

Annexure A

MATERIALS AND METHODS

A.1 MATERIALS

The reagents and chemicals used in the study are listed below:

Table A.1: List of all the chemicals and reagents used in the study

Chemicals/Reagents/ Materials	Make
Nessler's Reagent	Fischer scientific
COD estimation Reagents	Merck-Spectroquant kit
Nitrite estimation Reagents	Merck-Spectroquant kit
Basic salt chemicals	Fischer scientific/ Merck
Medium Supplements	Himedia, India
Asparagine nitrate media	Himedia, India
Bromothymol blue	Himedia, India
Nafion-117 Membrane	Nickunj Eximp Pvt. Ltd., Mumbai
Graphite felt (Electrode)	Nickunj Eximp Pvt. Ltd., Mumbai
PCR reagents	Himedia, India
High range standard DNA ladder	Himedia, India
Soil DNA purification kit	Himedia, India
Gel extraction kit	Himedia, India
Real-Time PCR Master Mix	Qiagen
Primers	Xcelaris, Ahmedabad
Glycerol 3 phosphate	Sigma-Aldrich
<i>p</i> -nitrophenol	Merck
pNPP (Para nitro phenyl Phosphate)	Sigma-Aldrich
Uranyl acetate	SP chemicals

A.2 METHODS

A.2.1 MFC Reactor design and fabrication

The study involved the use of two types of MFC reactors. These MFC reactors were fabricated in the lab. The detailed construction of MFC reactors are described below:

(a) Dual Chambered MFC with the Salt Bridge

Two identical airtight cylindrical chambers were connected using a glass tube (Length-7.5cm and Diameter-1cm) containing salt bridge. Graphite rod (Diameter-1.2 cm and length-5.5 cm) was placed in each chamber namely anode and cathode. The Salt bridge consisted of 2% agar-agar prepared in saturated KCl solution. Anode and cathode were connected using copper wire (Gauge-2mm) and a 100 Ω resistor was used for completing the circuit. The total volume of anodic and cathodic chamber was 0.150 L while working or net volume was 0.143 L. This MFC set up was used in the experiments described in chapter 3.

(b) Polyacrylic- Dual Chambered MFC

Double chamber MFC was constructed using transparent poly-acrylic sheets. Briefly, two rectangular chambers namely cathode and anode of equal dimensions were bolted together. The gaskets were inserted in between to ensure air tight arrangement. The schematic of the MFC system is depicted in the Figure-A1. The two chambers were separated physically by a proton exchange membrane (Nafion- 117). Every chamber was provided with 2 ports on the top for electrode wire and sampling. Graphite felt (Sainergy Fuel Cell India Pvt. Ltd., 3×4×0.58 cm) was used as anode and cathode electrode respectively. All the electrodes were pretreated by washing with 1N HCl and 1N NaOH to remove any type of contamination followed by washing with distilled water. The distance between anode and cathode electrode was 2.5cm. A copper wire was used to connect the anode and cathode. A fixed external resistance was plugged between both of the electrodes. The volume of the total cathodic and anodic compartment was 100ml. Both anode and cathode compartment were washed with ethanol and purged with oxygen-free nitrogen gas prior to the experiment. This MFC set up was used in the experiments described in chapter 4 and 5 (Figure-A2).

(c) Nafion Pre-Treatment

The Nafion-117 membrane was treated according to the process described previously [Ghasemi *et al.*, 2013]. In brief, the membrane was boiled (~80° C) in distilled water for 1 h followed by boiling in 3% hydrogen peroxide (H₂O₂). The membrane was washed by distilled water at 80° C, followed by boiling in 0.5 M sulphuric acid (H₂SO₄) for 1 h. The final step involved boiling in distilled water for 2 h. The membrane was stored in the distilled water until further use.

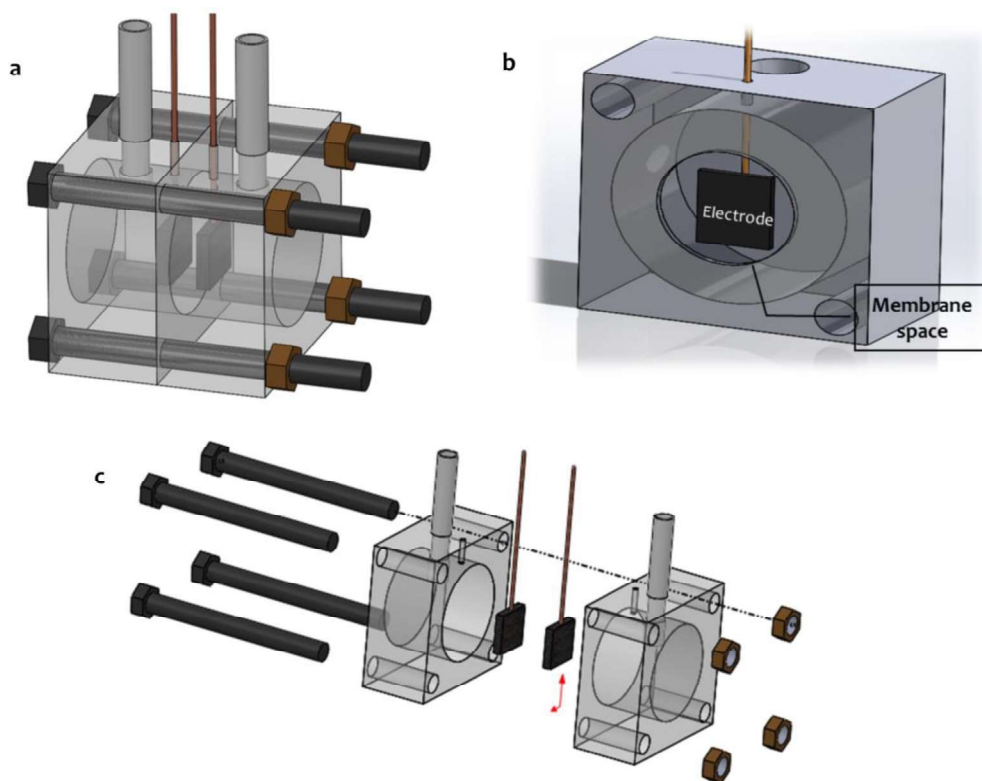


Figure A.1: MFC system used in the study (a- Assembled View, b- Half cell and c- exploded view)

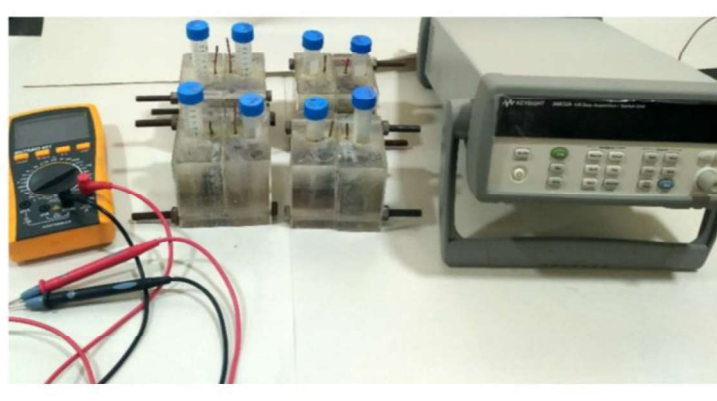


Figure A.2: The MFC reactors with data acquisition unit used in the study

A.2.2 Cow Manure, Soil, and Fruit Waste Collection and Characterization

Soil samples were collected from institute farmland (latitude and longitude 23.23 N and 73.06 E). The farm had been receiving nitrate fertilizers for over 6 years. The Soil sample was collected from 9 to 13 cm below the ground surface. The soil sample was passed through a 2-mm sieve to remove rocks and large particles. The sieved soil was mixed with normal saline. Sewage water was collected from a sewage collection pipe. Cow manure was collected from a local dairy farm. Fruit waste was collected from local fruit juice shop. Cow manure, soil and fruit waste were characterized for pH, conductivity, chemical oxygen demand (COD), total nitrogen ($\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$), total phosphorus and denitrifying Colony Forming Units (CFU/ml). In order to determine pH and conductivity, the sample was thoroughly mixed with double distilled water in 1: 1 ratio and allowed to settle in two layers. Measurements were made on the top liquid layer.

A.2.3 Analytical Techniques

A.2.3.1 Total Solids and Total Moisture Determination

Total solids and total moisture were determined by the standard method [APHA, 2005]. Briefly, a pre-weighed sample (in a thin layer) was subjected to 103°C for 1 h till a constant weight was achieved. Recorded weight was equivalent to the total solids and lost weight was equal to the total moisture content.

A.2.3.2 pH Measurement

pH was determined using the pH meter (make- Hanna Instruments, US). The pH of anodic and cathodic solutions was taken at a regular interval of time.

A.2.3.3 Chemical Oxygen Demand Estimation

Chemical oxygen demand (COD) was estimated as per the standard protocol [APHA, 2005]. This involved oxidizing the sample with acidified potassium dichromate solution in the presence of silver sulfate catalyst. For this, the 2.5 ml of sample is supplemented with 1.5 ml of potassium dichromate, mercury sulfate, and sulfuric acid solution, and 3.5 ml of silver sulfate solution in sulfuric acid. This is followed by digestion at 148°C in a COD digester (Merck) for 2 h in glass ampules. Ampules were cooled down and absorbance was taken at 600nm.

A.2.3.4 Nitrate Estimation

The nitrate test was done with Merck spectroquant kit. Briefly, the sample was added in a mixture of sulphuric and phosphoric acid solution (Reagent 1) in a ratio of 1:8. Then, 0.5 ml of 2, 6-dimethylphenol (DMP- Reagent 2) is added to this solution. After 10 minutes, the concentration of $\text{NO}_3^-\text{-N}$ was measured spectrophotometrically. The nitrate ions present in the

sample react with DMP to form 4-nitro-2, 2-dimethylphenol which is determined photometrically.

A.2.3.5 Nitrite Estimation

The nitrite test was done using Merck spectroquant kit. Briefly, nitrite ions react with sulfanilic acid to form a diazonium salt in acidic solution. This salt reacts with N-(1-naphthyl) ethylenediamine dihydrochloride to form a red-violet azo dye. This dye was determined photometrically.

A.2.3.6 Ammonium Estimation

Ammonium was estimated by Nesslerization spectrophotometric method. Briefly, 1ml of K-Na Tartarate (Rochelle salt solution) and 1ml Nessler reagent (Fischer scientific) was added in the sample (50ml-free of ammonia). After 5 min absorbance was taken at 425nm.

A.2.3.7 Phosphate Estimation

Phosphate estimation was done according to the standard protocol [APHA, 2005]. The process of total dissolved phosphorus estimation involves 2 steps. First the conversion of all types of phosphorus into orthophosphate, followed by estimation of total orthophosphate.

- i. **Conversion of total phosphorus into orthophosphate:-** For this step, 1 drop of phenolphthalein indicator solution was added into 50 ml of sample, followed by 1 ml of sulphuric acid solution & 0.4 g of ammonium persulfate. This mixture was heated on a hot plate. Then, 1 drop of phenolphthalein indicator was added in cooled sample & the solution was neutralized to a faint pink colour using NaOH solution. Further, deionized water was added to the solution in order to make up to 50 ml.
- ii. **Estimation of total phosphate:-** 1 drop of phenolphthalein indicator was added into 50 ml of previously treated sample. Then, 5N sulphuric acid was added in case of solution turns pink. Further, the solution was mixed with 8 ml of combined solution (mixture of 5N sulphuric acid, potassium antimonyl tartrate solution, ammonium molybdate solution, and ascorbic acid solution in a ratio of 10:1:3:6) & stirred vigorously. After 10 min OD was taken at 880 nm.

A.2.3.8 Uranium (VI) Estimation

Uranium assay was done using the method described by Appakuttan et al. [Appakuttan *et al.*, 2006]. In this assay, aliquots were taken at different time intervals and subjected to centrifugation at 12,000 X g for 10min. Residual uranium in the supernatant and uranium present in cell pellet were estimated using Arsenazo (III). A 0.1% solution of Arsenazo (III) was prepared by dissolving 0.2g of Arsenazo (III) in 180ml of 0.01N HCl and 20 ml of absolute ethanol (30 min of stirring) and filtered through Whatman No. 1 filter paper. For estimation of uranium 20µl of the supernatant was diluted to a total volume of 600µl. The samples were acidified by using 200µl of 0.01N HCl followed by addition of 20µl of Arsenazo (III). The resultant purple-coloured uranium-Arsenazo III complex was estimated spectrophotometrically at 655nm using uranyl acetate as the standard.

A.2.3.9 Phosphatase enzyme assay

Phosphatase activity assay was done as per the methods described previously [Martinez *et al.*, 2007] using *p*-nitrophenyl phosphate (*p*NPP) which liberates *p*-nitrophenol in the presence of phosphatase. Bacterial cells were centrifuged at 10,000g for 10min and washed twice with 100mM sodium acetate. The substrate 5mM *p*NPP prepared in 100mM sodium acetate buffer was added in the reaction volume of 0.5ml and incubated at 30°C for 0.5 h. The reaction was

terminated by the addition of 0.5ml of 0.02M NaOH. Accumulation of *p*-nitrophenol was observed at 405nm. Phosphatase activity was expressed as nmol of *p*-nitrophenyl liberated per minute per mg dry weight of cells.

A.2.3.10 Cultivable Denitrifying Bacteria Estimation

Cultivable denitrifying bacteria in both cow manure and soil were determined using the protocol as described previously [Takaya *et al.*, 2003]. Briefly, the samples (chapter-3) were serially diluted and plated in the denitrifier's enrichment medium containing 0.1% L-asparagine, 0.1% KNO₃, 0.1% KH₂PO₄, 0.005% FeCl₂·6H₂O, 0.02% CaCl₂·2H₂O, 0.1% MgSO₄·7H₂O, 1 ml of Bromothymol blue (BTB) l⁻¹ (1% in ethanol), 2% agar, and pH is 7.0 to 7.3. Plates with bacterial suspension were incubated for 3 days at 30° C under anaerobic conditions (Anaerobic Jar, Himedia). This method is based on the change in the pH of a medium due to nitrate depletion by denitrifying bacteria. The blue coloured colonies/halo indicated positive denitrification. The colonies were counted to determine denitrifying CFU/ml.

A.2.3.11 GC Analysis

Cathode head space nitrogen gas analysis was done using gas chromatography equipped with a Thermal Conductivity Detector (TCD) (YL 6500, Young Lin Instruments Korea). Stainless steel packed column with an internal coating of zeolite was used for the separation of gases. The oven and detector temperature were set at 35°C and 120°C respectively. Argon was used as carrier gas with the flow rate of 15ml/min. Also, nitrogen purging was replaced with argon purging for MFC operation in this experiment.

A.2.4 Statistical Analysis

All experiments were performed in triplicates. The results reported are the average values with the standard deviation ranging from 2-12%.

A.2.5 Electrochemical analysis

A.2.5.1 Measurement of Power Density and Voltage

Voltage and current measurements were made at different intervals of time using a digital multimeter (METRAVI-451). Power density (W m⁻³) was calculated by normalizing the power with anode chamber volume using the formula:

$$P = \frac{V \times I}{\text{Anode chamber volume}}$$

Where V is the potential in volts (V) and I is the current in amperes (A). The polarization curves were plotted by measuring potential and current at various external resistance from 50,000 Ω to 5 Ω. The readings were recorded after a stable value of potential and current were achieved. For stable potential value, MFC reactor was kept at open circuit until the value gets stabilized. The OCV was measured when the system is at infinite resistance. This is the maximum voltage MFC can achieve.

A.2.5.2 Columbic Efficiency

The columbic efficiency (ε) was calculated using the formula given by [Logan *et al.*, 2006].

$$\varepsilon = \frac{M \int_0^t I dt}{FnV_{An}\Delta COD}$$

where M is the molecular weight of nitrate, F is the Faraday's constant, n is the number of electrons exchanged per mole of nitrate when reduced completely to molecular

nitrogen, V_{An} is the Volume of anode liquid and ΔCOD is the difference between initial and final COD value after time t .

A.2.5.3 Cyclic Voltammetry

Cyclic voltammetry (CV) was performed using Autolab electrochemical workstation (PGSTAT 302N, Metrohm, The Netherlands) in MFCs with three electrode system. The anode was used as the working electrode, Ag/AgCl (+197 mV vs. SHE) as a reference electrode and cathode as a counter electrode. The CV was recorded in a voltage range of -0.8V to +0.8V and a scan rate of 1mV/sec. The plots of current (I) v/s potential (V) were recorded using software NOVA 1.10.

A.2.5.4 EIS Analysis

Electrochemical Impedance Spectroscopy (EIS) was carried out using the Autolab electrochemical workstation (PGSTAT 302N, Metrohm, The Netherlands) with three electrode system. The cathode was used as the working electrode, Ag/AgCl (+197 mV vs. SHE) served as a reference electrode and the anode served as a counter electrode. The EIS analysis was done in the frequency range of 10 kHz to 0.1Hz. The data obtained from EIS was analyzed using NOVA 1.10 software.

A.2.5.5 Chronoamperometry

Chronoamperometry was carried out using the Autolab electrochemical workstation (PGSTAT 302N, Metrohm, The Netherlands) with three electrode system. The cathode was used as the working electrode, Ag/AgCl (+197 mV vs. SHE) served as a reference electrode and the anode served as a counter electrode. Chronoamperometry analysis (CA) was performed at a fixed potential of 200mV to assess the magnitude of sustainable current.

A.2.6 Molecular Biology Techniques

A.2.6.1 Microbial Community Analysis by Next-Generation Sequencing

Biofilm sample was collected from the electrode of the cathode chamber after the last batch cycle. Genomic DNA was isolated from the electrode bio-film using the (Soil DNA purification kit, Himedia) as per manufacturer's instructions. The V3-V4 region of 16S rDNA amplicon was sequenced on the Illumina platform based Next Generation Sequencing (NGS) method at Xcelris Genomics facility (Ahmedabad, India) to analyze the bacterial diversity of the biofilm growing at the cathode electrode. The primer pair (Prokaryote-V3-forward: 5'CCTACGGGNGBGCASCAG3' and Prokaryote-V4-reverse: 5' GACTACNVGGGTATCTAATCC3') was used for the amplification of the V3-V4 hypervariable region of bacteria and archaea. The amplicon libraries were prepared from both the samples after amplifying the V3-V4 hypervariable region. QIIME (Quantitative Insight Into Microbial Ecology) tool was used to analyze the data generated from NGS platform. Similar sequences were put together into one representative taxonomic unit called as Operational Taxonomic Unit (OTU) on the basis of 97% sequence similarity using UCLUST algorithm. Taxonomic assignments were done at 90% threshold value using the greengenes database. Alpha-diversity indices such as Chao1, Shannon index (H) and observe species were calculated using QIIME. The OTU table is visualized as a heat map to see the relative abundance of each species. The OTU's having sequence more than 1200 (for Figure 4.11) and 3000 (for Figure 5.13) were considered for heatmap generation. The OTU's were log transformed and clustered by UPGMA hierarchical clustering method. Rarefaction curve was also plotted using QIIME. All the sequenced data (chapter-4) were submitted in the NCBI Sequence Read Archive (SRA) database with accession number SRP157016.

A.2.6.2 Quantification of Denitrifying Genes Using Real-Time PCR

Quantitative PCR of denitrifying genes was performed on a qPCR System (Agilent Technologies, Mx3005P) using SYBR Green detection method. Amplification was performed for three denitrifying genes i.e. *narG*, *nirS*, *nosZ* and compared with the 16S rDNA gene. DNA isolation was done as described above and quantified using a Nanodrop spectrophotometer. Amplification was carried out in a reaction mixture containing: 1µM of each primer, 10µl of SYBR Green PCR master mix (Applied Biosystems), 1-2 µl of DNA template, and DNase-free water to make up the volume (20µl). The sequence of the primers used and the PCR conditions are listed in Table A.2. The primers were used based on the previous reports [Vilar Sanz *et al.*, 2013]. All the amplicons were also checked for size correctness by agarose gel electrophoresis. *Paracoccus denitrificans* (MCC-2519) strain was used as a positive control and this culture was obtained from the Microbial Type Culture Collection NCCS, Pune. This bacterium has the ability to carry out complete denitrification by the virtue of having all the denitrifying genes in its genome as reported in the literature [Throback *et al.*, 2004].

A standard curve was created by tenfold serial dilution of the target gene of positive control (a PCR product) and the plots for threshold cycles (C_t) versus the log of gene copy number were obtained for each gene. A negative control (without template) was included in qPCR amplification reactions to rule out the presence of any false positives. The gene copy number was calculated as [Kim *et al.*, 2013]:

$$DNA \text{ (copy)} = \frac{6.02 \times 10^{23} \frac{\text{Copy}}{\text{mol}} \times DNA \text{ amount (g)}}{DNA \text{ length (bp)} \times 660 \left(\frac{\text{g}}{\text{mol}} \right) / \text{bp}}$$

Table A.2: List of primers used in the study

Gene	Primer Sequence (5'-3')	Expected amplicon size	PCR Conditions
<i>narG</i>	narG-F- TCGCCSATYCCGGCSATGTC narG-GAGTTGTACCAGTCRGCSGAYTCSG	173bp	95° C/7min; 35 cycles of 95° C/30s, 61° C/30 s, 72° C/1 min, and Final- 72° C/ 8 min
<i>nirS</i>	nirSCd3aFm- AACGYSAAGGARACSGG nirSR3cdm- GAS TTC GGR TGS GTC TTS AYG AA	425bp	95° C/2 min; 35 cycles of 95° C/30s, 53° C/30 s, 72° C 1 min, Final-72° C/8 min
<i>nosZ</i>	nosZ F- CGY TGT TCM TCG ACA GCC AG nosZ1622 R- CGS ACC TTS TTG CCS TYG CG	453bp	95° C/2 min; 35 cycles of 95° C/30 s, 53° C/30 s, 72° C/1 min, Final-72° C/8 min
16S rDNA	338F- GAC TCC TAC GGG AGG CAG CAG 518R- ATT ACG GCG GCT GCT GG	200bp	95° C/8min; 40 cycles of 95° C/30s, 54° C/30 s, 72° C/1 min, and Final- 72° C/8 min

A.2.7 SEM and EDX Analysis

Cathodic bio-film was examined using a scanning electron microscope (SEM, Made-Carl Zeiss, SEM EVO-18 special edition). SEM analysis is generally utilized for the surface morphology analysis. For SEM analysis electrode was completely dried overnight in the oven. The electrode was kept at the stub using the aluminum tape and it was fixed on the stub. The sample was charged with gold particles. The digital images were taken using SEM. The uranyl

phosphate precipitate generated at the MFC cathode (chapter-5) was analyzed by EDX to see the elemental components.

A.2.8 X-ray Diffraction (XRD) Analysis

XRD is used to identify the crystal structure of a powder sample. At the end of each batch cycle, Centrifugation at 12000 rpm for 10 min separated the catholyte into pellet and supernatant (chapter-5). Further steps involved washing the pellet with distilled water followed by drying at 80° C overnight. The dried sample was spread on the XRD sample disc. XRD analysis of this powder sample was done using X-ray powder diffraction instrument (XRD, Bruker Axs Automated Powder X-RAY Diffractometer System).

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