# *Chapter 2*

## *Materials and Methods*

"If you cannot do great things, do small things in a great way."

- Napoleon Hill

## 2.1 Sampling site description

Location map of the lower Mahi River basin with core drilling sites Chokari (CRD) (22°13’49"N, 72°55’41"E), Rayka (also known as Raika/Rayaka) (RYD) (22°26'55"N, 73°05'27"E) and Rampura (RMD) (22°23'0.3"N, 73°05'15"E) is shown in Fig. 2.1. Core locations lie within the Vadodara district of Gujarat (India) which is characterized by a semiarid climatic condition with a mean annual rainfall of ~800 to 973 mm (https://www.weather-ind.com/en/india/vadodara-climate; https://en.climate-data.org/asia/india/gujarat/vadodara 764414/; http://cgwb.gov.in/District_Profile/Gujarat/Vadodara.pdf). Core drilling site at Chokari lies on a younger terrace on the left bank of the Mahi River estuary and located at 17.3 km (linear distance) away from the coastline while RYD and RMD cores lie on an older terrace and located ~7.2 km apart from each other (Fig. 2.1).

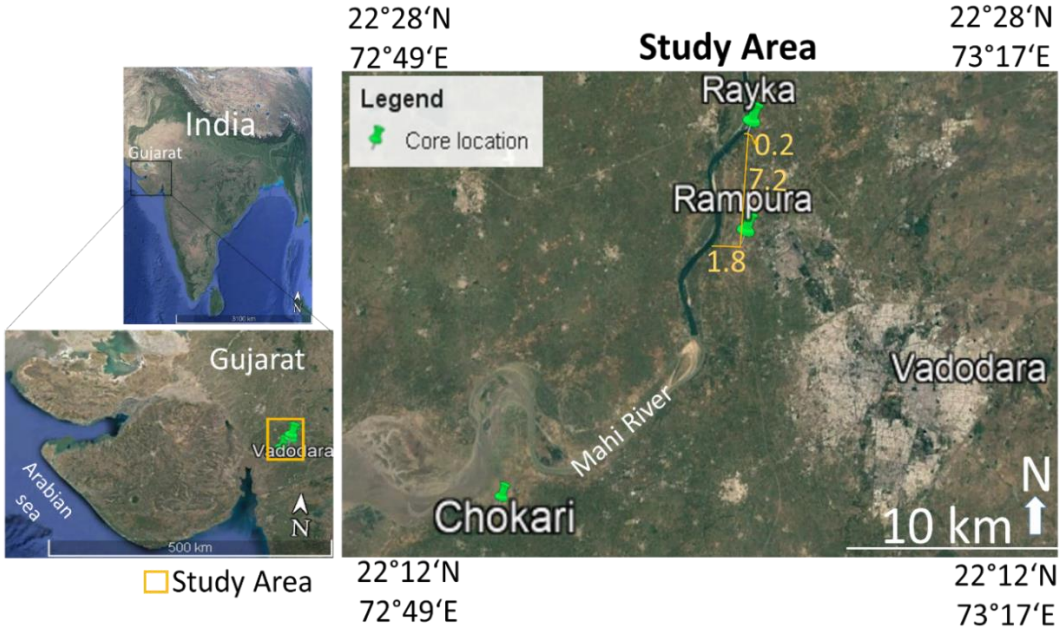


**Figure 2.1: Location map of core drilling sites and cross-sections showing core locations.** Location map of the lower Mahi River basin with core drilling sites (Source-www.googleearth.com). Values in yellow color indicate the linear distance (in km) between Rayka (RYD) and Rampura (RMD) sites and individual sites from the river channel.

## 2.2 Sediment sample collection by rotary drilling

Core samples were collected using the rotary drilling method with single tube core barrel (Rix et al. 2019). In this, mechanical rotation of drilling tool was utilized to create borehole

(drill hole), bentonite was used as drilling mud to seal the walls of the borehole to prevent a collapse of the drill hole, to cool the drill bit, and as a lubricant. During this process, drilling mud is picked up by the suction line of the mud pump and recirculated through the annulus between the drill string and the borehole wall (Kieft 2010). The drilling plastic core liner was used to collect core samples. After collection of core in the plastic core liner, drilling fluids was wiped off from the surface and labeled according to their depth. The plastic core liners with no visible fractures were selected for further studies to avoid the contamination of drilling fluids. These plastic core liners were then immediately kept under a cold condition in an icebox before being transported into the laboratory. The setup of the rotary drilling rigs at Chokari, Rayka, and Rampura are shown in Fig. 2.2a and sealed plastic core liners are shown in Fig. 2.2b. Core drilling was carried out up to depth ~17 m, ~28 m, and ~25 m depth respectively from Chokari, Rayka, and Rampura.

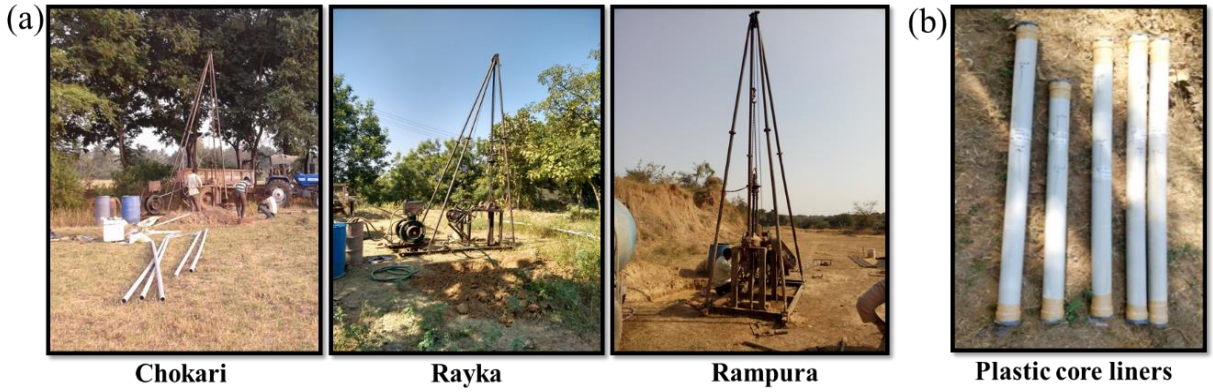


**Figure 2.2: (a) Rotary drilling rig at Chokari, Rayka, and Rampura (b) Labeled and sealed plastic core liners.**

### 2.3 Subsampling and preservation of sediment core samples

In the laboratory, plastic core liners were cut along their length using a small circular saw wiped with methanol. Geological formations/horizons (stratigraphic sequences) included within the core were identified based on physical examination and depth intervals for subsampling were decided accordingly. The lithologs for all three sediment core sections were prepared based on observed stratigraphic sequences and confirmed by grain size distribution data. The outer third to two-thirds portion of each core was aseptically pared away and only the centermost portion of the core was used for microbiological investigations. This was

accomplished using various sterilized implements: knives, spatulas, spoons, etc. Autoclaved plastic bags were used for storing the subsamples at -20 °C for DNA extraction and 4 °C for other microbial analysis. A total of 31 subsamples from CRD, 30 subsamples from RYD, and 29 subsamples from RMD have been used further for microbiological and physicochemical analysis. The locations of subsamples analyzed from each site are represented in the respective lithologs given in subsequent chapters. Each sample was homogenized thoroughly before proceeding for microbiological and physicochemical analysis.

### 2.4 Analysis of physicochemical characteristics of sediment samples

Grain size distribution analysis was carried out as described by Kunze and Dixon (1986). Salinity, pH, electrical conductivity (EC), and total dissolved solids (TDS) of sediment samples (1:5 w/v sediment to distilled water mixture was used to determine these parameters) were determined using the multi-parameter instrument Multi 3320 (WTW, Weilheim, Germany). The moisture, organic carbon and inorganic carbon contents were determined by measuring weight loss at temperatures 110 °C, 550 °C and 950 °C respectively (Subrahmanyam et al. 2021). Major element contents of bulk sediment samples were analyzed in form of their oxides using X-ray fluorescence spectrometer (S8 Tiger, Bruker) as described by Giles et al. (1995). Trace elements and rare earth elements (REEs) content of bulk sediment samples were analyzed by Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7700) as described by Subrahmanyam et al. (2021). These analyses were carried out in collaboration with Dr. Anupam Sharma, Birbal Sahni Institute of Palaeosciences (BSIP), Lucknow, India. Detailed information on these data is not shown here, but an overview of the physicochemical data of all three cores is given in the respective chapter.

### 2.5 Determination of microbial enzyme activities

Enzyme activities (involved in C, N, P, and S biogeochemical cycles) assayed included β-glucosidase activity (β-GA), dehydrogenase activity (DHA), alkaline phosphatase activity (alkaline PA), arylsulfatase activity (ASA), protease activity (PTA), and fluorescein diacetate hydrolysis activity (FDA HA).

Table 2.1 provides the details of the assay parameter for each of the enzymes studied. To measure β-GA, alkaline PA, and ASA, para-nitrophenol (*p*NP) based substrates were used and

the method was modified from that described in Taylor et al. (2000). In brief, 0.1 g core sediment sample with 400 µl of suitable buffer (Table 2.1) and 100 µl of the appropriate substrate (Table 2.1) vortexed for 1 min and incubated for 2 h at 30 °C, on an orbital shaker (200 rev $min^{-1}$). Controls containing 100 µl sterile distilled water (D/W) instead of substrate were also processed similarly. After incubation, 100 µl appropriate substrate was added in the controls and 100 µl sterile D/W to experimental tubes. Then immediately, 0.1 ml of 0.5 M $CaCl_2$ and 400 µl of 0.5 M NaOH were added to stop the reaction, and the resulting suspension was centrifuged at 6069 xg for 10 min. The yellow color of the pNP released was measured colorimetrically at 400 nm (Spectronic 20D+, Thermo Fisher Scientific, MA, USA). The optical density (OD) of the control was subtracted from the test OD to obtain the enzyme activity on the basis of µg pNP released per gram dry weight of sediment per 2 h. A standard curve was plotted using a para-nitrophenol concentration in the range of 5 to 30 µg $ml^{-1}$ D/W.

**Table 2.1: Sediment enzymes assay protocols for measuring microbial activities**

| Enzyme activity | Substrate | Buffer | Absorption wavelength | Unit | References |
|---|---|---|---|---|---|
| **β-GA** | 25 mM *p*-nitrophenyl *β* – D-glucopyranoside | 0.1 M maleate buffer - pH 6 | 400 nm | µg pNP $g^{-1}$ dry wt core sediment $2h^{-1}$ | Taylor et al. (2000) |
| **Alkaline PA** | 15 mM *p*-nitrophenyl phosphate | 0.1 M maleate buffer - pH 11 | 400 nm | µg pNP $g^{-1}$ dry wt core sediment $2h^{-1}$ | Taylor et al. (2000) |
| **ASA** | 25 mM *p*-nitrophenyl sulfate | 0.5 M acetate-buffer - pH 5.8 | 400 nm | µg pNP $g^{-1}$ dry wt core sediment $2h^{-1}$ | Taylor et al. (2000) |
| **DHA** | 1% 2, 3, 5-triphenyl tetrazolium chloride (TTC) | 0.1 M Tris buffer - pH 7.4 | 485 nm | µg TPF $g^{-1}$ dry wt core sediment $24h^{-1}$ | Subrahmanyam et al. 2016, 2011a |
| **PTA** | 2 % Na-caseinate | 0.05 M Tris buffer - pH 8.1 | 578 nm | µg tyrosine $g^{-1}$ dry wt core sediment $2h^{-1}$ | Subrahmanyam et al. 2011b |
| **FDA HA** | Fluorescein Diacetate (FDA) (2 mg $ml^{-1}$ in acetone) | phosphate buffer saline (PBS) pH 7.4 | 490 nm | µg fluorescein $g^{-1}$ dry wt core sediment $4h^{-1}$ | Adam and Duncan 2001 |

**Dehydrogenase activity (DHA)** was measured using 2, 3, 5-triphenyl tetrazolium chloride (TTC) as an artificial electron acceptor (Subrahmanyam et al. 2016, 2011a). To measure DHA, 1 g of sediment taken in 1.25 ml 1% TTC [prepared in 0.1 M Tris (hydroxymethyl aminomethane) buffer-pH 7.4] and vortexed for 1 min. After that, this reaction mixture was incubated at 30 °C for 24 h in an airtight vial under dark conditions (because of the light sensitivity of TTC). Controls were prepared with 1.25 ml 0.1 M Tris buffer (without TTC) and controls without core samples were also prepared. After incubation, released triphenyl formazan (TPF) was extracted using 8 ml acetone by shaking for 2 h under dark conditions. Extracted red color TPF was measured at 485 nm (Spectronic 20D+, Thermo Fisher Scientific, MA, USA). A standard curve was made using TPF and the DHA is expressed as μg of TPF per gram dry weight of sediment per 24 h (after subtracting the reading of the controls).

To measure **protease activity (PTA),** 0.5 g core sediment sample was incubated with 2.5 ml of 0.05 M Tris buffer (pH 8.1) and 2.5 ml of 2 % Na-caseinate at 50 °C for 2 h; after that 0.75 ml 15 % trichloroacetic acid (TCA) was added to stop enzymatic reaction via precipitating residual Na-caseinate. Subsequently, centrifugation was carried out at 8400 xg for 10 min. The aromatic amino acids released in the supernatant were quantified colorimetrically using 33% Folin-Ciocalteu reagent at 578 nm (Spectronic 20D+, Thermo Fisher Scientific, MA, USA). Two controls were prepared, one was without core samples and another one was without substrate. A standard was made using tyrosine and the PTA was calculated after subtracting the value of two controls and expressed on the basis of released μg tyrosine per gram dry weight of sediment per 2 h (Ladd and Butler 1972; Subrahmanyam et al. 2011b).

**FDA hydrolysis activity (FDA HA)** was determined by taking 0.5 g sediment sample in 8.33 ml phosphate buffer saline (PBS) (pH 7.4) and 42 μl FDA (Table 2.1). Controls were prepared with 42 μl D/W instead of FDA and controls without core samples were also prepared. After that vortexing was carried out and further reaction mixtures were incubated at 30 °C for 4 h. Then, tubes were centrifuged at 8400 xg for 5 min. The supernatant (2 ml) was mixed with 2 ml chloroform: methanol (2:1 v/v) and absorbance were measured at 490 nm (Spectronic 20D+, Thermo Fisher Scientific, MA, USA) (Adam and Duncan 2001). A standard graph was prepared using fluorescein concentrations ranging from 10 to 30 μg $ml^{-1}$. FDA HA activity is expressed as μg of fluorescein per gram dry weight of sediment per 4 h (after subtracting the reading of the controls).

## 2.6 Analysis of enzymatic stoichiometry

The following three approaches were used to investigate microbial nutrition limitations. The first approach was based on the method of Hill et al. (2012) that is the preparation of a scatter plot of ecoenzymatic stoichiometry, with N cycle enzyme/P cycle enzyme (i.e., β-GA/alkaline PA) as x-axis and C cycle enzyme/ N cycle enzyme (i.e., β-GA/PTA) as y-axis. Based on the deviation from the expected enzyme ratio of C:N (1:1) or N:P (1:1), this plot can show P limitation, N limitation, C&P limitation, and N&P limitation (Chen et al. 2019). In the second approach, the ratios of C cycle enzyme/ P cycle enzyme i.e. β-GA/Alkaline PA and N cycle enzyme/ P cycle enzyme i.e. PTA/Alkaline PA were calculated. Lower β-GA/Alkaline PA and PTA/Alkaline PA suggest higher P limitation (Waring et al. 2014). In the third approach, nutrition limitation was measured using the vector analysis of ecoenzymatic stoichiometry (Moorhead et al. 2016). Vectors length (L, unitless) and vector angle (A, degree) were calculated using following formulas. A relatively longer vector L indicates greater C acquisition, and the vector A < 45˚ and >45˚ indicate relative degrees of N and P acquisition, respectively.

Vector L= $\sqrt{(\ln\beta\text{-GA}/\ \ln\text{PTA})^2 + (\ln\beta\text{-GA}/\ \ln\text{Alkaline PA})^2}$

Vector A= Degrees (ATAN2 ((lnβ-GA/ lnAlkaline PA), (lnβ-GA/ lnPTA)))

## 2.7 Enumeration of culturable bacteria by plate counts and most probable number (MPN) technique

Total viable heterotrophic bacteria of subsurface sediment core were enumerated on Reasoner's 2A agar (R2A agar, HiMedia Pvt. Ltd., Mumbai, India) In brief, 0.5 g of sediment core sample was homogenized in 4.5 ml of 0.85% NaCl (w/v) and 10 fold dilutions of sediment suspensions were prepared in the same diluent. Aliquots (100 µl) were spread on triplicate plates of R2A agar and incubated at 30 °C for 7 days. Colony-forming unit (CFU) of heterotrophic bacteria per gram dry weight of sediment was calculated. Denitrifying and $N_2$ fixing bacteria were enumerated by the most probable number (MPN) method using casaminoacid-yeast extract broth (CY broth) (5 g casaminoacid; 5 g yeast extract; 6 g $KNO_3$; 3.5 g NaCl; 1000 ml D/W, pH 7.2±0.2) (Lorch et al. 1995) and Jensen's broth (HiMedia Pvt. Ltd., Mumbai, India) respectively. A series of ten-fold dilutions of the sample resuspended as above were prepared in N-saline and 0.5 ml of each dilution was used to inoculate three tubes containing 4.5 ml

medium. Gas production (i.e. formation of bubbles) in Durham vial was considered as a positive indicator for the presence of denitrifying bacterial population and visible turbidity of bacterial growth in nitrogen-free Jensen's broth was considered as positive for the presence of $N_2$ fixing bacterial population. Counts were derived based on the standard MPN table (de Man 1983).

### 2.8 Identification of heterotrophic bacteria

Criteria for the selection of distinct isolates for identification was based on their morphological characteristics and based on the abundance of individual isolates. For identification of bacterial strains by 16S rRNA gene sequence analysis, genomic DNA was extracted by CTAB method (Wilson 2001) and amplification of 16S rRNA gene was carried out using 27 F and 1107 R primers as described by Patel and Archana (2017). Sequencing of PCR products was performed using the services of AgriGenome Labs (India) and the sequence similarity search was carried out using the NCBI-BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990) as well as Sequence Match tool of Ribosomal Database Project (RDP) (https://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) (Wang et al. 2007). A distance matrix (phylogenetic) tree was constructed by the Neighbor-Joining method with the Jukes-Cantor algorithm in MEGA X (Kumar et al. 2018).

### 2.9 Community DNA extraction

Community DNA was extracted from a 0.25 g sediment core sample using the MoBio PowerSoil™ DNA Isolation Kit (MoBio Laboratories, USA) according to the manufacturer's instructions. From each sample, community DNA was extracted in singlet and the extracted DNA was quantified by spectrophotometry (NanoPhotometer™ P-Class, Implen, Germany).

### 2.10 Quantitative real-time PCR (qPCR)

To determine bacterial 16S rDNA, archaeal 16S rDNA, *nirS, dsrB, and czcA* gene abundance qPCR was carried out.

#### 2.10.1 qPCR based quantitation of gene abundance

Absolute quantification of bacterial 16S rDNA, archaeal 16S rDNA, *nirS, dsrB, and czcA* was achieved using Step One Real-Time PCR machine (Applied Biosystems, USA). qPCR cycles and primers used in the reaction are detailed in Table 2.2. All samples were run in

duplicates. Each reaction contained 5 µl of SYBR Green-I master mix (GoTaq™ qPCR Master Mix, Promega), 0.2 µl of forward and reverse primers (10 µM) (Sigma-Aldrich), 0.2 µl $MgCl_2$ (25mM) (Promega), 0.2 µl BSA (10 mg $ml^{-1}$), 1 µl/2 µl of template DNA (2 ng $\mu l^{-1}$) (1 µl for bacterial 16S rDNA and *nirS* while 2 µl for archaeal 16S rDNA, *dsrB and czcA*), 0.1 µl CXR reference dye and nuclease-free water to make up final reaction volume 10 µl. At the end of each qPCR run, a melt curve was conducted to confirm the specificity of the amplified product.

### 2.10.2 Preparation of standard curves for absolute quantification

To prepare standard curves, amplicons of 16S rDNA, archaeal 16S rDNA, *nirS*, *dsrB*, and *czcA* gene were obtained by doing PCR with their corresponding primers (Table 2.2). For bacterial 16S rRNA gene quantification amplicon was obtained from gDNA of *E. coli* WT, for *nirS* gene quantification amplicon was obtained from gDNA of *Pseudomonas sp*. CRD33.DN3 (MK163503) while for archaeal 16S rRNA gene, *dsrB* and *czcA*, the amplicon was obtained from extracted community DNA of sediment samples. Then, obtained amplicons were purified by the $SiO_2$ method (Sambrook and Russell 2001). Purified PCR products were ligated into pTZ57R/T cloning vectors (InsTAclone™ PCR Cloning, Fermentas) and transformed into competent *E. Coli* DH5α cells. The transformation was carried out by the calcium chloride ($CaCl_2$) method (Sambrook and Russell 2001). Transformed cells were grown on Luria-Bertani (LB) agar (HiMedia Pvt. Ltd., Mumbai, India) plates containing ampicillin (50 µg $ml^{-1}$), 40 µl of X-gal (20 mg $ml^{-1}$), and 40 µl of IPTG (100 mM). A single white colony containing the recombinant plasmids was picked up and inoculated into 5 ml LB broth (HiMedia Pvt. Ltd., Mumbai, India) with ampicillin (50 µg $ml^{-1}$) which were incubated at 37 °C for 12 h. Plasmid DNA was extracted by the alkaline lysis method (Sambrook and Russell 2001). The plasmid concentrations were determined spectrophotometrically (NanoPhotometer™ P-Class, Implen, Germany) by measuring absorbance at 260 nm and purity of plasmids was checked by observing 260/280 and 260/230 ratio. The presence of the appropriate insert in cloned plasmids was confirmed by performing sequencing of PCR amplicons (AgriGenome Labs, India) obtained by using standard M13/pUC forward and reverse primers. Similarity search of obtained different insert sequences was carried out using the NCBI-BLASTn and these sequences are depicted in the appendix (Table A-III, Appendix). Purified plasmids from the respective clones were used to prepare standard curves by taking $10^3$ to $10^9$ gene copies $\mu L^{-1}$ for

## Table 2.2: Primers and thermal profiles used in qPCR

| Target gene | Forward primer (5'→3') | Reverse primer (5'→3') | Thermal profile | No. of cycles | Template used for preparing standard clone | Reference |
|---|---|---|---|---|---|---|
| Bacterial 16S rRNA | 341F-CCTACGGGAGGCAGCAG | 534 R-ATTACCGCGGCTGCTGGCA | 95°C – 5 min | 1 | *E. coli* WT (Culture collection, Department of Microbiology and Biotechnology centre, The M. S. University of Baroda) | Muyzer et al. 1993 |
| | | | 95°C – 15 s/65 – 55°C – 30 s/72°C – 15s (touchdown; -0.5°C cycle$^{-1}$) | 20 | | |
| | | | 95°C – 15 s/55°C – 30 s/72°C – 15s | 20 | | |
| Archaeal 16S rRNA | A364Af - CGGGG**Y**GCA**S**CAGGCGCGAA | A934bR-GTGCTCCCCCGCCAATTCCT | 94°C – 5 min | 1 | Random individual PCR amplified clone from community DNA | Kemnitz et al. 2005 |
| | | | 94°C – 20 s/58°C – 30 s/72°C – 40s | 40 | | |
| *nirS* (encoded cytochrome $cd_1$-nitrite reductase) | Cd3aF-GT**S**AACGT**S**AAGGA**R**AC**S**GG | R3cd-GA**S**TTCGG**R**TG**S**GTCTTGA | 95°C – 3 min | 1 | Denitrifying *Pseudomonas* sp. CRD33.DN3 (GenBank Acc. No. MK163503) obtained from sediment core of Mahi river basin | Dandie et al. 2011; Petersen et al. 2012 |
| | | | 95°C – 30 s/63 – 58°C – 30 s/72°C – 40s (touchdown; -1°C cycle$^{-1}$) | 6 | | |
| | | | 95°C – 30 s/58°C – 30 s/72°C – 40s | 40 | | |
| *dsrB* (encoded sulfite reductase β-subunit) | DSRp2060F-CAACATCGT**Y**CA**Y**ACCCAGGG | DSR4R-GTGTAGCAGTTACCGCA | 94°C – 4 min | 1 | Random individual PCR amplified clone from community DNA | Geets et al. 2006; Liu et al. 2014 |
| | | | 94°C – 30 s/55°C – 30 s/72°C – 30s | 40 | | |
| *czcA* (encoded component of a Co/Zn/Cd efflux protein) | TCG ACG G**B**G CCG TGG T**SM** T**B**G TCG AGA A | GT**V** A**W**S GCC A**K**C GG**V** **B**GG AAC A | 95°C – 5 min | 1 | Random individual PCR amplified clone from community DNA | Roosa et al. 2014 |
| | | | 95°C – 30 s/63°C – 30 s/72°C – 30s | 40 | | |

Where, **R** = A/G, **S**=C/G, **Y**=C/T, **M**= A/C, **B**= G/T

determining corresponding $C_T$ values. PCR efficiency was calculated by using a linear regression line of standard curves through the following formula: $E = [10^{(-1/slope)}]-1\times100$ (Töwe et al. 2010). The amplification efficiency obtained from standard curves of the bacterial 16S rRNA, archaeal 16S rRNA, *nirS*, *dsrB* and *czcA* genes was 100.32%, 97.37%, 101.80%, 101.37% and 101.93%, respectively ($R^2$>0.95 for all) (Fig. A-I, Appendix).

**2.11 Community fingerprinting by denaturing gradient gel electrophoresis (DGGE)**

Bacterial 16S rRNA genes from sediment core DNA extracts were amplified using 341 F (possessing 40 bases GC clamp) and 534 R primers (Muyzer et al. 1993). The thermal cycler procedure used for amplification was the same as described in qPCR (Table 2.2). A total 50 µl reaction mixture was prepared which contained 5 µl of 10X Taq DNA polymerase buffer with $MgCl_2$, 2 µl dNTPs mix (2.5 mM each), 0.4 µl Taq DNA polymerase (5 U $\mu l^{-1}$) (New England Biolabs (NEB) Pvt. Ltd., Ipswich, MA),1 µl of forward and reverse primers (10 µM) (Sigma-Aldrich), 1 µl BSA (10 mg $ml^{-1}$), 5 µl of template DNA (2 ng $\mu l^{-1}$) and nuclease-free water to make up final reaction volume. DGGE was then carried out as described by Patel and Archana (2017). In brief, DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) was used with a denaturing gradient of 40% to 70% and 10% polyacrylamide gel. To resolve PCR products, gels were run at constant voltage (i.e. 50 V) for 16 h at 65 °C. After completion of the run, gels were stained with 1:10000 SYBR Gold Nucleic Acid Gel Stain (Invitrogen, UK). Gel photographs were taken using AlphaEase 4.0 software (Alpha innotech, USA). Sample relatedness based on microbial community fingerprinting or DGGE clustering was carried out by Dice similarity coefficient and unweighted pair group method with arithmetic means algorithm (UPGMA) using GelCompar II (Applied Maths NV).

**2.12 DGGE band excision and sequencing**

Selected DNA bands were carefully cut out from the gel and placed overnight into 100 µl sterile D/W at 4 °C. Eluted bands were amplified by PCR using primers 341F (without the GC clamp) and 534R primers. Amplified PCR products were purified and cloned into pTZ57R/T cloning vectors (InsTAclone™ PCR Cloning, Fermentas) as per manufacturer guidelines. From obtained positive clones representative single clones were picked to correspond to single DGGE band and from these clones inserted sequence was amplified using standard M13/pUC primers.

These amplified products were purified, sequenced (AgriGenome Labs, India), and analyzed using NCBI-BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990) database after removing flanking vector (plasmid) sequence by VecScreen tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/vecscreen/).

## 2.13 Bacterial community profiling by Illumina (MiSeq) based 16S rRNA gene amplicon sequencing

Sequencing of the V3-V4 region of the 16S rRNA gene was carried out using the Illumina MiSeq platform (metagenomics) (AgriGenome Labs, India). Read quality was checked followed by trimming of the spacer and conserved regions were performed. The average Phred score >30 and average GC content >50 was observed. The consensus V3-V4 region sequence was constructed using the FLASH program. Various filters such as conserved region filter and mismatch filtration were performed to obtain high quality V3-V4 region sequences for various downstream analyses. Chimeric contigs were also removed by using the *de novo* chimera removal method UCHIME implemented in the tool USEARCH (version 8.1.1861). The operational taxonomic units (OTUs) pickup and taxonomy classification was performed in QIIME v1.5.0. Pre-processed reads from all samples were pooled and clustering of *de novo* OTUs was performed using UCLUST with a cutoff value of 97% sequence identity. Singleton OTUs were removed for further analysis and representatives of resultant OTUs were aligned against Greengenes core set of sequences using the PyNAST program. Further, these representative sequences were aligned against reference chimeric data sets and after that taxonomic classification was performed using the RDP classifier against SILVA OTUs database. The sequences not showing any alignment against the taxonomic database were categorized as unknown.

## 2.14 Metagenomics data analysis and functional prediction

A biom file of OTUs abundance and their taxonomic profiles was fed to MicrobiomeAnalyst to rarefy data and filter out top fifteen abundant phyla and top thirty abundant genus (Dhariwal et al. 2017). A stacked bar plot of relative abundance of top fifteen phyla was prepared using Microsoft Excel and relative abundance of top 30 genera was represented as heatmap generated using the gplots package (Warnes et al. 2015) of statistical

software R (https://www.r-project.org/). The Venn diagram used for graphical descriptions of unshared and shared OTUs between samples was plotted using the Venn plotter at http://bioinformatics.psb.ugent.be/webtools/Venn/. METAGENassist matches the taxonomic data with the phenotype databases (Arndt et al. 2012) therefore used to predict metabolic capabilities of obtained bacterial community profiles. The relative percentage of resultant predicted metabolic capabilities were presented as circos plots or chord diagrams. Circos plots were generated using the circlize package (Gu et al. 2014) of statistical software R.

### 2.15 Assessment of metal tolerance ability of isolates

#### 2.15.1 Preparation of metal solutions

Metal ion solutions of Cu(II), Ni(II), Cd(II), Cr(VI), and Hg(II) were prepared by dissolving their respective salts namely $CuSO_4.5H_2O$, $NiCl_2.6H_2O$, $CdCl_2$, [obtained from HiMedia Pvt. Ltd., Mumbai, India], $K_2Cr_2O_7$, and $HgCl_2$ [obtained from Fisher Scientific]. Stock solutions (1000 ppm) were prepared in deionized water according to the following equation: metal equivalent to 1 g = (Molecular weight of metal salt × 100) / (Atomic weight of metal × Purity) and these stock solutions were sterilized by membrane filtration (pore size, 0.22 μm; Millipore Corp.). Metal stock solutions were further diluted in sterile deionized water to prepare various working stocks as per the range tested and were preserved at 4 $^oC$ for no longer than 1 month. Concentrations tested for Cu(II), Ni(II), and Cr(VI) ranged from 100 to 400 ppm for Cd(II) ranged from 20 to 100 ppm, and for Hg(II) ranged from 3 to 20 ppm based on their toxicity reported for most bacterial strains. To avoid metal contamination all glasswares were leached in 2 N $HNO_3$ and rinsed several times with double-distilled water before use (Hassen et al. 1998).

#### 2.15.2 Heavy metal tolerance study by the plate diffusion method

Heavy metal tolerance of each bacterial strain was determined by the plate diffusion method (Hassen et al. 1998; Nithya et al. 2011). Using the soft agar (0.8% agar) overlay method, R2A plates were inoculated with bacterial culture. Four wells of 8 mm in diameter and ~ 4 mm in depth were bored on solidified agar plates with a cork borer. Each well was loaded with 80 μl solution having 100, 200, 300, and 400 ppm of Ni(II), Cu(II), Cr(VI); 20, 50, 70, and 100 ppm of Cd(II); 3, 5, 10, and 20 ppm of Hg(II) individually. Plates were then incubated in the upright

position at 30 °C and after incubation heavy metal tolerance of bacterial strains for each metal was recorded in terms of the lowest concentration of metal tested at which growth inhibition was observed.

### 2.15.3 Determination of minimum inhibitory concentration of Cr (VI)

The minimum inhibitory concentration (MIC) of Cr(VI) (chromate) was determined for selected isolates by broth dilution methods (Wiegand et al. 2008) in which R2A broth tubes containing a different concentration of Cr(VI) ranging from 20 to 100 ppm were prepared. For *Streptomyces* sp. RMD 42.2B, 1% inoculum of spore suspension having ~$10^8$ spores $ml^{-1}$ was used. For other bacterial strains, each tube was inoculated with a 1% inoculum of 0.6 optical density at 600 nm ($OD_{600\ nm}$) cell suspension. Cultures were incubated at 30 °C for 24-48 h depending on the growth rates of each strain (based on the control tube which did not contain the metal). A positive control consisting of metal deficient medium inoculated with the bacterial strain and negative control of metal supplemented medium without inoculum were also prepared. The MIC of Cr(VI) for bacterial strains is reported here as a range of ppm between the highest concentration at which bacterial growth was visibly observed and the immediate next higher concentration tested at which no visible growth was observed.

### 2.16 Cr(VI) determination using S-diphenyl carbazide (DPC) reagent

Chromate removal was estimated as the decrease in Cr(VI) concentration in the supernatant using diphenyl carbazide (DPC) reagent (prepared in acetone 0.25% w/v) as described by Pillai and Archana (2012). In brief, the reaction mixture contains 100 or 200 µl samples or standard volume was made up to 1 ml using distilled water. Then 165 µl of 6 M $H_2SO_4$ and 200 µl of DPC were added. This reaction mixture was incubated for 5 min and the final volume was made up to 5 ml using distilled water. After that spectrophotometric measurement (Spectronic 200, Thermo Scientific, India) was made at $540_{nm}$. The standard curve for the Cr(VI) estimation was prepared using the different concentrations ranging from 0.1 to 1 ppm.

### 2.17 Determination of chromate removal ability of bacterial strains in liquid medium

The chromate removal ability of bacterial strains was determined in R2A broth and sediment extract (SE) + peptone broth (based on soil extract agar as given by Hamaki et al.

2005), both amended with 2, 5, 10, 15, and 20 ppm initial concentrations of Cr(VI). In order to prepare the sediment extract (SE), 100 gm (wet weight) sediment (here surface sediment samples were used) was mixed in 200 ml of distilled water and vortexed for 30 min followed by centrifuged at 3500×g for 10 min and the supernatant was collected. The supernatant was autoclaved three times for three consecutive days and used as a medium component. Broth consisted of SE supplemented with 5 g $l^{-1}$ peptone (Krishna and Philip 2005) which was found to support good growth of the bacterial strains under study.

Tubes with R2A broth and SE + peptone broth containing various initial concentrations of Cr(VI) individually were inoculated with 1% inoculum of 0.6 $OD_{600nm}$ culture (except for *Streptomyces* sp. RMD 42.2B where a 1% inoculum of spore solution having ~$10^8$ spores $ml^{-1}$ was used) and incubated at 30 °C for 48 h. After incubation, two aliquots (1 ml each) were collected from the broth media of which one aliquot was subjected to centrifugation (11,200×g for 10 min at 4 °C) to determine Cr(VI) concentration in the cell-free supernatant by using a diphenyl carbazide (DPC) reagent and the other aliquot was subjected to measurement of $OD_{600\ nm}$. The percentage chromate removal was calculated using the following formula: 1-[Cr(VI)F/ Cr(VI)I)]×100, where, Cr(VI)I is the concentration of Cr(VI) in the broth at the time of culture inoculation (i.e., Cr(VI) concentration in the medium); Cr(VI)F is the concentration of Cr(VI) in the cell-free supernatant after incubation. The percentage survival of the bacterium was calculated by using the formula, ($OD_{600\ nm}$ of bacterial suspension grown with metal×100/$OD_{600\ nm}$ of bacterial suspension grown without metal). For *Streptomyces* sp. RMD42.2B, the percentage of survival was calculated by measuring biomass (dry weight) instead of $OD_{600\ nm}$.

**2.18 Microcosm study for metal removal in packed bed column**

To perform the microcosm studies, an experimental setup shown in Fig. 2.3 was used. Surface (0-20 cm) sediment samples collected from Rayka and Rampura were mixed and subjected to grain size fractionation (Sieve shaker Octagon 200, Endecotts) to obtain sediment fractions of different consistency namely, medium sand (MS) (0.5-0.25 mm), fine sand (FS) (0.25-0.05 mm), and silt and clay (SC) (< 0.05 mm). Sediment samples MS, FS, and SC showed electrical conductivity (EC) values of 142.35 µS, 132.55 µS, and 229.05 µS respectively and pH values of 8.39, 8.45and 8.36 respectively (both measured as 1:5 w/v ratio of sediment: D/W). The MS, FS, and SC samples did not show any detectable levels of Cr(VI) as measured by the

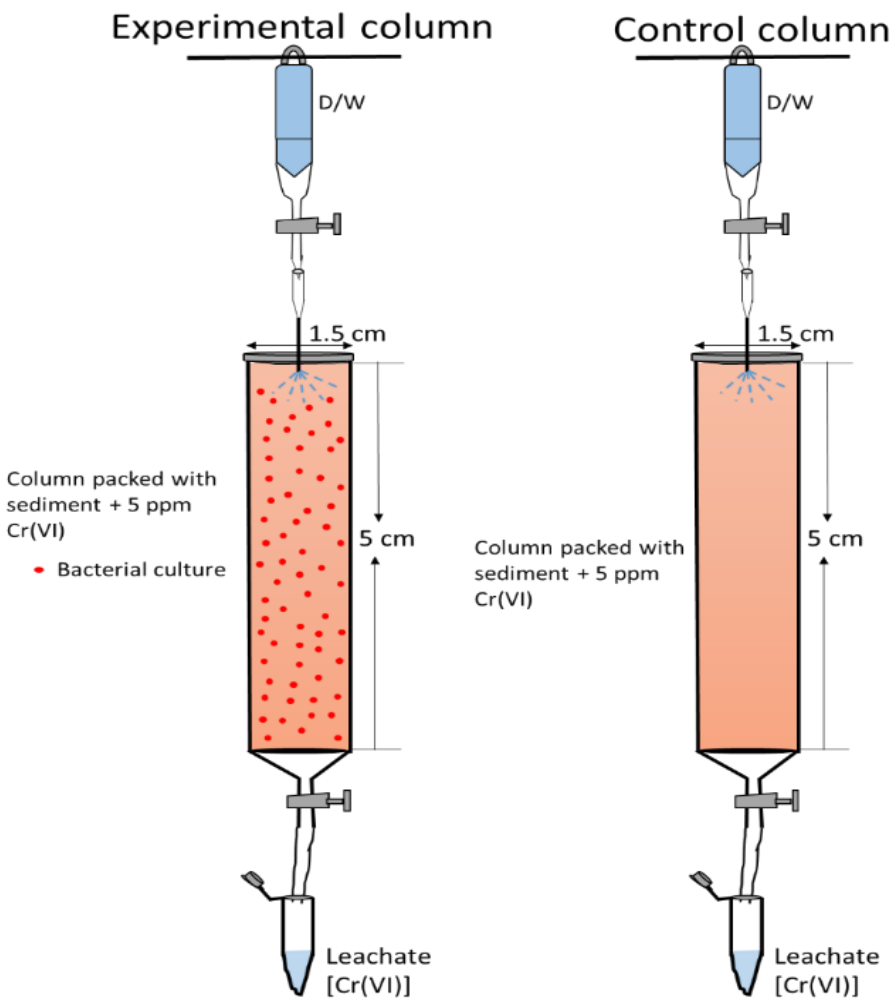


**Figure 2.3: Schematic diagram of the packed bed columns used in a microcosm study**. D/W: Distilled Water; Leachate fractions were subjected to Cr(VI) estimation using DPC reagent.

DPC method. The distilled water used in the analysis had an EC value of 16.15 µS and a pH of 6.8. Fractionated sediment samples of different consistency were artificially contaminated with 5 ppm of Cr(VI) and used to load the packed bed columns. A nitrocellulose filter membrane comprising pore size 0.22 µm (Merck-Millipore, India) was placed at the bottom of the column to avoid the outflow of sediment particles from the column. The columns were packed with 10 g sediment samples after homogeneous mixing with ~$2\times10^8$ bacterial cells suspended in 2 ml of 5 g $l^{-1}$ peptone solution (for control columns sediments were mixed with 2 ml peptone solution without bacterial cells). After packing, the columns were incubated for 3 days at room temperature (RT) after which D/W was applied to the top of the column at a flow rate of 0.5 ml

$min^{-1}$ and the leachate samples emerging from the basal outlet of the column were collected continuously in fractions of 1 ml. Each fraction was subjected to Cr(VI) estimation using the DPC reagent. This experiment was performed in duplicate. Percentage of Cr(VI) removal in the leachate fraction of the experimental column with respect to the control column was calculated using the following equation: $1-[Cr(VI)_{EC}/ Cr(VI)_{CC}]\times100$. Percentage of Cr(VI) removal in the leachate fraction of the experimental column with respect to applied Cr(VI) concentration was calculated using the following equation: $1-[Cr(VI)_{EC}/ Cr(VI)_{AC}]\times100$, where $Cr(VI)_{EC}$ is Cr(VI) concentration in the leachate of the experimental column, $Cr(VI)_{CC}$ is Cr(VI) concentration in the leachate of the control column, and $Cr(VI)_{AC}$ is the Cr(VI) concentration applied to the column (i.e., 5 ppm).

### 2.19 Statistical analysis

Welch's t-test (two-sided) was performed to determine the significant difference in individual variables of the upper region (up to ~5.5 m) and lower region (from ~5.5 m to 17 m) of the Chokari (CRD) core and also to determine the significant difference of individual variables between RYD and RMD cores. A Wilcoxon signed-rank test (two-sided) was performed to determine the significant difference between two different variables. A Tukey's multiple comparison test was performed to determine the significant differences in enzyme activity (single variable) between samples of the same core. These tests were performed in GraphPad Prism v 8.0.0 (GraphPad Software, San Diego, California, USA) and Box Plots were also prepared using GraphPad Prism v 8.0.0. Principal component analysis (PCA) was performed in PAST v 3.21 (Hammer et al. 2001) to determine the correlation (association) between physicochemical and microbial variables of CRD, RYD and RMD cores. All variables were normalized using the following formula: [(Variable-Mean)/ Standard Deviation] to perform PCA. Further, this correlation was confirmed by Pearson's correlation (two-sided). Significant differences between means were determined using either a paired t-test (two-tailed, $p<0.05$) or by one-way analysis of variance (ANOVA) with a post hoc Fisher's least significant difference (LSD) test using a probability level of $p<0.05$. These tests were performed in Microsoft Excel.