

SIGNIFICANCE OF SULFUR OXIDIZING BACTERIA FROM SALTPANS OF GOA AND THEIR ECOLOGICAL RELATIONSHIP WITH SULFATE REDUCING BACTERIA

A Thesis submitted in partial fulfillment for the Degree of

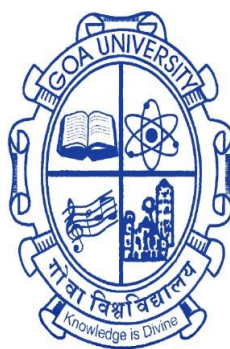
DOCTOR OF PHILOSOPHY

in

Biotechnology

School of Biological Sciences and Biotechnology

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By

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June 2024

DECLARATION

I, Ms. Priti Dattaram Gawas hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any other University or Institute for the award of any research degree.

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ACKNOWLEDGMENT

First and foremost, I am grateful to God for granting me the physical and mental strength to undertake and complete my research work. It is through his grace that I have been able to achieve all that I have today.

I am deeply grateful to **Prof. Savita Kerkar**, Senior Professor at the School of Biological Sciences and Biotechnology, for dedicating her valuable time to guide me. Her unwavering support, encouragement, and trust have profoundly shaped me both professionally and personally. Words cannot fully express my gratitude for her vast knowledge in the field, her positive attitude, and her unwavering commitment. Her generosity, patience, enthusiasm, and continuous guidance have been invaluable at every stage of my research.

I would also like to thank to **Prof. Harilal Menon**, the Vice Chancellor of Goa University, and **Prof. Varun Sahni**, the former Vice Chancellor of Goa University. Their provision of access to the University Laboratory, along with all the necessary apparatus, facilities, and infrastructure, enabled me to conduct my research effectively and efficiently.

I would like to express my gratitude to **Prof. Bernard Rodrigues**, the Dean of the School of Biological Sciences and Biotechnology, and **Prof. Savita Kerkar**, the former Dean of the Faculty of Life Sciences, for their valuable and encouraging support. I also extend my thanks to **Prof. Krishnan**, Vice-Dean (Research), for his guidance on official matters. I am extremely thankful to **Dr. Maria Judith Gonsalves** (Vice Chancellor's Nominee) and **Prof. Sanjeev Ghadi** (Vice Dean Academics and Vice Chancellor's Nominee) for their constant guidance and critical evaluation of my research. I extend my deepest gratitude to the University Grants Commission (UGC) for their financial support, which has significantly contributed to the successful completion of this research

A special thanks to Late. Dr. K. P. Krishnan, Principal Scientist at NCPOR Goa, for providing the sequencing facility. I am deeply grateful to Goa Business School, Goa University, with special thanks to **Prof. Jyoti Pawar** (Dean of Goa Business School), **Mr. S. Baskar** (Associate Professor), and **Mr. Ramrao Wagh** (Associate Professor) for providing the computer facility for my research work. I also thank **Mr. Akshay, Mr. Pranab, Mr. Aditya, and Mr. Rajendra**, programmers from Computer Sciences and Technology, for their assistance with the computer systems.

I take this opportunity to thank Programme Director of Biotechnology, **Dr. Meghnath Prabhu** for providing me with laboratories well equipped with the necessary instruments and chemicals needed for research. I also take this opportunity to thank **Prof. Usha Muraleedharan, Prof. Urmila Barros, Dr. Abhishek Mishra, Dr. Dharmendra Tiwari, Dr. Sanika Samant, Ms. Dviti Mapari, Dr. Samantha Fernandes, Ms. Snesha Bhomkar and Ms. Snigdha Mayekar** for their valuable advice and feedback throughout my tenure at the university.

I appreciate the ever-willing and cheerful assistance of the non-teaching staff **Mr. Serrao, Mrs. Sanjana, Dr. Sandhya, Mr. Parijat, Mr. Sameer, Mr. Rahul, Mr. Aashish, and Ms. Jaya**. I also fondly remember the very helpful Late. Mr. Martin who would go out of his way to help us out with anything, he will always be missed.

I was blessed to have a laboratory with helpful, and dedicated lab mates and friends with whom I have had the pleasure of spending all these years— **Dr. Kirti, Dr. Amruta, Dr. Srijay, Dr. Imran, Dr. Judith, Dr. Preethi, Dr. Michelle, Dr. Samantha, Dr. Alsiha, Dr. Ruchira, Dr. Manasi, Dr. Sreekala, Dr. Nicola, Perantho, Pingal, Delicia, Nuha, Deepti, Rakshita, Veda, Varsha, Anchit, Suman, Kunal, Hetika, Devika, Elaine, Fazila, Diksha, Trusha and Paresh**, thank you so much for all the help and support.

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Abbreviations

%	Percentage
°C	Degree Celsius
μL	Micro litre
μm	Micrometer
ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variance
BLAST	Basic Local Alignment Search Tool
BOD	Biological Oxygen Demand
CaCl	Calcium Chloride
CCA	Canonical Correspondence Analysis
Cd	Cadmium
COD	Chemical Oxygen Demand
cm	Centimeter
CuSO ₄	Copper Sulphate
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
Etc.	Etcetera
g	Gram(s)

g/L	Gram per litre
GPS	Global Positioning System
h	Hour(s)
H ⁺ ions	Hydrogen Ions
H ₂ O	Water
H ₂ O ₂	Hydrogen Peroxide
H ₂ S	Hydrogen Sulfide
HCl	Hydrochloric Acid
Km	Kilometer
K ₂ HPO ₄	Dipotassium Hydrogen Phosphate
KH ₂ PO ₄	Potassium Dihydrogen Phosphate
L	Liter
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram
mg/L	Milligram per Litre
mg/mL	Milligram per Millilitre
MgCl ₂	Magnesium Chloride
min	Minute(s)
mL	Millilitre
NaHS	Sodium Hydro Sulfide

$\text{Na}_2\text{S}_2\text{O}_3$	Sodium Thiosulfate
NEDE	N-(1-naphthyl)-ethylenediamine
ng/ μL	Nanograms per Microliter
$\text{S}_2\text{O}_3^{2-}$	Thiosulfate Ion
SO_4^{2-}	Sulfate Ion
$\text{S}_4\text{O}_6^{2-}$	Tetrathionate Ion
SOB	Sulfur Oxidizing Bacteria
SRB	Sulfate Reducing Bacteria
<i>Sqr</i>	Sulfide:quinone oxidoreductase
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NO_3^-	Nitrate Ion
NO_2^-	Nitrite Ion
nm	Nanometer
OD	Optical Density
ONPG	O-nitrophenylbeta-D-galactopyranoside
PAST	Paleontological Statistics Software
PCR	Polymerase Chain Reaction
pH	Hydrogen Ion Concentration

PICRUST	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PSU	Percentile Salinity Unit
QIIME2	Quantitative Insights Into Microbial Ecology 2
rRNA	Ribosomal Ribonucleic Acid
rpm	Rotations per Minute
sec	Second(s)
SEM	Scanning Electron Microscopy
sp.	Species
spp.	Species (plural)
SPSS	Statistical Package for the Social Sciences
TDS	Total Dissolved Solids
USA	United States of America
U	Units
v/v	Volume per Volume
w/v	Weight per Volume
WWTPS	Waste Water Treatment Plants

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Chapter 1

Introduction

1.1 Effects of Reduced Sulfur Compounds

Reduced inorganic sulfur compounds such as hydrogen sulfide (H_2S) and thiosulfate pose a significant challenge to various industries. Thiosulfate ($\text{S}_2\text{O}_3^{2-}$) is commonly found in effluents from diverse industrial sectors such as petroleum refineries, pulp and paper plants, photographic processing labs, etc. The presence of these compounds in wastewater poses a threat to natural ecosystems, as they contribute to both chemical oxygen demand (COD) and biochemical oxygen demand (BOD). Thus, in wastewater treatment plants, the controlled enzymatic oxidation of thiosulfate to sulfate (SO_4^{2-}) through bacterial metabolism offers a promising solution to detoxify these compounds (Millano and Sorber, 1986). Further, H_2S is an odorless gas that emits a pungent smell of rotten eggs. It is generated in anaerobic environments through the decomposition of organic matter, from natural occurrences such as petroleum and volcanic activity, as well as human activities like oil refining, wood pulp production, and the tanning industry (WHO 2000, Godoi et al. 2018). Notably, anaerobic wastewater treatment plants (WWTPs) and the degradation processes occurring in landfills represent substantial contributors to the release of H_2S into the environment (Godoi et al. 2018). Apart from its noticeable unpleasant smell, the primary concern associated with H_2S pollution lies in its documented toxicity to humans. The effects of H_2S pollution are dependent on the dosage and can have adverse impacts on the nervous, cardiovascular, and respiratory systems. Exposure to high concentrations of H_2S in the short term can result in eye damage, loss of smell perception, respiratory irritation, pulmonary

edema, convulsions, and even fatality (WHO 2000). Prolonged exposure to low concentrations of H_2S also negatively affects human health, manifesting as symptoms like nausea, headaches, and respiratory issues (Lebrero et al. 2011, Godoi et al. 2018).

The H_2S is produced by Sulfate-reducing bacteria (SRB) as metabolic products and is the major player in microbiologically influenced corrosion (MIC). SRB also contributes to corrosion due to the sulfide produced. Reports indicate that MIC contributes to approximately 20 % of the overall corrosion costs in X80 pipeline steel resulting in pipeline failure. X80 pipeline steel is valued for its high strength, superior toughness, and cost-effectiveness and is extensively utilized; however, it is particularly susceptible to MIC (Wang et al. 2022).

Reduced sulfur compounds cause problem, in both industrial and municipal waste streams, posing significant challenges in waste management. Over the years, numerous technologies and approaches have emerged to address this issue. Among them, biological methods have gained prominence due to their minimal operational demands and adaptable nature (Lin et al. 2018). Therefore, the reduced sulfur compounds formed need to be oxidized to products that are non-toxic and not corrosive. Therefore, the controlled oxidation of the sulfide or thiosulfate formed can lead to a reduction in both COD and BOD levels in the wastewater. Sulfate, being fully oxidized, does not impose an oxygen demand, unlike thiosulfate or sulfide. Therefore, understanding the biology of the sulfur cycle and the mechanism of oxidation by SOB is important.

1.2 Biological Sulfur Cycle

The ocean serves as the primary repository of sulfur (S) and ranks as the fourteenth most prevalent element in the Earth's crust (Steudel and Chivers 2019). Sulfur can be found in both organic and inorganic forms, such as dissolved sulfate, metal sulfides (pyrite, marcasite), and

sulfate-bearing minerals (gypsum, anhydrite), etc. (Sievert et al. 2007). Sulfur occurs in a various valence state, ranging from -2 (as in sulfide) to $+6$ (as in sulfate). Sulfides, polysulfides, thiosulfate, polythionates, elemental sulfur, bisulfite, and sulfate are the most common inorganic sulfur compounds that occur in natural environments. Sulfur is an essential element of life as it is a constituent of protein (S-containing amino acids, cysteine, and methionine), coenzymes (e.g. coenzyme A, biotin, thiamine) metalloproteins (in the form of iron-sulfur clusters), and in bridging ligands (for example binding in cytochrome c oxidase) (Sievert et al. 2007, Valiente et al. 2017). Sulfate being the most stable form of sulfur on today's oxic earth, is most abundant among other sulfur compounds in the form of dissolved sulfate and in sedimentary minerals (as gypsum and pyrite). Sulfide and sulfate cannot undergo any more reduction or oxidation and therefore, are the final products of most sulfur compound reduction or oxidation pathways. The microorganisms use sulfate as an electron acceptor and form sulfide in the process of assimilatory or dissimilatory (difference in the product formed) sulfate reduction. The assimilatory sulfate reduction process is typically used by microorganisms to form organic compounds like methionine and cysteine. In contrast, in dissimilatory sulfate reduction, the terminal product is hydrogen sulfide. The hydrogen sulfide formed can be cycled back to the sulfate or other sulfur compounds (elemental sulfur, polysulfides, thiosulfate, and sulfite) by microbial oxidation (Zhou et al. 2021, Kushkevych et al. 2020, Dahl 2020).

SRB are prime drivers in the sulfur biogeochemical cycle. SRB reduces sulfate (SO_4^{2-}) to hydrogen sulfide (H_2S) under anaerobic conditions. This process known as dissimilatory sulfate reduction, which results in the sulfide into the environment, which can further react with metals to form their metal sulfides. In salt pans, where sulfate concentrations are often high due to the presence of salt, SRB plays a crucial role in the sulfur cycle and in shaping the microbial community structure. Additionally, the production of sulfide by SRB can

influence the redox conditions of the environment, which further affect the microbial diversity and activity (Dahl 2020).

The sulfide produced by SRB can in turn serve as electron donors for bacteria such as SOB, that obtain the energy by oxidizing sulfide to sulfate, completing the sulfur cycle (Figure 1.1). This oxidation process can occur aerobically or anaerobically, either using oxygen or nitrate as an electron acceptor depending on environmental conditions. In salt pans, due to the high sulfate concentrations, SRB and SOB are prevalent and have a significant role in mediating the sulfur cycling and ecosystem functioning.

The disproportionation of inorganic sulfur compounds is another process that influences the biogeochemical sulfur cycle which is catalyzed microbiologically by chemolithotrophic processes. In this process, the intermediate sulfur compounds (sulfite, thiosulfate, and elemental sulfur) act as electron acceptors or donors resulting in the formation of sulfide and sulfate as end products (Finster et al. 2013).

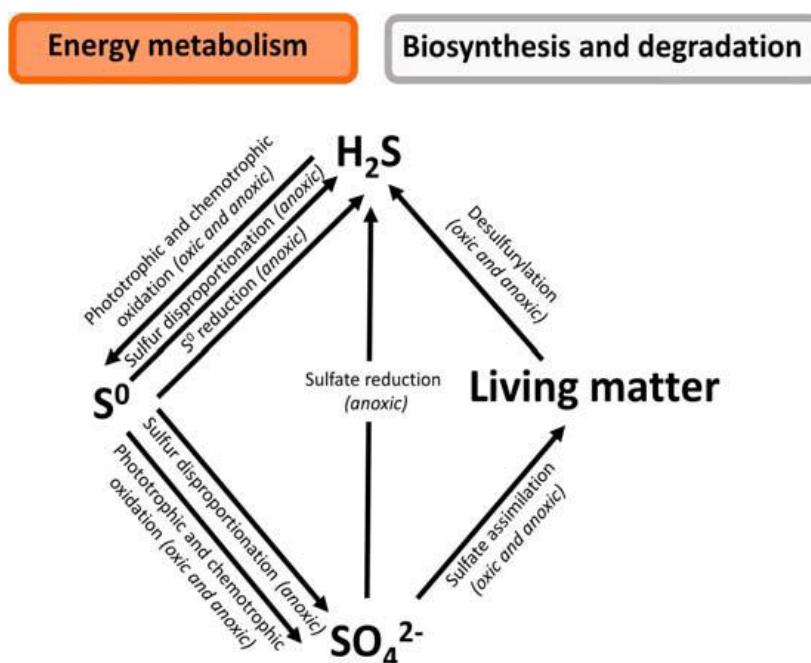


Figure 1.1: Biological Sulfur Cycle: Sulfate-reducing bacteria utilize sulfate as a respiratory electron acceptor, generating sulfide, the most reduced form of sulfur. Sulfur-oxidizing bacteria then oxidize sulfide under oxic or anoxic conditions. Elementary sulfur undergoes biological reduction or disproportionation (Adapted from Dahl 2020).

1.3 Sulfide Oxidation Biochemistry

Hydrogen sulfide (H_2S) along its ionic species resulting from deprotonation (HS^{2-} and S^{2-}) and R-SH in the organically bound form are the most reduced forms of sulfur commonly encountered in biological and chemical systems. These species have an oxidation state of -2 for the sulfur atom. Therefore, the removal of sulfide can be achieved by oxidation of these reduced sulfide species. The oxidation can be achieved naturally by the metabolism of some bacteria e.g. chemolithoautotrophic bacteria that occurs in the presence of oxygen (or nitrate/nitrite) and photoautotrophic bacteria in anoxic conditions as depicted in Figure 1.2 (Bharathi 2018, Cattaneo et al. 2023).

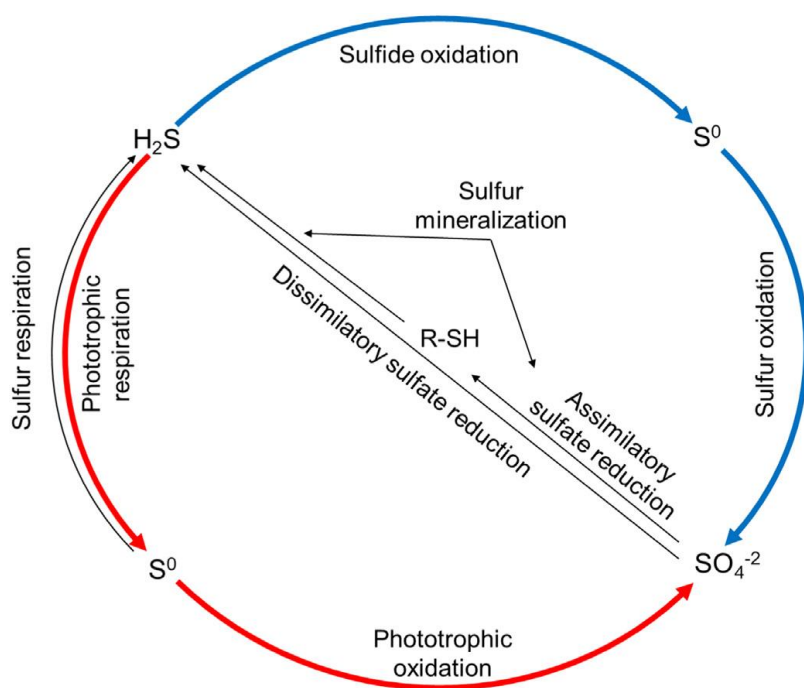


Figure 1.2: Diagrammatic illustration of sulfur cycling, with aerobic (or anoxic) metabolic processes represented in blue and anaerobic processes in red. (Adapted from Pepper et al., 2015.)

1.4 Sulfur Oxidation in SOB

The pathways involved in sulfur oxidation by the sulfur oxidizing bacteria (SOB) are quite intricate due to the diverse strategies these microorganisms employ to utilize reduced sulfur

compounds as an energy source. This takes place via three complex pathways (Figure 1.3) (Klatt and Polerecky 2015):

1. the Sox pathway mediated by the thiosulfate-oxidizing multi-enzyme (TOMES) Complex
2. the “branched” pathway
3. the tetrathionate pathway (SI4 pathway of thiosulfate oxidation)

1.4.1 Sulfide oxidation by Sox pathway

The Sox pathway begins with the binding of a SoxXA cytochrome, a triheme heterodimer, to thiosulfate. This oxidation reaction results in the transfer of the sulfane sulfur of thiosulfate to a SoxY-cysteine-sulfhydryl group of the SoxYZ complex, forming a cysteine S-thiosulfonate derivative. The sulfonate ($-\text{SO}_3^-$) group of the cysteine S-thiosulfonate derivative is then cleaved by the hydrolase SoxB and releases a sulfate ion. The remaining sulfane sulfur ($-\text{S}$) of the SoxY-cysteine persulfide is oxidized to cysteine-S-sulfate by the Sox (CD)₂ complex. SoxB again hydrolyzes the sulfonate group of the cysteine-S-sulfate, releasing another sulfate ion and regenerating the SoxYZ complex. In this oxidative processes, eight electrons are released. These electrons are transferred to a small c-type cytochrome of the electron transfer chain, ultimately contributing to cellular energy production (Dahl 2008, Friedrich et al. 2001, Cattaneo et al. 2023).

1.4.2 The branched pathway of H₂S oxidation

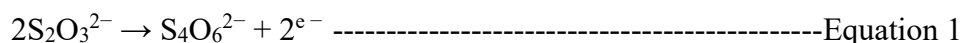
The “branched pathway” describes the oxidative pathway for sulfur metabolism in certain bacteria, particularly phototrophs and chemolithotrophic bacteria such as *Beggiatoa*, *Thiothrix*, and *Thiocapsa*.

The cytochrome complex SoxXA catalyzes the oxidation of thiosulfate, linking it to a cysteine-sulphydryl group of the SoxYZ complex. This process likely involves the transfer of the sulfane sulfur of thiosulfate to the SoxYZ complex. Following the oxidation of thiosulfate, the SoxB enzyme facilitates the hydrolysis of the sulfonate group, resulting in the release of a sulfate ion. The sulfane sulfur of thiosulfate is oxidized and transferred to growing sulfur globules which are subsequently oxidized to sulfite by Dsr proteins. This step involves the conversion of the sulfur species to sulfite, a more oxidized form of sulfur. Sulfite is further oxidized to sulfate in a series of enzymatic reactions. APS Reductase (Apr): Catalyzes the oxidative reaction where sulfite binds to adenosine monophosphate (AMP), producing adenosine-5'-phosphosulfate (APS). ATP Sulfurylase or APAT: Catalyzes the conversion of APS to sulfate. This reaction involves the binding of AMP to either pyrophosphate or phosphate, yielding ATP or ADP, respectively. This process ultimately results in the formation of sulfate from sulfite, which can be incorporated into cellular components or released into the environment. (Dahl 2008, Friedrich et al. 2001, Cattaneo et al. 2023).

1.4.3 The tetrathionate pathway (SI4 pathway of thiosulfate oxidation)

In contrast to the Sox pathway, which proceeds directly from thiosulfate to sulfate without the formation of intermediate compounds, the SI4 pathway involves the production of tetrathionate ($\text{S}_4\text{O}_6^{2-}$) and other polythionates as intermediates. This is followed by the further oxidation of tetrathionate to sulfate. Many proteins and genes involved in the S4I pathway, particularly those responsible for the oxidation of tetrathionate to sulfate, remain poorly understood (Hutt 2017). The overview of the key steps involved in the SI4 pathway is as follows:

The first step involves the oxidation of two molecules of thiosulfate into a single molecule of tetrathionate within the periplasm of the cells by the enzyme thiosulfate dehydrogenase (EC 1.8.2.2). During this oxidation, two electrons are transferred to cytochrome c, which enters the electron transport chain (Equation 1).



In the subsequent step, the hydrolysis of tetrathionate by tetrathionate hydrolase produces one molecule of thiosulfate, one molecule of sulfate, and another atom of elemental sulfur (as in the case of *A. ferroxidans* and *A. acidophilus*) (De Jong et al. 1997, Meulenberg et al. 1993), or only thiosulfate and pentathionate (Bugaytsova and Lindstrom 2004). The reaction products can vary among different species of sulfur-oxidizing microorganisms, and environmental factors such as pH. The final step involves the oxidation of sulfite produced from tetrathionate hydrolysis to sulfate. Four molecules of sulfate are produced for each molecule of tetrathionate formed by thiosulfate oxidation. A total of sixteen electrons from tetrathionate hydrolysis are released into the respiratory chain, including two electrons from the initial formation of tetrathionate. The energetics of ATP production in SI4 organisms remain poorly understood, indicating a need for further research to elucidate the mechanisms involved in energy generation in these organisms (Kelly et al. 1997, Kelly 1985, Hutt 2017).

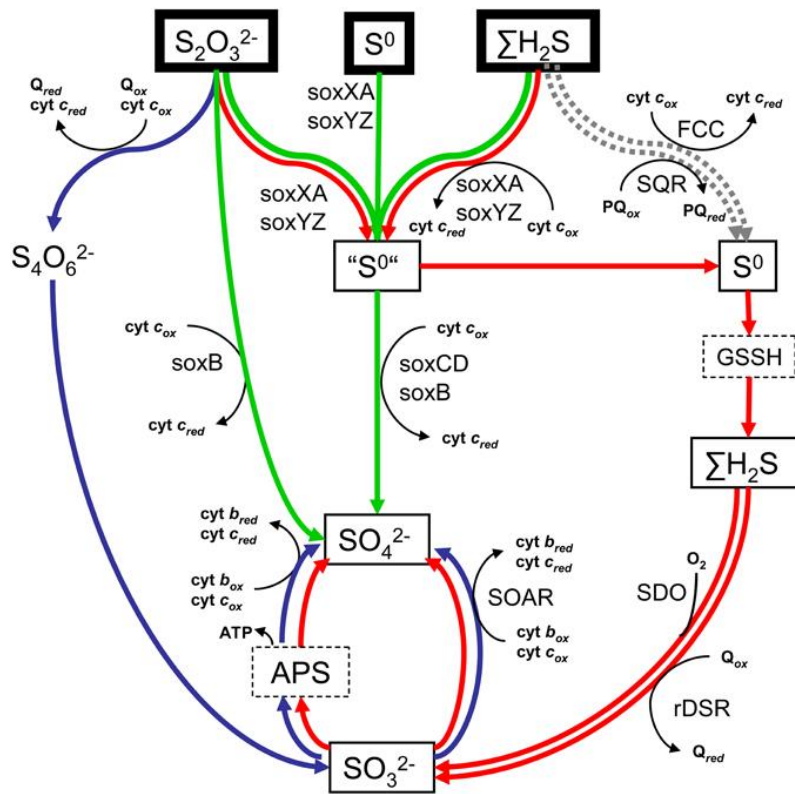


Figure 1.3: Pathways for oxidation of reduced sulfur compounds (Adapted from Klatt and Polerecky, 2015.) Green line: Sox pathway, Red line: Branched pathway, Blue line: Tetrathionate pathway

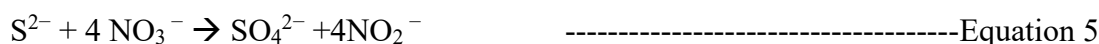
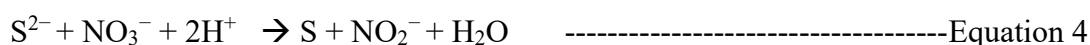
1.4.4 Chemolithotrophic sulfide oxidation

The primary sulfide oxidizers in natural environments are chemolithoautotrophs, also called sulfide/sulfur oxidizing bacteria (SOB) or colorless sulfur bacteria. The typical examples of this group include genera *Thiobacillus*, *Acidithiobacillus*, and *Beggiatoa*.

Oxidation mostly relies on O_2 as the final electron acceptor, though there are instances where exceptions occur. *Acidithiobacillus denitrificans*, for instance, is a facultative anaerobe capable of utilizing NO_3^- as a terminal electron acceptor yielding end product sulfur that is accumulated in the cell granules by the bacteria (Struk et al. 2020, Pepper et al 2015).

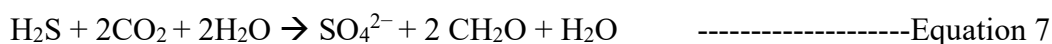
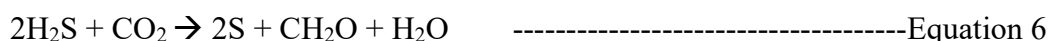
The biological desulfurization process, facilitated by chemolithotrophic bacteria, involves distinct reaction mechanisms for H_2S removal depending on the electron acceptors utilized. When oxygen (aerobic species) serves as the electron acceptor, different sulfur oxidation

products (Equations 2 and 3) are generated, influenced by the oxygen mole ratio (Guerrero et al. 2016, Cattaneo et al. 2023). Conversely, when nitrates (NO_3^-) or nitrites (NO_2^-) are employed as electron acceptors (anaerobic species), the reaction mechanism may yield sulfate, elemental sulfur, nitrogen, or nitrite, depending on the ratios of sulfide to nitrite or sulfide to nitrate mole ratios (Pudi et al. 2022, Cattaneo et al. 2023, Bharathi et al. 1997).



1.4.5 Anoxygenic photoautotrophic sulfide oxidation

Purple sulfur bacteria (PSB) and green sulfur bacteria (GSB) perform the anoxygenic photoautotrophic oxidation of reduced sulfur compounds. These microorganisms harness light energy to fix CO_2 and oxidize reduced sulfur compounds. The biochemistry and genetics of each bacterial group are intricate and varied and even belong to different phyla. The biological desulfurization process by phototrophic bacteria involves the removal of H_2S and the formation of elemental sulfur (Equation 6). Under conditions of low sulfide concentration and constant high light intensity, the reaction dynamics may shift, potentially resulting in the production of sulfates (Equation 7).



1.5 Potential of Hypersaline Bacteria as Oxidizers of Reduced Sulfur

Compounds

Salt pans are extreme environments with a wide range of extremophilic conditions viz. extreme salinities from 2-300 PSU, temperature up to 50°C, alkaline pH 7.2-10, low nutrient concentration, variable oxygen availability, and direct solar radiation. Salt pans, with their high salinity and extreme conditions, habitats a variety of microorganisms that have adapted to thrive in such high salinity and fluctuating environmental parameters (Kerkar 2003, Mani et al. 2012). Bacteria are the major group of microbes present in this hypersaline environment and play a part not only in element recycling and nutritional turnover but also their metabolic products are with great industrial significance. Therefore, it is vital to study them in this ecosystem. Salt pans host a wide array of halophilic microorganisms with significant potential for biotechnological applications due to their ability to thrive in extremely challenging environments (Fernandes et al. 2021, Fernandes et al. 2019, Kamat and Kerkar 2011, Ballav et al. 2015).

Researchers have highlighted the prevalence of the sulfur cycle, and the occurrence of sulfate reduction activity (SRA), SRB, and SOB in salt pans of Goa (Gawas and Kerkar 2023, Kerkar and Bharathi 2007). The existence of these sulfur bacteria presents substantial potential because they are a major part of the biogeochemical processes in the salt pan ecosystem. Secondly, the occurrence of SOB is reported to co-exist with SRB in hypersaline environments (Bharathi et al. 1997, Bharathi 2018). However, there are no reports on the oxidation potential of the SOB from Goan salt pans.

Thus, the current study aims to explore the diversity of sulfate-reducing bacteria and sulfur-oxidizing bacteria, a pivotal component of the biological sulfur cycle in the salt pan ecosystems. Specifically, the study investigates the utilization of SOB sourced from

extremophilic salt pan ecosystems to oxidize reduced sulfur compounds, notably thiosulfate and sulfide. Further, the research also investigates the bacterial diversity within the salt pan ecosystem, aiming to explore the diversity of the bacteria present in these extreme environments emphasizing the variation of the bacteria in different marine salt pans of Goa.

The study was carried out with the following objectives

1. Isolation of SOB and SRB from water and sediments and study of their ecology in Goan salt pans.
2. Phylogenetic relationship of SOB and SRB from salt pan ecosystems.
3. Identification of potential strains of SOB and their significance.
4. Bacterial diversity in three Goan salt pans with metagenomic approach.

Chapter 2

Review of Literature

Sulfur-oxidizing bacteria (SOB) are indeed studied extensively for their ability to oxidize sulfide compounds biologically, which are significant environmental pollutants in wastewater and the oil and gas industry. The biological removal of sulfide relies on the activities of photoautotrophic or chemolithotrophic SOB. Photoautotrophic SOB obtains energy for their metabolism from light, while chemolithotrophic SOB derive energy directly from oxidation reactions. In these reactions, oxygen (in oxic condition) or nitrates/nitrites (in anoxic condition) act as electron acceptors during sulfide oxidation (Janssen et al. 1998).

2.1 Issues Related with Sulfur Compounds

Anthropogenic sulfur compounds significantly pollute the environment, leading to odor issues in polluted surface waters, wastewater treatment plants, biogas plants, sewer systems, and landfills. These compounds also contribute to the corrosion of steel and concrete structures. The most severe problems are caused by sulfur in its reduced form, mainly sulfide, due to their strong odor and toxic characteristics (Pokorna and Zabranska 2015). Sulfide is the metabolic product of dissimilatory sulfate reduction by sulfate-reducing bacteria (SRB). SRB was first described by Beijerinck in 1895 whereas SRB was isolated in pure culture by van Delden in 1903. Later their physiology and biochemistry were thoroughly studied by Baars in 1930. Further, Von Wolzogen Kuhr and van der Vlugt (1934) identified their crucial role in the anaerobic corrosion of buried ferrous pipes. SRB are anaerobic microorganisms commonly found in anoxic environments. They are not only adaptable in utilizing a wide array of electron acceptors and donors, but can also thrive across various environmental conditions and are prevalent in numerous natural and engineered settings where sulfate is available (Kushkevych

et al. 2021, Muyzer et al. 2008). The presence of SRB is often indicated by the strong odor of hydrogen sulfide emissions. SRB have been detected or isolated from a wide range of environments, including marine sediments, marine sediment enrichment slurries (Webster et al. 2006), estuarine and brackish sediments (Boschker et al. 1998), marine Arctic sediments (Ravenschlag et al. 2000), hydrothermal vents (Jeanthon et al. 2002), and deep-sea mud volcanoes in the Black Sea (Stadnitskaia, A. et al. 2005). They are also abundantly present in hypersaline microbial mats (Minz et al. 1993, Rissati et al. 1994). Additionally, SRB have been found in oil fields and in engineered systems, such as anaerobic wastewater treatment plants. Genera such as *Desulfovibrio*, *Desulfobotulus*, *Desulfobulbus*, *Desulfohalobium*, and *Desulfomicrobium* are among the most widespread SRB in nature (Castro et al. 2000). Significant microbial pollution due to SRB growth has been documented in canals and ports, such as those in Venice, Italy, and Bruges, Belgium (Kushkevych et al. 2021a). Six major SRB genera: *Desulfomicrobium*, *Desulfovibrio*, *Desulfonema*, *Desulforegula*, *Desulfobacterium*, and *Desulfobulbus* spp. have been identified in wastewater biofilms. *Desulfobulbus* spp. exhibited the highest population density, reaching about 10^8 – 10^9 cells per cm^3 . SRB colonization poses significant issues in the oil and gas industry, as hydrogen sulfide produced by these bacteria can cause corrosion and contaminate hydrocarbon products. Corrosion of iron and ferrous alloys occurs in both aquatic and terrestrial environments, regardless of nutrient content, temperature, pressure, and pH. Concrete pipes are also susceptible to microbial corrosion due to SRB (Kushkevych et al. 2021a, Kushkevych et al. 2021b).

Minor concrete corrosion issues have been reported when the total sulfide concentration in wastewater ranges from 0.1 to 0.5 mg/L. Severe concrete corrosion can occur at sulfide concentrations starting from 2.0 mg/L. In Los Angeles County, around 10% of sewer pipes are significantly affected by sulfide corrosion, with rehabilitation costs estimated at roughly €400 million. The overall cost for restoring damaged sewer systems in Germany is estimated to be

about €100 billion per year. In Flanders (Belgium), biogenic sulfuric acid corrosion of sewers is estimated to cost around €5 million per year, representing about 10% of the total cost for wastewater collection and treatment.

Corrosion in petroleum refineries disrupts refinery operations, causing unplanned shutdowns, leaks, and product loss. Removing sulfur from petroleum products is one of the most crucial tasks in petroleum refining. In 2013, the global cost of corrosion was estimated at 2.5 trillion US dollars, accounting for 3–4% of global GDP (Zhang et al. 2008).

Hydrogen sulfide toxicity is a known risk for workers in the petroleum, sewer, maritime, and mining industries. A case study demonstrated the condition of a 34-year-old male sewer worker found unconscious approximately 20 feet below street level in a sewer line due to hydrogen sulfide exposure (Yalamanchili and Smith 2008). Therefore, various chemical and biological strategies have been employed to overcome the toxicity of sulfide.

2.2 Bioremediation of Sulfur Compounds

2.2.1 Chemical Method

Common chemical methods such as charcoal adsorption, incineration, and chemical washing has been applied (Cha et al. 1999). The use of metal salts for sulfide removal effectively eliminates dissolved sulfide from wastewater, thereby decreasing the amount available for release into the sewer atmosphere. Field investigations conducted by the Brisbane City Council in 1980 highlighted the importance of metal sulfide precipitation in the sulfur cycle (ASCE, 1989). Therefore, the precipitation of metals in wastewater was considered essential for controlling dissolved sulfide concentrations, but they are expensive and may disrupt downstream treatment processes. Metals such as iron, H_2O_2 , chlorine, and potassium permanganate are frequently used for this purpose. The cost of using these metal sulfides depends on the necessary chemical dosage and the sulfide levels in the wastewater. A summary of the literature on the use of these metals for sulfide removal and the associated costs is

provided in Table 2.1 (Zang et al. 2008). Hence, the use of biological techniques has attracted interest because a substantial amount of sulfide can be bioremediation.

Table 2.1: Summary of literature related to chemical technologies for hydrogen sulfide emission control in sewer systems (Adapted from Zang et al. 2008)

Chemicals	S ²⁻ concentration during upstream processes (mg L ⁻¹)	Average S ²⁻ elimination (%)	Ratio of chemicals to S ²⁻ (w/w)	Cost (€ kg ⁻¹ S)	References
FeCl ₂ . 4H ₂ O	More than 4.0	90	6.0–7.0:1	22.4–26.1	US EPA (1992)
FeSO ₄ . 7H ₂ O	18.0–25.0	95–97	1.7:1	4.8	Tomar and Abdullah (1994)
FeClSO ₄	18.0–25.0	88–98	1.2:1	4.5	Tomar and Abdullah (1994)
FeCl ₂ and FeCl ₃	6.4	97	2.5:1	7.2	Padival et al. (1995)
FeCl ₃	3.8	100	1.5:1	3.7	Nielsen et al. (2005)
H ₂ O ₂	15	85–90	4.0:1	10.6	Waltrip and Snyder (1985)
H ₂ O ₂	8.5	90–95	1.5–1.6:1	4.0–4.2	US EPA (1991)
H ₂ O ₂	20	87–100	1.3:1	3.5	Tomar and Abdullah (1994)
Cl ₂	18	100	9.0:1	2.7	Waltrip and Snyder (1985)
NaClO	20	96–100	2.0:1	2.6	Tomar and Abdullah (1994)
Ca(ClO) ₂	20	93–100	1.8:1	1.9	Tomar and Abdullah (1994)
NaClO and NaOH	18.2	100	1.0:1	1.9	Tomar and Abdullah (1994)
NaNO ₃	54	100	6.7:1	12.2	Jenneman et al. (1986)
NaNO ₃	1.37:1	10.2	100	2.5	Okabe et al. (2003b)
NaNO ₃	1.4–4.6:1	2.5–3.5	90–95	2.5–8.3	Yang et al. (2005)
Nutriox TM	0.88:1	5.1	63–95	2.1	Bentzen et al. (1995)
Nutriox TM	0.60:1	9.6	95	1.5	Hobson and Yang (2000)
Nutriox TM	2.50:1	70	95–100	6	Einarsen et al. (2000)
Nutriox TM	0.36:1	70	68–95	0.9	Garcia De Lomas et al. (2005)
Ca(NO ₃) ₂	1.92:1	2.6	100	4.4	Rodriguez-Gomez et al. (2005)

Note: NutrioxTM is the concentrated calcium nitrate, a commercially available solution of 8.8 % NO⁻³, 0.6 % NH⁺ and a density of 1.5 kg L⁻¹.

2.2.2 Biological Method

Researchers have thus explored biological methods using different types of SOB to treat wastewater and further control corrosion in treatment plants. For instance, Peighami et al. in 2022 investigated the ability of various SOB to screen those involved in the production of sulfur as the product of sulfide oxidation. SOB strains such as *Thioalkalimicrobium aerophilum*, *Thioalkalivibrio versutus*, *Thiobacillus thioparus*, *Thiobacillus novellus*, *Thiothrix nivea*, *Halothiobacillus neapolitanus*, and *Thiobacillus denitrificans* has been studied in detail to show the different end products formed after oxidation of sulfide and reduced sulfur compounds by these strains. Studies have demonstrated that controlling sulfide emissions after adding nitrate can be attributed to the activity of nitrate-reducing sulfur-oxidizing bacteria (NR-SOB), which oxidized the hydrogen sulfide (H₂S) produced by SRB. Research in this area was conducted in oil reservoir environments (Myhr et al. 2002, Nemati et al. 2001a, b) and sewage environments (Esoy et al. 2002, Bentzen et al. 1995, Jenneman et al. 1986; Jobbagy et al. 1994; Okabe et al. 1999, 2003a, b). Most experiments used pure cultures of SRB (Jenneman et al. 1986, Nemati et al. 2001a, b), with some conducted at pilot (Hobson and Yang 2000) or plant scale (Bentzen et al. 1995; Einarsen et al. 2000; Moody and Riek 1999; Garcia De Lomas et al. 2005). Research in oil reservoirs highlighted the crucial role of NR-SOB as key components in microbial communities when studying sulfide production inhibition through nitrate addition (Jenneman et al. 1986, Nemati et al. 2001b). Among the microorganisms implicated in these processes, *Thiomicrospira denitrificans* (Epsilonproteobacteria), and *Thiobacillus denitrificans* (betaproteobacterium) were identified (Kodama and Watanabe 2003; Voordouw et al. 1996). These bacteria oxidized sulfides only in the presence of nitrate, with the reduction of nitrate linked to the oxidation of sulfides and sulfur (Kelly et al. 1997; Kodama and Watanabe 2003). Further, in large-scale trials, the NR-SOB (Nitrate reducing-SOB), *Thiomicrospira denitrificans* ability to achieve sulfide removal efficiencies of up to 94% was demonstrated. Secondly, the

consortium primarily composed of *Arcobacter sp.*, successfully eliminated 99% of the dissolved sulfide in sewage waters (De Gusseme et al. 2009).

Immobilization methods for applying SOB have achieved high success in sulfide removal. Additionally, because immobilized microorganisms can reduce operation and maintenance costs, these methods have been utilized in various industrial applications. For example, *Thiobacillus novellus* SRM, isolated from sewage, was studied for sulfide removal in both immobilized and non-immobilized forms. The immobilized cells successfully removed 100% of 200 ppm sulfide from the medium. (Cha et al. 1999).

SOB has demonstrated its effectiveness not only in wastewater treatment but also in addressing sulfide issues in areas such as aquaculture water management, mining, and corrosion control. Recently SOB as a probiotic has been also used in aquaculture water to control sulfide concentration and other sulfur compounds. The three strains used in the probiotic consortium were *Enterobacter ludwigii* HS1-SOB, *Pseudomonas stutzeri* B6-SOB, and *Cytobacillus firmus* C8-SOB because of their high sulfate production potential (Shinde et al. 2022). In gold mining, conventional gold mining methods uses toxic chemicals like cyanide to separate gold from ore containing sulfide minerals. The excessive use of cyanide for gold dissolution poses environmental risks, therefore biological sulfide oxidizers are used. This bioleaching method harnesses SOB to oxidize sulfur, the primary compound present in the mineral ore within the gold-bearing rock. Various SOB species, including those from genera like *Leptospirillum*, *Thiobacillus*, *Bacillus*, *Beggiota*, *Thiothrix*, *Thermothrix*, *Thiovolum*, *Acidianus*, *Sulfolobus*, *Thioalcalimicrobium*, *Thioalkalividrio*, *Thiomonas*, *Sulfobacillus*, *Alicyclobacillus*, *Caldibacillus*, *Ferrimicrobium*, *Ferroplasma*, *Metallosphaera*, and *Sulforococcus*, have been identified for their roles in bioleaching via sulfide oxidation, as documented in previous studies. These techniques have also been employed in various countries such as Ghana, Australia,

Brazil, South Africa, and Peru (Fitriyani et al. 2018, Han et al. 2013, Rojas-Avelizapa et al. 2013, Okibe et al. 2003, Donati & Sand 2007).

2.3 Occurrence of SRB and SOB

Sulfate-oxidizing bacteria and sulfate-oxidizing bacteria exhibit extensive taxonomic diversity across several bacterial phyla. Sulfur oxidation-reduction is a crucial environmental reaction (Friedrich et al. 2005).

Table 2.2: Diversity of Sulfur-Oxidizing and Sulfur-Reducing Microorganisms Across Various Ecosystems (Adapted from Kumar et al. 2018).

Habitat	SOB and SRB	Oxidation/ Reduction	References
Agriculture ecosystem	<i>Beggiatoa</i> sp. (paddy soil)	Oxidation	Burke et al. (1974) and Joshi and Hollis (1976)
	<i>Thiobacillus denitrificans</i> (cotton and groundnut field)	Oxidation	Yousuf et al. (2014)
	<i>T. thioparus</i>	Oxidation	Wainwright (1984)
	<i>T. neapolitanus</i>	Oxidation	Wainwright (1984)
	<i>T. novellus</i>	Oxidation	Wainwright (1984)
	<i>Rhodovulum sulfidophilum</i>	Oxidation	Yousuf et al. (2014)
	<i>Betaproteobacteria</i>	Oxidation	Yousuf et al. (2014)
	<i>Marichromatium purpuratum</i>	Oxidation	Yousuf et al. (2014)

Aquatic ecosystem	<i>Beggiatoa</i> sp.	Oxidation	Wainwright (1984)
Barren terrestrial land ecosystem	<i>Rhodothalassium salexigens</i>	Oxidation	Yousuf et al. (2014)
	<i>Thiomicrospira crunogena</i>	Oxidation	Yousuf et al. (2014)
	<i>Paracoccus pantotrophus</i>	Oxidation	Bardischewsky et al. (2005)
Blacks Drain and Cudgen Lake	<i>Aquifex aeolicus</i>	Oxidation	Pelletier et al. (2008)
	<i>Paracoccus versutus</i>	Oxidation	Wodara et al. (1997)
	<i>Archaeoglobus profundus</i>	Reduction	Mander et al. (2004)
	<i>Thermodesulforhabdus norvegica</i>	Reduction	Larsen et al. (2001)
	<i>Desulfotomaculum thermocisternum</i>	Reduction	Larsen et al. (2001)
Coastal saline land, hypersaline habitats	<i>Rhodovulum sulfidophilum</i>	Oxidation	Tourova et al. (2011)
	<i>Thiomicrospira crunogena</i>	Oxidation	Tourova et al. (2011)
	<i>Spirochaeta</i> sp.	Oxidation	Tourova et al. (2011)
	<i>Rhodovillum adriaticum</i>	Oxidation	Tourova et al. (2011)
Coastal acid sulfate soil under	<i>Acidithiobacillus ferrooxidans</i>	Oxidation	Wakai et al. (2004)

sugarcane cultivation			
Costal ecosystem	<i>Thiomicrospira</i> sp., <i>Arcobacter sulfidicus</i> , and <i>Sulfurimonas denitrificans</i>	Oxidation	Kuenen and Tuovinen (1981)
Freshwater ecosystem	<i>Betaproteobacteria</i>	Oxidation	Wu et al. (2006)
Hot spring ecosystem	<i>Proteobacteria</i>	Reduction	Badhai et al. (2014)
	<i>Thermodesulfovibrio</i> sp.	Reduction	Badhai et al. (2014)
	<i>Thiobacillus ferrooxidans</i>	Oxidation	Wainwright (1984)
	<i>Thiobacillus organoparus</i>	Oxidation	Wainwright (1984)
	<i>Mycorrhizae</i>	Oxidation	Grayston and Wainwright (1988)
Hypersaline habitats	<i>Thiohalorhabdus denitrificans</i>	Oxidation	Sorokin et al. (2008)
Lihir Island	<i>Acidianus sulfidivorans</i> sp. nov.	Oxidation	Plumb et al. (2007)
Mangrove swamps	<i>Desulfovibrio desulfuricans</i>	Reduction	Sahoo and Dhal (2009)
Marine ecosystem	<i>Asteriomyces crucicatus</i>	Oxidation	Wainwright (1984)
	<i>Asteriomyces crucicatus</i>	Oxidation	Wainwright (1984)

	<i>Oscillochloris trichoides</i>	Oxidation	Dahl and Friedrich(2008)
Marine sediments	<i>Gammaproteobacteria</i>	Oxidation	Yousuf et al. (2014)
	<i>Archaeoglobus fulgidus</i>	Reduction	Mander et al.(2004)
	<i>Thioploca</i> sp.	Oxidation	Jørgensen and Nelson (2004)
	<i>Pseudoxanthomonas mexicana</i>	Oxidation	Krishnani et al.(2010)
Spruce forest ecosystem	<i>T. thiooxidans</i>	Oxidation	Wainwright (1984)
	<i>T. thioparus</i>	Oxidation	Wainwright (1984)
Sub-tropical rainforest and back swamps	<i>Ferrobacillus ferrooxidans</i>	Oxidation	Brunner et al.(2008)
Swamp ecosystem	<i>Aspergillus niger</i>	Oxidation	Grayston et al.(1986)
	<i>Mucor fiaous</i>	Oxidation	Grayston et al.(1986)

2.4 Hypersaline Environment as a Potential Source for Bioremediation

Hypersaline environments such as solar salterns, soda lakes, salt mines, and deep-sea salt brines were once thought to have low bacterial diversity, primarily inhabited by highly adapted Archaea. However as demonstrated by Sorokin et al. (2006), extreme habitats support complex microbial communities. Furthermore, the emergence of next-generation sequencing has facilitated the successful application of metagenomic techniques to explore microbial diversity in these extreme environments. Proteobacteria, known as a major phylum of gram-negative bacteria with high morphological, physiological, and metabolic diversity (Sogin et al. 2006). Numerous studies have documented the prevalence of Pseudomonadota in diverse ecosystems (Lee et al. 2020, Sogin et al. 2006, Priti and Kerkar 2023), highlighting their significant roles in carbon, nitrogen and sulfur mineralization and decomposition (Bernhard et al. 2012). Specifically, within solar salterns, Gammaproteobacteria are recognized as the predominant taxa, with Delta-, Alpha-, and Epsilonproteobacteria also present within the Proteobacteria phylum (Sogin et al. 2006). Halophiles are ideal candidates to be used in several industrial processes due to their remarkable stability and robust activity under extreme conditions, including pH, pressure, temperature, and low water activity. They are categorized based on their halotolerance into extreme, moderate, slight, and non-halophilic. Moderately halophilic bacteria, in particular, produce enzymes that remain stable and active in both medium and high salt concentrations, as well as in the absence of salt. This diverse group of microorganisms, encompassing species from genera such as *Halomonas*, *Marinococcus*, *Halobacterium*, *Flavobacterium*, *Chromobacterium*, *Paracoccus*, and *Pseudomonas*, exhibits traits like rapid growth, minimal nutritional requirements, and the ability to utilize a variety of compounds as sole carbon and energy sources. Consequently, the enzymes of moderately halophilic bacteria are being extensively studied for their biotechnological potential (Ventosa et al. 1998).

An unexpectedly high diversity of culturable, moderately, and extremely halophilic obligate chemolithoautotrophic SOB has been reported in the sediments of hypersaline habitats. They all belonged to Gammaproteobacteria. These habitats include chloride-sulfate lakes in Mongolia, Russia, and Ukraine, a sea saltern in Slovenia, and a deep-sea salt brine in the Mediterranean (Sorokin et al. 2006). Additionally, halophilic SOB was discovered in the salt brine from the former salt mine in Solivar (Presov, East Slovakia). These bacteria belong to the genera *Planococcus*, *Chromohalobacter*, *Halovibrio*, *Halomonas*, and *Marinobacter*, and could grow on thiosulfate medium (Nosalova et al. 2022). The coexistence of SOB and SRB helps maintain a balance of reduced sulfur compounds in hypersaline environments (Kerkar 2003). Consequently, SOB from hypersaline ecosystems could be of significance to manage sulfide and other reduced sulfur compounds, which would otherwise emit foul-smelling toxic sulfide gas, endangering personnel safety and equally contributing to corrosion.

The salt pans are characterized by high salt concentrations and are extreme habitats that host unique microbial communities. Bacteria from these hypersaline environments, known as halophiles, have adapted to thrive under high osmotic pressure. Salt pans are also found in Goa, India, and the salt produced, in the salt pan is highly consumed by locals (Kerkar 2003, Mani et al. 2012). Bacteria from such environments are particularly considered for their unique characteristics as the bacteria exhibit unique physiological and biochemical adaptations and can produce novel bioactive compounds. Additionally, extremophiles can degrade pollutants in harsh environments where conventional microorganisms cannot survive. Furthermore, enzymes derived from extremophiles, such as thermophilic or halophilic bacteria, are often more stable and efficient under extreme temperatures, pH levels, and salt concentrations (Manikandan and Senthilkumar 2017). Attempts have been made to isolate SOB from the salt pan of Goa (Kerkar 2003). The SOB from a hypersaline ecosystem could serve as a better candidate than that from other environments, to be applied in various harsh environmental conditions such as wastewater

treatment plants, biogas plant, oil and petroleum industry etc. to bioremediate sulfide and reduced sulfur compounds from polluted environments.

Chapter 3

Materials and Methods

Salt pan ecosystems in Goa are man-made impoundments of the ingress of seawater which are bound by bunds (mud borders) so that the entrapped water evaporates and the salt crystallizes when exposed to solar radiation. Depending on the proximity to the sea, the first pond is referred to primary pond, followed by the secondary, the tertiary, and the crystallizer pond which shows the variation in the salinity. The salinity of seawater is 32-35 PSU, which reflects in the primary pond with a gradual increase in the salinity levels due to evaporation from the secondary to the crystallizer pond, which reflects the salinity of over 250 PSU.

In Goa, the salt pans are prevalent in the north i.e. Bardez taluka, Tiswadi taluka and in Salcete taluka.

3.1 Sampling Sites

Three sampling sites of Goan salt pans were selected based on their distinct location where Agarwado salt pan is located in North Goa, Pernem taluka connected to the Chapora estuary whereas Curca salt pan lies on the Zuari estuary and Nerul salt pan lies on the Mandovi estuary (Figure 3.1). The Mandovi and the Zuari estuary are interconnected by the Cumbarjua canal.

The geographical location of the three sites was recorded using a Global Positioning System (GPS) [eTrexVista™ personal navigator®, Garmin International, Inc., USA]. Sampling was carried out seasonally in three phases viz. preparatory phase, ceased phase, and salt harvesting phase including the primary and crystallizer pond.

3.1.1 Agarwado Salt pan:

Agarwado village is in Pernem taluka of Goa, India. Salt pans of Agarwado are adjacent to the Chapora estuary which is 30 km long. This estuary is covered with small and scattered patches

of mangrove species. The salt pans are encircled by coconut plantations and the adjacent area is utilized for paddy cultivation. This study site was located at N15.640978, E73.76426. This salt pan is more pristine as compared to the other two salt pans and is away from anthropogenic influence.

3.1.2 Curca Salt pan:

Curca village is in Tiswadi taluka of Goa, India. These salt pans adjoin the Zuari estuary which is one of the major estuaries in Goa. This salt pan is bordered by human settlements with very few mangrove species surrounding the salt pans. The coordinates of the sampling site in Curca salt pans were N15.45798, E73.88065.

3.1.3 Nerul Salt pan:

Nerul, a village in the Bardez taluka of North Goa, is encircled on its north, west, and east sides by the Nerul River, which is connected to the Mandovi estuary, one of Goa's major estuaries. The riverbanks are bordered by human settlements, with a small patch of paddy fields on the opposite side. The sampling was conducted at the coordinates N15.51979, E73.78909.

3.2 Sampling Period

Salt pans are influenced by three phases namely: the salt harvesting phase (January- May), the ceased phase (June to September), and the preparatory phase (October to December). The samples were collected from the month of January to December 2019. Additionally, during the salt harvesting phase, the samples were collected from the primary and crystallizer ponds.

3.3 Sample Collection

Water samples were collected from the overlying salt pan water into sterile bottles. Sediment samples were obtained using a (5.5cm x 15 cm) sterile corer. To maintain anaerobic conditions in the corer, the corer was capped at both ends with a core cap. The aerobic sediment samples

were collected in open corers. All the samples were transported in ice-cold conditions to the laboratory for processing.



Figure 3.1: Goan Salt pans in study (A) Location of Agarwado, Curca, and Nerul Salt pans in Goa. Aerial image of (B) Agarwado salt pan (C) Curca salt pan (D) Nerul salt pan [Source: Google earth pro, 2023]

3.4 Analysis of Physico-Chemical Parameters

Temperature, salinity, and pH of the overlying salt pan water were recorded at the sampling site. A field thermometer (76 mm immersion, ZEAL, England) was used to measure the temperature and the pH was noted by a digital pH meter (pH 700, Eutech Instruments, Thermo Scientific, USA). A hand-held refractometer (S/Mill-E, ATAGO Co. Ltd., Japan) was used to record the salinity.,

Dissolved oxygen (DO), total dissolved solids (TDS), and conductivity of the water samples were measured using a EUTECH CyberScan PCD 650 multiparameter (Thermo Scientific, India). The physico-chemical parameters of the overlying water viz temperature, salinity, pH, DO, TDS, conductivity, sulfate, sulfide, nitrate, and nitrite were measured. In case of sediment samples, analysis was restricted to measurement of sulfate, sulfide, nitrate, and nitrite concentrations. Where the sediment pore water was pulled together by centrifugation of the sediment samples.

3.4.1 Sulfate

The sulfate of the water sample (1 mL) was analyzed by the turbidometry as described by Clesceri et al. (1998). The sample fixed with 4N HCl, was diluted to 25 mL with distilled water, and conditioning solution (1.25 mL) was added with constant agitation with a magnetic stirrer. BaCl₂, (2 mL) of 30 % was slowly added until a precipitate was formed within 1 min of stirring. A precipitate of barium sulfate was formed which was determined spectrophotometrically at 365 nm.

3.4.2 Sulfide

For determination of sulfide concentration in water (1 mL) and sediment (1 g), the samples were fixed in 2 %, 9 mL of zinc acetate. To this 5 mL of DMPD (N, N-dimethyl-p-phenylenediamine- Appendix II) and 0.25 mL of FAS (ferric ammonium sulfate- Appendix II) reagent were added and allowed to stand for 10 min. The volume of the sample was then brought

up to 50 mL with distilled water. The sulfide was determined spectrophotometrically at 670 nm (Cline, 1969 and Pachmayr, 1960) and the concentration of sulfide in the samples was calculated using a standard graph.

3.4.3 Nitrate

Cadmium (Cd) pellets were soaked in 1% HCl overnight. Then, the Cd pellets were transferred to a stoppered bottle and washed with distilled water, 3- 4 times until the water was clear. After removing the distilled water, 2 % CuSO₄ was added into a stoppered bottle and mix well for 30 min to coat the Cd pellets with CuSO₄. The pellets were washed again until a clear solution was obtained. The column was then filled with diluted ammonium chloride buffer solution (pH 8) and glass wool was placed at the bottom of the column using a glass rod. Then, the copper-coated Cd pellets were transferred into the column followed by another layer of glass wool. The column was activated by washing it with ammonium chloride buffer and the flow rate of 8 min per 100 mL was adjusted with distilled water.

Nitrate was estimated by introducing 100 mL of the sample through the Cd column, also referred to as the reducing column. The first 40 mL sample was discarded and the remaining 50 mL was collected. To this sulphanilamide (1mL) (Appendix II), 1mL of NEDE (N-1-naphthylamine ethylene diamine, Appendix II) were added and incubated for 15 min after thoroughly mixing the contents. The nitrate was spectrophotometrically estimated at an absorbance of 543 nm. The concentration was calculated using a standard graph of potassium nitrate solution (APHA, 2012).

3.4.4 Nitrite

Nitrite was estimated spectrophotometrically (APHA, 2012). Water and sediment pore water samples (25 mL) were taken in a stoppered test tube. Sulphanilamide reagent (0.5 mL) was added. Sulfanilamide reacts with nitrite resulting in the formation of diazo-compounds. The contents were mixed after 5 min and 0.5 mL of NEDE (N- (1-naphthyl)-1- ethylenediamine)

was added. The diazo-compound formed reacted with N- (1-naphthyl)-1- ethylenediamine and formed a pink colored azo-dye. Absorbance was measured at 543 nm by spectrophotometry (Shimadzu UV Spectrophotometer, Japan). The unknown concentration was calculated by using a standard graph of sodium nitrite.

3.5 Bacteriological Parameters

3.5.1 Retrievable Heterotrophic Bacterial Counts

Enumeration of culturable heterotrophic bacteria was carried out from water and sediment samples of the respective salt pans. Serial dilution and plate count methods were used to estimate the retrieved bacteria. For serial dilution, 10 mL of water sample was added to 90 mL of autoclaved salt pan water. Sediment sample, (10 g) was dissolved in 90 mL of sterile salt pan water, after serial dilutions were prepared, 100 μ L of dilutions were spread plated on Nutrient Agar (NA, Appendix I) plates. For the water and sediment samples dilutions of 10^{-3} , 10^{-4} , and 10^{-5} were plated on NA plates. The spread-plated plates were incubated at $30^{\circ}\text{C} \pm 2$ for 2 - 4 weeks.

3.5.2 Isolation of Anaerobic Sulfur Oxidizing Bacteria (SOB) and Sulfate Reducing Bacteria (SRB)

For enumeration and isolation of anaerobic SOB and SRB, Lieske's (Appendix I) and Hatchikian's medium (Appendix I) were used respectively (Bharati et al. 1988 and Kerkar 2003). Water (10 mL) and sediment (10 g) samples were added separately to 90 mL of sterile salt pan water and subsequently serial dilutions (up to 10^{-5}) were prepared. The respective agar media (12 mL) was dispensed in 15 mL screw-capped tubes. These tubes were further inoculated with 1 mL (10^{-2} , 10^{-3} , and 10^{-4}) dilutions, gently mixed, and allowed to solidify. Sterile paraffin wax (approximately 2 mL) was overlaid on the solidified media to promote an anaerobic environment in the culture tubes. The tubes were incubated at $30 \pm 2^{\circ}\text{C}$ for 15-30

days, the resulting colonies on Lieske's media were recorded as culturable SOB, and on the Hatchikian's media were recorded as culturable SRB.

3.5.3 Isolation of Aerobic Sulfur Oxidizing Bacteria

Aerobic SOB was isolated by spread plate method on Lieske's media, where dilutions of 10^{-3} , 10^{-4} , and 10^{-5} of water and sediment samples were used as inoculum and were spread plated (0.1 mL) on the petri plates with Lieske's media. These plates were then incubated at 30 ± 2 °C and the colonies were enumerated after 15-20 days of incubation.

3.6 Statistical Analysis

Data of physico-chemical and bacteriological parameters was statistically analyzed using a non-parametric test and correlation using IBM SPSS Statistics software [(Version 29.0.2.0 (20))].

3.7 Gram Staining and Cell Morphology

Gram staining of cultures was performed as described by Hans Christian Gram (1884).

The morphology of SOB and SRB isolates was visualized with Olympus microscope (BX60, Japan) under oil immersion.

3.8 Oxygen Tolerance Test

An oxygen tolerance test was carried out exclusively for the SOB isolated anaerobically from Curca and Agarwado salt pans. Anaerobic agar (Appendix I) was prepared, sterilized by autoclaving, and dispensed in screw-capped tubes. Bacterial culture (0.5 mL) was mixed in the molten anaerobic agar and was subsequently allowed to solidify. The tubes were capped tightly and incubated at 30 ± 2 °C for 15-20 days.

3.9 Growth of Anaerobic SOB on Lieske's Media Plates

To check whether the anaerobic SOB could grow in oxygenated conditions, they were inoculated onto Lieske's media plates. Pure cultures of anaerobic SOB were streaked on the media plates and were incubated under aerobic conditions at 30 ± 2 °C for 15-20 days.

3.10 Salinity Tolerance Test

Twenty-six SOB from Curca salt pan and 27 SOB from Agarwado salt pan were assessed for their salinity tolerance in Leiskes media. SOB isolates were grown in a medium with various salinity ranging from 0, 2.5, 5, 7.5, 10, 15, 20, 25, 30, 35, and 40 % prepared with crude salt in screw-capped tubes and incubated at 30 ± 2 °C. The growth of the SOB isolates on respective concentrations of salinity was noted.

3.11 Biochemical Characterization

3.11.1 Biochemical Characterization of SOB

Biochemical test viz. catalase, oxidase, sulfur and nitrate reduction, indole production, motility test, O-nitrophenylbeta-D-galactopyranoside (ONPG) and citrate utilization, Methyl Red-Voges Proskauer (MR-VP) test, and gelatinase test were carried out with isolated SOB.

A. Catalase Test

A single SOB colony was picked using a sterile nichrome loop and placed on a clean dry slide. A drop of H₂O₂ (3%) was added to it and the slide was observed for the production of effervescence as an indicator of a positive result.

B. Oxidase Test

A loopful of the actively grown pure culture was smeared on a sterile oxidase disc (Hi-Media DD018). A positive test was indicated by a change in color of the disc from white to purple-blue color within 30 sec of contact of the culture to the disc.

C. Sulfur Reduction, Indole Production and Motility (SIM) Test

Sulfur reduction, indole production, and motility of the SOB isolates was tested on SIM media (Hi-Media M181, Appendix I). SIM media was prepared, 5 mL was poured into each multiple test tubes, sterilized by autoclaving at 121°C, 15 lbs. pressure for 30 min, and cooled to form a butt. Using a sterile nichrome wire the stabs were inoculated with a 24 h grown SOB and were

incubated at 30 ± 2 °C. The tubes were checked after 48 h for formation of a black coloration which indicated a positive test for sulfur reduction.

The tubes were checked for motility after 48 h of incubation by observing the diffused growth pattern in the stabbed area of the agar butt.

A positive indole production was recorded after adding 1mL Kovacs reagent (1 mL) (Appendix II) onto the test tube with the SOB inoculated in the stab.

D. Nitrate Reduction Test

Nutrient broth with 1% potassium nitrate (Appendix I) was prepared in 5 mL vials and Durhams tubes were placed in the vials. The medium was sterilized by autoclaving at 121°C, 15 lbs. pressure for 30 min. Fresh bacterial culture was inoculated in the medium and incubated at 30 ± 2 °C. A positive nitrate reduction was observed after 24 -72 h of incubation taking into consideration the following four possibilities:

1. The turbidity in the tube was noted.
2. A bubble in the Durhams tube indicated the presence of both nitrate reductase and nitrite reductase.
3. In the absence of a bubble, nitrate reagent A (Sulfanilamide, Appendix II) and nitrate reagent B N-(1-naphthyl)-ethylenediamine, Appendix II] were added. A red color indicates the presence of nitrate reductase and the absence of nitrite reductase.
4. If no color change and the absence of a bubble in Durhams tube, indicates an absence of the enzymes in the bacteria.

E. ONPG Test

Sterile normal saline (0.2 mL) was inoculated with an ONPG disc (Hi-Media DD008). Further a pure SOB colony was inoculated in the test and vortexed thoroughly for 2 sec. The test tube was incubated at 30 ± 2 °C for 48 h and the color change from colorless to yellow was noted as a positive ONPG test.

F. Citrate Utilization Test

Sterile slants of Simmon Citrate Agar (Appendix I) were prepared in test tubes, cooled, and streaked with actively growing SOB. The slants were incubated at 30 ± 2 °C for 72 h and after incubation, the change in color of the media from green to blue was noted.

G. Methyl Red - Voges Proskauer (MR-VP) Test

MR-VP broth was prepared in 5 mL vials and sterilized by autoclaving at 121°C, 15 lbs. pressure for 30 min. The tube was inoculated with 20 µL of SOB culture suspensions. Further incubated at 30 ± 2 °C for 24-72 h. After incubation, 2.5 mL of the media was transferred to another tube and five drops of methyl-red indicator (Appendix II) was added and mixed thoroughly by rolling the vials between the palms. The color change from yellow to red was noted.

For the VP test, 2 mL of the above incubated MR-VP broth, was treated with 6 drops of Barritt's A (Appendix II) and 2 drops of Barritt's B (Appendix II). The development of a deep rose color in the vial after 15 min following the addition of Barritt's reagents was indicative of a positive test.

H. Gelatinase Test

Gelatin iron solid media (Appendix I) was prepared and dispensed (5 mL) into each tube. The tubes were then sterilized by autoclaving at 121°C, 15 lbs. pressure for 30 min. The media was then allowed to cool into stabs and subsequently stabbed with fresh grown SOB incubated at 30 ± 2 °C for 48 h. Subsequently, the tubes were refrigerated at 4 °C and checked for solidification/liquefaction of the medium. A positive gelatinous test indicated the liquefaction of the gelatin.

3.11.2 Biochemical Characterization of SRB

A. Catalase Test

A drop (0.1 mL) of SRB liquid culture was taken using a Pasteur pipette and placed on clean, dry slides. After being exposed to air for 30 minutes, a drop of 3% H₂O₂ was added to the culture. The slide was then observed for effervescence as an indicator of a positive result.

B. Oxidase Test

A 0.1 ml of the culture suspension was smeared on a sterile oxidase disc (Hi-Media DD018). A positive test was indicated by a change in color of the disc from white to purple-blue color within 30 sec of contact of the culture to the disc.

C. Substrate Utilization

SRB isolates were tested for growth on various substrates in Hatchikian's media. The following substrates were used at their respective final concentrations: sodium acetate (0.2% w/v), formate (0.01% w/v), benzoate (0.05% w/v), glucose (0.1% w/v) sodium pyruvate (0.22% w/v), propionate (0.07% w/v), palmitate (0.05% w/v), sodium lactate (0.6% w/v) and, ethanol (1% v/v). Growth was assessed by measuring the increase in optical density (OD) after 10-15 days of incubation.

D. Sporulation

Gram-stained slides were examined under 100X magnification using an Olympus BX60 microscope to detect the presence of spores.

E. Motility

The hanging drop method was used to determine the motility of the culture.

3.12 Molecular Characterization of SOB and SRB Isolates

3.12.1 Genomic DNA Isolation

The genomic DNA of the SOB and SRB was extracted using HiPurA Bacterial Genomic DNA purification kit MB554 (HiMedia, India) according to the instruction manual.

3.12.2 PCR (Polymerase chain reaction) of 16S rRNA Gene

PCR of 16S rRNA gene was carried out using 27'F (Forward primer, 5' AGA GTT TGA TCC TGG CTC AG 3') and 1492'R (Reverse primer, 5' TAC GGY TAC CTT GTT ACG ACT T 3'). PCR of the 16S rRNA gene was carried out using a thermal cycler (BIO-RAD) using a Hi-PCR Kit (HiMedia MBT075) and the following PCR conditions were used.

PCR reaction mixture

Molecular grade water

dNTPs	10 mM
Taq Buffer	10 X
27F primer	50 pmol
1492R primer	50 pmol
Template	50–500 ng
Taq polymerase	1 U

PCR profile

Initial denaturation	: 94°C - 5min	
Denaturation	: 94°C - 1min	} 25 cycles
Annealing	: 53°C - 1min	
Extension	: 72°C - 1min	
Final extension	: 72°C - 5min	

3.12.3 Quantification of PCR Product

The concentration of 16S rRNA gene PCR product was measured on NanoDrop 200c spectrophotometer (Thermo Scientific). Firstly, the lid and cuvette were cleaned with distilled water and clean tissue paper. Molecular grade water (2 μ L) was placed on the surface of the cuvette, the lid was placed and a blank was set. PCR product (2 μ L) was placed on the surface of the cuvette and the concentration (ng/ μ L) was recorded.

3.12.4 16S rRNA Gene Sequencing

The 16S rRNA gene PCR product was sequenced by G3500 Genetic Analyzer (Applied Biosystem, Hitachi Ltd., Japan). The forward and reverse raw sequences were double-checked and were assembled in contigs using DNA Baser sequence assembler software. NCBI-BLAST was then used to match these contigs with the GenBank database.

3.12.5 Phylogenetic Analysis

The forward and reverse sequences were double-checked using DNA Baser sequence assembler. Phylogenetic analysis was conducted in MEGA11 (Tamura et al. 2013). The evolutionary history was inferred using the Neighbor-Joining method (Saitou 1987).

3.13 Screening of Potential SOB Isolates for Thiosulfate Oxidation

Based on the results of molecular identification of SOB by 16S rRNA gene, SOB (7 isolates) belonging to different genera were selected for screening of the potential SOB isolates for thiosulfate oxidation. Scanning electron microscopy was carried out to observe the morphology and cell size of the selected seven SOB isolates. Freshly grown bacterial culture (10 μ L) was spread onto a clean glass slide (1 cm²) and air-dried. The cells were fixed with 2.5 % glutaraldehyde at 4 °C overnight. The next day, the excess glutaraldehyde was washed off with sterile PBS followed by successive dehydration with 10, 30, 50, 70, 80, 90 and 100 % acetone

(v/v) for 10 min each. The slides were subsequently attached to a stub, sputter coated with gold and viewed under a SEM with a magnification from 15 K to 40 K (Bratbak 2018).

The seven SOB isolates such as A*1, BJPS/7bB, DBPW/11, AMPW/13a, BJCW/14bB, AD-1-19a, BAUGPW/21b were selected to check their capability of thiosulfate oxidation. To set up this experiment, these SOB isolates were inoculated in Nutrient broth (Appendix I) and incubated at 30 ± 2 °C until the OD₆₀₀ equals 0.5. All the SOB isolates were checked for their thiosulfate oxidation capability at 30 mM of sodium thiosulfate anhydrous (Na₂S₂O₃, HI MEDIA- GRM1420) was used as substrate. The experiment was set up in duplicates in flasks. For each SOB isolate a positive control with substrate Na₂S₂O₃ and culture and a negative control without inoculation was also prepared. The test and the positive control flasks were inoculated with 1ml of SOB culture. All the test flasks, positive control and negative control flasks were incubated at 30 ± 2 °C. After 90 days the optical density for each bacterial isolate was noted. The residual thiosulfate in the media and sulfate produced were determined.

3.13.1 Estimation of Thiosulfate

The residual concentration of thiosulfate, was measured iodimetrically (Hansen, 1973). To 10 mL of sample, 2.5 mL of sulfuric acid (3 N), and 1.25 mL of 0.0125 N iodine solution were added and titrated immediately with standardized 0.0125 N Na₂S₂O₃.5H₂O until most of the iodine was consumed. A few drops of freshly prepared starch solution (1%) were added and titrated until the blue color disappeared. Following formula was used to determine the amount of thiosulfate utilized in the medium.

Titre value of blank (Avg) = A

Titre value of uninoculated media (Avg) = B

Average titre value of sample = S

$$\frac{A - S \times 1377.6}{\text{Volume of sample used for titration}} = \frac{B1}{\text{(thiosulfate measured in media)}}$$

$$\frac{A - S \times 1377.6}{\text{Volume of sample used for titration}} = \frac{S1}{\text{(thiosulfate measured in sample)}}$$

where 1377.6 = mol. wt. of S_2O_3 ($112 \times 0.0123 \text{ N} \times 1000$)

$B1 - S1 = D$ (amount of thiosulfate utilized in mg)

3.13.2 Estimation of Sulfate

The sulfate was analyzed by turbidometry as described by Clesceri et al. (1998).

3.14 Thiosulfate Oxidation by *Aloalcanivorax xenomutans* (A*1 isolate)

The A*1 (SOB isolate) was selected to further to examine its thiosulfate oxidation potential at different concentrations of thiosulfate from 30 – 160 mM. Lieske's media with 30, 40, 50, 60, 80, 100, 120, 140 and 160 mM of $Na_2S_2O_3$ were prepared in flasks in duplicates. All test flasks with different concentration of $Na_2S_2O_3$ and positive control were inoculated with 1 mL of actively grown culture (O.D = 0.5). The negative controls were maintained in duplicates for each concentration of $Na_2S_2O_3$ used in the experiment. Flasks were incubated at $30 \pm 2^\circ \text{C}$ for 120 days (4 months). Thiosulfate oxidation by the isolate A*1 was determined by estimating the thiosulfate utilized and sulfate produced after 120 days.

3.15 Sulfide Oxidation by *Alloalcanivorex xenomutans* (A*1 isolate)

Lieske's media with different concentrations of (100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mg/L) sodium hydro sulfide (NaHS - Sigma Aldrich 161527) was prepared as the substrate for sulfide oxidation and dispensed in serum bottles. This experiment was conducted

in duplicates. The test with different concentration of NaHS and the positive control bottles were inoculated with 1ml of actively grown culture (O.D = 0.5). The negative control was also maintained for each concentration of NaHS used in the experiment. Serum bottles were incubated at 30 ± 2 °C for 120 days (4 months). The amount of sulfide oxidized was then measured by estimation of the sulfate produced (Cline, 1969 and Pachmayr, 1960) and the residual sulfide concentration (Pachmayr, 1960) was recorded after 120 days.

3.16 Screening for Genes Involved in Thiosulfate and Sulfide Oxidation

Genomic DNA of the bacterial isolate A*1 was isolated using the HiPurATM Bacterial Genomic DNA Purification Kit (Hi-Media, India). This DNA was then used as a template for PCR amplification of genes involved in thiosulfate oxidation (*soxB*) and sulfide oxidation (*sqr*). PCR primer pair *soxB*432F/*soxB*1446B (Mayer et al. 2007) specific for *soxB* gene was selected with reference to literature studies. Similarly, two primer pairs such as SQR-G1-475FM/ SQR-G1-964R and SQR-G4-140FB/SQR-G4-840RM were selected for amplification of *sqr* gene (Pham et al. 2008). The sequences of the primers are listed in Table 3.1.

Table 3.1: Primer sequences used for amplification of *soxB* and *sqr* gene

Gene	Forward	Reverse
<i>soxB</i>	432F (5' GAY GGN GGN GAY ACN TGG 3')	1446B (5' CAT GTC NCC NCC RTG YTG 3')
<i>sqr</i>	G1-475FM (5' TGY TWY GGC CCG GCB TAY GA 3')	G1- 964R (5' GTS ACC ATS SWT TCR ATC AT 3')
<i>sqr</i>	G4-140FB (5' TGG ATT CCR TCR AAC ATT TGG GT 3')	G4- 840RM (5' AAT WAG CAT MGC RAA RTC GAA CTA 3')

PCR reaction mixture prepared for *soxB* and *sqr* gene (25 µL) contained 2.5 µL of 10x assay buffer, 1.25 µL of 50 mM MgCl₂, 2.5 µL of dNTP, 10.5 µL of nuclease-free water, 2 µL of template DNA and 2.5 µL of each forward and reverse primer (1 µM each). PCR was carried

out in a PCR thermal cycler (Sure cycler 8800, Agilent Technologies, Singapore) as per the reaction conditions given in Table 3.2 (A-C). 5 µL of each sample along with a DNA ladder (HiMedia) was loaded onto 1 % agarose gel and visualized with an ultraviolet illuminator (UV transilluminator, Fotodyne Incorporated, USA).

Table 3.2 A: PCR parameters used for amplification of *soxB* gene

Steps	Temperature	Time
Denaturation	95°C	2 min
Denaturation	95°C	30 sec
Annealing	43°C	30 sec
Elongation	72°C	1 min
Elongation	72°C	8 min

} 30 Cycles

Table 3.2 B: PCR parameters used for amplification of *sqr* gene (G1 primer set)

Steps	Temperature	Time
Denaturation	95°C	2 min
Denaturation	95°C	30 sec
Annealing	42°C	30 sec
Elongation	72°C	1 min
Elongation	72°C	8 min

} 30 Cycles

Table 3.2 C: PCR parameters used for amplification of *sqr* gene (G4 primer set)

Steps	Temperature	Time
Denaturation	94°C	5 min
Denaturation	94°C	30 sec
Annealing	53°C	30 sec
Elongation	72°C	1 min
Elongation	72°C	5 min

3.17 A Metagenomic Analysis of the Bacterial Diversity of Three Salt pan Sediments

Sediment samples from the crystallizer pond were collected in sterile Hi-media bags during the salt harvesting period using a 5.5 cm diameter corer. Sediment samples (0-2 cm deep) were collected from the salt pans of Curca village (N15.45798, E73.88065) situated at Tiswadi taluka, Agarwado village (N15.640978, E73.76426) situated at Pernem taluka and Nerul village (N15.51979, E73.78909) situated at Bardez taluka. The sediment samples were collected in triplicates from each salt pan site and were carried to the laboratory in the ice box and the triplicate samples were subsequently mixed together to obtain a single sediment sample and stored at -20°C until further processing. Sediment samples from Agarwado, Curca, and Nerul salt pans were indicated as AC, CC, and NC respectively. The temperature of the three salt pans sediment samples was recorded on-site using a thermometer (76 mm immersion, ZEAL, England). Pore water was obtained after centrifugation of sediment samples which was subsequently used to measure the pH, salinity, nitrate, nitrite, and sulfate. The pH was recorded using a pH meter (pH 700, Eutech Instruments, Thermo Scientific, USA) and the salinity was

measured by a refractometer (S/Mill-E, ATAGO Co. Ltd., Japan). The nitrite and nitrate were estimated spectrophotometrically according to APHA, (2012). Sulfate was estimated turbidometrically by measuring the precipitation of BaSO₄ (Clesceri et al., 1998). For the estimation of sulfide, 1 g of sediment sample was fixed in 2% zinc acetate and was further analysed spectrophotometrically according to Cline (1969).

3.17.1 Environmental DNA Extraction, Library Preparation, and Illumina Sequencing

Environmental DNA was extracted from each sediment (1g) sample using DNeasy PowerSoil Kit (Qiagen). The DNA obtained was checked for its concentration and quality using NanoDrop. The amplicon library was prepared using Nextera XT Index Kit (Illumina inc.) using bacterial specific forward primer 341F (GCCTACGGGNGGCWGCAG) and reverse primer 805R (ACTACHVGGGTATCTAATCC) for V3-V4 hypervariable region of 16S rRNA gene followed by adding Illumina adapters required for cluster generation. Amplicon libraries were purified by AMPureXP beads and quantified using a Qubit Fluorometer. Libraries were then loaded on MiSeq at 10-20 pM concentration for cluster generation and sequencing. The libraries were sequenced at Eurofins Genomics India Pvt. Ltd., Karnataka, India on the Illumina MiSeq platform using 2x300 bp chemistry to obtain paired-end reads.

3.17.2 Sequence Analysis by Bioinformatics

Raw paired-end reads of AC, CC, and NC were analysed by Quantitative Insights Into Microbial Ecology 2 (QIIME 2), version 2-2023.7 (Bolyen et al., 2019). Reads obtained after sequencing were denoised via the q2-dada2 plugin (Callahan et al., 2016) which involves correction of Illumina sequenced errors, merging of reverse and forward paired-end reads, and clustering them into ASVs (amplicon sequence variants). The rarefaction curve was plotted using QIIME 2. ASVs obtained were further taxonomically classified into different taxonomic

levels using the q2-feature-classifier plugin based on the SILVA (V.138) reference database (Quast et al., 2012). The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST2) method (Douglas et al., 2020) was utilized to predict the functional potential of the bacterial communities in the three salt pans mentioned above. The relative abundance of functional protein profiles in the three salt pans was represented in a heatmap constructed using PRIMER7.

3.17.3 Statistical Analysis

The alpha (intra-sample) diversity indices [such as Simpson index, Shannon index, Menhinick's richness index, and Pielou's evenness (equitability)] were calculated at the genus level for the three sampling sites, similarly the beta diversity was measured as described by Whittaker (Whittaker, 1960). Canonical Correspondence analysis (CCA) was done to understand the influence of environmental variables [Temperature (°C), pH, salinity (PSU), nitrate (mg/L), nitrite (mg/L), sulfate (mg/L), sulfide (mg/L)] on the bacterial genera. Alpha diversity, beta diversity and CCA analysis were performed by using Paleontological statistics software (PAST), version 4.13 (Hammer et al., 2001).

Chapter 4

Results

4.1 Physico-chemical Parameters of the Saltpans

The physico-chemical parameters of the Curca and Agarwado salt pans which were monitored during the salt harvesting phase (January- May), ceased phase (June-September), and the preparatory phase (October- December), including the primary and crystallizer pond were recorded as shown below:

4.1.1 Temperature

The overlying water sample of the two salt pans Curca and Agarwado recorded the highest temperatures during the salt-harvesting phase i.e. $32.7 \pm 0.2^{\circ}\text{C}$ in the Curca salt pan and $34.7 \pm 0.2^{\circ}\text{C}$ in the Agarwado salt pan, followed by the preparatory ($29.1 \pm 0.3^{\circ}\text{C}$ in the Curca salt pan and $30.8 \pm 0.5^{\circ}\text{C}$ in the Agarwado salt pan) and ceased phases ($28.5 \pm 0.2^{\circ}\text{C}$; $27.5 \pm 0.4^{\circ}\text{C}$) (Figure 4.1A and B). Temperature ranged from $27-35^{\circ}\text{C}$ and $27.6-36^{\circ}\text{C}$ in Curca and Agarwado salt pans respectively.

Particularly, during the months of the salt harvesting phase the temperature recorded in the samples of crystallizer ponds was higher than the primary pond (Figures 4.2 A and B).

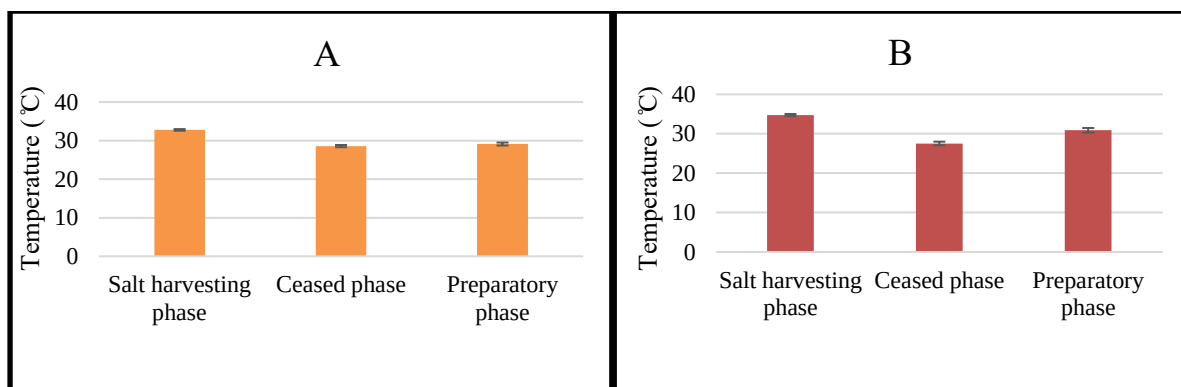


Figure 4.1: Temperature during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

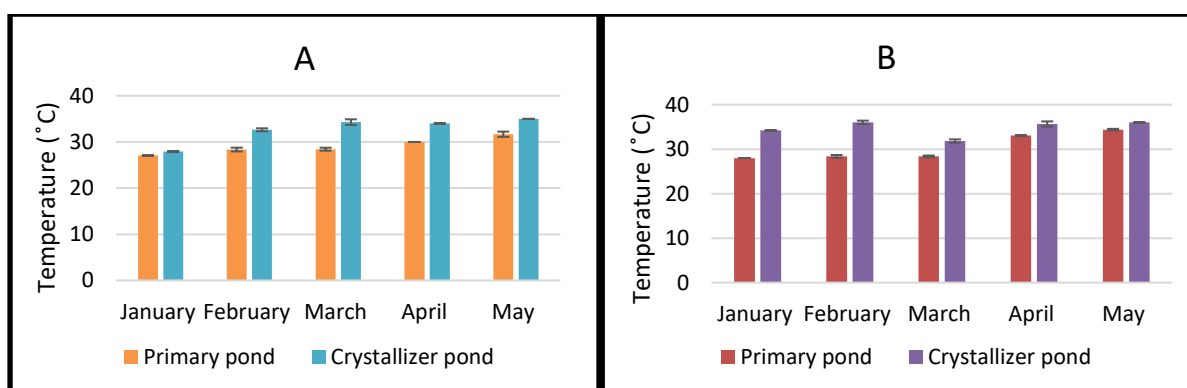


Figure 4.2: Temperature variation in the salt harvesting phase in Curca (A) and Agarwado (B) salt pans

4.1.2 Salinity

A prominent variation in the salinity was observed during the study period. The salinity was measured maximum during the salt-harvesting phase (specifically in the crystallizer pond) as compared to other two phases in both the salt pans (Figure 4.3 A and B). The salinity varied from 6 ± 0.5 to 250 ± 0 PSU and 6.9 ± 0.05 to 287 ± 1.7 PSU in Curca and Agarwado salt pans respectively during the three phases. The salinity recorded in month of April was 287 ± 1.7 PSU in the Agarwado salt pan while in Curca salt pan, the months of February, April, and May showed salinity of up to 250 PSU (Figure 4.4 A and B).

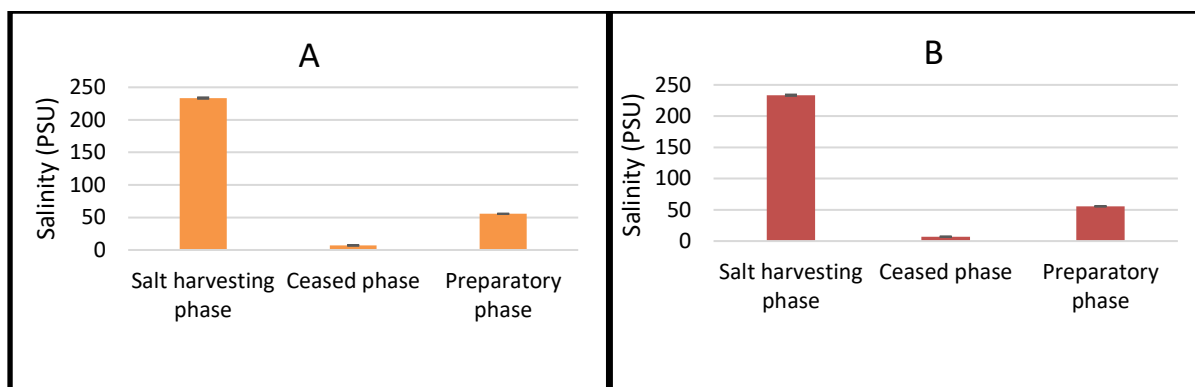


Figure 4.3: Salinity variation during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

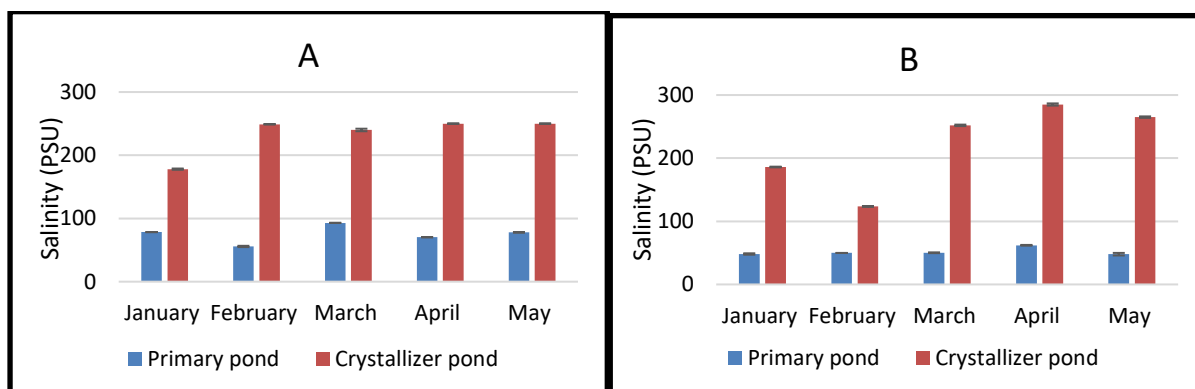


Figure 4.4: Salinity variation in the salt harvesting phase in Curca (A) and Agarwado (B)

4.1.3 Hydrogen Ion Concentration (pH)

The variation in the pH in Curca and Agarwado salt pan has been depicted in Figure 4.5 and 4.6. Unexpectedly the pH of the overlying water was most alkaline in preparatory phase in both the Curca (8.3 ± 0.06) and Agarwado (8.2 ± 0.07) salt pans, followed by the ceased phase and the salt-harvesting phases. The primary pond was observed to be alkaline as compared to crystallizer pond in both the salt pans during the entire period of salt harvesting.

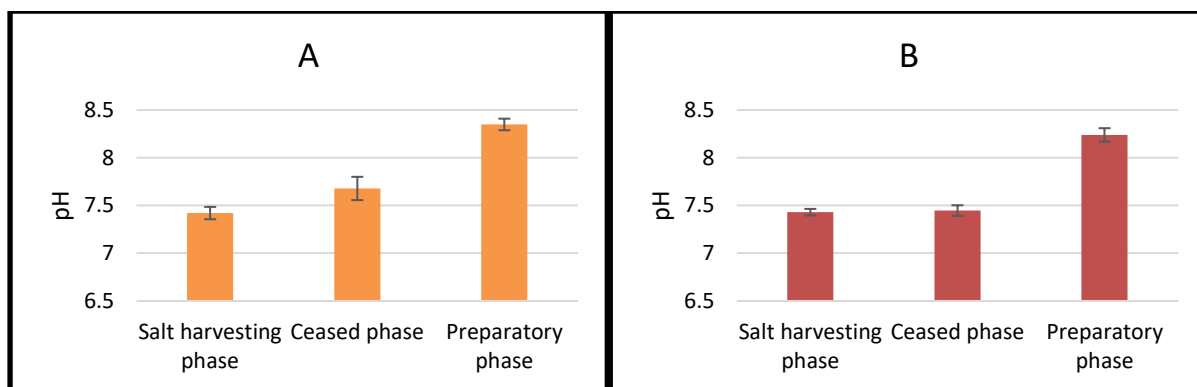


Figure 4.5: Variation in pH during three phases of salt pan in Curca (A) and Agarwado (B)

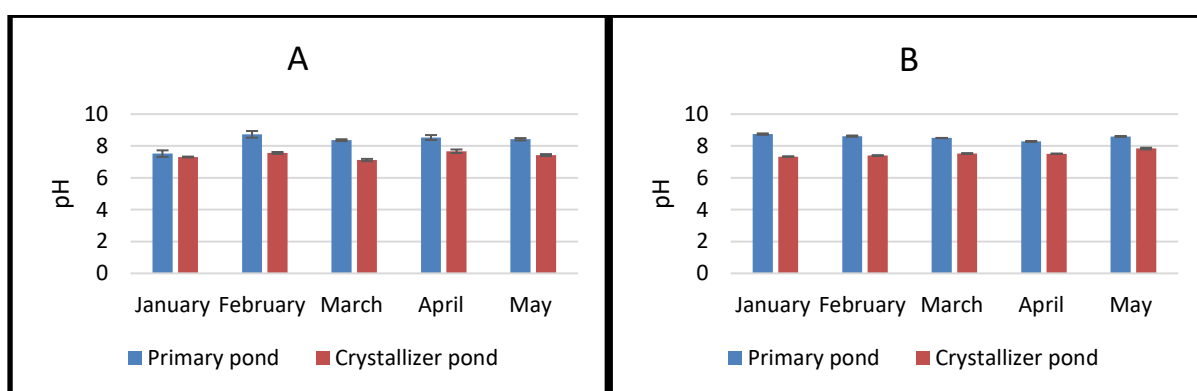


Figure 4.6: Variation in pH in the salt harvesting phase in Curca (A) and Agarwado (B) salt

4.1.4 Dissolved Oxygen (DO)

The dissolved oxygen of overlying water was maximum during the preparatory phase in both Curca (6.05 ± 0.63 mg/L) and Agarwado (5.2 ± 0.12 mg/L) salt pans whereas the lowest concentration was observed during the salt-harvesting phase (3.3 ± 0.12 mg/L in Curca and 2.3 ± 0.26 mg/L in Agarwado salt pans) (Figure 4.7A and B). The DO concentration in the primary pond (during the salt harvesting phase) was higher as compared to crystallizer pond (Figure 4.8A and B).

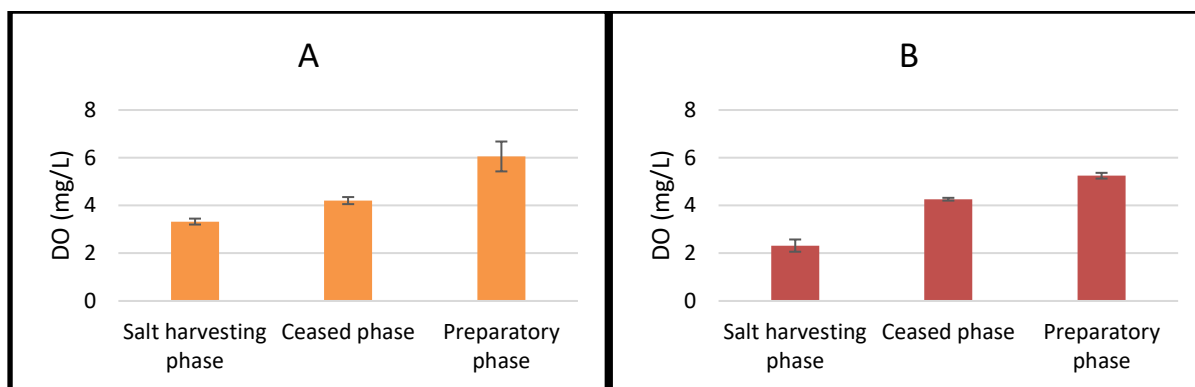


Figure 4.7: DO during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

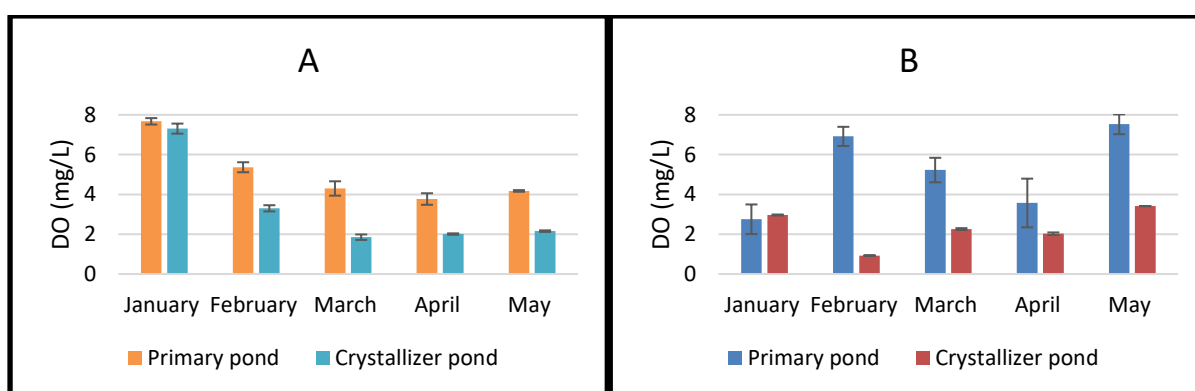


Figure 4.8: Variation in DO in salt harvesting phase in Curca (A) and Agarwado (B) salt

4.1.5 Conductivity and Total Dissolved Solids (TDS)

A similar pattern was also observed in the conductivity and the TDS. Conductivity (4.9 A and B) and TDS (Figure 4.11 A and B) of the salt pans (Curca and Agarwado) were highest during the salt-harvesting phase, followed by the preparatory and ceased phase. Furthermore, both these parameters were higher in the primary pond as compared to the crystallizer pond during the salt-harvesting phase as demonstrated in Figures 4.10 and 4.12.

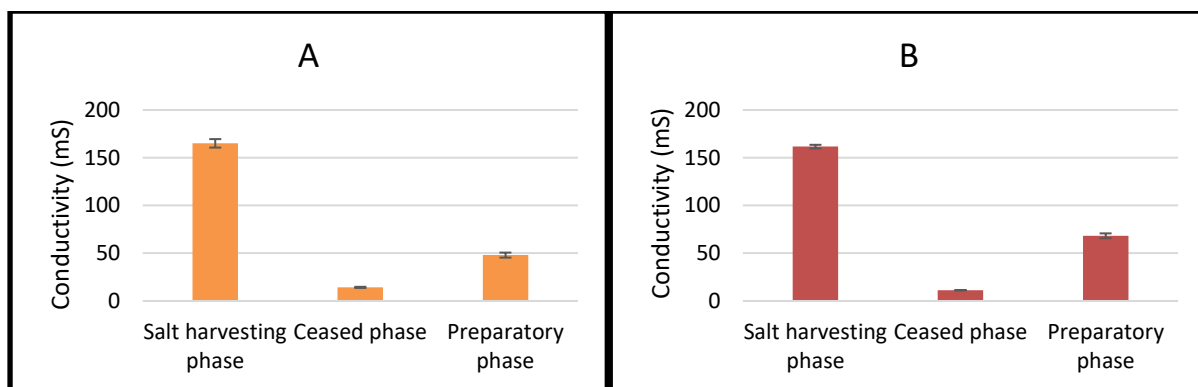


Figure 4.9: Variation in conductivity during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

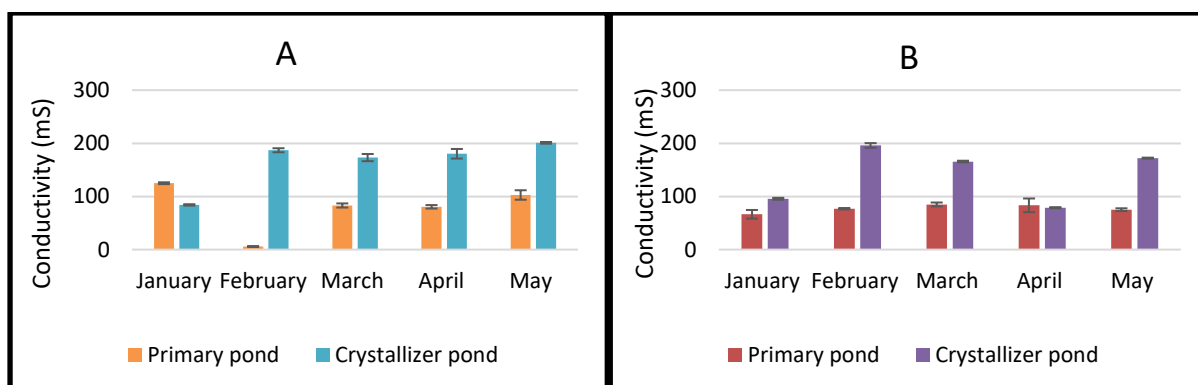


Figure 4.10: Variation in conductivity in the salt harvesting phase in Curca (A) and Agarwado (B) salt pans

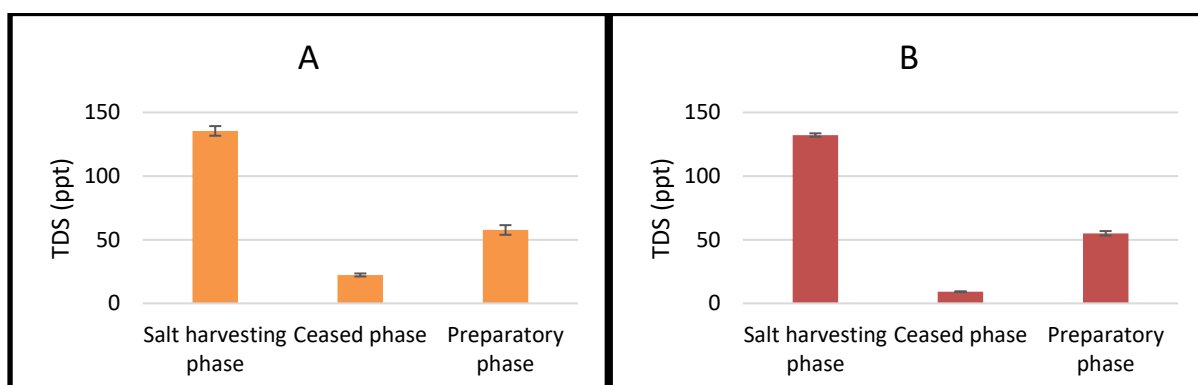


Figure 4.11: TDS variation during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

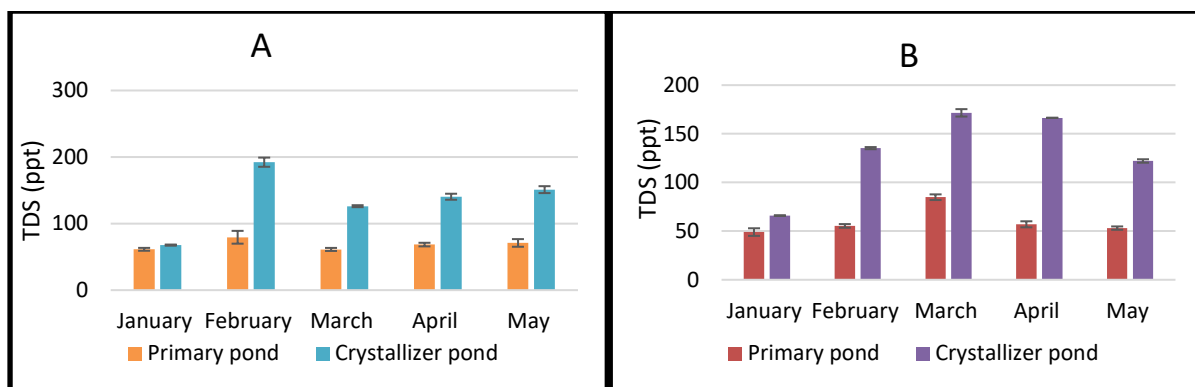


Figure 4.12: Variation in TDS in the salt harvesting phase in Curca (A) and Agarwado (B) salt

4.1.6 Sulfate

The concentration of sulfate in the overlying water in both salt pans reached its maximum during the salt-harvesting phase, followed by the preparatory and the ceased phase. The Kruskal Wallis test revealed significant differences in sulfate concentration across the three phases in the overlying water samples from both the Curca, with $H(2) = 23.17$, $P < 0.01$, and the Agarwado salt pan, with $H(2) = 23.15$, $P < 0.01$. In the Curca salt pans, the average sulfate concentrations during the salt-harvesting, preparatory, and ceased phases were 8.4 ± 0.5 mg/L, 2.6 ± 0.17 mg/L, and 0.9 ± 0.07 mg/L, respectively (Figure 4.13 A). Similarly, in the Agarwado salt pan, the concentrations were 7.7 ± 0.4 mg/L, 2.7 ± 0.13 mg/L, and 0.62 ± 0.02 mg/L, respectively (Figure 4.13B). Additionally, sulfate concentrations were higher in the overlying water and sediment samples of crystallizer pond as compared to the primary pond in both salt pans (Figures 4.14 and 4.16). The Mann-Whitney U test showed significant differences in sulfate concentration of overlying water between the primary and crystallizer ponds in Curca ($U = 63$, $z = 1.990$, $p = .047$, $r = .46$) and in Agarwado ($U = 81$, $z = 3.578$, $p < .001$, $r = .84$). Similarly, significant differences in the sulfate concentration were found between the ponds in sediment samples from Curca ($U = 79$, $z = 3.473$, $p < .001$, $r = .8$) and Agarwado ($U = 81$, $z = 3.599$, $p < .001$, $r = .84$).

In both Curca and Agarwado, the sulfate concentration in the sediments followed a similar trend with that of the overlying water, showing particularly high levels during the salt-harvesting phase in the crystallizer pond, as compared to the preparatory and ceased phases (Figures 4.15 and 4.16). Significant differences in sulfate concentration between the phases were also observed in sediment samples from Curca ($H(2) = 23.178, P < 0.01$) and Agarwado [$H(2) = 23.171, P < 0.01$].

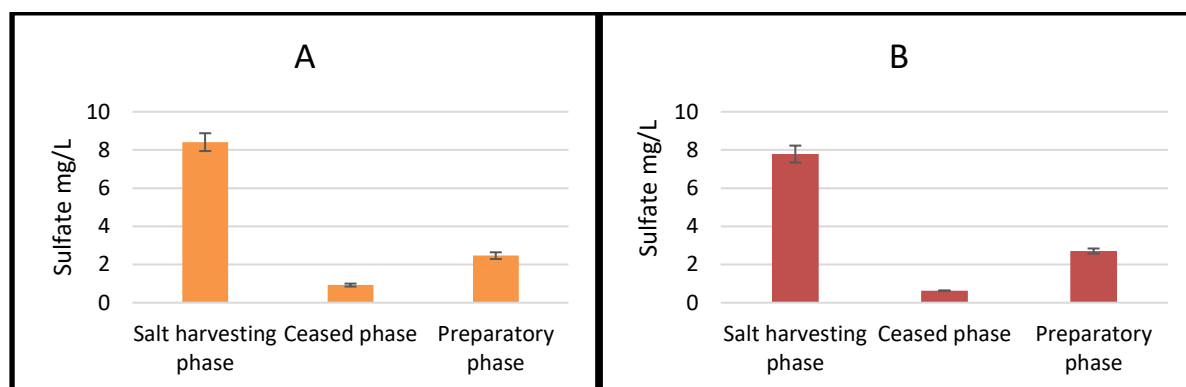


Figure 4.13: Variation in sulfate concentration in overlying water samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

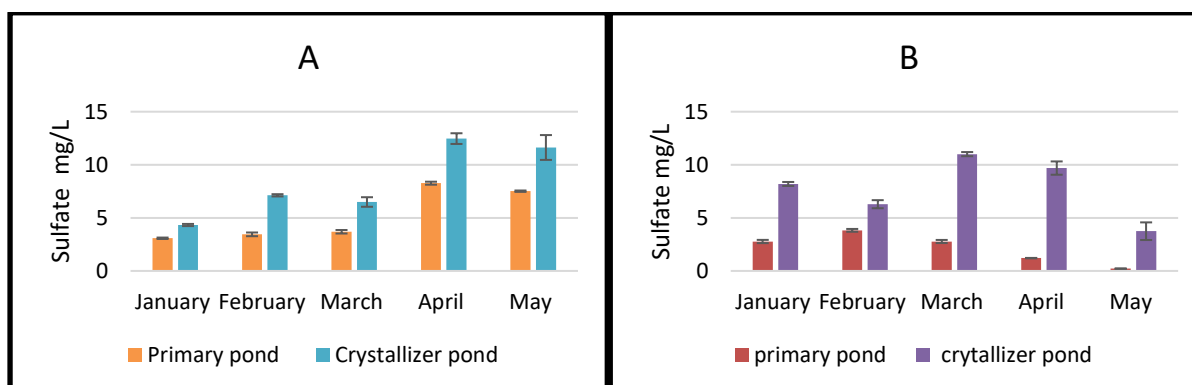


Figure 4.14: Variation in sulfate concentration in the overlying water samples during period of salt harvesting phase in Curca (A) and Agarwado (B) salt pans

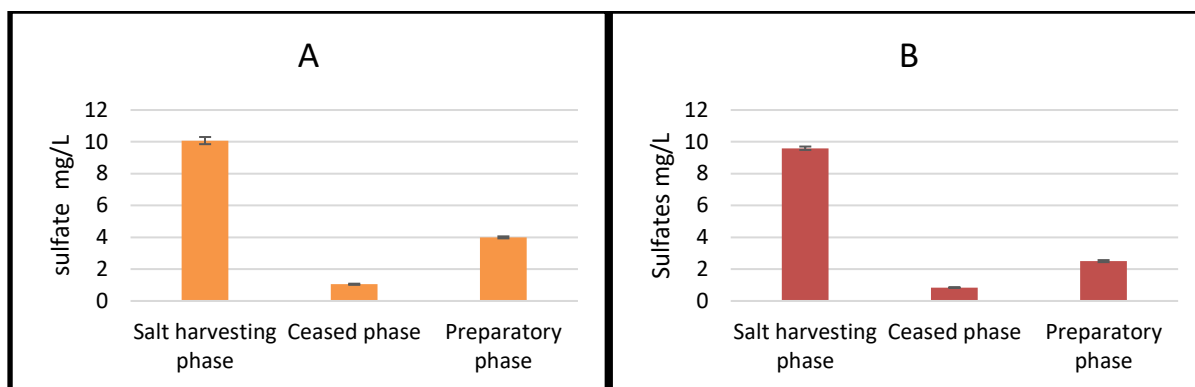


Figure 4.15: Variation in sulfate concentration in sediment during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

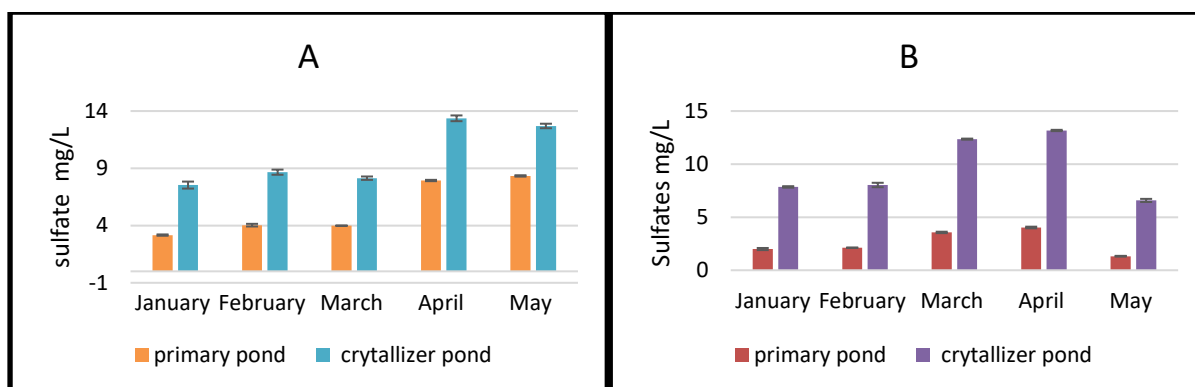


Figure 4.16: Variation in sulfate concentration in sediment samples during period of salt harvesting phase in Curca (A) and Agarwado (B) salt pans

4.1.7 Sulfide

Soluble sulfide is typically volatile in seawater, making its detection difficult. Nevertheless, we have attempted to measure its concentration. In overlying water samples of the Curca salt pan, the Kruskal-Wallis test indicated that the sulfide concentration in salt-harvesting phase was significantly different from both the ceased phase ($H(2) = 11, P = .003$) and the preparatory phase ($H(2) = 7, P = .61$), while the ceased phase was not significantly different from the preparatory phase ($H(2) = -4, P = .285$). Similar results were observed in overlying water samples of the Agarwado. The sulfide concentration in the overlying water peaked during the salt-harvesting phase and gradually decreased during the preparatory and the ceased phases in

both Agarwado and Curca. During the salt-harvesting phase, the average sulfide concentration was 8.3 ± 0.5 mg/L in Curca (Figure 4.17A), whereas it was notably higher in Agarwado, at 23.1 ± 0.6 mg/L (Figure 4.17 B). In the Agarwado salt pan's crystallizer pond, (Figure 4.18 B), sulfide levels were significantly higher as compared to the primary pond values ($U = 81$, $z = 3.582$, $p < .001$, $r = .84$). In Curca, elevated levels were only noted in February and March in the crystallizer pond (Figure 4.18 A), but the sulfide concentration was still significantly different between the two ponds ($U = 63$, $z = 1.993$, $p = .05$, $r = .47$).

In the sediment samples from both salt pans, sulfide concentration followed a similar trend with the overlying water (Figures 4.19 A and B). The statistical test showed significant differences in the sulfide concentration of sediment samples across all three phases in the Curca salt pan ($H(2) = 23.157$, $P < .001$), but no significant difference between the salt-harvesting phase and the preparatory phase in the Agarwado salt pan was observed ($H(2) = 5$, $P = .181$). Additionally, sulfide levels were higher in the crystallizer pond (Figures 4.20 A and B). The sulfide concentration between the ponds was significant in both Curca ($U = 81$, $z = 3.599$, $p < .001$, $r = .84$) and Agarwado ($U = 81$, $z = 3.591$, $p < .001$, $r = .84$) sediment samples.

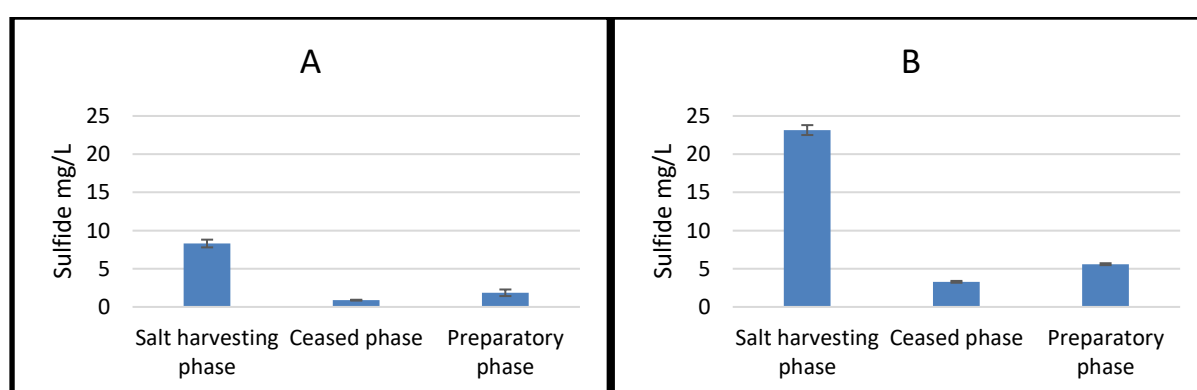


Figure 4.17: Variation in sulfide concentration in overlying water samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

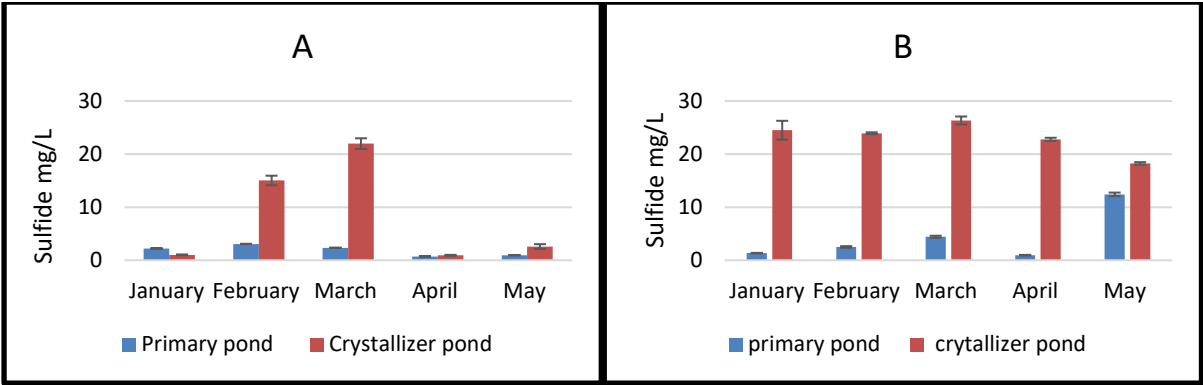


Figure 4.18: Variation in sulfide concentration in overlying water samples during period of salt harvesting phase in Curca (A) and Agarwado (B) salt pans

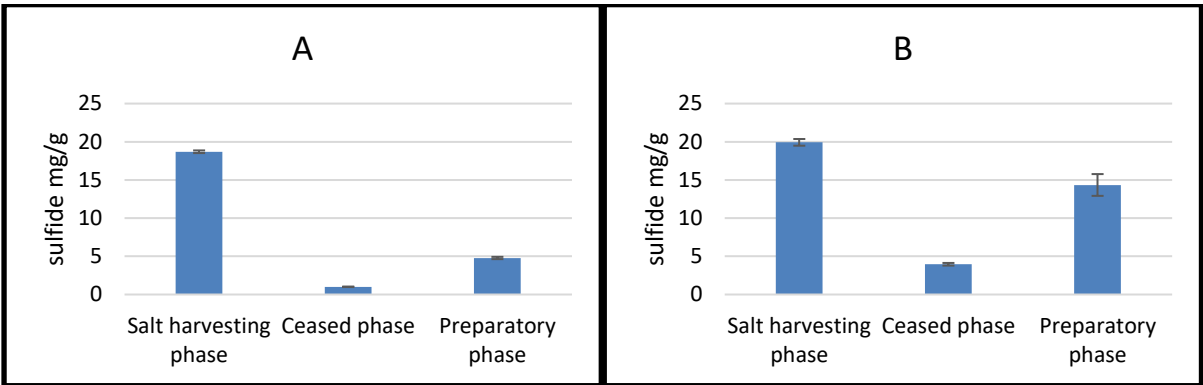


Figure 4.19: Variation in sulfide concentration in sediment samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

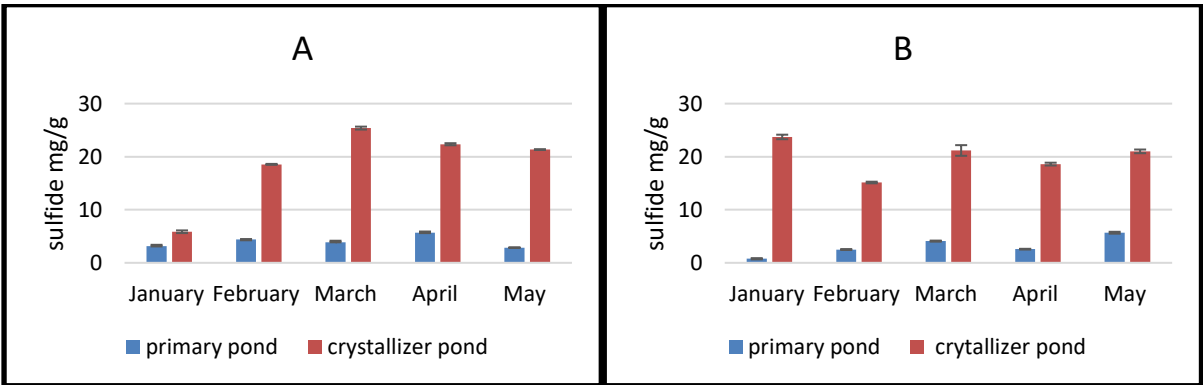


Figure 4.20: Variation in sulfide concentration in sediment samples during period of salt harvesting phase in Curca (A) and Agarwado (B) salt pans

4.1.8 Nitrate

The distribution of nitrate levels in the overlying water during the three phases in the Curca and Agarwado salt pans is shown in Figures 4.21(A and B). The highest nitrate concentrations were recorded during the salt-harvesting phase, with $0.3 \pm 0.02 \mu\text{M}$ in the Curca salt pan and $0.22 \pm 0.01 \mu\text{M}$ in the Agarwado salt pan, followed by the preparatory phase, and the lowest levels were found at the ceased phase. Statistical tests revealed a significant difference ($H(2) = 17.732, P < .001$) in nitrate concentration between all phases in the Curca salt pan, while in the Agarwado salt pan, the nitrate concentration between the salt-harvesting and preparatory phases was not significant ($H(2) = 5, P = .179$).

In sediment samples, nitrate concentration was minimal during the preparatory phase (Figures 4.23 A and B), in contrast with the levels observed in the overlying water samples. Comparing water and sediment samples (Figures 4.22 and 4.24) from the primary and crystallizer ponds, nitrate levels were higher in the crystallizer pond as compared to the primary pond in both Curca and Agarwado salt pans. Significant differences in nitrate concentration between the primary and crystallizer ponds were found in both water and sediment samples from Curca ($U = 74, z = 2.961, p = .02, r = .98$ in water; $U = 79, z = 3.374, p = .001, r = .79$ in sediment) and Agarwado ($U = 81, z = 3.582, p < .001, r = .84$ in water; $U = 81, z = 3.580, p < .001, r = .84$ in sediment).

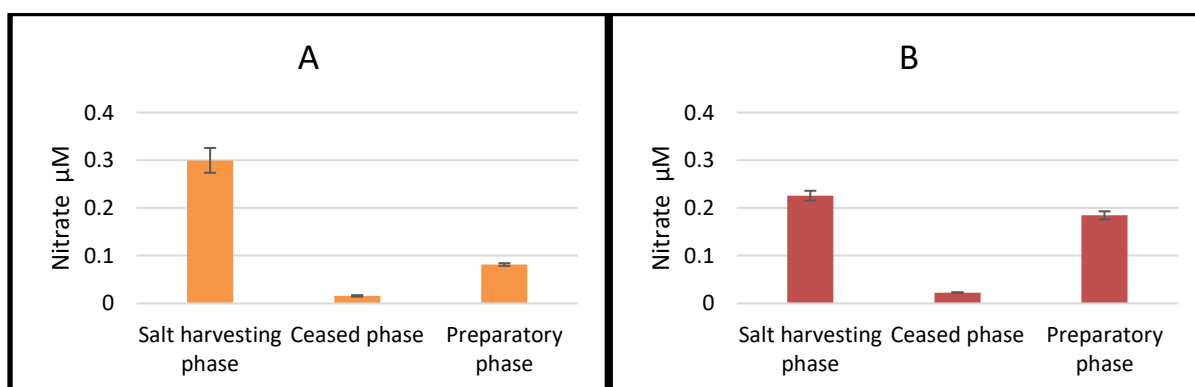


Figure 4.21: Nitrate variation in overlying water samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

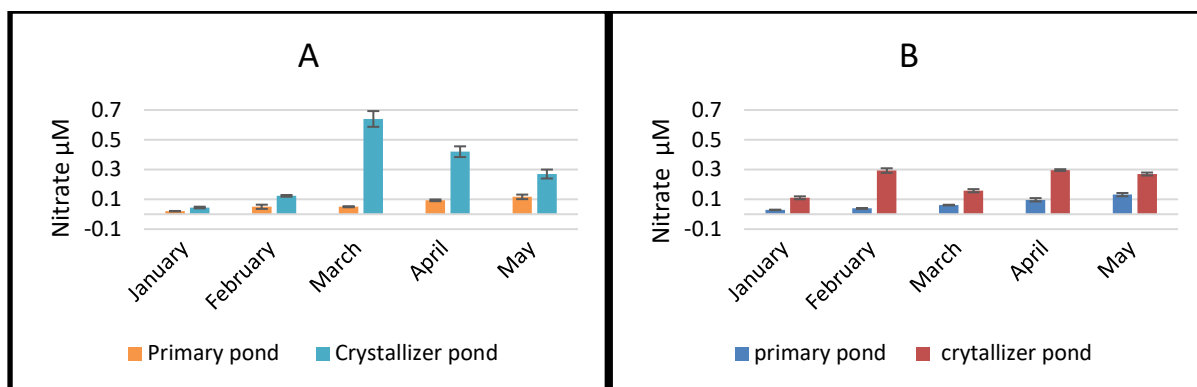


Figure 4.22: Variation in nitrate concentration in overlying water samples during period of salt harvesting phase in Curca (A) and Agarwado (B) salt pans

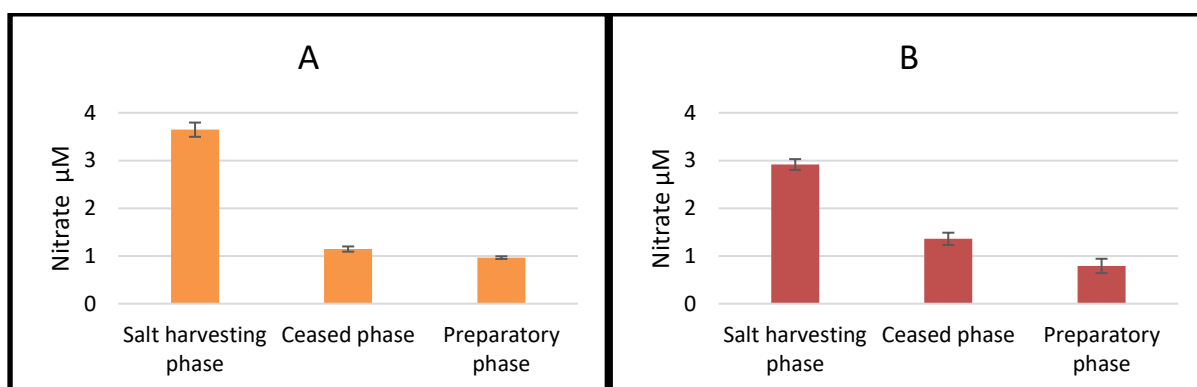


Figure 4.23: Variation in nitrate concentration in sediment samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

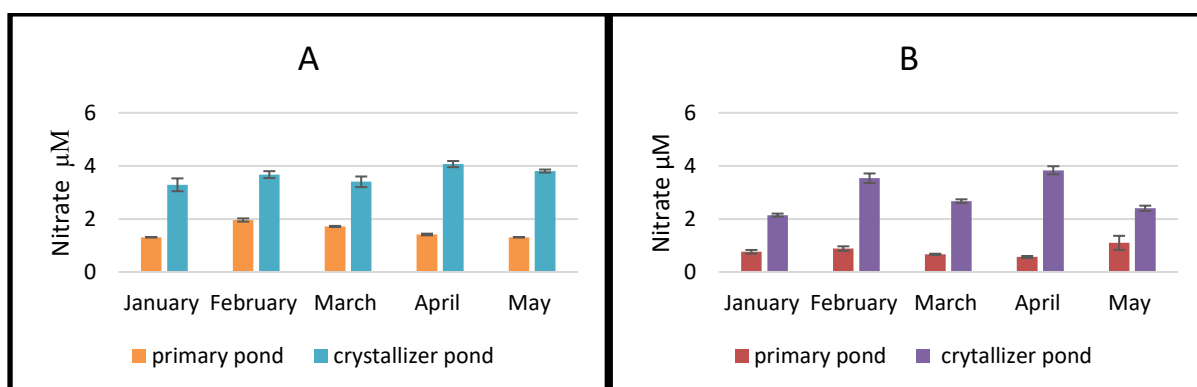


Figure 4.24: Variation in nitrate concentration in sediment samples during period of salt harvesting phase in Curca (A) and Agarwado (B) salt pans

4.1.9 Nitrite

A comparable pattern in the nitrite, with respect to nitrate concentrations were observed. The variation in nitrite levels across the three phases in both overlying water and sediment samples is shown in Figures 4.25- 4.27. The highest concentrations were recorded during the salt-harvesting phase, particularly in the crystallizer pond samples (Figures 4.26). Conversely, the lowest nitrite concentrations were found during the ceased phase in overlying water samples, while the preparatory phase showed the lowest levels in sediment in both salt pans.

Statistical analysis indicated that nitrite concentrations varied significantly across all three phases in both salt pans. Additionally, nitrite levels differed significantly between primary and crystallizer pond samples (both water and sediment) from both salt pans, except for the sediment samples from Agarwado, where no significant difference was found ($U = 56.5$, $z = 1.417$, $p = .161$, $r = .33$).

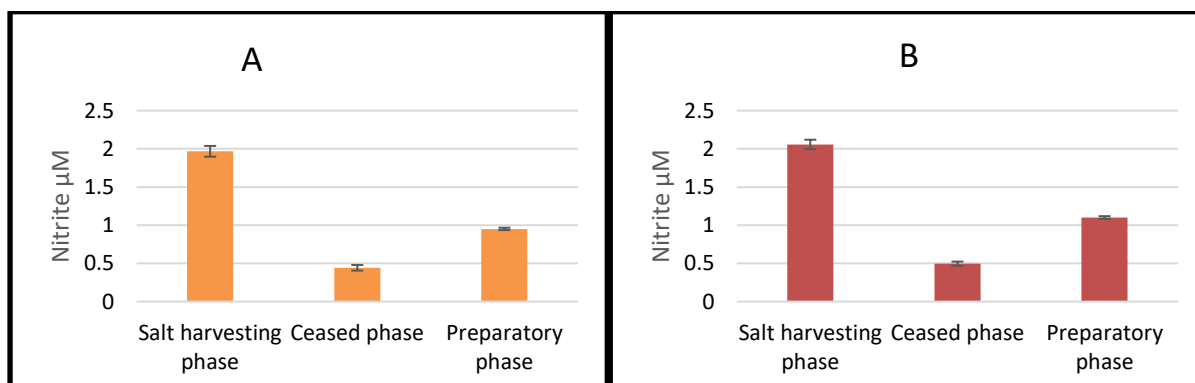


Figure 4.25: Variation in nitrite concentration in overlying water samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

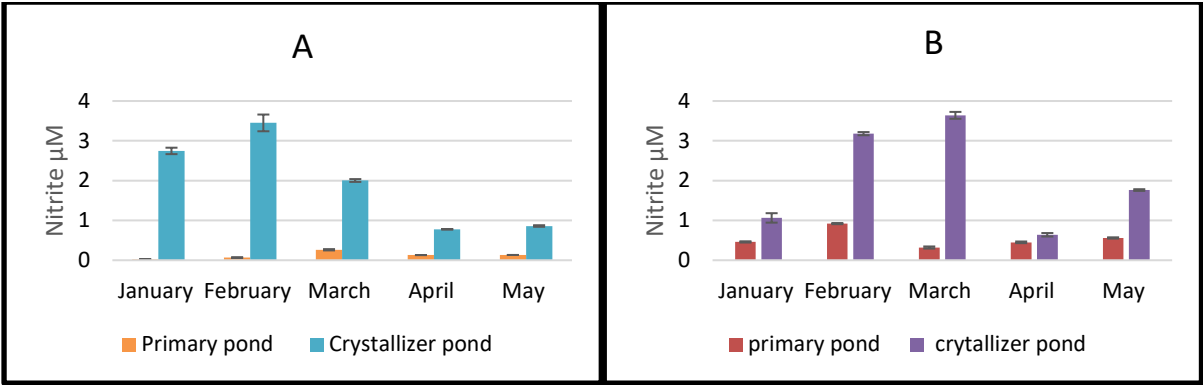


Figure 4.26: Variation in nitrite concentration in overlying water samples during the period of salt harvesting phase in Curca (A) and Agarwado (B) salt pans

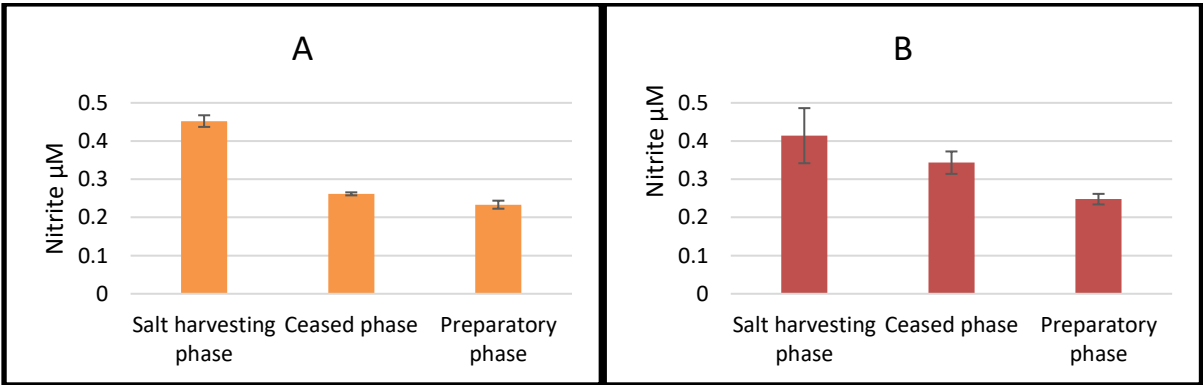


Figure 4.27: Variation in nitrite concentration in sediment samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

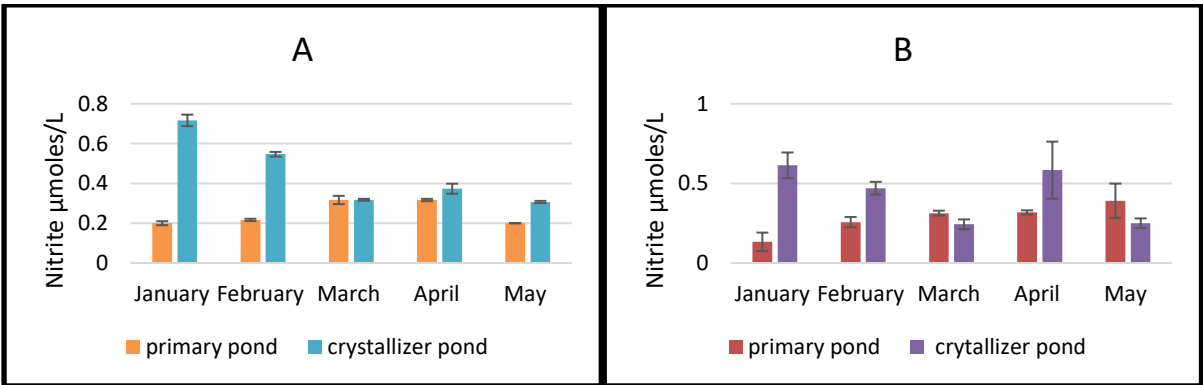


Figure 4.28: Variation in nitrite concentration in sediment samples during the period of salt harvesting phase in Curca (A) and Agarwado (B) salt pans

4.2 Bacteriological Parameters

A typical solar salt pan with its increasing salt concentration right from the primary to the crystallizer pond uses seawater as a source for the production of salt crystals ultimately by a process of evaporation and thus is called the thalassohaline system. In the present study the bacteria from primary and crystallizer ponds were sampled along with three different phases.

4.2.1 Retrievable Heterotrophic Bacterial Count

The heterotrophic count in the overlying water was found to be of the order 10^6 - 10^7 in both Curca and Agarwado salt pans (Figure 4.29 A and B). In the preparatory phase, the average heterotrophic count was higher in both Curca ($262.6 \pm 31.30 \times 10^5$ CFU mL⁻¹) and Agarwado ($82.4 \pm 4.5 \times 10^5$ CFU mL⁻¹) as compared to the other two phases. The heterotrophic counts in overlying water samples of Curca were higher than Agarwado during all three phases. The Kruskal Wallis test revealed that there is a significant difference in the heterotrophic counts in the three phases in the Curca water samples ($H(2) = 23.150$, $P < .001$). However, in Agarwado sample, the difference in heterotrophic bacterial counts between the preparatory phase and the salt-harvesting phase ($H(2) = 5$, $P = .181$) was not significant. Secondly, the primary pond showed higher heterotrophic counts in both the salt pans as compared to the crystallizer pond and the difference was statistically significant within Curca ($U = 74$, $z = 3.154$, $p = .001$, $r = .74$) and Agarwado ($U = 72$, $z = 2.787$, $p = .004$, $r = .65$) overlying water samples as confirmed by Mann Whitney U test.

In the sediment samples, the heterotrophic counts were of order 10^9 in both the salt pan samples (Figure 4.31 A and B). The counts observed in sediment samples were two order higher than the overlying water sample. The maximum count was observed in the Ceased phase $659 \pm 45 \times 10^7$ CFU g⁻¹ in Curca and $452.75 \pm 62.6 \times 10^7$ CFU g⁻¹ in Agarwado salt pan. The trend was ceased phase > salt harvesting phase > preparatory phase.

The heterotrophic counts in sediment samples of Curca and Agarwado salt pans were recorded two orders higher (10^9) as compared to overlying water and showed a similar trend to the overlying water samples being higher in the primary pond as compared to the crystallizer pond during the entire period of the salt-harvesting phase. However, the Mann Whitney U test revealed that the heterotrophic counts in the primary pond and the crystallizer pond were not significantly different in the sediment samples of both the salt pans (where, $U = 27$, $z = 1.193$, $p = .258$, $r = .28$ and $U = 27$, $z = 1.813$, $p = .077$, $r = .42$ in Curca and Agarwado sediment samples respectively).

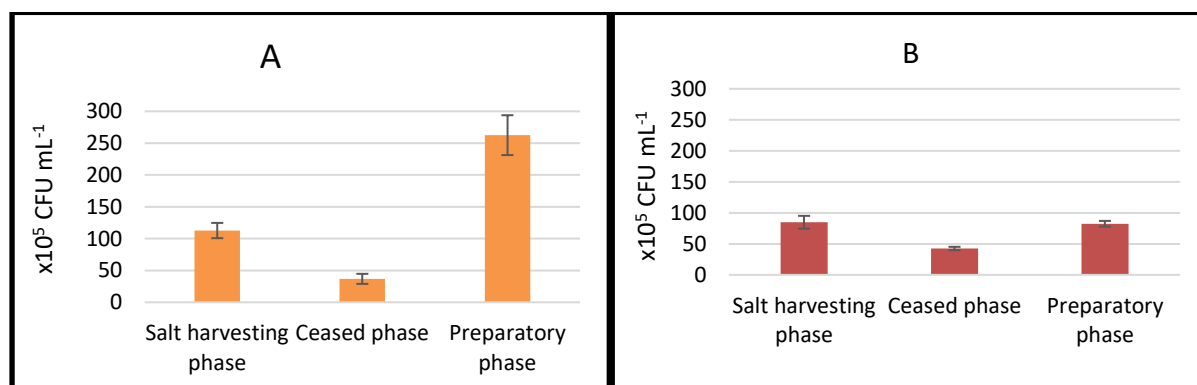


Figure 4.29: Abundance of heterotrophic bacteria in the overlying water samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

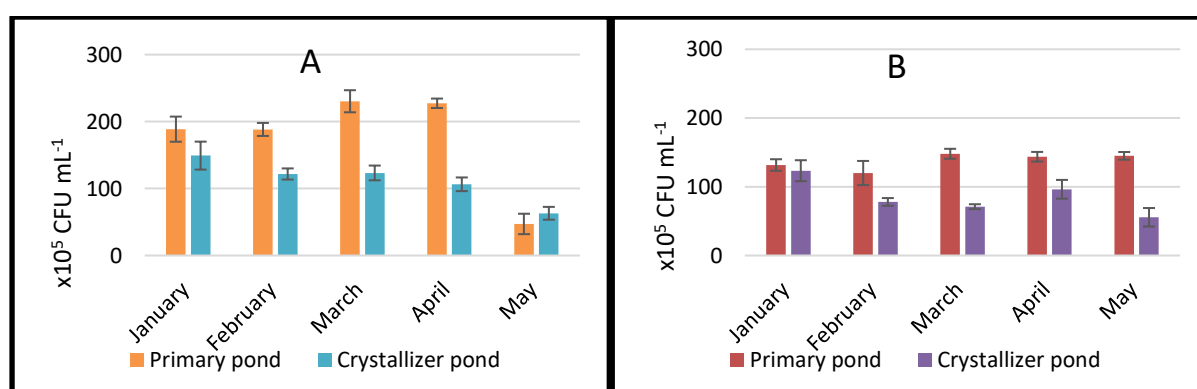


Figure 4.30: Abundance of heterotrophic bacteria in the overlying water samples of primary and crystallizer pond in Curca (A) and Agarwado (B) salt pans

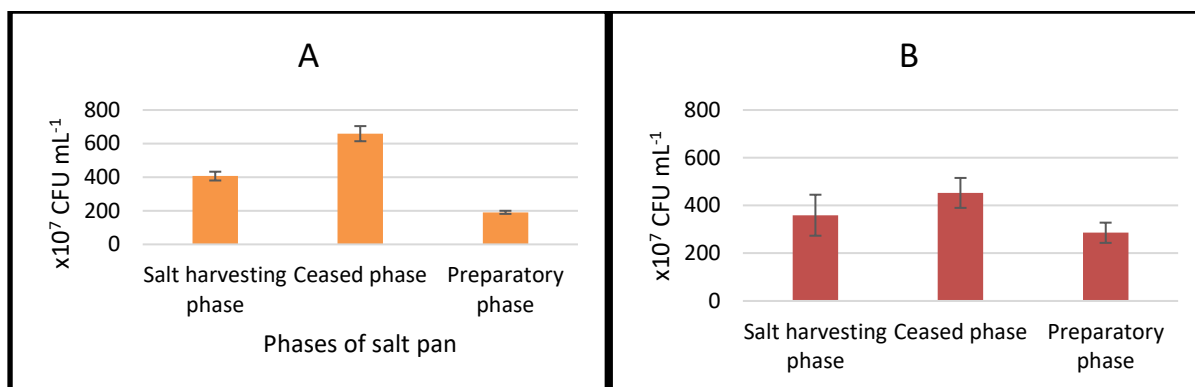


Figure 4.31: Abundance of heterotrophic bacteria in the sediment samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

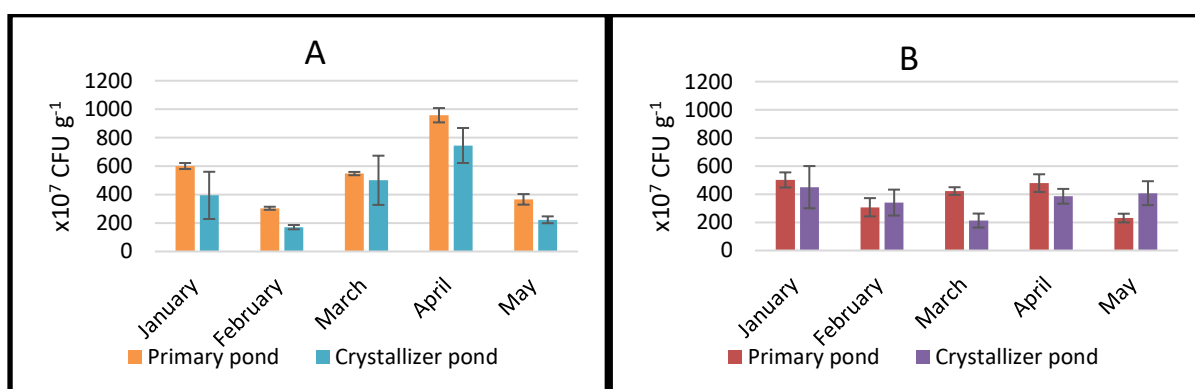


Figure 4.32: Abundance of heterotrophic bacteria in the sediment samples of primary and crystallizer pond in Curca (A) and Agarwado (B) salt pans

4.2.2 Sulfur Oxidizing Bacteria (SOB) Counts

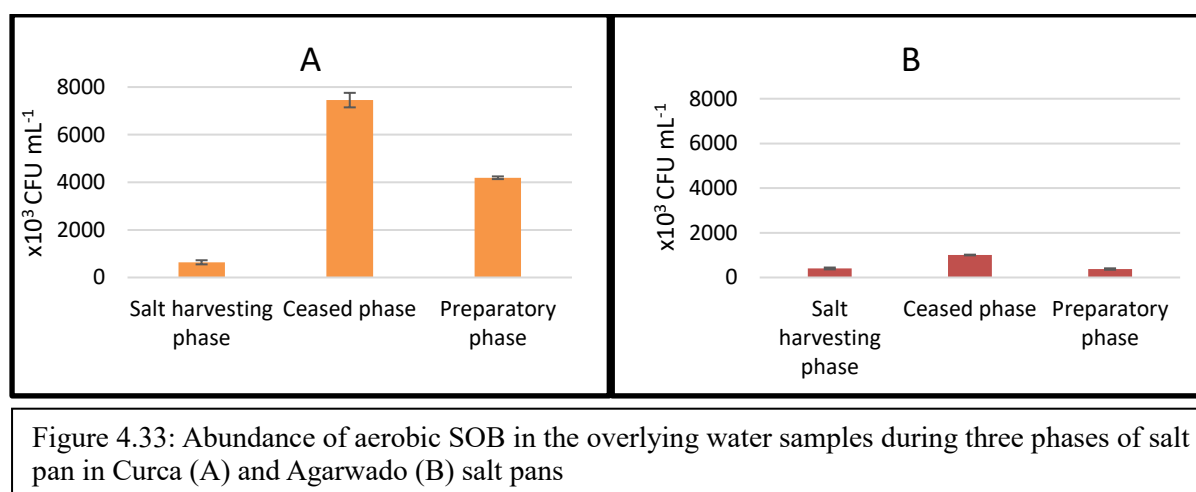
4.2.2.1 Aerobic SOB Count

The aerobic SOB population in overlying water samples in the two sampling sites were not comparable. The SOB counts were much higher in Curca salt pan as compared to Agarwado (Figure 4.33 A and B). Secondly, in Curca the aerobic SOB counts followed a trend where the ceased phase > preparatory phase > salt-harvesting phase with, 7451.70 ± 304.69 , 4189.54 ± 56.54 , and $637.92 \pm 86.46 \times 10^3$ CFU mL^{-1} respectively with a significant difference of $H(2) = 14$, $P = <.001$. Whereas in Agarwado, the trend was ceased phase > salt harvesting phase > preparatory phase with 1006.19 ± 16.53 , 542.26 ± 18.28 , and $378.74 \pm 31.43 \times 10^3$ CFU mL^{-1}

respectively. However, the non-significant difference in the counts was observed in these three phases in Agarwado overlying water sample with $H(2) = 2$, $P = .368$.

In Curca salt pan, the overlying water showed a maximum aerobic SOB population in the primary pond as compared to crystallizer pond during the entire salt-harvesting phase except in the month of January. However, in Agarwado salt pan, the above trend was observed only during the months of January March and May (Figure 4.34 A and B). However, this difference in the aerobic SOB counts in overlying water and sediment samples of primary and crystallizer ponds was not significant in both the salt pans.

In the sediment samples of Curca and Agarwado salt pans, the count of aerobic SOB was also of order $10^5 - 10^6$ (Figure 4.35 A and B). The ceased phase showed a high aerobic SOB count followed by the salt-harvesting phase and the lowest counts were observed in the preparatory phase. A significant difference was observed in the sediment samples during three phases in the Curca salt pan ($H(2) = 7.940$, $P = .019$) whereas there was no significant difference in counts during three phases of Agarwado ($H(2) = 3.372$, $P = .185$) salt pan in sediment samples.



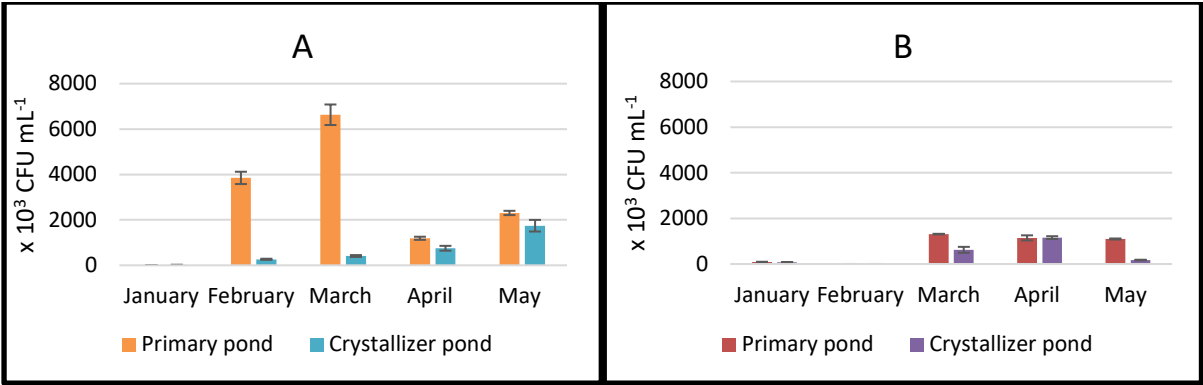


Figure 4.34: Abundance of aerobic SOB in the overlying water samples of primary and crystallizer pond in Curca (A) and Agarwado (B) salt pans

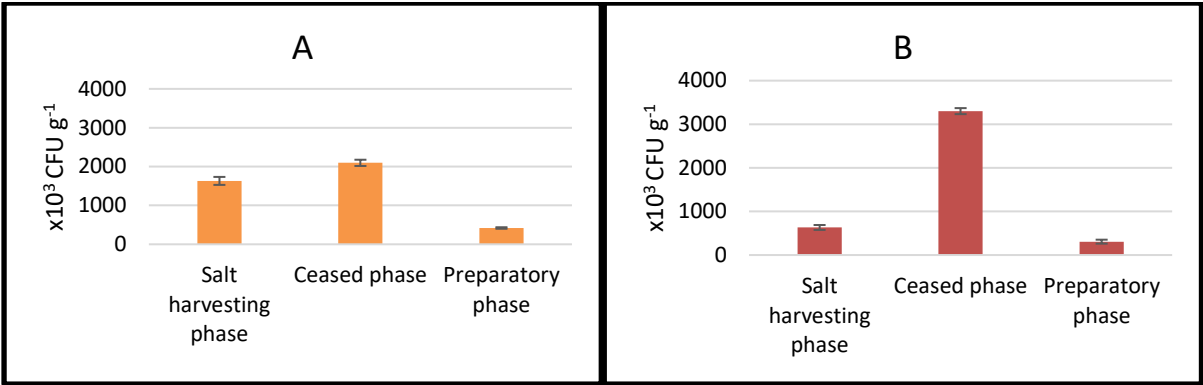


Figure 4.35: Abundance of aerobic SOB in the sediment samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

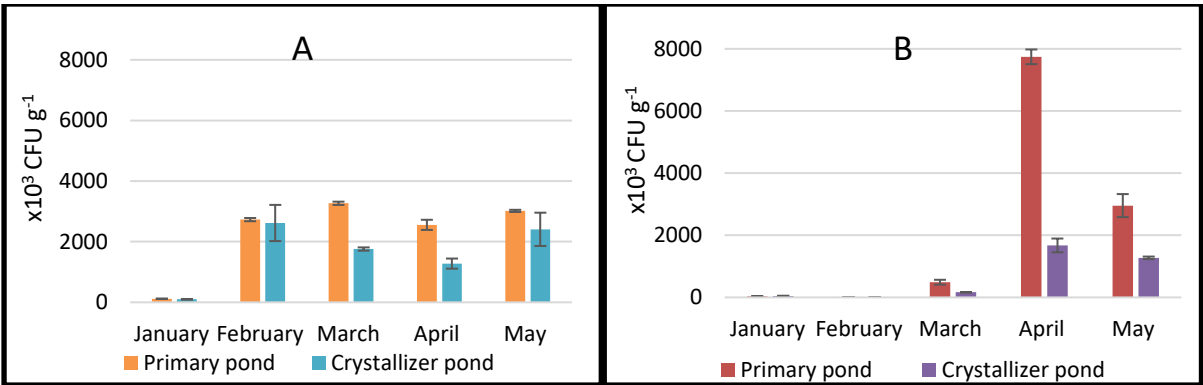


Figure 4.36: Abundance of aerobic SOB in the sediment samples of primary and crystallizer pond in Curca (A) and Agarwado (B) salt pans

4.2.2.2 Anaerobic SOB Counts

The anaerobic SOB count in the overlying water sample of the Curca and Agarwado salt pan was of the order of $10^5 - 10^6$. A similar count of 10^3 was noted in the sediment samples of both salt pans. The anaerobic counts in both the salt pans were highest in the ceased phase followed by salt-harvesting and preparatory phases (Figures 4.37 and 4.39). The highest count in the ceased phase observed was 7042.20 ± 374.59 and $406.25 \pm 24.45 \times 10^3 \text{ CFU mL}^{-1}$ in the Curca and Agarwado overlying water samples repetitively, whereas in sediment samples 2096.13 ± 80.53 and $3575.73 \pm 180.90 \times 10^3 \text{ CFU g}^{-1}$ were found in Curca and Agarwado respectively. However, the anaerobic SOB counts were not significantly different in the three phases in water and sediment samples of both Curca and Agarwado salt pans.

The primary pond showed the highest count of anaerobic SOB as compared to the crystallizer pond in both the samples of water and sediments of Curca and Agarwado salt pans (Figures 4.38 and 4.40). But statistically, the counts in the primary and crystallizer ponds in both the samples were not significant in both the salt pans.

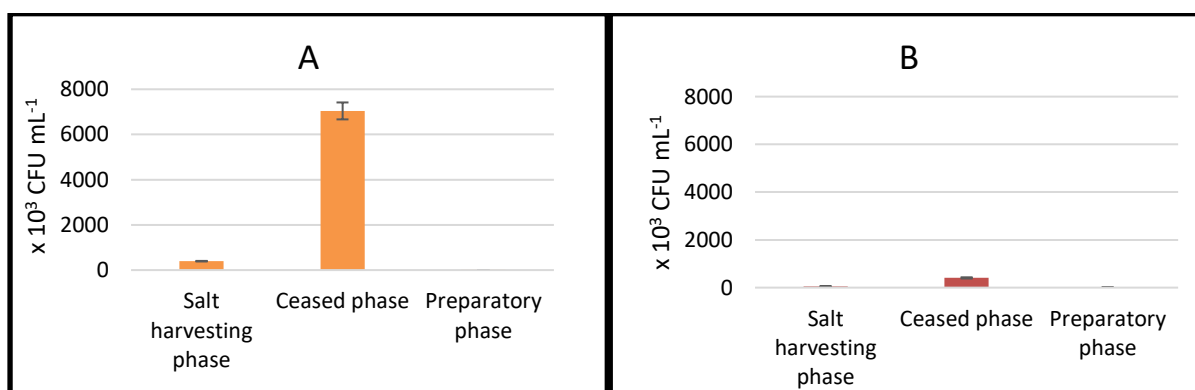


Figure 4.37: Abundance of anaerobic SOB in the overlying water samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

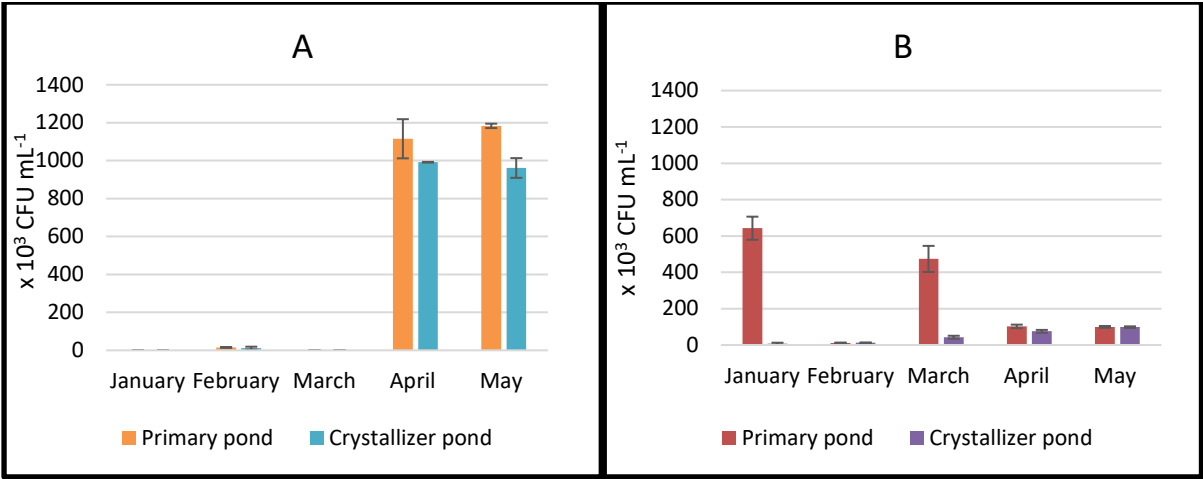


Figure 4.38: Abundance of anaerobic SOB in the overlying water samples of primary and crystallizer pond in Curca (A) and Agarwado (B) salt pans

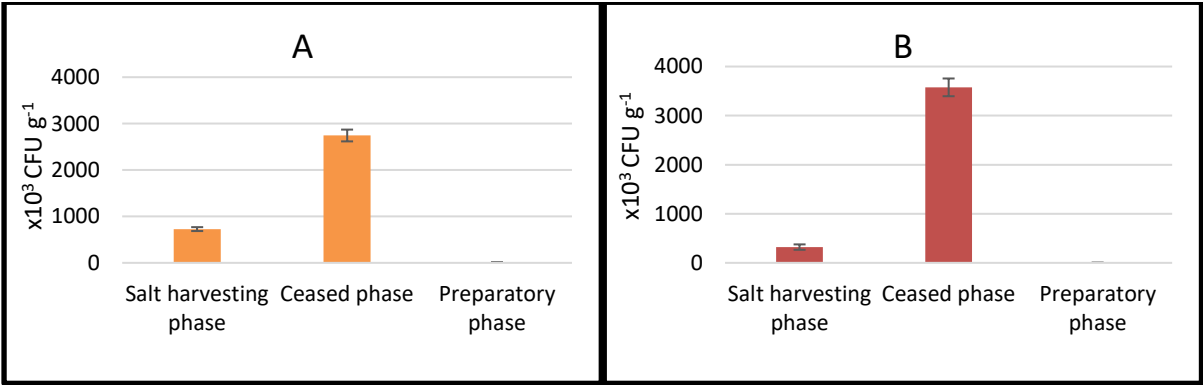


Figure 4.39: Abundance of anaerobic SOB in the sediment samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

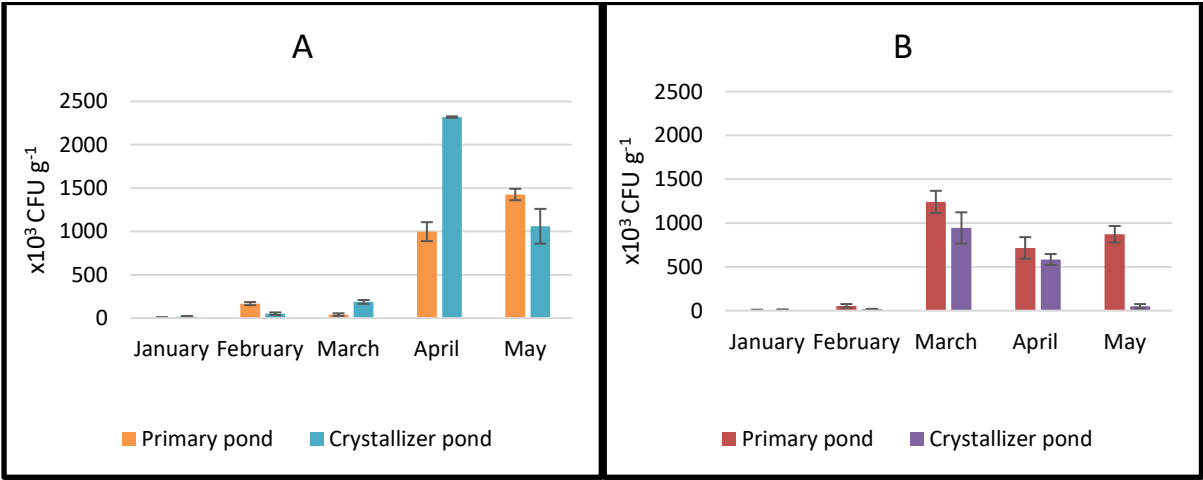


Figure 4.40: Abundance of anaerobic SOB in the sediment samples of primary and crystallizer pond in Curca (A) and Agarwado (B) salt pans

4.2.3 SRB Counts

The SRB counts in both water and sediments were in the order of 10^3 - 10^4 CFU mL⁻¹ at both sampling sites (Figure 4.41 and 4.43). The highest SRB counts were observed during the salt-harvesting phase, followed by the preparatory phase, with the lowest counts recorded during the ceased phase in water and sediment samples of the two salt pans. In overlying water samples, the SRB counts were highest in the crystallizer pond as compared to the primary pond. However, in the sediment, the counts were higher in the primary pond as compared to the crystallizer pond. Notably, the SRB counts were exceptionally high in the month of April.

In the overlying water samples, the SRB count was $43.05 \pm 2.95 \times 10^3$ CFU mL⁻¹ in the Curca salt pan and $32.93 \pm 1.34 \times 10^3$ CFU mL⁻¹ in the Agarwado salt pan during the salt-harvesting phase. During the ceased phase of both the salt pan, the SRB were not detected. Similarly, very low SRB counts were observed during the preparatory phase in both salt pans. Throughout the studies, the SRB counts were highest in the crystallizer pond as compared to the primary pond and exceptionally high in April (Figure 4.42).

In the sediment samples, the SRB count was highest with $107.53 \pm 35.78 \times 10^3$ CFU g⁻¹ in the Curca salt pan and $60.15 \pm 20.73 \times 10^3$ CFU g⁻¹ in the Agarwado salt pan during the salt-harvesting phase (Figure 4.43). The statistical analysis revealed that the SRB counts across the three phases were significant in Curca water ($H(2) = 8.447$, $P = .015$) and Agarwado sediment ($H(2) = 8.396$, $P = .015$) samples. Statistically, the counts were significantly different in the primary and crystallizer pond of Agarwado sediment ($U = 18$, $z = 1.996$, $p = .05$, $r = .47$) samples but was not significant in Curca water ($U = 27$, $z = 1.215$, $p = .258$, $r = .28$), Curca sediment ($U = 20$, $z = 1.815$, $p = .07$, $r = .42$) and Agarwado water ($U = 45$, $z = 0.474$, $p = .730$, $r = 0.11$).

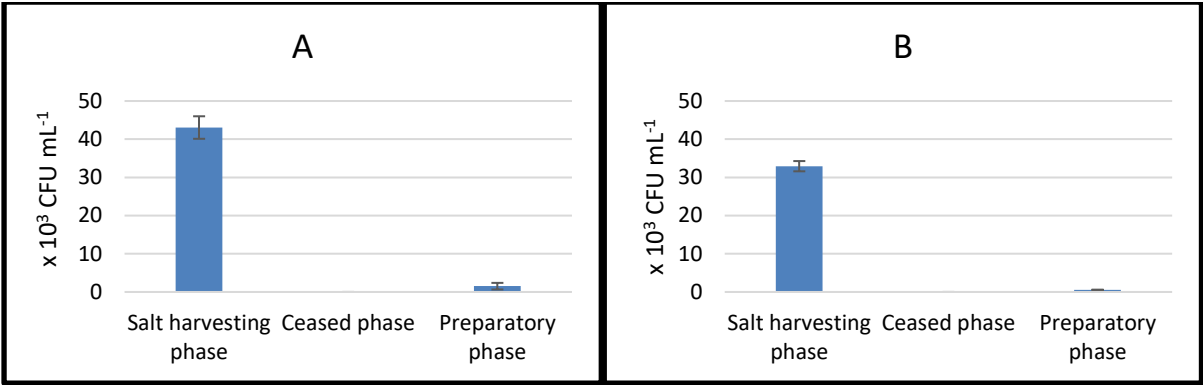


Figure 4.41: Abundance of SRB in the overlying water samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

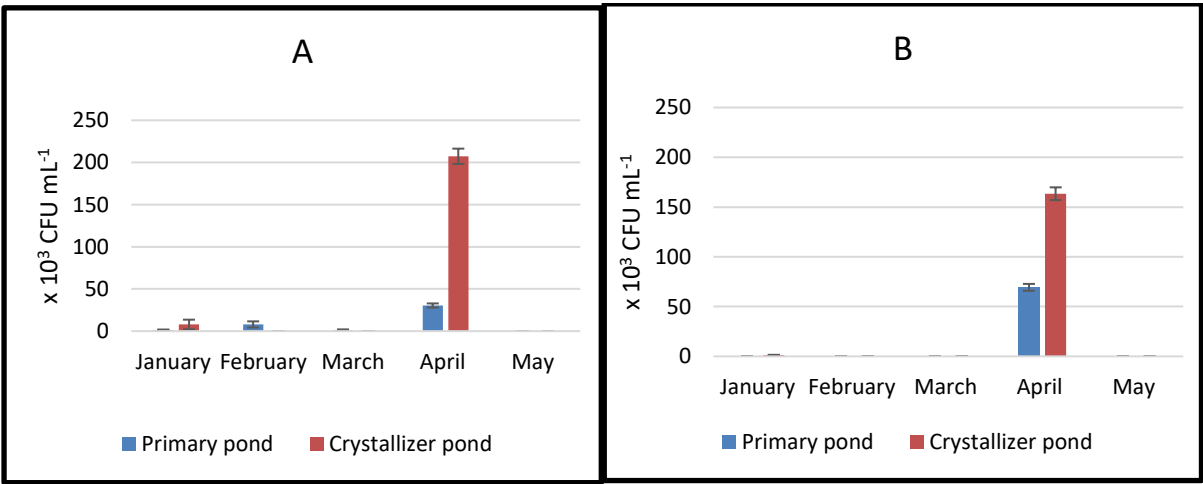


Figure 4.42: Abundance of SRB in the overlying water samples of primary and crystallizer pond in Curca (A) and Agarwado (B) salt pans

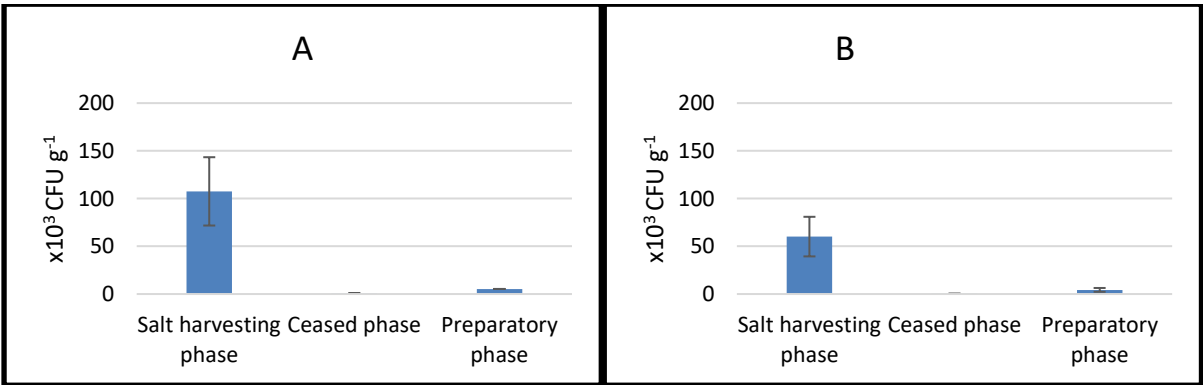


Figure 4.43: Abundance of SRB in the sediment samples during three phases of salt pan in Curca (A) and Agarwado (B) salt pans

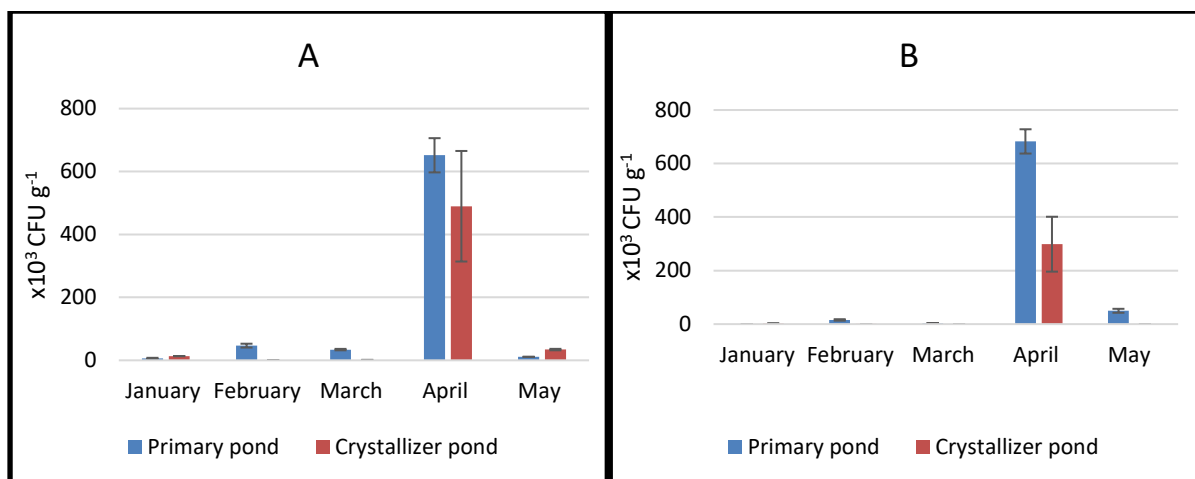


Figure 4.44: Abundance of SRB in the sediment samples of primary and crystallizer pond in Curca (A) and Agarwado (B) salt pans

4.3 Relationship Between Bacteriological and Chemical Parameters

The Pearson correlation matrix table for water and sediment samples from Curca and Agarwado are depicted in Appendix III.

4.3.1 Inter-relationship in the Water Sample

In Curca water, the aerobic sulfur oxidizing bacteria (SOB) showed a negative correlation with sulfate ($r = -.45$, $p = .01$), sulfide ($r = -.48$, $p = .01$), nitrate ($r = -.68$, $p < .001$), and nitrite ($r = -.59$, $p = .001$). Conversely, in Agarwado overlying water samples, aerobic SOB was negatively correlated with sulfide ($r = -.39$, $p = .04$) and nitrate ($r = -.43$, $p = .02$) concentrations. No significant relationship was found between anaerobic SOB and the chemical parameters in Curca waters. In Agarwado overlying water samples, a strong negative correlation was observed between anaerobic SOB and both heterotrophic counts ($r = -.50$, $p = .007$) and nitrate ($r = -.58$, $p = .001$). SRB displayed a positive correlation with sulfate in both Curca ($r = .46$, $p = .01$) and Agarwado ($r = .40$, $p = .03$) overlying water. Additionally, the heterotrophic count had a positive correlation with sulfate ($r = .54$, $p = .003$ in Curca, $r = .71$, $p < .001$ in Agarwado) and nitrite ($r = .58$, $p = .001$ in Curca, $r = .42$, $p = .02$ in Agarwado) in both salt pans.

4.3.2 Inter-relationship in the Sediment Sample

In Curca samples, aerobic SOB was positively correlated with anaerobic SOB ($r = .64$, $p = <.001$), nitrate ($r = .41$, $p = .03$), and nitrite ($r = .49$, $p = .008$). Anaerobic SOB also showed a positive correlation with nitrate ($r = .51$, $p = .006$). SRB correlated with the heterotrophic counts ($r = .40$, $p = .03$).

In Agarwado sediment samples, no correlation was found between aerobic SOB and other parameters. However, anaerobic SOB exhibited a positive correlation with heterotrophic counts ($r = .44$, $p = .01$) and nitrite ($r = .48$, $p = .01$). SRB had a positive correlation with both sulfate ($r = .47$, $p = .01$) and sulfide ($r = .59$, $p = <.001$). Heterotrophic counts were negatively correlated with sulfate ($r = -.54$, $p = .003$) and sulfide ($r = -.42$, $p = 0.02$) but positively correlated to anaerobic SOB ($r = .44$, $p = .01$) and nitrite ($r = .64$, $p = <.001$).

4.4 Isolation of Sulfur Oxidizing Bacteria (SOB) and Sulfate Reducing Bacteria (SRB)

A total of 26 SOB were isolated from Curca and 27 from Agarwado salt pan. Among 26 SOB isolates isolated from the Curca salt pan, 15 were aerobic SOB and 11 were anaerobic. From the Agarwado salt pan, 18 were aerobic SOB and 9 were anaerobic.

Furthermore, 25 SRB isolates were obtained from the Curca salt pan and 23 from the Agarwado salt pan. The bacterial isolate code, Gram characteristics, and morphology of SOB and SRB are depicted in Tables 4.1-4.3 below.

Table 4.1: Gram characteristics and morphology of aerobic SOB isolated from Curca and Agarwado salt pans.

Sampling sites	Aerobic SOB	Gram Characteristics	Morphology
Curca salt pans	BAugPW/23b, BAugPS/16b, BJCS/12-3-4, BDPW/12, BAugPW/19b, BAugPW/21b, BFPW/20, BJPS/2bB, BJPS/3b, BAugPW/18b, BDCS/2, BMCW/5, BF/9&10, DBPW/11	Gram negative (14 SOB)	Rods (15 SOB)
	BJCW/14bB	Gram positive (1 SOB)	
Agarwado salt pans	AMPW/13a, DAPW1/26, AN18-1/27, AFCS2/28, AJPS/7bA, AMPS/6bA, AJPS/2bA, AJPS/7bB, AMPS/5b, A*1, AD-1-19a, AW/25A, AW/27A, ACS/4a, AMPS/2a	Gram negative (15 SOB)	Rods (18 SOB)
	AD/16, AD/15, ADCS/28	Gram positive (3 SOB)	

Table 4.2: Gram characteristics and morphology of anaerobic SOB isolated from Curca and Agarwado salt pans.

Sampling sites	Anaerobic SOB	Gram Characteristics	Morphology
Curca salt pans	BJPS/7bB, BMPS/ 6bA, BMPS/5b, BJPS/8b, BJCW/15b, BJPS/1b, BMPS/6bB, BAugPW/ 24b, BJPS/7bA, BJPS/2bA, BJCW/13b	Gram negative (11 SOB)	Rods (11 SOB)
Agarwado salt pans	JlyAS/12, APW/8a, ACS/8, JlyAW/12ae, JlyAW/2, AW/19a,	Gram negative (6 SOB)	Rods (9 SOB)
	4A, ADPS/15, AJPS/16,	Gram positive (3 SOB)	

Table 4.3: Gram characteristics and morphology of anaerobic SRB isolated from Curca and Agarwado salt pans

Sampling sites	SRB	Gram Characteristics	Morphology
Curca salt pan	JBPS/1b, FBPS/2b, FBPS/3b, ApBPS/4b, BS/5b, BW/6, JBPS/7b, JBPS/8b, JBCS/9b, FBCS/10b, JBCS/11b, JBCS/12b, FBPW/13b, FBPW/14b, JBPW/15b, JBCW/17b, JBCW/18b, BCW1/ 19b, JBCW/20b, FBPW/21b, JBPS/22b, JBPS/23b, JBCW/24b, BCW/25b,	Gram negative (24 SRB)	Rods (25 SRB)
	BMPW/16b	Gram positive (1 SRB)	
Agarwado salt pan	JPS/1a, MyPS/3a, AFPS/4a, FAPS/5a, FAPS/6a, FAPS/7a, JACS/8a, JACS/9a, AS/11a, JlyAS/12a, JACW/13a, AFCW/15a, JAPW/16a, AFPS/17a, AFPS/18a, AFCW/19a, AFCW/20a, AMCS/21a, AMCS/22a, AMCS/23a, AFCW/14a,	Gram negative (21 SRB)	Rods (23)
	JAPS/2a, AS/10a,	Gram positive (2 SRB)	

4.5 Oxygen Tolerance of Anaerobic SOB

The anaerobic SOB isolated from both the salt pans were examined for their oxygen tolerance capability in an anaerobic agar medium supplemented with sodium thioglycolate (0.2%). All the anaerobic SOB isolates were found to be facultative anaerobes. Colonies grew along the length of the tube, congregating predominantly at the upper surface of the medium.

4.6 Aerobic Growth of Anaerobic SOB on Lieske's Media Plates

After an incubation period of 20 days, growth was observed on all Lieske's media plates streaked with pure cultures of SOB isolates.

Both experiments (section 4.5 and 4.6) confirmed that the SOB isolated from the salt pans of Curca and Agarwado could grow aerobically. Hence, subsequent experiments were conducted under aerobic conditions with all anaerobically isolated SOB. Suggestive that the anaerobic SOB are facultative in nature.

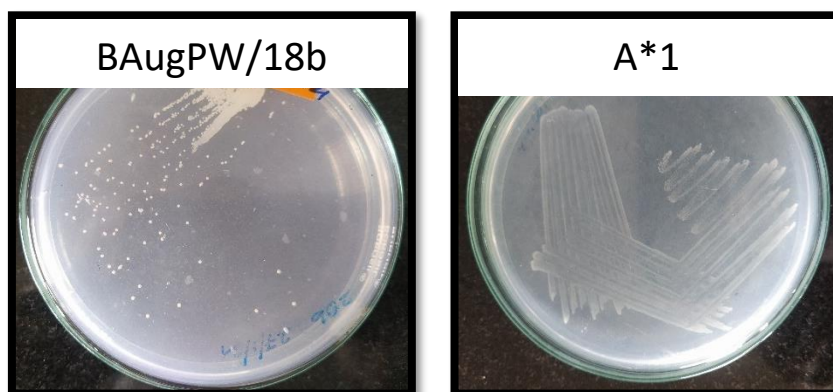


Plate 4.1: The growth of anaerobic SOB isolates on the Leiskes media plates after 20 days of incubation.

4.7 Salinity Tolerance

The salinity tolerance of 26 isolates from Curca and 27 from the Agarwado salt pan were assessed over a range of 0 to 40 % crude salt. All 26 SOB isolates from the Curca salt pan showed growth within the salinity ranges of 2.5 to 15 % crude salt. Out of these, only 6 (BDPW/12, BFPW/20, BJCW/15b, BJPS/7bA, BJCW/13b, DBPW/11) isolates could grow in a medium devoid of salt indicating their halotolerant nature while only two isolates (BAugPW/19b, BMPS/6bB) could tolerate a salinity range from 2.5 to 40 % crude salt (Table 4.4A and Figure 4.45A).

Furthermore, all the isolates from the Agarwado salt pan were able to tolerate a salinity range of 2.5 to 7.5 % crude salt. Among these, only 3 isolates (AN18-1/27, 4A, BJPS/16) were able to grow in a medium without salt. Notably, none of the isolates from the Agarwado salt pan exhibited tolerance to the extreme salinity of 40 % (Table 4.4 B and Figure 4.45 B). Therefore, this indicates the halotolerant nature of the SOB obtained from both salt pans.

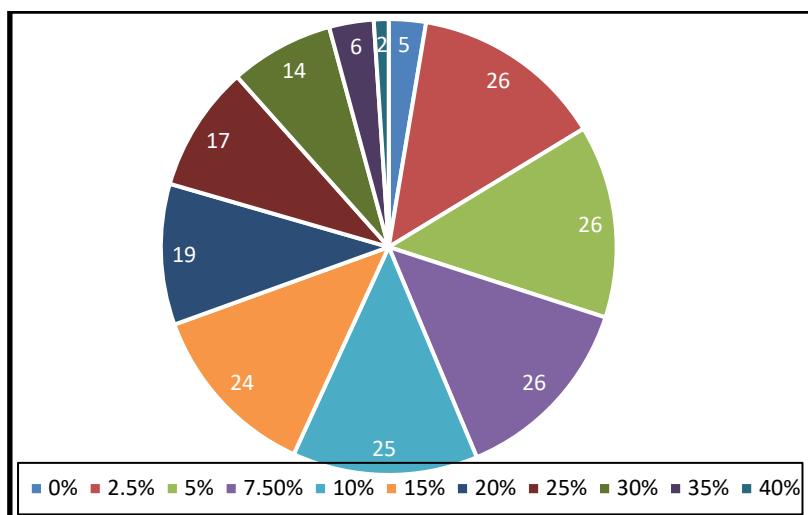


Figure 4.45A: Pie chart showing salinity tolerance by SOB isolates from Curca salt pans

Table 4.4A: Salinity Tolerance by SOB isolated from Curca salt pan

Sr. No	Isolates	0%	2.5%	5%	7.5%	10%	15%	20%	25%	30%	35%	40%
1	BAugPW/23b	-	+	+	+	+	+	+	+	+	+	-
2	BAugPS/16b	-	+	+	+	+	+	+	+	+	-	-
3	BJPS/7bB	-	+	+	+	+	+	-	-	-	-	-
4	BJCS/12-3-4	-	+	+	+	+	+	+	+	+	-	-
5	BDPW/12	+	+	+	+	+	+	+	+	+	-	-
6	BMPS/ 6bA	-	+	+	+	+	+	-	-	-	-	-
7	BAugPW/19b	-	+	+	+	+	+	+	+	+	+	+
8	BAugPW/21b	-	+	+	+	+	+	+	+	+	+	
9	BJCW/14bB	-	+	+	+	+	+	+	+	+	-	-
10	BFPW/20	+	+	+	+	+	+	+	+	-	-	
11	BJPS/2bB		+	+	+	+	+	+	-	-	-	-
12	BJPS/3b	-	+	+	+	+	+	-	-	-	-	-
13	BMPS/5b	-	+	+	+	+	+	+	+	-	-	-
14	BAugPW/18b	-	+	+	+	+	+	+	+	+	-	-
15	BJPS/8b	-	+	+	+	+	+	-	-	-	-	-
16	BFPW/20	-	+	+	+	+	+	+	+	+	-	-
17	BJCW/15b	-	+	+	+	+	+	-	-	-	-	-
18	BJPS/1b	-	+	+	+	+	+	+	+	+	+	
19	BMPS/6bB,	-	+	+	+	+	+	+	+	+	+	+
20	BAugPW/24b	-	+	+	+	+	+	+	+	+	-	-
21	BJPS/7bA	+	+	+	+	+	+	+	-	-	-	-
22	BJPS/2bA	-	+	+	+	+	-	-	-	-	-	-
23	BJCW/13b	+	+	+	+	-	-	-	-	-	-	-
24	BJCW/14bA	-	+	+	+	+	+	+	+	+	-	-
25	BJPS/4b	-	+	+	+	+	+	+	+	+	+	-
26	DBPW/11	+	+	+	+	+	+	+	+	-	-	-

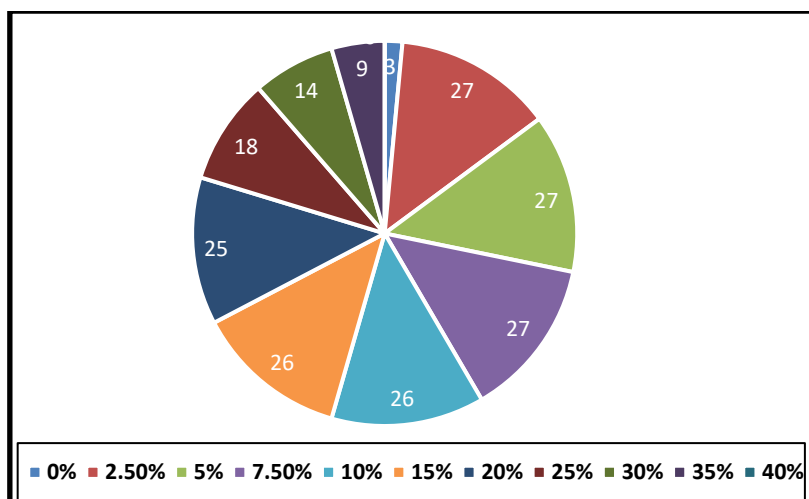


Figure 4.45B: Pie chart showing salinity tolerance by SOB isolates from Agarwado salt pan

Table 4.4B: Salinity Tolerance by SOB isolated from Agarwado salt pan

Sr. No	Isolates	0%	2.5 %	5%	7.5%	10%	15%	20	25%	30%	35%	40%
1	AMPW/13a	-	+	+	+	+	+	+	+	+	-	-
2	DAPW1/26	-	+	+	+	+	+	+	+	+	+	-
3	AN18-1/27	+	+	+	+	-	-	-	-	-	-	-
4	AFCS2/28	-	+	+	+	+	+	+	-	-	-	-
5	AJPS/7bA	-	+	+	+	+	+	-	-	-	-	-
6	AMPS/ 6bA	-	+	+	+	+	+	+	+	-	-	-
7	AJPS/2bA	-	+	+	+	+	+	+	+	+	+	-
8	AJPS/7bB	-	+	+	+	+	+	+	-	-	-	-
9	AMPS/5b	-	+	+	+	+	+	+	+	+	-	-
10	A*1	-	+	+	+	+	+	+	+	+	+	-
11	AD-1-19a	-	+	+	+	+	+	+	+	+	+	-
12	AW/25A	-	+	+	+	+	+	+	+	+	+	-
13	AW/27A	-	+	+	+	+	+	+	+	+	+	-
14	ACS/4a	-	+	+	+	+	+	+	+	-	-	-
15	AMPS/2a	-	+	+	+	+	+	+	-	-	-	-
16	AD/16,	-	+	+	+	+	+	+	+	+	+	-
17	AD/15	-	+	+	+	+	+	+	+	+	-	-
18	ADCS/28	-	+	+	+	+	+	+	-	-	-	-
19	4A	+	+	+	+	+	+	+	-	-	-	-
20	aDPS/15	-	+	+	+	+	+	+	+	-	-	-
21	aJPS/16	+	+	+	+	+	+	+	+	-	-	-
22	JlyAS/12	-	+	+	+	+	+	+	+	+	-	-
23	APW/8a	-	+	+	+	+	+	+	+	+	+	-
24	ACS/8	-	+	+	+	+	+	+	+	+	-	-
25	JlyAW/12ae	-	+	+	+	+	+	+	+	+	+	-
26	JlyAW/2	-	+	+	+	+	+	+	-	-	-	-
27	AW/19a	-	+	+	+	+	+	+	-	-	-	-

4.8 Biochemical Characterization of SOB and SRB Isolates

The results of biochemical test of SOB (Table 4.5A and 4.5B) and SRB (Table 4.6A and 4.6B) isolates from Curca and Agarwado are presented in Table 4.5- 4.6.

Table 4.5A: Results of biochemical test for SOB isolated from Curca salt pans

Sr. No	SOB Isolates	ONPG	Nitrate	MR	VP	Citrate Utilization	Sulfur reduction	Indole	Motility	Gelatin	Oxidase	Catalase
1	BAugPW/23b	—	+	—	—	—	—	—	—	—	+	+
2	BAugPS/16b	—	+	+	—	—	+	—	+	—	+	+
3	BJPS/7bB	—	+	+	+	—	+	—	—	—	+	+
4	BJCS/12-3-4	+	+	+	—	—	+	—	+	—	+	+
5	BDPW/12	+	+	+	+	—	+	+	—	—	—	+
6	BMPS/ 6bA	—	+	+	—	—	+	—	—	—	—	+
7	BAugPW/19b	—	+	—	—	—	+	—	—	—	+	+
8	BAugPW/21b	—	+	—	+	—	—	—	—	—	+	+
9	BJCW/14bB	+	+	—	—	+	—	+	—	—	+	+
10	BFPW/20	—	+	+	—	—	—	—	+	—	+	+
11	BJPS/2bB	—	+	+	—	—	—	—	—	—	—	+
12	BJPS/3b	—	+	—	—	+	—	—	—	—	—	+
13	BMPS/5b	—	+	—	+	—	—	—	—	—	+	+
14	BAugPW/18b	—	+	—	—	—	—	—	+	—	+	+
15	BJPS/8b	—	+	—	—	—	—	—	—	—	—	+
16	BFPW/20	—	+	—	—	—	—	—	—	—	+	+
17	BJCW/15b	—	+	—	+	—	—	—	—	—	+	+
18	BJPS/1b	—	+	—	+	+	—	—	—	—	+	+
19	BMPS/6bB,	+	+	—	—	—	—	—	+	—	+	+
20	BAugPW/24b	+	—	—	—	—	—	—	—	—	+	+
21	BJPS/7bA	—	—	—	—	—	—	—	—	—	+	+
22	BJPS/2bA	—	—	+	—	—	—	—	—	—	+	+
23	BJCW/13b	—	—	—	+	+	—	—	—	—	+	+
24	BJCW/14bA	—	—	—	—	—	—	—	+	—	+	+
25	BJPS/4b	—	—	—	—	—	—	—	+	—	+	+
26	DBPW/11	—	—	—	+	+	—	—	—	—	+	+

Table 4.5B: Results of biochemical test for SOB isolated from Agarwado salt pan

Sr. No	SOB Isolates	ONPG	Nitrate	MR	VP	Citrate Utilization	Sulfur reduction	Indole	Motility	Gelatin	Oxidase	Catalase
1	AMPW/13a	-	+	-	-	-	-	-	-	-	+	+
2	DAPW1/26	+	+	+	-	-	-	-	-	-	+	+
3	AN18-1/27	-	+	+	-	-	-	-	-	-	-	+
4	AFCS2/28	-	+	+	-	-	-	-	-	-	+	+
5	AJPS/7bA	-	+	+	-	-	-	-	-	-	-	+
6	AMPS/ 6bA	-	+	-	+	-	-	-	-	-	-	+
7	AJPS/2bA	-	+	-	+	-	-	-	-	-	+	+
8	AJPS/7bB	-	+	-	+	+	-	-	-	+	-	+
9	AMPS/5b	-	+	-	+	-	-	-	-	-	+	+
10	A*1	-	+	-	-	-	-	-	-	-	+	+
11	AD-1-19a	-	+	-	+	-	-	-	-	-	-	+
12	AW/25A	-	+	-	-	-	-	-	-	-	-	+
13	AW/27A	-	+	+	-	-	-	-	-	-	+	+
14	ACS/4a	+	+	-	-	-	-	-	-	-	+	+
15	AMPS/2a	-	+	-	-	-	-	-	-	-	-	+
16	AD/16,	-	+	+	-	-	-	-	-	-	+	+
17	AD/15	-	+	-	+	+	-	-	-	-	+	+
18	ADCS/28	+	+	-	-	-	-	-	+	-	+	+
19	4A	-	+	-	-	-	-	-	-	+	+	+
20	BDPS/15	-	+	+	-	-	-	-	-	-	-	+
21	BJPS/16	-	+	+	-	-	-	-	-	-	+	+
22	JlyAS/12	-	-	+	-	-	-	-	-	-	+	+
23	APW/8a	-	-	+	-	-	-	-	-	-	+	+
24	ACS/8	+	-	-	-	-	-	-	-	-	-	+
25	JlyAW/12ae	+	-	-	-	-	-	-	+	-	+	+
26	JlyAW/2	+	-	-	-	-	-	-	+	+	+	+
27	AW/19a	-	-	+	-	-	-	-	-	+	-	+

Table 4.6A: Results of biochemical test for SRB isolated from Curca salt pan

Sr. No	SRB Isolates	Oxidase	Catalase	Lactate	Acetate	Formate	Ethanol	Propionate	Glucose	Benzoate	Spores	Motility
1	JBPS/1b	-	-	-	+	-	-	-	-	+	-	+
2	FBPS/2b	-	-	+	+	-	-	-	-	+	-	+
3	FBPS/3b	-	-	+	-	-	+	-	-	+	-	+
4	ApBPS/4b	-	-	+	+	+	+	-	+	-	-	+
5	BS/5b	-	-	-	-	-	+	-	-	+	-	+
6	BW/6	-	-	+	+	-	-	-	-	+	+	+
7	JBPS/7b	-	-	+	-	-	+	-	-	+	-	+
8	JBPS/8b	-	-	-	+	-	-	-	-	+	-	+
9	JBCS/9b	-	-	+	+	-	-	-	-	-	-	+
10	FBCS/10b	-	-	+	+	-	-	-	-	-	-	+
11	JBCS/11b	-	-	+	+	-	-	-	-	-	-	+
12	JBCS/12b	-	-	+	+	+	-	-	-	+	+	+
13	FBPW/13b	-	-	-	+	+	-	+	-	+	-	+
14	FBPW/14b	-	-	+	+	-	-	+	+	-	-	+
15	JBPW/15b	-	-	+	+	-	-	-	+	+	+	+
16	JBCW/17b	-	-	-	+	+	+	-	-	-	-	+
17	JBCW/18b	-	-	+	+	-	+	-	-	-	-	+
18	BCW1/19b	-	-	+	-	-	-	-	-	+	-	+
19	JBCW/20b	-	-	+	+	+	-	+	-	+	-	+
20	FBPW/21b	-	-	+	+	-	+	+	-	+	-	+
21	JBPS/22b	-	-	-	-	+	-	-	-	-	-	+
22	JBPS/23b	-	-	-	+	+	-	-	+	-	-	+
23	JBCW/24b	-	-	-	+	+	-	-	+	-	-	+
24	BCW/25b	-	-	-	-	-	-	-	+	-	-	+
25	BMPW/16b	+	+	-	+	-	+	+	-	-	-	-

Table 4.6B: Results of biochemical test for SRB isolated from Agarwado salt pan

Sr. No	SRB Isolates	Oxidase	Catalase	Lactate	Acetate	Formate	Ethanol	Propionate	Glucose	Benzoate	Spores	Motility
1	JPS/1a	-	-	+	-	-	-	-	-	+	-	+
2	MyPS/3a	-	-	+	+	-	-	-	-	+	-	+
3	AFPS/4a	-	-	+	-	-	-	-	-	+	-	+
4	FAPS/5a	-	-	-	+	-	-	-	+	+	-	+
5	FAPS/6a	-	-	+	+	-	+	+	-	+	-	-
6	FAPS/7a	-	-	+	+	-	-	-	-	+	-	+
7	JACS/8a	-	-	+	+	-	-	-	-	-	-	+
8	JACS/9a	-	-	+	+	-	+	-	-	+	-	+
9	AS/11a	-	-	+	+	-	+	-	-	-	-	+
10	JlyAS/12a	-	-	+	+	-	-	-	-	-	-	-
11	JACW/13a	-	-	+	+	+	+	-	-	+	+	+
12	AFCW/14a	-	-	+	+	-	-	+	+	+	-	+
13	AFCW/15a	-	-	+	-	-	+	-	-	-	-	+
14	JAPW/16a	-	-	+	+	+	+	-	-	+	-	+
15	AFPS/17a	-	-	+	+	-	-	-	-	-	-	+
16	AFPS/18a	-	-	-	+	-	-	-	-		+	+
17	AFCW/19a	-	-	-	-	-	-	-	-	-	+	-
18	AFCW/20a	-	-	+	-	+	-	-	-	+	-	+
19	AMCS/21a	-	-	+	+	+	+	-	-			+
20	AMCS/22a	-	-	+	-	-	+	+	+	-	+	-
21	AMCS/23a	-	-	+	-	-	+	-	+	-	+	+
22	JAPS/2a	-	-	+	+	+	+	-	+	+	-	-
23	AS/10a	-	-	+	-	-	-	+	+	+	-	+

4.9 Identification of SOB and SRB Isolates

The genomic DNA was isolated from the SOB and SRB isolates. Subsequently, a 1.5 kb amplicon of the 16S rRNA gene was amplified, as shown in Figure 4.46. This amplicon was then sequenced and the forward and reverse sequences obtained were assembled using Bio Edit software.

Fifteen SOB isolates from Curca and sixteen from Agarwado were identified by comparing their contigs with the GenBank database via NCBI-BLAST (Table 4.7A and B). Additionally, SRB isolates were identified from both Curca (19 isolates) and Agarwado (22 isolates) salt pans (Table 4.8 A and B). Following identification, phylogenetic trees were constructed to illustrate the distinct genera present in each salt pan using the Mega 11 software tool by the Neighbor-Joining method.

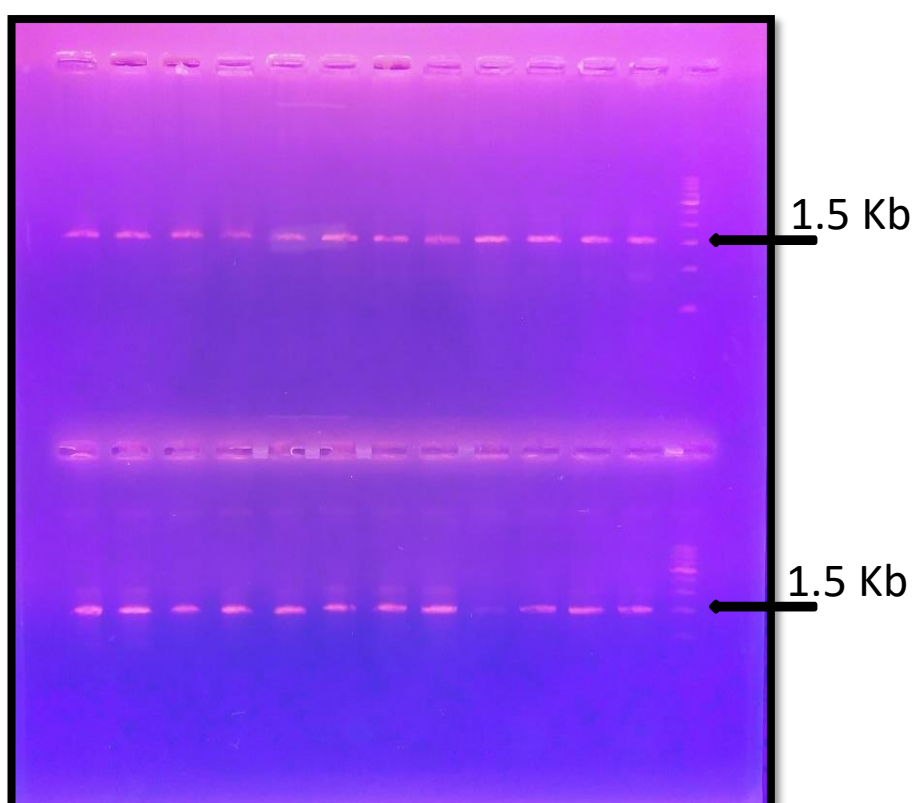


Figure 4.46: Gel image of 16S rRNA gene amplification (1.5 kb) of some SOB and SRB isolates

4.9.1 Molecular Phylogenetic Analysis of SOB

The phylogenetic trees for SOB isolates from both the salt pans showed two major clades belonging to two different phyla, viz. Pseudomonadota and Bacillota. The first clade, phylum Pseudomonadota contained two different classes, Gammaproteobacteria and Alphaproteobacteria and the second clade of Bacillota contained a class Bacilli.

Out of 15 SOB isolates identified from Curca, 7 distinct genera were obtained and in Agarwado, 8 distinct genera were obtained from 16 SOB isolates by Sanger dideoxy sequencing. The sequences of these isolates of distinct genera were submitted to GenBank and the accession number was obtained. The phylogenetic tree for SOB isolates from Curca and Agarwado salt pans are depicted in Figure 4.47 (A and B) respectively along with their accession numbers.

Pseudomonadota

Four bacterial taxa from the Curca salt pan and six from the Agarwado salt pan were clustered within the phylum Pseudomonadota. The genera *Alloalcanivorex*, *Marinobacter*, and *Chromohalobacter* spp. were common in both the salt pans. The genera *Vibrio*, *Halomonas*, and *Pseudoalteromonas* spp. were unique to the Agarwado salt and *Vreelandella* sp. to Curca. Among the observed genera, only *Marinobacter* sp. belonged to the class Alphaproteobacteria and was found in both the salt pans.

Bacillota

Three bacterial taxa from the Curca salt pan and two from the Agarwado salt pan were clustered under the phylum Bacillota and class Bacilli. The genus *Paenibacillus* sp. was found in both salt pans. The genera *Priestia* and *Cytobacillus* spp. were exclusive to the Curca salt pan, while *Bacillus* sp. was only found in the Agarwado salt pan.

Table 4.7A: Molecular identification of 15 SOB isolates from Curca salt pan

Sr.No	Isolates	Closest Match
1	BJCS/12-3-4	<i>Alloalcanivorax xenomutans</i> JC109
2	BAugPW/23b	<i>Alloalcanivorax dieselolei</i> B-5
3	BJCW/14bB	<i>Paenibacillus glucanolyticus</i> DSM 5162
4	BAugPW/18b	<i>Marinobacter nauticus</i> ATCC 27132
5	BAugPW/19b	
6	BJPS/2bB	<i>Vreelandella venusta</i> DSM 4743
7	DBPW/11	<i>Priestia flexus</i> strain IFO15715
8	BDPW/12	
9	BFPW/20	
10	BF/9&10	
11	BDSC/2	<i>Cytobacillus pseudoceanisediminis</i> BNO1
12	BMCW/5	<i>Chromohalobacter salexigens</i> DSM 3043
13	BJPS/3b	
14	BAugPS/16b	
15	BAugPW/21b	<i>Chromohalobacter israelensis</i> ATCC 43985

Table 4.7 B: Molecular identification of 16 SOB isolates from Agarwado salt pan

Sr.No	Isolates	Closest Match
1	AMPW/13a	<i>Marinobacter nauticus</i> strain ATCC 27132
2	DAPW1/26	<i>Pseudoalteromonas shioyasakiensis</i> SE3
3	AN18-1/27	<i>Vibrio alginolyticus</i> NC470
4	JlyAW/28	<i>Halomonas koreensis</i> SS20
5	AJPS/7bA	<i>Halomonas stevensii</i> S18214
6	AD/15	<i>Paenibacillus glucanolyticus</i> DSM 5162
	AD/16	
8	AMPS/6bA	<i>Chromohalobacter israelensis</i> ATCC 43985
9	AJPS/2bA	<i>Chromohalobacter salexigens</i> DSM 3043
10	AJPS/7bB	
11	AMPS/5b	
12	4A	<i>Bacillus cereus</i> ATCC 14579
13	AW/27A	<i>Alloalcanivorax dieselolei</i> strain B5
14	A*1	
15	AD-1-19a	<i>Alloalcanivorax xenomutans</i> strain JC109
16	AW/25A	

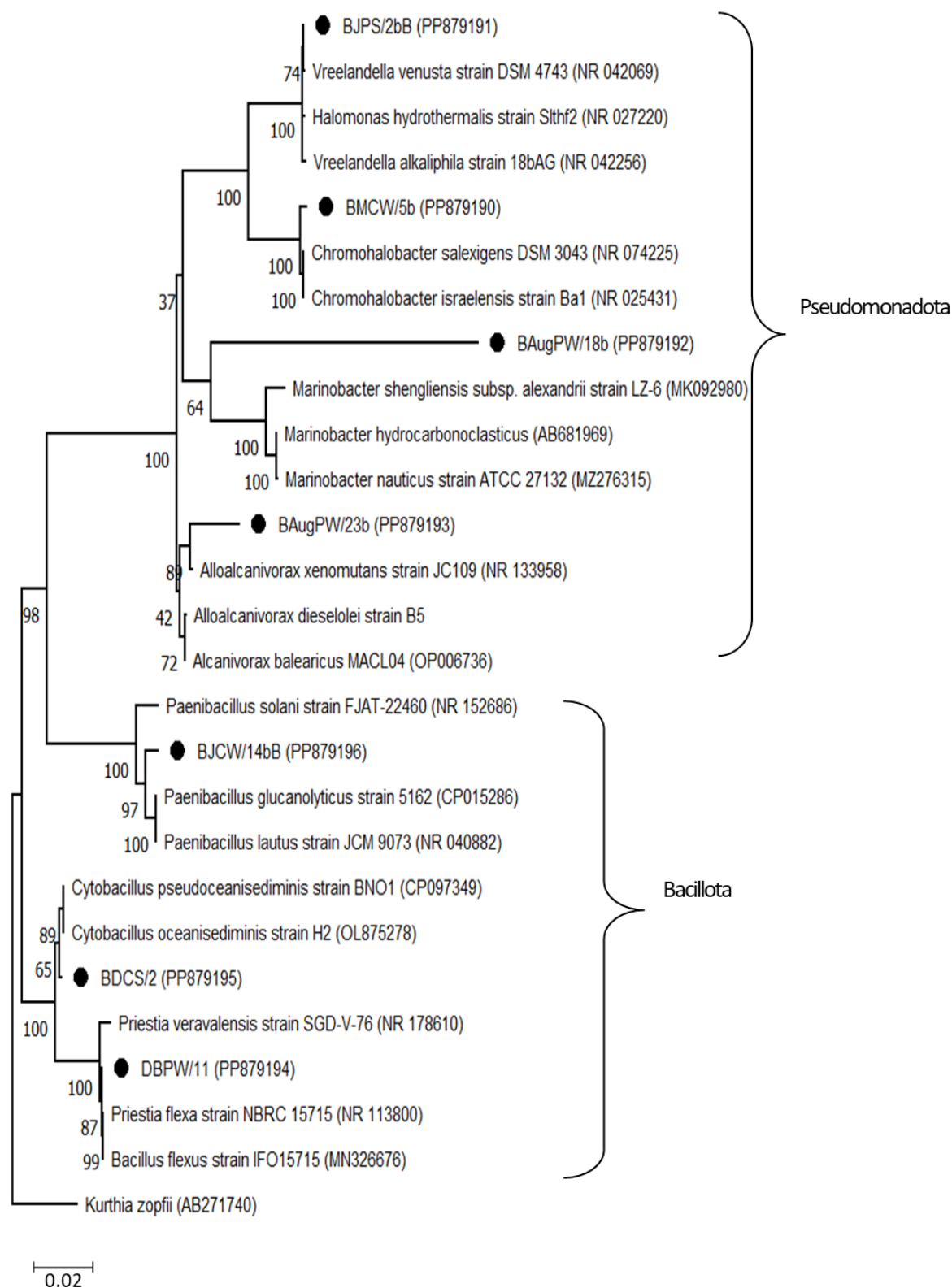


Figure 4.47A: 16S rRNA gene-based phylogenetic tree showing relationships of SOB isolates from Curca salt pans of Goa.

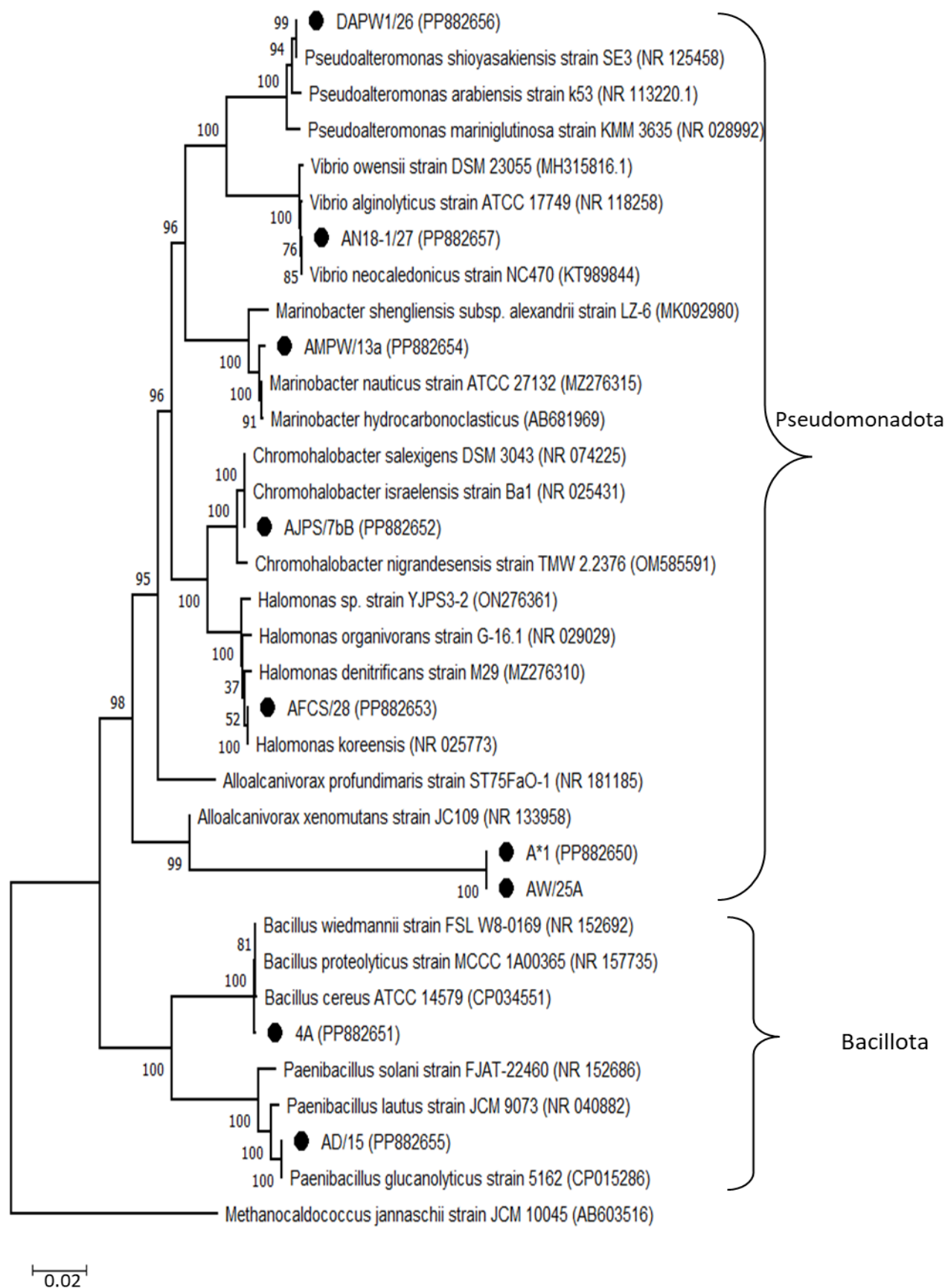


Figure 4.47B: 16S rRNA gene-based phylogenetic tree showing relationships of SOB isolates from Agarwado salt pans of Goa.

4.9.2 Molecular Phylogenetic Analysis of SRB

Figures 4.48 (A and B) display the phylogenetic trees for SRB isolates from the Curca and Agarwado salt pans, respectively. These trees revealed two major clades corresponding to two distinct phyla: Thermodesulfobacteriota and Bacillota in both the salt pans. In the Curca salt pan, the phylum Thermodesulfobacteriota contained the classes Desulfobacteria and Desulfovibrionia. In contrast, the Agarwado salt pan contained an additional class, Desulfobulbia. The phylum Bacillota included only one class, Clostridia, in both salt pans.

In the Curca salt pan, out of the 11 distinct genera observed, nine were classified under the phylum Thermodesulfobacteriota and two under Bacillota. In the Agarwado salt pan, among the 12 distinct genera identified, nine were categorized under Thermodesulfobacteriota and three under Bacillota.

Thermodesulfobacteriota

In the Curca salt pan, the class Desulfobacteria encompassed the genera *Desulfococcus*, *Desulfosarcina*, *Desulfobacter*, and *Desulfatiglans* spp. In the Agarwado salt pan, this class included the genera *Desulfonema* and *Desulfatiglans* spp. The genera *Desulfococcus*, *Desulfosarcina*, and *Desulfobacter* spp. were unique to the Curca salt pan, while the genus *Desulfonema* sp. was exclusive to the Agarwado salt pan.

Within the class Desulfovibrionia, the genera *Megalodesulfovibrio*, *Pseudodesulfovibrio*, *Desulfovermiculus*, and *Desulfomicrobium* spp. were common to both salt pans. However, the genus *Desulfovibrio* sp. was exclusive to Curca, whereas *Halodesulfovibrio* and *Nitratidesulfovibrio* spp. were unique to the Agarwado salt pan.

In addition, exclusive to Agarwado salt pan genus *Desulfopila* sp. was observed which belonged to the class Desulfobulbia.

Bacillota

The genera *Halanaerobium* and *Desulfosporosinus* spp., observed under the class Clostridia, were present in both salt pans. However, within the same class, the genus *Desulfofarcimen* sp. was unique to the Agarwado salt pan.

Table 4.8A: Molecular identification of 19 SRB isolates from Curca salt pan

Sr. No	Isolates	Closest Match
1	JBPS/1b	<i>Desulfococcus multivorans</i> DSM 2059
2	FBCS/5b	<i>Desulfosarcina variabilis</i> DSM 2060
3	ApBPS/10b	<i>Desulfobacter postgatei</i> 2ac9
4	BCS/2b	<i>Desulfovibrio piger</i> ATCC 29098
5	JBCS/13b	<i>Halanaerobium acetethylicum</i> EIGI
6	JBPS/3b	<i>Megalodesulfovibrio gigas</i> DSM 1382
7	JBCS/15b	<i>Pseudodesulfovibrio halophilus</i> DSM 5663
8	FBCS/4b	
9	JBCS/12b	
10	JBCS/11b	
11	FBPW/7b	<i>Desulfomicrobium baculatum</i> DSM 4028
12	FBPW/20b	
13	JBPW/9b	
14	BMPW/16b	<i>Desulfosporosinus orientis</i> DSM 765
15	JBCW/17b	<i>Desulfatiglans parachlorophenolica</i> DS
16	JBCW/18b	
17	BCW1/ 20b	
18	JBCW/14b	
19	FBPW/21b	

Table 4.8B: Molecular identification of 22 SRB isolates from Agarwado salt pan

Sr. No	Isolates	Closest Match
1	JCS/1a	<i>Desulfopila inferna</i> JS SRB250Lac
2	JACS/2a	<i>Halodesulfovibrio aestuarii</i> NCIMB 9335
3	AFPS/4a	<i>Desulfonema limicola</i> DSM 2076
4	FACS/5a	<i>Halanaerobium acetethylicum</i> EIGI
5	FACS/6a	<i>Megalodesulfovibrio gigas</i> DSM 1382
6	FACS/7a	<i>Pseudodesulfovibrio halophilus</i> DSM 5663
7	JACS/8a	
8	JACS/9a	
9	AS/10a	<i>Desulfofarcimen acetoxidans</i> DSM 771
10	AS/11a	<i>Desulfomicrobium baculatum</i> DSM 4028
11	JlyAS/12a	
12	JACW/13a	
13	AMCS/21a	
14	AFCW/15a	<i>Desulfatiglans parachlorophenolica</i> DS
15	JAPW/16a	
16	AFPS/17a	<i>Desulfovermiculus halophilus</i>
17	AFPS/18a	
18	AFCW/19a	
19	AFCW/20a	
20	AFCW/14a	<i>Desulfosporosinus orientis</i> DSM 765
21	AMCS/22a	<i>Nitratidesulfovibrio vulgaris</i>
22	AMCS/23a	

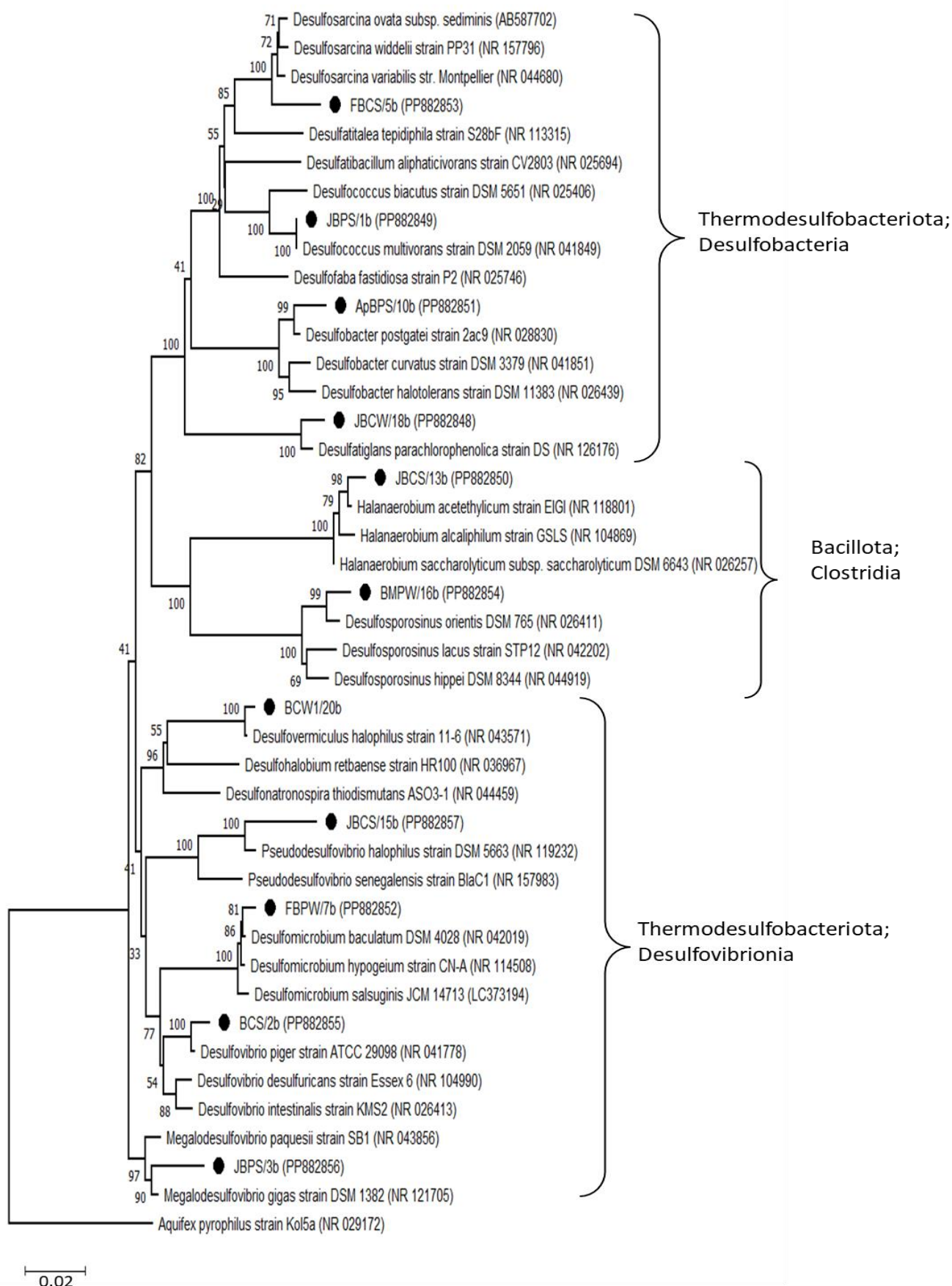


Figure 48A: 16S rRNA gene-based phylogenetic tree showing relationships of SRB isolates from Curca salt pans of Goa.

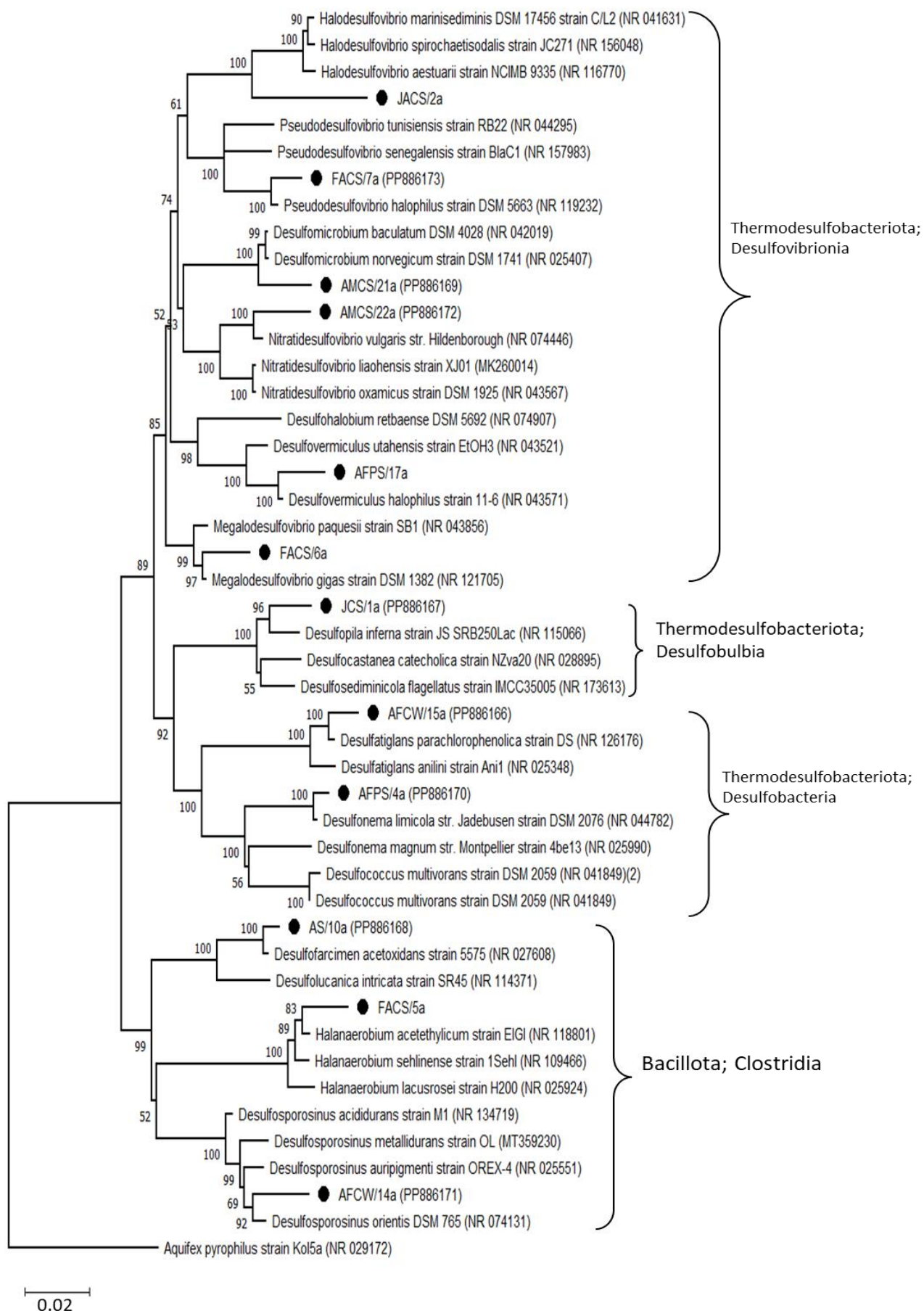


Figure 48B: 16S rRNA gene-based phylogenetic tree showing relationships of SRB isolates from Agarwado salt pans of Goa.

4.10 Screening of SOB cultures for thiosulfate oxidation

Based on the results of molecular identification of SOB by 16S rRNA gene, seven SOB isolates belonging to different genera were selected for screening their potential for thiosulfate oxidation (listed in Table 4.9). The morphology and cell size of these selected 7 SOB isolates by scanning electron microscopy were determined (Figure 4.49).

Table 4.9: Selected SOB isolates for screening thiosulfate oxidation ability

SOB isolates selected	Name	Average Cell Size
A*1	<i>Alloalcanivorax xenomutnas</i>	1.061 μm x 530 nm
BJCW/14bB	<i>Paenibacillus glucanolyticus</i>	2.523 μm x 504 nm
DBPW/11	<i>Priestia flexus</i>	2.416 μm x 805nm
BAugPW/21b	<i>Chromohalobacter salexigens</i>	917nm x 912.2 nm
AMPW/13a	<i>Marinobacter nauticus</i>	1.823 μm x 872 nm
AJPS/7bB	<i>Chromohalobacter israelensis</i>	2.204 μm x 734nm
AD-1-19a	<i>Alloalcanivorax xenomutans</i>	2.068 μm x 517 nm

After a duration of 90 days, the oxidation of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) by the SOB isolates was confirmed by estimating the residual thiosulfate and sulfate produced in the medium of test and control flasks. All the tested cultures were able to oxidize the thiosulfate in the medium. It was observed that sulfate concentration was higher in the test flasks as compared to the control.

Test culture A*1(*Alloalcanivorax xenomutans*) was able to oxidize the maximum amount of the thiosulfate in the medium i.e. 90 %, when compared with the residual thiosulfate amount in the control. The oxidation of thiosulfate observed in the 7 tested SOB cultures are depicted in Figure 4.50.

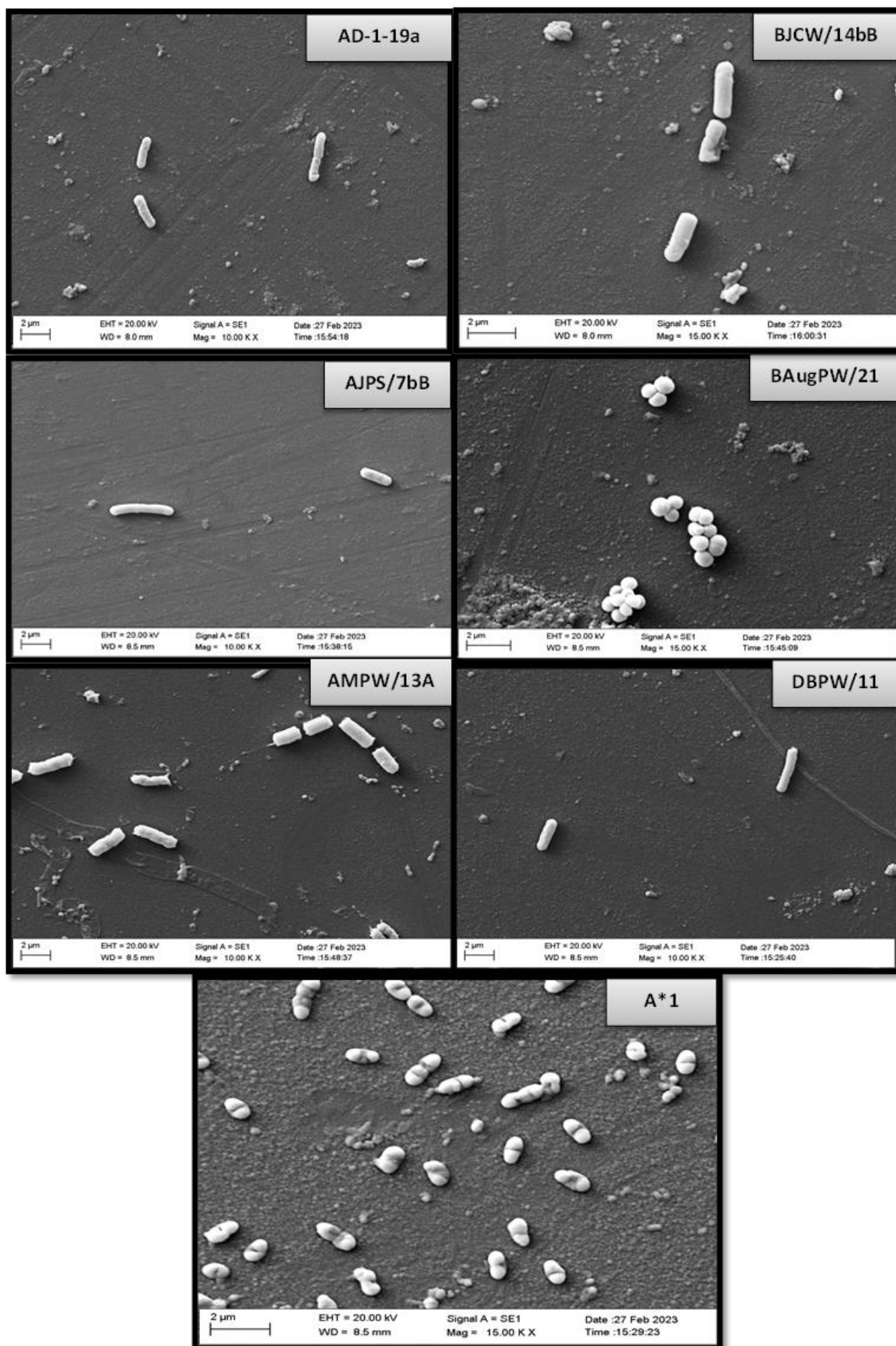


Figure 4.49: SEM images of selected SOB isolates for screening thiosulfate ability

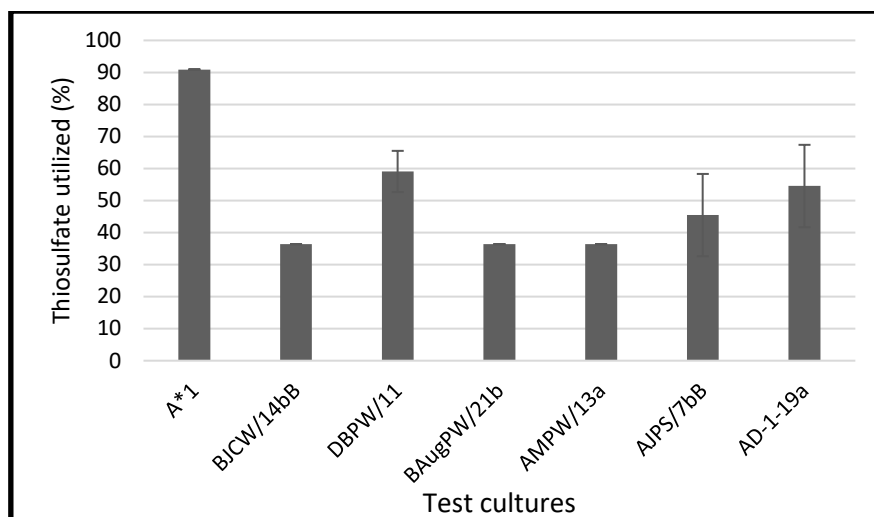


Figure 4.50: Amount of thiosulfate utilized (%) after oxidation of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) by 7 selected test cultures

Moreover, $79 \pm 1.57\%$ (19.05 ± 0.97 mg/L) of sulfate was produced by A*1 through biological thiosulfate oxidation (sulfate formed in the test flask is reported after subtracting it from the sulfate produced chemically in the control flask). Among the test cultures, A*1 produced the highest amount of sulfate following thiosulfate oxidation (Figure 4.50). Table 4.10 shows the amount of sulfate produced by DBPW/11, AD-1-19a, AJPS/7bB, BJCW/14bB, BAUGPW/21b, and AMPW/13a, listed in order of the highest sulfate production following oxidation. Hence, the most efficient biological thiosulfate oxidation activity was recorded in the A*1 isolate and was therefore selected for further experiments.

Table 4.10: Sulfate (mg/L) produced in the test flasks

Test cultures	Sulfate (mg/L) formed in test flasks
A*1	19.05465 ± 0.97
DBPW/11	6.0995 ± 0.15
AD-1-19a	5.2635 ± 0.17
AJPS/7bB	2.3595 ± 0.31
BJCW/14bB	1.6775 ± 0.18
BAUGPW/21b	1.6445 ± 0.23
AMPW/13a	0.5005 ± 0.09

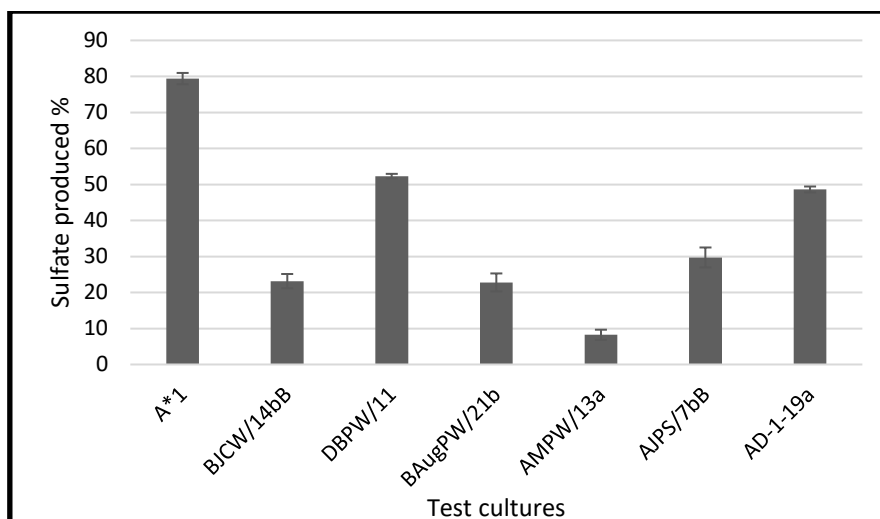


Figure 4.51: Amount of sulfate produced (%) after oxidation of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) by 7 selected test cultures.

4.11 Thiosulfate Oxidation by A*1 (*Alloalcanivorax xenomutans*)

The thiosulfate oxidation capability of A*1 (*Alloalcanivorax xenomutans*) was assessed at various sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) concentrations: 30, 40, 50, 60, 80, 100, 120, 140, and 160 mM. After a 120-day incubation period, both test and control flasks containing different concentrations of $\text{Na}_2\text{S}_2\text{O}_3$ were analyzed for residual thiosulfate and the concentration of sulfate produced. Thiosulfate oxidation was observed at all the concentrations tested, which resulted in the production of sulfate in the test flasks within 120 days. The highest oxidation activity was observed at 30 mM (with 90 % thiosulfate oxidation) and the lowest at 40 mM (20% thiosulfate oxidation) in the test flasks. The amount of sulfate produced was proportional to the amount of thiosulfate utilized at 30 mM and 140 mM concentrations, However, this conversion was not observed in the other concentrations. Particularly in a test flask with 30 mM of $\text{Na}_2\text{S}_2\text{O}_3$, 90 % of the thiosulfate was utilized, resulting in 79.3 ± 0.30 % sulfate through biologically thiosulfate oxidation by A*1 culture. Figure 4.52 below illustrate the percentage of thiosulfate utilized and sulfate produced by A*1 test culture after 120-day incubation and Figure 4.53 shows the sulfate production after oxidation at the tested concentrations of $\text{Na}_2\text{S}_2\text{O}_3$ compared to the respective controls.

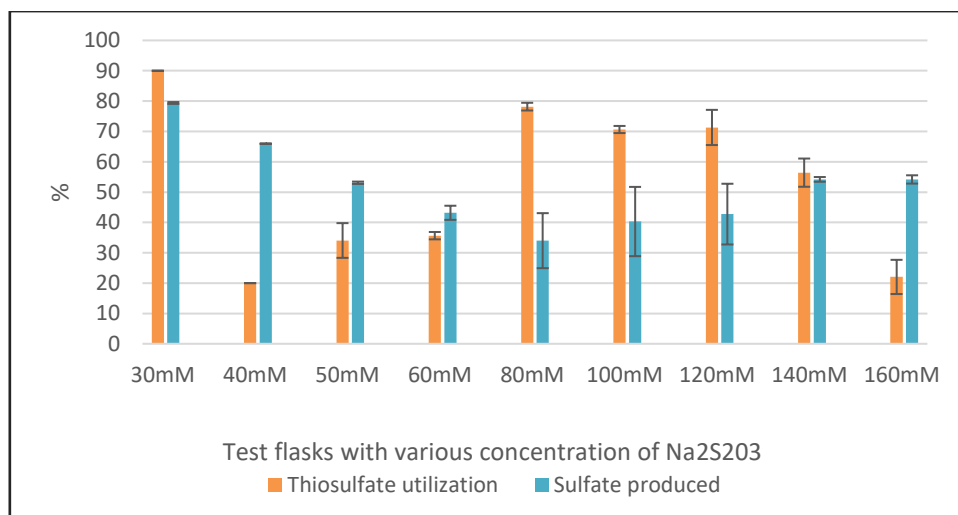


Figure 4.52: Percentage of thiosulfate utilized and sulfate produced by A*1 (*Alloalcanivorax xenomutans*) at various sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) concentrations.

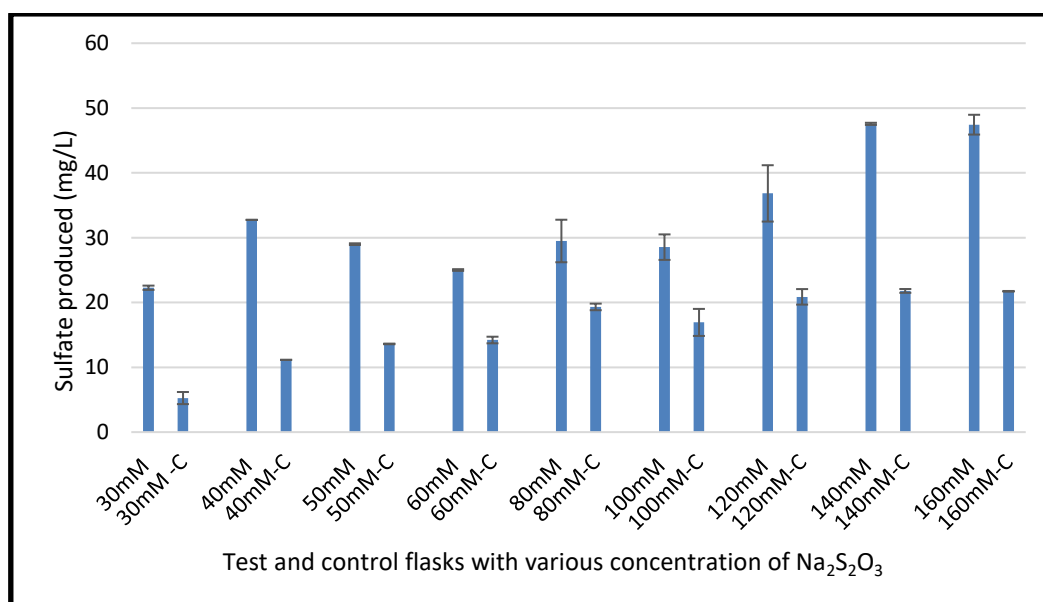


Figure 4.53: Sulfate (mg/L) produced A*1 (*Alloalcanivorax xenomutans*) at various sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) concentrations in test and control flasks.

4.12 Sulfide Oxidation by A*1 (*Alloalcanivorax xenomutnas*).

The sulfide oxidation in the test bottles was confirmed by comparing the amount of residual sulfide and sulfate formed in the medium of the test bottles with the control bottles. The A*1 oxidized NaHS (a sulfide source) at concentrations of 100 mg/L, 200 mg/L, and 300 mg/L in the test bottles, compared to the respective controls. However, no growth of the A*1 test culture or sulfide oxidation was observed at NaHS concentrations ranging from 400 to 1000 mg/L.

After 120 days, the residual sulfide content was assessed in test and control bottles of 100, 200, and 300 mg/L concentrations of sulfide. Sulfide was not detected in the test bottles, whereas it was present in the control bottles, indicating that sulfide oxidation occurred in the test bottles. Secondly, the sulfate produced in the test bottles was notably higher when compared to their corresponding control at the three concentrations of NaHS. Figure 4.54 (A-C) depicts the sulfate produced in the test and control bottles at 100, 200, and 300 mg/L concentrations of NaHS.

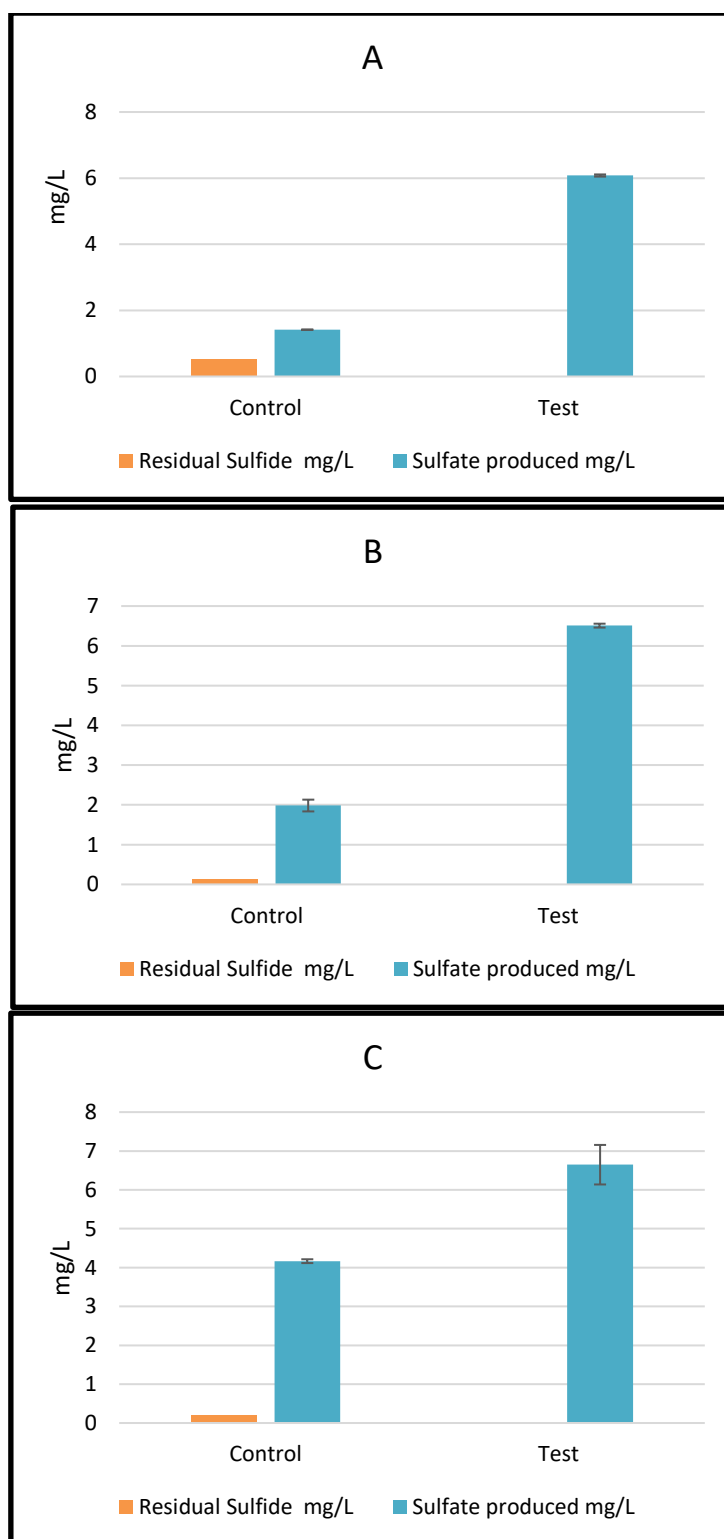


Figure 4.54: Sulfate produced at 100 (A), 200 (B) and 300 (C) mg/L of NaHS in test and control bottles by A*1 (*Alloalcanivorax xenomutans*).

4.13 Screening for Genes Involved in Thiosulfate and Sulfide Oxidation

Based on the positive results obtained for the oxidation of thiosulfate and sulfide by A*1 (*Alloalcanivorax xenomutans*), the presence of genes involved in these processes, specifically the *soxB* and *sqr* genes, was investigated using PCR analysis. The A*1 isolate showed successful amplification of the *soxB* gene but not the *sqr* gene. A band of approximately 1200 bp was observed when compared to ladder (L2–1Kb; L3–500bp), which corresponds to the expected product size of the *soxB* gene, typically ranging from 800 to 1200 bp as reported by Meyer et al. (2007) concluding the presence of *soxB* gene and the thiosulfate oxidizing potential of A*1.

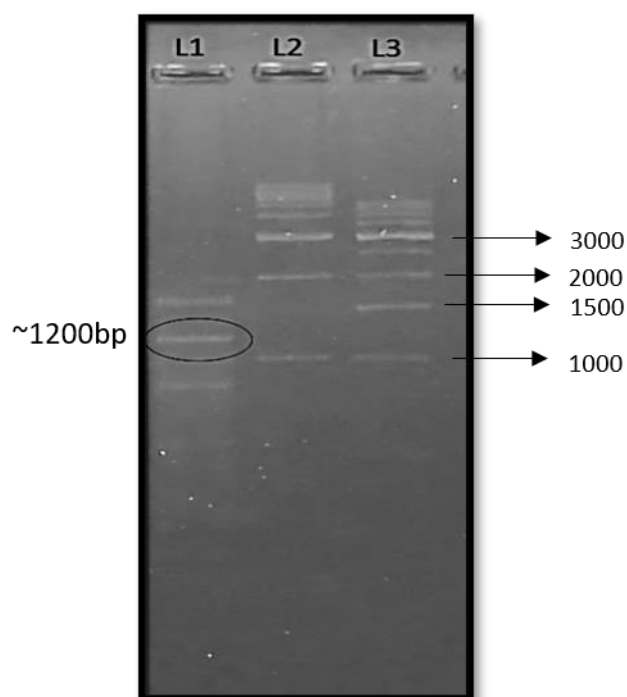


Figure 4.55: PCR amplification of *soxB* gene of A*1 (*Alloalcanivorax xenomutans*)

4.14 Analysis of Bacterial Diversity Using a Metagenomic Approach

4.14.1 Sediment Samples

The temperature of sediment samples collected from Agarwado (AC), Curca (CC), and Nerul (NC) salt pans was 35 °C (in CC) and 36 °C (AC and NC). The Salinity recorded was 450 PSU in AC and 400 PSU in CC and NC whereas the pH of the sediment samples was found to be 8.25 (AC), 7.6 (CC), and 8 (NC). The environmental DNA extracted from sediment samples AC, CC, and NC, concentrations were measured by Nanodrop and were found to be 28.6 ng/μl, 40.2 ng/μl, and 33.2 ng/μl respectively.

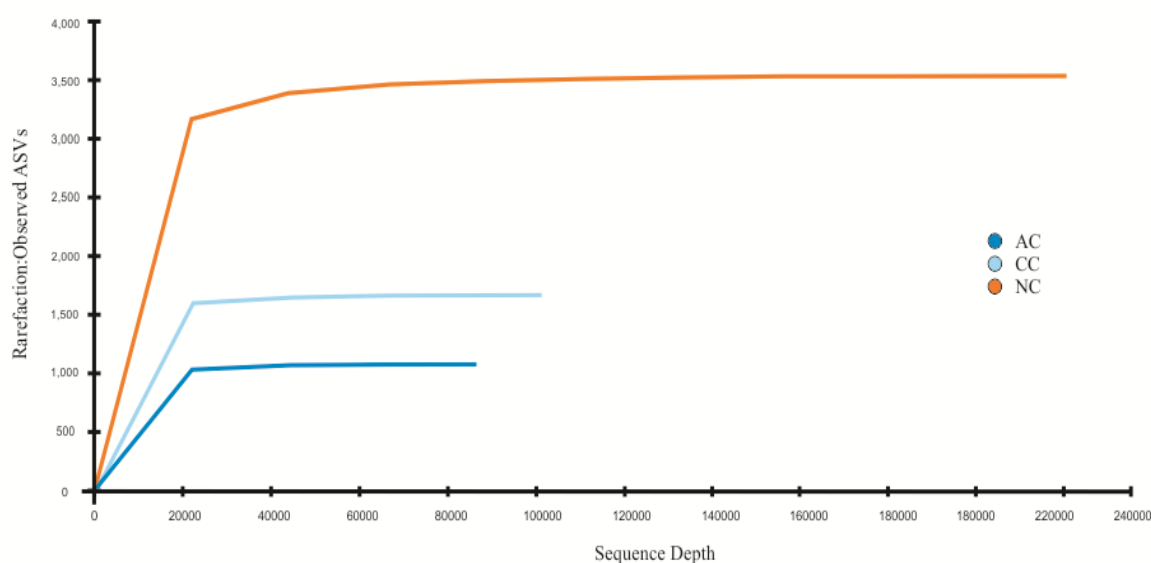


Figure 4.56. Rarefaction curve: Observed ASVs vs Sequencing depth for three salt pan samples namely Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India

4.14.2 Relative Abundance of Bacterial Community

A total of 95299, 104637, and 240563 reads were obtained in sediment samples of the Agarwado (AC), Curca (CC), and Nerul (NC) salt pan respectively. The rarefaction curve of all three samples reached a plateau at this sequencing depth, (Figure 4.56) indicating the information contained in the three samples, captured the majority of abundant phylotypes.

The bacterial community analysis of AC revealed 42 phyla and 201 genera. The abundant phyla such as *Pseudomonadota* (33 %), *Bacillota* (22 %), and "*Candidatus* Patescibacteria" (9.4 %), were observed (Figure 4.57A). Major contribution at the class level was by *Gammaproteobacteria* (30.7 %), and *Bacilli* (20.7 %). At the order level, *Pseudomonadales*, and *Bacillales* were found to be abundant with 26.8 % and 19.4 % relative abundance respectively. Predominant taxa observed at the family level were *Moraxellaceae* (26.5 %) and *Planococcaceae* (18.9 %) (Figure 4.58 A– C) and the major identified genera were *Psychrobacter* (26 %), *Halanaerobium* (3.4 %), and *Antarcticibacterium* (1.5 %) (Figure 4.57B).

In the CC sample, 50 phyla and 143 genera were recorded. The predominant phyla that contributed to the bacterial diversity include *Pseudomonadota* (20.8 %), *Desulfobacterota* (11.2 %), and *Bacteroidota* (9.8 %) (Figure 4.57 A). At the class level, *Gammaproteobacteria* (17 %) and *Bacteroidia* (8.4 %) were found dominant. At the order level, the predominance of *Desulfobulbales* (5 %) and *Desulfobacterales* (2.8 %) were detected. At the family level, *Desulfobulbaceae* (3.9 %) was relatively abundant (Figure 4.59 A-C). Lastly, at the general level, *Sulfurivermis* (1.3 %) and *Desulfatiglans* (1.1 %) presented a major proportion (Figure 4.57 B).

In the NC sediment sample, 53 phyla and 250 genera were identified. The dominant bacterial phyla include *Pseudomonadota* (14.7 %), *Planctomycetota* (13 %) and *Chloroflexota* (11.7 %) (Figure 4.57 A). The abundant classes observed were *Anaerolineae* (10.7 %) and

Gammaproteobacteria (9 %). At the order level, *Pirellulales* with 6.5 % relative abundance was found to be the highest followed by *Desulfobacterales* (3.8 %). At the family level, *Pirellulaceae* (6.5 %), was the major family obtained (Figure 4.60 A-C). Further, the most abundant genus identified was *Rhodopirellula* (1.2 %) (Figure 4.57 B).

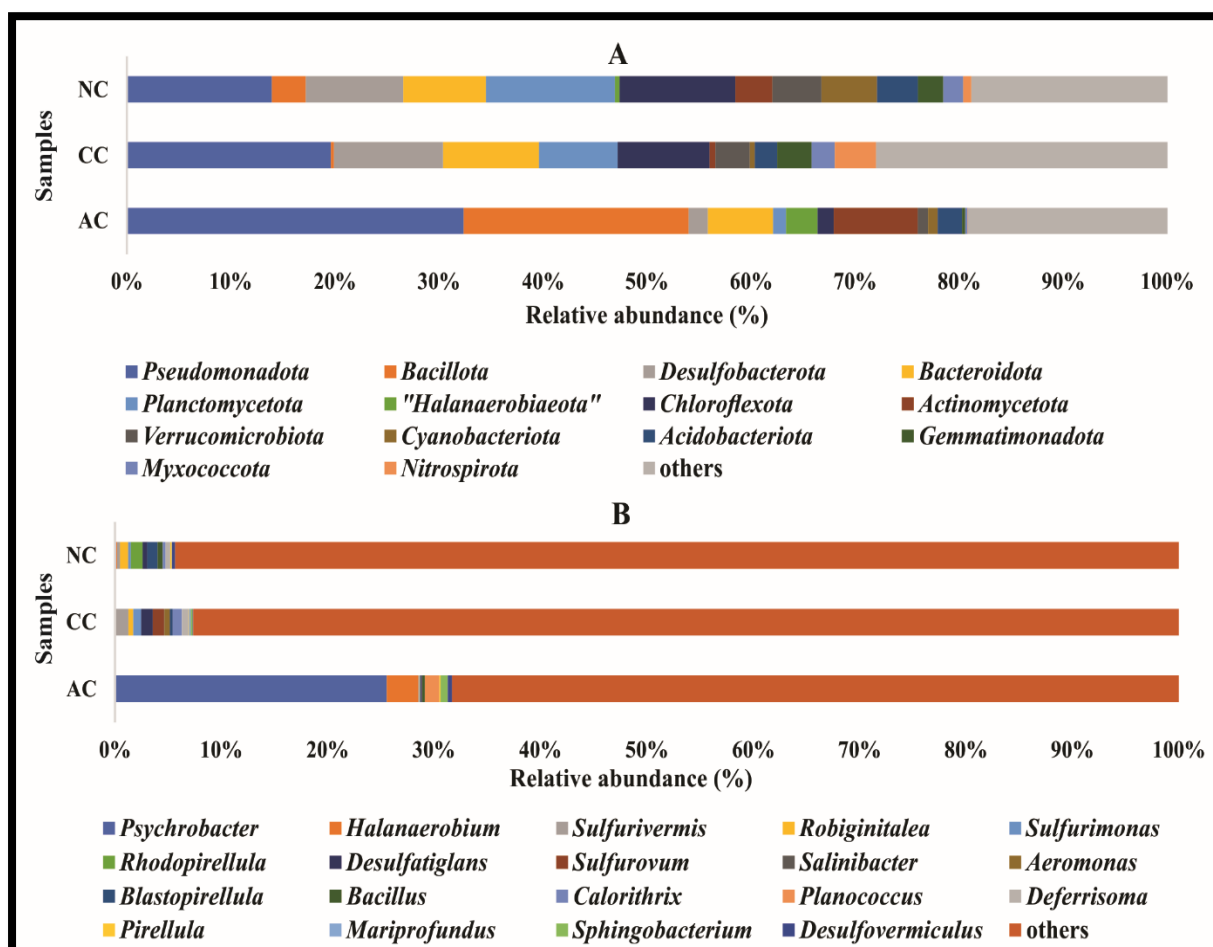


Figure 4.57: Relative abundance of bacterial communities at (A) phyla level and (B) genus level in sediment samples of Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India.

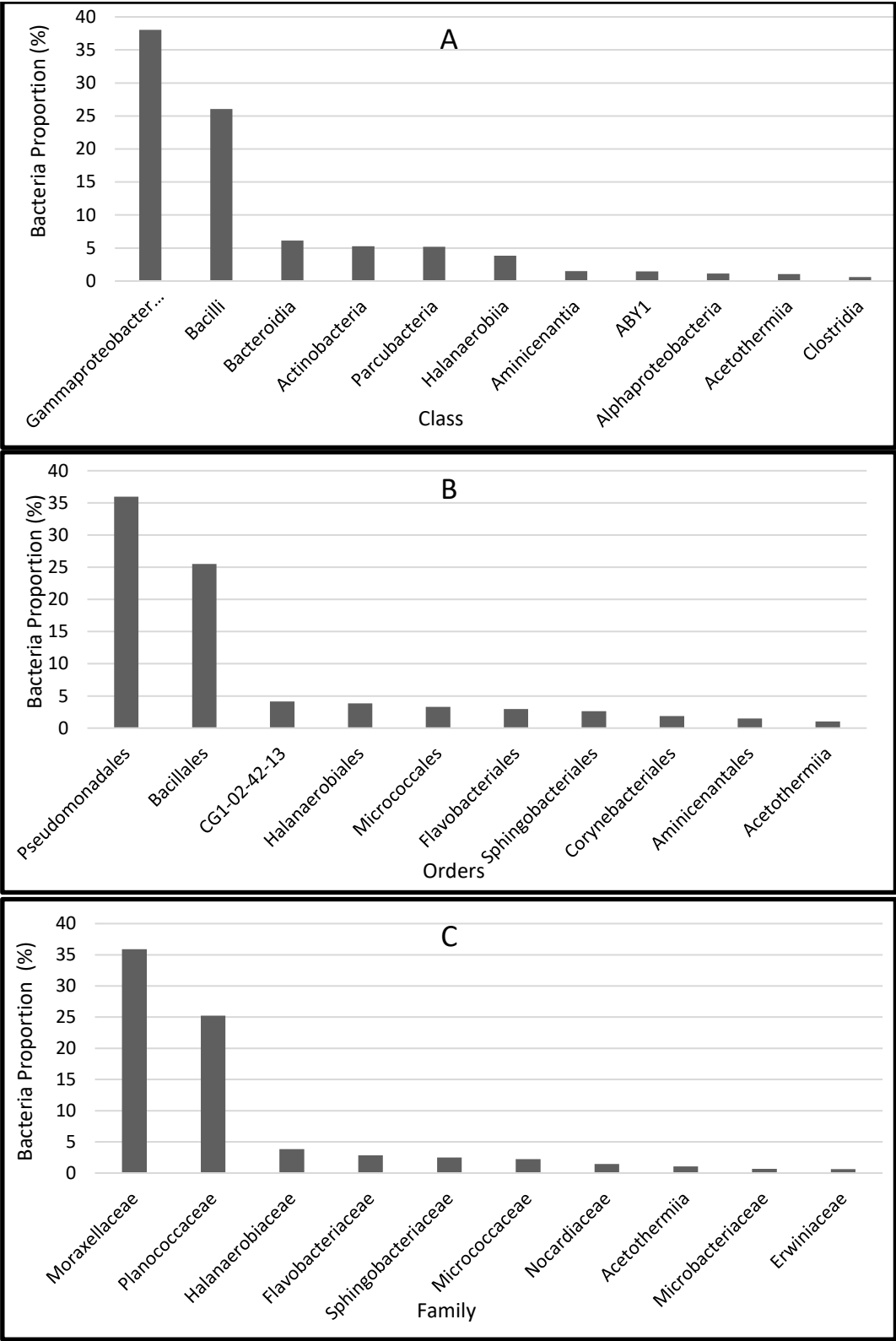


Fig 4.58. Major (A) class, (B) order, and (C) family identified in Agarwado saltpan

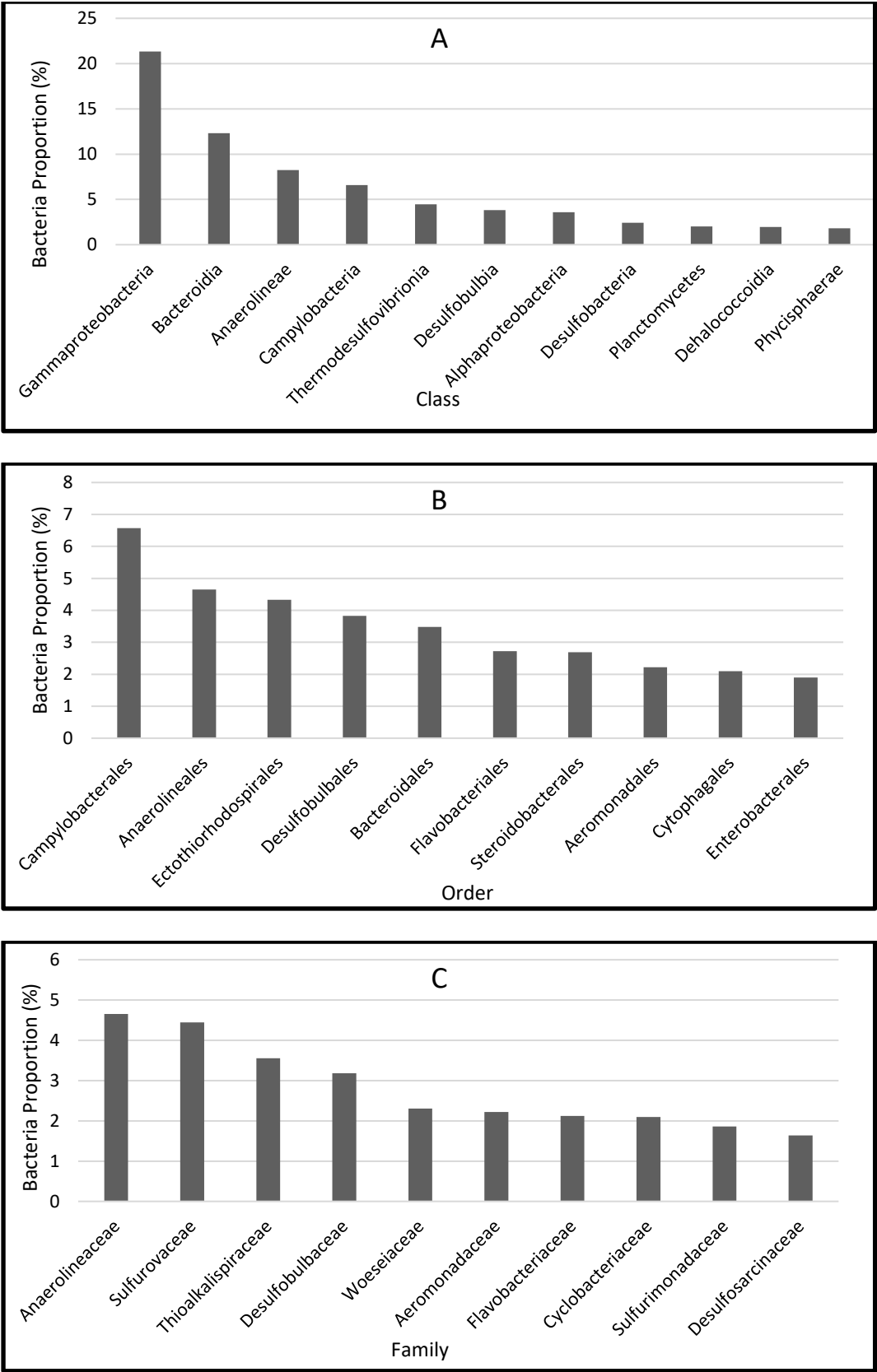


Fig 4.59. Major (a) class, (b) order, and (c) family identified in Curca saltpan (CC)

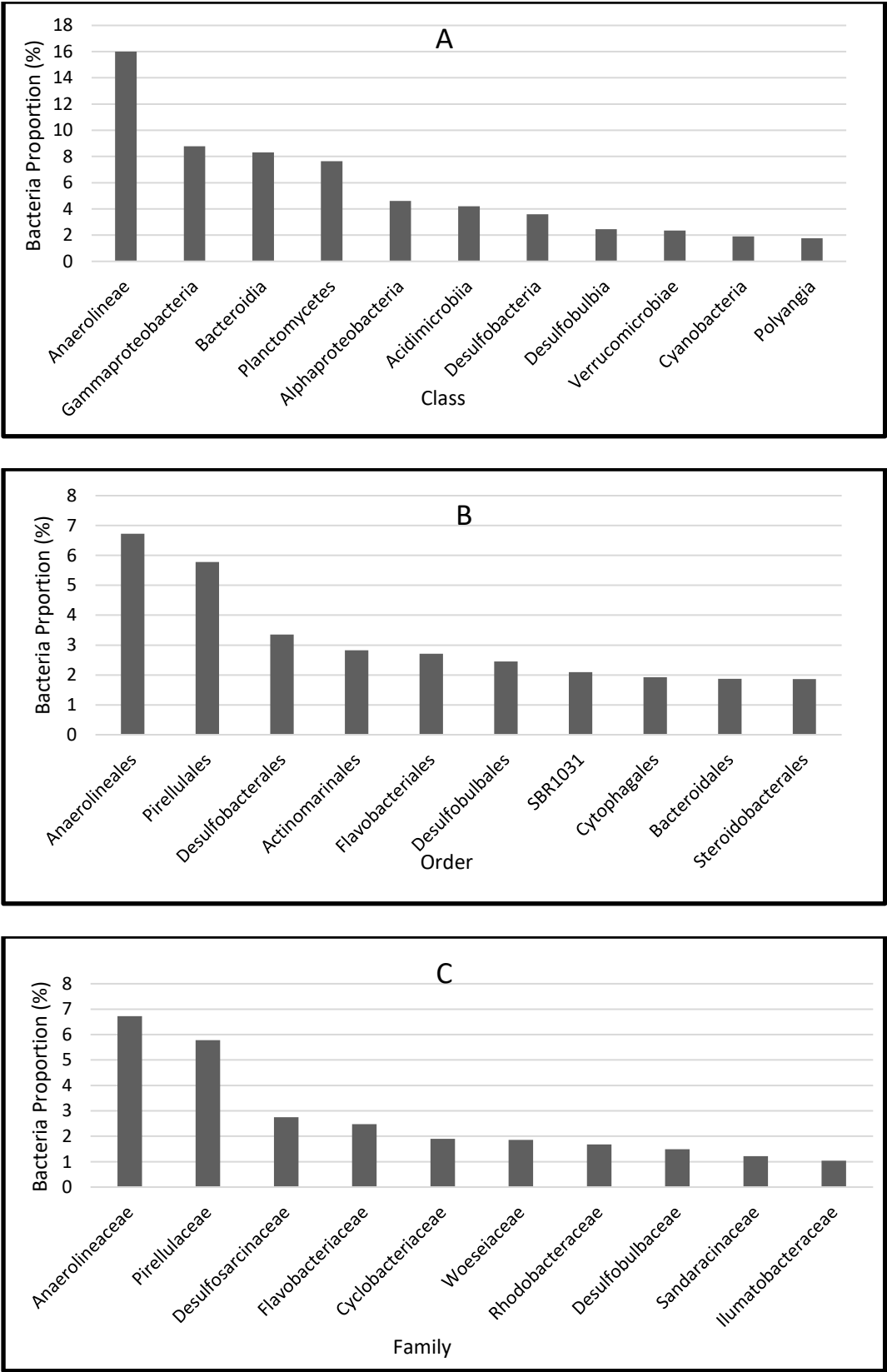


Fig 4.60. Major (a) class, (b) order, and (c) family identified in Nerul saltpan

4.14.3 Correlation of Bacterial Genera with Environmental Parameters

The effect of environmental parameters such as temperature, pH, salinity, nitrite, nitrate, sulfate, and sulfide were assessed with the bacterial genera using CCA. The CCA tripod explained 88.67 % of the variation in bacterial genera by environmental parameters (Figure 4.61). The result revealed the positive correlation between genera *Blastopirellula*, Sva0081 sediment group, *Rhodopirellula*, *Robiginitalea*, *Pirellula* and *Bacillus* with sulfide and sulfate. However, genera *Psychrobacter*, *Halanaerobium*, *Planococcus* and *Sphingobacterium* found abundant in AC were positively correlated with nitrite, nitrate, and salinity. Of all the environmental parameters sulfide and sulfate contributed significantly to the bacterial genera in NC.

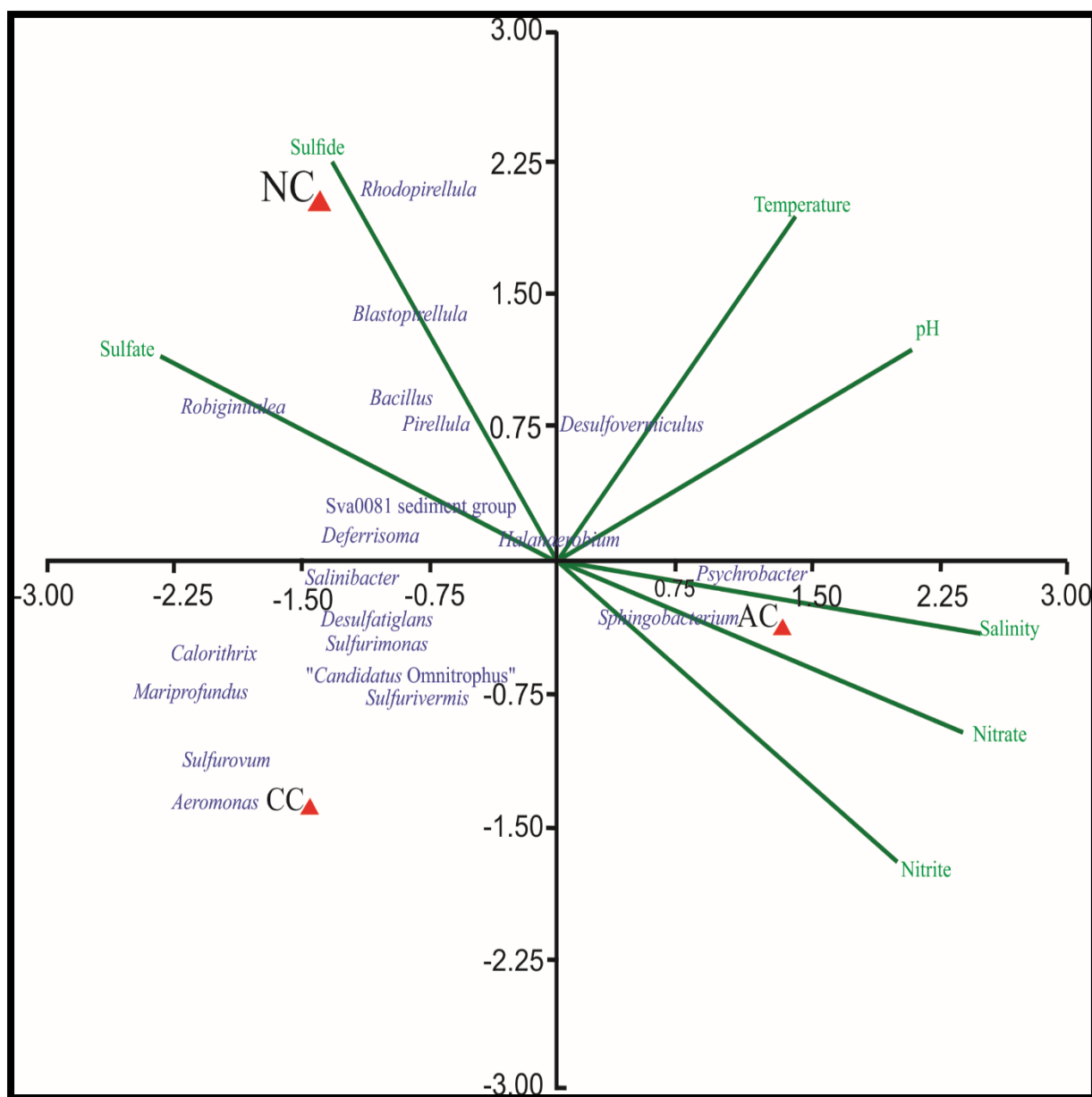


Figure 4.61: Canonical Correspondence Analysis ordination diagram of bacterial communities at generic-level at Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India associated with environmental parameters.

4.14.4 Alpha and Beta Diversity Analysis: Genus level

The alpha diversity indices such as Simpson diversity index, Shannon diversity index (Shannon-Wiener index), Dominance, Menhinick's richness index, and Pielou's evenness (equitability) were calculated and depicted in Table 4.11. Similarly, the beta diversity and its pair-wise comparison presented in Table 4.12 were performed using the mathematical expression $S/\bar{a}-1$, where S is the total number of genera and $\bar{a}$ is the average number of genera. The beta diversity was found highest and equal between AC and NC and AC and CC i.e., 0.73. The lowest beta diversity was observed between CC and NC (0.48).

Table 4.11: Alpha diversity indices in Agarwado salt pan (AC), Curca salt pan (CC) and Nerul salt pan (NC) of Goa at genera level.

Alpha diversity indices	AC	CC	NC
Taxa (no. of genera)	201	143	250
Simpson diversity index	0.67	0.97	0.98
Pielou's evenness (equitability)	0.38	0.87	0.86
Menhinick's richness index	0.6	1	1
Dominance	0.32	0.02	0.01
Shannon diversity index	2.03	4.3	4.8

Table 4.12: Pair-wise comparison of beta diversity of the salt pans of Goa: between Agarwado (AC), Curca (CC), and Nerul (NC) at the genus level.

	AC	CC	NC
AC	0	0.73	0.73
CC	0.73	0	0.48
NC	0.7	0.48	0

4.14.5 Unique and Overlapping Bacterial Communities Among the Salt pans

The Venn diagram (Figure 4.62A) represents the number of unique and common phyla in three salt pans. An acceptably small percentage of unique taxa was observed in AC, CC, and NC with 14.2 %, 6 %, and 5.6 % respectively. Contrastingly, the common taxa observed in all three salt pans were in a higher proportion ranging from 62 to 78 %. There were no common phyla found in CC and AC. The phyla found common among all three salt pans are listed in the Table 4.13.

At the genera level, a total of 201, 143, 250 genera were observed in AC, CC, and NC respectively. Although the number of genera was found highest in NC, the percentage of exclusivity was observed high in AC (61 %) > NC (47 %) > CC (19.4 %). The common genera found in all three salt pans ranged between 13 %- 23 %. The Venn diagram (Figure 4.62 B) depicts the number of unique and common genera in the three salt pans and the genera are listed in the Table 4.14.

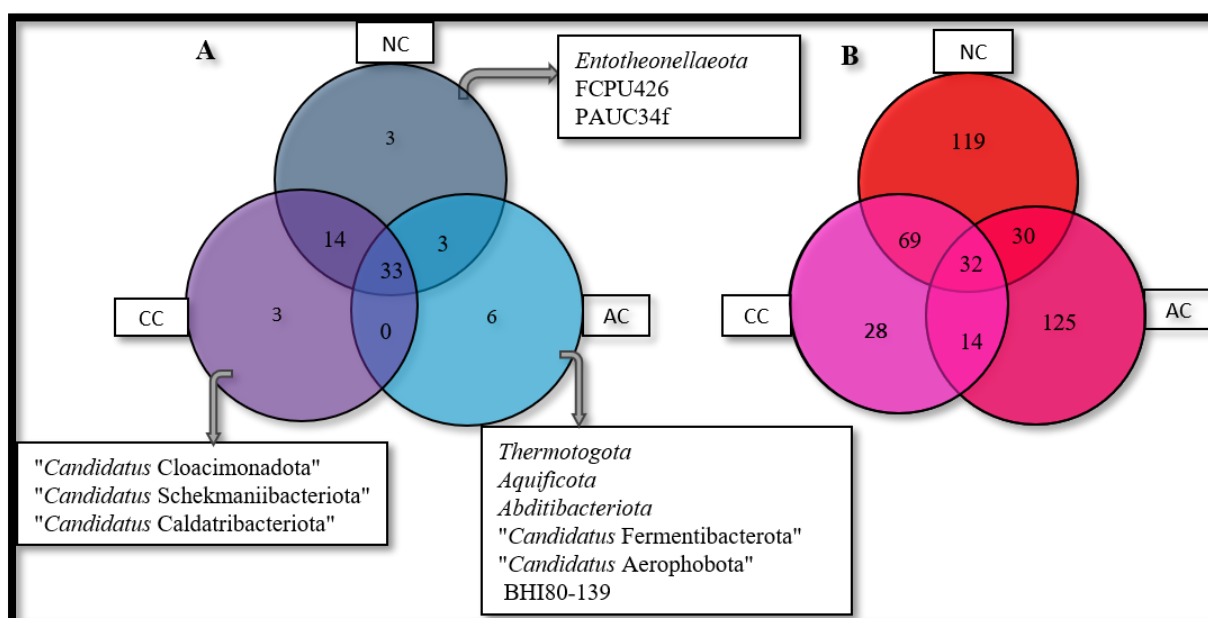


Figure 4.62: Venn diagram showing common and unique bacterial taxa at (A) phyla and (B) genus level between Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India.

Table 4.13: Common bacterial phyla found in Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India

Samples	Common phyla	Number of common phyla
AC, CC, NC	<i>Pseudomonadota</i> , <i>Bacillota</i> , <i>Cyanobacteriota</i> " <i>Candidatus</i> Patescibacteria", <i>Bacteroidota</i> , <i>Actinomycetota</i> , <i>Chloroflexota</i> , <i>Acidobacteriota</i> , <i>Planctomycetota</i> , " <i>Candidatus</i> Acetithermota", <i>Campylobacterota</i> , <i>Fusobacteriota</i> , <i>Myxococcota</i> , <i>Desulfobacterota</i> , <i>Nitrospirota</i> , <i>Calditrichota</i> , <i>Gemmatimonadota</i> , <i>Verrucomicrobiota</i> , <i>Spirochaetota</i> , " <i>Candidatus</i> Moduliflexota", " <i>Candidatus</i> Sumerlaeota", " <i>Candidatus</i> Zixiibacteriota", <i>Deinococcota</i> , <i>Fibrobacterota</i> , " <i>Candidatus</i> Babelota", " <i>Candidatus</i> Dadaibacteriota", <i>Bdellovibrionota</i> , " <i>Candidatus</i> Hydrogenedentota", <i>Elusimicrobiota</i> Groups at phylum level: WS2, TA06, NB1-j, SAR324 clade (Marine group B)	33
AC NC	<i>Halanaerobiaeota</i> , " <i>Candidatus</i> Methyloirabilota" Groups at phylum level: NKB15	3
CC NC	<i>Thermodesulfobacteriota</i> , " <i>Candidatus</i> Latescibacterota", <i>Nitrospinota</i> , " <i>Candidatus</i> Poribacteriota", <i>Caldiseriicota</i> , <i>Armatimonadota</i> , " <i>Candidatus</i> Margulisiibacteriota", " <i>Candidatus</i> Neomarinimicrobiota" Groups at phylum level: NKB15, 10bav-F6, MBNT15, LCP-89, WOR-1, Sva0485, CK-2C2-2	14

Table 4.14: Common and unique bacterial genera found in Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India

Samples	Common genera	Number of genera
AC, CC, NC	<i>Robiginitalea</i> , <i>Spirochaeta</i> , <i>Sulfurimonas</i> , <i>Sulfurivermis</i> , <i>Pseudohalaea</i> , <i>Blastopirellula</i> , <i>Fusibacter</i> , <i>Desulfatiglans</i> , <i>Desulfovermiculus</i> , <i>Pelolinea</i> , <i>Desulfobacca</i> , <i>Streptococcus</i> , <i>Marinobacter</i> , <i>Woeseia</i> , <i>Haliangium</i> , " <i>Candidatus Sumerlaea</i> ", <i>Flavobacterium</i> , <i>Truepera</i> , <i>Nitrospira</i> , <i>Thiohalorhabdus</i> , " <i>Candidatus Omnitrophus</i> ", <i>Pirellula</i> , <i>Bryobacter</i> , <i>Peredibacter</i> , <i>Bdellovibrio</i> Groups at genus level: SM23-31, Subgroup 10, SM1A02, P3OB-42, Pir4 lineage, Subgroup 23, JTB255 marine benthic group	32
AC, CC	<i>Sphingobacterium</i> , <i>Sulfurovum</i> , <i>Chryseobacterium</i> , " <i>Candidatus Paceibacter</i> ", <i>Serratia</i> , <i>Enhydrobacter</i> , <i>Pseudomonas</i> , <i>Staphylococcus</i> , <i>Desulfatirhabdium</i> , <i>Delftia</i> , " <i>Candidatus Obscuribacter</i> ", <i>Burkholderia</i> , <i>Tenacibaculum</i> Groups at genus level: CL500-3	14
AC, NC	<i>Psychrobacter</i> , <i>Halanaerobium</i> , <i>Antarcticibacterium</i> , <i>Pedobacter</i> , <i>Rhodopirellula</i> , <i>Bacteroides</i> , <i>Bacillus</i> , <i>Planctomicrobium</i> , <i>Acinetobacter</i> , <i>Prevotella</i> , <i>Haemophilus</i> , <i>Guyarkaria</i> , <i>Acanthopleuribacter</i> , <i>Pontibacillus</i> , <i>Tangfeifania</i> , <i>Faecalibacterium</i> , <i>Tumebacillus</i> , <i>Paenibacillus</i> , <i>Simkania</i> , <i>Hydrogenispora</i> , <i>Lachnospira</i> , <i>Halopectonella</i> , <i>Halanaerobacter</i> , <i>Cerasicoccus</i> , <i>Holdemanella</i> , <i>Desulfovibrio</i> , <i>Salisaeta</i> , <i>Succinoclasticum</i> , <i>Thalassobaculum</i> Groups at genus level: BBMC-4	30
CC, NC	<i>Actibacter</i> , <i>Deferrisoma</i> , <i>Propionigenium</i> , " <i>Candidatus Anammoximicrobium</i> ", <i>Calorithrix</i> , <i>Gimesia</i> , <i>Pelagibius</i> , <i>Ilumatobacter</i> , " <i>Candidatus Tenderia</i> ", <i>Sandaracinus</i> , <i>Thiohalophilus</i> , <i>Thiogramum</i> , <i>Andersenella</i> , <i>Magnetovibrio</i> , <i>Roseibacillus</i> , " <i>Candidatus Electrothrix</i> ", " <i>Candidatus Thiobius</i> ", <i>Desulfosarcina</i> , <i>Limibaculum</i> , <i>Ardenticatena</i> , <i>Nitrospina</i> , <i>Geothermobacter</i> , <i>Inmirania</i> , <i>Fulvivirga</i> , <i>Magnetospira</i> , <i>Mariprofundus</i> , <i>Pseudohongiella</i> , <i>Phaeodactylibacter</i> , <i>Desulfobulbus</i> , <i>Thermomarinilinea</i> , <i>Varunaivibrio</i> , <i>Draconibacterium</i> , <i>Spirochaeta</i> , <i>Imperialibacter</i> , <i>Owenweeksia</i> , <i>Malaciobacter</i> , <i>Nitrosomonas</i> , <i>Luteolibacter</i> , <i>Desulfobacter</i> , <i>Ignavibacterium</i> , <i>Phormidium</i> , <i>Halobacteriovorax</i> , <i>Vicingus</i> , <i>Turneriella</i> , <i>Salinirepens</i> , <i>Fluviicola</i> , <i>Thiomicrothrix</i> , <i>Phycisphaera</i> , <i>Oceanococcus</i> , <i>Carboxylicivirga</i> , <i>Caldithrix</i> , " <i>Candidatus Latescibacter</i> ",	69

	<i>Oleiphilus</i>, <i>Phaselicystis</i>, <i>Salinibacter</i>, <i>Aureispira</i>, <i>Thermoanaerobaculum</i> Groups at genus level: R76-B128, Sva0081 sediment group, Urania-1B-19 marine sediment group, ADurb.Bin120, IS-44, IheB3-7, OM27 clade, A4b, MSBL7, GWE2-31-10, BD1-7 clade, LCP-80	
AC	<i>Planococcus</i>, <i>Rhodococcus</i>, <i>Pantoea</i>, <i>Exiguobacterium</i>, <i>Sphingomonas</i>, <i>Planomicrobium</i>, <i>Micrococcus</i>, <i>Paracoccus</i>, <i>Microbacterium</i>, <i>Rothia</i>, <i>Massilia</i>, <i>Corynebacterium</i>, <i>Devosia</i>, <i>Pontibacter</i>, <i>Dietzia</i>, <i>Thiohalospira</i>, <i>Mycobacterium</i>, <i>Aerococcus</i>, <i>Microvirga</i>, <i>Methylobacterium</i>, <i>Desulfofustis</i>, <i>Actinomyces</i>, <i>Hyphomicrobium</i>, <i>Methylibium</i>, <i>Roseomonas</i>, <i>Rubrobacter</i>, <i>Pontimonas</i>, <i>Dongia</i>, <i>Chthoniobacter</i>, <i>Porphyromonas</i>, <i>Leptolyngbya</i>, "<i>Candidatus Udaeobacter</i>", <i>Gordonia</i>, <i>Granulicatella</i>, <i>Gemella</i>, <i>Rubellimicrobium</i>, <i>Nocardioide</i>s, <i>Cetobacterium</i>, <i>Chryseolinea</i>, <i>Ferruginibacter</i>, <i>Chryseomicrobium</i>, <i>Rhodocytophaga</i>, <i>Veillonella</i>, <i>Gelidibacter</i>, <i>Abditibacterium</i>, <i>Fusobacterium</i>, <i>Deinococcus</i>, <i>Blastocatella</i>, <i>Tunicatimonas</i>, <i>Nonlabens</i>, <i>Lysobacter</i>, <i>Acidaminococcus</i>, <i>Nibrella</i>, <i>Taibaiella</i>, <i>Desulfocarbo</i>, <i>Ornithinimicrobium</i>, <i>Arcicella</i>, <i>Megamonas</i>, <i>Aridibacter</i>, <i>Cupriavidus</i>, <i>Solimonas</i>, <i>Desulfotignum</i>, <i>Domibacillus</i>, <i>Prostheco</i>bacter, <i>Solirubrobacter</i>, <i>Leptotrichia</i>, <i>Gaetbulibacter</i>, <i>Weeksella</i>, <i>Flavitalea</i>, <i>Lysinibacillus</i>, <i>Rufibacter</i>, <i>Panacagrimonas</i>, <i>Herbaspirillum</i>, <i>Glutamicibacter</i>, <i>Enterococcus</i>, <i>Brevibacillus</i>, <i>Brevibacterium</i>, <i>Dyadobacter</i>, <i>Virgisporangium</i>, <i>Bergeyella</i>, <i>Anaerolinea</i>, <i>Ottowia</i>, <i>Treponema</i>, <i>Hymenobacter</i>, <i>Verticiella</i>, <i>Monoglobus</i>, <i>Carnobacterium</i>, <i>Halothiobacillus</i>, <i>Natranaerobius</i>, <i>Alloprevotella</i>, <i>Desulfotobacterium</i>, "<i>Candidatus Protochlamydia</i>", <i>Wenzhouxiangella</i>, <i>Prosthecochloris</i>, <i>Pseudonocardia</i>, <i>Arcticibacter</i>, "<i>Candidatus Halysiosphaera</i>", <i>Propioniceclava</i>, <i>Singulisphaera</i>, <i>Ohtaekwangia</i>, <i>Ahniella</i>, <i>Jeotgalicoccus</i>, <i>Alkalibacterium</i>, <i>Halarsenatibacter</i>, <i>Nubsella</i>, <i>Neochlamydia</i>, <i>Pajaroellobacter</i>, <i>Streptomyces</i>, <i>Anaerococcus</i>, <i>Gaiella</i>, <i>Cutibacterium</i>, <i>Ezakiella</i>, <i>Chloronema</i>, <i>Mycoplasma</i> Groups at genus level: TM7, RS25G, Ellin516, AKYG587, SH-PL14, Ellin6067, SWB02, SC103, MND1, RB41, <i>Christensenellaceae</i> R-7 group	125
CC	<i>Aeromonas</i>, <i>Klebsiella</i>, <i>Stenotrophomonas</i>, <i>Bradyrhizobium</i>, <i>Ralstonia</i>, <i>Allorhizobium</i>, <i>Endomicrobium</i>, <i>Porticoccus</i>, <i>Litoribrevibacter</i>, <i>Sulfuriflexus</i>, <i>Hyphococcus</i>, <i>Roseivirga</i>, <i>Methylophaga</i>, <i>Hydrogenovibrio</i>, <i>Litorivivens</i>, <i>Oleibacter</i>, <i>Desulfomicrobium</i>, <i>Saccharopolyspora</i>, <i>Gracilimonas</i>, "<i>Candidatus Actinomarina</i>", <i>Desulfurivibrio</i>, <i>Chlamydia</i>, <i>Alterococcus</i>, "<i>Candidatus Kuenenia</i>"	28

	Groups at genus level: BCF9-17 termite group, ADurb.Bin063-1, SUP05 cluster, MSBL3	
NC	<i>Arthrospira</i>, <i>Defluviicoccus</i>, <i>Clostridiisalibacter</i>, <i>Thiohalomonas</i>, <i>Oceanirhabdus</i>, <i>Methyloceanibacter</i>, <i>Silicimonas</i>, <i>Roseivivax</i>, <i>Sporacetigenium</i>, <i>Bradymonas</i>, <i>Orenia</i>, <i>Roseicyclus</i>, <i>Iodidimonas</i>, <i>Limibacillus</i>, <i>Rubripirellula</i>, <i>Oscillatoria</i>, <i>Parahaliaea</i>, <i>Pullulanibacillus</i>, <i>Haloferula</i>, <i>Pelagicoccus</i>, <i>Desulfosalsimonas</i>, <i>Cyanobacterium</i>, <i>Sporosalibacterium</i>, <i>Natranaerovirga</i>, <i>Tepidibacillus</i>, <i>Lentimonas</i>, "Candidatus Thiodiazotropha", <i>Crocinitomix</i>, <i>Ruminiclostridium</i>, <i>Desulfomonile</i>, <i>Ferrimonas</i>, <i>Desulfofaba</i>, <i>Limisphaera</i>, <i>Vulcanibacillus</i>, <i>Marinobacterium</i>, <i>Halobacillus</i>, <i>Anaerophaga</i>, <i>Haloplasma</i>, <i>Balneola</i>, <i>Magnetococcus</i>, <i>Gottschalkia</i>, <i>Dichotomicrobium</i>, <i>Kordiimonas</i>, <i>Halofilum</i>, <i>Geoalkalibacter</i>, <i>Alicyclobacillus</i>, <i>Microbulbifer</i>, <i>Maricaulis</i>, <i>Rhodovibrio</i>, <i>Synechocystis</i>, <i>Asteroleplasma</i>, <i>Izimaplasma</i>, <i>Maritimimonas</i>, <i>Succinivibrio</i>, <i>Clostridium</i>, <i>Thermoflexus</i>, <i>Cyanothece</i>, <i>Bythopirellula</i>, <i>Waddlia</i>, <i>Oceanicaulis</i>, <i>Aliifodinibius</i>, <i>Pleurocapsa</i>, <i>Halodesulfovibrio</i>, <i>Roseimaritima</i>, <i>Brachyspira</i>, <i>Filomicrobium</i>, <i>Puniceicoccus</i>, <i>Sunxiuqinia</i>, <i>Anaeromicrobium</i>, <i>Alcanivorax</i>, <i>Geminocystis</i>, <i>Coxiella</i>, <i>Sedimenticola</i>, <i>Bifidobacterium</i>, <i>Geitlerinema</i>, <i>Fodinibius</i>, <i>Winogradskyella</i>, "Candidatus Endecteinascidia", <i>Levilinea</i>, <i>Dehalobacterium</i>, <i>Desulfospira</i>, <i>Muricauda</i>, <i>Parabacteroides</i>, <i>Vibrio</i>, <i>Microscilla</i>, <i>Lewinella</i>, <i>Marinilabilia</i>, <i>Escherichia</i>, <i>Halanaerobaculum</i>, <i>Desulfitibacter</i>, <i>Dialister</i>, <i>Alistipes</i>, <i>Megasphaera</i>, <i>Marinicella</i>, <i>Pseudobacteriovorax</i>, <i>Halomonas</i>, <i>Aquicella</i>, <i>Rubidibacter</i>, <i>Amphiplicatus</i>, <i>Anaerovibrio</i>, <i>Schleiferia</i>, "Candidatus Nucleicultrix", <i>Heliophilum</i> Groups at genus level: SBZC-1223, YC-ZSS-LKJ90, Cm1-21, RBG-16-49-21, SCGC AAA164-E04, RCP1-48, M2PT2-76 termite group, YC-ZSS-LKJ63, UCG-002, Sva0996 marine group, B2706-C7, UCG-012, BMS9AB35, C1-B045, JL-ETNP-F27, MAT-CR-P4-C12	119

4.14.6 Functional Gene Prediction in Three Salt pans

Many functional genes involved in various biological processes, including cellular metabolism and adaption to extreme environmental conditions, have been found in the indigenous bacterial communities of all three salt pans. Figure 4.63 depicts the most abundant genes that were found in all the three salt pans. Further, numerous genes involved in the nitrogen, sulfur, and carbon cycles have also been found to be part of the overall functional gene profile (Figures 4.64 A-C). According to the heatmap, the genes Acetyl-CoA C-acetyltransferase (K00626) and Glyceraldehyde 3-phosphate dehydrogenase (K00134) were most abundant in AC, as compared to CC and NC (Figure 72). Genes involved in the carbon cycle, such as ABC-2 type transport system permease protein (K01992), ABC-2 type transport system ATP binding protein (K01990), and RNA polymerase sigma 70-factor ECF subfamily (K03088), were abundant in AC and CC salt pans (Figure 4.64A). Nitrogen cycle genes, including nitrite reductase (NADH) small and large subunit (K00362, K00371), and assimilatory nitrate reductase catalytic subunit (K00372), were abundant in AC salt pans (Figure 4.64 B). Sulfur cycle genes, like phosphoadenosine phosphosulfate reductase (K00390) and adenylyl sulfate kinase (K00860), sulfate adenylyl transferase subunit-2 (K00957) were dominant in NC and CC (Figure 4.64 C).

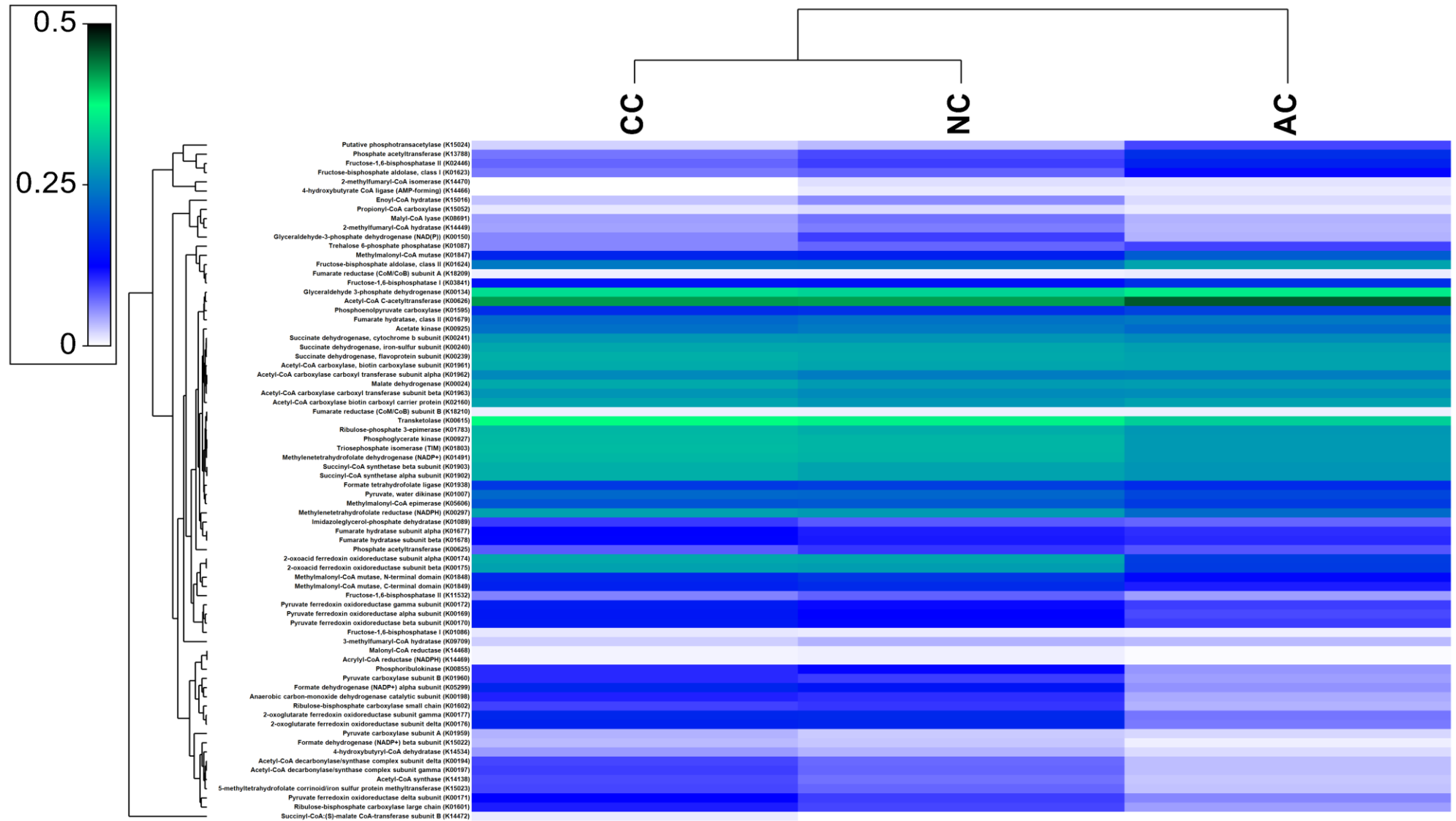


Figure 4.63: Heatmap showing most abundant genes predicted by using PICRUST2 algorithm in the sediment samples of Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India.

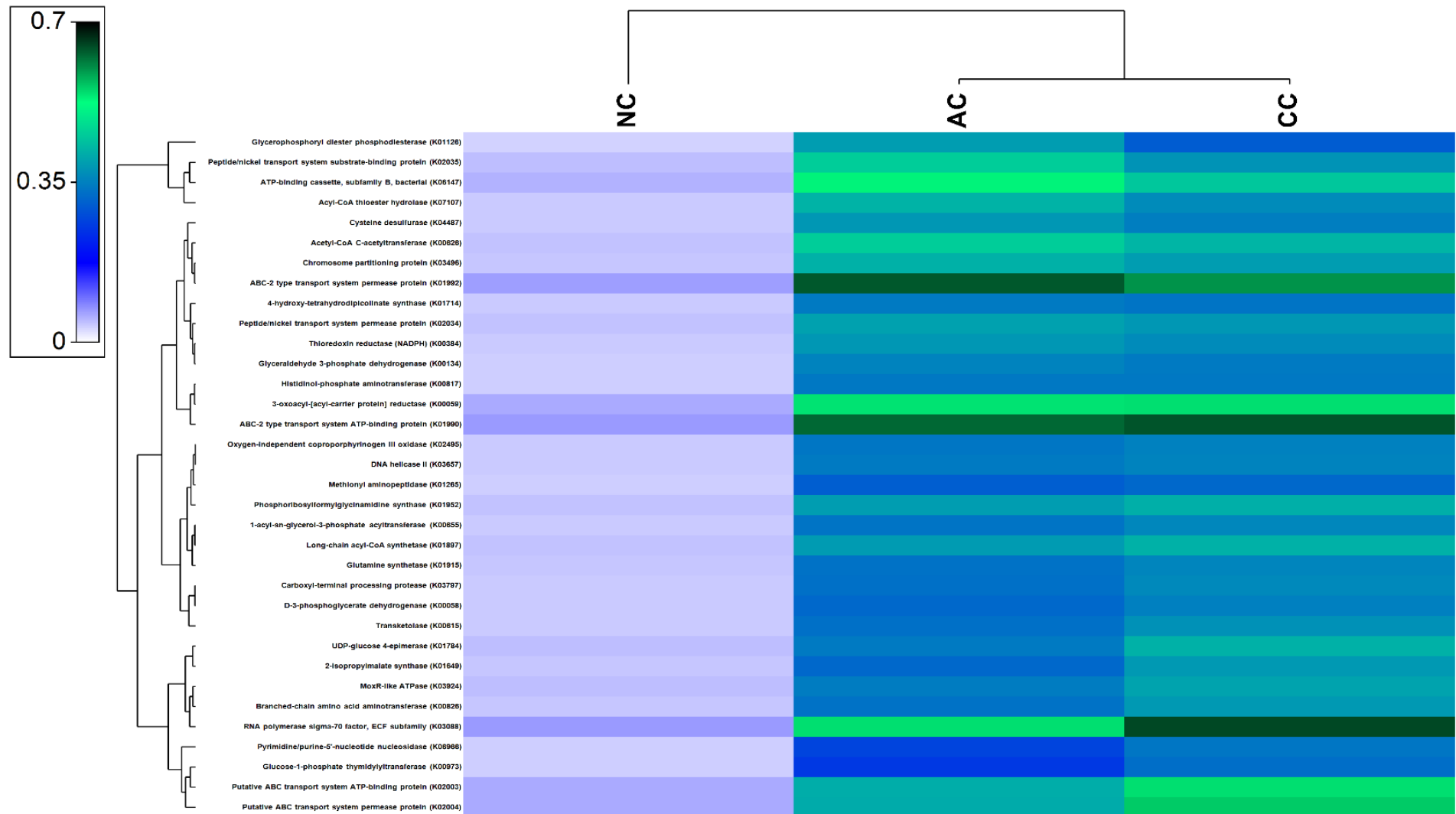


Figure 4.64 A: Heatmap showing genes associated with carbon metabolism predicted by using PICRUST2 algorithm in the sediment samples of Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India.

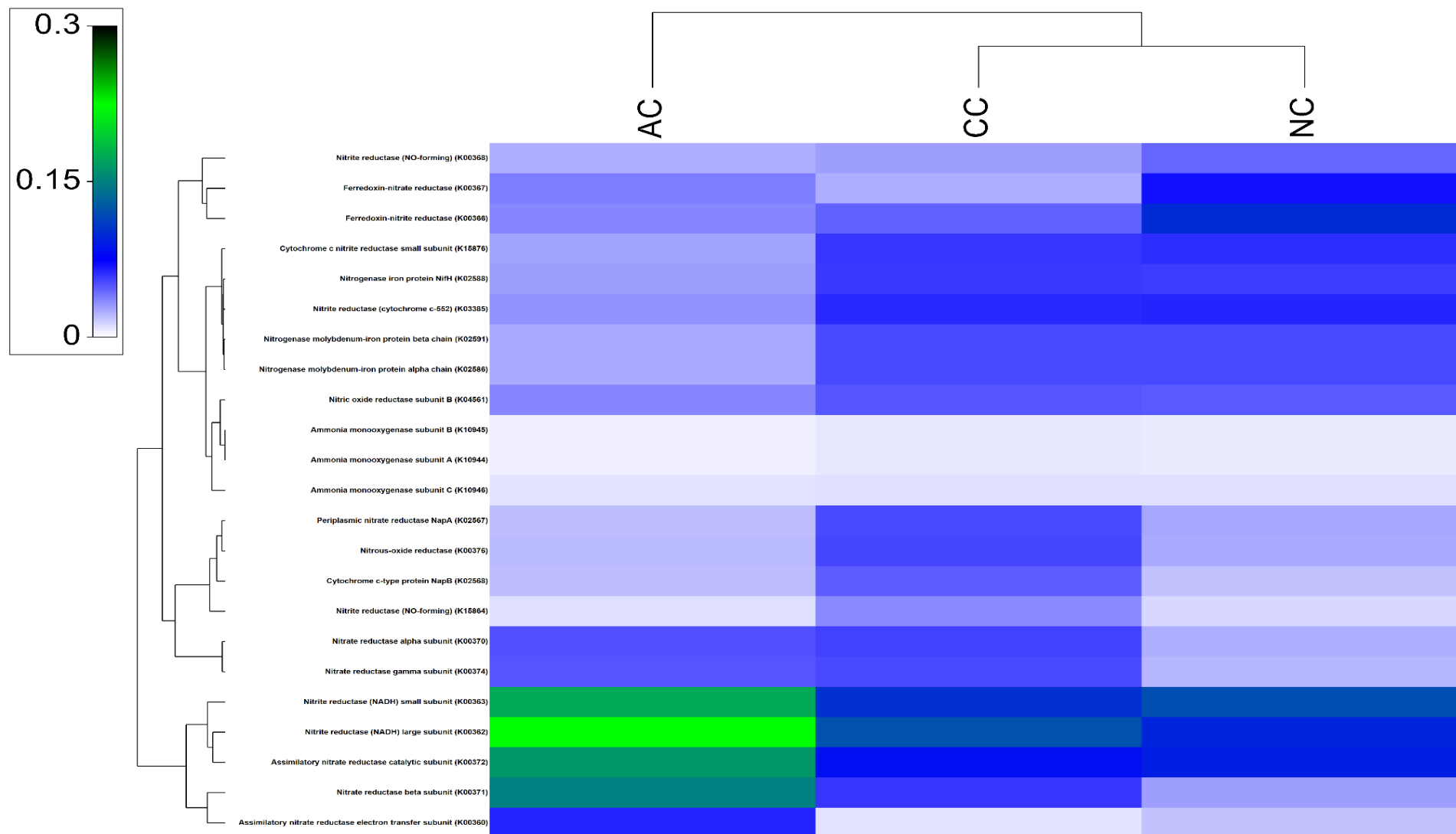


Figure 4.64 B: Heatmap showing genes associated with nitrogen metabolism predicted by using PICRUST2 algorithm in the sediment samples of Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India.

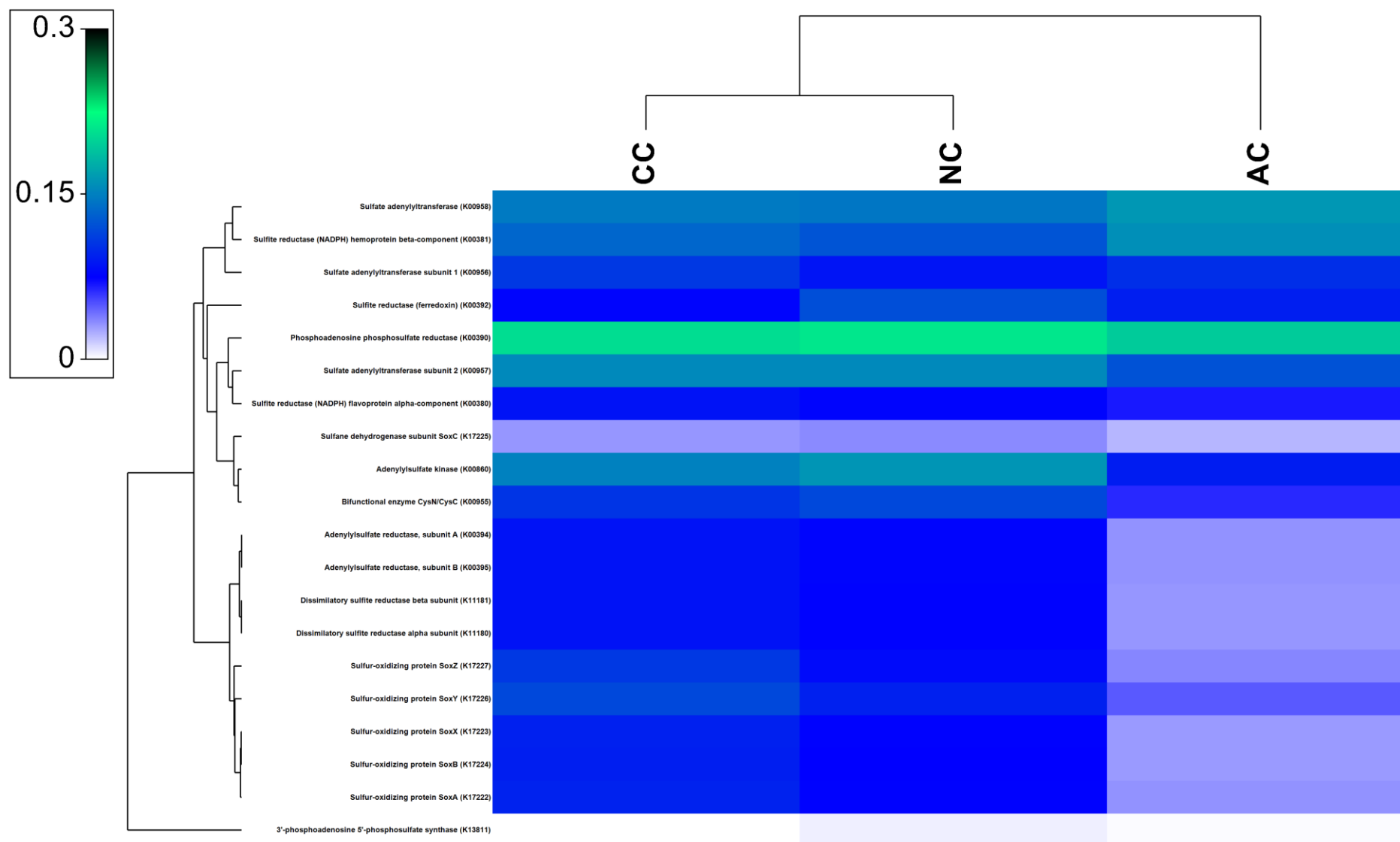


Figure 4.64 C: Heatmap showing genes associated with sulfur metabolism predicted by using PICRUST2 algorithm in the Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India.

Chapter 5

Discussion

5.1 Physical Parameters in Salt pan

Salt production in Goa, India is an aged-old traditional method designed by farmers known as the Mittkars. This unique occupation has thrived in the state of Goa due to its extensive 107 km long coastline, abundant sunlight, and warm climate, which are the ideal conditions for the evaporation process essential in salt-making. Traditional salt pans, with their high salinity and extreme conditions, serve as habitats for hypersaline bacteria. These bacteria have adapted to survive and flourish in environments with high salt concentrations and fluctuating conditions of salinity (0-450 PSU). They have developed specialized strategies and cellular systems to manage salt stress, making them well-suited to the challenging environment of the salt pans.

The study sites, Agarwado and Curca, were chosen due to their distinctive geographical locations. In our study, we observed significant fluctuations in the environmental parameters in these salt pans, with temperatures ranging from 27–35°C in Curca and 27.6–36° C in Agarwado. Since the sample collection was restricted from 10 a.m. to 10.30 a.m., the temperatures were normally lower than the afternoon diurnal temperatures which are reported upto 41°C in the Ribander salt pan (Das 2018). Salinity levels varied from 6 to 287 PSU in both locations, and pH ranged from 7 to 8. Kerkar, (2003) has also reported an alkaline pH from 7.38 to 9.03 in the salt making season where the pre-monsoon (salt-harvesting phase) season was relatively the most alkaline period in the Ribander salt pan in Goa in all the three phases of the salt pan in the period of one year. The frequency of variations of these parameters in the hypersaline environment is substantial. As noted by Mani et al. (2020), in the Siridao saltern of Goa, the

initial salt-harvesting phase shows differences in physicochemical parameters such as temperature, pH, and salinity as compared to the peak salt-harvesting phase, which occurs just 20-25 days after the initial phase. They have reported a temperature of 38.4°C, pH of 7.8, and salinity of 280 PSU from the same salt pans, aligning with our results during the salt-harvesting phase at both the study sites. The significant differences in temperature, and salinity observed in our study between the primary pond and the crystallizer pond (Figure 4.2 and 4.4) were also noted in comparison to other studies. Both the previous and the present research reported higher temperatures and salinity levels in the crystallizer pond as compared to the primary pond (Das 2018, Mani et al. 2020). These fluctuating conditions can indeed make the microbes in this habitat robust and versatile, enhancing their adaptability.

5.2 Abundance of SOB and SRB

The amount of sulfate in marine systems remains relatively stable, due to the combined activities of sulfate-reducing bacteria (SRB) and sulfide-oxidizing bacteria (SOB) in natural marine ecosystems. Therefore, when quantifying SRB from either pristine environments or polluted areas, it is also important to measure the bacteria involved in the oxidative turnover of sulfur to understand the natural purification processes (Loka 1989).

Sulfur compounds are mainly used by SOB and SRB, which are crucial for sulfur metabolism. Additionally, SOB converts the toxic sulfide produced by SRB into inorganic sulfate, which can then be used by other microorganisms. Due to the limited knowledge of the ecological role of the SOB and SRB complementing each other in these hypersaline environments, we have hypothesized the following questions: (1) Do the three phases of salt pan affect the abundance of SOB and SRB? (2) Do the chemical compounds viz. sulfate, sulfide, nitrate, and nitrite influence the abundance of SOB and SRB? (3) Which are the indigenous species/ genera of

SOB and SRB in the salt pan ecosystem? (4) Can the SOB from the hypersaline ecosystem be potentially utilized for the bioremediation of reduced sulfur compounds?

Biological sulfur oxidation plays a crucial role in the sulfur cycle within natural environments. SOB utilize sulfur compounds like elemental sulfur, sulfide, and thiosulfate as energy sources (Luo et al., 2013). Further, the sulfur cycle is found to be most prevalent in hypersaline environments (Kerkar and Bharathi, 2007). Hence, SOB and SRB being the most important factors in the S cycle, were explored across three distinct phases: the ceased phase, preparatory phase, and salt-harvesting phase that influence the Curca and Agarwado salt pan ecosystem throughout the year. The Curca and Agarwado salt pans similar trends in terms of their physicochemical and biological parameters, however, the variations in the nutrient concentrations and the dominance of certain chemical parameters influenced the distribution of the bacteria.

The ceased phase, which corresponds to the monsoon season in Goa, showed a significant abundance of aerobic and anaerobic SOB, with a culturable count of 10^6 CFU mL⁻¹ or g⁻¹ in both water and sediment samples of Curca and Agarwado salt pans (Figure 4.33, 4.35, 4.37, 4.39). While considering the limitation in availability of reports on the abundance of SOB in hypersaline environments, their populations have been extensively studied in marine tropical mangrove sediments in the Mandovi estuary along India's west coast and in water samples of Arabian Sea off India's southwest coast, where the abundance of 10^5 - 10^6 was reported (SamKamaleson 2019a, 2019b, SamKamaleson & Gonsalves 2019). Conversely, SRB exhibited their highest abundance during the salt-harvesting phase, a period characterized by peak salinity levels ranging from 170 to 287 PSU, with counts reaching upto 10^4 CFU mL⁻¹ in water and 10^5 CFU g⁻¹ in sediments (Figure 4.41 and 4.43). Notably, in the present study, the culturable SRB was not detected during the ceased phase in these both salt pans. The highest observed SRB abundance during the salt-harvesting phase aligns with findings reported by

Kerkar and Loka (Kerkar and Loka 2007, Kerkar 2003) from the Ribandar saltern of Goa, where SRB counts (10^2 CFU mL⁻¹ in water and 10^4 CFU g⁻¹ in sediment) were recorded at a salinity of 123 PSU. A relatively dense population of halophilic SRB with 10^6 CFU g⁻¹ was reported at a salinity of 475 PSU from Kulunda soda lakes in southeastern Siberia, Russia (Foti et al. 2007). This justifies the increase in abundance of SRB with respect to the high salinity in the salt pans.

The co-existence of SRB and SOB observed in salt pans is crucial for regulating the sulfur cycle, and maintaining constant sulfate levels in the marine ecosystem (Loka 1989; Kerkar 2003; Bharathi and Chandramohan 1990). Sulfide oxidation is most intense in surface sediments at the oxic-anoxic interface due to high rates of sulfate reduction and the abundant availability of oxidants that can drive sulfide oxidation (Jørgensen et al. 2019, Qun et al. 2023) as compared to surficial waters in the water bodies. Secondly, the high abundance of SRB was also reported in surface sediment of 0- 2 cm (10^4 CFU g⁻¹) by Kerkar and Loka in 2007. In the present work, we observed a positive correlation between aerobic and anaerobic SOB ($r = .64$, $p < .001$) in the top 0-2 cm of Curca salt pan sediments, indicating their prominent role in sulfide oxidation. Some studies classify these bacteria as aerobic and anaerobic *Thiobacillus denitrificans*, based on their electron donors for respiration (Bharathi and Chandramohan 1990). SOB oxidize reduced sulfur compounds under both aerobic and anaerobic conditions, using oxygen and nitrate as electron acceptors, respectively (Bharathi and Chandramohan 1990; Fike et al. 2016).

Further, we also report a significant positive correlation between anaerobic SOB and nitrate concentrations ($r = .51$, $p = .006$) in Curca salt pan sediments, and between anaerobic SOB and nitrite ($r = .48$, $p = .01$) in Agarwado salt pan sediments, suggesting that these bacteria are possibly involved in nitrate reduction coupled with sulfur oxidation. This relationship was also reported by Sam Kamaleson and Gonsalves et al. in 2019. SRB showed positive correlations with both sulfate ($r = .47$, $p = .01$) and sulfide ($r = .59$, $p < .001$) in Agarwado sediment samples,

establishing their active role as primary drivers of the sulfur cycle through dissimilatory sulfate reduction in the Goan salt pans (Jørgensen et al. 2019; Jørgensen and Kasten 2006). During the salt-making process, an initial routine of mixing and skimming are repeatedly carried out every day to collect the salt crystals in heaps, which are subsequently deposited on the bunds (mud borders) of the salt pans, making the overlying water of the salt pans a highly dynamic ecosystem. This in turn affects the anaerobic environment which is crucial for sulfur metabolism, resulting in the inability to obtain concurrent results on the relationship of SOB and SRB with sulfate, sulfide, nitrate, and nitrite in water samples. Thus, due to these man-made processes of disturbing the overlying water ecosystem in the salt pans, steady and repeatable values of the physico-chemical parameters were not possible, and hence the assessment of these parameters was deduced with the further analysis of the physico-chemical parameters of the salt pan sediments.

The concentration of chemical parameters that affect the abundance of SOB and SRB viz sulfate, sulfide, nitrate, and nitrite was found maximum during the salt-harvesting phase in the crystallizer pond (which is attributed to the process of evaporation). The same trend was reported by Das in 2018 in the Ribandar salt pan. The lower concentrations of the above chemical parameters in the primary pond as compared to the crystallizer pond, is due to the influx of fresh seawater from the estuary into the primary pond that resulted in lower concentrations of sulfate, sulfide, nitrate, and nitrite in both the water and sediment samples (Das 2018).

5.3 Taxonomic Diversity of SRB

Sulfate-reducing bacteria are physiologically unique among the kingdom of Bacteria due to their ability to reduce sulfates to sulfides. Besides this function, they can also reduce sulfites, thiosulfates, and elemental sulfur to sulfides. SRB are ubiquitous in almost all types of soil and

water bodies, and they are widely distributed in the marine environments. Sulfate-reducing bacteria and sulfate-reducing Archaea show extensive phylogenetic diversity, exhibiting six bacterial phyla namely Desulfobacterota (earlier known as Deltaproteobacteria), Bacillota (synonym: Firmicutes), Thermodesulfobacteriota, Actinobacteriota, Nitrospirota, and Caldiseicota and three Archaeal phyla viz: Euryarchaeota, Crenarchaeota, and Aigarchaeota. Among these, the phylum Desulfobacterota, which includes over 80 genera of SRB, is the largest group containing SRB (Song et al. 2021, Waite et al. 2020, Anantharaman et al. 2018). Analysis of the identified DsrAB-encoding genomes for genes involved in dissimilatory sulfur metabolism revealed that approximately half of the bacterial genomes were affiliated with the Desulfobacterota (35%), Bacillota (11%), Nitrospirota (5%), and Thermodesulfobacteriota (0.2%) phyla. These phyla collectively encompass all cultured and well-characterized bacterial SRB (Diao et al. 2023). In the current study of culturable SRB from the Curca and Agarwado salt pans of Goa, we identified genera from two different lineages: Thermodesulfobacteriota and Bacillota. In both the salt pans, nine different genera were found within the Thermodesulfobacteriota phylum.

In the Bacillota lineage, the Curca salt pan contained the genera *Halanaerobium* and *Desulfosporosinus*, while the Agarwado salt pan included *Halanaerobium*, *Desulfofarcimen*, and *Desulfosporosinus*. *Halanaerobium* found in both the salt pans under study, is an anaerobic sulfate-reducing bacteria that belongs to the family Halanaerobiaceae. Members of the Halanaerobiaceae family have been isolated from various saline environments, including the Dead Sea, marine salterns, cyanobacterial mats, and hypersaline lakes (Das et al. 2017, Abdeljabbar et al. 2013 and Ollivier et al. 1994). In the Ribandar salt pan, this genus was found in the crystallizer pond where the salinity was 300 PSU. Moreover, our study using next-generation sequencing identified *Halanaerobium* as the second most abundant genus in the sediments of the Agarwado salt pan, where the salinity of the sediment was 400 PSU (Gawas

and Kerkar, 2023). The genera *Desulfosporosinus* and *Desulfofarcimen* observed in our study are spore-forming, sulfate-reducing bacteria that have not been previously reported in hypersaline habitats. They have been isolated from soil, deep aquifers, piggery waste, and other environments (Panova et al. 2021, Vatsurina et al. 2008, and Ranchou-Peyruse et al. 2021, Spring et al. 2009).

In contrast, members of the Thermodesulfobacteriota are thermophilic, sulfate-reducing bacteria. Using culture-dependent techniques, some genera, including *Desulfococcus*, *Desulfosarcina*, *Desulfobacter*, and *Desulfovibrio*, were found to be unique to the Curca salt pans. These genera have also been reported in earlier studies from the Ribander salt pans of Goa (Das, 2018) and other hypersaline ecosystems (Fourçans et al., 2008; Caumette et al., 1991; Roychoudhury et al. 2013). However, a metagenomic approach revealed the presence of *Desulfosarcina*, *Desulfobacter*, and *Desulfovibrio* also in the salt pans of Agarwado and Nerul (Gawas and Kerkar, 2023). Notably, *Desulfococcus* was not detected in any of the salt pans studied using the culture-independent technique. Thus, both culturable and non-culturable techniques provide a comprehensive view of microbial ecosystems. Culturing allows the detection and study of certain genera that may be overlooked or under-represented in non-culturable approaches. Using both approaches, helps to overcome the limitations inherent in each method, offering a more complete picture of the microbial diversity. Further, the genera *Desulfopila*, *Halodesulfovibrio*, *Nitratidesulfovibrio*, and *Desulfonema*, isolated from the Agarwado salt pan, were found to be exclusive to Agarwado. Additionally, genera *Megalodesulfovibrio*, *Pseudodesulfovibrio*, *Desulfomicrobium*, *Desulfatiglans*, and *Desulfovermiculus* were also noted in our study in both Agarwado and Curca salt pans. These SRB have been reported from various locations worldwide, underscoring their widespread distribution and ecological significance (Roychoudhury et al. 2013, Cao et al. 2016, Da Silva et al. 2021, Marshall et al. 2019, Kjeldsen et al. 2007, Foti et al. 2007).

5.4 Taxonomic Diversity of SOB

Traditionally, hypersaline environments were thought to have low bacterial diversity, primarily inhabited by highly adapted Archaea. However, recent studies have revealed that these extreme habitats harbor complex microbial communities, including chemolithotrophic sulfur-oxidizing bacteria (Nosalova et al. 2022, Sorokin et al. 2006). These bacteria thrive in hypersaline conditions due to the significant energy yield from oxidizing reduced sulfur compounds. A relatively high cultivable diversity of halophilic SOB has been found in lakes in, Russia, China, Ukraine, Mongolia, and the Mediterranean Sea (Yang et al. 2013, Tourova et al. 2010, Yakimov et al. 2007, Nosalova et al. 2022). The cultivable SOB identified in this study belonged to three different classes: Gammaproteobacteria, Alphaproteobacteria, and Bacilli. The expression of key sulfur oxidation genes was reported to be dominant in Gammaproteobacteria, which comprises the greatest number of SOB (Baltar et al. 2023, Nosalova et al. 2022 and Sorokin et al. 2007). The genera *Alloalcanivorax*, *Vreelandella*, and *Chromohalobacter* spp. were identified under Gammaproteobacteria in both the Curca and Agarwado salt pans. So far, only one report has demonstrated the ability of sulfur oxidation by *Alloalcanivorax* from marine environments (Huang Honghui Gu Xiaoli Meng Xia Liao Xiuli, 2011). However, in-depth knowledge of which reduced sulfur compounds are utilized for energy production by *Alloalcanivorax* sp. is lacking. Under Alphaproteobacteria, a genus *Marinobacter* was identified in both the salt pans. In Curca salt pan, the genera *Paenibacillus*, *Priestia* (Synonyme: *Bacillus*), and *Cytobacillus* spp. were observed under Bacilli, while in the Agarwado salt pan, the genera *Paenibacillus*, *Pseudoalteromonas*, *Bacillus*, and *Vibrio* spp. were noted. The presence of these genera in salt pans of the states of Goa and Gujarat, India has been documented (Gawas and Kerkar 2023, Sen and Mukhopadhyay 2019) and they are potential oxidizers of sulfur compounds. Nosalova et al. (2022) demonstrated the ability of *Halomonas*, *Marinobacter*, and *Chromohalobacter*, isolated from Solivar salt brine, to oxidize reduced sulfur

compounds. Additionally, genera identified viz *Priestia*, *Paenibacillus*, *Cytobacillus*, *Chromohalobacter* and *Marinobacter* in our study have also been reported to have sulfur oxidation capabilities (Nosalova et al. 2022, Pérez-Ibarra et al. 2007, Shinde et al. 2022, Teske et al. 2000, Dou et al. 2022, Chen et al. 2023)

5.5 Biological Oxidation of Reduced Sulfur Compounds

In our study, we have evaluated the thiosulfate oxidation capability (using sodium thiosulfate as the substrate) of various genera identified, including *Alloalcanivorax* (A*1, AD-1-19a), *Paenibacillus* (BJCW/14bB), *Priestia* (DBPW/11), *Marinobacter* (AMPW/13a), and *Chromohalobacter* (BAugPW/21b, AJPS/7bB) spp. All tested genera were capable of oxidizing thiosulfate at a concentration of 30 mM [Sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$)] within 90 days. Notably, the genus *Alloalcanivorax*, particularly the A*1 isolate, showed the highest oxidation potential, oxidizing 90 % of 30 mM $\text{Na}_2\text{S}_2\text{O}_3$ and producing 24.6 mg/L of sulfate through biological oxidation (Figure 4.50 and 4.51). In comparison, thiosulfate oxidation by *Cytobacillus* and *Pseudomonas* species, as reported by Shinde et al. (2022), resulted in the production of 6.4 mM (614 mg/L) and 3.3 mM (317 mg/L) of sulfate, respectively. Although the sulfate production by A*1 isolate was lower than previously reported values, it still achieved 90 % substrate utilization. The low sulfate production could be due to the incomplete oxidation of thiosulfate into intermediates such as polythionates, sulfite, or elemental sulfur, which we could not measure since they were intermediary unstable compounds (Ahmad et al. 2015, Grabarczyk and Berks 2017, Zhang et al. 2020). Further, the same isolate exhibited thiosulfate oxidation at various sodium thiosulfate concentrations (30 mM - 160mM), and the activity was the highest at 30 mM of sodium thiosulfate (Figure 4.52). The thiosulfate oxidation potential of A*1 was also confirmed by the identification of the *SoxB* gene with a band size of approximately 1200 bp by polymerase chain reaction (PCR). Petri et al. (2001) investigated the phylogeny and distribution of the *soxB* gene among thiosulfate-oxidizing bacteria and observed

that PCR amplification typically produced a *soxB* fragment of approximately 1000 bp in most cases. Later, Meyer et al. (2007) suggested that the expected size of the *soxB* PCR product for most thiosulfate-oxidizing bacteria range between 800 and 1200 base pairs (bp).

Thiosulfate is utilized by most SOB as a supplementary energy source (Tourna et al. 2014, Kelly 1999). This stable sulfur compound, with an intermediate oxidation state [the two sulfur atoms of thiosulfate exist in the oxidation state of sulfate (+6) and sulfide (−2)], plays a crucial role in the natural sulfur cycle (Grabarczyk and Berks 2017, Mahmood et al. 2009;). The oxidation of thiosulfate to sulfate represents the oxidation of reduced sulfur compounds, including intermediates, and thus reflects the oxidation efficiency of the isolates (Petri et al. 2001). Consequently, thiosulfate oxidation was further used to screen practically feasible SOB strains for sulfide oxidation.

The A*1 isolate (*Alloalcanivorax*) demonstrated sulfide oxidation at concentrations of 100, 200, and 300 mg/L of NaHS [NaHS is a donor of sulfide (Huang et al. 2021)] within 120 days under aerobic conditions. The absence of detectable sulfide in the test, indicated complete oxidation or utilization of the substrate (sulfide) in 120 days. In contrast, the control which was assessed for chemical oxidation of sulfide showed a lower concentration of sulfide than initially added (Figure 4.54 A-C). Our study aligns with the similar observations of Bharathi et al. (1997), who also noted a decrease in sulfide concentration in controls. This is because sulfide added to the medium undergoes partial chemical oxidation even without the presence of culture. However, SOB can catalyze sulfide oxidation at rates significantly higher than chemical oxidation alone (Bharathi et al. 1997, Jørgensen et al. 2019).

Additionally, the product sulfate was observed in the test was 4-5 times higher as compared to the control at three concentrations 100, 200, and 300 mg/L of NaHS (Figure 4.54A-C). The presence of sulfide and lower sulfate in the control as compared to the test which contained no

detectable sulfide but high sulfate production, indicated the sulfide oxidation potential of the A*1 isolate under aerobic conditions. Sulfide oxidation under aerobic and anaerobic conditions has previously been demonstrated by other researchers as an effective sulfide oxidation (Vaiopoulou et al. 2005, De Gusseme 2009, Hou 2018, Peighami 2022). Thus, this report may encourage the use of SOB in sulfide stripping from polluted environments. These SOB isolates can thus serve as alternative options to chemical stripping methods to detoxify sulfide from the environment. Moreover, given their existence in nature, they could be easily employed for in-situ sulfide oxidation to further extrapolate the application of this potential SOB in marine as well as hypersaline environments (since SOB are halotolerant and can grow at various salinities ranging from 0-300 PSU). In a salt pan ecosystem, they play a synergistic role with the sulfate-reducing bacterial population where they complement the function of SRB (that converts sulfate to sulfide) and SOB convert this toxic sulfide concentration to sulfate, thus controlling the water and the sediment sulfide levels. Our study reveals their potential role of detoxifying the sulfide ions in the salt pan ecosystem.

Detoxification is a promising technology in today's world to overcome the effect of pollutants and to neutralize the harmful pollutants from contaminated sites using microorganisms. The aim of the present study was to assess the physicochemical properties of the salt pan ecosystem together with the sulfate-oxidizing bacteria and their relationship with sulfate-reducing bacteria. The bacterial species were isolated, characterized as per standard methods of cultivation, and using a metagenomic approach to establish their function and the dominant groups that coexist concerning their abundance in the salt pans of Goa. Our study reports the average concentration of sulfides to be 0.8-23 mg/L and the average sulfate concentration to be 0.8-10 mg/L in the salt pans of Goa. Considering these relatively stable concentrations of sulfide and sulfate in the salt pan ecosystem (the permissible limit for sulfates in water is 250mg/L (WHO 2011) and sulfide is up to 10 mg/L as reported by OSHA standards,(U.S), we have established certain SOB strain

isolated from Agarwado salt pans that can efficiently strip off 100-300mg/L of dissolved sulfide concentrations and we have established the dominant function of selected sulfur oxidizing bacteria which have a major role in environmental biotechnology.

5.6 Bacterial Diversity Analysis of Three Salt pan Sediments by a Metagenomic Approach

The sediment samples from three salt pan sites *viz.* Agarwado (AC), Curca (CC), and Nerul (NC) were analyzed for metagenomic analysis of bacterial diversity. For decades, these three distinct salterns have been conventionally used to produce local salt by traditional methods in Goa. The previous work which has been reported by our study has concentrated on two salt pans *viz.* Agarwado and Curca, however, we have included a third salt pan of Nerul which was over the years, a very productive salt pan and highly important salt pan concerning the highly consumed salt in Goa due to its nutritive value and taste. This aroused our curiosity to also include the salt pan of Nerul in the Metagenomic study. The temperature of 35 °C - 36 °C, recorded from these salt pans during the salt harvesting period favors the evaporation process in the crystallizer pond to achieve the salinity required for salt production. The salinity of the sediments in this salt pans was found to be in the range of 400 – 450 PSU and pH 7.6-8.25 indicating a halophilic and alkaline nature of the Goan salt pans.

5.6.1 Comparative Analysis of the Bacterial Community in the Salt pans

The bacterial composition of the sediment samples of the salt pans of Goa revealed that the salt pans were dominated by phylum Pseudomonadota. These results were parallel to the work reported by Gorrasi et al., (2021) in other solar salterns. Within the Pseudomonadota phylum in the AC salt pan, only two classes such as Gammaproteobacteria and Alphaproteobacteria were observed. In addition to these two classes, Zetaproteobacteria was identified in CC and NC. Interestingly the only genera found in our study within the Zetaproteobacteria class was

Mariprofundus. This class of bacteria is a model organism for microbial iron oxidation in marine environments (McAllister et al., 2019). The detection of genera *Mariprofundus* in CC and NC perhaps originated from the transport of iron ore due to mining activities in Goa through the Mandovi estuary which has led to an increase in the iron concentrations in this estuary. Secondly, the Cumbarjua canal interconnects the Mandovi and Zuari estuaries (adjacent to NC and CC respectively) and forms a network that has served as a major route for the transportation of iron ore. Additionally, the discharge of mining rejects from the mining sites has also concentrated iron particles in these estuaries (Gauns, 2011). This demonstrates how the distribution of microorganisms in a particular area is affected by their chemical composition. Similarly, other environmental parameters like salinity, nitrite, nitrate, sulfate, and sulfide also have an influence on bacterial distribution and abundance as depicted by CCA.

The dominant phyla observed in our study such as Pseudomonadota, Bacteriodota, Chloroflexota, etc. are found to have ubiquitous genetic machinery and survival weapons (Mehta et al., 2021). Hence, understanding the microbiome composition, evolving genetic machinery, and adaptive mechanisms evolved by these extremophiles make it important in biotechnology industries. The genera *Psychrobacter*, belonging to the family *Moraxellaceae*, was the most abundant genera and family, respectively, in AC with approximately 25.5 % relative abundance. Like our studies, the presence of *Psychrobacter* was also reported in the solar saltern of Bohai Bay, China, revealed by DGGE of the 16S rRNA gene (Zhang et al., 2016). The isolation of sulfidogenic bacteria *Halanaerobium*, reported from the Ribandar salt pan of Goa was observed to be the second abundant genera in AC (Das et al., 2017). The abundant genera observed in the CC and NC salt pans were *Sulfurivermis* and *Rhodopirellula* respectively. The presence of *Sulfurivermis* shows the occurrence of active sulfur cycles in the salt pan being a sulfur oxidizing bacterium (SOB) that uses nitrate as an electron acceptor in the rTCA pathway (Kojima et al., 2017).

5.6.2 Alpha and Beta Diversity Analysis of the Salt pans

The diversity of any ecosystem depends on two components “richness and evenness” (Soininen et al., 2012). In the Menhinicks richness index, an equal richness value was observed for NC and CC and was higher in comparison to AC, signifying a higher community richness in Nerul and Curca salt pans. Similarly, the Pielou’s evenness index for NC and CC was found to be higher as compared to AC, which indicates the distribution of an equal number of taxa in both salt pan (NC and CC) ecosystems as compared to the Agarwado salt pan. Further, dominance in AC was higher than NC and CC, demonstrating the presence of a more dominant taxon in the Agarwado salt pan community. Lower evenness and higher dominance observed in Agarwado indicate lower alpha diversity. However, in Nerul and Curca salt pans high evenness and lower dominance indicate high alpha diversity. A similar observation was shown by the Shannon diversity index which considers both the richness and evenness of a community. The alpha diversity measured by the Shannon index was $NC > CC > AC$.

Interestingly equal dissimilarity diversity (beta diversity) was observed between AC and NC and AC and CC, which further indicated that both CC and NC are quite comparable to each other but highly divergent to AC. The similarity between NC and CC was further confirmed by the presence of high number of common genera in both salt pans (represented in Figure 4.62) in addition to the beta diversity. This is due to the connection between the adjoining estuaries of Nerul and Curca salt pans, and therefore share a uniform environmental characteristic (Qasim and Sen Gupta, 1981) influencing the bacterial composition.

5.6.3 Bacterial Taxa in Ecological Roles

Pseudomonadota, Planctomycetota, and Chloroflexota observed in the three salt pans are representatives of the commonly occurring phyla that are involved in a variety of ecological roles. The sulfur cycle advances in tandem with the carbon cycle and is most prevalent in hypersaline environments. Using sulfate as an electron acceptor, the sulfate-reducing bacteria

break down organic molecules in an anoxic environment thereby cycling carbon anaerobically (Kerkar and Bharathi, 2007). In the current anticipated functional metagenome, genes involved in assimilatory and dissimilatory sulfate reduction and oxidation of sulfur compounds were found. The reductive nature of the salt pans sulfur cycle in the current work is indicated by the presence of the genera *Desulfovermiculus* and *Desulfatiglans* (Begmatov et al., 2021) in addition to genes such as adenylylsulfate reductase, subunits A (K00394) and B (K00395), dissimilatory sulfite reductase alpha subunit (K11180), dissimilatory sulfite reductase beta subunit (K11181), and sulfate adenylyltransferase (K00958). Likewise, different genes of the Sox system that are involved in sulfur oxidation: SoxA (K17222), SoxX (K17223), SoxB (K17224), SoxY (K17226), SoxZ (K17227), SoxC (K17225) and Sulfane dehydrogenase subunit were observed in the functional gene prediction (Figure 4.64 C). *Thiohalospira* is a genus of halophilic, chemolithoautotrophic SOB that utilizes sulfur compounds as electron donors (Sorokin et al., 2020); it has only been found in the Agarwado (AC) salt pan. Secondly, the SOB genus, *Sulfuriflexus* (class: *Gammaproteobacteria*) (Kojima and Fukui, 2016; Rana et al., 2020), was exclusive to Curca (CC) sediments. *Sphingobacterium* and *Woeseia* functional in carbon-fixing (Lohmann et al., 2020) were also observed in these salt pans along with numerous genes required in carbon metabolism (Figure 4.64 A). Unique to the Nerul (NC) salt pan, a group at genus level Cm1-21 (family: Nitrosococcaceae) was recently reported as ammonium oxidizers involved in the nitrification process of the nitrogen cycle (Semedo et al., 2021). Besides functional genes (Figures 4.64 A-C), bacterial genera like *Desulfovibrio* (observed in AC and NC sediment samples), *Pseudomonas* (AC and CC), *Nitrospina* (CC and NC), and *Nitrospira* (AC, CC, and NC) noted in our study are implicated in the carbon, nitrogen, and sulfur cycles (Megonigal et al., 2003; Meng et al., 2022; Sun et al., 2022), which illustrates that the movement of nutrients necessary to support the persistent life in these man-made salt pan ecosystems are governed by the presence of its indigenous microbes.

Heatmaps represent a potent tool for the visualization of microarray data. A comparative heatmap of the salt pans of Goa is seen in Figure 4.64 (A-C) which depicts the most dominant functional genes which may be attributed to the nutrient levels in these salt pans during the salt harvesting season when the samples were collected. The high nitrate and nitrite concentration in Agarwado reveals the presence of abundant nitrite reductase (NADH) small and large subunits and assimilatory nitrate reductase catalytic subunit.

The nutrient level of the crystallizer pond appears to be highly correlated with the indigenous bacteria population and the diversity of the bacterial population in different salt pans, may also correlate to adaptation to the changing nutrient levels due to the changes in the seawater ingress into the salt pans through the sea. Thus, the nutrient composition of seawater highly influences the composition of nutrients in the salt pans and in turn, influences the diverse bacterial population.

5.6.4 Significance of Hypersaline Bacterial Communities Inhabiting the Goan salt pans

Salinity is a key factor that governs the microbial community in hypersaline ecosystems such as solar salt pans. High salinity imposes stress on microbial cells, challenging their survival (Fei Xi et al., 2019, Song et al., 2022). Numerous genes associated with osmoprotective mechanisms, such as salt-in and salt-out mechanisms, membrane modification, protein adaptation, DNA repair, etc. acquired by residents of hypersaline environment for normal growth and development of cells were observed to be prevalent in our study. These genes include ABC-2 type transport system permease protein (K01992), ABC-2 type transport system ATP-binding protein (K01990), ATP-binding cassette, subfamily B, bacterial (K06147), 1-acyl-sn-glycerol-3-phosphate acyltransferase (K00655), 4-hydroxy-tetrahydrodipicolinate synthase

(K01714), and DNA helicase II / ATP-dependent DNA helicase PcrA (K03657) (Yu et al., 2018, Schmitz et al., 2020, Sleator and Hill, 2002).

Salt pans in Goa are influenced by three seasons: pre-monsoon, monsoon, and post-monsoon, where salinity ranges from 30-300 PSU (Mani et al., 2012). Therefore, bacteria with different salt tolerance like slightly, moderately, and extremely halophilic bacteria are expected to inhabit these ecosystems. In the present study, we have denoted these seasonal variations as three phases of the salt pan viz. salt-harvesting, ceased, and the preparatory phase. The sampling was carried out during the salt-harvesting phase along with the metagenomic analysis. Bacteria within the moderately halophilic group are very important as they occupy a large proportion of the halophiles. As adaptive measures, the production of compatible solutes and hydrolytic enzymes to acclimatize to salinity variations is attracting industries (Baati et al., 2010; Ramos-Cormenzana, 2020). For example, the moderate halophilic genus *Micrococcus* synthesizes catalase that plays a role in maintaining color and flavour in cured meats (García-López et al., 1999, Onishi and Kamekura, 1972). Secondly, *Rhodopirellula*, an abundant genus observed in NC sample is a moderate halophile and was reported to have a role as a food supplement to *Daphnia magna* (indicator used for testing toxicity of water) (da Conceição Marinho et al., 2019).

In our studies, we have isolated 8 different genera that have an immense role in detoxifying reduced sulfur compounds from aqueous impoundments, namely, *Alloalcanivorex*, *Chromohalobacter*, *Paenibacillus*, *Marinobacter*, *Priestia*, *Halomonas*, *Cytobacillus* and *Pseudoalteromonas*. Further exploring their specialized function in the detoxification of marine waters may be of utmost value in industrial wastewater treatment. Therefore, profound knowledge and discovery of halophilic bacteria in such an ecosystem will help us to disclose numerous properties, enzymes, and proteins of halophiles that could be of substantial use to various biotechnological industries.

Summary

1. Agarwado salt pan is in Pernem taluka adjoining the pristine Chapora estuary and Curca salt pans in Bardez taluka connected to Mandovi estuary influenced by anthropogenic factors were selected for the study.
2. Physicochemical parameters (salinity, temperature, conductivity, TDS, sulfate, sulfide, nitrite, and nitrate) in the two salt pans peaked during the salt-harvesting phase, except pH and DO, which were alkaline/highest during the preparatory phase.
3. The abundance of SOB and SRB in both water and sediment samples was of order 10^6 and 10^{4-5} respectively, whereas the heterotrophic count was 10^{6-7} in water and 10^9 in sediments in both salt pans.
4. A total of 26 (15 aerobic, 11 anaerobic) SOB isolates were isolated from the Curca and 27 (18 aerobic, 9 anaerobic) from the Agarwado salt pan.
5. All the anaerobic SOB isolates were found to be facultative anaerobes and were able to grow aerobically.
6. Twenty-five SRB were isolated from the Curca saltpan and 23 from the Agarwado salt pan.
7. All 26 SOB isolates from the Curca salt pan were able to grow in salinity ranges of 2.5 to 15 %. Among these, only 6 isolates (BDPW/12, BFPW/20, BJCW/15b, BJPS/7bA, BJCW/13b, DBPW/11) could grow in a salt-free medium, and only two isolates (BAugPW/19b, BMPS/6bB) could tolerate salinity from 2.5 to 40 %.
8. Out of the 27 isolates from the Agarwado salt pan, all could tolerate a salinity range of 2.5 to 7.5 % crude salt. Only 3 isolates (AN18-1/27, 4A, BJPS/16) could grow in a salt-free medium. None of the isolates from the Agarwado salt pan were able to grow at 40%.

9. Fifteen SOB from Curca belonged to the genera *Alloalcanivorax*, *Paenibacillus*, *Marinobacter*, *Cytobacillus*, *Vreelandella*, *Priestia* and *Chromohalobacter* spp.
10. From Agarwado salt pans, 16 were identified to be of genera *Alloalcanivorax*, *Paenibacillus*, *Marinobacter*, *Halomonas*, *Bacillus*, *Chromohalobacter*, *Pseudoalteromonas* and *Vibrio* spp.
11. Nineteen and 22 SRB isolates were identified by molecular approach from Curca and Agarwado salt pans respectively.
12. Genera identified in the Curca salt pan included *Desulfococcus*, *Desulfosarcina*, *Desulfobacter*, *Desulfovibrio*, *Halanaerobium*, *Megalodesulfovibrio*, *Pseudodesulfovibrio*, *Desulfomicrobium*, *Desulfosporosinus*, *Desulfatiglans* and *Desulfovermiculus* spp.
13. In Agarwado salt pan, some unique genera viz. *Desulfopila*, *Halodesulfovibrio*, *Desulfonema*, and *Nitratidesulfovibrio* were observed in addition to *Halanaerobium*, *Megalodesulfovibrio*, *Pseudodesulfovibrio*, *Desulfofarcimen*, *Desulfomicrobium*, *Desulfosporosinus*, *Desulfatiglans* and *Desulfovermiculus* spp.
14. Seven different SOB isolates: A*1, BJCW/14bB, DBPW/11, BAUGPW/21b, AMPW/13a, BJPS/7bB, AD-1-19a belonging to different genera were selected for screening their potential for thiosulfate oxidation.
15. Among the 7 isolates, A*1 showed the highest activity of thiosulfate oxidation at 30mM of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) with 79.39 ± 1.57 % of sulfate production and 90.9 % thiosulfate oxidation after incubation of 90 days, followed by DBPW/11, AD-1-19a, AJPS/7bB, BJPS/7bB, BJCW/14bB, BAUGPW/21b, and AMPW/13a.
16. Thiosulfate oxidation by A*1 (*Alloalcanivorax* sp.) was observed in all the concentrations tested (30-160mM) and was highest at 30 mM.

17. The A*1 could oxidize the sulfide (NaHS was used as substrate) of 100 mg/L, 200 mg/, and 300 mg/L. Whereas the oxidation was not observed at 400-1000 mg/L of sulfide.
18. A total of 95,299 reads were obtained from the Agarwado (AC) sediment samples, 104,637 from Curca (CC), and 240,563 from Nerul (NC).
19. The greatest alpha diversity was seen in Nerul salt pan 4.8, followed by Curca (4.3), and Agarwado (2.03). Beta diversity, was highest between Agarwado and the other two salt pans (0.73), namely Nerul and Curca. The lowest dissimilarity was recorded between Nerul and Curca salt pans (0.48).
20. Phylum Pseudomonadota dominated the salt pans of Nerul (13.9 %), Curca (19.6 %), and Agarwado (32.4 %). The predominant genera in the sediment samples of Nerul, Curca and Agarwado salt pans were *Rhodopirellula* (1.12%), *Sulfurivermis* (1.28 %), and *Psychrobacter* (25.5 %) respectively. Agarwado salt pan exhibited 125 unique genera, while Nerul had 119, and Curca showed 28 distinct genera.
21. Various functional genes found abundant were ABC-2 type transport system permease protein (K01992), 1-acyl-sn-glycerol-3-phosphate acyltransferase (K00655), 2-isopropylmalate synthase (K01649), 3-oxoacyl-[acyl-carrier protein] reductase (K00059), 4-hydroxy-tetrahydronicotinate synthase (K01714), ABC-2 type transport system ATP-binding protein (K01990). Other genes participating in diverse biological functions, such as cellular metabolism and adaptation to harsh environmental conditions, have been identified within the bacterial populations across all three salt pans.

Conclusion

- This study reveals the potential of sulfur oxidizing bacteria isolated from Goan salt pans in the oxidation of sulfide and thiosulfate, which is useful for stripping the sulfide concentration from water bodies.
- The research provides a comparative and comprehensive metagenomic insight into the bacterial diversity and community structure in three salt pans of Goa namely, Agarwado, Curca and Nerul.

Bibliography

1. Abdeljabbar, H., Cayol, J. L., Ben Hania, W., Boudabous, A., Sadfi, N., & Fardeau, M. L. (2013). *Halanaerobium sehlinense* sp. nov., an extremely halophilic, fermentative, strictly anaerobic bacterium from sediments of the hypersaline lake Sebkha. *International journal of systematic and evolutionary microbiology*, 63(Pt_6), 2069-2074.
2. Æsøy, A., Østerhus, S. W., & Bentzen, G. (2002). Controlled treatment with nitrate in sewers to prevent concrete corrosion. *Water Science and Technology: Water Supply*, 2(4), 137-144.
3. Ahmad, N., Ahmad, F., Khan, I., & Khan, A. D. (2015). Studies on the oxidative removal of sodium thiosulfate from aqueous solution. *Arabian Journal for Science and Engineering*, 40, 289-293.
4. Anantharaman, K., Hausmann, B., Jungbluth, S. P., Kantor, R. S., Lavy, A., Warren, L. A., ... & Banfield, J. F. (2018). Expanded diversity of microbial groups that shape the dissimilatory sulfur cycle. *The ISME journal*, 12(7), 1715-1728.
5. APHA (2012). Standard Methods for the Examination of Water and Waste Water. 22nd ed. American Public Health Association, American Water Work Association, Water Environment Federation.
6. ASCE (1989). Sulfide in Wastewater Collection and Treatment Systems. Manuals and Reports on Engineering Practice 69. American Society of Civil Engineers, New York.
7. Baars, J. K. (1930). Over sulfaatreductie door bacterien. PhD. Thesis, University of Delft.

8. Baati, H., Amdouni, R., Gharsallah, N., Sghir, A., & Ammar, E. (2010). Isolation and characterization of moderately halophilic bacteria from Tunisian solar saltern. *Current microbiology*, 60, 157-161. doi: 10.1007/s00284-009-9516-6.
9. Ballav, S., Kerkar, S., Thomas, S., & Augustine, N. (2015). Halophilic and halotolerant actinomycetes from a marine saltern of Goa, India producing anti-bacterial metabolites. *Journal of bioscience and bioengineering*, 119(3), 323-330.
10. Baltar, F., Martínez-Pérez, C., Amano, C., Vial, M., Robaina-Estévez, S., Reinthaler, T., ... & González, J. M. (2023). A ubiquitous gammaproteobacterial clade dominates expression of sulfur oxidation genes across the mesopelagic ocean. *Nature Microbiology*, 8(6), 1137-1148.
11. Bardischewsky F, Quentmeier A, Rother D, Hellwig P, Kostka S, Friedrich CG (2005) Sulfurdehydrogenase of *Paracoccus pantotrophus*: the heme-2 domain of the molybdoprotein cytochrome c complex is dispensable for catalytic activity. *Biochemistry* 44:7024–7034.
12. Begmatov, S., Savvichev, A. S., Kadnikov, V. v., Beletsky, A. v., Rusanov, I. I., Klyuvitkin, A. A., et al. (2021). Microbial Communities Involved in Methane, Sulfur, and Nitrogen Cycling in the Sediments of the Barents Sea. *Microorganisms* 9, 2362. doi: 10.3390/microorganisms9112362.
13. Beijerinck, M. W. (1895). Über *Spirillum desulfuricans* als ursache von sulfatreduktion. *Zentralblatt Bakteriologie*, 1, 1-9.
14. Bentzen, G., Smit, A. T., Bennett, D., Webster, N. J., Reinholt, F., Sletholt, E., & Hobsont, J. (1995). Controlled dosing of nitrate for prevention of H₂S in a sewer network and the effects on the subsequent treatment processes. *Water Science and Technology*, 31(7), 293-302.

15. Bernhard, A. E., Marshall, D., & Yiannos, L. (2012). Increased variability of microbial communities in restored salt marshes nearly 30 years after tidal flow restoration. *Estuaries and Coasts*, 35, 1049-1059.
16. Bharathi, L., & Chandramohan, D. (1990). Sulfate-Reducing Bacteria from the Arabian Sea—Their Distribution in Relation to Thiosulfate-Oxidizing and Heterotrophic Bacteria. *Bulletin of Marine Science*, 47(3), 622-630.
17. Bharathi, P. A., Nair, S., & Chandramohan, D. (1997). Anaerobic sulfide-oxidation in marine colorless sulfur-oxidizing bacteria. *Journal of Marine Biotechnology*, 5(2-3), 0172-0177.
18. Bharathi, P. L., Chadramohan, D., & Nair, S. (1988). A preliminary study of anaerobic thiosulfate-oxidizing bacteria as denitrifiers in the Arabian Sea. *Geomicrobiology journal*, 6(3-4), 195-207.
19. Bharathi, P. L. (2008). Sulfur cycle. *Encyclopedia of Ecology*. Elsevier, pp. 192–199 [https:// doi.org/10.1016/B978-0-444-63768-0.00761-7](https://doi.org/10.1016/B978-0-444-63768-0.00761-7).
20. Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., ... & Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature biotechnology*, 37(8), 852-857. doi: 10.1038/s41587-019-0209-9.
21. Boschker, H. T. S., Nold, S. C., Wellsbury, P., Bos, D., De Graaf, W., Pel, R., ... & Cappenberg, T. E. (1998). Direct linking of microbial populations to specific biogeochemical processes by ¹³C-labelling of biomarkers. *Nature*, 392(6678), 801-805.
22. Bratbak, G. (2018). Microscope methods for measuring bacterial biovolume: epifluorescence microscopy, scanning electron microscopy, and transmission electron

- microscopy. In *Handbook of methods in aquatic microbial ecology* (pp. 309-317). CRC Press.
23. Brunner, B., Yu, J. Y., Mielke, R. E., MacAskill, J. A., Madzunkov, S., McGenity, T. J., & Coleman, M. (2008). Different isotope and chemical patterns of pyrite oxidation related to lag and exponential growth phases of *Acidithiobacillus ferrooxidans* reveal a microbial growth strategy. *Earth and Planetary Science Letters*, 270(1-2), 63-72.
 24. Bugaytsova, Z., & Lindström, E. B. (2004). Localization, purification and properties of a tetrathionate hydrolase from *Acidithiobacillus caldus*. *European journal of biochemistry*, 271(2), 272-280.
 25. Burke, M. E., Gorham, E., & Pratt, D. C. (1974). Distribution of purple photosynthetic bacteria in wetland and woodland habitats of central and northern Minnesota. *Journal of Bacteriology*, 117(2), 826-833.
 26. Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods*, 13(7), 581-583. doi: 10.1038/nmeth.3869.
 27. Cao, J., Gayet, N., Zeng, X., Shao, Z., Jebbar, M., & Alain, K. (2016). *Pseudodesulfovibrio indicus* gen. nov., sp. nov., a piezophilic sulfate-reducing bacterium from the Indian Ocean and reclassification of four species of the genus *Desulfovibrio*. *International journal of systematic and evolutionary microbiology*, 66(10), 3904-3911.
 28. Castro, H. F., Williams, N. H., & Ogram, A. (2000). Phylogeny of sulfate-reducing bacteria. *FEMS Microbiology Ecology*, 31(1), 1-9.
 29. Cattaneo, C. R., Muñoz, R., Korshin, G. V., Naddeo, V., Belgiorno, V., & Zarra, T. (2023). Biological desulfurization of biogas: A comprehensive review on sulfide

- microbial metabolism and treatment biotechnologies. *Science of The Total Environment*, 164689.
30. Caumette, P., Cohen, Y., & Matheron, R. (1991). Isolation and characterization of *Desulfovibrio halophilus* sp. nov., a halophilic sulfate-reducing bacterium isolated from Solar Lake (Sinai). *Systematic and applied microbiology*, 14(1), 33-38.
 31. Cha, J. M., Cha, W. S., & Lee, J. H. (1999). Removal of organo-sulphur odour compounds by *Thiobacillus novellus* SRM, sulphur-oxidizing microorganisms. *Process Biochemistry*, 34(6-7), 659-665.
 32. Chen, L., Li, W., Zhao, Y., Zhang, S., Meng, L., & Zhou, Y. (2023). Characterization of sulfide oxidation and optimization of sulfate production by a thermophilic *Paenibacillus naphthalenovorans* LYH-3 isolated from sewage sludge composting. *Journal of Environmental Sciences*, 125, 712-722.
 33. Clesceri, L.S., (1998). Standard methods for examination of water and wastewater. American public health association, 9.
 34. Cline, J. D. (1969). Spectrophotometric determination of hydrogen sulfide in natural waters¹. *Limnol Oceanogr* 14, 454–458. doi: 10.4319/lo.1969.14.3.0454.
 35. da Conceição Marinho, M., Lage, O. M., Sousa, C. D., Catita, J., and Antunes, S. C. (2019). Assessment of *Rhodopirellula rubra* as a supplementary and nutritional food source to the microcrustacean *Daphnia magna*. *Antonie Van Leeuwenhoek* 112, 1231–1243. doi: 10.1007/s10482-019-01255-x.
 36. Da Silva, T. F., Gomes, S. B. C., Da Silva, F. S., Stojanović, K., Castro, R. N., Mendonça Filho, J. G., & Santos, M. (2021). Lipid composition of the microbial mat from a hypersaline environment (Vermelha Lagoon, Rio de Janeiro, Brazil). *Journal of Sedimentary Research*, 91(4), 349-361.

37. Dahl C, Friedrich RW (2008) Inorganic sulfur compounds as electron donors in purple sulfur bacteria, vol 168. Institut für Mikrobiologie Biotechnologie, Bonn, p D-53115.
38. Dahl, C. (2020). A biochemical view on the biological sulfur cycle. *Environmental technologies to treat sulfur pollution: principles and engineering*, 2, 55-96.
39. Das, K. R., Kerkar, S., Meena, Y., & Mishra, S. (2017). Effects of iron nanoparticles on iron-corroding bacteria. *3 Biotech*, 7, 1-9. doi: 10.1007/s13205-017-1018-9.
40. De Gusseme, B., De Schryver, P., De Cooman, M., Verbeken, K., Boeckx, P., Verstraete, W., & Boon, N. (2009). Nitrate-reducing, sulfide-oxidizing bacteria as microbial oxidants for rapid biological sulfide removal. *FEMS microbiology ecology*, 67(1), 151-161.
41. de Jong, G. A., Hazeu, W., Bos, P., & Kuenen, J. G. (1997). Polythionate degradation by tetrathionate hydrolase of *Thiobacillus ferrooxidans*. *Microbiology*, 143(2), 499-504.
42. Diao, M., Dykema, S., Koeksoy, E., Ngugi, D. K., Anantharaman, K., Loy, A., & Pester, M. (2023). Global diversity and inferred ecophysiology of microorganisms with the potential for dissimilatory sulfate/sulfite reduction. *FEMS Microbiology Reviews*, 47(5), fuad058.
43. Donati ER, & Sand W. (2007). *Microbial Processing of Metal Sulfides*. Dordrecht: Springer.
44. Dou, L., Zhang, M., Pan, L., Liu, L., & Su, Z. (2022). Sulfide removal characteristics, pathways and potential application of a novel chemolithotrophic sulfide-oxidizing strain, *Marinobacter* sp. SDSWS8. *Environmental Research*, 212, 113176.
45. Douglas, G.M., Maffei, V.J., Zaneveld, J.R. et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat Biotechnol* 38, 685–688. doi: 10.1038/s41587-020-0548-6.

46. Edition, F. (2011). Guidelines for drinking-water quality. WHO chronicle, 38(4), 104-8.
47. Einarsen, A. M., Æsøy, A., Rasmussen, A. I., Bungum, S., & Sveberg, M. (2000). Biological prevention and removal of hydrogen sulphide in sludge at Lillehammer wastewater treatment plant. *Water science and technology*, 41(6), 175-187.
48. Fei Xi, X., Wang, L., Jun Hu, J., Shu Tang, Y., Hu, Y., Hua Fu, X., et al. (2014). Salinity influence on soil microbial respiration rate of wetland in the Yangtze River estuary through changing microbial community. *Journal of Environmental Sciences* 26, 2562–2570. doi: 10.1016/j.jes.2014.07.016.
49. Fernandes, M. S., & Kerkar, S. (2017). Microorganisms as a source of tyrosinase inhibitors: a review. *Annals of Microbiology*, 67(4), 343-358.
50. Fernandes, S., Kerkar, S., D'Costa, A., Costa, M., Mishra, A., Shyama, S. K., & Das, K. R. (2021). Immuno-stimulatory effect and toxicology studies of salt pan bacteria as probiotics to combat shrimp diseases in aquaculture. *Fish & Shellfish Immunology*, 113, 69-78.
51. Fike, D. A., Bradley, A. S., & Leavitt, W. D. (2016). Geomicrobiology of sulfur. *Ehrlich's geomicrobiology*, 6, 635.
52. Finster, K. W., Kjeldsen, K. U., Kube, M., Reinhardt, R., Musmann, M., Amann, R., & Schreiber, L. (2013). Complete genome sequence of *Desulfocapsa sulfexigens*, a marine deltaproteobacterium specialized in disproportionating inorganic sulfur compounds. *Standards in genomic sciences*, 8, 58-68.
53. Fitriyani, N. L. L., Irianto, A., & Pramono, H. (2018). Potential of Chemolitotrophic Bacteria From Gold Mining Area in Sulfur Oxidation Process. *Biosaintifika: Journal of Biology & Biology Education*, 10(3), 526-532.

54. Foti, M., Sorokin, D. Y., Lomans, B., Mussman, M., Zacharova, E. E., Pimenov, N. V., ... & Muyzer, G. (2007). Diversity, activity, and abundance of sulfate-reducing bacteria in saline and hypersaline soda lakes. *Applied and environmental microbiology*, 73(7), 2093-2100.
55. Fourçans, A., Ranchou-Peyruse, A., Caumette, P., & Duran, R. (2008). Molecular analysis of the spatio-temporal distribution of sulfate-reducing bacteria (SRB) in Camargue (France) hypersaline microbial mat. *Microbial ecology*, 56, 90-100.
56. Friedrich, C. G., Rother, D., Bardischewsky, F., Quentmeier, A., & Fischer, J. (2001). Oxidation of reduced inorganic sulfur compounds by bacteria: emergence of a common mechanism?. *Applied and environmental microbiology*, 67(7), 2873-2882. <https://doi.org/10.1128/AEM.67.7.2873-2882.2001>.
57. García de Lomas, J., Corzo, A., Gonzalez, J. M., Andrades, J. A., Iglesias, E., & Montero, M. J. (2006). Nitrate promotes biological oxidation of sulfide in wastewaters: Experiment at plant-scale. *Biotechnology and bioengineering*, 93(4), 801-811.
58. Gauns, R. (2011). Mining havoc: Impact of mining on water resources in Goa - Article from Dams, Rivers and People.
59. Gawas, P., & Kerkar, S. (2023). Bacterial diversity and community structure of salt pans from Goa, India. *Frontiers in Microbiology*, 14, 1230929.
60. Godoi, A. F. L., Grasel, A. M., Polezer, G., Brown, A., Potgieter-Vermaak, S., Scremin, D. C., ... & Godoi, R. H. M. (2018). Human exposure to hydrogen sulphide concentrations near wastewater treatment plants. *Science of the total environment*, 610, 583-590.
61. Gorrasi, S., Franzetti, A., Ambrosini, R., Pittino, F., Pasqualetti, M., and Fenice, M. (2021). Spatio-Temporal Variation of the Bacterial Communities along a Salinity

- Gradient within a Thalassohaline Environment (Saline di Tarquinia Salterns, Italy). *Molecules* 26, 1338. doi: 10.3390/molecules26051338.
62. Grabarczyk, D. B., & Berks, B. C. (2017). Intermediates in the Sox sulfur oxidation pathway are bound to a sulfane conjugate of the carrier protein SoxYZ. *PLoS One*, 12(3), e0173395.
 63. Gram, C. (1884). Ueber die isolirte Färbung der Schizomyceten in Schnitt-und Trockenpräparaten. *Fortschritte der Medicin*, 2, 185-189.
 64. Grayston SJ, Wainwright M (1988) Sulphur oxidation by soil fungi including some species of mycorrhizae and wood-rotting basidiomycetes. *FEMS Microbiol Ecol* 4:1–8.
 65. Guerrero, L., Montalvo, S., Huiliñir, C., Campos, J.L., Barahona, A., Borja, R., 2016. Advances in the biological removal of sulphides from aqueous phase in anaerobic processes: a review. *Environ. Rev.* 24 (1), 84–100.
 66. Hammer, D. A. T., Ryan, P. D., Hammer, Ø., and Harper, D. A. T. (2001). Past: Paleontological Statistics Software Package for Education and Data Analysis. Available at: http://palaeo-electronica.orghttp://palaeo-electronica.org/2001_1/past/issue1_01.htm.
 67. Han Y, Ma X, Zhao W, Chang Y, Zhang X, Wang X, Wang J, & Huang Z. (2013). Sulfur-oxidizing bacteria dominate the microbial diversity shift during the pyrite and low-grade pyrolusite bioleaching process. *Journal of Bioscience and Bioengineering*, 116, 465-471.
 68. Hanson, N. W. (1973). Official, standardised and recommended methods of analysis.
 69. Hobson, J., & Yang, G. (2000). The ability of selected chemicals for suppressing odour development in rising mains. *Water science and technology*, 41(6), 165-173.

70. Hou, N., Xia, Y., Wang, X., Liu, H., Liu, H., & Xun, L. (2018). H₂S biotreatment with sulfide-oxidizing heterotrophic bacteria. *Biodegradation*, 29, 511-524.
71. Huang Honghui Gu Xiaoli Meng Xia Liao Xiuli (2011). Marine sulfur oxidizing alcanivorax bacterial strain HGMS16 (Homeotic Genic Male Sterile) and application thereof (Chinese patent No. CN102399720A).
72. Huang, D., Huo, J., & Liao, W. (2021). Hydrogen sulfide: Roles in plant abiotic stress response and crosstalk with other signals. *Plant Science*, 302, 110733.
73. Hutt, L. P. (2017). Taxonomy, physiology and biochemistry of the sulfur Bacteria (Doctoral dissertation, University of Plymouth).
74. Hydrogen sulfide, U.S. DEPARTMENT OF LABOR, Occupational Safety and Health Administration (OSHA). <https://www.osha.gov/hydrogen-sulfide/hazards>.
75. Janssen, A. J. H., Meijer, S., Bontsema, J., & Lettinga, G. (1998). Application of the redox potential for controlling a sulfide oxidizing bioreactor. *Biotechnology and bioengineering*, 60(2), 147-155.
76. Jeanthon, C., L'Haridon, S., Cueff, V., Banta, A., Reysenbach, A. L., & Prieur, D. (2002). *Thermodesulfobacterium hydrogenophilum* sp. nov., a thermophilic, chemolithoautotrophic, sulfate-reducing bacterium isolated from a deep-sea hydrothermal vent at Guaymas Basin, and emendation of the genus *Thermodesulfobacterium*. *International Journal of Systematic and Evolutionary Microbiology*, 52(3), 765-772.
77. Jenneman, G. E., Mcinerney, M. J., & Knapp, R. M. (1986). Effect of nitrate on biogenic sulfide production. *Applied and environmental microbiology*, 51(6), 1205-1211.
78. Jørgensen, B. B., & Nelson, D. C. (2004). Sulfide oxidation in marine sediments: geochemistry meets microbiology.

79. Jørgensen, B. B., & Kasten, S. (2006). Sulfur cycling and methane oxidation. In *Marine geochemistry* (pp. 271-309). Berlin, Heidelberg: Springer Berlin Heidelberg. doi: 10.1007/3-540-32144-6_8.
80. Jørgensen, B. B., Findlay, A. J., & Pellerin, A. (2019). The biogeochemical sulfur cycle of marine sediments. *Frontiers in microbiology*, 10, 436320.
81. Joshi MM, Hollis JP (1976) Rapid enrichment of Beggiatoa from soil. *J Appl Bacteriol* 40:223–224.
82. Kamaleson, A. S., Gonsalves, M. J., Kumar, S., Jineesh, V. K., & LokaBharathi, P. A. (2019b). Spatio-temporal variations in sulfur-oxidizing and sulfate-reducing bacterial activities during upwelling, off south-west coast of India. *Oceanologia*, 61(4), 427-444.
83. Kamat, T., & Kerkar, S. (2011). Pharmaceutical potentials of bacteria from salt pans of Goa.
84. Kelly, D. P. (1999). Thermodynamic aspects of energy conservation by chemolithotrophic sulfur bacteria in relation to the sulfur oxidation pathways. *Archives of Microbiology*, 171, 219-229.
85. Kelly, D. P. (1985). Physiology of the thiobacilli: elucidating the sulphur oxidation pathway. *Microbiological Sciences*, 2(4), 105-109.
86. Kelly, D. P., Shergill, J. K., Lu, W. P., & Wood, A. P. (1997). Oxidative metabolism of inorganic sulfur compounds by bacteria. *Antonie Van Leeuwenhoek*, 71, 95-107.
87. Kerkar Savita. (2003). Studies on bacteria of dissimilatory reproductive processes of the sulphur cycle from the salt pans of Goa. Ph.D. Thesis.
88. Kerkar, S., & Loka Bharathi, P. A. (2007). Stimulation of sulfate-reducing activity at salt-saturation in the salterns of Ribandar, Goa, India. *Geomicrobiology Journal*, 24(2), 101-110.

89. Kjeldsen, K. U., Loy, A., Jakobsen, T. F., Thomsen, T. R., Wagner, M., & Ingvorsen, K. (2007). Diversity of sulfate-reducing bacteria from an extreme hypersaline sediment, Great Salt Lake (Utah). *FEMS microbiology ecology*, 60(2), 287-298.
90. Klatt, J. M., & Polerecky, L. (2015). Assessment of the stoichiometry and efficiency of CO₂ fixation coupled to reduced sulfur oxidation. *Frontiers in Microbiology*, 6, 133345. <https://doi.org/10.3389/fmicb.2015.00484>.
91. Kodama, Y., & Watanabe, K. (2003). Isolation and characterization of a sulfur-oxidizing chemolithotroph growing on crude oil under anaerobic conditions. *Applied and Environmental Microbiology*, 69(1), 107-112.
92. Kojima, H., & Fukui, M. (2016). *Sulfuriflexus mobilis* gen. nov., sp. nov., a sulfur-oxidizing bacterium isolated from a brackish lake sediment. *International journal of systematic and evolutionary microbiology*, 66(9), 3515-3518. doi: 10.1099/ijsem.0.001227.
93. Kojima, H., Watanabe, M., & Fukui, M. (2017). *Sulfurivermis fontis* gen. nov., sp. nov., a sulfur-oxidizing autotroph, and proposal of Thioprofundaceae fam. nov. *International journal of systematic and evolutionary microbiology*, 67(9), 3458-3461. doi: 10.1099/ijsem.0.002137.
94. Krishnani, K. K., Kathiravan, V., Natarajan, M., Kailasam, M., & Pillai, S. M. (2010). Diversity of sulfur-oxidizing bacteria in greenwater system of coastal aquaculture. *Applied Biochemistry and Biotechnology*, 162, 1225-1237.
95. Kuenen, J. G., & Tuovinen, O. H. (1981). The genera *Thiobacillus* and *Thiomicrospira*. In *The prokaryotes: a handbook on habitats, isolation, and identification of bacteria* (pp. 1023-1036). Berlin, Heidelberg: Springer Berlin Heidelberg.
96. Kumar, U., Panneerselvam, P., Gupta, V. V., Manjunath, M., Priyadarshinee, P., Sahoo, A., ... & Annapurna, K. (2018). Diversity of sulfur-oxidizing and sulfur-reducing

- microbes in diverse ecosystems. *Advances in Soil Microbiology: Recent Trends and Future Prospects: Volume 1: Soil-Microbe Interaction*, 65-89.
97. Kushkevych, I., Cejnar, J., Tremel, J., Dordević, D., Kollar, P., & Vítězová, M. (2020). Recent advances in metabolic pathways of sulfate reduction in intestinal bacteria. *Cells*, 9(3), 698. doi: 10.3390/cells9030698.
 98. Kushkevych, I., Hýžová, B., Vítězová, M., & Rittmann, S. K. M. (2021). Microscopic methods for identification of sulfate-reducing bacteria from various habitats. *International Journal of Molecular Sciences*, 22(8), 4007.
 99. Kushkevych, I., Kovářová, A., Dordevic, D., Gaine, J., Kollar, P., Vítězová, M., & Rittmann, S. K. M. (2021). Distribution of sulfate-reducing bacteria in the environment: cryopreservation techniques and their potential storage application. *Processes*, 9(10), 1843.
 100. Larsen Ø, Lien T, Birkeland NK (2001) A novel organization of the dissimilatory sulfite reductase operon of *Thermodesulforhabdus norvegica* verified by RT-PCR. *FEMS Microbiol Lett* 203:81–85.
 101. Lebrero, R., Bouchy, L., Stuetz, R., Muñoz, R., 2011. Odour assessment and management in wastewater treatment plants: a review. *Environ. Sci. Technol.* 41, 915–950.
 102. Lee, H., Heo, Y. M., Kwon, S. L., Yoo, Y., Lee, A. H., Kwon, B. O., ... & Kim, J. J. (2020). Recovery of the benthic bacterial community in coastal abandoned saltern requires over 35 years: A comparative case study in the Yellow Sea. *Environment international*, 135, 105412.
 103. Lin, S., Mackey, H. R., Hao, T., Guo, G., Van Loosdrecht, M. C., & Chen, G. (2018). Biological sulfur oxidation in wastewater treatment: a review of emerging opportunities. *Water research*, 143, 399-415.

104. Lohmann, P., Benk, S., Gleixner, G., Potthast, K., Michalzik, B., Jehmlich, N., et al. (2020). Seasonal Patterns of Dominant Microbes Involved in Central Nutrient Cycles in the Subsurface. *Microorganisms* 8(11), 1694. doi: 10.3390/microorganisms8111694.
105. Loka Bharathi, P. A. (1989). The occurrence of denitrifying colourless sulphur-oxidising bacteria in marine waters and sediments as shown by the agar shake technique. *FEMS Microbiology Ecology*, 5(6), 335-342.
106. Luo, J., Tian, G., & Lin, W. (2013). Enrichment, isolation and identification of sulfur-oxidizing bacteria from sulfide removing bioreactor. *Journal of environmental sciences*, 25(7), 1393-1399.
107. Mahmood, Q., Zheng, P., Hu, B., Jilani, G., Azim, M. R., Wu, D., & Liu, D. (2009). Isolation and characterization of *Pseudomonas stutzeri* QZ1 from an anoxic sulfide-oxidizing bioreactor. *Anaerobe*, 15(4), 108-115. doi: 10.1016/j.anaerobe.2009.03.009.
108. Mander, G. J., Pierik, A. J., Huber, H., & Hedderich, R. (2004). Two distinct heterodisulfide reductase-like enzymes in the sulfate-reducing archaeon *Archaeoglobus profundus*. *European journal of biochemistry*, 271(6), 1106-1116.
109. Mani, K., Salgaonkar, B. B., Das, D., and Bragança, J. M. (2012). Community solar salt production in Goa, India. *Aquat Biosyst* 8, 30. doi: 10.1186/2046-9063-8-30.
110. Mani, K., Taib, N., Hugoni, M., Bronner, G., Bragança, J. M., & Debroas, D. (2020). Transient dynamics of archaea and bacteria in sediments and brine across a salinity gradient in a solar saltern of Goa, India. *Frontiers in microbiology*, 11, 514630.
111. Manikandan, P., & Senthilkumar, P. K. (2017). On overview of saltpan halophilic bacterium. *Antimicrob. Agents*, 3, 1-5.
112. Marshall, I. P., Ren, G., Jaussi, M., Lomstein, B. A., Jørgensen, B. B., Røy, H., & Kjeldsen, K. U. (2019). Environmental filtering determines family-level structure of

- sulfate-reducing microbial communities in subsurface marine sediments. *The ISME journal*, 13(8), 1920-1932.
113. McAllister, S. M., Moore, R. M., Gartman, A., Luther III, G. W., Emerson, D., & Chan, C. S. (2019). The Fe (II)-oxidizing Zetaproteobacteria: historical, ecological and genomic perspectives. *FEMS microbiology ecology*, 95(4), fiz015. doi: 10.1093/femsec/fiz015.
 114. Megonigal, J. P., Hines, M. E., and Visscher, P. T. (2003). “Anaerobic Metabolism: Linkages to Trace Gases and Aerobic Processes,” in *Treatise on Geochemistry* (Elsevier), 317–424. doi: 10.1016/B0-08-043751-6/08132-9.
 115. Mehta, P., Yadav, M., Ahmed, V., Goyal, K., Pandey, R., & Chauhan, N. S. (2021). Culture-independent exploration of the hypersaline ecosystem indicates the environment-specific microbiome evolution. *Frontiers in Microbiology*, 12, 686549. doi: 10.3389/fmicb.2021.686549.
 116. Meng, S., Peng, T., Liu, X., Wang, H., Huang, T., Gu, J.-D., et al. (2022). Ecological Role of Bacteria Involved in the Biogeochemical Cycles of Mangroves Based on Functional Genes Detected through GeoChip 5.0. *mSphere* 7. doi: 10.1128/msphere.00936-21.
 117. Meulenberg, R., Scheer, E. J., Pronk, J. T., Hazeu, W., Bos, P., & Gijs Kuenen, J. (1993). Metabolism of tetrathionate in *Thiobacillus acidophilus*. *FEMS microbiology letters*, 112(2), 167-172.
 118. Meyer, B., Imhoff, J. F., & Kuever, J. (2007). Molecular analysis of the distribution and phylogeny of the soxB gene among sulfur-oxidizing bacteria—evolution of the Sox sulfur oxidation enzyme system. *Environmental Microbiology*, 9(12), 2957-2977.
 119. Millano, E. F., & Sorber, C. A. (1986). Treatment of thiosulfate-containing wastewater in activated sludge systems. *Journal (Water Pollution Control Federation)*, 917-923.

120. Minz, D. et al. Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis of dissimilatory sulfite reductase genes. *Appl. Environ. Microbiol.* 65, 4666–4671 (1999).
121. Minz, D., Flax, J. L., Green, S. J., Muyzer, G., Cohen, Y., Wagner, M., ... & Stahl, D. A. (1999). Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis of dissimilatory sulfite reductase genes. *Applied and Environmental Microbiology*, 65(10), 4666-4671.
122. Muyzer, G., & Stams, A. J. (2008). The ecology and biotechnology of sulphate-reducing bacteria. *Nature reviews microbiology*, 6(6), 441-454.
123. Myhr, S., Lillebø, B. L., Sunde, E., Beeder, J., & Torsvik, T. (2002). Inhibition of microbial H₂S production in an oil reservoir model column by nitrate injection. *Applied microbiology and biotechnology*, 58, 400-408.
124. Nemati, M., Jenneman, G. E., & Voordouw, G. (2001). Impact of nitrate-mediated microbial control of souring in oil reservoirs on the extent of corrosion. *Biotechnology progress*, 17(5), 852-859.
125. Nemati, M., Jenneman, G. E., & Voordouw, G. (2001). Mechanistic study of microbial control of hydrogen sulfide production in oil reservoirs. *Biotechnology and bioengineering*, 74(5), 424-434.
126. Nielsen, A.H., Lens, P., Vollertsen, J., Hvitved-Jacobsen, T., 2005a. Sulfide–iron interactions in domestic wastewater from a gravity sewer. *Water Res.* 39, 2747–2755.
127. Nosalova, L., Piknova, M., Bonova, K., & Pristas, P. (2022). Deep subsurface hypersaline environment as a source of novel species of halophilic sulfur-oxidizing bacteria. *Microorganisms*, 10(5), 995.

128. Okabe, S., Ito, T., Satoh, H., & Watanabe, Y. (2003). Effect of nitrite and nitrate on biogenic sulfide production in sewer biofilms determined by the use of microelectrodes. *Water science and technology*, 47(11), 281-288.
129. Okabe, S., Itoh, T., Satoh, H., & Watanabe, Y. (1999). Analyses of spatial distributions of sulfate-reducing bacteria and their activity in aerobic wastewater biofilms. *Applied and Environmental Microbiology*, 65(11), 5107-5116.
130. Okabe, S., Santegoeds, C. M., & De Beer, D. (2003). Effect of nitrite and nitrate on in situ sulfide production in an activated sludge immobilized agar gel film as determined by use of microelectrodes. *Biotechnology and bioengineering*, 81(5), 570-577.
131. Okibe N, Gericke M, Hallaberg KB, & Johnson DB. (2003). Enumeration and characterization of acidophilic microorganisms isolated from a pilot plant stirred-tank bioleaching operation. *Applied and Environmental Microbiolog*, 69, 1936- 1943.
132. Ollivier, B., Caumette, P., Garcia, J. L., & Mah, R. A. (1994). Anaerobic bacteria from hypersaline environments. *Microbiological reviews*, 58(1), 27-38.
133. Onishi, H. and Kamekura, M., (1972). *Micrococcus halobius* sp. n. *International Journal of Systematic and Evolutionary Microbiology*, 22, 233-236. doi.org/10.1099/00207713-22-4-233.
134. Pachmayr F (1960) Vorkommen und Bestimmung von Schwefelverbindungen in Mineralwasser. PhD Thesis University Munich.
135. Padival, N. A., Weiss, J. S., & Arnold, R. G. (1995). Control of Thiobacillus by means of microbial competition: implications for corrosion of concrete sewers. *Water environment research*, 67(2), 201-205.
136. Panova, I. A., Ikkert, O., Avakyan, M. R., Kopitsyn, D. S., Mardanov, A. V., Pimenov, N. V., ... & Karnachuk, O. V. (2021). *Desulfosporosinus metallidurans* sp. nov., an acidophilic, metal-resistant sulfate-reducing bacterium from acid mine

- drainage. *International Journal of Systematic and Evolutionary Microbiology*, 71(7), 004876.
137. Peighami, R., Rasekh, B., Motamedian, E., Yazdian, F., & Khodaverdi, H. (2022). Investigating the ability of sulfur oxidizing strains in biodesulfurisation of sulfide-containing streams, screening the most capable strain and determining the optimum condition for elemental sulfur recovery from sulfide using response surface method. *Fuel*, 309, 121985.
 138. Pelletier, N., Leroy, G., Guiral, M., Giudici-Orticoni, M. T., & Aubert, C. (2008). First characterisation of the active oligomer form of sulfur oxygenase reductase from the bacterium *Aquifex aeolicus*. *Extremophiles*, 12, 205-215.
 139. Pepper, I. L., Gerba, C. P., & Gentry, T. J. (2015). Global emerging microbial issues in the Anthropocene Era. In *Environmental Microbiology* (pp. 677-688). Academic Press.
 140. Pérez-Ibarra, B. M., Flores, M. E., & García-Varela, M. (2007). Isolation and characterization of *Bacillus thioparus* sp. nov., chemolithoautotrophic, thiosulfate-oxidizing bacterium. *FEMS microbiology letters*, 271(2), 289-296.
 141. Petri, R., Podgorsek, L., & Imhoff, J. F. (2001). Phylogeny and distribution of the sox B gene among thiosulfate-oxidizing bacteria. *FEMS Microbiology Letters*, 197(2), 171-178. doi:10. 1016/ S0378- 1097(01) 00111-2.
 142. Pham, V. H., Yong, J. J., Park, S. J., Yoon, D. N., Chung, W. H., & Rhee, S. K. (2008). Molecular analysis of the diversity of the sulfide: quinone reductase (sqr) gene in sediment environments. *Microbiology*, 154(10), 3112-3121.
 143. Plumb, J. J., Haddad, C. M., Gibson, J. A., & Franzmann, P. D. (2007). *Acidianus sulfidivorans* sp. nov., an extremely acidophilic, thermophilic archaeon isolated from a solfatara on Lihir Island, Papua New Guinea, and emendation of the genus

- description. *International journal of systematic and evolutionary microbiology*, 57(7), 1418-1423.
144. Pokorna, D., & Zabranska, J. (2015). Sulfur-oxidizing bacteria in environmental technology. *Biotechnology Advances*, 33(6), 1246-1259.
 145. Pudi, A., Rezaei, M., Signorini, V., Andersson, M. P., Baschetti, M. G., & Mansouri, S. S. (2022). Hydrogen sulfide capture and removal technologies: A comprehensive review of recent developments and emerging trends. *Separation and Purification Technology*, 298, 121448. doi: 10.1016/j.seppur.2022.121448.
 146. Qasim, S. Z., & Gupta, R. S. (1981). Environmental characteristics of the Mandovi-Zuari estuarine system in Goa. *Estuarine, Coastal and Shelf Science*, 13(5), 557-578.
 147. Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., ... & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, 41(D1), D590-D596. doi: 10.1093/nar/gks1219.
 148. Cao, Q., Liu, X., Wang, Q., Gao, W., Xu, W., Xia, Y., ... & Liu, H. (2023). Heterotrophic bacteria dominate the sulfide oxidation process in coastal sediments. *Environmental Technology & Innovation*, 32, 103450. doi:10.1016/j.eti.2023.103450.
 149. Ramos-Cormenzana, A. (2020). “Ecology of Moderately Halophilic Bacteria,” in *The Biology of Halophilic Bacteria Edition* (CRC Press), 55–86.
 150. Rana, K., Rana, N., and Singh, B. (2020). “Applications of sulfur oxidizing bacteria,” in *Physiological and Biotechnological Aspects of Extremophiles* (Elsevier), 131–136. doi: 10.1016/B978-0-12-818322-9.00010-1.

151. Ranchou-Peyruse, M., Guignard, M., Casteran, F., Abadie, M., Defois, C., Peyret, P., ... & Ranchou-Peyruse, A. (2021). Microbial diversity under the influence of natural gas storage in a deep aquifer. *Frontiers in Microbiology*, 12, 688929.
152. Rathakrishnan, D., & Gopalan, A. K. (2022). Isolation and characterization of halophilic isolates from Indian salterns and their screening for production of hydrolytic enzymes. *Environmental Challenges*, 6, 100426.
153. Ravensschlag, K., Sahm, K., Knoblauch, C., Jørgensen, B. B., & Amann, R. (2000). Community structure, cellular rRNA content, and activity of sulfate-reducing bacteria in marine Arctic sediments. *Applied and Environmental Microbiology*, 66(8), 3592-3602.
154. Risatti, J. B., Capman, W. C., & Stahl, D. A. (1994). Community structure of a microbial mat: the phylogenetic dimension. *Proceedings of the National Academy of Sciences*, 91(21), 10173-10177.
155. Rodríguez-Gómez, L. E., Delgado, S., Álvarez, M., & Elmaleh, S. (2005). Inhibition of sulfide generation in a reclaimed wastewater pipe by nitrate dosage and denitrification kinetics. *Water Environment Research*, 77(2), 193-198.
156. Rojas-Avelizapa NG, Gomes-Ramirez M, Hernandez- Gama R, Aburto J, & Garcia de Leon R. (2013). Isolation and selection of sulphur-oxidizing bacteria for the treatment of sulfur-containing hazardous waste. *Chemical and Biochemical Engineering Quarterly*, 27, 109-117.
157. Roychoudhury, A. N., Cowan, D., Porter, D., & Valverde, A. (2013). Dissimilatory sulphate reduction in hypersaline coastal pans: an integrated microbiological and geochemical study. *Geobiology*, 11(3), 224-233.
158. Sahoo, K., & Dhal, N. K. (2009). Potential microbial diversity in mangrove ecosystems: a review. *Indian Journal of Marine Sciences*. 38:249–256.

159. Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution*, 4(4), 406-425.
160. Sam Kamaleson, A., Gonsalves, M. J., & Nazareth, D. R. (2019a). Interactions of sulfur and methane-oxidizing bacteria in tropical estuarine sediments. *Environmental monitoring and assessment*, 191, 1-18.
161. SamKamaleson, A., & Gonsalves, M. J. (2019). Role of sulfur-oxidizing bacteria on the ecology in tropical mangrove sediments. *Regional Studies in Marine Science*, 28, 100574.
162. Schmitz, R. A., Dietl, A., Müller, M., Berben, T., Op den Camp, H. J., & Barends, T. R. (2020). Structure of the 4-hydroxy-tetrahydrodipicolinate synthase from the thermoacidophilic methanotroph *Methylophilum thermophilum* SolV and the phylogeny of the aminotransferase pathway. *Acta Crystallographica Section F: Structural Biology Communications*, 76(5), 199-208. doi: 10.1107/S2053230X20005294.
163. Semedo, M., Lopes, E., Baptista, M. S., Oller-Ruiz, A., Gilabert, J., Tomasino, M. P., & Magalhães, C. (2021). Depth profile of nitrifying archaeal and bacterial communities in the remote oligotrophic waters of the North Pacific. *Frontiers in microbiology*, 12, 624071. doi: 10.3389/fmicb.2021.62407.
164. Sen, U., & Mukhopadhyay, S. K. (2019). Microbial community composition of saltern soils from Ramnagar, West Bengal, India. *Ecological Genetics and Genomics*, 12, 100040.
165. Shinde, A. H., Sharma, A., Doshi, S., Kumar, M. A., & Haldar, S. (2022). Isolation and screening of sulfur-oxidizing bacteria from coast of Bhavnagar, India, and formulation of consortium for bioremediation. *Environmental Science and Pollution Research*, 29(36), 54136-54149.

166. Sievert, S. M., Kiene, R. P., & Schulz-Vogt, H. N. (2007). The sulfur cycle. *Oceanography*, 20(2), 117-123.
167. Sleator, R. D., & Hill, C. (2002). Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. *FEMS microbiology reviews*, 26(1), 49-71.
168. Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M., Neal, P. R., ... & Herndl, G. J. (2006). Microbial diversity in the deep sea and the underexplored “rare biosphere”. *Proceedings of the National Academy of Sciences*, 103(32), 12115-12120.
169. Soininen, J., Passy, S., & Hillebrand, H. (2012). The relationship between species richness and evenness: a meta-analysis of studies across aquatic ecosystems. *Oecologia*, 169, 803-809. doi: 10.1007/s00442-011-2236-1.
170. Song, J., Hwang, J., Kang, I., & Cho, J. C. (2021). A sulfate-reducing bacterial genus, *Desulfosediminicola* gen. nov., comprising two novel species cultivated from tidal-flat sediments. *Scientific Reports*, 11(1), 19978.
171. Song, T., Liang, Q., Du, Z., Wang, X., Chen, G., Du, Z., et al. (2022). Salinity Gradient Controls Microbial Community Structure and Assembly in Coastal Solar Salterns. *Genes (Basel)* 13, 385. doi: 10.3390/genes13020385.
172. Sorokin, D. Y., Tourova, T. P., Galinski, E. A., Muyzer, G., & Kuenen, J. G. (2008). *Thiohalorhabdus denitrificans* gen. nov., sp. nov., an extremely halophilic, sulfur-oxidizing, deep-lineage gammaproteobacterium from hypersaline habitats. *International journal of systematic and evolutionary microbiology*, 58(12), 2890-2897.
173. Sorokin, D. Y., Merkel, A. Y., & Muyzer, G. (2015). *Thiohalospira*. *Bergey's Manual of Systematics of Archaea and Bacteria*, 1-7. . doi: 10.1002/9781118960608.gbm01694.

174. Sorokin, D. Y., Tourova, T. P., Braker, G., & Muyzer, G. (2007). *Thiohalomonas denitrificans* gen. nov., sp. nov. and *Thiohalomonas nitratreducens* sp. nov., novel obligately chemolithoautotrophic, moderately halophilic, thiodenitrifying Gammaproteobacteria from hypersaline habitats. *International journal of systematic and evolutionary microbiology*, 57(7), 1582-1589.
175. Sorokin, D. Y., Tourova, T. P., Lysenko, A. M., & Muyzer, G. (2006). Diversity of culturable halophilic sulfur-oxidizing bacteria in hypersaline habitats. *Microbiology*, 152(10), 3013-3023.
176. Spring, S., Lapidus, A., Schröder, M., Gleim, D., Sims, D., Meincke, L., ... & Han, C. (2009). Complete genome sequence of *Desulfotomaculum acetoxidans* type strain (5575 T). *Standards in genomic sciences*, 1, 242-252.
177. Stadnitskaia, A., Muyzer, G., Abbas, B., Coolen, M. J. L., Hopmans, E. C., Baas, M., ... & Damsté, J. S. (2005). Biomarker and 16S rDNA evidence for anaerobic oxidation of methane and related carbonate precipitation in deep-sea mud volcanoes of the Sorokin Trough, Black Sea. *Marine Geology*, 217(1-2), 67-96.
178. Steudel, R., & Chivers, T. (2019). The role of polysulfide dianions and radical anions in the chemical, physical and biological sciences, including sulfur-based batteries. *Chemical Society Reviews*, 48(12), 3279-3319.
179. Struk, M., Kushkevych, I., & Vítězová, M. (2020). Biogas upgrading methods: recent advancements and emerging technologies. *Reviews in Environmental Science and Bio/Technology*, 19(3), 651-671.
180. Sun, X., Kong, T., Li, F., Häggblom, M. M., Kolton, M., Lan, L., ... & Sun, W. (2022). *Desulfurivibrio* spp. mediate sulfur-oxidation coupled to Sb (V) reduction, a novel biogeochemical process. *The ISME Journal*, 16(6), 1547-1556. doi: 10.1038/s41396-022-01201-2.

181. Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, 30(12), 2725-2729.
182. Teske, A., Brinkhoff, T., Muyzer, G., Moser, D. P., Rethmeier, J., & Jannasch, H. W. (2000). Diversity of thiosulfate-oxidizing bacteria from marine sediments and hydrothermal vents. *Applied and Environmental Microbiology*, 66(8), 3125-3133.
183. Tomar, M., & Abdullah, T. H. (1994). Evaluation of chemicals to control the generation of malodorous hydrogen sulfide in waste water. *Water research*, 28(12), 2545-2552.
184. Tourna, M., Maclean, P., Condon, L., O'Callaghan, M., & Wakelin, S. A. (2014). Links between sulphur oxidation and sulphur-oxidising bacteria abundance and diversity in soil microcosms based on soxB functional gene analysis. *FEMS microbiology ecology*, 88(3), 538-549. doi:10.1111/1574-6941.12323.
185. Tourova, T. P., Kovaleva, O. L., Bumazhkin, B. K., Patutina, E. O., Kuznetsov, B. B., Bryantseva, I. A., ... & Sorokin, D. Y. (2011). Application of ribulose-1, 5-bisphosphate carboxylase/oxygenase genes as molecular markers for assessment of the diversity of autotrophic microbial communities inhabiting the upper sediment horizons of the saline and soda lakes of the Kulunda Steppe. *Microbiology*, 80, 812-825.
186. Tourova, T. P., Kovaleva, O. L., Sorokin, D. Y., & Muyzer, G. (2010). Ribulose-1, 5-bisphosphate carboxylase/oxygenase genes as a functional marker for chemolithoautotrophic halophilic sulfur-oxidizing bacteria in hypersaline habitats. *Microbiology*, 156(7), 2016-2025.
187. US Environmental Protection Agency, 1991. Hydrogen sulfide corrosion in wastewater collection and treatment system. Technical Report, 430/09-91-010.

188. US Environmental Protection Agency, 1992. Detection, Control, and Correction of Hydrogen Sulfide Corrosion in Existing Wastewater System. Office of Wastewater Enforcement and Compliance, Washington, DC, 20460.
189. Vaiopoulou, E., Melidis, P., & Aivasidis, A. (2005). Sulfide removal in wastewater from petrochemical industries by autotrophic denitrification. *Water Research*, 39(17), 4101-4109.
190. Valiente, N., Carrey, R., Otero, N., Gutiérrez-Villanueva, M. A., Soler, A., Sanz, D., ... & Gómez-Alday, J. J. (2017). Tracing sulfate recycling in the hypersaline Pétrola Lake (SE Spain): A combined isotopic and microbiological approach. *Chemical Geology*, 473, 74-89.
191. Van Delden, A. (1903). Beitrag zur Kenntnis der Sulfatreduktion durch bakterien. *Zentralbl. Bakteriologie. Parasitenkd. Infektionskr. Hyg. Abt. II*, 11, 81-94.
192. Vatsurina, A., Badrutdinova, D., Schumann, P., Spring, S., & Vainshtein, M. (2008). *Desulfosporosinus hippei* sp. nov., a mesophilic sulfate-reducing bacterium isolated from permafrost. *International journal of systematic and evolutionary microbiology*, 58(5), 1228-1232.
193. Ventosa, A., Nieto, J. J., & Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. *Microbiology and molecular biology reviews*, 62(2), 504-544. doi: 10.1128/MMBR.62.2.504-544.1998.
194. von Wolzogen Kühr, C. A. H., & Van der Vlugt, L. S. (1934). The graphitization of cast iron as an electrobiochemical process in anaerobic soil. *Water*, 18(16), 147-165.
195. Voordouw, G., Armstrong, S. M., Reimer, M. F., Fouts, B., Telang, A. J., Shen, Y. I. N., & Gevertz, D. (1996). Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and

- sulfide-oxidizing bacteria. *Applied and Environmental Microbiology*, 62(5), 1623-1629.
196. Wainwright M (1984) Sulfur oxidation in soils. *Adv Agron* 37:349–396.
 197. Waite, D. W., Chuvochina, M., Pelikan, C., Parks, D. H., Yilmaz, P., Wagner, M., ... & Hugenholtz, P. (2020). Proposal to reclassify the proteobacterial classes Deltaproteobacteria and Oligoflexia, and the phylum Thermodesulfobacteria into four phyla reflecting major functional capabilities. *International Journal of Systematic and Evolutionary Microbiology*, 70(11), 5972-6016.
 198. Wakai, S., Kikumoto, M., Kanao, T., & Kamimura, K. (2004). Involvement of sulfide: quinone oxidoreductase in sulfur oxidation of an acidophilic iron-oxidizing bacterium, *Acidithiobacillus ferrooxidans* NASF-1. *Bioscience, biotechnology, and biochemistry*, 68(12), 2519-2528.
 199. Waltrip, G. D., & Snyder, E. G. (1985). Elimination of odor at six major wastewater treatment plants. *Journal (Water Pollution Control Federation)*, 1027-1032.
 200. Wang, Q., Zhou, X., Su, H., Zhang, M., Li, Z., & Wu, T. (2022). Accelerated sulfate reducing bacteria corrosion of X80 pipeline steel welded joints under organic carbon source starvation. *npj Materials Degradation*, 6(1), 82.
 201. Webster, G., Watt, L. C., Rinna, J., Fry, J. C., Evershed, R. P., Parkes, R. J., & Weightman, A. J. (2006). A comparison of stable-isotope probing of DNA and phospholipid fatty acids to study prokaryotic functional diversity in sulfate-reducing marine sediment enrichment slurries. *Environmental Microbiology*, 8(9), 1575-1589.
 202. Whittaker, R. H. (1960). Vegetation of the Siskiyou mountains, Oregon and California. *Ecological monographs*, 30(3), 279-338. doi: 10.2307/1943563.
 203. WHO, World Health Organization, (2000). Air Quality Guideline for Europe. Ed 2. Regional Publications, European Series, No. 91, Copenhagen.

204. Wodara, C., Bardischewsky, F., & Friedrich, C. G. (1997). Cloning and characterization of sulfite dehydrogenase, two c-type cytochromes, and a flavoprotein of *Paracoccus denitrificans* GB17: essential role of sulfite dehydrogenase in lithotrophic sulfur oxidation. *Journal of bacteriology*, 179(16), 5014-5023.
205. Wu, Q. L., Zwart, G., Schauer, M., Kamst-van Agterveld, M. P., & Hahn, M. W. (2006). Bacterioplankton community composition along a salinity gradient of sixteen high-mountain lakes located on the Tibetan Plateau, China. *Applied and Environmental Microbiology*, 72(8), 5478-5485.
206. Xie, S., Wang, S., Li, D., Shao, Z., Lai, Q., Wang, Y., ... & Jiang, L. (2021). *Sulfurovum indicum* sp. nov., a novel hydrogen-and sulfur-oxidizing chemolithoautotroph isolated from a deep-sea hydrothermal plume in the Northwestern Indian Ocean. *International Journal of Systematic and Evolutionary Microbiology*, 71(3), 004748. doi: 10.1099/ijsem.0.004748.
207. Yakimov, M. M., La Cono, V., Denaro, R., D'auria, G., Decembrini, F., Timmis, K. N., ... & Giuliano, L. (2007). Primary producing prokaryotic communities of brine, interface and seawater above the halocline of deep anoxic lake L'Atalante, Eastern Mediterranean Sea. *The ISME journal*, 1(8), 743-755.
208. Yalamanchili, C., & Smith, M. D. (2008). Acute hydrogen sulfide toxicity due to sewer gas exposure. *The American Journal of Emergency Medicine*, 26(4), 518-e5.
209. Yang, J., Jiang, H., Dong, H., Wu, G., Hou, W., Zhao, W., ... & Lai, Z. (2013). Abundance and diversity of sulfur-oxidizing bacteria along a salinity gradient in four Qinghai-Tibetan lakes, China. *Geomicrobiology Journal*, 30(9), 851-860.
210. Yang, W., Vollertsen, J., & Hvitved-Jacobsen, T. (2005). Anoxic sulfide oxidation in wastewater of sewer networks. *Water science and technology*, 52(3), 191-199.

211. Yousuf, B., Kumar, R., Mishra, A., & Jha, B. (2014). Unravelling the carbon and sulphur metabolism in coastal soil ecosystems using comparative cultivation-independent genome-level characterisation of microbial communities. *PLoS One*, 9(9), e107025.
212. Yu, J., Loh, K., Song, Z. Y., Yang, H. Q., Zhang, Y., & Lin, S. (2018). Update on glycerol-3-phosphate acyltransferases: the roles in the development of insulin resistance. *Nutrition & diabetes*, 8(1), 34. doi: 10.1038/s41387-018-0045-x.
213. Zhang, J., Liu, R., Xi, S., Cai, R., Zhang, X., & Sun, C. (2020). A novel bacterial thiosulfate oxidation pathway provides a new clue about the formation of zero-valent sulfur in deep sea. *The ISME Journal*, 14(9), 2261-2274.
214. Zhang, J., Ma, G., Deng, Y., Dong, J., Van Stappen, G., & Sui, L. (2016). Bacterial diversity in Bohai Bay solar saltworks, China. *Current microbiology*, 72, 55-63. doi: 10.1007/s00284-015-0916-5.
215. Zhang, L., De Schryver, P., De Gussemé, B., De Muynck, W., Boon, N., & Verstraete, W. (2008). Chemical and biological technologies for hydrogen sulfide emission control in sewer systems: a review. *Water research*, 42(1-2), 1-12.
216. Zhou, L., Ou, P., Zhao, B., Zhang, W., Yu, K., Xie, K., & Zhuang, W. Q. (2021). Assimilatory and dissimilatory sulfate reduction in the bacterial diversity of biofoulant from a full-scale biofilm-membrane bioreactor for textile wastewater treatment. *Science of The Total Environment*, 772, 145464

Appendix

APPENDIX I MEDIA COMPOSITION

Lieske's media

Ingredients	g/L
NaCl	31 g
Na ₂ S ₂ O ₃ 5H ₂ O	5 g
KNO ₃	5g
NaOH CO ₃	1 g (filter sterilize auto separately)
NaOH PO ₄	0.2 g
MgCl ₂ .6H ₂ O	0.1 g
NH ₄ Cl	2g
CaCl ₂ 6H ₂ O	10 mg
FeCl ₃ 6H ₂ O	10 mg
NaHS	0.5 mL in 200 mL

Lieske's Media: last minute additions (200 ml):

2 ml of NaHCO₃ (1.5 g/15 ml Distilled water) autoclave separately

0.05 ml of vitamin solution 2 drops

NaHS solution of desired ppm just before distribution.

Vitamin Solution	Litre
Calcium Pantothenate	100 mg
Pyridoxine HCl	40 mg
Thiamine HCl	20 mg
Riboflavin	20 mg
Nicotinamide	20 mg
Biotin	0.001
Calcium Pantothenate	100 mg
Pyridoxine HCl	40 mg
Thiamine HCl	20 mg

Hatchikian's Media

Ingredients	g/L
NH ₄ Cl	2 g
NaCl	62 g
K ₂ HPO ₄	0.2 g
Yeast Extract	1 g
Sea Water	1000 mL
Trace Elements	5 mL
Agar (for solid media)	0.8%

Trace metal	g/L
ZnSO ₄ 7H ₂ O	10 mg
MnCl ₂ 4H ₂ O	3 mg
H ₃ BO ₃	30 mg
CoCl ₂ 6H ₂ O	20 mg
CuCl ₂ 2H ₂ O	1 mg
NiCl ₂ 6H ₂ O	2 mg
Na ₂ MoO ₄ 2H ₂ O	3 mg
CaCl ₂ 2H ₂ O	20 mg
D/W	500 mL

liquid media additions:

Ingredients	g/L
FeSO ₄ . 7H ₂ O	20 mg/50 ml distilled water acidified with 1 drop of H ₂ SO ₄ (1 ml to be added to 200 ml media)
Na ₂ S.9H ₂ O	50% stock made in 10 ml test tubes (0.5 ml to be added to 200 ml of media)

Solid media additions:

Ingredients	g/L	Volume
FeSO ₄	10% solution	1 ml/200 ml
Na–thioglycolate	0.6 g/10 ml	2 ml/200 ml
NaOH	0.5 N	0.8 ml/200 ml

Sealer:

Ingredients	g/L
Wax – paraffin	100 ml melted
Paraffin oil	200 ml

Cabon Source added:

Ingredients	g/L
Sodium Acetate	20 g + 100 ml Distilled water
Sodium lactate	1 ml added to 100 ml media
Propionic acid	7 g + 100 ml Distilled water
Benzoic acid	5 g + 100 ml Distilled water
Ethanol	1 ml added to 100 ml media
Formate (Formic acid)	1 ml to 100 ml media

Nutrient Broth (NB) Hi-Media M002

Ingredients	g/L
Peptone	5.0
Sodium chloride	5.0
HM Peptone B	1.5
Yeast Extract	1.5
Final pH (at 25°C)	7.4±0.2

Nutrient Agar (NA) Hi-Media M001

Ingredients	g/L
Peptone	5.0
Sodium chloride	5.0
HM Peptone B	1.5
Yeast Extract	1.5
Agar	15.0
Final pH (at 25°C)	7.4±0.2

SIM Media Hi-Media M181

Ingredients	g/L
HM Peptone B #	3.000
Peptone	30.000
Peptonized iron	0.200
Sodium thiosulphate	0.025
Agar	3.000
Final pH (at 25°C)	7.3±0.2

Gelatin media

Ingredients	g/L
Peptone	5
Beef Extract	3
Gelatin	120
Final pH (at 25°C)	6.8±0.2

Anerobic Agar Hi-Media M228

Ingredients	g/L
Tryptone	20.000
Dextrose (Glucose)	10.000
Sodium chloride	5.000
Sodium thioglycollate	2.000
Sodium formaldehyde Sulfoxylate	1.000
Methylene blue	0.002
Agar	20.000

APPENDIX II

REAGENTS

Conditioner Solution: Dissolved 75 g NaCl in 300 mL of d/w to which is added 100 mL of 95 % ethanol or isopropyl alcohol. To this add 30 mL concentrated HCl and 50 mL of glycerine. Filter the solution if turbid.

Ferric Ammonium Sulphate: 50 g iron III ammonium sulfate, $(\text{NH}_4)\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in a 500 ml volumetric flask add 10 ml concentrated H_2SO_4 and fill up to 500 ml with d/w.

N, N-Dimethyl-p-phenylenediamine dihydrochloride: 2g dimethyl para-phenylenediamine sulfate in 1-liter volumetric flask. Add 200 mL d/w and then slowly add 200 mL concentrated H_2SO_4 (sp. wt. 83), place in ice box after cooling to room temperature fill up to 1 liter with d/w.

Sulfanilamide: 1g of crystalline sulphanilamide is dissolved in 10 mL of concentrated HCl and 60 mL of distilled water after cooling. The solution is made up to 100 mL.

N-(1-naphthyl) ethylene diamine hydrochloride (NEDE):

0.1g is dissolved in 50mL of distilled water and make up the volume to 100mL. Store in amber colored bottle in the refrigerator

Concentrated NH_4Cl buffer solution: 10g in 1 Litre, make pH to 8.5.

When column is unused it is soak in NH_4Cl buffer to maintain an alkaline solution.

1X Phosphate buffered saline (PBS)

Ingredients	g/L
Sodium chloride	8.00
Potassium chloride	0.20
Disodium hydrogen phosphate	1.44
Potassium dihydrogen phosphate	0.24
Final pH (at 25°C)	7.4±0.2

Kovac's Reagent Hi-Media R008

p-dimethylamino benzaldehyde	5.0 g
Amyl alcohol	75 mL
Hydrochloric acid, concentrated	25 mL

Barrit's Reagent A Hi-Media R029

α -Naphthol (1-Naphthol)	5.0 g
Absolute ethanol	100 mL

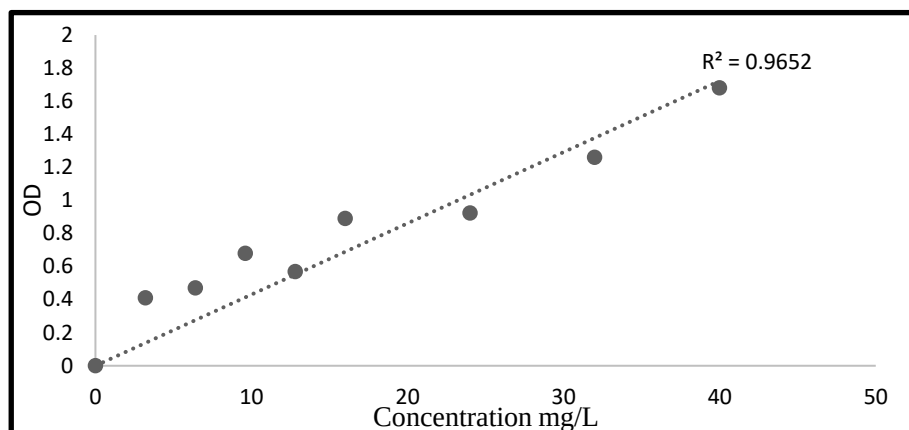
Barrit's Reagent B Hi-Media R030

Potassium hydroxide	40.0 g
DW	100

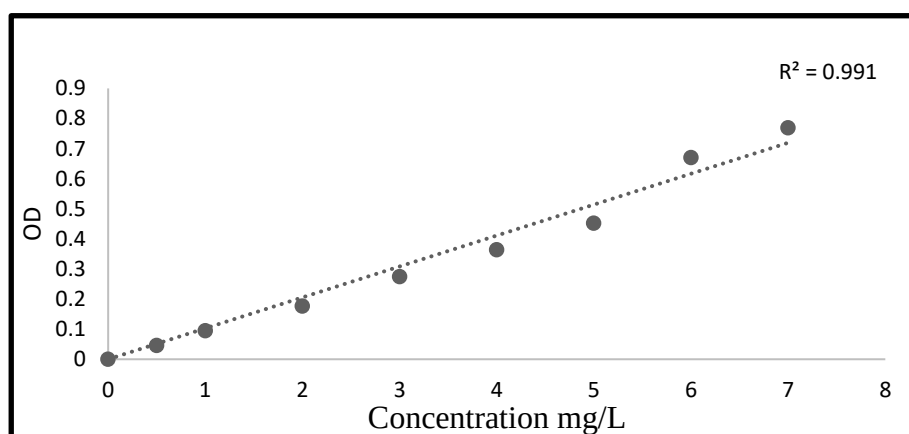
APPENDIX III

STANDARD GRAPHS

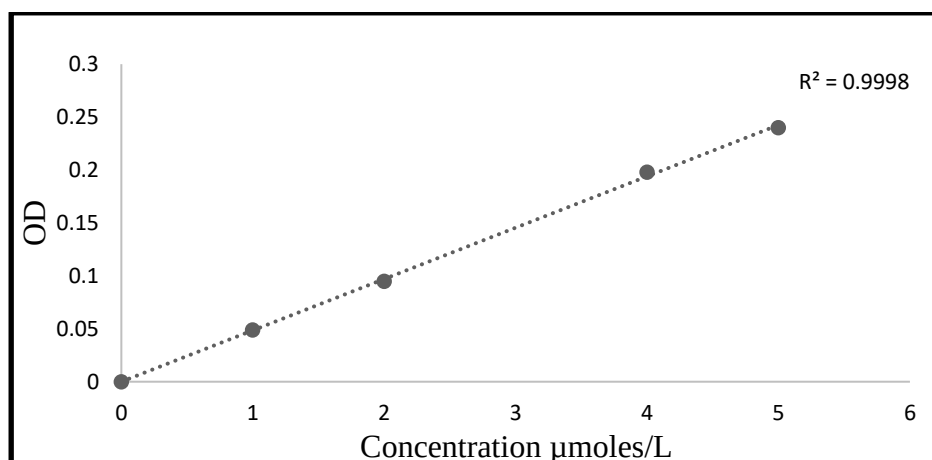
Ammonium Sulfate standard graph for sulfate estimation



Sodium HydroSulfide standard graph for sulfide estimation



Sodium nitrite standard graph for nitrite estimation



Correlation matrix of Curca Water

	Aerobic SOB	Anerobic SOB	SRB	Heterotrophic Counts	Sulfate	Sulfide	Nitrate	Nitrite
Aerobic SOB	1							
Anerobic SOB	-0.049	1						
SRB	-0.128	0.041	1					
Heterotrophic	-0.214	-0.331	.508**	1				
Sulfate	-.455*	0.169	.463*	.549**	1			
Sulfide	-.482*	-0.049	-0.367	0.184	.455*	1		
Nitrate	-.687**	0.184	0.29	0.368	.764**	.682**	1	
Nitrite	-.592**	-0.157	-0.059	.485*	.599**	.749**	.653**	1

Correlation matrix of Curca Sediment

	Aerobic SOB	Anerobic SOB	SRB	Heterotrophic Counts	Sulfate	Sulfide	Nitrate	Nitrite
Aerobic SOB	1							
Anerobic SOB	.647**	1						
SRB	-0.056	0.244	1					
Heterotrophic	-0.043	0.325	.407*	1				
Sulfate	0.19	0.16	0.135	-0.302	1			
Sulfide	0.247	0.153	0.182	-0.219	.961**	1		
Nitrate	.412*	.517**	0.139	0.152	.779**	.807**	1	
Nitrite	.497**	0.174	-0.016	0.048	.410*	.535**	.600**	1

Correlation matrix of Agarwado Water

	Aerobic SOB	Anerobic SOB	SRB	Heterotrophic Counts	Sulfate	Sulfide	Nitrate	Nitrite
Aerobic SOB	1							
Anerobic SOB	0.182	1						
SRB	0.213	-0.079	1					
Heterotrophic	-0.246	-.507**	0.165	1				
Sulfate	-0.248	-0.352	.405*	.710**	1			
Sulfide	-.393*	-0.091	.435*	.471*	.876**	1		
Nitrate	-0.295	-.588**	0.272	.651**	.752**	.497**	1	
Nitrite	-.438*	-0.169	-.448*	.420*	.575**	.442*	.570**	1

Correlation matrix of Agarwado Sediment

	Aerobic SOB	Anerobic SOB	SRB	Heterotrophic Counts	Sulfate	Sulfide	Nitrate	Nitrite
Aerobic SOB	1							
Anerobic SOB	0.37	1						
SRB	0.097	-0.181	1					
Heterotrophic	0.355	.448*	-0.091	1				
Sulfate	-0.251	-0.019	.479*	-.548**	1			
Sulfide	-0.001	-0.065	.599**	-.424*	.746**	1		
Nitrate	-0.121	0.349	0.269	0.051	.582**	.564**	1	
Nitrite	0.108	.481*	0.21	.646**	-0.03	0.001	.508**	1

*. Correlation is significant at the 0.05 level.

**. Correlation is significant at the 0.01 level.

Publications

Manuscripts published:

Gawas P and Kerkar S (2023) Bacterial diversity and community structure of salt pans from Goa, India. *Front. Microbiol.* 14:1230929. doi: 10.3389/fmicb.2023.1230929.

Abstracts published in International and National conferences:

Presented a poster on “Oxidation of toxic hydrogen sulfide by sulfur-oxidizing bacteria from the marine salterns of Goa” at 6th Annual International Conference on IP Global Trends in IPR: Patenting & Beyond Dec. 1-2, 2021, organised by the Goa College of Pharmacy, Panjim, Goa India.

Presented a poster on “Salt pan TDLO as scavengers of environmental sulfide” at National Conference on Harnessing Microbial Wealth For Start-Ups In Life Sciences organized by Department of Microbiology, Savitribai Phule Pune University in collaboration with Association of Microbiologists of India, Pune Unit on 29-31 January 2020.



OPEN ACCESS

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RECEIVED 29 May 2023

ACCEPTED 17 November 2023

PUBLISHED 04 December 2023

CITATION

Gawas P and Kerkar S (2023) Bacterial diversity
and community structure of salt pans from
Goa, India.
Front. Microbiol. 14:1230929.
doi: 10.3389/fmicb.2023.1230929

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Bacterial diversity and community structure of salt pans from Goa, India

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In Goa, salt production from the local salt pans is an age-old practice. These salt pans harbor a rich diversity of halophilic microbes with immense biotechnological applications, as they tolerate extremely harsh conditions. Detecting the existence of these microbes by a metabarcoding approach could be a primary step to harness their potential. Three salt pans viz. Agarwado, Curca, and Nerul adjoining prominent estuaries of Goa were selected based on their unique geographical locations. The sediments of these salt pans were examined for their bacterial community and function by 16S rRNA amplicon-sequencing. These salt pans were hypersaline (400–450 PSU) and alkaline (pH 7.6–8.25), with 0.036–0.081 mg/L nitrite, 0.0031–0.016 mg/L nitrate, 6.66–15.81 mg/L sulfate, and 20.8–25.6 mg/L sulfide. The relative abundance revealed that the *Pseudomonadota* was dominant in salt pans of Nerul (13.9%), Curca (19.6%), and Agarwado (32.4%). The predominant genera in Nerul, Curca, and Agarwado salt pan sediments were *Rhodopirellula* (1.12%), *Sulfurivermis* (1.28%), and *Psychrobacter* (25.5%) respectively. The highest alpha diversity (Shannon-diversity Index) was observed in the Nerul salt pan (4.8) followed by Curca (4.3) and Agarwado (2.03). Beta diversity indicated the highest dissimilarity between Agarwado and the other two salt pans (0.73) viz. Nerul and Curca and the lowest dissimilarity was observed between Nerul and Curca salt pans (0.48). Additionally, in the Agarwado salt pan, 125 unique genera were detected, while in Nerul 119, and in Curca 28 distinct genera were noted. The presence of these exclusive microorganisms in a specific salt pan and its absence in the others indicate that the adjacent estuaries play a critical role in determining salt pan bacterial diversity. Further, the functional prediction of bacterial communities indicated the predominance of stress adaptation genes involved in osmotic balance, membrane modification, and DNA repair mechanisms. This is the first study to report the bacterial community structure and its functional genes in these three salt pans using Next-Generation Sequencing. The data generated could be used as a reference by other researchers across the world for bioprospecting these organisms for novel compounds having biotechnological and biomedical potential.

KEYWORDS

salt pan, halophilic, Bacteria, diversity, next generation sequencing

Introduction

Salt production is an aged-old traditional method designed by farmers called *Mittkars* for over 150 decades. Salt pans are habitats inhabiting a huge community of extremophiles, thriving in extreme salinity, temperature, pH, and minimum nutrient availability (Mani et al., 2012). Halophilic bacteria from these environments have developed strategies and

cellular systems that could cope with salt stress. Salt-in and salt-out are the two major strategies observed in halophiles to escape stress. In the salt-in strategy, the intracellular system is adapted to the salt concentration of the surrounding but the cellular energy consumed here is huge. Whereas, in salt-out strategies, organisms avoid adaptation and thereby energy loss by an accumulation of organic compatible solutes, also called a compatible solute strategy (Oren, 1999).

Halophilic bacteria from Goan salt pans are widely studied as a potent source of bioactive compounds (Tonima and Savita, 2011; Fernandes et al., 2019). The potential microorganisms in the salt pans of Goa have attracted the attention of the public as well as the researchers to conserve these habitats, *mittkars*, and their traditional knowledge of salt making. The culture-dependent approach examines a very minuscule microbial population. Modern methods such as high throughput sequencing and *in silico* analysis allow us to fill this gap (Janda and Abbott, 2007). The bacterial diversity of Agarwado, Curca, and Nerul salt pans and its comparative analysis has not been elucidated previously by next-generation sequencing. Hence, the present study is the first report accomplished to understand the diversity, distribution, and abundance with a comparative analysis of the bacterial community in these salt pans.

Materials and methods

Sample collection

Sediment samples were collected during the salt harvesting period from the crystallizer pond using a 5.5 cm diameter corer in sterile bags from the salt pans of Curca village (N15.45798, E73.88065) situated at Tiswadi taluka, Agarwado village (N15.640978, E73.76426) situated at Pernem taluka and Nerul village (N15.51979, E73.78909) situated at Bardez taluka (Figures 1A–E). Sediment sample refers to the sediments collected about 2 cm from a surface layer of salt pan. The sediment

samples collected in triplicates from each salt pan site were mixed to obtain a single sediment sample. Sediment samples from Agarwado, Curca, and Nerul salt pans were indicated as AC, CC, and NC, respectively. Samples were transported in ice-cold condition to the laboratory and stored at -20°C until further processing. The temperature was recorded on-site using a thermometer of the three salt pan sediment samples. The pore water obtained after centrifugation of sediment samples was used to measure pH, salinity, nitrate, nitrite, sulfate, and sulfide. The pH was recorded by pH meter (Eutech, Germany) and the salinity was measured by refractometer (ATAGO, Japan). The nitrite and nitrate were estimated spectrophotometrically according to APHA (2012). Sulfate was estimated turbidometrically by measuring the precipitation of BaSO_4 (Clesceri et al., 1998). For the estimation of sulfide, the sample was fixed in 2% zinc acetate and was further analysed spectrophotometrically (Cline, 1969).

Environmental DNA extraction, library preparation, and Illumina sequencing

Environmental DNA was extracted from 1 g of each sediment sample using DNeasy PowerSoil Kit (Qiagen). The DNA obtained was checked for its concentration and quality using NanoDrop. The amplicon library was prepared using Nextera XT Index Kit (Illumina inc.) using bacterial specific forward primer 341F (GCCTACGGGNGGCWGCAG) and reverse primer 805R (ACTACHVGGGTATCTAATCC) for V3–V4 hypervariable region of 16S rRNA gene followed by adding Illumina adapters required for cluster generation. Amplicon libraries were purified by AMPureXP beads and quantified using a Qubit Fluorometer. Libraries were then loaded on MiSeq at 10–20 pM concentration for cluster generation and sequencing. The libraries were sequenced at Eurofins Genomics India Pvt. Ltd., Karnataka, India on the Illumina MiSeq platform using 2×300 bp chemistry to obtain paired-end reads.

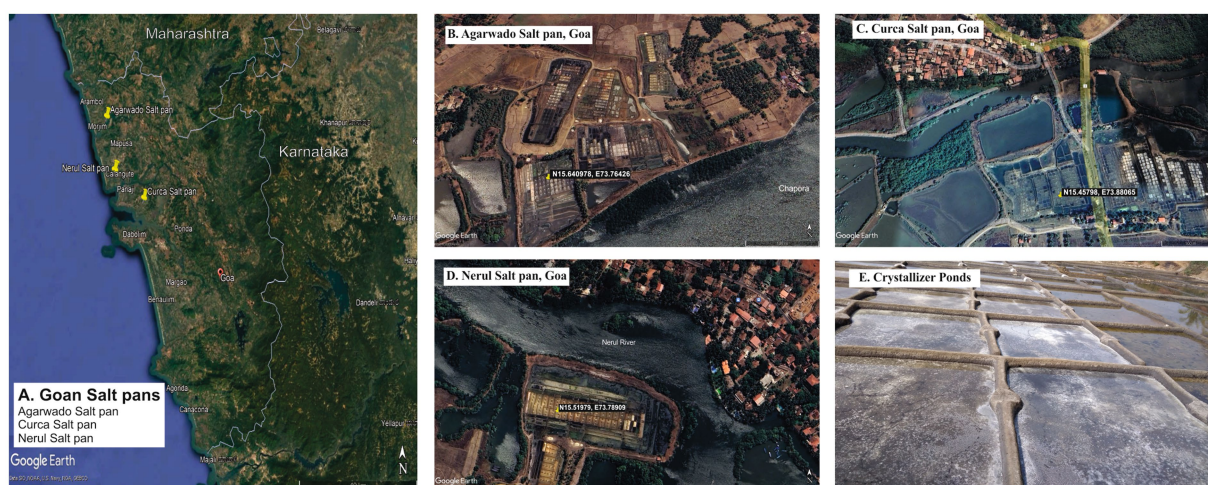


FIGURE 1

Goan Salt pans in study (A) Location of Agarwado, Curca, and Nerul Salt pans in Goa. Aerial image of (B) Agarwado salt pan (C) Curca salt pan (D) Nerul salt pan [Source: Google earth pro, 2023] (E) Image of crystallizer pond under solar evaporation at Agarwado salt pan.

Sequence analysis by bioinformatics

Raw paired-end reads of AC, CC, and NC were analysed by Quantitative Insights Into Microbial Ecology 2 (QIIME 2), version 2–2023.7 (Bolyen et al., 2019). Reads obtained after sequencing were denoised via the q2-dada2 plugin (Callahan et al., 2016) which involves correction of Illumina sequenced errors, merging of reverse and forward paired-end reads, and clustering them into ASVs (amplicon sequence variants). The rarefaction curve was plotted using QIIME. ASVs obtained were further taxonomically classified into different taxonomic levels using the q2-feature-classifier plugin based on the SILVA (V.138) reference database (Quast et al., 2012). The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST2) method (Douglas et al., 2020) was utilized to predict the functional potential of the bacterial communities in the three salt pans mentioned above. The relative abundance of functional protein profiles in the three salt pans was represented in a heatmap constructed using PRIMER7.

Statistical analysis

The alpha (intra-sample) diversity indices [such as Simpson index, Shannon index, Menhinick's richness index, and Pielou's evenness (equitability)] were calculated at the genus level for the three sampling sites, similarly the beta diversity was measured as described by Whittaker (1960). Canonical Correspondence analysis (CCA) was done to understand the influence of environmental variables [Temperature (°C), pH, salinity (PSU), nitrate (mg/L), nitrite (mg/L), sulfate (mg/L), sulfide (mg/L)] on bacterial genera. Alpha diversity, beta diversity and CCA analysis were performed by using Paleontological statistics software (PAST), version 4.13 (Hammer et al., 2001).

Results

Sediment sample analysis

The physical parameters of sediment samples collected from Agarwado (AC), Curca (CC), and Nerul (NC) salt pans possessed temperatures of 35°C (in CC) and 36°C (AC and NC). Salinity observed was 450 PSU in AC and 400 PSU in CC and NC whereas the pH of the sediment samples was found to be 8.25 (AC), 7.6 (CC), and 8 (NC). The environmental DNA extracted from sediment samples AC, CC, and NC were measured by Nanodrop for its concentration and was found to be 28.6 ng/μl, 40.2 ng/μl, and 33.2 ng/μl, respectively.

Relative abundance of bacterial community

A total of 95,299, 104,637, and 240,563 reads were obtained in sediment samples of the Agarwado (AC), Curca (CC), and Nerul (NC) salt pan, respectively. The rarefaction curve of all three samples reached a plateau at this sequencing depth (Figure 2) indicating the information contained in the three samples captured the majority of abundant phylotypes.

In the bacterial community analysis of the AC sample, 42 phyla and 201 genera were identified. The abundant phyla such as

Pseudomonadota (33%), *Bacillota* (22%), and “*Candidatus* Patescibacteria” (9.4%), were observed (Figure 3A). Major contribution at the class level was by *Gammaproteobacteria* (30.7%), and *Bacilli* (20.7%). At the order level, *Pseudomonadales*, and *Bacillales* were found to be abundant with 26.8 and 19.4% relative abundance, respectively. Predominant taxa observed at the family level were *Moraxellaceae* (26.5%) and *Planococcaceae* (18.9%) and the major identified genera were *Psychrobacter* (26%), *Halanaerobium* (3.4%), and *Antarcticibacterium* (1.5%) (Figure 3B).

In the CC sample, 50 phyla and 143 genera were noted. The predominant phyla that contributed to bacterial diversity include *Pseudomonadota* (20.8%), *Desulfobacterota* (11.2%), and *Bacteroidota* (9.8%), (Figure 3A). At the class level, *Gammaproteobacteria* (17%) and *Bacteroidia* (8.4%) were found dominant. At the order level, the predominance of *Desulfobulbales* (5%) and *Desulfobacterales* (2.8%) were detected. At the family level, *Desulfobulbaceae* (3.9%) was relatively abundant. Lastly, at the general level, *Sulfurivermis* (1.3%) and *Desulfatiglans* (1.1%) presented a major proportion (Figure 3B).

In the NC sediment sample, 53 phyla and 250 genera were recognized. The dominant bacterial phyla include *Pseudomonadota* (14.7%), *Planctomycetota* (13%) and *Chloroflexota* (11.7%) (Figure 3A). The abundant classes observed were *Anaerolineae* (10.7%) and *Gammaproteobacteria* (9%). At the order level, *Pirellulales* with 6.5% relative abundance was found to be the highest followed by *Desulfobacterales* (3.8%). At the family level, *Pirellulaceae* (6.5%), was the major family obtained. Further, the most abundant genus identified was *Rhodopirellula* (1.2%) (Figure 3B).

Correlation of bacterial genera with environmental parameters

The effect of environmental parameters such as temperature, pH, salinity, nitrite, nitrate, sulfate, and sulfide were assessed on the bacterial genera using CCA. The CCA tripod explained 88.67% of the variation in bacterial genera by environmental parameters (Figure 4). The result revealed the positive correlation between genera *Blastopirellula*, Sva0081 sediment group, *Rhodopirellula*, *Robiginitalea*, *Pirellula* and *Bacillus* with sulfide and sulfate. However, genera *Psychrobacter*, *Halanaerobium*, *Planococcus* and *Sphingobacterium* found abundant in AC were positively correlated with nitrite, nitrate, and salinity. Of all the environmental parameters sulfide and sulfate contributed significantly to bacterial genera in NC.

Alpha and beta diversity analysis at the genus level

The alpha diversity indices such as Simpson diversity index, Shannon diversity index (Shannon-Wiener index), Dominance, Menhinick's richness index, and Pielou's evenness (equitability) were calculated and depicted in Table 1. Similarly, the beta diversity and its pair-wise comparison presented in Table 2 were performed using the mathematical expression $S/\bar{a}-1$, where S is the total number of genera and $\bar{a}$ is the average number of genera. The beta diversity was found highest and equal between AC and NC and AC and CC, i.e., 0.73. The lowest beta diversity was observed between CC and NC (0.48).

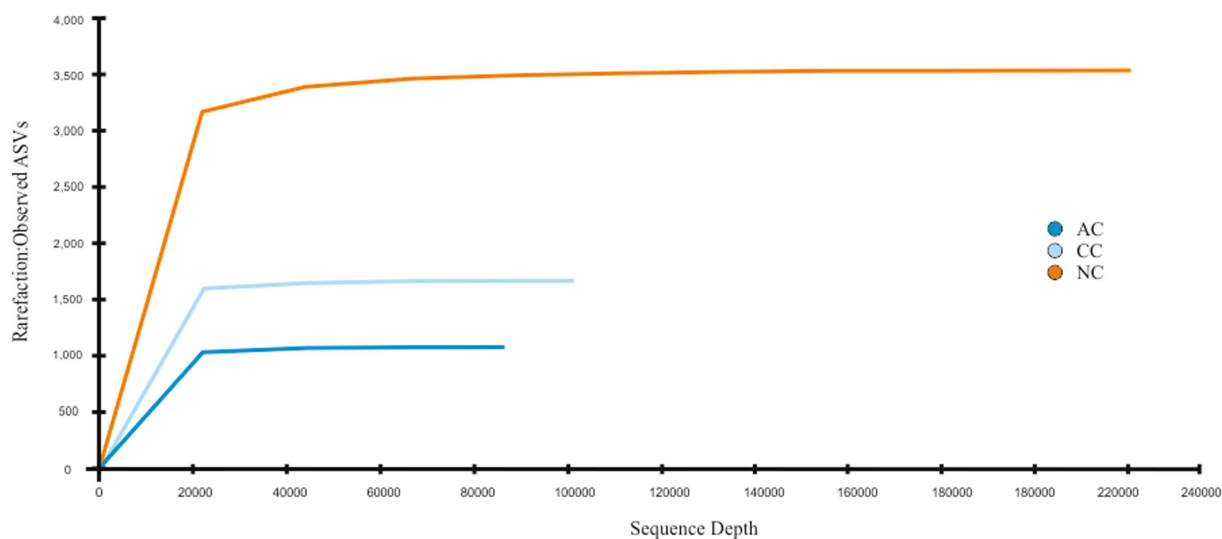


FIGURE 2

Rarefaction curve: Observed ASVs vs. Sequencing depth for three salt pan samples namely Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India.

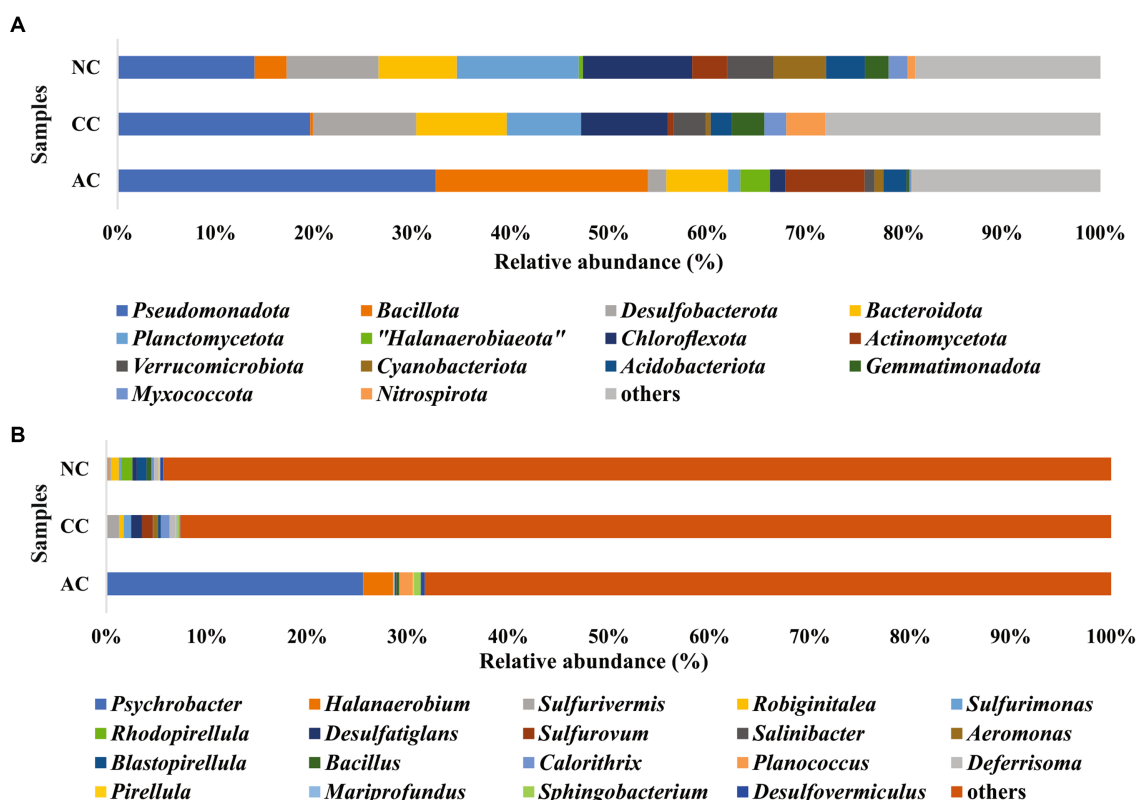


FIGURE 3

Relative abundance of bacterial communities at (A) phyla level and (B) genus level in sediment samples of Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India.

Unique and common bacterial communities among the salt pans

The Venn diagram (Figure 5A) represents the number of unique and common phyla in three salt pans. An acceptably small percentage of

unique taxon was observed in AC, CC, and NC with 14.2, 6, and 5.6%, respectively. Contrastingly, the common taxa observed in all three salt pans were in a higher proportion ranging from 62–78%. There were no common phyla found in CC and AC. The phyla found common among all three salt pans are listed in the [Supplementary Table S1](#).

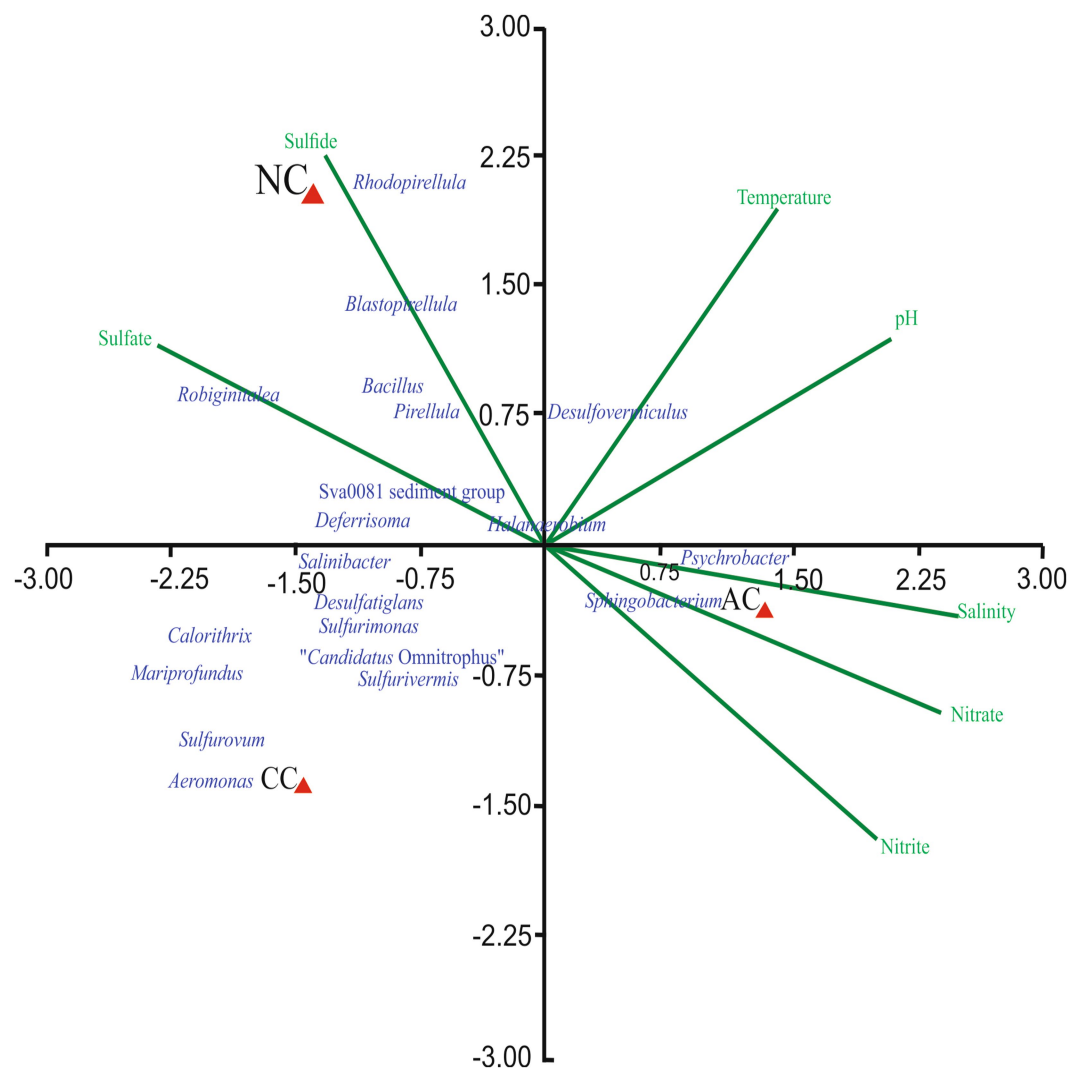


FIGURE 4
Canonical Correspondence Analysis ordination diagram of bacterial communities at generic-level at Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India associated with environmental parameters.

TABLE 1 Alpha diversity indices in Agarwado salt pan (AC), Curca salt pan (CC) and Nerul salt pan (NC) of Goa, India at genera level.

Alpha diversity indices	AC	CC	NC
Taxa (no. of genera)	201	143	250
Simpson diversity index	0.67	0.97	0.98
Pielou's evenness (equitability)	0.38	0.87	0.86
Menhinick's richness index	0.6	1	1
Dominance	0.32	0.02	0.01
Shannon diversity index	2.03	4.3	4.8

At the genera level, a total of 201, 143, 250 genera were observed in AC, CC, and NC, respectively. Although the number of genera was found highest in NC, the percentage of exclusivity was observed high in AC (61%) > NC (47%) > CC (19.4%). The common genera found in all three salt pans ranged between 13 - 23%. The Venn diagram (Figure 5B) depicts the number of unique

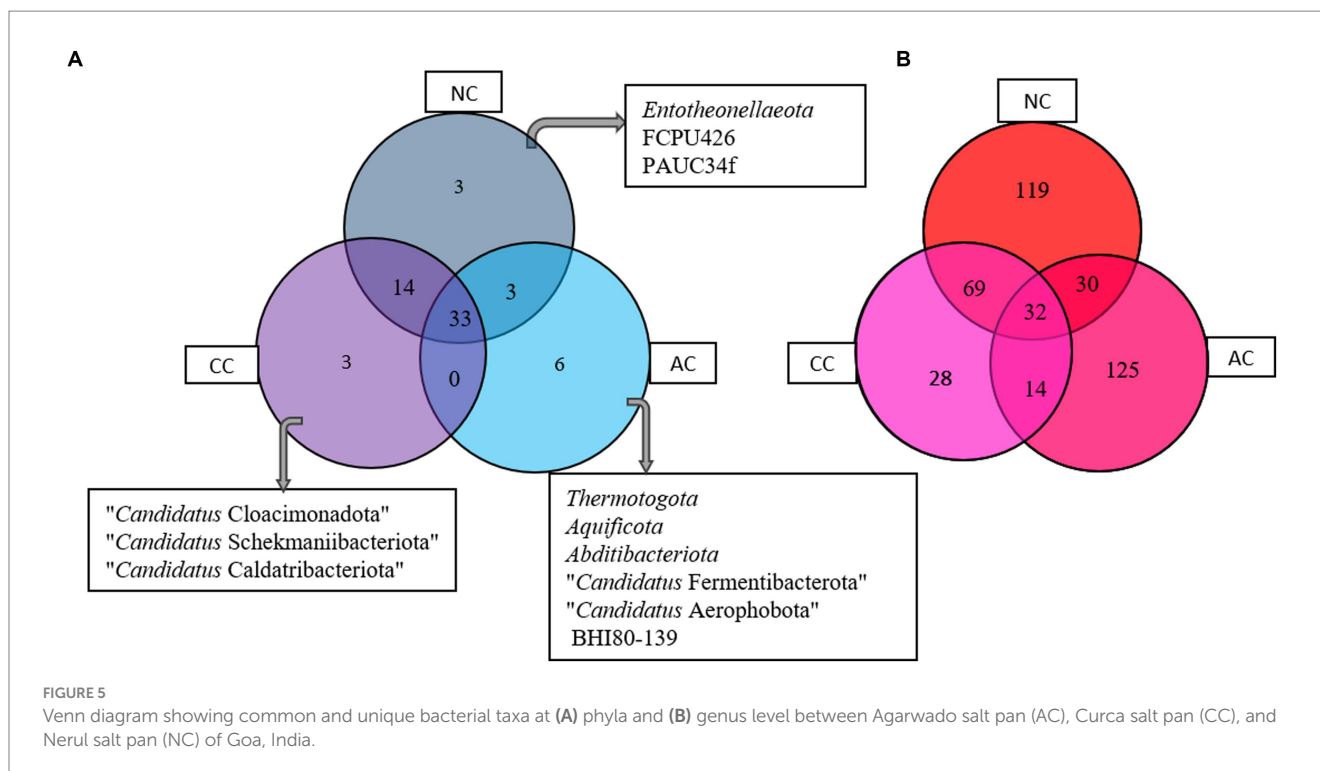
TABLE 2 Pair-wise comparison of beta diversity between Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India at the genus level measured by Whittaker using the mathematical expression $S/\bar{a}-1$, where S is the total number of genera and $\bar{a}$ is the average number of genera.

	AC	CC	NC
AC	0	0.73	0.73
CC	0.73	0	0.48
NC	0.7	0.48	0

and common genera in the three salt pans and the genera are listed in the [Supplementary Table S2](#).

Rare taxa in the three salt pans

Rare taxa are defined as those having a relative abundance of $\leq 0.2\%$ (Hugoni et al., 2013). At the phylum level, rare phyla such as



Bdellovibrionota (0.06%), *Nitrospirata* (0.07%) and "Candidatus Sumerlaeota" (0.07%) were found in AC. In CC, "Candidatus Moduliflexota," *Fusobacteriota*, and "Candidatus Acetithermota" are rare taxa with 0.04, 0.07, and 0.06% relative abundance, respectively. Further in NC, "Candidatus Babelota," *Elusimicrobiota*, and *Deinococcota* are a few minor phyla with 0.11, 0.08, and 0.081% relative abundance, respectively. Secondly, "Candidatus Dadaibacteriota" was the only rare ($\leq 0.2\%$) bacterial phyla found common in all three salt pans. Further, at the genus level only 11 taxa were detected common in all three salt pans (AC, CC, NC) with $\leq 0.2\%$ of relative abundance (Figure 6).

Functional gene prediction in three salt pans

A large number of functional genes involved in various biological processes, including cellular metabolism and adaption to extreme environmental conditions, have been found in the indigenous bacterial communities of all the three salt pans. Figure 7 depicts the most abundant genes that were found in all the three salt pans. Further, numerous genes involved in the nitrogen, sulfur, and carbon cycles have also been found to be part of the overall functional gene profile (Supplementary Figures S1–S3).

Discussion

Traditional salt-making is one of the major and unique occupations of Goa due to its lengthy coastline of 107 Km. The three study sites viz. Agarwado, Curca, and Nerul salt pans were selected based on their unique geographical locations. The Agarwado salt pan

is in Pernem taluka adjoining the pristine Chapora estuary in the extreme north of Goa. The Nerul and Curca salt pans are connected to the two major lifeline estuaries of Goa, i.e., Mandovi in Bardez taluka and Zuari in Tiswadi taluka, respectively. Seawater from the estuary gushes into the salt pans through a sluice gate during high tide and is evaporated to produce salt. For decades, these three distinct salterns have been conventionally used to produce local salt by traditional methods in Goa. The temperature of 35°C - 36°C , noted from these salt pans during the salt harvesting period favors the evaporation process in the crystallizer pond to achieve the salinity required for salt production. The salinity of these salt pans was found to be in the range of 400–450 PSU and pH 7.6–8.25 indicating a halophilic and alkaline nature.

Comparative analysis of the bacterial community in the salt pan

The bacterial composition of the salt pans of Goa revealed that the three sediment samples of salt pans were dominated by phylum *Pseudomonadota*. These results were parallel to the work reported by (Gorrasi et al., 2021) in other solar salterns. Within the *Pseudomonadota* phylum in the AC salt pan, only two classes such as *Gammaproteobacteria* and *Alphaproteobacteria* were observed. In addition to these two classes, *Zetaproteobacteria* was identified in CC and NC. Interestingly the only genera found in our study within the *Zetaproteobacteria* class was *Mariprofundus*. This class of bacterium is a model organism for microbial iron oxidation in marine environments (McAllister et al., 2019). The detection of genera *Mariprofundus* in CC and NC perhaps originated from the mining activities at Mandovi estuary which has deposited several tonnes of iron ore. Secondly, the Cumbarjua Canal interconnects the Mandovi

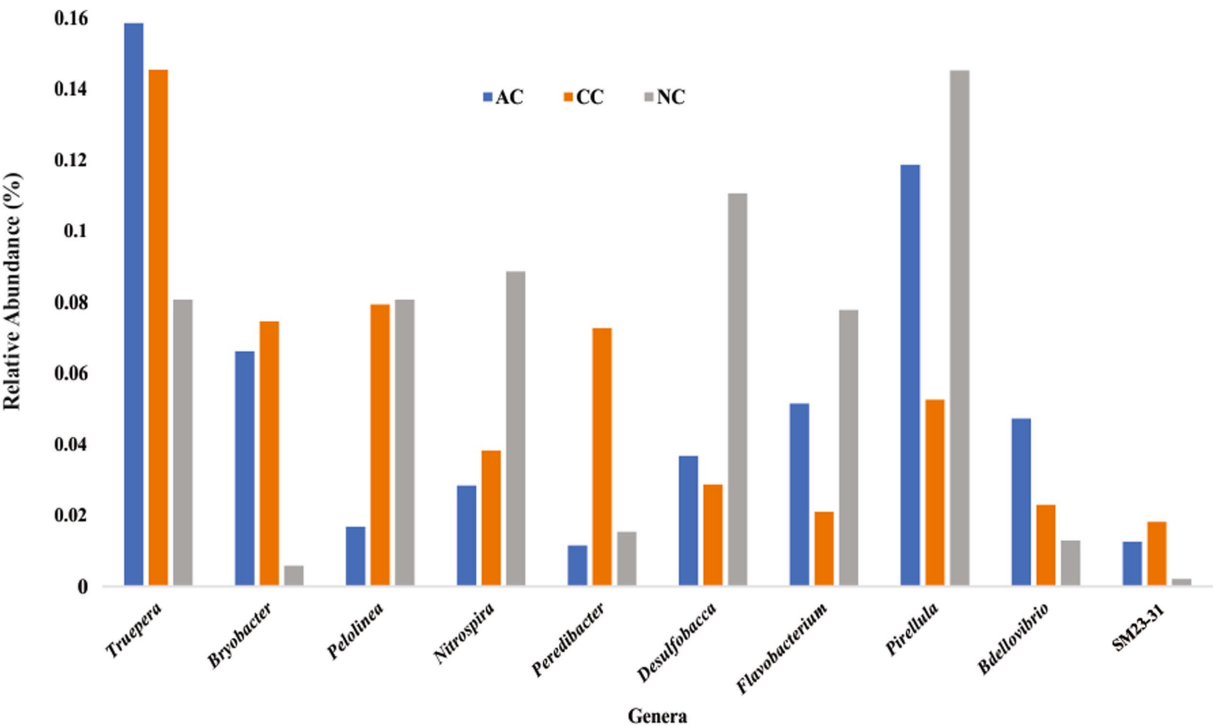


FIGURE 6
Rare bacterial genera ($\leq 0.2\%$ relative abundance) found common in all three sediment samples of Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India.

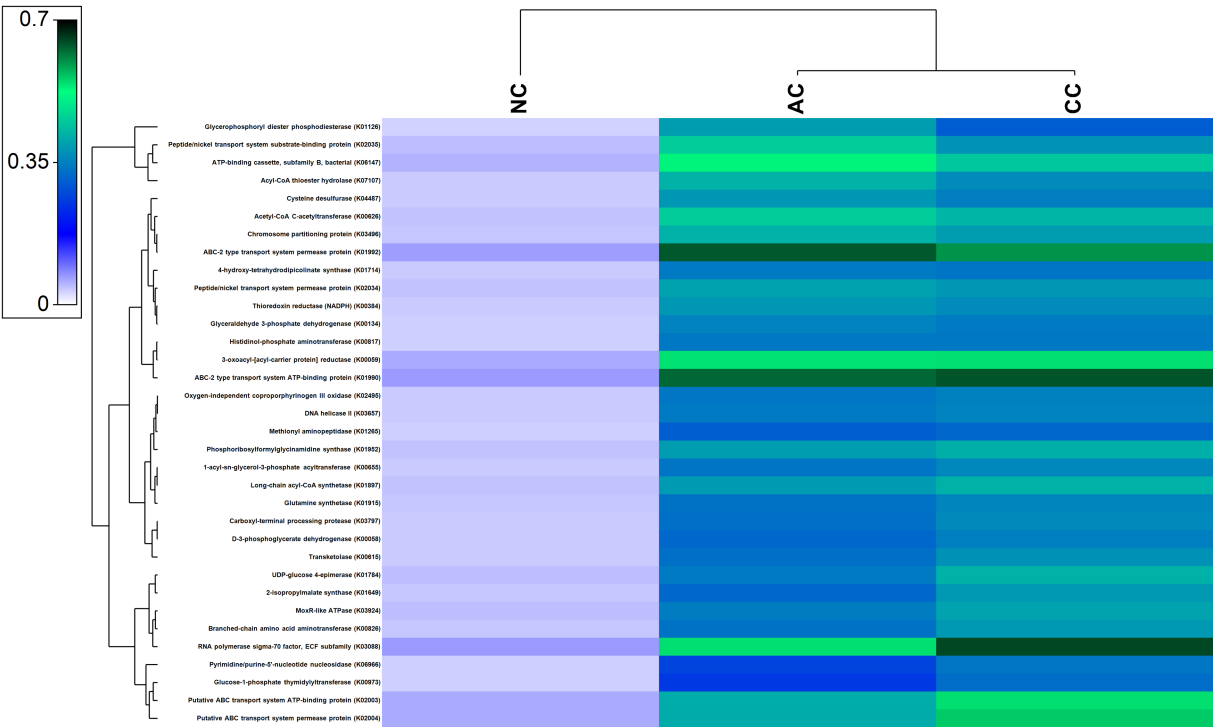


FIGURE 7
Heatmap showing the abundant functional genes predicted by using PICRUST2 algorithm in the sediment samples of Agarwado salt pan (AC), Curca salt pan (CC), and Nerul salt pan (NC) of Goa, India.

and Zuari estuaries (adjacent to NC and CC respectively) and forms a network that has served as a major route for the transportation of iron ore. Additionally, the discharge of mining rejects from the mining sites has also concentrated iron particles in these estuaries (Gauns, 2011). This demonstrates how the distribution of microorganisms in a particular area is affected by their chemical composition. Similarly, other environmental parameters like salinity, nitrite, nitrate, sulfate, and sulfide also have an influence on bacterial distribution and abundance as depicted by CCA. The dominant phyla observed in our study such as *Pseudomonadota*, *Bacteroidota*, *Chloroflexota*, etc. are found to have ubiquitous genetic machinery and survival weapons (Mehta et al., 2021). Hence, understanding the microbiome composition, evolving genetic machinery, and adaptive mechanisms evolved by these extremophiles make it important in biotechnology industries. The genera *Psychrobacter*, belonging to the family *Moraxellaceae*, was the most abundant genera and family, respectively, in the AC with approximately 25.5% relative abundance. Like our studies, the presence of *Psychrobacter* was also reported in the solar saltern of Bohai Bay, China, revealed by DGGE of the 16S gene (Zhang et al., 2016). The isolation of sulfidogenic bacteria *Halanaerobium*, reported from the Ribandar salt pan of Goa was observed to be the second abundant genera in AC (Das et al., 2017). The abundant genera observed in the CC and NC salt pans were *Sulfurivermis* and *Rhodopirellula*, respectively. The presence of *Sulfurivermis* shows the occurrence of active sulfur cycles in the salt pan being a sulfur oxidizing bacterium (SOB) that uses nitrate as an electron acceptor in the rTCA pathway (Kojima et al., 2017).

Alpha and Beta diversity analysis of the salt pans

The diversity of any ecosystem depends on two components “richness and evenness” (Soininen et al., 2012). In the Menhinicks richness index, an equal richness value was observed for NC and CC and was higher in comparison to AC, evident for higher community richness in Nerul and Curca salt pans. Similarly, the Pielou’s evenness index for NC and CC was found to be higher as compared to AC, which indicates the distribution of an equal number of taxa in both salt pan (NC and CC) ecosystems than in the Agarwado salt pan. Further, dominance in AC was higher than NC and CC, demonstrating the presence of a more dominant taxon in the Agarwado salt pan community. Lower evenness and higher dominance observed in AC indicate lower alpha diversity. However, in Nerul and Curca salt pans high evenness and lower dominance indicate high alpha diversity. A similar observation was shown by the Shannon diversity index which considers both the richness and evenness of a community. The alpha diversity measured by the Shannon index was NC > CC > AC.

Interestingly equal dissimilarity diversity (beta diversity) was observed between AC and NC and AC and CC, which further indicated that both CC and NC are quite comparable to each other but highly divergent to AC. The similarity between NC and CC was further confirmed by the presence of high number of common genera in both (represented in Figure 5B) in addition to the beta diversity. This is due the adjoining estuaries at Nerul and Curca salt pans that are connected by the Cumbarjua canal and therefore share uniform environmental characteristic (Qasim and Gupta, 1981) influencing bacterial composition.

Bacterial taxa in ecological roles

Pseudomonadota, *Planctomycetota*, and *Chloroflexota* observed in the three salt pans are representatives of the commonly occurring phyla that are involved in a variety of ecological roles. The sulfur cycle advances in tandem with the carbon cycle and is most prevalent in hypersaline environments. Using sulfate as an electron acceptor, the sulfate-reducing bacteria break down organic molecules in an anoxic environment thereby cycling carbon anaerobically (Kerkar and Bharathi, 2007). In the current anticipated functional metagenome, genes involved in assimilatory and dissimilatory sulfate reduction and oxidation of sulfur compounds were found. The reductive nature of the salt pans sulfur cycle in the current work is indicated by the presence of the genera *Desulfovermiculus* and *Desulfatiglans* (Begmatov et al., 2021) in addition to genes such as adenylylsulfate reductase, subunits A (K00394), adenylylsulfate reductase, subunits B (K00395), dissimilatory sulfite reductase alpha subunit (K11180), dissimilatory sulfite reductase beta subunit (K11181), and sulfate adenylyltransferase (K00958). Likewise, different genes of the Sox system that are involved in sulfur oxidation: SoxA (K17222), SoxX (K17223), SoxB (K17224), SoxY (K17226), SoxZ (K17227), SoxC (K17225) and Sulfane dehydrogenase subunit were observed in the functional gene prediction (Supplementary Figure S3). *Thiohalospira* is a genus of halophilic, chemolithoautotrophic SOB that utilizes sulfur compounds as electron donors (Sorokin et al., 2020); it has only been found in the Agarwado (AC) salt pan. Secondly, the SOB genus, *Sulfuriflexus* (class: *Gammaproteobacteria*) (Kojima and Fukui, 2016; Rana et al., 2020), was exclusive to Curca (CC) sediments. *Sphingobacterium* and *Woeselia* functional in carbon-fixing (Lohmann et al., 2020) were also observed in these salt pans along with numerous genes required in carbon metabolism (Supplementary Figure S1). Unique to the Nerul (NC) salt pan, a group at genus level Cm1-21 (family: *Nitrosococcaceae*) was recently reported as ammonium oxidizers involved in the nitrification process of the nitrogen cycle (Semedo et al., 2021). Besides functional genes (Supplementary Figures S1–S3), bacterial genera like *Desulfovibrio* (observed in AC and NC sediment samples), *Pseudomonas* (AC and CC), *Nitrospina* (CC and NC), and *Nitrospira* (AC, CC, and NC) noted in our study are implicated in the carbon, nitrogen, and sulfur cycles (Megonigal et al., 2003; Meng et al., 2022; Sun et al., 2022), which illustrates that the movement of nutrients necessary to support the persistent life in these man-made salt pan ecosystems are governed by the presence of its indigenous microbes.

Significance of halophiles

Salinity is a key factor that governs the microbial community in hypersaline ecosystems such as solar saltern. High salinity imposes stress on microbial cells, challenging their survival (Fei Xi et al., 2014; Song et al., 2022). Numerous genes associated with osmoprotective mechanisms, such as salt-in and salt-out mechanisms, membrane modification, protein adaptation, DNA repair, etc. acquired by residents of hypersaline environment for normal growth and development of cells were observed to be prevalent in our study. These genes include ABC-2 type transport system permease protein (K01992), ABC-2 type transport system ATP-binding protein (K01990), ATP-binding cassette, subfamily B, bacterial (K06147),

1-acyl-sn-glycerol-3-phosphate acyltransferase (K00655), 4-hydroxy-tetrahydrodipicolinate synthase (K01714), and DNA helicase II / ATP-dependent DNA helicase PcrA (K03657) (Sleator and Hill, 2002; Yu et al., 2018; Schmitz et al., 2020).

Salt pans in Goa are influenced by three seasons: pre-monsoon, monsoon, and post-monsoon, where salinity ranges from 30–300 PSU (Mani et al., 2012). Therefore, bacteria with different salt tolerance like slightly, moderately, and extremely halophilic bacteria are expected to inhabit this ecosystem. Bacteria within the moderately halophilic group are very important as they occupy a large proportion of halophiles. As adaptive measures, the production of compatible solutes and hydrolytic enzymes to acclimatize to salinity variations is attracting industries (Baati et al., 2010; Ramos-Cormenzana, 2020). For example, the moderate halophilic genus *Micrococcus* synthesizes catalase that plays a role in maintaining colour and flavor in cured meats (Onishi and Kamekura, 1972; García-López et al., 1999). Secondly, *Rhodopirellula*, an abundant genus observed in NC sample is a moderate halophile and was reported to have a role as a food supplement to *Daphnia magna* (indicator used for testing toxicity of water) (da Conceição Marinho et al., 2019). Therefore, profound knowledge and discovery of halophilic bacteria in such an ecosystem will help us to disclose numerous properties, enzymes, and proteins of halophiles that could be substantially important to different biotechnological industries.

Conclusion

Salt pans being polyextremophilic, are dynamic habitats of the resident bacteria potentially capable of tolerating extremely stressful environmental factors, i.e., high salinity (30–450 PSU), high alkalinity (pH 7.6–8.25) and fluctuating temperatures (20°C – 45°C). This is the first study profiling the bacterial communities in the Agarwado, Curca, and Nerul salt pans of Goa. The data reveals that the highest bacterial genera prevail in the Nerul salt pan sediment (250) as compared to Agarwado (201) and Curca (143). However, in the sediments of Agarwado salt pan, 125 distinct genera were found, followed by Nerul (119) and Curca (28) which were exclusively unique to these salt pans. These hypersaline natives are resistant to osmotic stress, demonstrated by the presence of genes in the predicted functional gene profile, that allows them to actively participate in the carbon, nitrogen, and sulfur cycles in these hypersaline environments. A wide microbial community and its function remains unknown, as “unculturable” as it is impractically impossible to mimic these diverse physical, chemical, biological, and mechanical factors which contribute to simulating the salt pan ecosystem in a laboratory. The high throughput sequencing has made it possible to provide an insight of the bacterial community and most of its functions in the salt pans, which could not be explored earlier. Further, a deep understanding of their functional mechanisms in this habitat will help us to assess the existence of these unique bacterial communities, and the adaptive cellular machinery/ biomolecules involved in making this hypersaline environment a conducive environment to thrive in.

References

APHA (2012). *Standard methods for the examination of water and waste water*. 22 American Public Health Association, American Water Work Association, Water Environment Federation. Washington

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

PG: writing original draft preparation, methodology, and software analysis. SK: supervision, experimental design, review and editing. All authors contributed to the article and approved the submitted version.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by a Junior Research Fellowship for a Ph.D. scholar (award letter number: 19/06/2016(i) EU-V), funded by University Grant Commission (UGC), New Delhi, India.

Acknowledgments

The authors are thankful to the School of Biological Sciences and Biotechnology (Discipline of Biotechnology) and Goa Business School (Discipline of Computer Science and Technology), Goa University for the necessary facilities. We are grateful to Venkatachalam S., Project Scientist II at NCPOR, Goa, India for the technical assistance provided.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2023.1230929/full#supplementary-material>

Baati, H., Amdouni, R., Gharsallah, N., Sghir, A., and Ammar, E. (2010). Isolation and characterization of moderately halophilic Bacteria from Tunisian solar Saltern. *Curr. Microbiol.* 60, 157–161. doi: 10.1007/s00284-009-9516-6

- Begmatov, S., Savvichev, A. S., Kadnikov, V. V., Beletsky, A. V., Rusanov, I. I., Klyuvitkin, A. A., et al. (2021). Microbial communities involved in methane, sulfur, and nitrogen cycling in the sediments of the Barents Sea. *Microorganisms* 9:2362. doi: 10.3390/microorganisms9112362
- Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9
- Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869
- Clesceri, L. S., Greenberg, A. E., and Eaton, A. D. (1998). Standard methods for examination of water and wastewater. *American Public Health Association*, Washington.
- Cline, J. D. (1969). Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol. Oceanogr.* 14, 454–458. doi: 10.4319/lo.1969.14.3.0454
- da Conceição Marinho, M., Lage, O. M., Sousa, C. D., Catita, J., and Antunes, S. C. (2019). Assessment of *Rhodospirillum rubrum* as a supplementary and nutritional food source to the microcrustacean *Daphnia magna*. *Antonie Van Leeuwenhoek* 112, 1231–1243. doi: 10.1007/s10482-019-01255-x
- Das, K. R., Kerkar, S., Meena, Y., and Mishra, S. (2017). Effects of iron nanoparticles on iron-corroding bacteria 7, 385. doi: 10.1007/s13205-017-1018-9
- Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 685–688. doi: 10.1038/s41587-020-0548-6
- Fei Xi, X., Wang, L., Jun Hu, J., Shu Tang, Y., Hu, Y., Hua Fu, X., et al. (2014). Salinity influence on soil microbial respiration rate of wetland in the Yangtze River estuary through changing microbial community. *J. Environ. Sci.* 26, 2562–2570. doi: 10.1016/j.jes.2014.07.016
- Fernandes, S., Kerkar, S., Leitao, J., and Mishra, A. (2019). Probiotic role of salt Pan Bacteria in enhancing the growth of Whiteleg shrimp, *Litopenaeus vannamei*. *Probiotics Antimicrob. Proteins* 11, 1309–1323. doi: 10.1007/s12602-018-9503-y
- García-López, M.-L., Santos, J.-Á., and Otero, A. (1999). MICROCOCCLUS. *Encyclopedia of Food Microbiology* (Elsevier). 20, 1344–1350. doi: 10.1006/rwfm.1999.1025
- Gauns, R. (2011). *Mining havoc: Impact of mining on water resources in Goa - article from dams, Rivers, and People*. SANDRP, 10–12.
- Gorrasí, S., Franzetti, A., Ambrosini, R., Pittino, F., Pasqualetti, M., and Fenice, M. (2021). Spatio-temporal variation of the bacterial communities along a salinity gradient within a Thalassohaline environment (saline di Tarquinia Salterns, Italy). *Molecules* 26:1338. doi: 10.3390/molecules26051338
- Hammer, D. A. T., Ryan, P. D., Hammer, Ø., and Harper, D. A. T. (2001). Past: paleontological statistics software package for education and data analysis. *Palae. Elec.* 4, 1–9.
- Hugoni, M., Taib, N., Debroas, D., Domaizon, I., Jouan Dufournel, I., Bronner, G., et al. (2013). Structure of the rare archaeal biosphere and seasonal dynamics of active ecotypes in surface coastal waters. *Proc. Natl. Acad. Sci.* 110, 6004–6009. doi: 10.1073/pnas.1216863110
- Janda, J. M., and Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J. Clin. Microbiol.* 45, 2761–2764. doi: 10.1128/JCM.01228-07
- Kerkar, S., and Bharathi, P. A. L. (2007). Stimulation of sulfate-reducing activity at salt-saturation in the salterns of Ribandar, Goa, India. *Geomicrobiol. J.* 24, 101–110. doi: 10.1080/01490450701266597
- Kojima, H., and Fukui, M. (2016). *Sulfuriflexus mobilis* gen. Nov., sp. nov., a sulfur-oxidizing bacterium isolated from a brackish lake sediment. *Int. J. Syst. Evol. Microbiol.* 66, 3515–3518. doi: 10.1099/ijsem.0.001227
- Kojima, H., Watanabe, M., and Fukui, M. (2017). *Sulfurivermis fontis* gen. Nov., sp. nov., a sulfur-oxidizing autotroph, and proposal of Thioprofundaceae fam. Nov. *Int. J. Syst. Evol. Microbiol.* 67, 3458–3461. doi: 10.1099/ijsem.0.002137
- Lohmann, P., Benk, S., Gleixner, G., Potthast, K., Michalzik, B., Jehmlich, N., et al. (2020). Seasonal patterns of dominant microbes involved in central nutrient cycles in the subsurface. *Microorganisms* 8:1694. doi: 10.3390/microorganisms8111694
- Mani, K., Salgaonkar, B. B., Das, D., and Bragança, J. M. (2012). Community solar salt production in Goa. *India. Aquat. Biosyst* 8:30. doi: 10.1186/2046-9063-8-30
- McAllister, S. M., Moore, R. M., Gartman, A., Luther, G. W., Emerson, D., and Chan, C. S. (2019). The Fe(II)-oxidizing Zetaproteobacteria: historical, ecological and genomic perspectives. *FEMS Microbiol. Ecol.* 95:fiz015. doi: 10.1093/femsec/fiz015
- Megonigal, J. P., Hines, M. E., and Visscher, P. T. (2003). Anaerobic metabolism: linkages to trace gases and aerobic processes. *Treatise on Geochemistry* (Elsevier). 8, 317–424.
- Mehta, P., Yadav, M., Ahmed, V., Goyal, K., Pandey, R., and Chauhan, N. S. (2021). Culture-independent exploration of the hypersaline ecosystem indicates the environment-specific microbiome evolution. *Front. Microbiol.* 12:686549. doi: 10.3389/fmicb.2021.686549
- Meng, S., Peng, T., Liu, X., Wang, H., Huang, T., Gu, J.-D., et al. (2022). Ecological role of Bacteria involved in the biogeochemical cycles of mangroves based on functional genes detected through GeoChip 5.0. *mSphere* 7:21. doi: 10.1128/msphere.00936-21
- Onishi, H., and Kamekura, M. (1972). *Micrococcus halobius* sp. n. *Int. J. Syst. Evol. Microbiol.* 22, 233–236. doi: 10.1099/00207713-22-4-233
- Oren, A. (1999). Bioenergetic aspects of Halophilism. *Microbiol. Mol. Biol. Rev.* 63, 334–348. doi: 10.1128/MMBR.63.2.334-348.1999
- Qasim, S. Z., and Gupta, R. S. (1981). Environmental characteristics of the Mandovi-Zuari estuarine system in Goa. *Estuar. Coast. Shelf Sci.* 13, 557–578. doi: 10.1016/S0302-3524(81)80058-8
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219
- Ramos-Cormenzana, A. (2020). Ecology of moderately halophilic Bacteria. *The Biology of Halophilic Bacteria Edition* (CRC Press), 55–86. doi: 10.1201/9781003069140-3
- Rana, K., Rana, N., and Singh, B. (2020). Applications of sulfur oxidizing bacteria. *Physiological and Biotechnological Aspects of Extremophiles*, (Academic Press), 131–136.
- Schmitz, R. A., Dietl, A., Müller, M., Berben, T., Op den Camp, H. J., and Barends, T. R. (2020). Structure of the 4-hydroxy-tetrahydrodipicolinate synthase from the thermoacidophilic methanotroph *Methylobacterium fumariolicum* SolV and the phylogeny of the aminotransferase pathway. *Acta Crystallogr. Sect. F: Struct. Biol. Commun.* 76, 199–208. doi: 10.1107/S2053230X20005294
- Semedo, M., Lopes, E., Baptista, M. S., Oller-Ruiz, A., Gilabert, J., Tomasino, M. P., et al. (2021). Depth profile of nitrifying archaeal and bacterial communities in the remote oligotrophic waters of the North Pacific. *Front. Microbiol.* 12, 1–18. doi: 10.3389/fmicb.2021.624071
- Sleator, R. D., and Hill, C. (2002). Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. *FEMS Microbiol. Rev.* 26, 49–71. doi: 10.1111/j.1574-6976.2002.tb00598.x
- Soininen, J., Passy, S., and Hillebrand, H. (2012). The relationship between species richness and evenness: a meta-analysis of studies across aquatic ecosystems. *Oecologia* 169, 803–809. doi: 10.1007/s00442-011-2236-1
- Song, T., Liang, Q., Du, Z., Wang, X., Chen, G., Du, Z., et al. (2022). Salinity gradient controls microbial community structure and assembly in coastal solar Salterns. *Genes (Basel)* 13:385. doi: 10.3390/genes13020385
- Sorokin, D. Y., Merkel, A. Y., and Muyzer, G. (2020). *Thiohalospira*. *Bergey's Manual of Systematics of Archaea and Bacteria*. John Wiley & Sons, Inc.
- Sun, X., Kong, T., Li, F., Häggblom, M. M., Kolton, M., Lan, L., et al. (2022). *Desulfurivibrio* spp. mediate sulfur-oxidation coupled to Sb(V) reduction, a novel biogeochemical process. *ISME J.* 16, 1547–1556. doi: 10.1038/s41396-022-01201-2
- Tonima, K., and Savita, K. (2011). Pharmaceutical potentials of bacteria from saltpans of Goa. *Intern. J. App. Pharmaceutics* 2, 150–154.
- Whittaker, R. H. (1960). Vegetation of the Siskiyou Mountains, Oregon and California. *Ecol. Monogr.* 30, 279–338. doi: 10.2307/1943563
- Yu, J., Loh, K., Song, Z. Y., Yang, H. Q., Zhang, Y., and Lin, S. (2018). Update on glycerol-3-phosphate acyltransferases: the roles in the development of insulin resistance. *Nutr. Diabetes* 8:34. doi: 10.1038/s41387-018-0045-x
- Zhang, J., Ma, G., Deng, Y., Dong, J., van Stappen, G., and Sui, L. (2016). Bacterial diversity in Bohai Bay solar Saltworks, China. *Curr. Microbiol.* 72, 55–63. doi: 10.1007/s00284-015-0916-5