate (0 to 50 mM), acetate (0 to 100 mM), pyruvate (0 to 50 mM), α-lactoglutarate (0 to 50 mM), lactate (0 to 50 mM), oxalate (0 to 50 mM), succinate (0 to 50 mm) were injected and their retention time and area under the peaks compared with the samples. The data was processed with chromatography interface module and Winchrom Ver. 2.00.

7.2.4.4. Amplified rDNA Restriction Analysis (ARDRA) and 16S rRNA sequencing

Carbonate dissolving bacteria were grown on DB broth for 48 h and the genomic DNA was extracted using CTAB method as described (Chapter 2). Genomic DNA was amplified with the eubacterial 16Sr rRNA universal primers. Conditions for 16S rRNA PCR amplification as well as the protocol for the ARDRA were given in the previous Chapter 2. Based on the ARDRA profile, representative isolates were chosen for sequencing analysis. Out of the sequence, the best read from the chromatogram was taken and converted to FASTA format. With these sequences NCBI BLAST analysis as well as RDP homology search was carried out. Multiple sequences alignment was carried out in RDP by taking some standard database sequences. A phylogenetic tree was constructed for all the isolates using neighbour joining method in MEGA 5.0 (Tamura et al., 2011) programme.

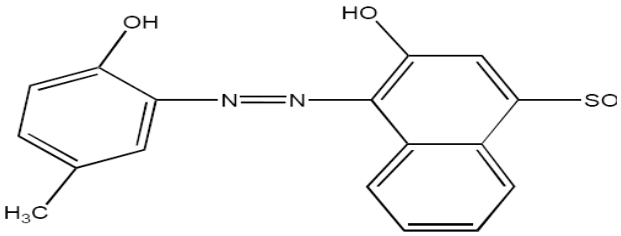
7.2.4.5. Effect of different carbon sources on dissolution of miliolite

Different carbon sources such as glucose, lactose, sucrose and glycerol were tested for their influence in release of Ca from miliolite granules. Two controls i.e. DB medium without carbon source, and only D/W (no medium) were established. Miliolite granules (size around 4 mm) were added to DB medium at a concentration of 5 g L^{-1}. Different carbon sources mentioned above were added in such a way that final molar concentration remains same as glucose (5g L^{-1}, i.e. 27 mM) in case of DB

medium. Each sugar containing broth was incubated at 30°C for 6 d. At regular time interval samples were collected and released of Ca^{+2} from the miliolite was checked by using Cal-red method (Harris, 2003).

7.2.4.5.1. Monitoring Ca^{+2} release

The calmagite indicator, often referred to as cal-red because it produces a wine red colour when bound to metal ion, has following chemical structure:-.



The structure pictured above is denoted H_2In- and is red in colour. Upon further deprotonation, the indicator will change from red to blue forming HIn^{2-} and then from blue to orange forming In^{3-}. The calgamite indicator will form a wine-red complex with metal ions. This is illustrated in the following reaction:

$$Ca^{2+} + \underset{\text{(orange)}}{In^{3-}} \rightarrow \underset{\text{(wine red)}}{Ca\text{-}In^{-}}$$

To quantitatively determine the amount of calcium present, a standardized solution of EDTA is used as titrant. The EDTA binds calcium much strongly then does calgamite, allowing reaction to occur:

$$\underset{\text{(wine red)}}{Ca\text{-}In^{-}} + EDTA \rightarrow Ca\text{-}EDTA^{2-} + \underset{\text{(blue)}}{HIn^{2-}}$$

Titration of Ca-In-solution to a blue colour indicates that sufficient EDTA was added to bind all the Ca^{2+} and the titrant has reacted its end point. Description about the

Reagents preparation

Ammonia buffer was made by dissolving 17.5 g NH_4Cl in 142 ml Liq.NH_3 and 80 ml D/W and made the final volume to 250 ml using D/W. Cal-Red (calmagite) indicator stock solution (0.1 g /ml) was made by dissolving 0.125 g of cal-red indicator in 40 ml of distilled water and made the final volume to 50 ml in a volumetric flask with distilled water. Calcium standard, (0.1 g/L) was made by taking 0.02497 g of $CaCO_3$, few dropes of concentrated hydrochloric acid was added until it completely dissolved (until no CO_2

evolution observed, usually 4-5 drops of concentrated HCl). After that it was diluted to 100 ml in a volumetric flask with D/W.

Procedure

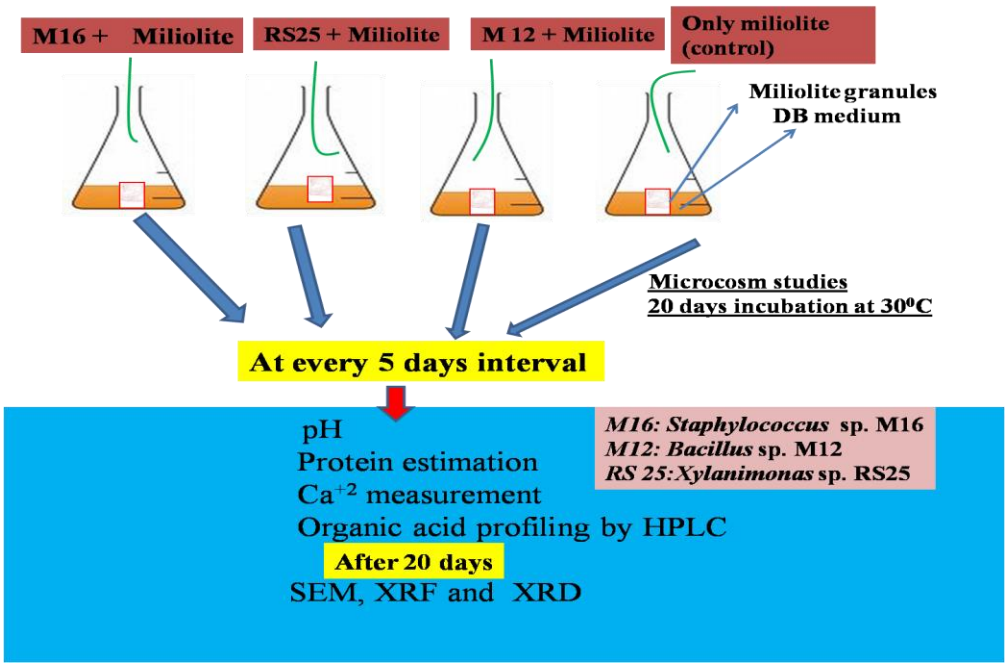
In five test tubes 1ml of calcium standard solution of was added. In sixth test tube add 1ml of unknown sample was taken, 4ml D/W was added to each test tube. 2 ml ammonia buffer and 0.25 ml of cal-red indicator solution was added (mixed well) to a test tube. Mixed properly, and titrated with 0.01EDTA until a wine-red to purple to blue transition observed. A persistent blue color indicates the end point. The concentration of calcium in broth was determined using the 0.01 EDTA concentrations as mentioned in below formula.

$$\text{Calcium (mg /ml):} \quad \frac{400 \text{ (EDTA volume)}}{\text{Volume of sample}}$$

7.2.5. Short term microcosm studies for elucidating bacterial potentiality in dissolution of miliolite

In order to explore the potential of the bacteria in dissolution of miliolite, short term microcosm experiments were conducted. The flowchart for the microcosm studies is shown in the following Fig. 7.4.

Fig. 7.4: *Generalized flowchart representing short term microcosm studies for elucidation of bacterial potentaility in dissolution of miliolite.*



7.2.5.1. Preparation of medium for microcosm studies

DB medium 150 ml contained glucose (for isolate RS25) and sucrose (for isolates M16 and RS25) were prepared in 250 ml Erlenmeyer culture flasks in triplicates. Sterilized miliolite granules 10 g were added to each flask. Cultures (M16, M12 and RS25) were inoculated in 2 ml DB broth and incubated for overnight. After several round of washing with normal saline, a OD of 0.3 was adjusted for all cultures. 0.5% of culture suspension was inoculated in respective medium and incubated at 30°C for 20 days. A control flask was made without inoculating bacteria (only medium constituents + miliolite granules).

7.2.5.2. Preparation of miliolite granules

Miliolite samples were broken with a hammer, and fresh pieces devoid of visible weathering were crushed using mortar and pestle**.** The crushed rock was sieved to obtain about the 8 mm size fraction. Sieved samples were surface sterilized using 0.2% HgCl2 solution for 15 minutes. Sample were treated with70% ethyl alcohol for 15 min for further surface sterilization. After several round of washing with sterile D/W, it was autoclaved at 15 psi for 1h. These miliolite granules are added to the above mentioned DB medium before bacterial inoculation.

7.2.5.3. Monitoring, Ca^{+2} release, organic acid profile, geochemistry and bacterial colonization

At regular time interval samples were collected and its released Ca^{+2} was estimated using cal-red method as described above section 7.2.4.5.1. To identify the quality and quantity of organic acids, HPLC analysis was carried out as mentioned above section 7.2.4.3.4. Micro and macro elements of the miliolite was investigated with powdered XRF in Department of Geology, University of Pune, Pune. X-ray diffraction (XRD) studies were performed on M16 treated and untreated samples, to monitor and highlight mineralogical modifications. X-ray diffraction (XRD) studies were conducted in Department of Earth Science, Indian Institute of Technology (IIT)-Bombay, Mumbai. Scanning electron microscopy for analyzing bacterial colonization and calcite ($CaCO_3$, a major mineral in miliolite rock) dissolution was done in Indian Institute of Technology (IIT)-Bombay, Mumbai.

7.2.6. DNA sequence accession numbers

Nucleotide sequences (bacterial 16S rRNA gene fragments) retrieved from this study have been deposited in the GenBank and the details are mentioned in Appendix-I

7.2.7. Statistical analysis

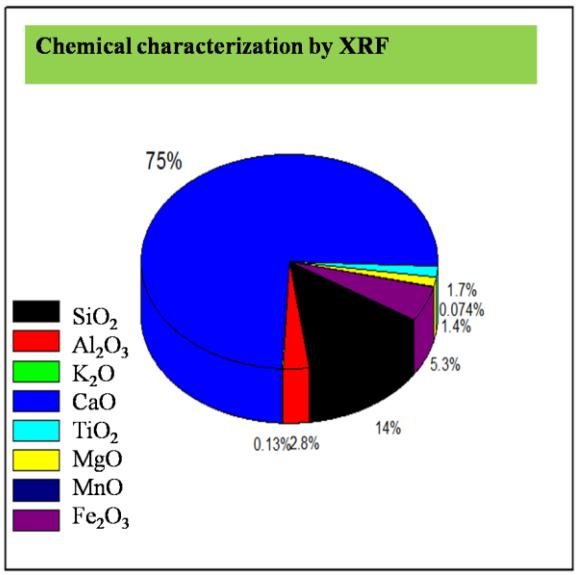
All statistical analyses were performed using SPSS version 15.0 and SigmaStat 3.4. Pairwise comparisons and quantitative differences between means of microbial activity were analyzed by one way analysis of variance by fisher least significant test (LSD). In this study, $p<0.05$ was considered to be statistically significant.

7.3. Results

7.3.1. Miliolite chemical analysis

Chemical characters of the miliolite are shown with Fig. 7.5. Ca was found to be the most abundant element in miliolite and its proportion was found to be 75 ± 6.71%. In view of the fact that miliolite is a bioclastic limestone, a relatively higher amount of organic matter (0.82%) was found than in red soil (0.54%). pH of the miliolite as well as surface red soils were found to be 7.9 and 7.5 respectively.

Fig. 7.5: *Chemical composition (major elements) of the miliolite by powdered XRF.*

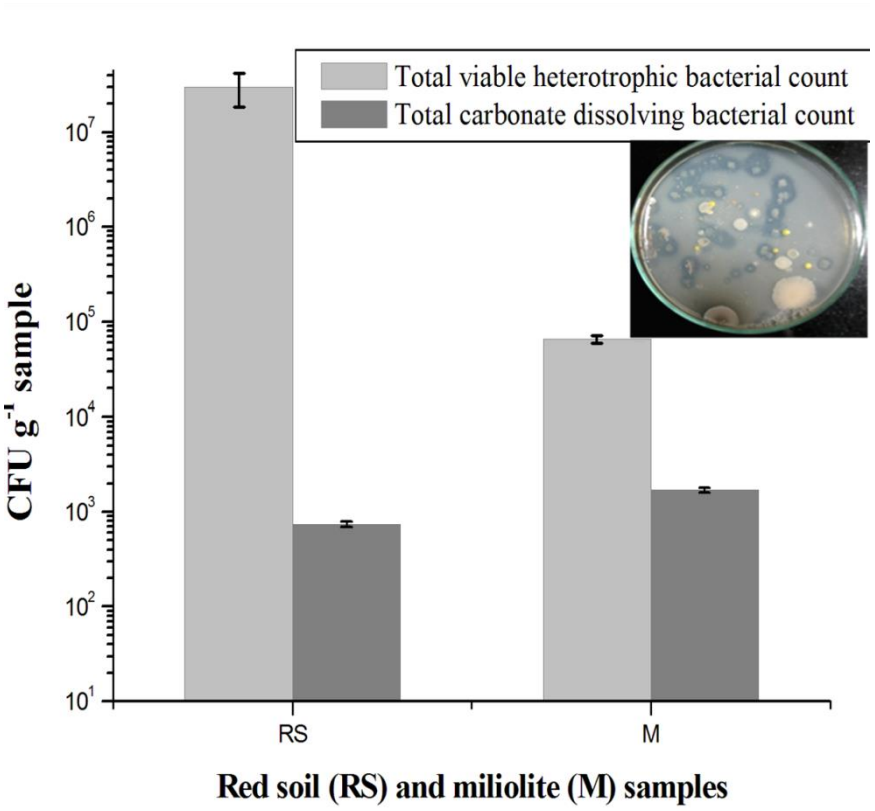


7.3.2. Isolation and enumeration of THB and TCB

Relatively higher numbers of total heterotrophic bacteria (THB) were found in red soil (3.1×10^7, Fig. 7.6) than in miliolite (6.5×10^4), whereas a higher number of

carbonate-dissolving bacteria (TCB) (1.7×10^3, Fig. 7.6) were noticed in miliolite than in red soil (7.4×10^2).

Fig. 7.6: *Enumeration of total viable heterotrophic bacteria (THB) and total carbonate-dissolving bacteria (TCB) from miliolite (M) and weathered red soil (R). The results are shown for three independent measurements and error bars depict standard deviation (SD). A representative plate with carbonate-dissolving bacterial colonies on DB medium is shown in the inset. Clear halo zone around the bacterial colony indicates carbonate solubilization.*



7.3.3. Miliolite dissolution by bacterial isolates

Initially based on the colony abundance and zone of clearance on Devenze –Bruni (DB) agar medium, 12 isolates (M1, M2, M12, M14, M16, M17, M18, M22, M23, M25, M26 and M28) from miliolite and 9 isolates (RS1, RS2, RS3, RS10, RS12, RS23, RS25, RS33 and RS34) from red soil were selected for further studies. These isolates were further screened their CZ/CS ratio on DB medium containing $CaCO_3$ and miliolite agar. Most isolates showed decrease in CZ/CS ratio and some showed no zone of clearance on miliolite agar possibly because of its complex composition. Finally, 14 isolates showing significant of CZ/CS ratio (zone of clearance/colony size) on DB medium containing $CaCO_3$ and miliolite agar were selected for further characterization. Fig. 7.7 shows zone of clearance and Fig. 7.8 shows CZ/CS ratio of selected 14 bacterial isolates on DB medium and miliolite agar.

Fig. 7.7: *Miliolite and red soil isolates zone of clearance on DB medium and miliolite agar plates. Clear halo zone around the colony can be seen.*

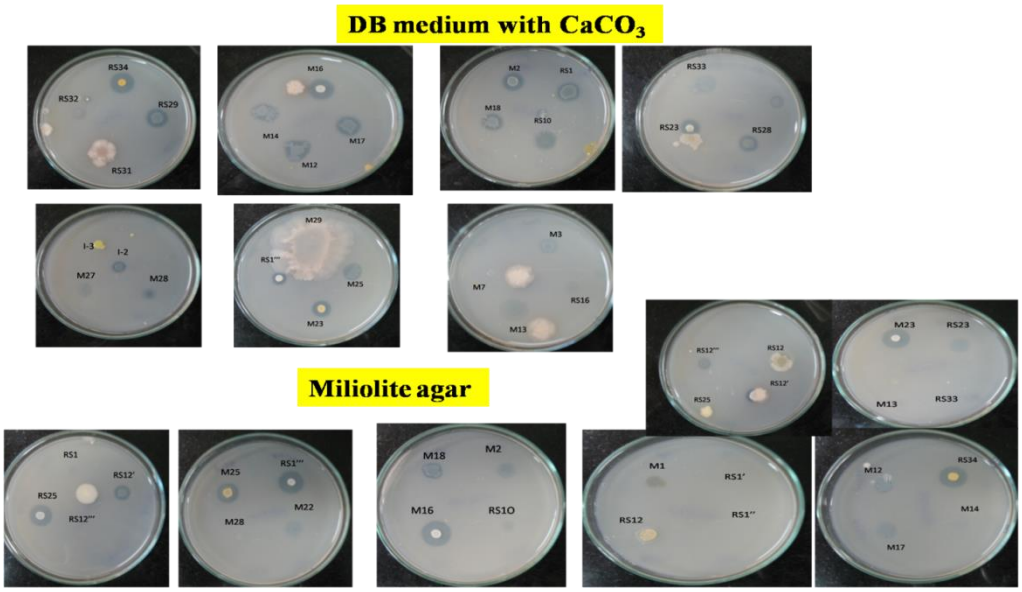
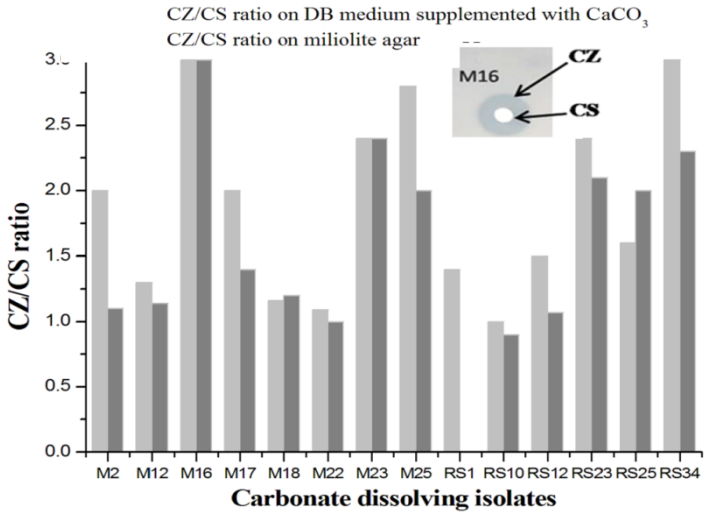


Fig. 7.8: *Zone of clearance (CZ)/colony size (CS) ratios of carbonate-dissolving bacteria on DB medium and miliolite agar. Inset shows CZ and CS of one of the isolates (M16) on miliolite agar.*



All these 14 carbonate dissolving isolates were found to be gram positive in nature.

7.3.4. Phosphate solubilization and siderophore production of miliolite and red soil isolates

CZ/CS ratios indicate that M16, M25 and RS1 are the good phosphate solubilizers on Pikovaskyas agar (Table 7.2). Isolates M22, RS10, and RS25 did not grow on Pikovaskya agar. Isolate RS25, M25, M16 were found to be high siderophore producers, where as others did not show any siderophore production on CAS plates.

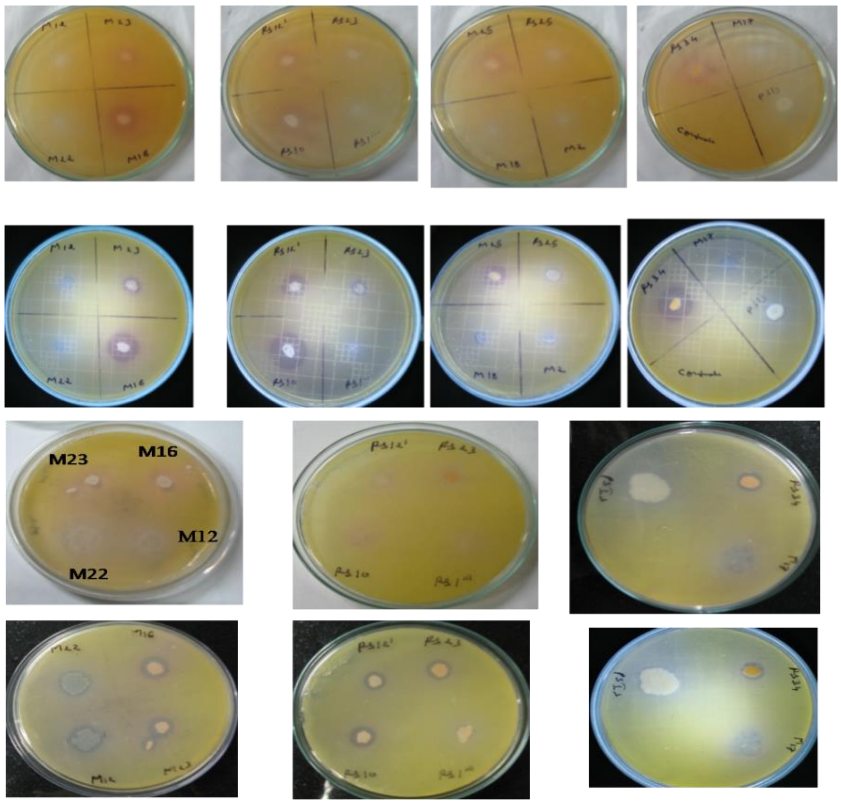
Table 7.2: *Phosphate solubilization and siderophore production by miliolite and red soil isolates.*

Isolate no	Colony size (CS)	Zone of clearance (CZ)	CZ/CS	Siderophore production
M16	0.3	0.8	2.66	++
M25	0.4	0.8	2.00	+++
M23	0.4	0.6	1.5	+
M18	0.7	1.1	1.57	-
RS1	0.3	0.8	2.67	-
RS12	0.4	0.6	1.5	-
RS23	0.3	0.6	2.0	-
RS34	0.4	0.9	2.25	-
M2	0.4	0.5	1	-
M12	0.3	0.6	2	+
M17	0.3	0.5	1.67	+
M22	-	-	-	+
RS10	-	-	-	-
RS25	**0.4**	**0.5**	**1.25**	++++

7.3.5. Ability of bacterial isolates to lower pH on buffered and unbuffered MR-miliolite agar plates.

Appearance of red color on MR-miliolite agar plates represents pH drop where as appearance of clear halo zone around the colony indicated dissolution of miliolite powder. Isolates M16, M17, M23, M25, RS34 and RS 10 are able to acidify the medium and dissolve carbonates in both buffered (50 mM Tris) and unbuffered conditions (Fig. 7.9). At 100 Mm Tris-buffered DB media, neither pH drop nor zones of clearance were noticed. Relatively M16, M17, M23, M25, RS34 and RS 10 were shown higher CZ/CS ratios (Table 7.3)

Fig. 7.9: *Methyl red impregnated miliolite agar plates show pH drop ability in un buffered (I st and 2nd lane) and buffered (50 mM tris, 3rd and 4th lane) media. Red colour zone around the colony indicates pH drop. Zone of clearance around the colony indicates dissolution of miliolite powder.*



7.3.6. Ability of bacterial isolates to lower pH of buffered and unbuffered DB broth

In order to detect the organic acid production ability of the bacterial isolates, experiments were conducted to study the ability to the bacteria reduce the pH of liquid media (Fig. 7.10). Most of the isolates showed a pH drop between 1 to 5 days after that the pH of the broth increased.

The isolates brought about a decrease in pH from pH 8.0 to around pH 5.5, indicating release of acids. In unbuffered conditions isolates M16, M23, RS1 and RS34 were found to bring about a large decrease in pH. Similarly, at 50 mM-Tris M16, M23, RS10 and RS34 were found to bring about high acidification of the medium. Although all organisms showed growth in the media, no correlation between pH drop and whole cell protein content (Fig. 7.11) of the isolates was observed.

Table 7.3: *CS/CZ ratios of isolates in miliolite agar plates. Values for both unbuffered and buffered (50 mM Tris-base) miliolite agar plates are shown*

Isolate no	**Unbuffered medium**			**50 mM Tris buffered medium**		
	Colony size (CS)	**zone size (CZ)**	**CZ/CS ratio**	**Colony size (CS)**	**zone size (CZ)**	**CS/CZ ratio**
M2	0.7	0.9	1.28	0.7	0.8	1.14
M12	0.7	0.9	1.28	1.2	1.6	1.33
M16	0.65	2.2	3.38	0.7	1.2	1.79
M17	0.4	1.0	2.5	1.1	1.2	1.09
M18	0.6	0.8	1.33	1.2	1.35	1.12
M22	0.7	1.0	1.42	1.1	1.4	1.27
M23	0.4	0.8	2.0	0.6	1.1	1.8
M25	0.7	2.2	3.14	0.6	0.8	1.3
RS1	0.6	1.3	2.16	0.6	0.8	1.3
RS10	0.8	2.4	3.0	0.6	1.1	1.8
RS12	0.7	1.8	2.57	0.6	0.9	1.5
RS23	0.8	1.5	1.87	0.6	1.0	1.5
RS25	0.9	1.2	1.33	1.0	-	-
RS34	0.7	2.4	3.42	0.7	1.2	1.7

Unit for the values mentioned above table is cm.

Fig. 7.10: *pH profile of miliolite dissolving isolates on different media A and B are isolates from miliolite (M Series) and C and D are isolates from red soil (RS series). A and C are on unbuffered DB broth and B and D on 50 mM Tris-base buffered DB broth. .*

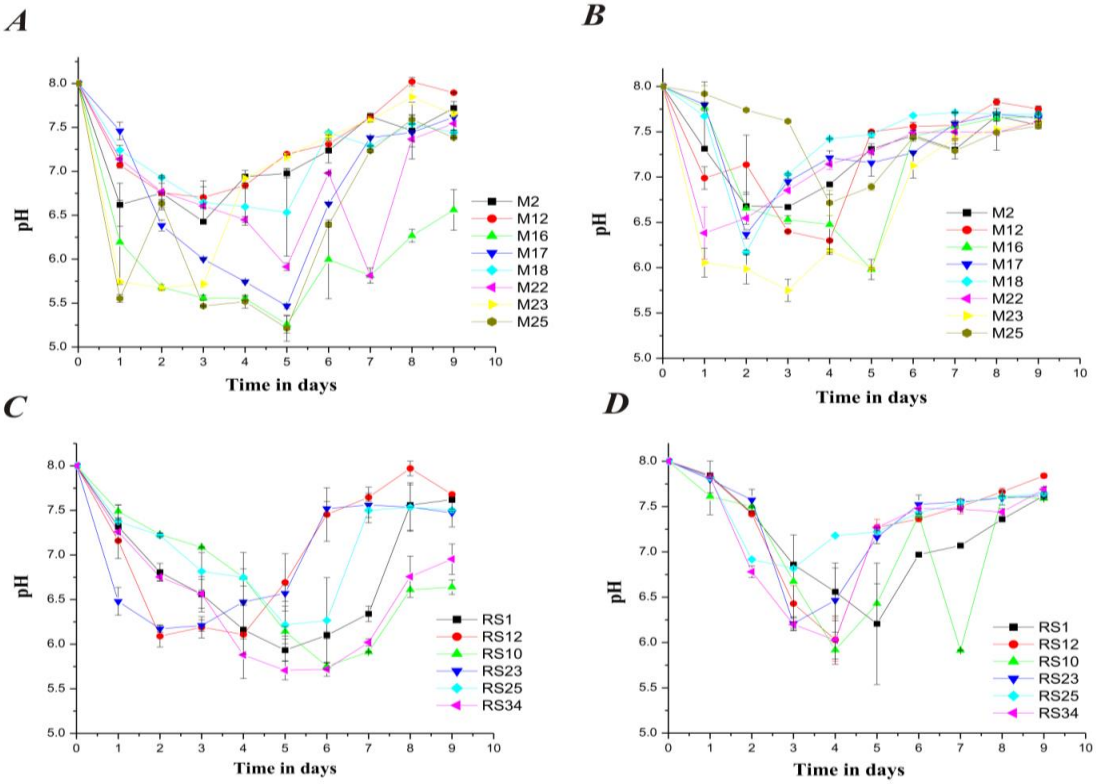
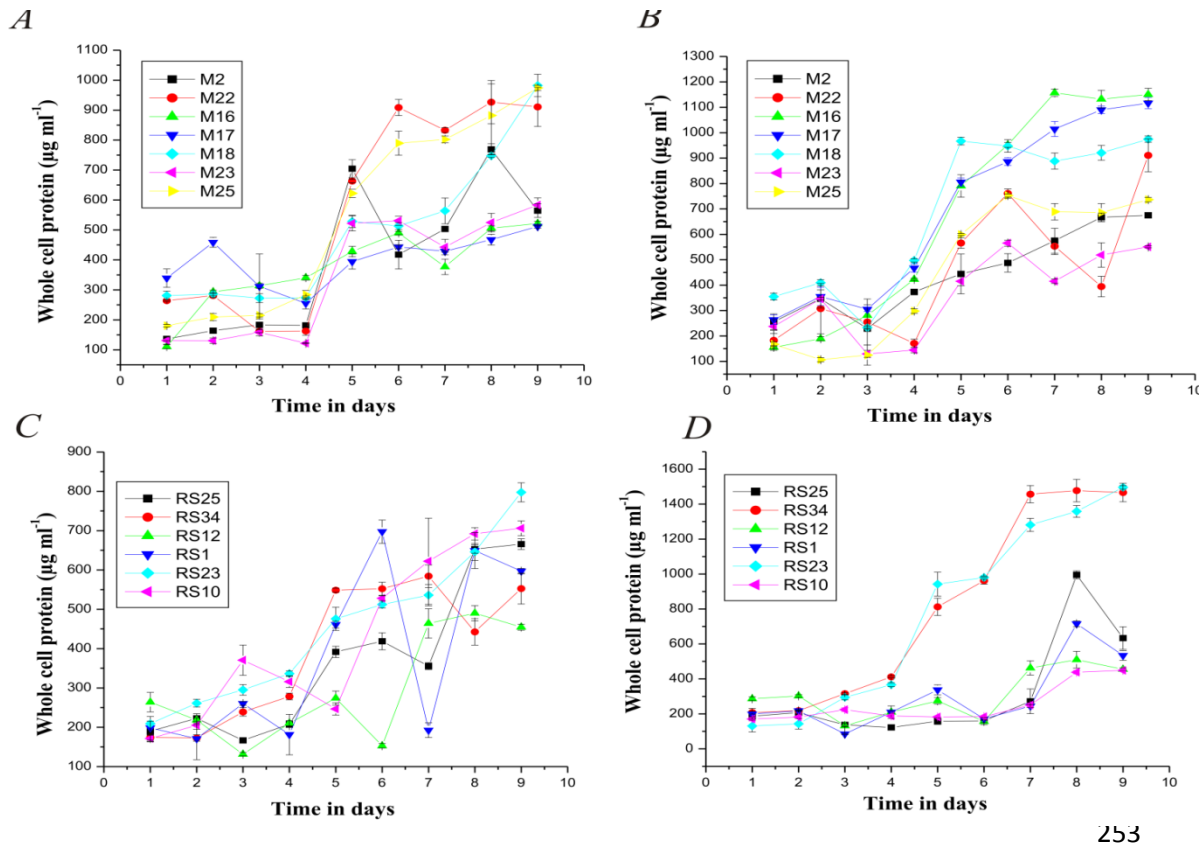


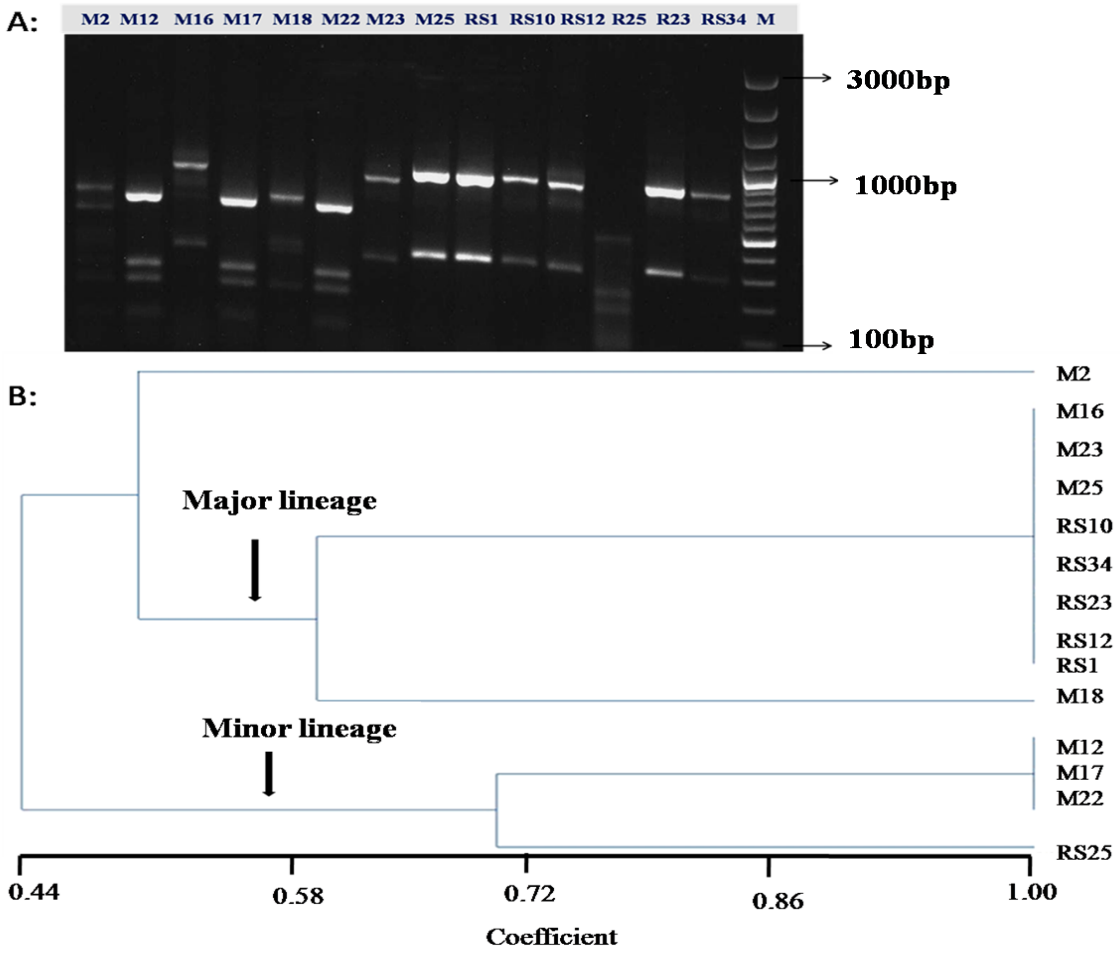
Fig. 7.11: *Bacterial growth as measured by whole cell protein profile of miliolite dissolving isolates on different media. A and B are isolates from miliolite (M Series) and C and D are isolates from red soil (RS series). A and C are on unbuffered DB broth and B and D on 50 mM Tris-base buffered DB broth.*



7.3.7. ARDRA analysis of the carbonate dissolving isolates

The ARDRA pattern of carbonate-dissolving bacteria (Fig. 7.12A) revealed that the isolates, depicted as operational taxonomic units (OTUs), were distributed in 5 different clades at a similarity coefficient of 0.85 (Fig. 7.12B). It was also noted that most of the OTUs were distributed in major evolutionary lineages at a similarity coefficient of 0.54. Around 9 isolates were located in a major lineage whereas minor lineages consisted of 4 isolates.

Fig. 7.12: *ARDRA pattern of the carbonate-dissolving bacteria (A) and their phylogenetic relationship (B). M indicates miliolite isolates, whereas RS indicates red soil isolates.*

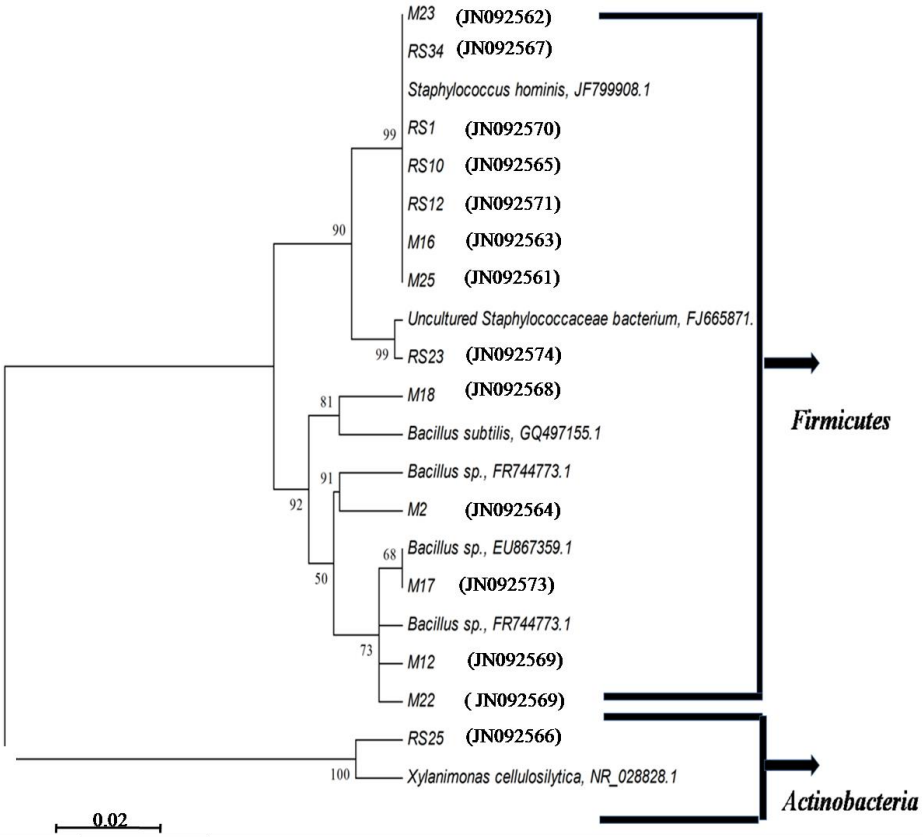


7.3.8. Identification of the carbonate dissolving isolates

16S rRNA sequencing results largely corroborated to ARDRA results with some variations. 16S rRNA sequence analysis helped to decipher that the majority of carbonate-dissolving bacteria were affiliated to phyla *Firmicutes* (Families

Bacillaceae and *Staphylococcaceae*) and *Actinobacteria* (Family *Promicromonosporaceae*) (Fig 7.13 and Table 7.4).

Fig. 7.13: *16S rRNA gene sequence-based phylogenetic tree of carbonate-dissolving bacteria. The evolutionary history was inferred using the UPGMA method. Phylogenetic analysis was conducted using MEGA4 (28). M indicates miliolite isolates, whereas RS indicates red soil isolates. Genbank accession numbers of the sequences reported in this study are shown in parentheses.*



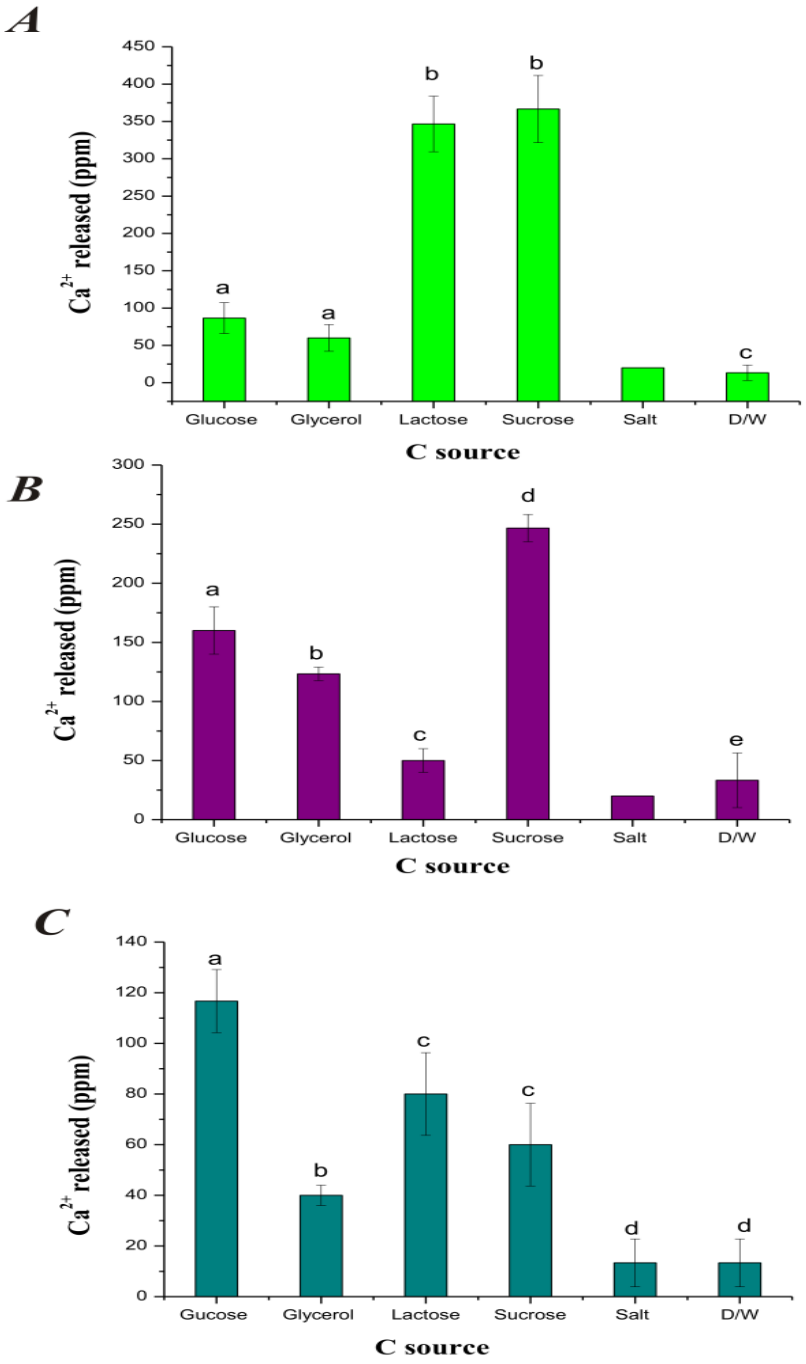
7.3.9. Effect of different carbon sources on Ca^{2+} release:

Effect of different carbon sources on Ca^{2+} released by carbonate dissolving bacteria after 6 d is shown in Fig 7.14. Significant higher (P <0.01) Ca^{2+} was released by *Staphylococcus* sp. M16 in sucrose and lactose contain media. Similarly higher Ca^{2+} was released by Bacillus sp. M12 in sucrose and glucose containing tubes. Glucose was found to increase Ca^{2+} release by *Xylanimonas* sp RS25. Relatively higher Ca^{2+} (366 ± 45 ppm) was released by *Staphylococcus* sp. M16 than others.

Table 7.4: *16S rRNA gene sequence similarity of miliolite-dissolving bacteria. Isolates designated with suffix M were obtained from miliolite sample and RS, from red soil*

Isolate	Genbank accession number	Closest match in Genbank	% Similarity	Genbank accession number of closest match	Eubacterial Phylum	Eubacterial Family
M25	JN092561	*Staphylococcus hominis*	99	JF799908.1	*Firmicutes*	*Staphylococcaceae*
M23	JN092562	*Staphylococcus hominis*	98	JF799908.1	*Firmicutes*	*Staphylococcaceae*
M16	JN092563	*Staphylococcus hominis*	99	JF799908.1	*Firmicutes*	*Staphylococcaceae*
M2	JN092564	*Bacillus* sp.	96	DQ275174.1	*Firmicutes*	*Bacillaceae*
RS10	JN092565	*Staphylococcus hominis*	99	JF799908.1	*Firmicutes*	*Staphylococcaceae*
RS25	JN092566	*Xylanimonas cellulosilytica*	100	NR_028828.1	*Actinobacteria*	*Promicromonosporaceae*
RS34	JN092567	*Staphylococcus hominis*	99	JF799908.1	*Firmicutes*	*Staphylococcaceae*
M18	JN092568	*Bacillus subtilis*	100	GQ497155.1	*Firmicutes*	*Bacillaceae*
M12	JN092569	*Bacillus* sp.	100	FR744773.1	*Firmicutes*	*Bacillaceae*
RS1	JN092570	*Staphylococcus hominis*	99	JF799908.1	*Firmicutes*	*Staphylococcaceae*
RS12	JN092571	*Staphylococcus hominis*	99	JF799908.1	*Firmicutes*	*Staphylococcaceae*
M22	JN092572	*Bacillus* sp.	100	FR744773.1	*Firmicutes*	*Bacillaceae*
M17	JN092573	*Bacillus* sp.	100	EU867359.1	*Firmicutes*	*Bacillaceae*
RS23	JN092574	Uncultured *Staphylococcaceae* bacterium	100	FJ665871.1	*Firmicutes*	*Staphylococcaceae*

Fig 7.14: *Effect of different carbon sources on Ca^{2+} release. A, B and C: isolates M16, M12 and RS25 respectively. Values are mean of three independent replicates (n = 3). Means accompanied with different letters are significantly different at P<0.05 (LSD). "D/W" indicates culture inoculated in distilled water. "Salt" indicates DB media contained only salts and miliolite granules (not any carbon source). Systems D/W and Salt are used as controls.*



7.3.10. Bacterial dissolution studies of miliolite by short-term microcosms

Bacterial potential in dissolution of miliolite was investigated with 20 d short term microcosm studies. Microcosm experiments revealed the bacterial activity in dissolution of calcite (Fig. 7.15). Compared to control, all the three organisms were found to significantly solubilize miliolite. Higher Ca^{2+} was released by *Staphylococcus* sp. M16. The dissolution of miliolite is attributed to organic acid production (Table 7.5). Acetate and lactate were found to be major organic acids during miliolite dissolution (Table 7.5). Relatively higher amount of lactate is produced by *Staphylococcus* sp. M16, where as higher acetate was produced by *Bacillus* sp. M12. The relation between organic acids and Ca^{2+} release was observed with principle component analysis (PCA) (Fig. 7.16). The first three factors had Eignen values more than 1 indicates significance of the test (Fig. 7.16A). Positive relation between lactate, acetate and Ca^{2+} release in the microcosm was noticed in the three dimensional ordination plot (Fig. 7.16B).

XRF results clearly indicate the dissolution of Ca^{2+} from miliolite by inoculated bacteria (Fig. 7.17). Bacterium *Staphylococcu*s sp. M16 had prominent effect on dissolution of miliolite where initial 75% calcium oxide was reduced to 67%. Similarly, *Bacillus* sp. M12 and *Xylanimonas* sp. RS25 reduced to 68% in both. This was in agreement with Ca^{2+} release results (Fig. 7.15). Scanning electron microscopy (SEM) images of these bacteria during dissolution of miliolite are shown in Fig. 7.18. All the three bacterial strains colonized the mineral surface. In control miliolite samples, clear cut hexagonal calcite crystals are seen. However, in the bacteria treated miliolite, calcite crystals have undergone erosion, disintegration and bacterial induced pits are seen. Obvious biofilm formation on the surface of miliolite by *Staphylococcus* sp. M16 was seen (Fig. 7.18B). Bacteria appear to have a strong influence on miliolite weathering by forming pits (about 10 µm in size) and widened micropores. Further XRD results indicate the reduction of calcite in bacterial treated miliolite samples (Fig. 7.19).

Fig. 7.15: *Bacterial effect on release of Ca^{2+} from miliolite during microcosm experiment.*

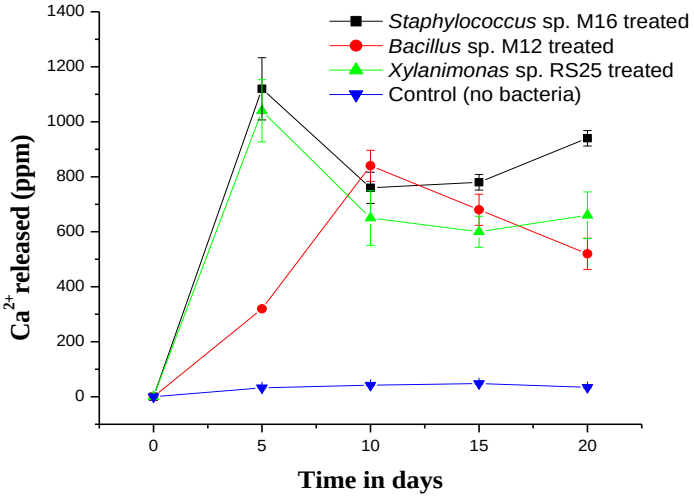


Table 7.5: *Organic acids released during miliolite dissolution after 20 d of microcosm incubation.*

Time in d	M16		Organic acid (mM)				
	Lactate	Acetate	Gluconate	Malate	Oxalate	Pyruvate	Citrate
5	169.3a	47.28a	0	9.5a	1.19a	18.5 a	0
10	71.5b	38.46b	0.1	0	0.87a	0.5 b	0.7
15	27.06c	21.74c	0	0	0.2b	0.58b	0.78
20	16.65d	23.15c	0	5.5b	0.87a	0.1 c	0
Time in d	**M12**		**Organic acid (mM)**				
	Lactate	Acetate	Gluconate	Malate	Oxalate	Pyruvate	Citrate
5	23.48a	125.09a	0	0.5a	0.02b	12.9a	0
10	58.35b	157.26b	0	0.5a	0	10.9 a	10a
15	43.95c	126.45a	0	0.3a	0.6a	0.75 b	4.4 b
20	15.02d	34.37c	0	6.7b	0.9a	0.78 b	0.9 c
Time in d	**RS25**		**Organic acid (mM)**				
	Lactate	Acetate	Gluconate	Malate	Oxalate	Pyruvate	Citrate
5	46.3a	154.7a	0	5.71a	0	2.31a	0.63 a
10	7.18b	41.66b	0	0.3b	0.5	0.59b	3.76 b
15	27.06c	21.96c	0	0.23c	0	0.5 b	0
20	16.65d	0.73d	0	0	0	0	0

Fig. 7.16: *A: Relation between organic acids and Ca^{2+} release; B: Effect of pure organic acids on release of Ca^{2+} (mg ml^{-1}).*

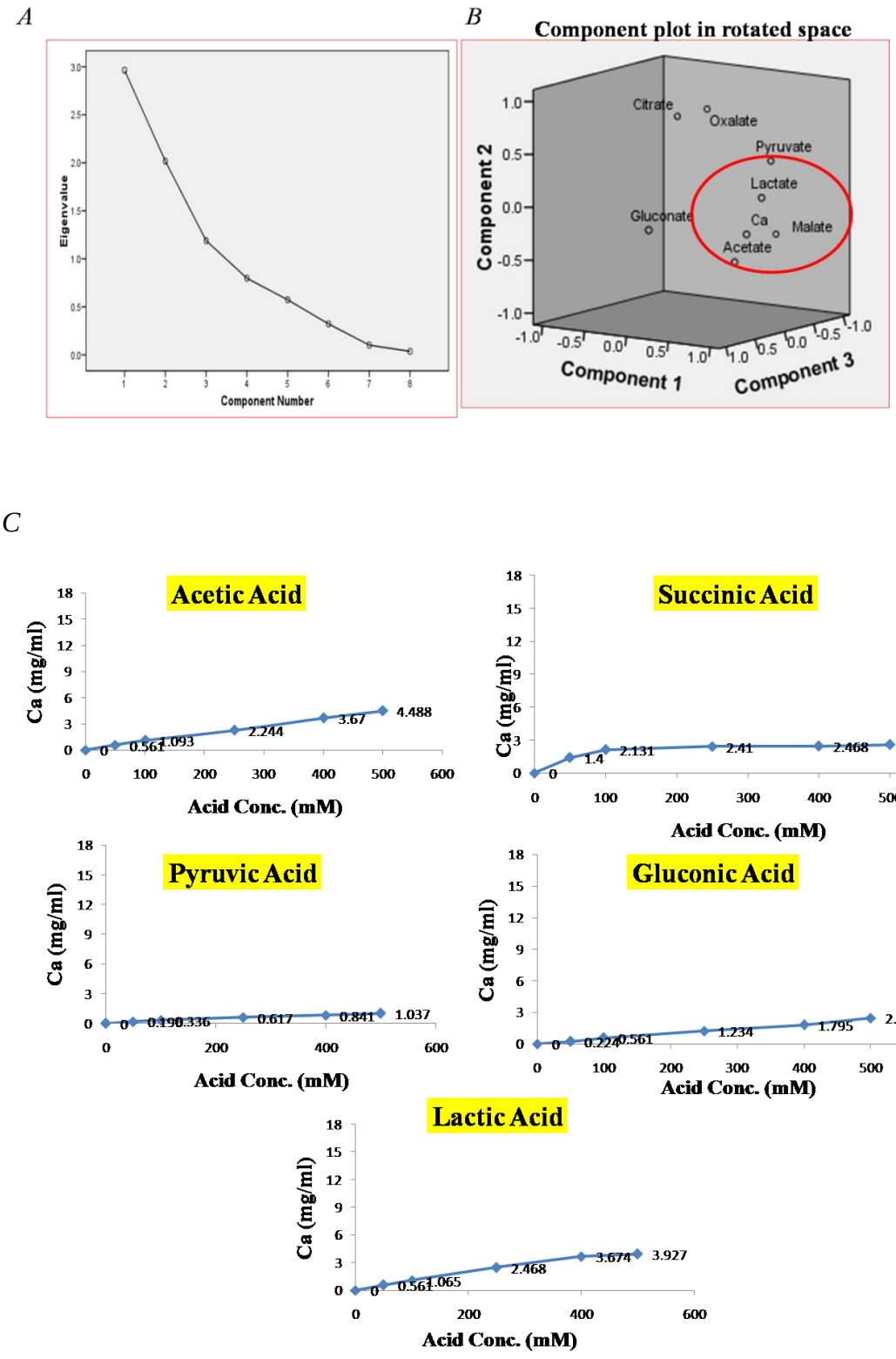


Fig 7.17: *Chemical composition (major elements) of the bacterial treated miliolite samples during microcosm experiments. The results are obtained by X-ray fluorescence (XRF) studies.*

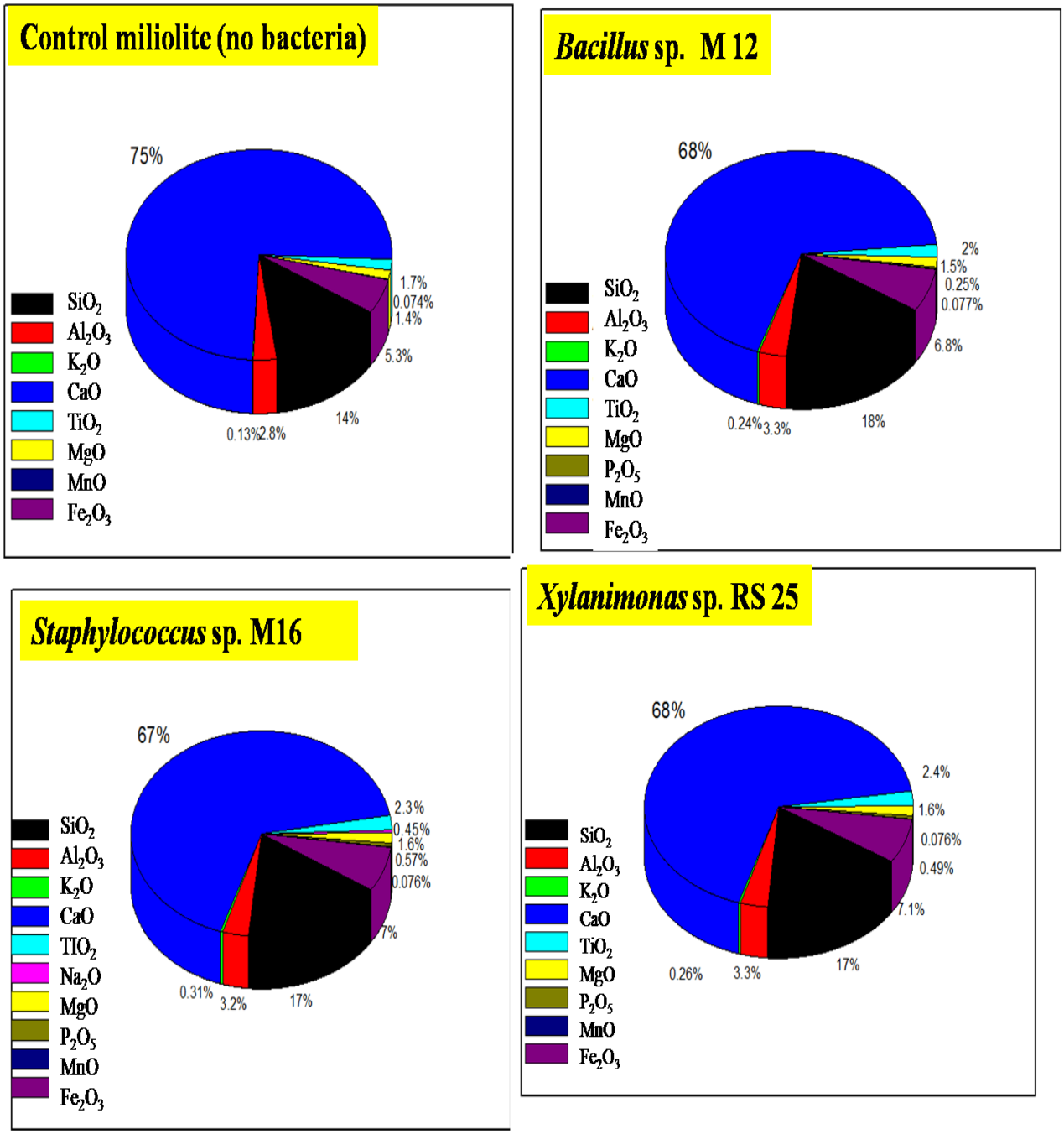
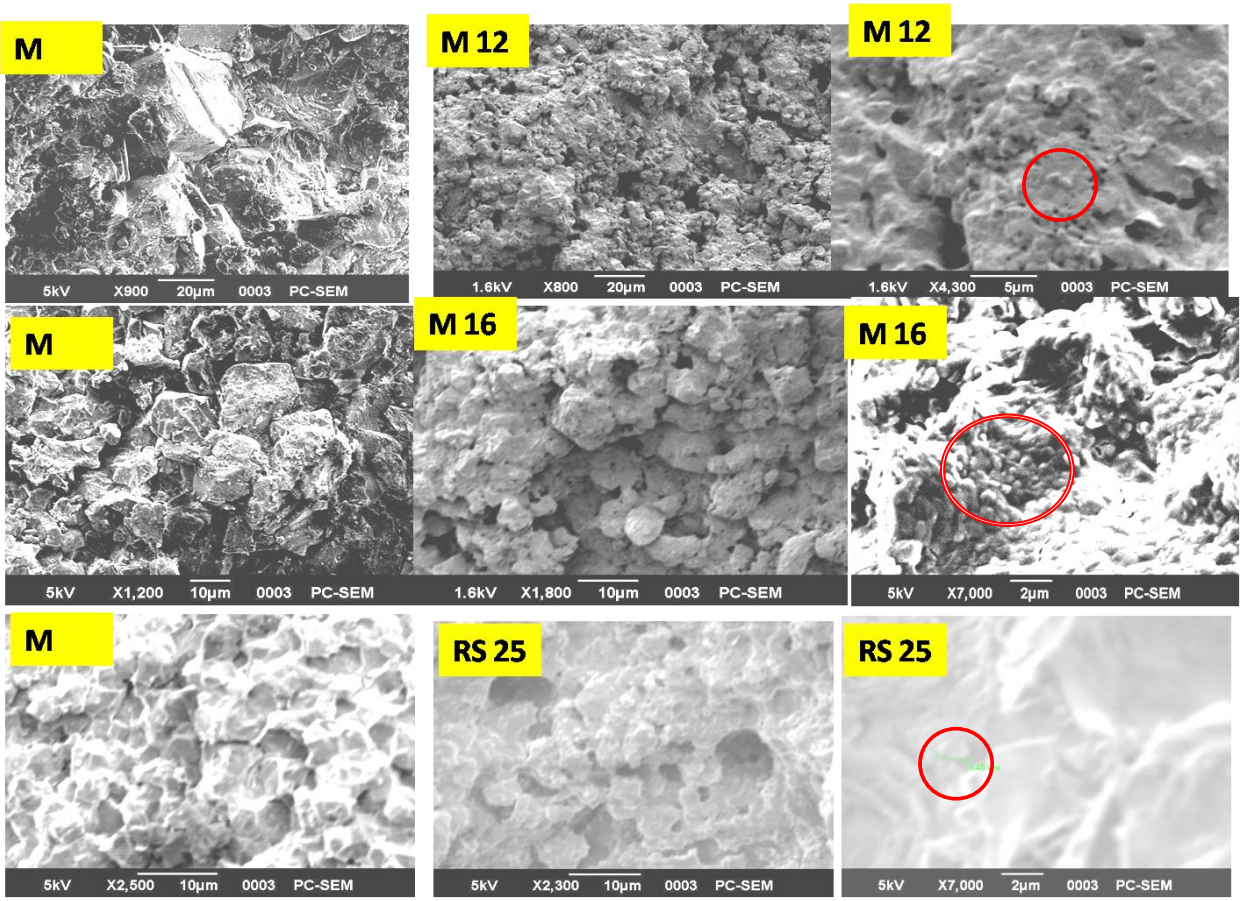


***Fig. 7.18**: Scanning electron microscopic images of miliolite samples retrieved from insitu microcosm experiments. Letter "M" indicates control miliolite sample (sterile sample). A: Letters M12, M16 and RS25 indicates Bacillus sp. M12, Staphylococcus sp. M16 and Xylanimonas sp. RS 25 respectively. Typical bacterial cells are shown with red circle for ready reference. B: typical biofilm formation of Staphylococcus sp. M16*

A



B:

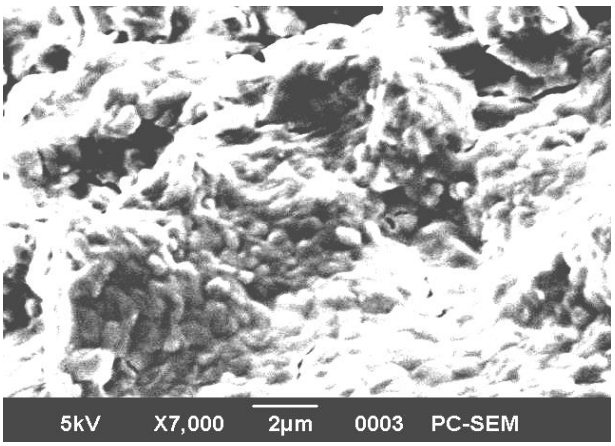
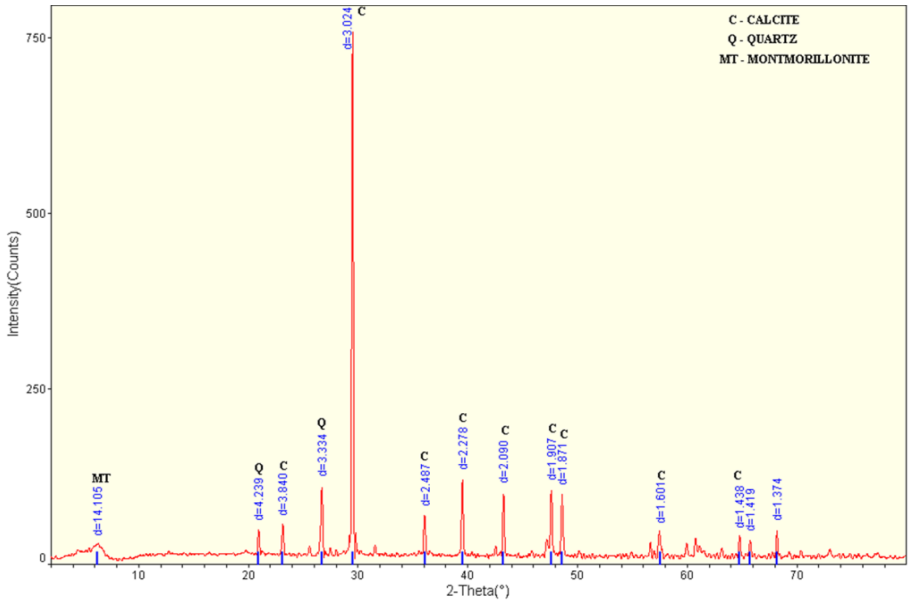
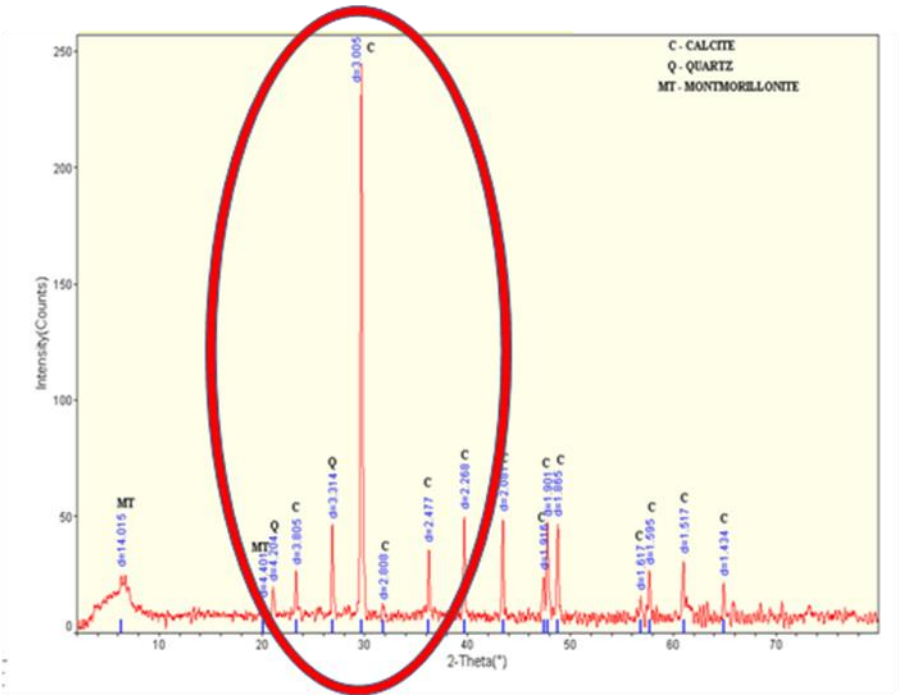


***Fig. 7.19**: X-ray diffraction studies of miliolite samples from microcosm experiments. A: control miliolite sample where 750 counts of calcite peak seen. B: Staphylococcus sp. M16 treated miliolite sample where decreased calcite peak (250 counts) can be seen.*

A



B:



7.4. Discussion

Quaternary carbonate deposits known as miliolites are deposited in the semiarid environments of Saurashtra and Kachchh provinces of Gujarat and appear as patchy outcrop inlands up to 180 km from the miliolite-bearing coast. Upon weathering, miliolite forms red soil sequences, which are important landscapes for agriculture in the regions of Saurashtra and Kachchh. Although geomorphological, chronological and geochemical studies of these deposits are well established, almost nothing is known about the potential role of microbes in biogeochemical processes of this environment. Since Ca-bearing mineral calcite ($CaCO_3$) is the major mineral phase in miliolite (Bhatt et al., 2003), it was thought that bacteria that have the ability to dissolve carbonate (and release Ca^{2+}) may be keystone species in miliolite weathering. In the present investigation, such species were isolated, characterized and their phylogeny was established.

X-ray fluorescence (XRF) results indicated that Ca (75%) was the most abundant element in this bioclastic rock miliolite, which is in general agreement with the previous report (Bhatt et al., 2003). The percentage of the TCB to THB was significantly (P = 0.001) higher in miliolite (27.17%) than in red soil (0.002%), indicating a higher abundance of carbonate-dissolving bacteria in miliolite than in red soil. This discrepancy is likely due to the geochemistry of the surroundings, since calcium carbonate is a major mineral phase (40-80%) in miliolite; the microorganisms need to dissolve carbonate to extract their nutritional requirements; as a result, high TCB were associated with miliolite (Bennett et al., 2001; Warren et al., 2001). In addition, the relatively high amount of organic matter and possibly nitrogen content of miliolite may support the high amount of TCB in miliolite. Previously, it is shown that microbes readily acquire nutrients by weathering of minerals (Mailloux et al., 2009).

Acidolysis (mineral dissolution owing to acidification of the medium) and production of siderophores are primarily responsible mechanizes for dissolution of wide variety minerals such as phosphate, apatite, hydroxyapatite, hornblende and hematite (Page, 1987; Kalinowski et al., 2000; Liermann et al., 2000; Welch et al., 2002; Hameeda et al., 2006; Ben Farhat et al., 2009, Urz et al., 2009). Therefore, the miliolite dissolving isolates were characterized for the production of low molecular

weight organic acids and siderophores. In the present study lactate and acetate are found to be the major organic acids in dissolving miliolite by these isolates. Previously geoecological role of gluconic acid is discussed (Hameeda et al, 2006), however, geomicrobiological role of lactate and acetate in carbonate dissolution is first time reported here. Putative mechanisms of action of these organic acids in rock weathering are reviewed by Uroz et al (2009). In brief, organic acids serve as mineral weathering agents by acidolysis, chelation and oxidoreduction of minerals being acted upon. The protons associated with organic acid molecules decrease the pH of surroundings therefore, induce the releasing of cations such as calcium, iron, potassium and magnesium. Chelating molecules might increase the dissolution rates of cations by forming strong bonds with them or with mineral surfaces. Oxidoreduction reactions of organic acids occurring at the surface of complex minerals, such as silicates, require direct contact of the bacterial membrane and the mineral surface to occur.

Organism's ability to drop pH in buffering conditions has an advantage in better acquisition of nutrients as natural terrestrial ecosystems are buffering in nature. Isolates M16 and RS23, RS10 and RS 34 are found as good acid producers in both buffered and unbuffered conditions. Interesting all these isolates later phylogenetically identified as *Staphylococcus* sp. under phylum *Firmicutes*. Moreover, all these isolates dissolve both mineral phosphate and calcite. The zone of clearance on MR-miliolite agar and the red colour in MR-miliolite broth is attributed to the reduction in pH drop by the organisms.

It was found that all the 14 carbonate-dissolving isolates belonged to phyla *Firmicutes* and *Actinobacteria*, indicating the prominent role of these taxa in miliolite dissolution. These two phyla were found to be major representatives of the bacterial communities in carbonated environments such as karst and caves, indicating their ability of biomineralization (Song et al., 2007 and 2010; Portillo et al., 2008 and 2009). Because of their elite mineral composition, rocks could be considered as primary ecosystems, where only a few adapted microbes, with distinct mineral-weathering capacities, can survive and grow (Uroz et al., 2009). It can be inferred that *Firmicutes* and *Actinobacteria* might have been highly adopted in this particular environment than other bacterial genera. Earlier 16S rRNA-based culture-independent studies demonstrated that Gram-negative bacteria belonging to the phylum

Proteobacteria contributed markedly to the bacterial diversity in carbonated environments (Loez-Garcia et al., 2005; Zhou et al., 2007, Engel and Randall , 2011; Tang et al., 2012); however, proteobacterial ability in carbonate dissolution is not yet known. In the present investigation, we did not notice any representative members of *Proteobacteria* which could solubilize carbonate or miliolite.

Most of the carbonate-dissolving isolates showed high 16S rRNA gene similarity (98-99%) to *Staphylococcus* sp. (accounting for 57%) and to (96-100%) *Bacillus* sp. (accounting for up to 35.7%). Comparison of CZ/CS ratios suggested that *Staphylococcus* species (M16, RS34 and M23) were more proficient at carbonate dissolution than *Bacillus* sp. (M17). This was further confirmed in our short-term microcosm studies. The involvement of *Bacillus* sp. in carbonate dissolution is known **(**Li et al., 2007; Song et al., 2007 and 2011); however, this is the first report indicating the geomicrobial role of *Staphylococcus* sp. in carbonate dissolution. The sole isolate belonging to *Actinobacteria*, showed high 16S rRNA gene identity (100%) to a newly identified (Rivas et al., 2003) *Xylanimonas* sp. in the family *Promicromonosporaceae*.

Microcosm experiment revealed the bacterial activity in dissolution of calcite. Bacterial colonization, erosion and pitting of calcite crystals were well documented by SEM. Calcite dissolution was further confirmed by XRD pattern and XRF analysis. The order of bacterial ability in dissolution of miliolite was in the following order *Staphylococcus* sp. M16 > *Bacillus* sp. M12 > *Xylanimonas* sp. RS25. SEM images indicate that bacterial activity increased the *surface area of calcite*, so that the process of chemical and biological *weathering* would be further accelerated. SEM images show that calcite mineral surfaces are aggressively coated with a thick complex microbial biofilm by *Staphylococcus* sp. M16. Mineral surfaces underneath these microbial extracellular polymers were often extensively engraved. Previously, colonization of mineral particles by bacteria was reported for different minerals such as biotitte, granite, limestone, apatite (calcium phosphate), plagioclase and quartz. (Barker et al., 1998; Gorbushina, 2007; Uroz et al., 2009). Biofilm formation ability may give *Staphylococcus* sp. M16 to competitive edge in adapting and colonizing extreme environments that are not tolerated by other organisms (Gorbushina, 2007). Further, the attachment of bacteria to mineral surfaces creates microenvironments that protect bacteria against environmental stresses. In these microenvironments, bacteria

may extract inorganic nutrients and obtain energy directly from the mineral matrix. Colonization of bacteria on mineral surface can also induce biomechanical deterioration of rocks and minerals through penetration, boring and burrowing into decaying material and along crystal planes (e.g. hexagonal plane in calcitic rocks) (Golubic et al., 2005; Smits, 2006; Gadd, 2007, 2008 and 2009; Cockell and Herrera, 2008 and 2009).

The present study improves our conceptual understanding of microbial communities as important players in carbonate weathering, which has a wide range of implications; from the elucidation of biogeochemical cycles to the potential impact of atmospheric CO_2 sinks (Liu et al., 2000).The present investigation deals specifically with the culturable bacterial population that dissolves miliolite, it does not preclude the possibility of as yet uncultivable carbonate-dissolving bacteria for which we do not have any information from this study. Application of molecular tools and isotope techniques may help to extend the knowledge about total carbonate-dissolving microbial communities and their specific function *in situ*.

7.5. Conclusions

Firmicutes and *Actinobacteria* represent two keystone culturable carbonate-dissolving heterotrophic bacterial phyla involved in *in vitro* carbonate dissolution and attendant miliolite weathering. Microcosm experiments demonstrated the dissolution of miliolite by bacteria. The order of bacterial ability in dissolution of miliolite was in the following order *Staphylococcus* sp. M16 > *Bacillus* sp. M12 > *Xylanimonas* sp. RS25. Lactate and acetate are the two major organic acids in dissolving miliolite by the organisms. The efficacy of organic acids in dissolution of miliolite was found to be in following order: Acetate> lactate> succinate> gluconate> pyruvate. This study extends the knowledge on geomicrobiology of carbonates in semiarid region.

APPENDICES

Appendix I

Information about nucleotide sequences obtained from the present study and their corresponding GenBank accession numbers.

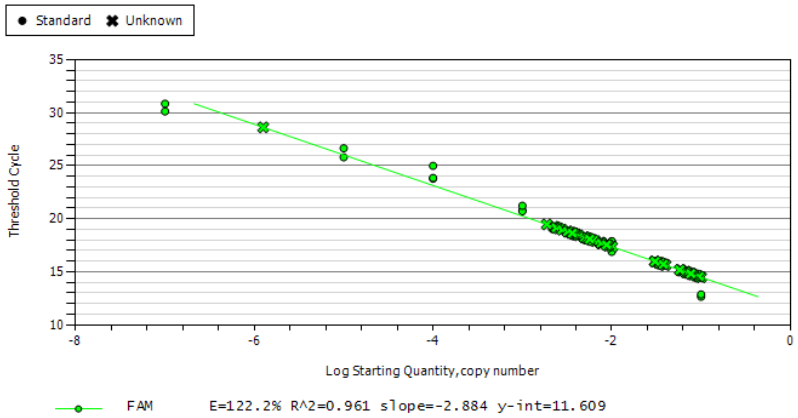
Chapter	Ecosystem	Gene	Source of the gene	Number of sequences	GenBank accession numbers
4	Agricultural and pristine soils	16S-rRNA	Isolate genomic DNA	16	KC748475 to KC748490
5	Flood affected and unaffected soils	16S-rRNA	Isolate genomic DNA	10	KC763642 to KC763651
	Long-term industrial effluent polluted soil and uncontaminated soils	16S-rRNA	Soil community DNA, DGGE bands	26	JX504051 to JX504076
		AOA-*amoA*	Soil community DNA, DGGE bands	15	JX570739 to JX570753
		AOB-*amoA*	Soil community DNA, DGGE bands	10	JX556912 to JX556921
	Long-term industrial effluent polluted sediment	16S rRNA	Soil community DNA, DGGE bands	8	KC462741 to KC462748
	Heavy metals Cd and As spiked acidc soils	AOA-*amoA*	Soil community DNA, AOA-*amoA* functional gene clone library	400	JX416592 to JX416690 and JX427105 to JX427403
6	Long-term industrial effluent polluted sediment	16S-rRNA	Isolate genomic DNA	6	JN936958 to JN936963
7	Carbonated rock miliolite and surface red soils	16S-rRNA	Isolate genomic DNA	14	JN092561 to JN092574
			Total number of sequences	**505**	

Appendix II

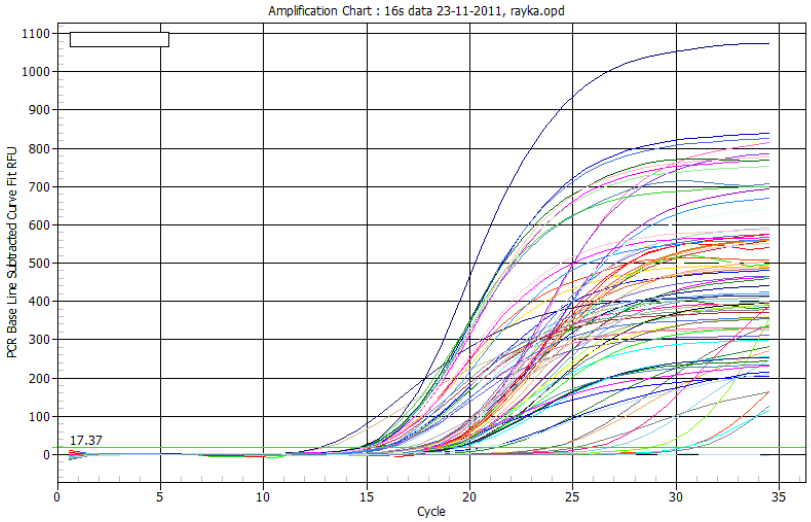
Stadared curves, amplification charts and melting curve profiles (for SYBR chemistry) of q-PCR used for estimating eubacterial 16S rRNA, archaeal 16S rRNA, AOA-*amoA* and AOB-*amoA* gene copy numbers from heavy metals spiked soils.

1. Eubacterial 16SrRNA standard curve (A) and amplification chart (B).

A

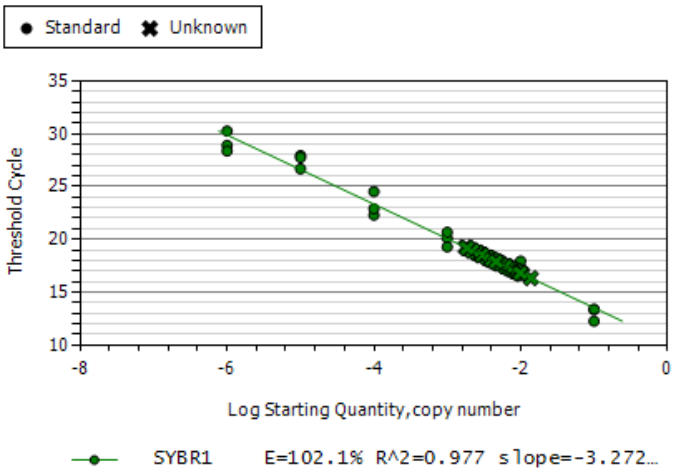


B

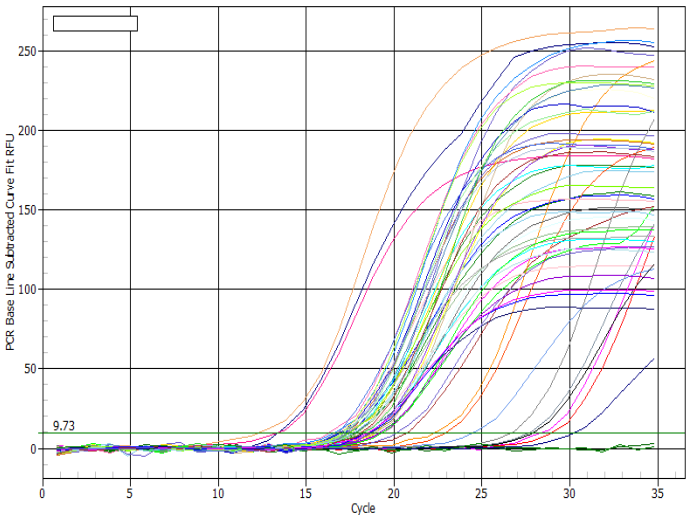


2: Archaeal 16S rRNA standard curve (A), amplification chart (B) and melting curve profiles (C)

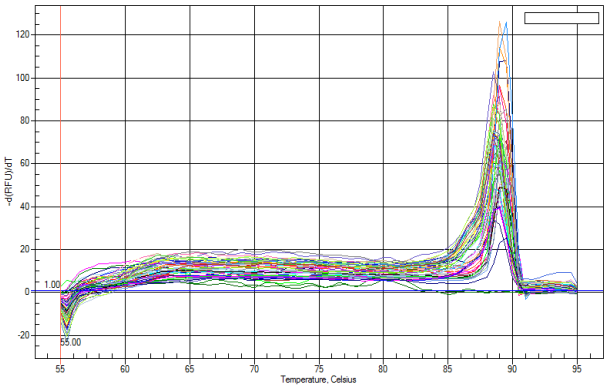
A



B

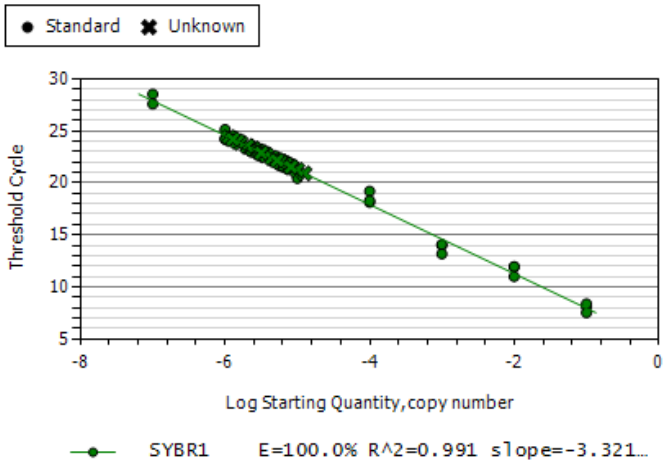


C

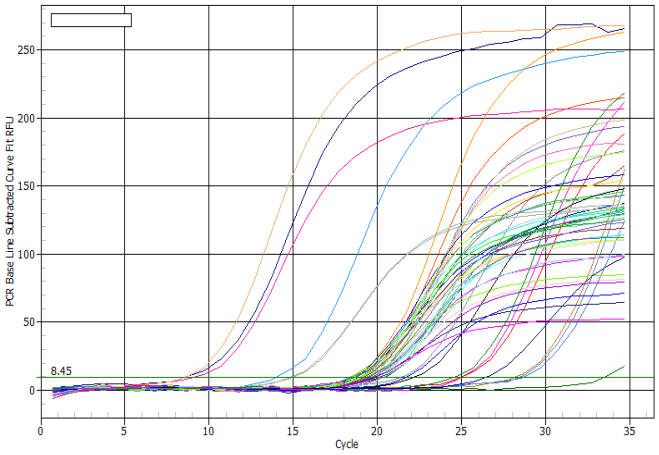


3. AOB-*amoA gene* standard curve (A), amplification chart (B) and melting curve profiles (C)

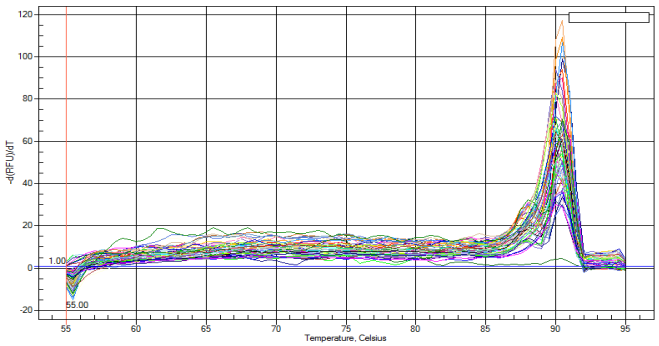
A



B

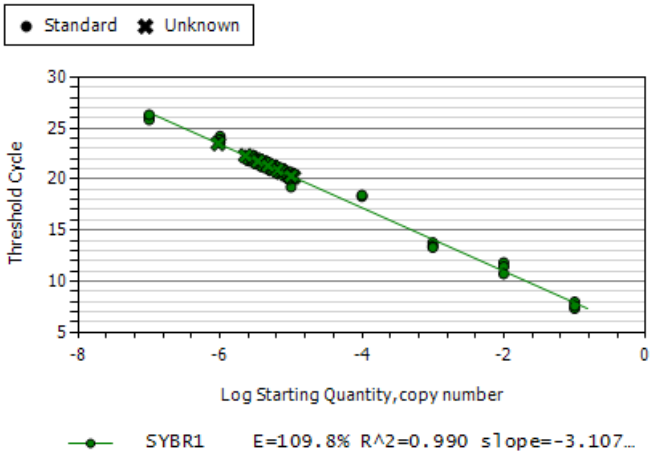


C

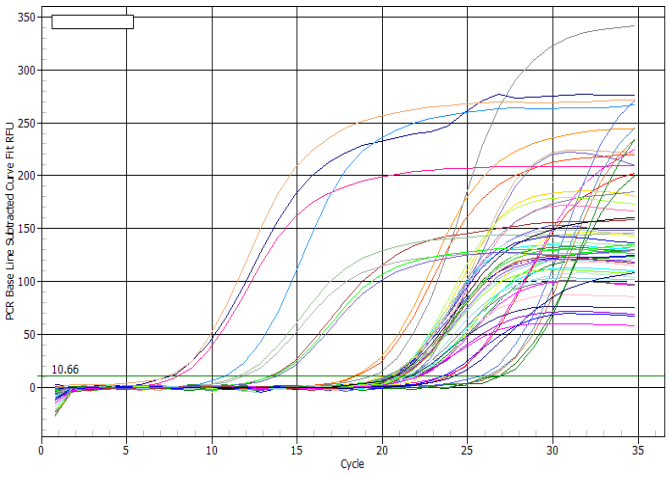


4. AOA-*amoA gene* standard curve (A), amplification chart (B) and melting curve profiles (C)

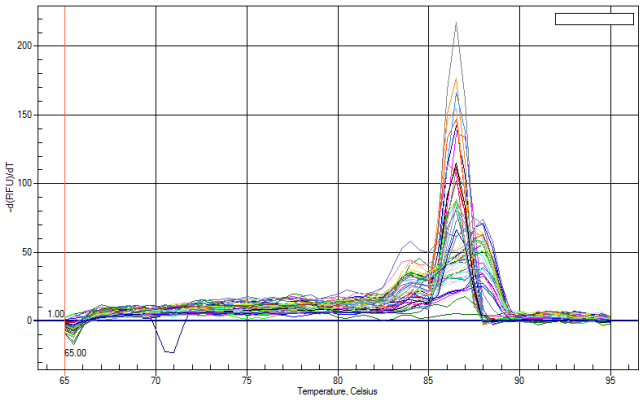
A



B



C



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SUMMARY

Summary and overview

Soil microbes are vital for soil quality and soil ecosystem processes due to their involvement in organic matter dynamics, nutrient cycling, decomposition of organo-pollutants and soil formation by rock weathering. Characterizing microbial diversity and activity in soils, would enable a better management of the soil ecosystem functions, because the ability of an ecosystem to withstand serious disturbances may be reflected in its microbial component. How soil microbial activities and communities are affected by anthropogenic and environmental perturbations in semiarid alluvial regions of Indian subcontinent, which occupy about 37 % of the total geographic area of the country, is not well understood.

The present study investigated the effect of anthropogenic and environmental parameters on soil microbial activity and diversity in semiarid alluvial soils (developed on River sediment) around Mahi River region, western India. The soils along the region studied are well characterized geomorphologically and presented several unique features that are of interest in studying from a microbiological point of view. First, the alluvial soils have been dated and can be classified as relatively recent and older (last 30 ka to 45 ka); secondly, they have been mapped as agriculturally fertile or badlands, some of them of same chronological age and of common sediment origin; third, there are certain spots along the estuarine part of the river where treated industrial effluent is discharged in to the water bodies; fourth, some regions along the river margin have succumbed to seasonal floods and suffer regular inundation; lastly several exposed sections of the sediments up to several meters in depth are accessible which have been extensively characterized geochemically and geomorphologically and stratigraphy correlated with chronological dating of the sedimentation events is available. These exposed sections allowed access to deeper and older sediments many of which are buried soils or palaeosols. Several of the features of the region under study have been exploited in this work to compare microbiological activity and diversity and provided an opportunity to investigate different geomorphologic settings under a single climatic zone (semiarid). Further, the study was extended to coastal soils developed on carbonated rocks under same climatic zone wherein the microbial weathering of parental rock “miliolite” was studied.

The approach used for the different studies here was polyphasic, involving cultivation-dependent as well as molecular studies and *in-situ* activity measurements. Qualitative and quantitative components of microbial community structure have been assessed, which made it possible to understand to some extent the relationship between structure of functional microbial guilds and the soils parameters in the ecosystem. Following section provides a brief summary of the thesis, chapterwise, for the five Chapters (Chapters 3-7) which present the research findings of this work.

Chapter 3. *Establishing a link between soil characteristics and microbial activity processes in semiarid Mahi River basin by polyphasic approach*

Estuarine and alluvial soils were compared for microbial activity (as an index of soil enzymes i.e. dehydrogenase and protease) in relation with soil properties such as soil organic matter (SOM), soil moisture content (SMC), soil texture (sand, silt and clay) and soil pH has been addressed. The study was conducted at 7 sites spread over 3 locations (2 were from alluvial zone and 1 was from estuarine zone), each sampled at interval of 25 cm from the three exposed sections along a 30 km long stretch of the Mahi River in western India. High microbial activity was noticed in estuarine soils than in alluvial soils. Dehydrogenase activity in both alluvial and estuarine soils indicated positive correlations with SOM, SMC and a moderate correlation with clay content. On the contrary, the protease activity showed poor correlation with SOM, SMC and clay content of alluvial soils, however significant positive correlations were noticed in estuarine soils. No correlation was observed between these two enzymes. A negative correlation existed between soil depth and both the enzymes at P level < 0.001. The findings demonstrate that SOM and SMC, clay content and soil depth are the important determinants for dehydrogenase activity (indicative of organic matter transformation) in both alluvial and estuarine soils, whereas the soil depth is the lone determinant for protease in alluvial soils and its correlation with other properties in estuarine soils is site specific.

Investigations on palaeosols intercalated within the late Quaternary continental sequences of Mahi River basin indicates that two palaeosols P1 and P2 have been dated back to ~ 45 and 30 ka, respectively. The two palaeosols were compared for their soil and microbiological characteristics. High microbial biomass carbon, soil respiration, microbial quotient, metabolic quotient (q-CO_2) and

dehydrogenase activity revealed that the organic matter decomposition and carbon flow to the atmosphere were much higher in P1 palaeosol. Soil *in-situ* enzymes, specific enzymatic quotients (SEQ) and specific enzyme indices (SPI) revealed that P1 has been more productive than P2. The order of biogeochemical cycling in P1 was N >P ≥C whereas P2 showed N >P >C. 16S rRNA gene profiling using denaturing gradient gel electrophoresis (DGGE) indicated greater heterotrophic bacterial diversity and higher carrying capacity of P1 compared to P2. The contrasting microbial activities and diversity in these palaeosols point to different environments during palaeosol formation and are in general agreement to the climatic inferences drawn earlier workers.

A study involving the microbiological profiling of an entire exposed section (approximately 30 m in height) was carried out to explore critical physiochemical determinants that govern microbial activity in the different strata of exposed subsurface soils and sediments along the Mahi River bank. A total of 51 triplicate composite samples were collected at an interval of 25 cm from exposed soils and sediments at a subsurface section measuring about 28 m length near Rayka. Enzyme activities (dehydrogenase [DHA] and phosphatase [APA]), were varied particularly with the sampling depth and nature of the soil. Along the section, 5 microbial activity hot spots distributed at a depth of 2 to 2.5 m, 4.75 m, 6.5 m and 7.5 m were found. High microbial diversity as indicated by 16S rRNA-DGGE was noticed in sandy silt, silt with bedded calcrete, brown soil and surface top soil. DGGE based α-proteobacterial diversity studies revealed vertisol had higher alpha proteobacteria diversity which is an indicative of good soil health. Multivariate statistical analysis such as principle component analyses (PCA), canonical correspondence analysis (CCA), linear regression models and Pearson linear correlations were carried out to ascertain the critical geochemical, mineralogical and other soil parameters that may determine the microbial activity. Results indicated that organic carbon (OC), SMC, fungal spores (FS), black carbon (BC), structured carbon (SC), Al, Fe, Mg, Mn and Zr are the critical determinants for Rayka soil and sediment microbial activity at P <0.05. Further, microbial activity (as an index of enzymes and respiration) and their relation with various phosphorus fractions were deciphered. Significant positive relation (r = 0.8; P <0.001) between APA and organic phosphorous % (OP %) was observed. A weak negative correlation (r = 3.9) between inorganic phosphorous (IP) and APA also

noticed at P <0.05. Basal soil respiration (BSR) was positively correlated with both IP (r = 0.55, P <0.05) and OP (r = 7.8, P <0.01). Although there was a significant (P <0.05) relation between OP and DHA, the strength of the relation is (r = 3.9) weak. A negative correlation was observed between apatite inorganic phosphorous (AIP %) and BSR (r = -0. 58).

Chapter 4. *Microbial activity and diversity profiles of agricultural, pristine, flood affected and unaffected soils of common sedimentary origin: Case studies from Rayka, Mujpur and Pilludra Gujarat, western India.*

Agricultural (Agri) and pristine (Pri) soils (considered as badlands) studied in the present investigation were collected from two different fields situated near Rayka. Geologically both the sites were evolved simultaneously and fall under the same sedimentary origin. Significantly higher (P <0.05, LSD) values of both C_{mic} and BSR were found in agricultural soils. An increase in soil *in situ* enzymes was noticed with agricultural soils (P <0.05, LSD, N = 3). Enzymes such as DHA, APA, GSA, and PSA were reduced to 29.1%, 43.4%, 18.9% and 25.78% respectively in pristine soils. Bacterial diversity of investigated with 16S rRNA based DGGE molecular fingerprinting indicated very complex but not very contrasting microbial diversity in both agricultural and pristine soils. Relatively high total 16S rRNA based microbial diversity was noticed in agricultural soils than in pristine soils.

A large discrepancy in total viable count of diazotrophs was noticed. The counts were 2.5 x 10^6 (in agri soil) to 4.5 x 10^4 (in pri soil). Amplified ribosomal DNA restriction analysis (ARDRA) indicated that agricultural soil diazotrophic bacterial isolates fall in 12 different phylogenetic groups where as pristine soil isolates fall in 9 different groups at a similarity coefficient of 0.90. These results tentatively indicate that there was relatively higher diversity in culturable diazotrophs of agricultural soil than in non-agricultural soil. Most of the agricultural soil isolates are represented under phyla *Proteobacteria, Actinobacteria* and group *Firmicutes w*hereas in pristine soil, most of the isolates were affiliated to *Firmicutes*. 16S rRNA based diazotrhophic community was established with culture dependent -DGGE (CD-DGGE) and activity was determined based on their *in-situ* respiration activity. Higher diazotrophs diversity was noticed with agricultural soil than pristine soil by CD-DGGE. Relatively high amount of BSR activity (88.24± 9.3 µg CO_2-C ml broth^{-1})

was noticed with agriculture soil consortium than in pristine soil based consortium (52.84± 9.3 µg CO_2-C ml broth^{-1}).

Effect of annual floods (environmental impact) on soil microbial diversity and activity in agricultural soils has been studied. Three soil samples named as S1 (0 -10 cm), S2 (10-20cm) and S3 (20-30cm) were collected with an interval of 10 cm from an agriculture field near Mujpur which was inundated with fluvial sediment. Control reference soil named S4 was collected from nearby unaffected agricultural field from Pilludra. Relatively high amount of heavy metals were detected in the flood affected soils. Inundated fluvial sediment significantly decreased (P <0.05) the soil biochemical properties (C_{mic}, BSR and SIR) in this very particular region. Microbial metabolic potential (q-CO_2) was higher in S1and S2 samples. The detrimental effect of fluvial sediment on soil enzymes (DHA, GSA, APA and PSA) was noticed. Significant lower amount of total cultivable bacteria was observed in flood affected soils. Most probable number (MPN) indicates that high amount of the nitrogen fixers, denitrifiers, phosphate solubilising bacteria, cellulose degrading bacteria were observed in unaffected agricultural soil (S4). High amount of H_2S producers were indentified only with flood effected S1. Majority of the dominant bacterial phyla in S4 samples were *Proteobacteria*, *Actinobacteria* and *Firmicutes*; wile dominant phyla in S1 samples are found to be *Firmicutes, Actinobacteria* and *Acidobacteria*. Bacterial genera found in S4 sample were *Bacillus (*Family *Bacillaceae), Naxibacter (*Family *Oxalobacteraceae, Arthrobacter (*Family *Micrococcaceae*) *and Beijerinckia (*Family *Beijerinckiaceae).* Bacterial isolates affiliated to genera *Bacillus, Promicromonospora*. *(*Family *Promicromonosporaceae*) and *Granulicella* (Family *Acidobacteriaceae)* were found in S1 sample. The 16SrRNA gene based bacterial diversity as detected by DGGE was higher in S4 as compared to flood affected samples. Statistical analysis indicated an apparent negative impact of sand and heavy metals on microbial activity parameters. A strong positive correlation among SMC, TOC, clay and soil microbial activity parameters was noticed in this particular environment.

Chapter 5. *Microbial activity and diversity profiling in pristine and long term industrial effluent contaminated semiarid landscapes*

Soil pollution by elevated heavy metals is a major concern in alluvial plains. In the present study microbial activity, diversity and microbial ammonia oxidative functions in treated industrial waste effluent (IWE) polluted soils were investigated by polyphasic approach. Soil samples were chosen from three different locations under semiarid climate. Sample R1 is a natural pristine soil without any contamination, collected from a site nearby Ryaka. Samples R2 and R3 were collected from 2 different sites situated along the contaminated Mini River basin. These sites have been receiving mixed bag of contamination for several years by episodic streams of Mini River containing industrial waste water. R4 is a sediment sample collected from IWE settling lagoon near Sarod.

ICP-AES analysis revealed the elevated levels of heavy metals in samples R2, R3 and R4. Particularly elevated heavy metals such as Cu, As, Cr and Pb were found in R3 and R4 samples. Significantly lesser (P <0.05, LSD) values of soil C_{mic}, BSR and SIR and *in-situ* microbial enzymes such as DHA, APA, ß-glucosidase (GSA), and protease (PSA) were found in metal polluted soils (R2, R3 and R4). Higher q-CO_2 values found in contaminated soils. Contrasting eubacterial community shift along the contamination gradient was noticed by 16S rRNA based DGGE molecular finger printing. No significant (P <0.05) difference among the diversity indices H^I and E^I was observed. However E^I values were relatively high in R2 and R3 samples. A total of 26 bands were excised from the DGGE gel, reamplied, cloned and sequenced for phylogenetic analysis.

Major eubacterial phyla in R1 soil were affiliated to *Poteobacteria and Actinobacteria,* where as phyla *Actinobacteria, Firmicutes* and *Acidobacteria* were dominant in R2 and R3 soils. Phylum *Spirochaeta* was found only in R2 soil. Classes *Alphaproteobacteria* and *Betaproteobacteria* were found to be specific to R1 where as classes *Epsilonproteobacteria* and *Gammaproteobacteria* were specific to R2 and R3 samples. Major euabcterial phyla in contaminated sediment R4 were found to be *Actinobacteria, Firmicutes and Acidobacteria.* Dominance of unclassified bacteria was observed in contaminated soils (R2 and R3) and sediment (R4).

Potential nitrification rate (PNR) was found to be highly sensitive to soil contamination. Quantitative real time PCR (q-PCR) results indicate that relatively, high amount of bacterial 16S rRNA, archaeal 16S rRNA, ammonia monooxygenase

gene (*amoA*) of ammonia oxidizing archaea (AOA-*amoA)* and of ammonia oxidizing bacterial (AOB-*amoA)* copies (log transformed) were found in uncontaminated R1 soil than contaminated R2 and R3 soils. Both AOA and AOB-amoA gene copies significantly decreased in contaminated soils. In R1 the ratio of AOA-*amoA* to AOB-*amoA* gene was found to be high (36.82 ± 6.75), however the ratios significantly (*P* <0.05, LSD) decreased in R2 and R3 soils. DGGE analysis of *amoA* gene diversity indicated complex and contrasting AOA-*amoA* gene and AOB-*amoA* gene profiles along the contamination. Relatively, higher AOA-*amoA* and AOB-*amoA* gene diversity was noticed in pristine R1 soil than contaminated R2 and R3 soils. Phylogenetic analysis of the nucleotide sequences retrieved from DGGE bands revealed that thaumarchaeal "group 1.1b" was dominant AOA in these soils. Thaumarchaeal "group 1.1a" was found only in contaminated soils (R2 and R3) where as thaumarchaeal "group 1.1a associated" was found only in pristine soil R1. All the 10 AOB-*amoA* DGGE band clones were affiliated to *Nitrosospira*-like sequences and *Nitrosomona*s-like sequences. Nevertheless, *Nitrosospira*-like sequences were dominant ones, consisted of 70% of total sequences. *Nitrosomonas nitrosa* cluster-8 was found only in contaminated soils, whereas *Nitosospira* cluster-11 was specific to pristine soil. *Nitrosospira* cluster-3a is common for all three soil samples. In addition to this, *Nitosospira* cluster-1, 2, and 4 were also noticed among soil AOB-amoA genetic profile.

Microcosm studies were conducted to investigate the initial response of AOB and AOA to heavy metals Cu and As. Two different acidic alfisols *viz*. RA and GZ spiked with different concentrations of As, Cu and As+Cu were incubated for 10 weeks. Significant reduction (*P* <0.05) in copy numbers of archaeal 16S rRNA, bacterial 16S rRNA and AOB-*amoA* genes was noticed at elevated metal concentrations, while reduction in AOA-*amoA* gene copy numbers was minimal. AOA was found to be more abundant than AOB in all the treatments and the ratio of AOA to AOB ranged among 94-303 in RA soils and 21-39 in GZ soils. The soil PNR differed across different treatments, and significantly (*P* <0.05) decreased with increasing As and Cu-concentrations. PNR was significantly correlated with both AOA and AOB, nevertheless the correlation was much stronger for AOA. DGGE of functional AOA- *amoA* gene revealed no apparent community shift for AOA even at higher concentrations of As and Cu. Phylogenetic analysis of AOA-*amoA* gene from

4 clone libraries indicated that all the AOA-*amoA* gene sequences (400) were placed within 3 distinct clusters from soil and sediment group 1.1b. Cluster-III of group 1.1b was found to be the most dominant one containing 51%-59% of total sequences, indicating their potential role in nitrification of acidic alfisols polluted with As and Cu.

Chapter 6. *Exploring multimetal resistant plant growth promoting bacteria (PGPB) for enhancing plant growth in heavy metal polluted soils.*

Metal resistant bacteria were isolated form sediment contaminated with industrial effluent R4. Isolates were identified and screened for their plant growth promoting (PGPR) traits. Gnotobiotic plant inoculation experiments were conducted with the most promising bacterial isolate identified as *Enterobacter* sp. (by 16S rRNA gene sequence phylogeny) to evaluate its plant growth promoting potential under elevated levels of Cr (VI) (up to 400 mg kg^{-1}soil) and Pb (II) (up to 400 mg kg^{-1}soil). *Enterobacter* sp. C1D was found to be multi-metal resistant in nature with multiple plant growth promoting traits. In order to know the mechanisms involved in plant growth promotion under elevated metal concentrations, PGPR traits were studied in presence of metal.

Cr (VI) toxicity was noticed in above and below ground biomass of *Vigna radiata* GM4 during plant experiments. However, plant inoculation studies with *Enterobacter* sp. C1D significantly increased (P = 0.05) root length, shoot length, shoot weight, root weight and chlorophyll content of the plants in a range of Cr (VI) treatments. Plant tolerance towards Cr (VI) measured as effective concentration (EC50) showed higher values with *Enterobacter* sp. C1D treated plants as compared to the uninoculated plants at all concentrations of Cr (VI). High amount of acidic and alkaline periplasmic phosphatases, elevated IAA production and ACC deaminase activities probably enable *Enterobacter* sp. C1D to enhance plant growth in Cr (VI) amended soils.

Chapter 7. *Bacteriology of miliolite, a bioclastic lime stone: Bacterial diversity, activity and potential role of bacterial in weathering of miliolite*

Miliolite and surface red soil sequences were collected from the miliolite-bearing natural section in Gopnath, Gujarat, India. Calcium was found to be the most

abundant element in miliolite and its proportion was found to be 75 ± 6.71% as revealed by X-ray fluorescence XRF. Potential carbonate dissolving bacteria were isolated, characterized and screened their ability to disintegrate miliolite. A total of 14 bacterial isolates showed significant Cz/Cs ratio (zone of clearance/colony size) on DB medium containing $CaCO_3$ and miliolite agar. Further, these isolates were chosen for organic acid profiling by HPLC. Lactic acid and acetic acid are found to be major organic acids, while pyruvic acid and malic acids are minor ones in assisting miliolite dissolution by these organisms. Phylogenetic diversity of carbonate dissolving bacteria was deciphered by ARDRA and 16S rRNA sequencing analysis. *Firmicutes* (Families *Bacillaceae* and *Staphylococcaceae*) and *Actinobacteria* (Family *Promicromonosporaceae*) represent two keystone culturable carbonate-dissolving heterotrophic bacterial phyla involved in carbonate dissolution and attendant miliolite weathering. Carbon sources had contrasting effects on bacteria mediated Ca^{2+} release miliolite. Short-term microcosm experiments were conducted with isolates *Staphylococcus* sp. M16, *Bacillus* sp. M12 and *Xylanimonas* sp. RS25 for demonstration of bacterial mediated miliolite weathering. Results clearly indicated the bacterial metabolic activity in dissolution of calcite. Bacterial colonization, erosion and pitting of calcite crystals was well documented by scanning electron microscopy SEM. Calcite dissolution was further confirmed by XRD pattern and XRF analysis. The order of bacterial ability in dissolution of miliolite was found in the following order *Staphylococcus* sp. M16 > *Bacillus* sp. M12 > *Xylanimonas* sp. RS25. SEM images indicate that bacterial activity increased the *surface area of calcite*, so that the process of chemical and biological *weathering* would be further accelerated. Calcite mineral surfaces were aggressively coated with a thick complex microbial biofilm by *Staphylococcus* sp. M16. The present study improves our conceptual understanding of microbial communities as important players in carbonate weathering, which has a wide range of implications; from the elucidation of biogeochemical cycles to the potential impact of atmospheric CO_2 sinks.

Salient research findings of the work

Table S1 provides a summary of the different microbial activity and diversity parameters analyzed in various settings. The key findings that have emerged from this work are emphasized below.

- Relatively estuarine zone showed high microbial activity as compared to alluvial zone in the semiarid Mahi River region. Two soil enzymes dehydrogenase and protease showed different relationships with soil properties depending upon the alluvial and estuarine zones. SOM, SMC, clay content and soil depth are the main determinants for soil dehydrogenase activity and subsequent organic matter transformation in both zones. Soil protease activity and its critical determinates were found to be site specific.
- The order of microbial activity in biogeochemical cycling in chronologically older (45 ka) P1 palaeosol was found to be N > P ≥ C, whereas in P2, relatively younger (30 ka) palaeosol it was N > P > C. P1 showed high heterotrophic bacterial diversity and broader carrying capacity thanP2. Contrasting microbial activity and diversity in these Mahi River palaeosols indicate two different palaeo-environments and are in agreement with differences in climatic inferences drawn by earlier workers.
- Microbial activity and diversity are affected by contrasting soil or sediment physicochemical gradient in the exposed section of nearly 30 m in the semiarid region. Multivariate statistical analysis indicated that OC, OP, SMC, FS, BC, SC, Al, Fe, Mg, Mn and Zr are the critical determinants for microbial activity in exposed shallow subsurface soils and sediments of Mahi River basin.
- Soil management practices influenced soil physical and chemical characteristics and brought about changes in the soil microbial activity, community structure and function. Semiarid soil quality has been improved by sustainable agricultural practices. Higher bacterial diversity of eubacteria in agricultural soil as compared to pristine soil. Agriculture practice enhanced the diazotophic bacterial population size. Floods have led to distinct alterations of the soil ecosystem functioning. Microbial mediated organic matter dynamics, nutrient cycling and decomposition processes in agriculture fields are decreased by fluvial sediment inundation. The soil health in the flood affected field is in endangered which suggest the importance of modifying management for sustainable agriculture. Enzymes, q-CO_2 values, bacterial phyla *Firmicutes*, *Actinobacteria* and *Acidobacteria* can be used as sensitive biological measure to assess or detect the soil disturbances lead by environmental perturbations in semiarid soils.

- Contamination by industrial waste effluent (IWE) had significant negative impacts on bacterial abundance, activity and community structure in semiarid soil/sediments. Microbial parameters such as C_{mic} and extracellular enzymes are found to be good responsive measures for monitoring the toxicity of IWE deposited in the environment. It can be infered that dehydrogenase, q-CO_2 and bacterial phyla *Firmicutes*, *Actinobacteria* and *Acidobacteria* could be used as soil indicative parameters for soil contamination by mixed bag of pollutants.
- Nitrification activity was found to be significantly (P <0.05) decreased by IWE in semiarid soils. Relatively, AOB-*amoA* gene diversity is more decreased than AOA-amoA diversity by IWE. Thaumarchaeal "group 1.1b" might be playing potential role in ammonia oxidation of pristine and IWE contaminated soils.
- Soil potential nitrification rate (PNR) was significantly decreased by heavy metals As and Cu in acidic alfisols. AOA were found to be more abundant than AOB in As and Cu spiked acidic alfisols. Together these results may implicate the dominant role of AOA over AOB in ammonia oxidation observed in Cu and As amended acidic alfisols. AOA-community did not respond to As and Cu in acidic alfisols. Thaumarchaeal AOA group 1.1b might be playing potential role in ammonia oxidation of oligotrophic acidic soils. These results could support further the hypothesis that physiological differences between AOA and AOB may facilitate them to occupy distinct ecological niches. These results indicate that soil PNR could be used as soil quality indicator for heavy metal perturbations.
- Heavy metals significantly reduced plant growth parameters of of *Vigna radita* GM4 (mung bean) plants. The bacterial strain *Enterobacter* sp. C1D exerted substantial growth promoting effects on the *Vigna radiata* var GM4 plants in Cr (VI), Pb (II) stress and unstressed conditions. Plant tolerance towards both Cr (VI) and Pb (II) has been significantly increased by *Enterobacter* sp. C1D. High amount of acidic and alkaline periplasmic phosphatases, elevated IAA production and sufficient ACC-deaminase activities probably enable *Enterobacter* sp. C1D to enhance plant growth in Cr (VI) and Pb (II) amended soils.

- Bacterial phyla *Firmicutes* and *Actinobacteria* represent two keystone culturable carbonate-dissolving heterotrophic bacteria involved in *in-vitro* carbonate dissolution and attendant miliolite weathering. The order of bacterial ability in dissolution of miliolite was in the following order *Staphylococcus* sp. M16 > *Bacillus* sp. M12 > *Xylanimonas* sp. RS25. Lactate and acetate are two major organic acids in dissolving miliolite by the organisms. The efficacy of organic acids in dissolution of miliolite was found to be in following order: acetate > lactate > succinate > gluconate > pyruvate.

Other contributions in terms of tangibles obtained through this work are as follows:

- About 500 new nucleotide sequences have been added to the GenBank database. Out of these 80 are 16S rRNA gene sequences from cultivated microbes as well as clones. They represent bacteria present in hitherto unexplored environmental settings. Remaining 425 are sequences of AOA-*amoA* and AOB-*amoA* genes. These sequences represent potential ammonia oxidizing microbes in polluted soils.
- A collection of novel bacterial isolates have been obtained. They have interesting properties such as N fixation, multimetal resistance, miliolite (carbonate) dissolution. A particular multi-metal resistant bacterial isolate *Enterobacter* C1D, has excellent potential as a biofertiliser for plant growth promotion in metal polluted soils. Carbonate dissolving bacterial isolates obtained could be further utilized in applied biotechnological aspects (e.g. dissolution of carbonate clogs from irrigation pipes)

Table S1: Summary of the microbial activity and diversity parameters of discrete soil ecosystems of Mahi River basin. Rows with same colour are compared with each other.

Sample	DHA	APA	PSA	GSA	BSR	C_{mic}	SIR	q-CO_2	DGGE Diversity	Dominant bacterial phyla[¥]
Agricultural soils	High* (172.4)	High * (378.7)	High * (607.4)	High * (292.7)	High * (2.78)	High (484.5)	High* (16.5)	N. S. (0.137)	High*	Proteobacteria,Actinobacteri Firmicutes
Pristine soils	low* (131.6)	low* (214.1)	low* (450.7)	low* (237.3)	low* (1.91)	low (373.8)	low* (10.7)	N.S. (0.133)	low*	Firmicutes
Industrial effluent contaminated soils	low* (55.1 to 73.1)	low* (89.7 to 177.7)	low* (203.5 to 334)	low* (91.4 to 142.5)	low* (0.9 to 1.22)	low (148.4 to 236.9)	High (14.6 to 11.1)	High* (0.146 to 0.13)	N. S. (16S rRNA), low (amoA)*	Acidobacteria, Actinobacteria Firmicutes
Unaffected pristine soil	High* (98.37)	High* (246.1)	High* (412.7)	High* (185.2)	High* (1.33)	High (298.2)	low (12.69)	low* (0.107)	High* AO [ϒ]	Bacterioidetes, Actinobacteria, VerrucomicrobiaProteobacteria
Flood affected agricultural soils	low* (40.3 to 74.2)	low* (95 to 174.2)	low* (308.7 to 370.2)	low* (62.8 to 132)	low* (1.86 to 2.3)	low (217.1 to 332)	low* (9.2 to 13.3)	High* (0.2 to 0.166)	low*	Acidobacteria, Actinobacteria and Firmicutes
Unaffected agricultural soils	High* (94.8)	High* (218.9)	High* (424.8)	High* (175.7)	High* (2.71)	High (397.8)	High* (19.8)	High* (0.163)	High*	Proteobacteria, Actinobacteria and Firmicutes
Subsurface	High*	High*	-	-	High		High	-	-	-

Vertisols	**(60 to 65)**	**(250 to 500)**			**(3 to 5)**		**(6 to 8)**			
Red soils	**Low (40 to 60)**	**low* (120 to 300)**			**low* (0.82 to 2)**		**low* (5 to 6.25)**			**-**
Burried Palaeosol P1	**High* (81.5)**	**High* (180)**	**High* (502.3)**	**High* (166.5)**	**High* (3.05)**	**High* (282.6)**	**High* (7.3)**	**High* (0.25)**	**High ***	**-**
Burreid Palaeosol P2	**High* (47.6)**	**low* (129.9)**	**low* (358)**	**low* (68.1)**	**low* (1.78)**	**low* (221)**	**low* (3.26)**	**low* (0.19)**	**low***	**-**
Estuarine soils	**High* (20-80)**	**-**	**High* (500 to 1800)**	**-**	**-**		**-**	**-**	**-**	
Alluvial pristine soisl	**low* (12 to 50)**		**low (200 to 1200)**	**-**	**-**		**-**	**-**	**-**	

DHA: dehydrogenase; APA: phosphatase; PSA: protease; GSA: glucosidase; C_{mic} : Microbial biomass C; BSR: basal soil respiration; SIR; substrate induced respiration; q-CO_2: metabolic quotient; High and low indicate relative comparison where * indicates significant at *P* <0.05). ¥ deciphered by 16S rRNA sequencing. Actual values are provided (in parenthesis) for allover comparison. The units for the values are - DHA: µg of TPF g^{-1} soil h^{-1}; APA: µg p-NP g^{-1} soil h^{-1}, PSA: µg tyrosine g^{-1} soil 2 h^{-1}; GSA: µg p-NP g^{-1} soil h^{-1}; C_{mic} : mg kg^{-1} soil; BSR: µg CO_2-C gm^{-1} soil h^{-1}; SIR: µg CO_2-C gm^{-1} soil h^{-1}; qCO_2: µg CO_2-C mg C_{mic} $24h^{-1}$; N.S.: Not significant. ϒ: Higher ammonia oxidizers (AOA and AOB) diversity.

PUBLICATIONS

AND PRESENTATIONS

Published / Accepted articles

1. Subrahmanyam G, Vaghela R, Bhatt N and Archana G. (2012). Carbonate dissolving bacteria from 'miliolite', a bioclastic limestone, from Gopnath, Gujarat, western India. *Microbes and Environments* **27:** 334-337

2. Subrahmanyam G, Archana G and Chamyal LS. (2011). Microbial activity and diversity in the late Pleistocene paleosols of alluvial Mahi River basin, Gujarat, western India. *Current Science*. **101:** 202-209

3. Subrahmanyam G, Archana G and Chamyal LS. (2011). Soil microbial activity and its relation to soil indigenous properties in semiarid alluvial and estuarine soils of Mahi River basin, Western India. *International Journal of Soil Science* **6:** 224-237.

4. Subrahmanyam G, Khonde N, Maurya DM, Chamyal LS and Archana G. (2013). Microbial activity and culturable bacterial diversity in the sediments of Great Rann of Kutch, a unique ecosystem, Western India is accepted for publication in *Pedosphere*. Manuscript number: PEDOSPHERE121201399

Articles under review

1. Subrahmanyam G, Hang-Wei Hu, Yu-Rong Liu, Li-Mei Zhang, Yuan-Ming Zheng, Archana G and Ji-Zheng He (2013). Effects of As and Cu on ammonia oxidizing archaea and bacteria in two acidic alfisolsis is under review in *Chemosphere*. Manuscript number: CHEM27347.

Articles under communication

1. Subrahmanyam G, Archana G and Chamyal LS. (2013). Multivariate statistical analysis of microbial activity, diversity and it's relation to soil physiochemical characters of exposed vadosezone around Mahi River basin, western India is under preparation.

2. Subrahmanyam G and Archana G (2013). "Multimetal resistant *Enterobacter* sp. C1D enhances plant growth in Cr (VI) amended soils by IAA production and ACC deaminase activity" is under review is under preparation

3. Subrahmanyam G, Hang-Wei Hu, Yu-Rong Liu, Archana G, and Ji-Zheng He. (2013) Activity and diversity of ammonia oxidizing archaea (AOA) and bacteria (AOB) in long-term industrial effluent polluted soils, western India is under preparation

4. Subrahmanyam G and Archana G. (2013). Microbial diversity and activity in flood effected fields of Mahi River basin, western India is under preparation

5. Subrahmanyam G and Archana G. (2013). Characterization of carbonate dissolving heterotrophic bacteria on dissolution of miliolite: A short term microcosm study is under preparation.

Articles published in international conference proceedings

1. Subrahmanyam G and Archana G. (2011). Plant growth promoting activity of *Enterobacter* sp. C1D in heavy metal contaminated soils. pp. 436-442. Proceedings of the 2nd Asian PGPR Conference, August 21-24, 2011, Beijing, PR China.

2. Subrahmanyam G, Ravi V, Bhatt N, Archana G. (2010). Bacteriology of miliolite, a bioclastic lime stone: bacterial diversity and activity in weathering of miliolite. pp. 119-122. Proceedings of initial ecosystems, MYRES, Sep-20-24, BTU, Cottbus, Germany. (**www.tu-cottbus.de/sfb_trr/Conf_2010/pic/Proceedings_InitialEcosystemDevelopment_2010.pdf**)

Papers presented in national and internal conferences

International conferences

1. Subrahmanyam G and Archana G, "Plant growth promoting activity of *Enterobacter* sp. C1D in heavy metal contaminated soils" was presented as a **poster** in 2nd Asian PGPR congress held at China Agricultural University, during August 21-24, 2011, Beijing, PR China.

2. Subrahmanyam G and Archana G, "Bacteriology of miliolite, a bioclastic limestone: Diversity and their possible potential activity in weathering of miliolite" presented as **oral presentation (Invited lecture)** in MYRES, held at Brandenburg University of Technology, during September 20-24. 2010, Cottbus, Germany.

3. Subrahmanyam G and Archana G, "Plant growth promoting activity of metal resistant *Enterobacter sp. C1D* isolated from heavy metal polluted site, Sarod, western India" was presented as a **poster** in International workshop on Rhizosphere Biology of Agriculture, Horticulture and Forestery: Present and Future held at J.B. Pant Agricultural University and Technology (J.B.P.U.&T.) during February 25th-27th, 2010, Pantnagar- 263 145, India.

4. Subrahmanyam G and Archana G. Diazotrophic bacterial diversity and activity in semi-arid agricultural and pristine soils of Mahi river basin, Western India was presented as a **poster** in 1st Asian PGPR congress held at Acharaya N.G. Ranga Agricultural University, during June 21st to 24th 2009, Hyderabad**,** India.

5. Subrahmanyam G and Archana G, "Microbial diversity and activity in Paleosols of Mahi River basin, western India" was presented as a **oral presentation (Invited lecture)** in International Conference and Field Workshop on Paleopedology: Paleosols, Geomorphic Evolution of Landscape and paleoclimate change held at Anna University, January 10th-14th, 2008, Chennai- 600 044, India.

National conferences

1. **Subrahmanyam G,** Naresh kumar G and Archana G. Multimetal resistant *Enterobacter* sp. C1D enhances plant growth promotion of *Vigna radiata* GM4 in Pb and Cr amended silty soils was presented as a **oral presentation** in Recent trends in sustainable agriculture, Indian group meeting of Asian PGPR society during October 22-23rd, 2012, Department of Biochemistry, The M.S. University of Baroda, Vdodara-390002, India.

2. Subrahmanyam G, Bhat A, Bhatt N and Archana G. Organic acid producing heterotrophic bacteria dissolving miliolite, A bioclastic limestone, Saurashtra, western India was presented as a **oral presentation** in Gujarat Science Congress-XVI held at M.S. University of Baroda during 26th **2012**, Vadodara-390002, India.

3. Subrahmanyam G and Archana G "Microbial diversity and activity of semiarid soils around Mahi River bank, western India" was presented as a **oral presentation** in in DST PAMC meeting on science of shallow subsurface (SSS) held at NGRI during October 12th-14th, **2009**, Hyderabad- 500 606, India.

4. Subrahmanyam G and Archana G, "Assessment of bacterial diversity and activity in a semiarid Holocene flood effected field by polyphasic approach" presented as a **poster** in Association of Microbiologists of India (AMI), 48th annual conference held at Indian Institute of Technology (IIT) during December 18th-21th **2007**, Chennai- 600 044, India.

5. Subrahmanyam G and Archana G, "Microbiology of miliolite" was presented as **oral presentation** in DST Field Workshop on Quaternary Carbonate Deposits of Saurashtra and Katchchh, Gujarat held at Diu, December 4th-10th, **2006**, Diu, India.

Awards

1. **Secured prestigious TWAS-CAS/PG international fellowship** for the year 2010 for conducting final year PhD in Chinese academy of science (CAS) institute, Research Centre for Eco-Environmental Sciences (RCEES), Beijing under the guidance Prof. Ji-Zheng He (jzhe@rcees.ac.cn).

2. Junior research fellowship (2006-2009) and senior research fellowships (2009-2010) from Department of Science and Technology (DST), New Delhi, India.

3**. Best oral presentation** award in Indian group meeting of Asian PGPR, 'Recent Trends in Sustainable Agriculture'. October 22-23, 2012, at Department of Biochemistry, M. S.University of Baroda, Vadodara-390002, India.

4. **Best oral presentation award** in DST PAMC meeting on science of shallow subsurface (SSS), October 12-14, 2009, National Geophysical Research Institute (NGRI), Hyderabad- 500 606, India.

5. Best poster presentation award in International workshop on Rhizosphere Biology of Agriculture, Horticulture and Forestery: Present and Future., February 25-27, 2010, J.B. Pant agricultural university and technology (J.B.P.U.&T.), Pantnagar- 263 145, India.

6. Selected as a potential young earth scientist by MYRES, 2010, Cottbus, Germany. **Both travel and accommodation grants** were secured**.**