SPATIO - TEMPORAL METAGENOMIC PROFILING OF BACTERIAL DIVERSITY OF AQUACULTURE SEDIMENTS

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THESIS

Submitted to the

Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli

In partial fulfillment of requirements for degree of

MASTER OF FISHERIES SCIENCE

IN

FISH BIOTECHNOLOGY

BY

UTKARSH SURENDRA CHACHARKAR

B.F.Sc.

Under the guidance of

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CANDIDATE'S DECLARATION

I hereby declare that this thesis or part thereof has not been submitted by me or other person to any other University or Institute for a Degree or Diploma.

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(**Utkarsh Surendra Chacharkar**)

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सारांश

व्यावसायिक गोड्या पाण्यातील मत्स्यपालन (IMC) आणि निमखाऱ्या पाण्यातील न्यातिल कोळंबी संवर्धन आवासातल्या जिवाणू समुदायांचे मेटाजीनोमिक प्रोफायलिंग पद्धतीचा वापर करून विश्लेषण केले गेले. 16S rRNA सिक्वेंसिंग करून जीवाणू समुदायांची ओळख पटवली गेली. एकुण ११२४२१२ QIIME QC फिल्टर्ड सिक्वेंसिंग रिड्स पासून दोन्हीं मत्स्यपालन आवासातून एकंदरीत ३४१६ OTU (Operational taxonomic unit) ओळखले गेले. प्रस्तूत अभ्यासामध्ये मत्स्यपालनाच्या दोन्ही प्रकारात प्रोटीयोबैक्टीरिया जीवानुंचे ~ ९९% प्राबल्य आढळले. त्यापाठोपाठ क्लोरोफ्लक्झी (०.०२%) आणि इतर जीवानुंचे घटते क्रमाने आढळले. मत्स्य आणि कोळंबी शेतीत २७.५% समान OTUs आढळले. म्हणजेच, एकंदरित OTU पैकी ६४.६% OTU गोड्या पान्यातिल IMC प्रणालीसाठी तर ३५.४% OTUs हे निमखाऱ्या पाण्यातिल प्रणालीसाठी अद्वितीय ठरले. कोळंबी संवर्धन विविध स्तरांमधील तुलनात्मक १२.८% (एसएस / एस 1), ६.३% (एस 1 / एस 2), आणि सामायिक ओटीयूच्या ४.४% (एसएस / एस 2) दर्शविल्या आहेत. कोलंबीपालनात (एसएस → एस 1 → एस 2) पासून मिळालेल्या 'इनिशियल' आणि 'फाइनल' मेटाजीनोमिक प्रोफाइलमध्ये सांख्यिकीय तुलना यांनी मान-व्हिटनी यू-टेस्ट (पी> ०.०५) वर आधारित कोणतेही लक्षणीय फरक दिसले नाहीत. प्रतिगमन विश्लेषणाच्या आधारे मात्र संवर्धनकाळादरम्यान OTU मध्ये झालेले बदल हे 'मोठया' प्रकारात मोडतात. त्याचप्रमाणे, अनुक्रमे फायलाम-आणि ऑर्डर-लेव्हलमध्ये, सुमारे ७३% आणि ८०% प्रोफाइल 'मोठ्या' प्रभाव आकार मध्ये ट्रेंड दर्शवितात. अभ्यासातून असे आढळून आले आहे की मत्स्यशेती पद्धतीमुळे जिवाणूतिल घटकांची रिलेटिव्ह बहुसंख्या प्रभावित होते आणि प्रोटीयोबैक्टीरिया (गॅमाप्रोटीयोबैक्टीरिया) मत्स्यशेती अवसदाच्या आरोग्यासाठी महत्त्वाचे आहे.

Abstract

Bacterial communities in commercial freshwater (IMC) and brackishwater shrimp aquaculture pond sediments were analysed using the culture-independent metagenomic profiling approach. The bacterial communities were identified using V3-V4 paired hyper variable regions of 16S rRNA combined with next-generation sequencing. The assay revealed the bacterial community structure of the sediments and identified 3416 OTUs from 1124212 QIIME QC filtered sequencing reads for bottom sediments of both aquaculture activities including source for shrimp aquaculture activity. The present study found the predominance of phylum Proteobacteria (~ 99%) from both aquaculture types followed by Chloroflexi (0.02%), and others in decreasing order. At order level, about 50% the bacterial population remained unclassified with Aeromonadales, Alteromonadales, Enterobacteriales, Pseudomonadales, aaa34a10, Rhodocyclales, Rhizobiales, Desulfuromonadales, Rhodospirillales, and others as the remaining major orders. Comparison of the fish and shrimp farm OTUs revealed 27.5% sharing of OTUs between the two aquaculture activities. Thus, only about 64.6% of the OTUs were unique for the freshwater IMC system. Comparisons between different stages of shrimp farms indicated 12.8% (SS/S1), 6.3% (S1/S2), and 4.4% (SS/S2) of shared OTUs. Statistical comparisons between the 'initial' and 'final' metagenomic profiles obtained from shrimp farm sediments (SS→S1→S2) revealed no significant differences based on the Mann-Whitney U-test ($P > 0.05$). Regression analyses showed that the overall trends with respect to OTUs (total, shared, or unique) had a 'large' 'effect size'. Similarly, at phylum- and order-level respectively, about 73% and 80% of the profiles displayed trends that translated into a 'large' ES. The study reveals that aquaculture practices influence the relative abundance of bacterial entities and that the Proteobacteria (Gammaproteobacteria) are important for the health of aquaculture sediments.

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1. Introduction

India ranks second in the world aquaculture production after China (FAO, 2018). The culture of food fishes seems to be the only way to fulfill the increasing demand for aquatic products given the stagnating or declining capture fisheries production. In 2014, the production of food fishes from aquaculture has surpassed the production of capture fisheries (FAO, 2016). This is the first such incidence in the history of fish industry when the aquaculture production surpassed the capture fisheries production. Thus, in the being future aquaculture is the only remaining source for supply of food fishes at the least. There are various important aspects for getting the yield of aquatic organisms in a captive condition, i.e. the water quality, soil quality, balance between biogeochemical cycles in a pond ecosystem. These are the basic common parameters required of all type of aquaculture systems.

Various factors have contributed to the success of aquaculture including an array of domesticated species suited for various environmental conditions, nutritional breakthroughs, disease management, etc. however, the major factor for the sustained success of aquaculture around the globe has been good husbandry practices leading to the success of every crop for of all commercially species. In this, water supply, water quality, and water quality management of aquaculture facilities is of prime importance throughout the history of the aquaculture industry. Soil or pond sediment health is also key factor in this (Boyd, 1995). In the pond based aquaculture systems sediments or bottom of the pond is the zone which is highly dynamics and where the greatest number of aquatic microbes are present (Al-Harbi and Naim, 2006). The pond water quality is therefore influenced by the exchange of substances at the soil-water interface (Boyd, 1995). Concentrations of nutrients, organic matter and

microorganism density at the pond bottom is much greater than in water. Intensive organic matter degradation at the pond bottom leads to the anoxic conditions in the sediments and also at the sediment-water interface. Shrimps normally live on or near the bottom are exposed to these conditions may result in reduced feeding, slower growth rates, higher sensitivity to various vital diseases and possibly to mass mortality (Avnimelech and Ritvo, 2003). All biogeochemical and metabolic processes are caused in aquaculture ecosystem is done by the microscopic life is present in it. Bacterial content of the sediments affects the water quality as well as the quality of fish and fish products (Moriarty, 1997). Therefore, most fish and shrimp have farmers realized that maintaining good sediment quality is as important as the water quality in pond aquaculture (Boyd, 2004).

Pond sediment microbes need a special mention here owing to their significance in pond bottom dynamics. Pond sediment microorganisms are a highly diverse group of organisms comprising the greatest number of individual organisms on the earth and also constitute about 60% of total earth's biomass (Singh *et al.*, 2009). In general, microorganisms are the most essential components of earth's biodiversity as they play a critical role in natural biogeochemical cycles, such as the degradation of hydrocarbons in the environment (Leahy and Colwell, 1990). Bacteria in the sediment ecosystems are well known for the transformation of organic matter and in biogeochemical cycling of primary elements such as nitrogen, sulfur, phosphorus, and iron (Cheng *et al.*, 2013; Newton *et al.*, 2011). While several researchers are engaged in exploring unseen microbial diversity from various environments the microbial diversity that has been reported less than 1% of the known bacterial species since 99% of bacterial diversity remain unculturable (Martinez-Porchas and Albores, 2015). Therefore, conventional culture-dependent

techniques of bacterial diversity assessment fall too short of capturing the complex microbial dynamics as expected under microbial ecology. The development of new sequencing technologies such as 454 pyrosequencing, Illumina (Solexa) sequencing and ABI SOLiD (Sequencing by Oligonucleotide Ligation and Detection) provide much broader taxonomic coverage of the unknown and often unculturable microbial communities (Malik *et al*., 2013).

During last two decades, metagenomics has emerged as a promising scientific tool to analyze the complex genomes contained within microbial communities (Martinez-Porchas and Albores, 2015). The term 'metagenomics' was first used by Handelsman *et al*., (1998). Metagenomics has been a rapidly growing field in molecular genetics and ecological studies. The genome analysis of whole environmental microbiome on their functional and sequence-based analysis in any given environment enables us to explore and understand the vast and still unseen genetic potential of microbial communities. With a much deeper understanding about population structure, genetic diversity, and their ecological roles played by the particular groups of microorganisms (Bashir *et al*., 2014). Introduction of high-throughput sequencing techniques has provided new opportunities in the studies of the genetic structure of microbial communities, but at the same time highlighting the significant difficulties, arising particularly during the investigation of the soil metagenome (Pershina *et al*., 2013).

Using of signature RNA sequence (16S rRNA & 23S rRNA) data of small subunits of prokaryotic ribosome combined with refined molecular technologies to support microbial community identification have been investigated for long since it is known that these sequences are ubiquitous among all prokaryotic life forms, have

slow evolution rates, and more importantly consist both the conserved and variable regions (Man *et al*., 2013). In 2013, Pershina and colleagues stated that soil is the most densely populated habitat on the planet, can contain up to 1,000 Gbps of genetic information per gram leading to the great misfortune during the analysis of the soil metagenome.

Study and management of microorganisms in aquaculture have become a most famous practice from the last few decades. They have a great importance in fulfilling different roles in aquaculture ecosystem (Tilia *et al*., 2016). Use of microorganisms in aquaculture as environmental biomarkers, effluent-bioremediation, probiotics, and as a direct food source for the cultured species has expanded in the last decade (Ezemonye *et al*., 2009; Caruso, 2013; Martinez-Cordova *et al*., 2014). Bacterial communities responsible for many of the chemical processes that occur in earthen fish ponds are either the primary or secondary pathogens in many infectious diseases (Lightner, 1996). Management of pond sediment may be a key factor influencing the bacteriology of earthen fish ponds and health of fish during the culture period. For this, information about the microbial load and types of bacteria in pond sediments is essential (Al-Harbi and Naim, 2006).

Despite all these enormous potential, knowledge of microbial dynamics with respect to freshwater IMC culture systems and brackish water *Litopenaeus vannamei* culture systems is still remains unknown. The identification of such microorganisms those already harbored in the culture system can provide us a wide lens to understand; monitoring and accurate management of the essential processes carried by particular group of organisms, and it will surely lead to the better aquaculture practices to raise

the production of the cultured aquatic animals in the various aquaculture systems. The present study was conducted with following objectives:

I. To identify the different bacterial communities inhabiting sediments associated with fish and shrimp aquaculture activities, and

II. To understand the variations in the bacterial diversity in relation to culture duration.

2. Review of Literature

2.1 Metagenomics

Given that less than 1% of the total bacterial diversity is culturable, the adoption of newer culture-independent studies is on the rise. Metagenomic profiling is one such approach which offers rapid characterizing of clones corresponding to unidentified bacterial species based either on the sequence-based screening and/or function-based screening. The sequenced-based analysis involves sequencing of phylogenetic anchors that will indicate the taxonomic groups of microbial communities in particular environment. Sequencing of phylogenetic anchors can provide a link of phylogeny with the functional gene. While the function based screening of a microbial community does not require any marker/signature or targeted genes to identify the groups of microbes it totally dependent on the specific expression of any gene such as antibiotic resistance genes, degrading enzymes, Na+(Li+)/H+ transporters (Handelsman, 2004).

Many efforts have been made with traditional approaches such as modified culture media, culturing and isolation techniques, imaging or morphology to reveal information related to microbial diversity and dynamics; none of these are able to detect the vast unknown diversity of culture-independent microorganisms (Handelsman, 2004). Many efforts have been made with traditional approaches such as modified culture media, culturing and isolation techniques, imaging or morphology to reveal information related to microbial diversity and dynamics; none of these are able to detect the vast unknown diversity of culture-independent microorganisms (Handelsman, 2004).

The bacterial communities are generally identified by 16S rRNA gene sequencing. Its slow evolution rate and ubiquitous among all prokaryotic life forms serves it as a 'universal target' gene for bacterial identification and it is large enough (1,500 bps approx.) to contain statistically relevant information. The 16S rRNA gene consists of both conserved and variable regions. Conserved regions are used for designing the amplification primers and nine hyper variable regions (V1–V9) are used to identify phylogenetic characteristics of the communities (Man *et al*., 2013). 16S rRNA gene sequence is most widely used as signature gene sequence for profiling bacterial communities (Yang *et al*., 2016).

Taxonomic classification and phylogenetic analysis of sequences were done on the basis of similarity and dissimilarity of hyper variable regions of 16S rRNA gene. So the selection of the most efficient hyper variable regions 16S rRNA gene is the most important task in profiling of bacterial communities. It has been estimated that the V4 and V6 were the most reliable regions for representing the full-length 16S rRNA sequences in the phylogenetic analysis of most bacterial phyla, while V2 and V8 were the least reliable regions (Yang *et al*., 2016).

Molecular methods involving PCR amplification and cloning of 16S rRNA gene are the most effective technique available for describing the composition of complex microbial communities. Metagenomics in collaboration with next-generation sequencing technology has proven as a potent approach for providing the widest, unbiased view of microbial diversity in any environment, related to taxonomy and potential functioning (Basak *et al*., 2015). Metagenomic methods, especially targeting the 16S rRNA, have eliminated the limitations of traditional microbiological culturing methods as a means for describing the composition of the complex microbial

communities (Yakimov *et al*., 2005). Application of these technologies provides large datasets for microbial communities from various environments such as soil and ocean water (Simon *et al*., 2009).

However, some culture-independent studies were done for investigation of microbial diversity in various types of aquaculture systems based on 16S rRNA sequencing are, Grass carp culture pond (Zhou *et al*., 2012), Saline-alkali ponds of common carp *Cyprinus carpio* (Huang *et al*., 2014), Marine fish farms (Bissett *et al*., 2005), Polyculture of carps (Zhou *et al*., 2017), Comparision between sediment and fish gut of inland saline fish culture pond (Tyagi and Singh, 2017), freshwater farming of *Litopenaeus vannamei* (Tang *et al*., 2014). Various studies were successfully done from other environments like Mangrove sediments (Ghosh *et al*., 2010; Andreote *et al*., 2012; Basak *et al*., 2015), Lake sediments (Meena *et al*., 2015), Sediments from lakes, rivers and glaciers (Foong *et al*., 2010), Sea sediments (Verma *et al*., 2016; Nair *et al*., 2017), Seaport sediments (Pramanik *et al*., 2015).

2.2 Environmental metagenomics

All ecosystems functioning greatly depend on the different microbial load present in it. Microorganisms, especially bacteria play an important role in the smooth functioning and maintaining the balance in every ecosystem, by conducting all the vital biogeochemical processes of primary elements such as nitrogen, sulfur, phosphorus, and iron (Zhang *et al*., 2014). Moreover, the soil microorganisms represent a large portion of genetic diversity of the earth which still remains unknown largely (Whitman *et al*., 1998). It has been estimated that one gram of soil can hold up to 10 billion microorganisms of possibly thousands of different species (Roesch *et al*., 2007). However, up to 99% of bacterial species present in most of the environments

are not readily culturable (Martinez-porchas and Albores, 2015). Therefore, the vast part of microscopic life and its diversity is not accessible (Streit and Schmitz, 2004). Over the last decade, metagenomics has been used to understand the microbial communities by DNA sequencing (Mitra *et al*., 2011). Metagenomics is proving as a promising scientific tool to analyze the complex genomes contained within microbial communities (Nielsen *et al*., 2014).

Metagenomics reveals the previously hidden diversity of microscopic life as present in the environment. Microbial community structure analysis can provide a better understanding about the microbial population and their interactions in defined geographical regions (Ghosh *et al*., 2010). It depends on collecting DNA directly from environmental samples along with amplification of a signature sequence of organisms using polymerase chain reaction (PCR) with the help of second-generation sequencing (Wooley *et al*., 2010). Metagenomics can provide information about the diversity of the microorganisms thriving in a certain area and about their functions and biological roles (Martinez-Porchas and Albores, 2015). Such analysis is important with respect to understanding the microbial load in an ecosystem and defining the role of various microorganisms that are involved in ecological processes. This approach can avoid the limitations of the traditional culturing techniques for assessing the microbial diversity in the natural environments based on the use of general and selective media (Ghosh *et al*., 2010).

Metagenomic approach was successfully employed by Ghosh *et al*. (2010) for the identification of microbial communities in the mangrove sediments using a culture-independent approach. Bacterial communities were identified by comparing clones of partial 16S rRNA gene sequences with available 16S rRNA gene sequences

in the public database, for species-level identification at the similarity of ≥ 97% with that of the prototype strain sequence in the GenBank. The diversity of the partially sequenced 16S rRNA gene sequences was revealed by phylogenetic analysis. In this approach, they detected eight different phyla of the bacterial domain in the sediments with the dominance of Gammaproteobacteria in the mangrove sediments.

Another metagenomic survey showed the dominance of Gammaproteobacteria in mangrove sediments (Andreote *et al*., 2012) and indicated that the microbial core involved in methane, nitrogen, and sulphur metabolism consists mainly of *Burkholderiaceae*, *Planctomycetaceae*, *Rhodobacteraceae,* and *Desulfobacteraceae.*

Basak *et al*., (2015) performed 16S rRNA gene amplicon sequencing for profiling the bacterial community thriving at variable depths of mangrove soil sediment. The bacterial diversity in the mangrove sediments was analyzed by PCR amplification of V1–V3 regions of bacterial 16S rRNA gene. Metagenomic approach recovered 61,301 sequences of 2746 species belonging to 33 different phyla revealing the dominance of Proteobacteria, Firmicutes, Chloroflexi, Bacteroidetes, Acidobacteria, Nitrospirae, and Actinobacteria with 32.0 Mbps and 55.6% G + C content.

Meena *et al*., (2015) performed metagenomics analysis for the group wise diversity of methylotrophs of lake sediments. Metagenomic DNA from the sediments were amplified using GC clamp mxaF primers and resolved through DGGE, which revealed the diversity within the unculturable methylotrophic bacterium Methylobacterium organophilum, Ancyclobacter aquaticus, Burkholderiales and Hyphomicrobium sp. were reported. About % 90 of the methylotrophs found were unculturable.

Foong *et al*., (2010) determined the bacterial population of sediments and soils from the lakes, river, and glaciers using metagenomic approach. Using the 16S rRNA PCR, the predominant bacterial groups were found to be Bacteroidetes, Proteobacteria, Acidobacteria, Gemmatimonadetes, Nitrospira, Firmicutes, Actinobacteria, Chloroflexi, Cyanobacteria, Spirochaetes, Deinococcus-Thermus, WS3, and BRC. About 15% of the obtained operational taxonomic unit (OTUs) did not group into any of the existing phyla in the Ribosomal Database Project (RDP) and the OTUs had a similarity of < 0.90 compared to the GenBank sequences probably was a novel bacterium specific to that location.

Bacterial community composition in deep-sea sediment was explored by using V3 hyper-variable region of bacterial 16S rRNA gene by Verma *et al*., (2016). High-throughput sequencing of the 16S rRNA gene sequences obtained from the sediments revealed the presence of >44,000 OTUs in each sample, suggesting high bacterial diversity in the deep sea sediments. The complex composition of the bacterial communities in deep-sea sediments was dominated by the phylum Actinobacteria representing >20% of the taxonomically assignable OTUs, which were followed by Firmicutes, Proteobacteria, and Chloroflexi. The obtained 16S rRNA gene sequences, bacterial species, genus, and family/class were classified into OTUs at 3%, 5%, and 10% genetic distance or dissimilarity, respectively.

Pramanik *et al*., (2015) explored the bacterial diversity of seaport sediments based on the V1–V3 regions of the bacterial 16S rRNA genes. The study revealed 34,121 sequences of about 11,705 species belonging to forty different phyla with 56.3% G + C content. Community metagenome sequencing analysis showed the dominance of Bacteroidetes (23%) followed by Firmicutes (19%), and Proteobacteria

(17%), Spirochaetes (10%), Nitrospirae (8%), Actinobacteria (7%) and Acidobacteria (3%) was harbored in the port sediments.

2.3 Aquaculture metagenomics

Aquaculture can be considered as an artificial media for the proliferation of microorganisms (Martinez-Porchas and Albores, 2015). The continuously increasing development of world aquaculture requires new strategies and alternatives to achieve sustainability (Martinez-Cordova *et al*., 2014). Identification of bacterial communities associated aquaculture bottom may prove as one of the most viable strategies to achieve a sustainable aquaculture. For better husbandry, it is necessary to understand the bacterial and other microbial load that is associated with the aquaculture pond eco-systems which would directly or indirectly affect the farmed organisms. Metagenomic analysis of pond ecosystems can provide us a better understanding of communities harbored within pond ecosystem. Metagenomics is not yet commonly used in aquaculture sectors (Martinez-Porchas and Albores, 2015). However, some studies were done for investigating prokaryotic microbial diversity in various types of aquaculture systems by 16S rRNA sequencing. Grass carp culture pond (Zhou *et al*., 2012), Saline-alkali ponds of common carp *Cyprinus carpio* (Huang *et al*., 2014), Marine fish farms (Bissett *et al*., 2005), Polyculture of carps (Zhou *et al*., 2017), Comparison between sediment and fish gut of inland saline fish culture pond (Tyagi and Singh, 2017), freshwater farming of *Litopenaeus vannamei* (Tang *et al*., 2014). As outlined below.

Bacterial communities from the water column and sediments in grass carp (*Ctenopharyngodon idellus*) ponds were assayed by culture-independent method using V3 region of bacterial 16S rRNA sequence followed by PCR amplification

(Zhou *et al*., 2012). Thirty two different bacterial species were obtained from the samples belonging to seven phyla: Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria, Acidobacteria, Fibrobacteres, and Fusobacteria. Especially from the sediment samples, 12 ribotypes, were retrieved. Comparison of identified sequences to the sequences in genebank found 100% & 96% similarity to Pedobacter sp. & Terrimonas sp. respectively, both of them were grouped under the phylum Bacteroidetes. Sequences closest to Uncultured Acidobacterium sp. at 99% similarity was under the phylum Acidobacteria. Sequences closest to Uncultured Fusobacteriales bacterium found 98% similar with phylum Fusobacteria. Several Sequences were grouped into different genera with various similarity levels and fall under phylum Proteobacteria, similarity at 100% of Pseudomonas syringae, Asticcacaulis benevestitus, Rhodoferax sp. likewise Uncultured Geobacter sp. at 87% similarity (Zhou *et al*., 2012).

Huang *et al*., (2014) identified the bacterial diversity in saline-alkali ponds rearing common carp (*Cyprinus carpio*) by culture-independent technique, using the 16S rRNA gene clone libraries. The metagenomic analysis reveals most of the sequences in the saline-alkali rearing ponds were have low similarity with known bacterial 16S rRNA genes, and suggesting that these sequences may represent the novel bacteria. Most common and dominant sequences recovered for Proteobacteria (α–, β–, γ–), Actinobacteria, Cyanobacteria, Planctomycetes, Fibrobacteres, Bacteroidetes, Chloroflexi and unclassified bacteria. Sequence analysis showed that the bacterial diversity of the sequences belonging to β–Proteobacteria, α–Proteobacteria, and Actinobacteria were predominant in saline-alkali ponds rearing Common carp.

Bissett *et al*., (2005) used 16S rRNA gene clone library for analyzing bacterial diversity and community structure within organically enriched and unimpacted, nearshore marine sediments at two fish farms in southern Tasmania, Australia. Over 900 clones were analyzed and grouped into 631 different phylotypes suggesting a very high level of microbial diversity. The Sandy sediment below the cage aquaculture was dominated by the Cytophaga-Flavobacteria-Bacteroides (CFB) group but during the 3 month following period, the CFB group decreased and the Delta- and Alphaproteobacteria took on more dominant roles. The bacterial communities at Organic sediment cage were more reduced and had a higher background carbon level, showed a different response to organic load than those at Sandy sediment. Libraries were statistically different from one another ($P < 0.05$) and many of them did not group with cultured bacteria.

Tyagi and Singh, (2017) compared two 16S rRNA variable (V3 and V4) region-specific primer Pairs to understand microbial diversities in sediment and fish (*Labeo rohita*) gut of inland saline fish culture pond using next-generation sequencing (NGS) of 16S rRNA gene. Observed a biased characteristic of data sets generated separately from V3–V4 regions primers. Only 11 phyla were detected in a V3 dataset, while V4 primer dataset reported much diverse taxonomic compositions consisting of 62 phyla. The dominance of Proteobacteria (41%) followed by Verrucomicrobia (11%) and OD1 (11%). Among the phylum Proteobacteria in V4 dataset of sediment sample Deltaproteobacteria (45%), Gammaproteobacteria (26%) and Alphaproteobacteria (23%) were the dominant classes. And strictly anaerobic sulphur and sulphate-reducing bacteria were dominant in class Deltaproteobacteria. Other important phyla like Acidobacteria, Chloroflexi, Tenericutes, and Firmicutes were also detected in this approach.

Litopeneaus vannamei are highly susceptible to pathogenic microorganisms and interactions among shrimp pathogenic microorganisms and the aquaculture environment may results in infectious disease to the cultured organisms this will lead to severe economic losses in the shrimp aquaculture industries. So the amount and kinds of pathogenic microorganisms in the aquaculture environment are key factors in shrimp disease occurrence (Engering *et al*., 2013).

Tang *et al*., (2014) characterized the aquatic bacteria present in the water samples by polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) and 16S rRNA pyrosequencing using PCR amplification of the V3 variable region of the bacterial 16S rRNA gene. Aimed to reveal effects of environmental temperature on bacterial community composition in freshwater aquaculture system farming of *Litopenaeus vannamei* (FASFL). Bacterial population structure with variation over the seasonal changes Suggests that environmental temperature was a key driver of the bacterial population in the FASFL.

In another major study by Zhang *et al*., (2016) recovered 31 phyla, 66 classes, 90 orders, 213 families and 697 bacterial genera from the water and sediment of *L. vannamei* culture ponds using 16S rRNA gene sequencing to analyze Bacterial communities in the aquaculture environment. Sequences were clustered into OTUs defined at the 97% similarity threshold, bacterial communities in the sediments were classified in 31 phyla, 66 classes, 87 orders, 204 families and 619 genera with the dominance of Proteobacteria followed by Cyanobacteria, Firmicutes, Acidobacteria, Chloroflexi and Bacteroidetes suggesting higher microbial diversity in the sediment than in the water samples. Zhang *et al*., (2016) have also concluded that the bacterial richness increases with the culture duration.

3. Material and Methods

3.1 Aquaculture facilities selected for metagenomic profiling

The objectives of the present study were: (a) to identify the different bacterial communities inhabiting sediments associated with fish and shrimp aquaculture activities, and (b) to study changes in the communities with respect to culture duration, if any. Two different commercial aquaculture systems were targeted for metagenomic profiling, namely the IMC (Indian major carp) and *Litopenaeus vannamei* farms. The selection was done on the basis that the two systems represent diverse habitats freshwater and brackishwater. Both the fish and shrimp farms were selected from Andhra Pradesh- the aquaculture hub of India. In particular, while the IMC farms were selected from Krishna district, the *Litopenaeus vannamei* farms were selected from Bhimawaram area of the West Godavari district of Andhra Pradesh.

3.2 Sediment sample collection plan

Given the economically intensive nature of obtaining metagenomic profiles and also given the exploratory aim of this study, single composite sediment samples were prepared separately representing the select fish and shrimp farms. Each composite sample, in turn, represented approximately 8–10 sediment samples collected per facility per sampling event for the fish and/or shrimp farms (Meena *et al*., 2015). Utmost care was exercised while fixing the sediment collection spots for each pond for both the fish and shrimp farms. In particular, care was taken to avoid the feeding lines and/or areas where the unconsumed feed tended to accumulate within each individual pond. Furthermore, all sediment samples were collected just before the next scheduled feeding, especially for the final sampling. Both the fish and

shrimp farms were sampled twice during their respective farming cycle (Table 3.1 and 3.2). The first (initial) sample was obtained one day prior to the stocking of fish and/or shrimp seed in the respective farms. The second (final) sample was obtained one day prior to harvesting of the respective fish/shrimp crop. Thus, the final sediment sample for IMC (F2) was collected after 11 months and for *Litopenaeus vannamei* (S2) on the 115th DOC (day of culture). In order to compare the changes in the pond sediment microflora with respect to that of the native microflora, feeder or sediment samples of the source or feeder/supply canals were also obtained at the same time as that of collecting the first (initial) samples for both facilities. However, since the freshwater IMC farms were fed from artificial canals, no sediments were collected from the source in this case. The coding details of the samples collected during the present study are provided in Table 3.1 and 3.2.

3.3 Sample collection, handling, and storage

Samples were obtained with the help of a corer having a 50 mm diameter and 1.5 m length. The corer was inserted perpendicularly in the bottom sediments so as to obtain a core (sample) of about 15 cm of the top sediment (Ghosh *et al*., 2010). However, about 5 cm of the top soil was discarded from each core sample to avoid contamination due to accumulated faeces and/or unconsumed feed. Thus, about 10 cm of the bottom core was retained from each sample for making one composite sample each representing the fish and shrimp farm. Composite samples were prepared on the farm itself. For this, the collected samples were immediately laid on a large piece of HDPE sheet, where the individual samples were mixed thoroughly using a large spatula. About 100 g of the final composite (homogeneous) sample was finally weighed and sealed in fresh polythene bag and held on ice (to maintain the moisture

Table 3.1 Sediment sample from freshwater (IMC) fish farm (Krishna District, Andhra Pradesh)

Sr. no	Sample name	Sample code	Day of culture
1.	Initial	F1	0
2.	Final	F2	335

Table 3.2 Sediment sample from *Litopenaeus vannamei* farm Bhimawaram (West Godavari), Andhra Pradesh.

Sr. no	Sample name	Sample code	Day of culture
1.	Initial (Source)	SS	0
2.	Initial	S1	0
3.	Final	S2	110

content) till further processing (Zhang *et al*., 2009). On reaching the laboratory, the samples were maintained at 4^0C pending further analysis (Meena *et al*., 2015).

3.4 DNA extraction

DNA extraction from sediment samples was performed as described by Wilson (2001) with slight modifications. Briefly, 0.2–0.3 g of sediment sample was taken in a 1.5 ml of eppendorf tube and suspended in 200 µl of (100 mg/ml) lysozyme solution. The mixture was vortexed for 5 minutes and incubated overnight at 150 rpm and 37^0C. On the following day, the tubes were centrifuged at 14000 rpm for 20 minutes and the supernatant were carefully discarded. Thereafter, 400 µl of Edward's buffer and 20 µl of (20 mg/ml) of proteinase K were added to the tubes and vortexed for 5 minutes. Further the tubes were incubated at 55^0C for 2 hrs and centrifuged at 4000 rpm for 5 minutes. This was followed by collection of the supernatant which was carefully transferred to new centrifuge tube. Thereafter, 600 µl of phenol/chloroform/isoamyl alcohol mixture (25:24:1) was added to each tubes and centrifuged for 15 minutes at 10000 rpm at 4^0C. Again, the supernatant were collected in fresh centrifuge tube which was further treated with equal volume of chloroform-isoamyl alcohol mixture (24:1) followed by centrifugation for 15 minutes at 10000 rpm at 4^0C. The aqueous phase was carefully transferred to fresh centrifuge tube. Finally, DNA was precipitated by the addition of equal volume of chilled isopropanol. Resultant DNA pellets were recovered by centrifugation at 10000 rpm for 20 minutes at 4^0C. DNA pellets were washed twice with 200 µl of 70% ethanol. Further, the pellets were dried at 37^0C for 1 hr and final dilution was done in 30 µl of TE Buffer, and kept at 37^0C for 10 minutes. For each sample, DNA was extracted in triplicate.

3.5 DNA purification

About 3 µl of 3M sodium acetate along with 75 µl absolute alcohol was added in eppendorf tubes containing extracted DNA from soil samples and dissolved by vortexing for 10 minutes followed by the centrifugation at 10000 rpm for 30 minutes at 4^0C and further incubated for 10 minutes at 4^0C. The supernatant (approximately 98 µl) was then extracted and washed by adding 100 µl of 70% ethanol and gently vortexed briefly followed by centrifugation at 11000 rpm for 30 minutes at 4^0C. The DNA was then kept overnight for incubated at 37^0C overnight.

3.6 DNA quantification

Quality and quantity of the purified DNA obtained for each replicate was determined by loading about 2 µl of the DNA sample on spectrophotometer and by measuring the absorbance at 260/280 nm (for DNA/protein) and 260/230 nm (for DNA/humic acid) using Nanodrop ND–1000 spectrophotometer (JH BIO Innovations Pvt. Ltd.). This step was essentially performed to check for protein and humic acid contamination of the DNA.

3.7 16S rRNA amplification

16S rRNA amplification was performed for V3-V4 the paired hyper variable region of the 16S rRNA gene using region-specific primers with overhang adapters. The primers used for amplification of both hyper variable regions are given in Table 3.3.

Table 3.3 Primers used for the amplification of V3-V4 region of 16S rRNA gene

Name	Primer Sequence 5'- 3'	Reference
Forward Primer	TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGCCTACGGGNGGCWGCAG	Klindworth *et al*. (2013)
Reverse Primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGGACTACHVGGGTATCTAATCC	Klindworth *et al*. (2013)

3.7.1 PCR reaction mixture

Component	Volume
Microbial DNA (5 ng/µl)	2.5 µl
Amplicon PCR Forward Primer 1 µM	5 µl
Amplicon PCR Reverse Primer 1 µM	5 µl
KAPA HiFi Ready Mix	12.5 µl
Total	25 µl

3.7.2 Thermal regime of PCR

Thermal regime of PCR consisted of an initial denaturation step performed at 95^0C for 3 minutes followed by 25 cycles of denaturation at 95^0C for 30 seconds, primer annealing at 55^0C for 30 seconds, extension at 72^0C for 30 seconds. Final extension was given at 72^0C for 5 minutes and then held at 4°C.

3.8 16S rRNA sequencing and analysis

Sequences were recovered from the both aquaculture activities and quality metrics for raw reads were determine using FastQC. Low quality bases (Q = 30) and adapter (forward reads – u24 Reverse reads – u24) were removed using Cutadapt. The paired-end reads were assembled using PEAR. Chimeric sequences were removed from filtered reads using VSEARCH. Non-chimeric filtered sequences were processed further using QIIME (Quantitative Insights into Microbial Ecology). Reference-based OTU (operational taxonomic units) picking (97% identity) was done by using Uclust method based on SILVA reference database. Taxonomic assignment

to each representative OTU was done by the RDP classifier software with a 80% confidence threshold. The Biome Table was then constructed and rarefied for further analysis (Bhute *et al*., 2017).

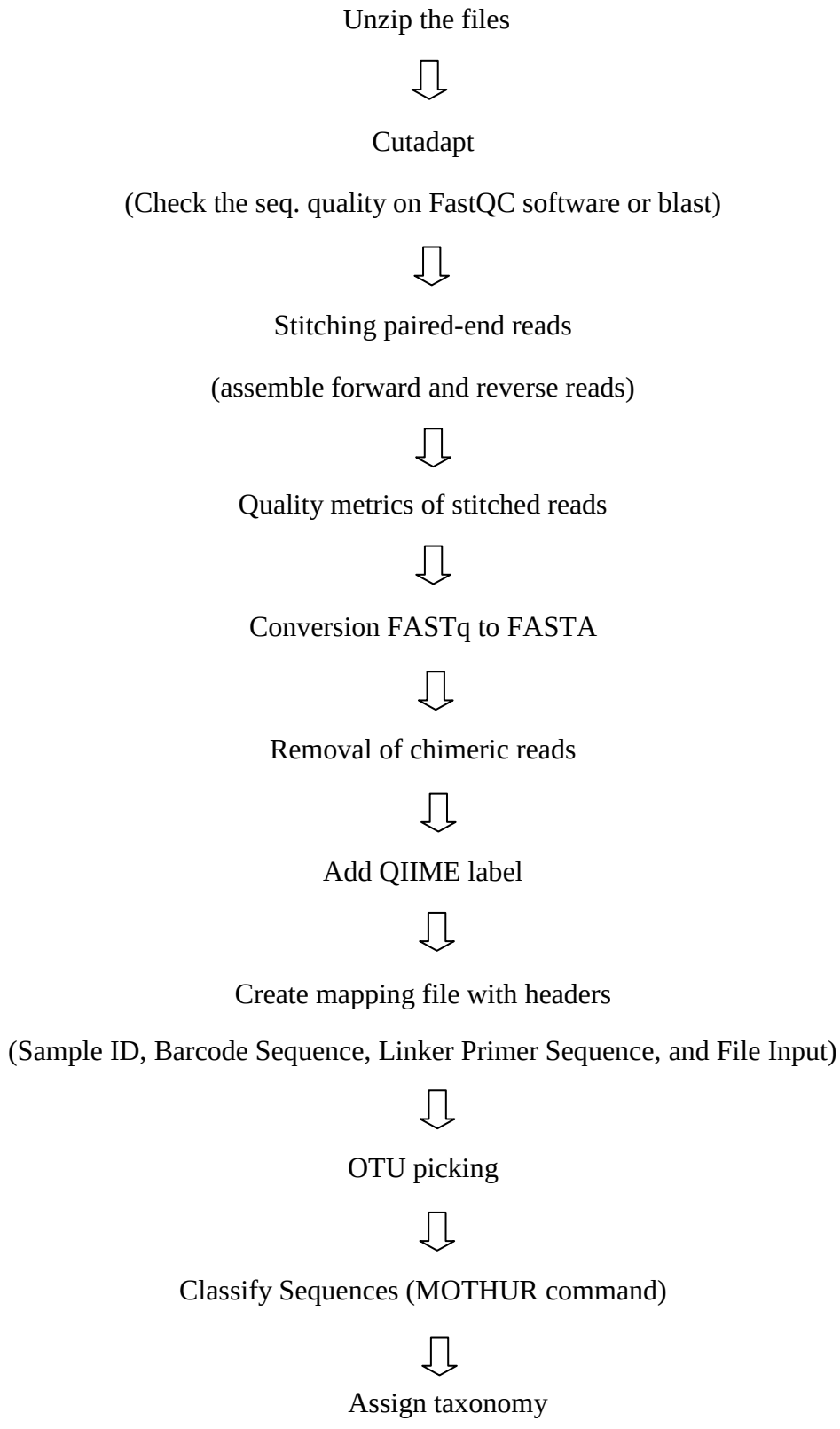
3.9 Bioinformatic analysis of the 16S rRNA marker gene sequences

The 16S rRNA sequences obtained were analyzed using QIIME software. QIIME is especially used for analyzing microbial communities based on 16S/18S rRNA gene amplicon sequencing data by clustering the marker gene nucleotide sequences into OTUs. Further, the software taxonomically annotates the OTUs by looking for sequences similar to them on a reference taxonomic database. The main output from the QIIME pipeline is the OTU table, which describes the microbial OTUs and their abundances in each of the samples. The *in-silico* pipeline for raw data analysis using QIIME is given in Fig. 3.1.

3.10 Statistical analysis

All bacterial abundance profiles were tested for normality of distribution using the Shapiro-Wilk's W-test. Accordingly, none of the profiles were found to conform to normality of data. Thus, statistical comparisons of the 'intial' and 'final' metagenomic profiles were performed using the non-parametric Mann-Whitney U-test. Furthermore, the trends for changes in the number of bacterial OTUs, phyla, and orders were performed using regression analysis. Obtained profiles and/or data on OTU, phyla, and order abundance were regressed on culture duration. For the shrimp farm, SS values were used as the initial reference. Thus, the trends were regressed using SS→S1→S2 as the reference time intervals. The magnitude of the trends (positive or negative slopes) was adjudged using the 'effect size' (ES) metric i.e., 'r^2' and based on the ES criteria as defined by Cohen (1998). No such comparisons i.e.,

Fig.3.1 *In-silico* pipeline for raw data analysis in QIIME



initial/final and/or regression analysis could be performed for the IMC farm samples since only a single metagenomic profile (F1) was successfully obtained. All analyses were performed an SAS (v.9.3).

4. Results

In this study, a total of five sediment samples were collected and assayed for their metagenomic profiles (Table 3.1 and 3.2). Despite meeting the stringent pre-quality checks and assaying each sample in duplicate, sample F2 (IMC farm, final) could not be processed successfully. Metagenomic profiles, thus, could be successfully obtained for sample F1 (IMC farm, initial), S1 (shrimp farm, initial), S2 (shrimp farm, final), and SS (shrimp farm, water source).

Metagenomic analyses of the four samples revealed the presence of a total of 3416 OTUs (operational taxonomic units) obtained from 1124212 QIIME QC filtered sequencing reads. The 3416 OTUs were partitioned as follows: 2094 OTUs (F1; IMC farm, initial), 1059 OTUs (SS; shrimp farm, water source), 1345 OTUs (S1; shrimp farm, initial), and 1298 OTUs (S2; shrimp farm, final) (Table 4.1). Given the singular nature of the IMC farm OTU profile, it was not possible to compare it (F1; IMC farm, initial) with that of the final (F2; IMC farm final) profile. Nonetheless, though altogether disparate in nature, the F1 profile was compared with that of S1 with a view to find out the number of OTUs shared or common between these two different aquaculture systems (Table 4.1). Surprisingly, the two systems were found to share about 27.5% common OTUs between them (Fig. 4.1a). This made up of about 35.4% of the total 2094 OTUs of F1. Thus, only about 64.6% of the F1 OTUs were entirely unique to the freshwater system. Comparisons conducted to find out the sharing of OTUs from within the shrimp farm samples revealed the following (Table 4.1). The SS/S1, S1/S2, and SS/S2 comparative profiles respectively shared 303 (12.8%), 149 (6.3%), and 103 OTUs (4.4%) in common. Furthermore, about 392 OTUs (16.6%)

Table 4.1 OTUs and their sharing between or within different culture systems

Sample	OTUs total	OTUs shared			OTUs unique	
		No.	%	with	No.	%
F1	2094	741	27.5	S1	1353	72.5
SS	1059	798	75.3	S1, S2	261	24.7
S1	1345	846	62.8	SS, S2	501	37.2
S2	1298	644	49.6	SS, S1	654	50.4

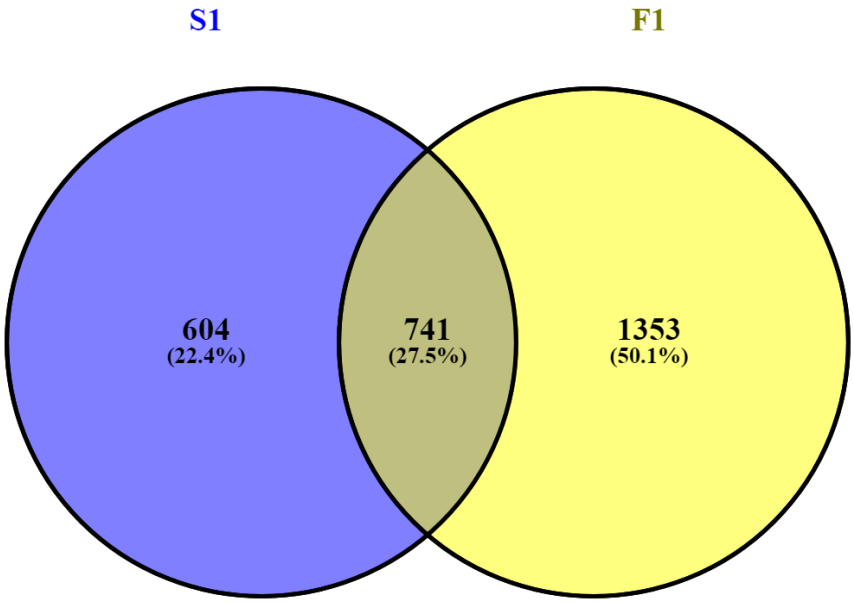


Fig. 4.1a Sharing of OTU's between fish farm (F1) and shrimp farm (S1)

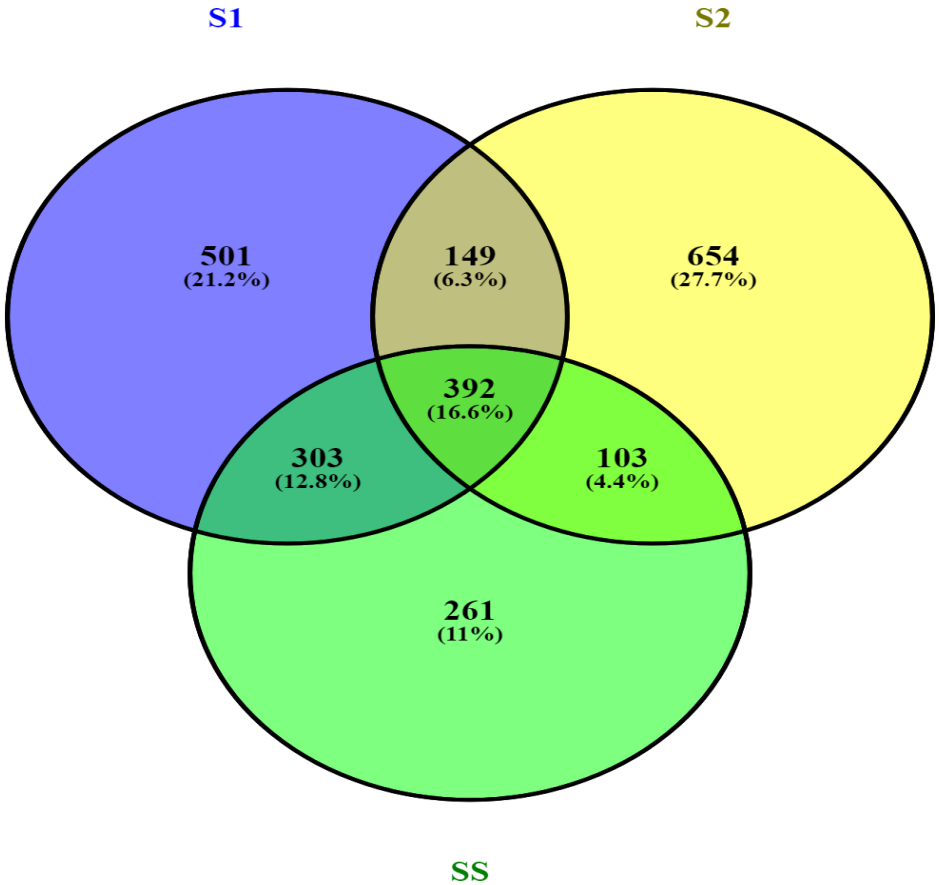


Fig. 4.1b Sharing of OTU's between shrimp farm samples

were common for the brackishwater environment as a whole i.e., considering SS, S1, and S2 as a single environment (Fig. 4.1b).

At phylum level, the QIIME analysis revealed that both the fish and shrimp farms were 99% dominated by Proteobacteria followed by Chloroflexi (0.2%), and others (Table 4.2; Fig. 4.2). Within the Proteobacteria, the Gammaproteobacteria was the most abundant class in both the freshwater and brackishwater environments with a relative abundance of about 95%. The Gammaproteobacteria were then followed by the Betaproteobacteria (1.9%), Alphaproteobacteria (0.7%), Deltaproteobacteria (0.5%) in terms of relative abundance (Table 4.2; Fig. 4.2). All phyla having relative abundance of greater than 0.01% were designated as major phyla and those with abundance less than 0.01% each were all clubbed together and denoted as 'Other_phylum' (Table 4.2 and Fig. 4.2).

At order level, about 50% of diversity remained "unclassified" for both aquaculture types, and this major group was designated as 'Unclassified_order' in both Table 4.3 and Fig. 4.3. Besides this, the other major orders contributing to the bacterial community structure of the pond sediments of both system types were: Aeromonadales (23.7%), Alteromonadales (8.30%), Enterobacteriales (7.82%), Pseudomonadales (3.6%), aaa34a10 (0.58%), Rhodocyclales (0.55%), Rhizobiales (0.29%), Desulfuromonadales (0.20%), Vibrionales (0.05%), and other minors groups (Table 4.3; Fig. 4.3). Statistical comparisons between the 'initial' and 'final' metagenomic profiles could not be undertaken for the IMC farm sediments since no profile could be obtained for the F2 sample (IMC farm, final). Similar comparisons between SS/S1, SS/S2, and S1/S2 revealed no significant difference based on the Mann-Whitney U-

Table 4.2 Phylum level relative abundance (%) of bacterial communities in collected samples

Sr. no	phylum	SS	S1	S2	F1
1	Proteobacteria_Gammaproteobacteria	95.68	96.22	97.57	95.73
2	Proteobacteria_Betaproteobacteria	3.58	0.72	0.08	0.38
3	Proteobacteria_Alphaproteobacteria	0.27	0.06	0.09	2.43
4	Proteobacteria_Deltaproteobacteria	0.04	1.82	0.02	0.11
5	Other_unclassified	0.11	0.61	0.23	0.68
6	Proteobacteria_Other	0.26	0.43	0.28	0.29
7	Chloroflexi	0.00	0.04	0.73	0.04
8	Actinobacteria	0.01	0.02	0.45	0.22
9	Firmicutes	0.04	0.05	0.12	0.06
10	Nitrospirae	0.00	0.00	0.10	0.01
11	Planctomycetes	0.00	0.02	0.02	0.03
12	Acidobacteria	0.00	0.00	0.03	0.01
13	Aminicenantes	0.00	0.00	0.03	0.01
14	Gemmatimonadetes	0.00	0.00	0.02	0.00
15	Other_phylum (pooled phyla with a relative abundance of < 0.01% each)	0.00	0.00	0.04	0.00

Fig. 4.2 **Phylum level relative abundance (%) of bacterial communitiesin collected samples**

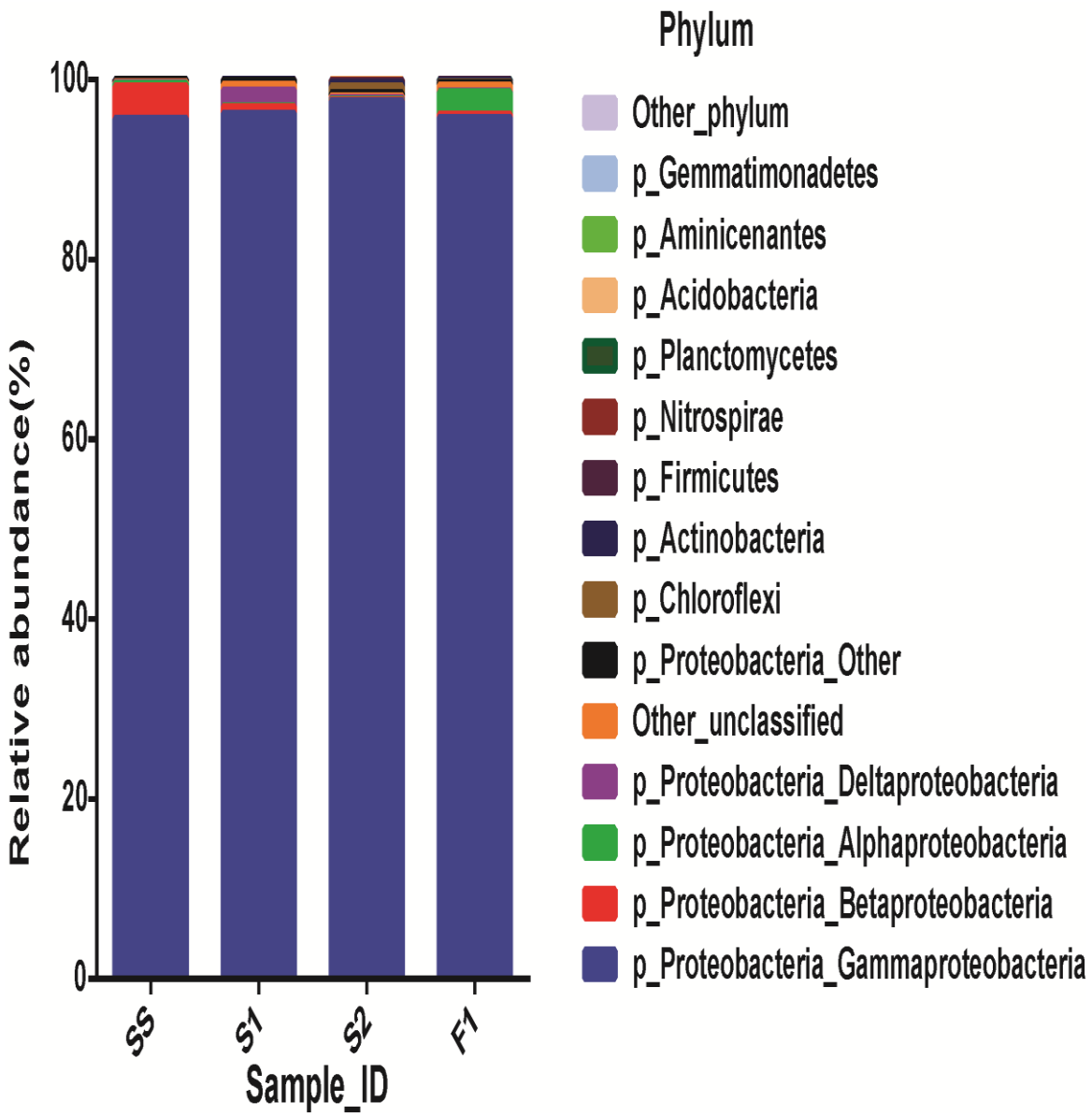


Table 4.3 Order level relative abundance (%) of bacterial communities in collected samples

Sr. no	Order	SS	S1	S2	F1
1	Unclassified_orders	50.00	50.00	50.00	50.76
2	Aeromonadales	36.42	29.99	18.00	11.01
3	Alteromonadales	0.75	0.47	26.28	5.79
4	Enterobacteriales	1.15	6.12	0.52	23.63
5	Other (uncultured; classified only at order level)	5.51	3.28	2.87	6.02
6	Pseudomonadales	4.22	6.95	1.39	2.11
7	aaa34a10	0.02	1.86	0.01	0.47
8	Rhodocyclales	1.72	0.33	0.01	0.16
9	Rhizobiales	0.00	0.00	0.01	1.16
10	Desulfuromonadales	0.02	0.80	0.01	0.00
11	Vibrionales	0.02	0.00	0.06	0.15
12	Rhodospirillales	0.12	0.02	0.01	0.06
13	Acidimicrobiales	0.00	0.00	0.08	0.01
14	Bacillales	0.01	0.01	0.05	0.02
15	Anaerolineales	0.00	0.01	0.07	0.00
16	Chromatiales	0.00	0.00	0.07	0.00
17	MSBL5	0.00	0.00	0.07	0.00
18	Xanthomonadales	0.00	0.01	0.03	0.04
19	Gaiellales	0.00	0.00	0.05	0.01
20	Uncultured soil bacterium	0.00	0.00	0.06	0.00
21	Desulfovibrionales	0.00	0.05	0.00	0.01
22	Nitrospirales	0.00	0.00	0.05	0.00
23	Desulfobacterales	0.00	0.01	0.02	0.02
24	Propionibacteriales	0.00	0.00	0.04	0.00
25	Myxococcales	0.00	0.00	0.02	0.01
26	Hydrogenophilales	0.00	0.00	0.03	0.00
27	Sva0485	0.00	0.00	0.02	0.00

28	Rhodobacterales	0.00	0.00	0.03	0.00
29	Syntrophobacterales	0.00	0.01	0.01	0.01
30	Clostridiales	0.00	0.01	0.01	0.00
31	Coriobacteriales	0.00	0.00	0.02	0.00
32	Cellvibrionales	0.00	0.02	0.00	0.00
33	Solirubrobacterales	0.00	0.00	0.00	0.01
34	Planctomycetales	0.00	0.01	0.00	0.01
35	SZB30	0.00	0.01	0.00	0.00
36	Burkholderiales	0.01	0.00	0.00	0.00
37	uncultured actinomycete	0.00	0.00	0.01	0.01
38	Pla1 lineage	0.00	0.00	0.01	0.00
39	Desulfarculales	0.00	0.00	0.01	0.00
40	Other_order (pooled order with a relative abundance of < 0.01% each)	0.02	0.02	0.08	0.05

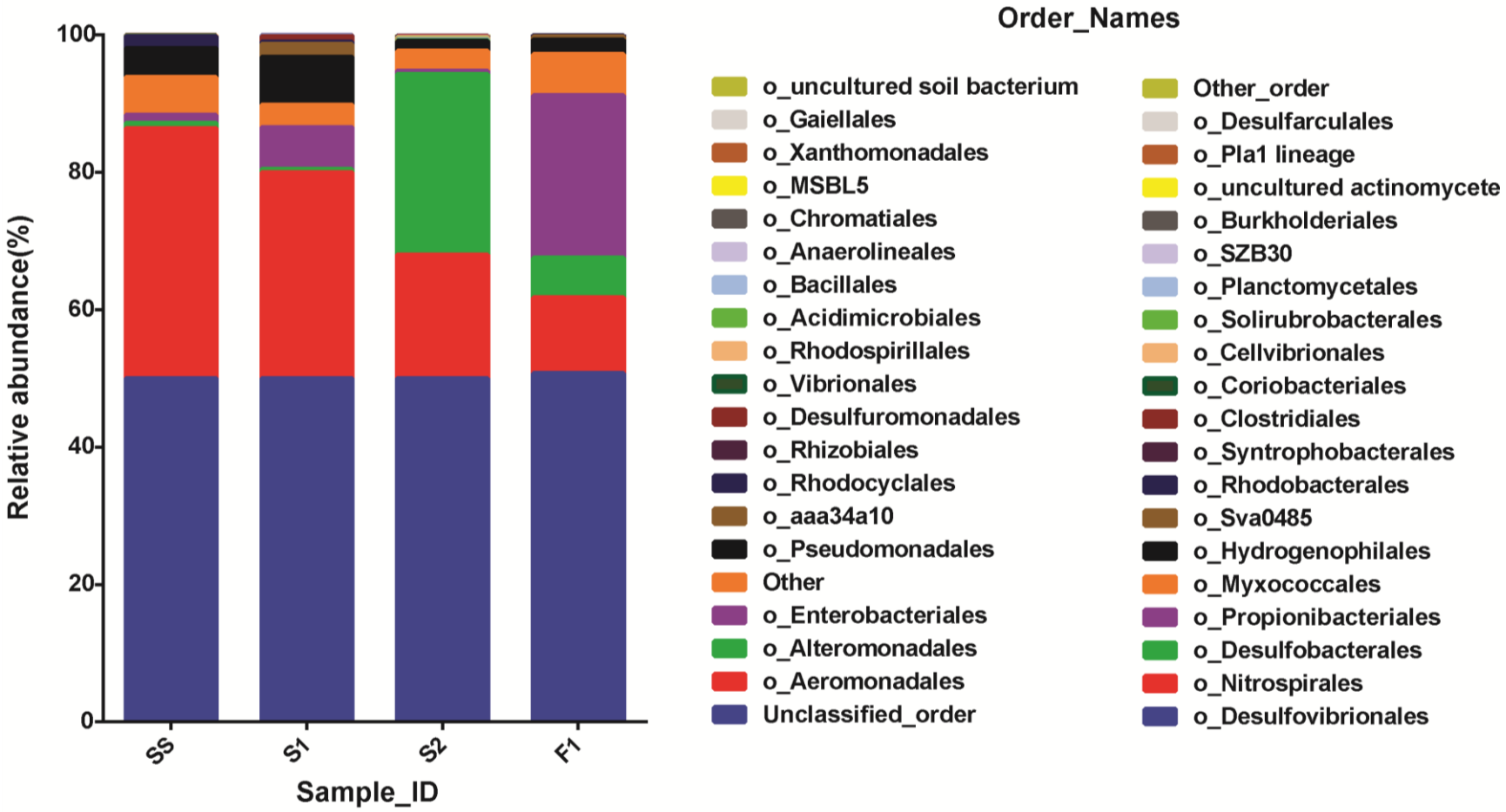


Fig. 4.3 Order level relative abundance (%) of bacterial communities in collected samples

test ($P > 0.05$). However, trend analyses with respect to culture duration were performed with respect to the shrimp farm sediments (S1 and S2) taking the shrimp farm source sediment (SS) as the starting (reference) profile. Thus, changes in the overall trends in the OTUs (total, shared, and unique), in the bacterial phyla, and in the bacterial orders were regressed over the culture duration for shrimp farm were recorded for (SS→S1→S2) (Table 4.4). Furthermore, trends with respect to changes in the relative abundance with reference to the culture duration both at phylum level (Table 4.5) and order level (Table 4.6) were recorded for all identified phyla and orders. Also, the ES magnitudes for the obtained r^2 values based on Cohen (1988) for the three regression analyses have been given for: (a) overall trends in OTUs, phyla, and orders (Table 4.4), (b) phylum-specific trends (Table 4.5), and (c) order-specific trends (Table 4.6). All these trends were analysed over the SS→S1→S2 culture duration for the shrimp farm sediments. All the overall trends displayed a 'large' ES with respect to OTUs (total, shared, or unique), phyla, and orders (Table 4.4). However, about 73% and 80% of the phylum- (Table 4.5) and order-based (Table 4.6) profiles accounted for 'large' ES, respectively.

Table 4.4 Overall trends in the bacterial OTUs, phyla, and orders with respect to shrimp culture duration (SS →S1→S2)

Sr. no	Particulars	Slope	r^2	Effect size magnitude
1	OTUs (overall)	119.5	0.60	L
2	OTUs (shared)	– 77.0	0.53	L
3	OTUs (unique)	196.5	0.98	L
4	Phylum (all with relative abundance > 0.01%)	– 0.4	0.80	L
5	Order (all with relative abundance > 0.01%)	– 0.4	0.77	L

Table 4.5 Phylum wise trends in the relative bacterial abundance for shrimp farm (SS →S1→S2) profiles

Sr. no	Phylum	Slope	r^2	Effect size magnitude
1	Proteobacteria_Gammaproteobacteria	0.94	0.94	L
2	Proteobacteria_Betaproteobacteria	– 1.75	0.88	L
3	Proteobacteria_Alphaproteobacteria	– 0.09	0.61	L
4	Proteobacteria_Deltaproteobacteria	0.08	0.01	S
5	Other_unclassified	0.06	0.06	S
6	Proteobacteria_Other	0.09	0.01	S
7	Chloroflexi	0.36	0.78	L
8	Actinobacteria	0.22	0.07	M
9	Firmicutes	0.04	0.87	L
10	Nitrospirae	0.05	0.74	L
11	Planctomycetes	0.01	0.88	L
12	Acidobacteria	0.02	0.82	L
13	Aminicenantes	0.01	0.75	L
14	Gemmatimonadetes	0.01	0.80	L
15	Other_phylum (pooled phyla with a relative abundance of < 0.01% each)	0.02	0.84	L

Table 4.6 Order wise trends in the relative bacterial abundance for shrimp farm (SS →S1→S2) profiles

Sr. no	Order	Slope	r^2	Effect size magnitude
1	Unclassified_order	< 0.001	0.25	L
2	Aeromonadales	– 9.21	0.97	L
3	Alteromonadales	12.76	0.74	L
4	Enterobacteriales	– 0.32	0.01	S
5	Other	– 1.39	0.86	L
6	Pseudomonadales	– 1.42	0.26	L
7	aaa34a10	– 1.00	< 0.001	S
8	Rhodocyclales	– 0.86	0.88	L
9	Rhizobiales	0.003	0.69	L
10	Desulfuromonadales	– 0.01	0.00	L
11	Vibrionales	0.02	0.49	L
12	Rhodospirillales	– 0.06	0.86	L
13	Acidimicrobiales	0.04	0.79	L
14	Bacillales	0.02	0.74	L
15	Anaerolineales	0.03	0.80	L
16	Chromatiales	0.03	0.70	L
17	MSBL5	0.03	0.78	L
18	Xanthomonadales	0.02	0.85	L
19	Gaiellales	0.03	0.76	L
20	uncultured soil bacterium	0.03	0.77	L
21	Desulfovibrionales	< 0.001	0	-
22	Nitrospirales	0.02	0.74	L
23	Desulfobacterales	0.01	0.95	L
24	Propionibacteriales	0.02	0.75	L
25	Myxococcales	0.01	0.87	L
26	Hydrogenophilales	0.01	0.75	L
27	Sva0485	0.01	0.88	L

28	Rhodobacterales	0.01	0.80	L
29	Syntrophobacterales	0.00	0.99	L
30	Clostridiales	0.00	0.18	M
31	Coriobacteriales	0.01	0.75	L
32	Cellvibrionales	< 0.001	0	-
33	Solirubrobacterales	< 0.001	0	-
34	Planctomycetales	0.00	0.04	S
35	SZB30	0.00	0.01	S
36	Burkholderiales	– 0.005	0.75	L
37	uncultured actinomycete	0.00	0.96	L
38	Pla1 lineage	0.00	0.95	L
39	Desulfarculales	0.01	0.79	L
40	Other_order (pooled order with a relative abundance of < 0.01% each)	0.03	0.79	L

5. Discussion

The present study was conducted to explore the different bacterial communities associated with the fish and shrimp aquaculture activities, and to understand, if possible, the variations in bacterial diversity (relative abundance) in relation to culture duration. A total of four metagenomic profiles were obtained: F1 (IMC farm, initial), SS (shrimp farm, water source), S1 (shrimp farm, initial), and S2 (shrimp farm, final).

Depending on the type of environment (e.g., freshwater or brackishwater), the fish farm sediment sample (F1) returned the highest numbers of OTUs (operational taxonomic units) at 2094. Thus, 61.3% of the total OTUs reported in the present study were represented by the freshwater IMC fish farm (initial) sediments. For the brackishwater shrimp farm, the source water sediments (SS) displayed the least number of OTUs (31%) for all shrimp farm sediment samples considered together. The S1 and S2 contributed 39.3% and 38% of the OTUs respectively. It has been estimated that about 1g of soil can hold up to 2000 to 5000 OTUs (Roesch *et al*., 2007). The 2094 OTUs were encountered in about 0.2g of the sediment sample. Several researchers have recovered thousands of OTUs with respect to different aquaculture activities using 16s rRNA gene sequencing. For example, Tyagi and singh, (2017) recovered 6679 OTUs by applying next-generation sequencing (NGS) metagenomics approach in carp (*Labeo rohita*) aquaculture sediments. In another study on four different types of freshwater aquaculture systems, about 3701 and 11,150 OTUs were identified from the water and sediment samples respectively by Zheng *et al*. (2016). Zhang *et al*., (2016) obtained 5039 OTUs from the shrimp aquaculture sediments, which was quite high as compared to the mean number of

OTUs of 1235 for the SS, S1, and S2 samples taken together in the present study. However, no any justification for such lower representation could be ascribed based on the currently available inputs.

Phylum level comparisons of obtained profiles revealed the predominance of Proteobacteria with a minimum and maximum relative abundance of 97.95% (S2) and 99.59% (SS), respectively (Table 4.2). The phylum was represented by four major classes namely Gammaproteobacteria, Betaproteobacteria, Alphaproteobacteria, Deltaproteobacteria. However, the Gammaproteobacteria was the most dominant class accounting for about 95.68% (SS) to 97.57% (S2) of the total bacterial abundance. Numerous studies have reported the dominance of Proteobacteria in different environments of up to 83.6% in freshwater lake (Zhang *et al*., 2014), up to 62.3% in sea sediments (Liao *et al*., 2008), up to 71% mangrove sediments (Ghosh *et al*., 2014). Andreote *et al*. (2012) found major abundance of Proteobacteria (47.1–56.3%) followed by Firmicutes (10.5–13.8%), Actinobacteria (5.4–12.2%), Bacteroidetes (3.8–11.8%), and Chloroflexi (1.3–5.4%) in mangrove environment. Among Proteobacteria, the most frequent class was the Gammaproteobacteria (32.6–42.6%), followed by Deltaproteobacteria (29.5–40.0%), Alphaproteobacteria (7.5–18.6%), Betaproteobacteria (2.2–9.3%), and Epsilonproteobacteria (2.3–20.0%). In general, the freshwater grass carp ponds (Zhou *et al*., 2012), freshwater fish polyculture ponds (Zheng *et al*., 2016), and *Litopenaeus vannamei* ponds (Zhang *et al*., 2016) all have reported the predominance of Proteobacteria in the pond environments, which was also true for the fish and the shrimp farms in the present study.

At order level, about 50% of the bacterial diversity remained unclassified for both fish and the shrimp farms (Table 4.3). Statistical comparisons did not reveal any

differences in the profiles based on culture duration for the shrimp farm sediments ($P > 0.05$). In specific, the Mann-Whitney U-test did not reveal any differences in the related profiles. The same was also true for the earlier phylum level comparisons.

Despite this, the present study, however, provided some new insights into the bacterial microbiome associated with the two aquaculture systems. With respect to the OTUs, the freshwater environment turned out to be more diverse as compared to the brackishwater with 2094 OTUs. Another surprising observation was that the freshwater and the brackishwater environments, though entirely diverse, shared about 27.5% of OTUs in common. Thus, only about 1353 (72.5%) OTUs were entirely unique to the freshwater system (Table 4.1). Furthermore, for the shrimp farm related sediments, the number of unique OTUs for the final (S2) sample was almost double that of the source water (SS) sediment sample (Table 4.1).

Trend analysis were performed for shrimp farm samples to analyse some important and ecologically meaningful changes occurring in the aquaculture microbiomes with respect to the overall number of bacterial OTUs (total/shared/unique), number of phyla, and number of orders (Table 4.4), relative abundance of phyla in shrimp farm (Table 4.5), and relative abundance of various bacterial orders (Table 4.6). Both Table 4.5 and 4.6 are in relation to the shrimp farm culture duration. The significance of the trends was adjudged using the ES benchmarks as defined by Cohen (1988). For r^2 values, the benchmarks are: small ($r^2 = 0.01$), medium ($r^2 = 0.09$), and large ($r^2 = 0.25$). In short, ES metrics are independent of the sample size and denote the magnitude or strength at which a phenomenon exists in the population rather than its sample (Murphy *et al*., 2014). With respect to the OTUs, there was an increasing trend in the number of overall

OTUs and in the number of unique OTUs when regressed on the shrimp culture duration and both these trends accounted for a 'large' effect size with r^2 of 0.60 and 0.98, respectively (Table 4.4). At the same time, the culture duration had a large effect in terms of reduction in the number of shared OTUs. From this a couple of things become clear: (a) the shrimp farm preparation activities alter the normal microbiome (SS→S1), and (b) that the culture duration further modifies the microbiome (S1→S2) thereby completely altering the original OTU makeup. At phylum level, 15 phyla displayed large effect sizes with respect to their trends in relation to culture duration (SS→S1→S2). Thus about 73% of the identified phyla (11 out of 15) appear to have a significant ecological role in aquaculture environments. Comprehensive negative trend for all phyla (having relative abundance of > 0.01%) taken together also displayed a large ES for SS→S1→S2 comparisons (Table 4.4). At order level, 32 (out of 40) i.e., about 80% of the bacterial orders displayed a large effect size (Table 4.4). For all orders taken together, the comprehensive trend was negative but with a large ES having r^2 of 0.77.

The ecological significance of these prominently 'large' trends is especially difficult to interpret given the complex and varied nature of the species identified in terms of OTUs but with little or no specific information to go about. Also, about one-half of the bacterial classes (50%) remained unclassified, which further complicated any concrete deduction. Table 5.1 provides additional insights into the complexity of the problem. Based on the same it is evident that the Proteobacteria (Gammaproteobacteria), which was the most dominant class for the both aquaculture environments, consists of four Orders, 21 families, 156 genera and 59,697 species (NCBI, 2018). Thus, for a group this complex it is only possible to highlight very broad based and synoptic primary ecological role(s) in relation to the aquatic

Table 5.1 Details of major bacterial orders encountered in the present study based on NCBI database (https://www.ncbi.nlm.nih.gov/) [accessed on June 30th, 2018]

Phylum	Class	Order	Family	Genera	Species
Proteobacteria	Gammaproteobacteria	Aeromonadales	2	11	2975
		Alteromonadales	8	40	9368
		Enterobacteriales	8	87	15719
		Pseudomonadales	3	18	31635
	Betaproteobacteria	Rhodocyclales	3	20	466
	Deltaproteobacteria	Desulfuromonadales	2	7	125
	Alphaproteobacteria	Rhodospyrilales	3	45	2093
Firmicutes	Bacilli	Bacillales	9	130	45961

environments. Further, Table 5.2 highlights some such key ecological roles ascribed against the important groups identified in the present study.

Given the exceptionally high numbers of unclassified groups (orders) of bacteria in the present study (Table 4.3), and also given the meager information available with respect to the functions of several classified and/or unclassified groups with respect to aquatic environments, further detailed studies targeting each of these bacterial groups separately are warranted. Such studies only would enable us to understand whether the trends as observed in the present study at the given magnitudes (Table 4.5 and 4.6) are conducive to aquaculture productivity of these systems, or otherwise. Also, a lot of standardization is still required with respect to obtaining bacterial DNA from the sediments for such analysis to be successful. For example, despite several attempts at obtaining DNA from F2 sample (IMC farm, final) no DNA could be obtained even after working with replacement samples. It is possible that humic acid and other contaminants may possibly be interfering with the DNA isolation.

Overall, the present study which was largely of an exploratory nature has provided us some novel insights into the aquaculture microbiome. Some concrete observations emanating from this study are that: (a) the routine culture practices and/or culture durations modify or alter the pond sediment microbiome, (b) the Phylum Proteobacteria is the most dominant group in both the fish and shrimp aquaculture farms at about ~ 99%, (c) the Class Gammaproteobacteria is the most among the Proteobacteria (~ 95%), and (d) the changes in the microbiome at the level of bacterial OTUs, phyla, and orders are of a ‘large’ magnitude in terms of their effect

Table 5.2 Broad functions of the major bacterial groups encountered in the present study.

Phylum	Class	Major functions	Reference
Proteobacteria	Gammaproteobacteria	• Oil & hydrocarbon degraders	Ghosh *et al*., 2010
		• Sulfur oxidizers • Bioconversion of sulphur • Organic carbon oxidation	Nair *et al.*, 2017
	Betaproteobacteria	• Nitrogen transformations	Andreote *et al*., 2012
	Deltaproteobacteria	• Sulfur and sulphate reducing bacteria	Andreote *et al*., 2012; Ghosh *et al*., 2010
	Alphaproteobacteria	• Nitrogen transformations	Nelson *et al*., 2016
		• Organic matter degradation	Nair *et al.*, 2017
Firmicutes	Bacilli	• Methane transformations	Andreote *et al*., 2012
Chloroflexi		• Fermentative metabolism	Mcllroy *et al*., 2017
		• Hydrocarbon decomposer	Zhang *et al*., 2017
		• Carbon metabolism	Krzmarzick *et al*., 2013

size. Further studies would eventually make it possible for us to understand and enable us to suitably manage/modify the aquaculture microbiome in more beneficial ways thereby adding to the overall sustainability and productivity of the various aquaculture systems. Thus, metagenomic profiling of aquaculture sediments appears to be a promising tool with great potential for favorably monitoring and/or manipulating the sediment microbial ecology of the various pond-based aquaculture systems and with respect to different environments, species, and culture durations.

6. Summary

Culture of food fishes seems to be the only way to fulfill the increasing demand for aquatic products given the stagnating or declining capture fisheries production. In 2014, the production of food fishes from aquaculture surpassed the production of capture fisheries (FAO, 2016). Proper husbandry practices are necessary for the successful culture of all commercial species. In the pond-based aquaculture systems, sediment or pond bottom is the zone which is highly dynamic and where the greatest numbers of aquatic microbes are found (Al-Harbi and Naim, 2006). All biogeochemical and metabolic processing of waste in aquaculture systems is carried out by the pond microbiome. Thus, the bacterial composition of the pond sediments affects the water quality as well as the quality of the aquaculture produce (Moriarty, 1997). Therefore, most fish and shrimp farms resort to maintaining sediment health in addition to water quality for pond aquaculture (Boyd, 2004).

During last two decades, metagenomic profiling has emerged as a promising scientific tool to analyze the complex genomes contained within microbial communities (Martinez-Porchas and Albores, 2015). A deeper understanding about population structure, genetic diversity, and the ecological roles played by particular groups of microorganisms (Bashir *et al*., 2014) helps in better management of different aquaculture system. In this study, metagenomic profiling of bacterial diversity of both aquaculture facilities was done by sequencing of V3–V4 hyper variable regions of 16S rRNA gene with the following objectives:

I. To identify the different bacterial communities inhabiting sediments associated with fish and shrimp aquaculture activities, and

II. To understand the variations in the bacterial diversity in relation to culture duration.

16S rRNA gene sequencing revealed total 3416 OTUs from both aquaculture activities including water source (SS). Since the F2 (IMC farm, final) sample could not return quality DNA it was not possible to compare the 'initial' (F1) and 'final' (F2) bacterial profiles for the IMC aquaculture activity. However, comparison of 'initial' profiles of fish (F1) and shrimp (S1) farms revealed the presence of about 27.5% shared OTUs (operational taxonomic units). OTU comparisons between the shrimp farm samples indicated 12.8% (SS/S1), 6.3% (S1/S2), and 4.4% (SS/S2) common OTUs, respectively.

Both aquaculture activites were found to be dominated by phylum Proteobacteria (99%), Chloroflexi (0.2%), and other minor groups. In Proteobacteria, the Gammaproteobacteria occupied 95% of the total abundance followed by Betaproteobacteria (1.9%), Alphaproteobacteria (0.7%), and Deltaproteobacteria (0.5%). At order level, about 50% of bacterial profiles remained unclassified. The average relative abundance of other major orders were: Aeromonadales (23.7%), Alteromonadales (8.30%), Enterobacteriales (7.28%), Pseudomonadales (3.6 %), aaa34a10 (0.58%), Rhodocyclales (0.55%), Rhizobiales (0.29%), Desulfuromonadales (0.20%), and others. Statistical comparisons between the 'initial' and 'final' metagenomic profiles (SS→S1→S2) revealed no significant difference based on the Mann-Whitney U-test ($P > 0.05$). Thus, trends with respect to changes in the relative abundance with reference to the culture duration both at phylum level and order level were recorded in terms of regression analysis. In short, the overall trends in the OTUs (total, shared, and unique), in the bacterial phyla, and in the bacterial

orders were regressed over the culture duration of shrimp farm. All the trends displayed a ‘large’ ES with respect to OTUs (total, shared, or unique), phyla, and orders. Additionally, about 73% and 80% of the phylum- and order-based trends with respect to culture duration accounted for ‘large’ ES, respectively.

Overall, the present exploratory study has provided us some novel insights into the aquaculture microbiome. Some concrete observations emanating from this study are that: (a) the routine culture practices and/or culture durations modify or alter the pond sediment microbiome, (b) the Phylum Proteobacteria is the most dominant group in both the fish and shrimp aquaculture farms at about ~ 99%, (c) the Class Gammaproteobacteria is the most among the Proteobacteria (~ 95%), and (d) the changes in the microbiome at the level of bacterial OTUs, phyla, and orders are of a ‘large’ magnitude in terms of their effect size.

Further detailed studies are necessary to understand and enable us to suitably manage/modify the aquaculture microbiome in more beneficial ways thereby adding to the overall sustainability and productivity of the various aquaculture systems. Thus, metagenomic profiling of aquaculture sediments appears to be a promising tool with great potential for favorably monitoring and/or manipulating the sediment microbial ecology of the various pond-based aquaculture systems and with respect to different environments, species, and culture durations.

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