MOLECULAR CHARACTERISATION OF MICROBIAL COMMUNITIES ASSOCIATED WITH LIVE FEED AND FISH LARVAE IN A COMMERCIAL FISH FARMING SYSTEM

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JUNE 2014

MOLECULAR CHARACTERISATION OF MICROBIAL COMMUNITIES ASSOCIATED WITH LIVE FEED AND FISH LARVAE IN A COMMERCIAL FISH FARMING SYSTEM

By

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A dissertation submitted to the Department of Chemical Engineering,
Faculty of Engineering and Science,
Universiti Tunku Abdul Rahman,
in partial fulfillment of the requirements for the degree of
Master of Science
June 2014

Dedication

I would like to dedicate this piece of work to my love ones who encouraged and supported me in the journey of completing this work.

ABSTRACT

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Tan Eng Tiek

The characterisation of microbial communities associated with live feed and fish larvae in a commercial fish farming system is an important step to understand the diversity of microbes, how they function and their benefits to the system or the harm that they bring. Characterisation of such microbial communities in recent years have shifted to molecular methods therefore provides a more thorough understanding of the microbial communities within a particular environment and allows the detection of unculturable microorganisms. There has been lack of research on the microbial communities associated with live feed and fish larvae in the commercial fish farming environment which is crucial for the maintenance of a healthy environment for fish larvae to survive and reduce mortalities. In this study, water source, live feed and fish larvae were being examined to understand the diversity and effect of the microbial community in the system in which adult fish faeces have been recycled as food source for the live feed (*Moina* sp.) and the live feed was subsequently fed to fish larvae. Clone libraries of Groundwater, *Moina* Culture Water, Fish Culture Water, *Moina* sp., Fish Larvae and Broodstock Culture Water were subjected to richness estimator, S_{Chao1} to determine the suitability of 16S rDNA library size where all samples from this study achieved an asymptotic curve which indicated that the clone library established was sufficient. Detection of bacteria from the phylum of

Actinobacteria, Armatimonadetes, Bacteroidetes, Candidate division OP10 which is closely related to *Armatimonadetes, Chloroflexi, Cyanobacteria, Fibrobacteres, Firmicutes, Planctomycetes, Proteobacteria*, *Spirochates* and *Verrumicrobia* shows the diversity of phylotypes commonly detected in a commercial fish farming system. The highest diversity of phylotypes was seen in *Moina* Culture Water followed by *Moina* sp., Broodstock Culture Water, Fish Culture Water, Fish Larvae and finally Groundwater. The presence of bacteria based on their phylum was observed from one tank to the other especially between *Moina* Culture Water, Fish Culture Water, *Moina* sp. and Fish Larvae suggest a carry-over of microbes. A clear indication of carry-over effect was found where *Morganella morganii* was detected in both *Moina* sp. and Fish Larvae samples. The presence of *Acinetobacter* in Groundwater samples, *Moina* Culture Water samples, Fish Culture Water samples and Fish Larvae samples indicate the ingestion of bacteria by fish larvae and carry-over of microbial communities. This can be either from the water source or through live feed. The presence of *Actinobacteria*, *Chytophagia*, *Erysipelotrichi*, *Planctomycetia* and *α-Proteobacteria* in *Moina* Culture Water samples were not detected in *Moina* sp. samples might imply that the bacteria were digested by *Moina* sp. Based on the findings, there were no pathogenic bacteria linked to the microbial communities identified in this study however, there were opportunistic pathogens detected. It is strongly suggested that a sustainable and cost-effective fish farming practice could be established by recycling fish faeces as food source for live feed and feeding of live feed to fish larvae.

ACKNOWLEDGEMENT

To be able to complete this project after three years has been a bitter sweet experience. Towards the end of this project during writing up, I personally felt that the experiences and knowledge gained throughout the entire process was abundance. There is no full stop in life and gaining knowledge as long as we are breathing.

I would sincerely like to thank my project supervisor, Assoc. Prof. Dr. Alan Ong Han Kiat for his unparalleled patience and invaluable guidance. Without his advice and guidance, the entire project would not be complete and may not have been completed. My sincere thanks to my co-supervisor, Assoc. Prof. Dr. Hii Siew Ling who does not say much normally but when she does, her advice and opinions were highly valuable. Many thanks as well to Assoc. Prof. Dr. Gideon Khoo who provided assistance and precious advices during the initial stages of this project.

Not to forget Prof. Dr. Tom Smith and Mr. Patrick Harrison from Sheffield Hallam University for their valuable tips and ideas during their visit to Malaysia as well as my short stint in Sheffield Hallam University. I would like to extend my thanks to Dr. Malcolm Lock and Ms. Claire Bradshaw for their tips and warm hospitality during my trip in Sheffield Hallam University too.

Last but not least, I would like to pen down my gratitude and thanks to my parents, Mdm. Lillian Ho and Mr. Robert Tan for their unconditional love, patience and guidance who made me who I am today. I would like to thank a very good friend and companion Mr. Ryan Tai for accompanying and assisting me on many occasions.

Special thanks to my friends and family, especially Mr. Ivan Chye, Mr. Vincent Loong, Mdm. Juwairiyah Ho and Dr. Ghazali Taib who provided me moral support and Ms. Swan Sow who had assisted me throughout the project.

I would like to thank the management of Recirculating Aquaculture System Sdn. Bhd. in Semenyih for their assistance during sample collection, laboratory officers and staffs in UTAR attached to Faculty of Engineering and Science, Setapak; Faculty of Science, Kampar and Faculty of Medicine and Health Sciences, Sungai Long. My sincere apologies and thanks to those whom I failed to mention here or crossed boundaries, nevertheless your assistance and encouragement has made this possible.

Finally, my thanks to Universiti Tunku Abdul Rahman (UTAR) for making this project possible by providing research funding under UTARRF Vote No. 6200/A04.

APPROVAL SHEET

This thesis entitled "**MOLECULAR CHARACTERISATION OF MICROBIAL COMMUNITIES ASSOCIATED WITH LIVE FEED AND FISH LARVAE IN A COMMERCIAL FISH FARMING SYSTEM"** was prepared by TAN ENG TIEK and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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SUBMISSION OF DISSERTATION

It is hereby certified that **TAN ENG TIEK** (ID No: **09UEM09182**) has completed this dissertation entitled **"MOLECULAR CHARACTERISATION OF MICROBIAL COMMUNITIES ASSOCIATED WITH LIVE FEED AND FISH LARVAE IN A COMMERCIAL FISH FARMING SYSTEM"** under the supervision of **ASSOCIATE PROFESSOR DR. ALAN ONG HAN KIAT** (Supervisor) from the Department of Pre-Clinical Sciences, Faculty of Medicine and Health Sciences, and **ASSOCIATE PROFESSOR DR. HII SIEW LING** (Co-Supervisor) from the Department of Chemical Engineering, Faculty of Engineering and Science.

I understand that the University will upload softcopy of my dissertation in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(TAN ENG TIEK)

DECLARATION

I, TAN ENG TIEK hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____
(TAN ENG TIEK)

Date _____

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
δ	Delta
ε	Epsilon
%	Percent
SO_4^{2-}	Sulphate
AMO	Ammonia Monooxygenase
amoA	Ammonia-Oxidising Gene A
AOB	Ammonia Oxidising Bacteria
AOB	Ammonia Oxidising Bacteria
BCW	Broodstock Culture Water
BLAST	Basic Local Alignment Search Tool
bp	Basepair
CCA	Canonical Correspondence Analysis
cDNA	Complementary Deoxyribonucleic Acid
CO_2	Carbon Dioxide
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
EDTA	Ethylenediaminetetraacetic acid
F	Fish Larvae
FAO	Food and Agriculture Organization of the United Nations
FAOSTAT	FAO Statistics

FCW	Fish Culture Water
gDNA	Genomic Deoxyribonucleic Acid
GW	Groundwater
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilo basepair
LB	Luria-Bertani
M	*Moina* sp.
MCW	*Moina* Culture Water
MEGA	Molecular Evolutionary Genetics Analysis
MEGAN	Meta Genome Analyzer
MSA	Multiple Sequence Alignment
n.d	No date
NAP3	Third National Agriculture Policy
NCBI	National Centre of Biotechnology Information
NH3	Ammonia
NJ	Neighbour-Joining
NO_2	Nitrite
NO_3	Nitrate
O_2	Oxygen
OD	Optical Density
P	Phosphorus
PAUP	Phylogenetic Analysis Using Parsimony
PCA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
PyNAST	Python Nearest Alignment Space Termination

RAS	Recirculating Aquaculture System
rDNA	Ribosomal Deoxyribonucleic Acid
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolution per minute
rRNA	Ribosomal Ribonucleic Acid
sdH_2O	Sterile distilled water
SDS	Sodium Dodecyl Sulfate
SINA	Silva Incremental Aligner
SOC	Super Optimal broth with Catabolite repression
sp.	Species
TAN	Total Ammonia Nitrogen
TBE	Tris-Borate-EDTA
TE	Tris Ethylenediaminetetraacetic acid
T-RFLP	Terminal Restriction Fragment Length Polymorphism
U/µL	Unit per microlitre
UV	Ultra violet
V	Volt
vol	volume
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside
ZIA	Aquaculture Industrial Zone

CHAPTER 1

INTRODUCTION

Aquaculture or also known as fish farming has become more important in recent years. The very purpose of aquaculture is to meet the demand for fishes by society. Fishes are seen as a natural source of protein and it is a healthier choice compared to other types of meat available. Aquaculture system has been a more viable choice because culturists have more control over the fish culture environment and they are able to grow fish all year round under optimal conditions (Dunning et al., 1998) as compared to traditional pond culture which consumes large quantities of water (Losordo, Masser & Rakocy, 1992).

There are many factors that need to be taken into consideration in order to ensure that the fishes in the aquaculture industry are healthy and fit for human consumption. These factors include but are not limited to the quality of water source used for culturing, culture environment, fish feed, microflora content and other physiochemical properties. These factors give an indication as to how well the fishes are adapting to the environment. Care must be employed in every stage of the fish growth to avoid the fishes from premature death. As such, this study aims to enhance and deepens knowledge on fish farming at the beginning of the fish cycle which is the fish larvae culture stage.

In this study, the larvae of *Clarias gariepinus* were used. *Clarias gariepinus* is also commonly known as African catfish and alternatively known to the locals of Malaysia as *Ikan Keli Afrika*. Catfish is a popular freshwater fish among the natives of Malaysia especially Malays and it is commonly available in other ASEAN countries such as Brunei, Cambodia, Indonesia, Laos, Myanmar, the Philippines, Singapore, Thailand and Vietnam. Catfishes are highly sought after due to their reasonable and affordable price.

Culturing of fish larvae requires certain amount of knowledge, extensive preparations and care from the operator because fish larvae are very fragile and they are exposed to many environmental threats. This is essential to culture healthy fish larvae. Due to the high risk and requirement in culturing fish larvae, it is considered one of the most profitable stages in fish farming because prices of fish larvae depends on the availability of stocks and the risk involved. Live feed was used as an initial food for the larvae. This is because live feed contributes to faster growth and survival. Besides that, live feed is also more attractive to the larvae as compared to commercial pellets.

Moina macrocopa which was fed with recycled fish faeces has been chosen as the live feed based on the study conducted by Loh et al. (2009) because of its availability and potential to be enriched with fish faeces. Moreover, it is a cheaper source compared to other cladocerans. *Moina macrocopa* has also been reported as a potential bio-carrier to dispense medication to fishes (Wiwattanapatapee et al., 2002). From this study, it is hope that there will be discovery of any carry-over of bacteria from the live

feed to the fish larvae as the live feed has been enriched by adult fish faeces.

The survival of fish larvae culture is very dependent on the environment and in addition to the use of live feed that has been enriched using adult fish faeces carries a whole range of possibilities which includes but not limited to the microbial communities that flourishes under the culture conditions. The fact that environmental factors and culturing method may cause carry-over of microbial communities from one tank to the other warrants a study to determine if there are any risk involved.

The aim of the study is to characterise the microbial communities in the commercial fish farming system associated with larvae culture fed with live feed and in water culture. Molecular characterisation of such microbial communities allow better understanding towards the system and better control of associated microflora may result in more cost efficient and higher quality larvae.

With the characterisation of microbial communities in the system, a correlation between enrichment source for live feed, the live feed itself and fish larvae mortality should be observed. Once established, a sustainable system could be in place whereby the fish faeces can be safely used to culture live feed and the enriched live feed will be used to feed fish larvae.

Main Objectives:

- Detection of bacterial communities in various water sources used in the system
- Detection of bacterial communities in live feed (*Moina sp*.) and fish larvae.
- Comparison of bacterial diversity or species richness within and among water sources, live feed and fish larvae.
- Identification of the potential pathogens and beneficial bacteria in culture environment and system.
- Identify carry-over effect of bacterial communities between water sources, live feed and fish larvae.

CHAPTER 2

LITERATURE REVIEW

2.1 Aquaculture

Aquaculture is referred to as the systematic cultivation of natural produce of aquatic habitats (Kinsey, 2006). According to Food and Agriculture Organization of the United Nations (FAO), aquaculture growth has been tremendous which makes it probably the fastest growing food-producing sector.

At the moment, aquaculture accounts for nearly 50 percent of the world's food fish as reported by FAO. Based on FAOSTAT, fish consumption in Malaysia was 1,365,708 tonnes in 2003 but the marine fishes supplied were only at 1,283,256. There is a difference of 82,452 tonnes. Data from FAO for year 2010 in Malaysia sees an increase in both captured and cultured fishes whereby 1,433,427 tonnes of fishes, crustaceans and molluscs were captured and 373,151 tonnes were produced by aquaculture method. This proves that aquaculture is the key to supply the protein need of the citizens.

The aquaculture sector has been given a tremendous boost in Malaysia based on the Third National Agriculture Policy (NAP3) which has been running since 1998. An Aquaculture Industrial Zone (ZIA) has been created to zone land and coastal areas that have been identified as suitable for the development of commercial scale aquaculture projects (Department of Fisheries, 2009).

The policy aims to increase the production of fishes, prawn and shell fish. Another important objective of this policy is to increase the net income of aquaculturist to a minimum of RM3000 per month (Department of Fisheries, 2009). Besides increasing the production, the aquaculture industry is also targeted to contribute to national income and exportation and last but not least it is to alleviate poverty by maximizing producers' income (Othman, 2006).

According to the Department of Fisheries Malaysia, aquaculture in Malaysia is divided into freshwater ponds, cages, ex-mining pools, cement tanks, canvas tanks and pen culture (Department of Fisheries, 2007). The focus of this research will be on the commercial fish farming system where the larvae fish are being cultured using tanks in association with live feed and bacterial communities.

2.1.1 Fish Aquaculture

Fish farming has been widely carried out in the aquaculture field. It is very important for operators and researchers to understand the life cycle of fishes. This is simply because at every stage of the fish life cycle, the fishes are subjected to physical and environmental threats. Fish life cycle includes the growth of fish from eggs to larval stage, larval to fry, then to juvenile and finally to adult or also known as grow out stage (Michigan Sea Grant, 2008). Figure 2.1 shows production cycle of *Clarias gariepinus* (FAO, 2010), while Figure 2.2 shows the stages of fish from egg to juvenile (Johnson & Allen, 2005).

During the life cycle of the fish, fishes are exposed to many types of threats typically from the physical conditions of the environment. These threats include but are not limited to changes in water temperature and oxygen levels, flooding or sedimentation, predators and diseases (Michigan Sea Grant, 2008). The main focus is on the fish larvae culture stage of catfish.

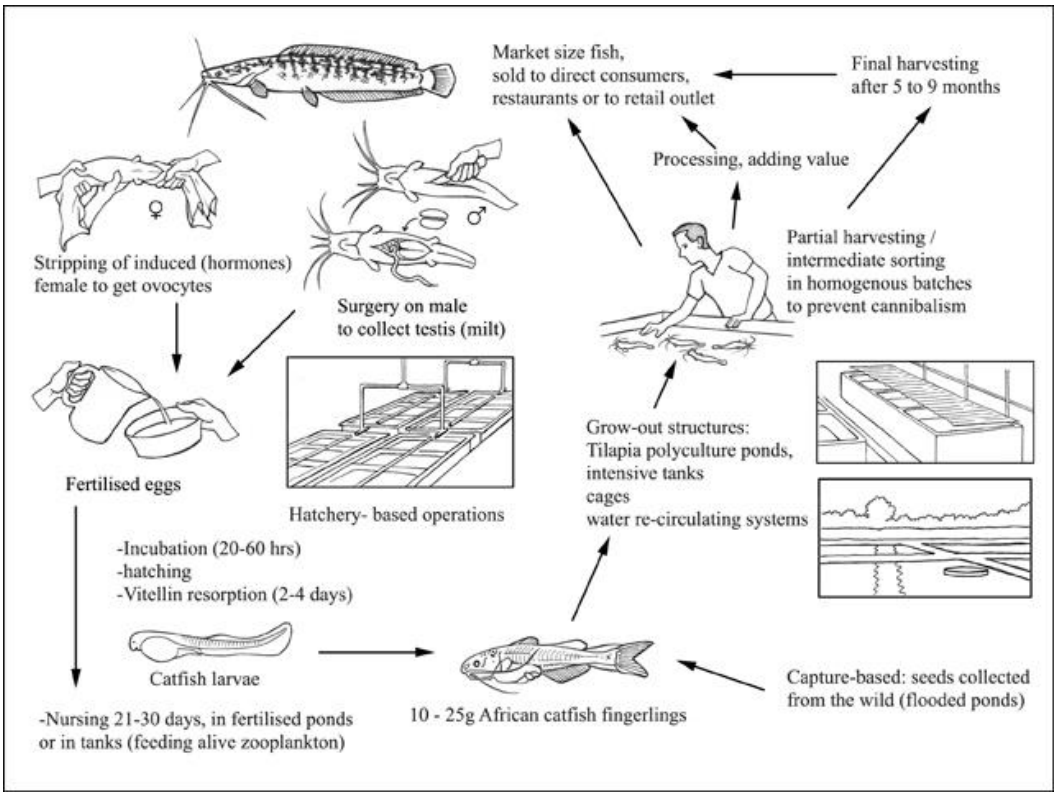


Figure 2.1: Production Cycle of *Clarias gariepinus* (FAO, 2010).

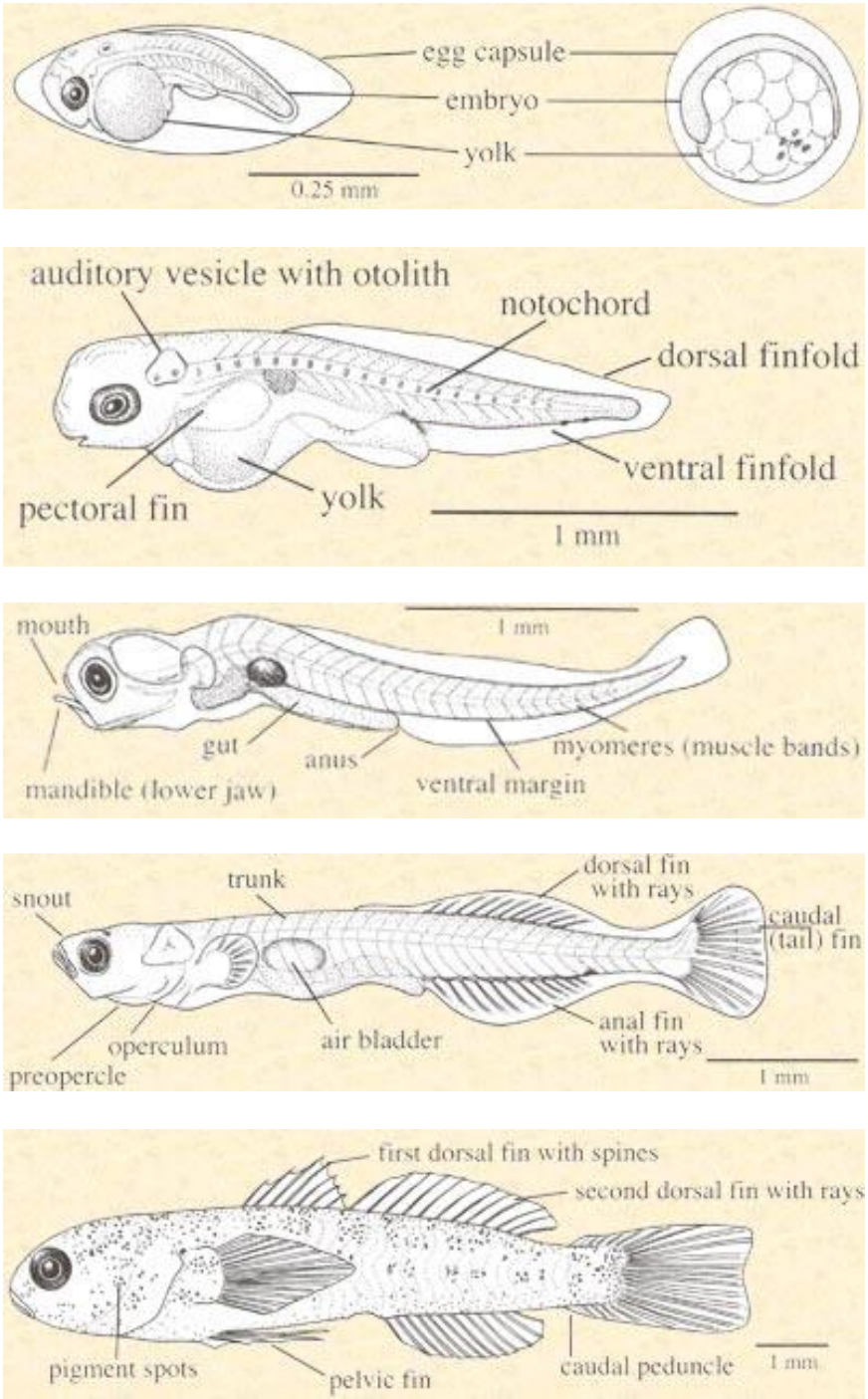


Figure 2.2: *Gobiosomo bosc.* life stages from egg to juvenile stage (Johnson & Allen, 2005).

Fish larval is considered the most difficult stage to establish large number of juveniles for aquaculture as the fish larvae do not have the ability to feed on larger food such as commercial pellet (Matsuda & Takenouchi, 2007). Besides that, there are certain physical conditions such as presence of pathogenic bacteria and water conditions which affect the growth of the larvae. Matsuda and Takenouchi (2007) has also expressed the same idea as Ludwig (1999) indicating that larval fish culture is considered one of the riskiest phases for culture of freshwater fishes but can be one of the most profitable stages. According to Ludwig (1999), operators must secure a dependable larvae supply and facility appropriate for fry and fingerlings, right kind and quantity of food. These steps are taken to ensure that the larval fish survives through the early stage, grow into healthy juveniles and finally into an adult.

2.2 *Clarias gariepinus* (North African Catfish / African Sharptooth Catfish)

Clarias gariepinus or better known as the African catfish has become an important species farmed around tropical and subtropical freshwater. The catfish has been widely introduced in the world. It can be found from South Africa and northern Africa. Besides that, they have been introduced to Europe, the Middle East and parts of Asia. *Clarias gariepinus* migrate within streams and rivers which are known as potamodromous. They are normally found in quiet waters, lakes and pools but may also be found in fast flowing rivers (Appelbaum & Kamler, 2000).

The catfish is very adaptive to extreme environmental conditions and able to live in pH ranging from 6.5 to 8.0. Catfishes have the ability to live in turbid water and tolerant to temperature from as low as 8°C to as high as 35°C (Gunder & Fink, 2004). Figure 2.3 refers to the classification of *Clarias gariepinus.*

Kingdom: Animalia (animals)

Phylum: Chordata (Chordates)

Subphylum: vertebrata (Vertebrates)

Superclass: Gnathostomata (Jawed vertebrates)

Class: Actinopterygii (ray-finned fishes)

Subclass: Neopterygii

Superorder: Ostariophysi

Order: Siluriformes (catfishes)

Family: Clariidae (airbreathing catfishes)

Genus: Clarias (walking catfishes)

Species: Gariepinus (North African catfish)

Figure 2.3: Classification of *Clarias gariepinus* (Myers et al., 2008).

Clarias gariepinus (Burchell 1822) or African catfish is one of the most important species currently being farmed. It is a species native to tropical and subtropical fresh waters has also been farmed in heated waters not within its native range (Appelbaum et al., 2000). It has rapid growth rate even at high densities, has the ability to breathe air, able to withstand poor water quality

conditions, and has tasty flesh makes *Clarias gariepinus* is an excellent candidate for aquaculture. Figure 2.4 is an image of *Clarias gariepinus*.

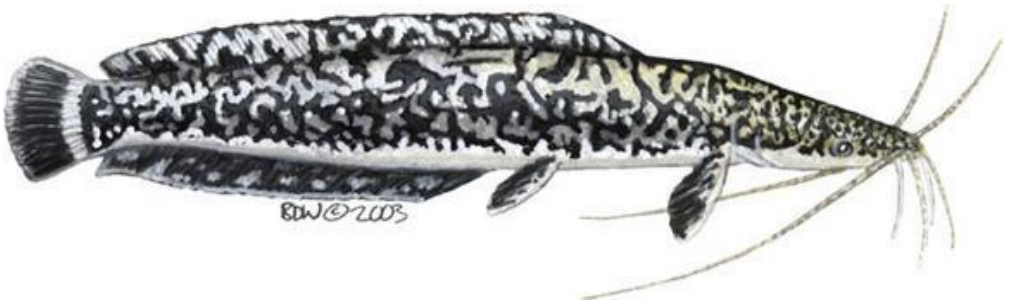


Figure 2.4: *Clarias gariepinus* (Myers et al., 2008).

2.3 Live Feed

In order to maintain high survival rate of larval fishes, the right type of food needs to be supplied to them in fish farming. Normally, fish pellets are fed to fishes that are in their juvenile stages. This is simply because commercial pellets do not fit into the mouth of fish larvae and they are not attractive enough for the smaller fishes. As for larval fishes of catfish, they are normally fed on live feed which are primarily aquatic insects (Wellborn, 1988).

Zooplankton is one of the primary foods for larval fish in nature and the two most dominant zooplankton groups used as feed are Rotifera (rotifers) and a sub-class of the Crustacea, Copepoda (copepods) (Treece & Davis, 2000). Based on Treece (2000), *Artemia* which is a zooplankton is used as live feed in aquarium trade and for marine finfish and crustacean larval culture. Besides *Artemia*, copepods, daphnia (*Moina micrura*) are also used as live feed for larval fish. Treece (2000) has reported that *Artemia* is one of the

easiest to prepare and it is the most nutritious food available for rearing larval finfish and crustaceans despite the pricing.

Loh et al. (2009), has indicated that several classes of zooplanktonic organisms such as rotifers, brine shrimp, copepods and cladocerans (water fleas) occupies an important position in aquatic food web of tropical countries as live feed to fish and crustacean larvae. According to Loh et al. (2009) too, the water flea *Moina macrocopa* fed on recycled fish faeces, showed great potential as live feed for the larviculture industry.

While Wang et al. (2008) reported that larval loach (*Misgurnus anguillicaudatus*) fed with *Moina micrura* treated with microparticle diets had the highest survival rate compared to microparticle itself and untreated *Moina micrura* and *Moina micrura* treated with live chlorella (*Chlorella pyrenoidosa*). Fish faeces are not only seen as a cultivation medium for *Moina* but recycling the natural waste products help to conserve the environment better as lesser waste from commercial fish farms need to be treated.

The usage of live feed such as *Moina* sp. fed with recycled fish faeces for larval fishes can be applied to maintain a sustainable fish farming or aquaculture system as it is environmental friendly and cost effective. Besides being used as a live feed, *Moina macrocopa* has been used by Wiwattanapatapee et al. (2002) as a novel biocarrier for norfloxacin (antibiotic treatment) in aquaculture due to the fact that conventional method of

dispensing drugs into the system has some disadvantages, as such practice promotes resistance of bacterial strains.

However, the practice of incorporating live feed into the culture system can unintentionally introduce pathogenic bacteria into the system (Ringø & Birkbeck, 1999). Thus, additional care or precaution has to be taken into consideration in order to minimise the risk of culturing.

2.4 Aquatic Bacterial Diversity

Aquatic bacterial diversity are usually the natural flora which exists in the water used, bacteria introduced through live feed or other means such as handling of fish farming system which includes but not limited to the treatment or culture conditions. In non-extreme aquatic habitats, usually bacteria represents >90% of the microorganisms (Hahn, 2006).

2.4.1 Inland Waters Bacterial Diversity

According to Hahn (2006), inland waters include lakes, ponds, rivers, streams, wetlands and groundwater which can either consist of freshwater or saline water. Based on a study by Nishimaru and Nagata (2007), the most abundant group of bacteria in their study at Lake Biwa was *α-Proteobacteria* with a lesser presence of *β-Proteobacteria* and *Actinobacteria*. Chloroplast, *Cyanobacteria*, *Deinococcus-Thermus*, *Bacteroidetes*, *β-Proteobacteria* and *Nitrospirae* were found in river biofilms (Lyautey et al., 2005).

Briée et al. (2007) through their study on archael and bacterial community from suboxic freshwater pond detected five subdivisions of *Proteobacteria* (α, β, γ, δ and ε) and other groups of bacteria such as *Acidobacteria, Actinobacteria, Bacteroidetes, Chlorobi, Chloroflexi, Fibrobacteres, Firmicutes, Gemmatimonadetes, Planctomycetes, Spirochaetes, Verrucomicrobia* and candidate divisions OD1, OP11, TM6, WS1, WS6 and Termite Group 1 ("*Endobacteria*''). These results are somehow similar to the study carried out by Kawahara et al. (2009) where most clones fell into the six major lineages of domain Bacteria which are α-, β-, ε-*Proteobacteria*, *Acidobacteria*/ *Halophaga*, *Bacteriodetes* and *Plantomycetes*.

2.4.2 Commercial Fish Farming (Close System) Bacterial Diversity

Close system fish farming methods include individual compartment fish culturing or recirculating aquaculture system (RAS). Common bacterial communities found in a commercial fish farming system may include heterotrophic bacteria (α-*Proteobacteria* and β-*Proteobacteria* in general), nitrifiers, pathogens, opportunistic bacteria and probionts (Blancheton et al., 2013). In a close commercial fish farming system facility, bacteria are commonly available due to environmental factors or they are being introduced into the system. There are a few channels where different groups of bacteria are being introduced to the system which includes but are not limited to feeding and culturing techniques.

Feeding of live feed to the fish larvae are very common, Skjermo and Vadstein (1993) have investigated the bacterial density and composition associated with mass cultivated rotifers (*Brachionus plicatilis*, SINTEF-strain) to prepare rotifers as live feed for use in marine cold water fish larvae. In their investigation, there was an increase in bacterial number of 50-150% after feeding rotifer with squid meal. The dominant bacterial composition shift was induced by enrichment of cultures from *Cytophaga/ Flavobacterium* to *Pseudomonas/ Alcaligenes*. Due to the dominance of *Pseudomonas* genus, it may have detrimental effect on the fish larvae and may contribute to the poor reproducibility in terms of survival and growth between the replicates.

Ringø et al. (2007) mentioned that commercial fish farming creates a favourable condition for the development of infectious diseases in fish. Certain *Vibrio anguillarum* strains possess neurotoxin capability of triggering neuroexcitatory responses (Faris et al., 1994). These pathogenic bacteria will endanger fish larval and thus has big economic impact as the production of fishes dropped.

Not all bacteria in the system are pathogens. Kindaichi, Ito and Okabe (2004) reported that many types of microflora such as nitrifying bacteria and heterotrophic bacteria are able to flourish in the aquaculture systems as these systems have suitable nutrients for autotrophic and heterotrophic bacteria growth. Efficient food web or carbon metabolism in autotrophic nitrifying biofilm allows utilisations of soluble microbial products produced by nitrifiers are used by heterotrophic bacteria (Kindaichi, Ito & Okabe, 2004).

Ammonia-oxidising bacteria (AOB) have also been detected in many recirculating aquaculture systems (RAS). They play an important role in nitrogen cycle by aerobically converting ammonia to nitrite and gaining energy at the same time (Purkhold et al., 2000; Purkhold et al., 2003). There are two subclass of AOB which are the beta subclass of *Proteobacteria* and the gamma subclass which includes two species of the genus *Nitrosococcus* (Coci, Bodelier, & Laanbroek, 2008).

AOB converts ammonia to nitrite in a two-step process, whereby oxidation of ammonia to hydroxylamine is being catalysed by the enzyme ammonia monooxygenase (AMO) first then only to nitrite (Hornek et al., 2006). You and Chen (2008) reported that oxidising of ammonia to nitrite is the key step in biological nitrogen removal process by autotrophic bacteria such as *Nitrosomonas* and *Nitrobacter* in the aerobic tank and further denitrified into gaseous nitrogen (N_2) by heterotrophic bacteria such as *Pseudomonas* and *Bacillus* in the anoxic tank.

The use of 16S rDNA has proven useful to distinguish between nitrosococci and nitrosomonads, but the outcome has become confusing for a single genus such as *Nitrosospira* because there are no specific regions to identify them (Calvó et al., 2005). The current study of microbial diversity will be able to help in identifying the harmful bacteria or beneficial bacteria. Once the microorganisms are identified, there are possibilities that probiotics can be applied to the system in order to maintain or improve the culture condition of the fish larvae.

Probiotics according to FAO/WHO is “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host”. Probiotics have been used to control pathogenic bacterial flora growth in hatcheries can lead to improvements in the growth and survival of larval fish and fishes treated with probiotics had a significant accumulation of lipid as compared to controls (Plante et al., 2007).

2.5 Detection of Microbial Communities

There have been many methods reported to detect microbial communities in the recirculating system and to characterise them. Detection of microbial communities can be carried out through microbiology identification methods which includes culturing the bacteria or non-microbiological methods (molecular methods). Molecular methods have been a more preferred choice in the recent years starting late 1980s (Kemp & Aller, 2004a).

This is due to the fact that aquatic bacteria are very difficult to culture. Furthermore, culturing method is time consuming and tedious due to the slow growth rate of such bacteria (Kemp & Aller, 2004a; Purkhold et al., 2003; Gonzales et al., 2003). Moreover, microbiological methods needs enrichment and isolation strategies which might fail to recover the entire diversity because only a small fraction of total microbial diversity in the environment can be cultured (Bafana et al., 2008; Purhold et al., 2003).

Molecular methods on the other hand provides a more accurate description of microbial populations and a clearer overvie of microbial

diversity because it is able to distinguish among different organisms represented by the primers used (Bafana et al., 2008; Mahmood, Freitag & Prosser, 2006; Kemp & Aller, 2004a).

2.5.1 Molecular Detection

To detect the presence of microorganisms by molecular method, the first step is to extract the genomic DNA from the samples. Most of the extraction was carried out by standard method with some minor modifications (Cho et al., 2003; Regan et al., 2002; Purkhold et al., 2000). Besides that, bead beating technique through glass beads has also been used (Sugita, Nakamura, Shimada, 2005; Purkhold et al., 2003).

DNA extraction kits and boiling method were also used to extract genomic DNA (Wang, Chen & Li, 2007; Hornek et al., 2006; Kindaichi, Ito & Okabe, 2004). Subsequently after the successful extraction of genomic DNA, polymerase chain reaction plays a pivotal role in amplifying desired target region. This would largely depend on the primer being designed for the target region.

The bacterial 16S rRNA gene contains nine hypervariable regions (V1-V9) that demonstrates considerable sequence diversity. These hypervariable regions are flanked by conserved regions in most bacteria. This has enabled PCR amplification of gene of interest using universal primers but could not capture the whole diversity (Chakravorty et al., 2007; Baker, Smith & Cowan, 2003).

According to Chakravorty et al. (2007), region V2, and V3 were the most suitable region to differentiate genus level of all bacterial species with the exception for closely related *Enterobacteriaceae*. Different primers have been used by various researchers to amplify the 16S rDNA. This is because the 16S rDNA primers are able to highlight a wealth of information represented in the 16S rDNA libraries (Kemp & Aller, 2004a).

The usage of 27F-1492R primer set by Hiraishi (1992) has indicated that 16S rRNA gene primer with approximate 1500 bp PCR products may be applied to a wide variety of bacterial taxa and facilitates systematic studies. The usages of 16S rDNA primers provide a basis for cultivation-independent methods to investigate the diversity and community composition of microorganism because these primers are not specific to certain bacteria species but it is able to amplify most bacteria (Purkhold et al., 2000). Table 2.1 is a tabulation of 16S rDNA bacterial primers used by some researchers.

Table 2.1: 16S rDNA PCR primer sequences and position in *E. coli.*

Primer Name	Primer Sequence	*E. coli* position
11f	5'-GTTTGATCCTGGCTCAG-3'	Position 11 to 27 [2,4]
1492r	5'-TACCTTGTTACGACTT-3'	Position 1492 to 1507 [2,4]
616F	5'-AGAGTTTGATYMTGGCTCAG-3'	Position 8 to 27 [1]
630R	5'-CAKAAAGGAGGTGATCC-3'	Position 1529 to 1545 [1]
27F	5'-GAGTGGATCCTGGCTCAG-3'	Position 8 to 27 [3]
1492R	5'-AAGGAGGGGATCCAGCC-3'	Position 1508 to 1527 [3]
27f	5'-AGAGTTTGATCCTGGCTCAG-3'	Position 8 to 27 [5]
685r	5'-TCTACGCATTTCACCGCTAC-3'	Position 685 to 704 [5]

[1]Juretschko et al. (1998); [2]Regan et al. (2002); [3]Cho et al. (2003); [4] Kindaichi, Ito and Okabe (2004); [5]Itoi, Niki and Sugita (2006)

2.5.2 16S rDNA Library Analysis

Restriction Enzymes (RE) are used to break down the DNA strands into a few fragments depending on the restriction site. The analysis of polymorphism for the same group of bacteria can then be carried out by agarose gel electrophoresis or also known as Restriction Fragment Length Polymorphism (RFLP). A total of 23 informative sites were reported by Urakawa et al. (2006) whereby 12 were from the 16S rDNA and 11 from the *amoA* genes were used to generate a distance matrix by neighbour-joining (NJ) using PAUP software package.

You and Chen (2008) digest the successfully amplified *amoA* PCR product using *Hae*III (GG'CC) and *Hha*I (GCG'C) restriction enzyme whereby the polymorphism shows some similarity and differences between the samples. Terminal Restriction Fragment Length Polymorphism (T-RFLP) was also carried out to analyse the AOB (Regan et al., 2002; Regan et al., 2003). There are some limitations to RFLP as it is unable to tell us the genus or the species of the bacteria. Through the polymorphism of the bands it is possible to tell whether the amplified products from different samples are similar or different. The information provided through RFLP could only be used as an initial screening tool before other molecular techniques can be applied such as cloning and sequencing to identify the genus, species and strain of the bacteria using relevant software available.

2.5.3 Databases and Species Richness Diversity

Cloning has been carried out due to the availability of the clones or genetic material to be used for other processes such as identifying noble gene, insertion of gene to obtain transgenic products which are beneficial and more. Most importantly, cloning enable researchers to manipulate the plasmids extracted. While cloning seems to be a better choice, creating clone libraries could prove to be an extensive work and it is not able to capture the whole diversity.

This has been the concern of recent research mainly on the size of the clone libraries. Kemp and Aller (2004b) reported a few ways to determine when the clone libraries are enough using S_{ACE} and S_{Chao1} estimation method.

In their study too, they have recommended the usage of S_{Chao1} estimator as it appears to be suitable for estimating phylotype richness from prokaryotic 16S rDNA libraries. The estimator S_{Chao1} is non-parametric and the estimation is based mark-release-recapture technique. It can be calculated manually using the below formula:

$$S_{Chao1} = S_{obs} + \frac{F_1^2}{2(F_2 + 1)} - \frac{F_1 F_2}{2(F_2 + 1)^2}$$

Where S_{obs} refers to number of phylotypes observed in library, F_1 and F_2 are number of phylotypes which occurs once or twice (Kemp & Aller, 2004b).

Another downstream processing that has been used by a number of researchers is Denaturing Gradient Gel Electrophoresis (DGGE) method. According to Gonzalez et al. (2003), molecular fingerprinting techniques are commonly used to detect microbial diversity in natural samples. Molecular fingerprinting techniques such as DGGE and T-RFLP, skips the extensive work to create clone libraries and maintaining the clones. By producing bands of interest with better resolution, the bands can be excised and directly send for sequencing because of the nature of denaturing gradient gel is to separate the bands by temperature profile.

The limitation to the DGGE work is that the short sequences obtained from the excised gel could not provide a thorough review. This is because of the limited phylogenetic information from the short excised fragments as smaller bands are not able to be separated or they may diffuse which may lead to incomplete interpretations (Gonzalez et al., 2003). The sequence results

obtained from the short fragments even after matching with software such as BLAST from National Centre of Biotechnology Information (NCBI) could not return with a convincing suspected organism as the query coverage would be limited due to the short fragment being matched against a larger fragment in the database. A higher percentage of query coverage seems to be more convincing as the number of bases matched against will indicate the accountability of the percentage identity.

Silva Incremental Aligner (SINA), an online accurate high-throughput multiple sequence alignment (MSA) of rRNA genes was evaluated by Pruesse, Peplies, and Glöckner (2012) with other commonly used MSA programs such as PyNAST and mothur. SINA was able to achieve higher accuracy than PyNAST and mothur with BRAliBase III benchmarks MSA obtaining 99.3%, 97.6% and 96.1% respectively (Pruesse, Peplies, & Glöckner, 2012). Subsequently, MSA data would require analysis in order to extract important information such as evolution and distance, species richness and abundance, interaction between physiochemical properties and population of bacterial communities.

MetaGenome Analyzer (MEGAN) is a tool use to analyse both targeted 16S rRNA sequencing and random shotgun sequencing for environmental samples (Mitra, Stärk & Huson, 2011) is able to provide taxanomic information obtained through SINA or other MSA programs. Molecular Evolutionary Genetics Analysis (MEGA) software is a commonly

used program to conduct sequence alignment, infer phylogenetic tree and testing evolutionary hypotheses (Tamura et al., 2011).

Yang and Rannala (2012) in their review on molecular phylogenetics: principle and practice discussed on the strength and weaknesses on maximum parsimony whereby it is simple and computational efficient but lack of explicit assumptions. On the other hand, maximum likelihood model assumptions are explicit but it is computationally demanding. They have also concluded that maximum likelihood has an advantage over distance and parsimony methods when the aim of the study is to understand the process of sequence evolution.

UniFrac has also been used by researchers to compare microbial communities using phylogenetic information. UniFrac has been demonstrated to cluster multiple environments and to test the significant differences between those environments (Lozupone, Hamady & Knight, 2006). Relationships between biological data of species and environment can be explained using canonical correspondence analysis (CCA) (Braak & Verdonschot, 1995). CCA can be carried out using Canoco 4.5 software.

2.6 Physical Conditions

There are other conditions that need to be taken note of in order to make sure the fishes are healthy. Monitoring of physical conditions of the system provides us valuable information as it is associated to the growth of the fishes and bacterial diversity. As the culture condition is normally exposed to the

environment in a commercial fish farming system, the physical condition allows us to determine the growth of certain microbial communities.

The dominance of such microbial communities depends largely on the culture conditions as if the culture conditions are medium to cultivate bacteria. Urakawa et al. (2008) mentioned that physical conditions such as salinity can affect the population shift of dominant ammonia-oxidising bacteria. In their research they have investigated the effect of low oxygen and high ammonia concentrations in which extremely high concentrations of ammonia select a novel nitrifier population but a moderate concentration (3.6 to 7.1 mM) did not endanger bacterial population change.

The condition that need to be considered include concentration of dissolved oxygen, carbon dioxide, nitrate concentration, pH, temperature and total ammonia nitrogen (TAN) (Losordo, Masser & Rakocy, 1992; Rakocy et al., 2006; Ebeling et al., 1995). These conditions must be maintained at an optimum level to ensure the optimal performance of the system. The temperature for culturing of catfish is preferably between 25°C to 29°C (Swann, 1997; Wyatt et al., 2006; Chapman, 2012). Mollah (1984) mentioned that growth and survival of young fishes are dependent on water temperature and at a lower temperature; consumption is lower due to the slower rate of digestion.

High concentration of total suspended solids is one of the sources of many problems pertaining to stream health and aquatic life and it affects the dissolved oxygen due to high bacterial activity which uses more oxygen. Increase of surface water temperature is due to absorption of heat from sunlight by the particles (Murphy, 2007).

The amount of dissolved oxygen in the tank should be maintained by artificial aeration with atmospheric oxygen (air) or pure gaseous oxygen injection (Losordo, Masser & Rakocy, 1992). This is because as the dissolved oxygen (DO) is depressed by the solid faecal waste, carbon dioxide (CO_2) and ammonia will be produced (Rakocy et al., 2006).

The proportion of toxic TAN increases as the temperature and pH of the water increases (Durborow et al., 1997). Fishes excrete TAN through their gills when they digest feed and the organic waste solids are decomposed by bacteria. Concentration of un-ionised ammonia-nitrogen in the system should not exceed 0.05 mg/l (Losordo, Masser & Rakocy, 1998).

CHAPTER 3

MATERIALS AND METHOD

3.1 Commercial Fish Farming System

Samples were obtained from a commercial fish farming system facility in Semenyih, Kajang. Figure 3.1 is an image of the commercial fish farm facility. The fish farming facility where samples were taken consist of a groundwater source stored in barrels, fish larvae culture tank, *Moina* sp. culture tank and broodstock culture tank (Figure 3.2). Groundwater from the storage tank (Figure 3.2 (i)) was used as a water source to culture *Moina* sp. (Figure 3.2 (ii)) and fish larvae (Figure 3.2 (iii)). Fish faeces from the adult fish tank was being fed to *Moina* sp. (Figure 3.2 (a)) and cultured *Moina* sp. was given as live feed to fish larvae (Figure 3.2 (b)). Aeration was delivered through an air pump for the fish larvae culture and brood stock culture tank. Figure 3.3 is an overview of the recirculating aquaculture system being used in the commercial farm.

Figure 3.1: Image of Commercial Fish Farming Facility

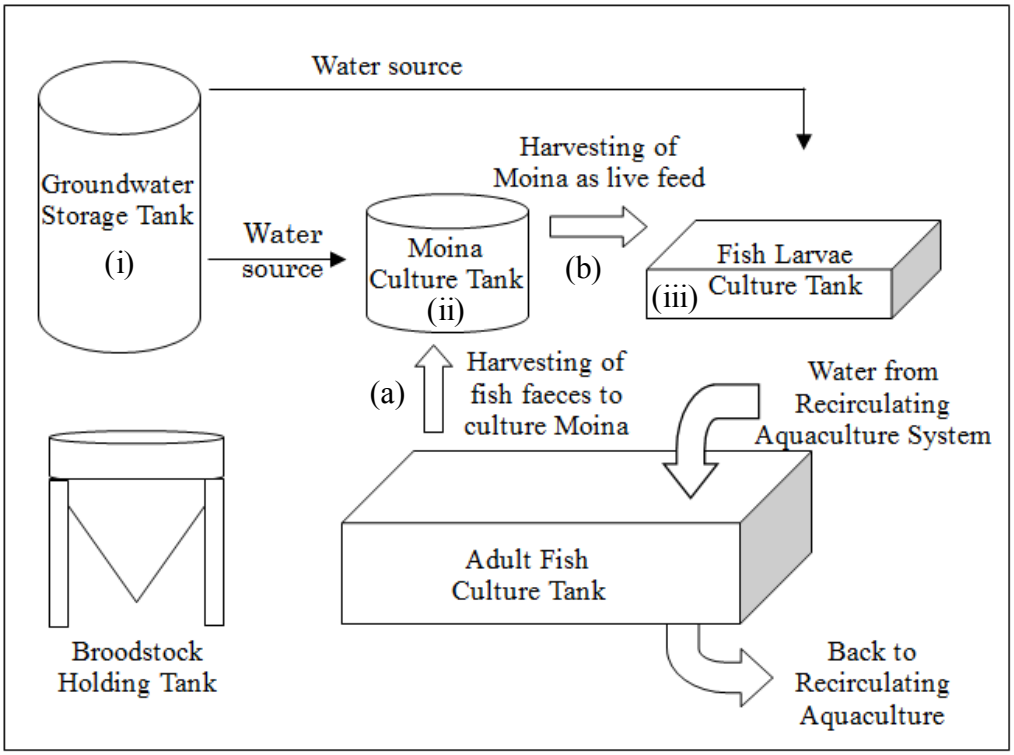


Figure 3.2: Layout of Commercial Fish Farming System in Semenyih

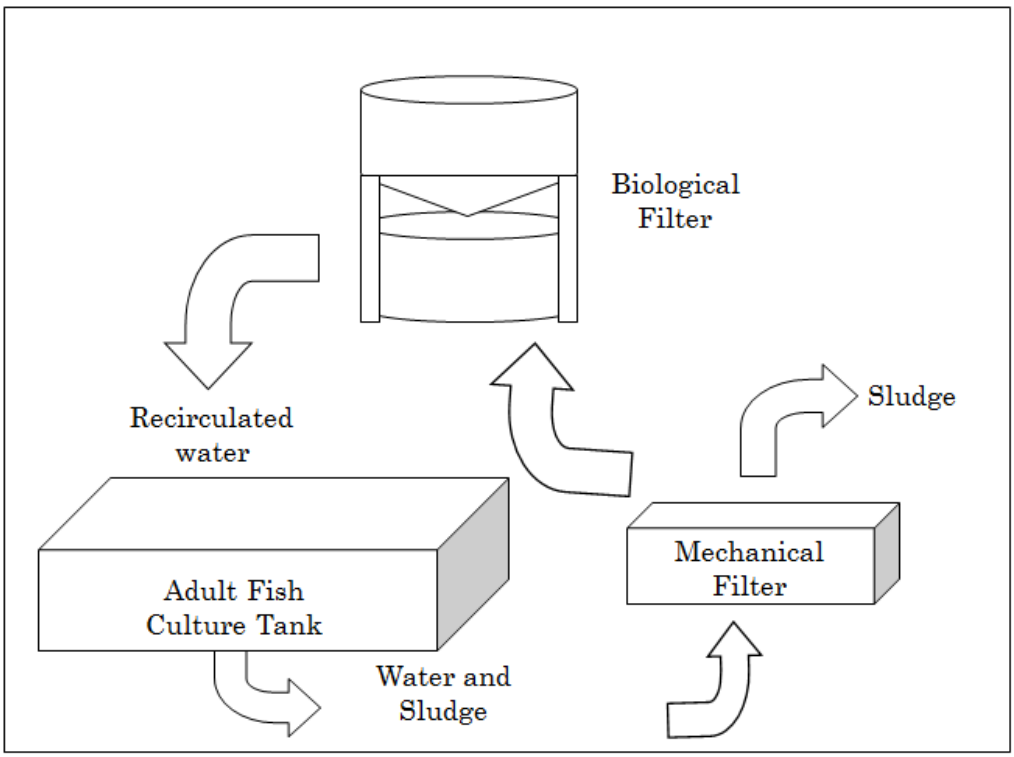


Figure 3.3: Layout of Recirculating Aquaculture System (RAS) in which

faeces from adult fishes were obtained as food source for *Moina* sp. (live feed)

3.2 Sample Collection

Samples were collected from a few sources namely Groundwater (GW), *Moina* Culture Water (MCW), Fish Culture Water (FCW), *Moina* sp. (M), Fish Larvae (F) and Broodstock culture water (BCW). Collection of samples was carried out in a monthly process for two consecutive months. Samples were collected at Day 0, Day 30 and Day 60. This is because fish larvae or also known as fry is between week one and week six after hatching (Graaf & Janssen, 1996). In order to maintain the condition of sampling, sample collections were all carried out before 10 a.m.

3.3 Water Quality Analysis

Collected samples from GW, MCW and FCW were tested using DR/890 colorimeter (Hach, USA) and multi-parameter probe HI 9828/4 (Hanna Instruments, USA) at the site of collection. Samples were tested for temperature, pH, turbidity, suspended solids, ammonia (NH_3) concentration, nitrite (NO_2) concentration, nitrate (NO_3) concentration, oxygen (O_2) concentration, phosphorus (P) concentration and sulphate (SO_4^{2-}) concentration.

3.4 Pre-treatment of Samples

Prior to genomic DNA extraction, samples were being pre-treated in order to ease the extraction process and to remove any unwanted samples or contamination of the desired product.

3.4.1 Water Samples

Water samples from each compartment were first sampled. About 1200 mL of water sample from GW, MCW, FCW and BCW individually were centrifuged using Avanti J-E High Performance Centrifuge (Beckman Coulter, USA) at 13000 rpm for 15 minutes at 4°C. The supernatant was decanted and 250 µL of the precipitate was then transferred to a 1.5 mL microcentrifuge tube for further processing.

3.4.2 *Moina* sp. Samples

Collected *Moina* sp. samples were first rinsed using sterile distilled water (sdH_2O) to wash off any surface bacteria for at least three times and drained off. Thereafter, 1 mL of harvested *Moina* sp. was grinded into fine powder using liquid nitrogen in a pre-chilled mortar and pestle. The powdered *Moina* sp. was then transferred into a 1.5 mL microcentrifuge tube for further processing.

3.4.3 Fish Larvae Samples

Fifteen fish larvae were rinsed continuously with sdH_2O and placed in a 250 mL Schott bottle. The Schott bottle was then shook vigorously to remove any surface bacteria that may be present. Subsequently, the fish larvae were ground into fine powder by pouring liquid nitrogen in a pre-chilled mortar and pestle. The fish larvae powder was then transferred to a microcentrifuge tube for further processing.

3.5 Genomic DNA Extraction

All the microcentrifuge tubes were added with 500 mL of TE buffer (Tris 0.1 mM, EDTA 0.1 mM, pH 8.0). Following that, 50 µL of lysozyme (10 mg/mL) was added and incubated for 30 minutes at 37°C. Then, 50 µL of Proteinase K (20 mg/mL) was added into the mixture and was further incubated for another 30 minutes at the same temperature. Subsequently, 75 µL of 20% SDS solution was added into each microcentrifuge tubes and incubated at 65°C for 2 hours. Upon incubation, 1 vol of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added into the microcentrifuge tubes and were inverted slowly for 10 minutes.

All the microcentrifuge tubes were then centrifuged at 13000 rpm for 3 minutes at room temperature. Supernatant from each tube was then transferred into new microcentrifuge tubes individually. 1 vol of chloroform: isoamyl alcohol (24: 1) was added into the supernatant and was inverted slowly for 10 minutes. After inversion, all the tubes were centrifuged at 13000 rpm for 3 minutes. The supernatant were then transferred into new microcentrifuge tubes with the addition of 1 vol of isopropanol individually to precipitate the DNA. The mixtures were centrifuged at 13000 rpm for 3 minutes at room temperature.

The supernatants were then decanted and 1 mL of 70% ice-cold ethanol was added to each tube. It was then centrifuged again with the same condition. Finally, the precipitate was dried using MicroVac vacuum for 20 minutes. Subsequently, each tube was then resuspended in 50 µL of sdH_2O. Genomic

DNA was quantified using SmartSpec 3000 UV/Vis Spectrophotometer (Biorad, USA).

3.6 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was carried out for all genomic DNA samples. A few sets of primers were designed to target the gene of interest for subsequent manipulation.

3.6.1 Primer Design

Specific primers were used to obtain the desired PCR products. Table 3.1, refers to the primers that were being used in this study.

Table 3.1: Primer Sequences and its Position in *E. coli*

Primer name	Sequence	Position in *E. coli*
27F[1,2]	5'-AGAGTTTGATCCTGGCTCAG-3'	8 – 27
1492R[1,2]	5'-GGTTACCTTGTTACGACTT-3'	1529 – 1545
704R[3]	5'-TCTRCGNATTTCACCNCTAC-3'	685 – 704

[1]Hirashi (1992); [2]Cho et al. (2003); [3]Yoon and Park (2000)

Referring to Table 3.1, two pairs of primers were identified. The primer pairs were 27F/1492R and 27F/704R which produces about 1500 bp fragment and 700 bp fragment respectively. The primers were selected based on literature review and suitability of the study to characterise 16S rDNA fragments. The primer pairs are the most commonly used primer and spans more inclusive hypervariable regions (Hirashi, 1992; Cho et al., 2003; Yoon & Park, 2000).

3.6.2 Polymerase Chain Reaction (PCR) Protocol

The PCR reaction mixture was prepared following the standard method with minor modifications (Hirashi, 1992). A 50 µL of PCR reaction mixture was prepared by first preparing a master mix of 5 µL of buffer (10×) with a final concentration of 1×, 1.5 µL of *Taq* polymerase (1U/µL) with a final concentration of 1.5 U/µL, 2.5 µL of $MgCl_2$ (50 mM) with a final concentration of 2.5 mM, 1 µL of dNTP mix (10 mM each) with a final concentration of 0.2 mM each. Next, a sample mix was prepared. The sample mix consisted of 10 ng/µL of genomic DNA, 2.5 µL (10 µM) forward primer with a final concentration of 0.5 µM, 2.5 µL (10 µM) reverse primer with a final concentration of 0.5 µM and finally top up to 50 µL with sddH_2O. A negative control was also prepared by excluding the genomic DNA and replacing it with sdH_2O.

3.6.3 Amplification Cycle

All amplifications were run using Biometra's TPersonal Thermocycler (Germany) with 48 wells and the lid temperature was set at 105°C with preheating mode turned on.

The amplification started with the predenaturation step (1 cycle) at 95°C for 3 min. This was continued by the main programme (25 cycles) which consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min. The end programme (1 cycle) was at 72°C for 2 min. Part of the PCR product was used to run the agarose gel electrophoresis and the rest was stored at -20°C.

3.7 Quantification and Qualitative Analysis of Genomic DNA

Two methods were employed to check on the quality and quantity of the extracted genomic DNA. It is essential to check these two parameters in order to ascertain the quality of the starting material before downstream processing can be carried out. The two methods employed were spectrophotometry and agarose gel quantification.

3.7.1 Spectrophotometric Determination

Absorption of UV by nucleotides provides an accurate yet simple estimation of the concentration of DNA in a sample before downstream processing. The standard absorption of nucleic acid is around 260 nm. To assess the quality of the DNA, a ratio of OD_{260}:OD_{280} was recorded to check for the purity and amount of DNA present in the sample. Quantification of DNA was carried out using Biorad's (USA) SmartSpec 3000 UV/Vis Spetrophotometer. 50 µL of sdH_2O was pipetted into a cuvette as a blank. Subsequently, 1 µL of sample was mixed with 49 µL sdH_2O. The optical density (OD) readings were then recorded. The amount of DNA was quantified using the below formula.

$$\text{DNA concentration (µg/mL)} = \frac{OD_{260} \times 100 \text{ (dilution factor)} \times 50 \text{ µg/mL}}{1000}$$

3.7.2 Agarose Gel Quantification

Agarose gel electrophoresis was carried out for each of the steps starting from the extraction of genomic DNA and PCR. This technique was employed to assist in determining the results. Different percentage and voltage was used in

accordance with the expected size of the DNA fragments. For genomic DNA, 1% agarose gel was used and the voltage was fixed at 75 V for 45 minutes. As for the PCR products, a 2% agarose gel was run at 80 V for 1 hour. Three percent of agarose gel was run at 80V for about 3 hours for the RFLP products. The gel loaded with sample was stained with GelRedTM (Biotium, USA).

The agarose powder in the conical flask was top up with 1 X TBE buffer and heated in the microwave until the powder dissolves full in the solution. After a clear solution was obtained, it was allowed to cool to about 50°C depending on the percentage of the gel before it was poured into the casting tray. The solution with attached comb was allowed to solidify for about 45 minutes. After that, the comb was removed and the gel was placed into the tank with 1X TBE buffer covering the entire surface of the gel.

3.8 Cloning and Sequencing

PCR products were concentrated on agarose gel and excised in order to be purified before cloning. Excised bands for GW, MCW, FCW, M, F and BCW were cloned using pSTBlue-1 **A**ccep**T**or™ Vector Cloning Kits (Merck, Germany) following standard protocol that was recommended by the manufacturer. Two µL of PCR product was mixed with 1 µL of **A**ccep**T**or™ Vector (50 ng/µL), 5 µL of Clonables™ 2X Ligation Premix and 2 µL of Nuclease-free Water with a total of 10 µL in a microcentrifuge tube. The ligation reaction was mixed slowly with a pipette tip. Thereafter, the ligation reactions were incubated at 16°C for 2 hours prior to transformation.

Transformation was carried out by first removing tubes of NovaBlue Singles™ Competent Cells from storage and immersed immediately on ice except for the cap. The cells were thawed on ice for about 2 minutes and were gently flicked to check for thorough thawing. To the cells, 1 µL of ligation reaction was added and was mixed before returning the tube to ice. All tubes were incubated on ice for 5 minutes. Directly after incubation, the tubes were placed in a water bath which was set at 42°C exactly for 30 seconds.

The tubes were returned on ice for another 2 minutes and 250 µL of room temperature SOC medium was added into each tube. The tubes were then incubated for 45 minutes in a shaking incubator set at 250 rpm and 37°C prior to plating as an outgrowth step. LB agar plates with ampicillin (10 µg/mL) were used to plate the transformed cells. 35 µL of 50 mg/mL X-gal and 20 µL of 100 mM IPTG were spread on the plates and left for about 15 minutes. 50 µL of SOC medium were added with 20 µL of transformation mixture to the plate and was spread evenly.

The same step was repeated for all ligation mixtures. All plates were incubated at 37°C for 16 hours to screen for blue-white colonies. Blue-white screening is a technique to detect transformed recombinant bacteria. Cells that are successfully transformed with vectors containing recombinant DNA will produce white colonies. Only white colonies were subjected to PCR using primer set T-7 promoter and U-19 mer to check for insert. Band size with inserts was expected at about 900 bp on the agarose gel. Successfully transformed cells were then picked up and grown in 5 mL of LB broth with

ampicillin (10 µg/mL) for 18 hours at 37°C and 250 rpm for plasmid extraction. Extracted plasmids were subjected to agarose gel quantification before sending out for sequencing. All sequencing was carried out by Bioneer, Korea using ABI 3730XL DNA Analyzer (USA).

3.9 Databases, Analysis and Phylogenetic Studies

Sequencing data was analysed using SILVA rRNA Database which provides comprehensive datasets of aligned 16S/18S small subunit and 23S/28S large subunit rRNA sequences for Bacteria, Archaea and Eukarya. Sequences obtained are aligned by SINA (v1.2.9) using SILVA SEED as reference alignment. Alignment results were then analysed using MEGAN (v4.61.5). Upon grouping of bacteria using MEGAN, a table was built to analyse the species diversity in each compartment or sample. Evaluation of clone library size and species richness was calculated based on S_{Chao1} calculations available at http://www.aslo.org/lomethods/free/2004/0114a.html as described by Kemp and Aller (2004a).

Subsequently, MEGA5 (Tamura et al., 2011) was used to build phylogenetic trees. Bootstrap consensus tree from 500 replicates was taken to represent the phylogenetic tree. Phylogenetic trees were then subjected to UniFrac analysis using Principal Coordinates Analysis (PCA) to determine the significant differences between environments (Lozupone, Hamady & Knight, 2006) and Canonical Correspondence Analysis (CCA) was employed to analyse bacterial diversity relationship from physiochemical properties.

CHAPTER 4

RESULTS

4.1 Water Quality Analysis

Water quality monitoring was carried out for period of sampling from November 2009 to March 2010. The water quality monitoring results are stated in Table A.1 (Appendix A). The water quality analysis includes temperature, turbidity, suspended solids, ammonia, nitrite, nitrate, oxygen, pH, phosphorus and sulfate. Water quality is affected by fish activities such as nitrogen metabolism and respiration which in return determines how well the fishes grow and their survival (Buttner et al., 1993).

Temperature for GW was slightly higher than of MCW and FCW but all of them are within the normal range. Turbidity and suspended solids in MCW was the highest followed by FCW and GW has the lowest reading. There was an increased in turbidity and suspended solids observed for MCW on the second sampling point. Very high level of ammonia was observed for MCW throughout the sampling, while ammonia content for FCW decreases over time and no ammonia was detected for GW. Nitrite for MCW was higher when compared to FCW and there was a spike in nitrite content on the last day of sampling for MCW. In contrast, a dip was observed for nitrate content on the same day for MCW. Oxygen level in MCW has the lowest reading as compared to GW and FCW. pH readings were within range for GW, MCW and FCW even though GW pH reading is slightly lower compared to the rest. Phosphorus content and sulfate content was very high in MCW as compared to

the rest. Figure 4.1 to Figure 4.10 are comparison of each physiochemical properties in GW, MCW and FCW with their respective sampling period.

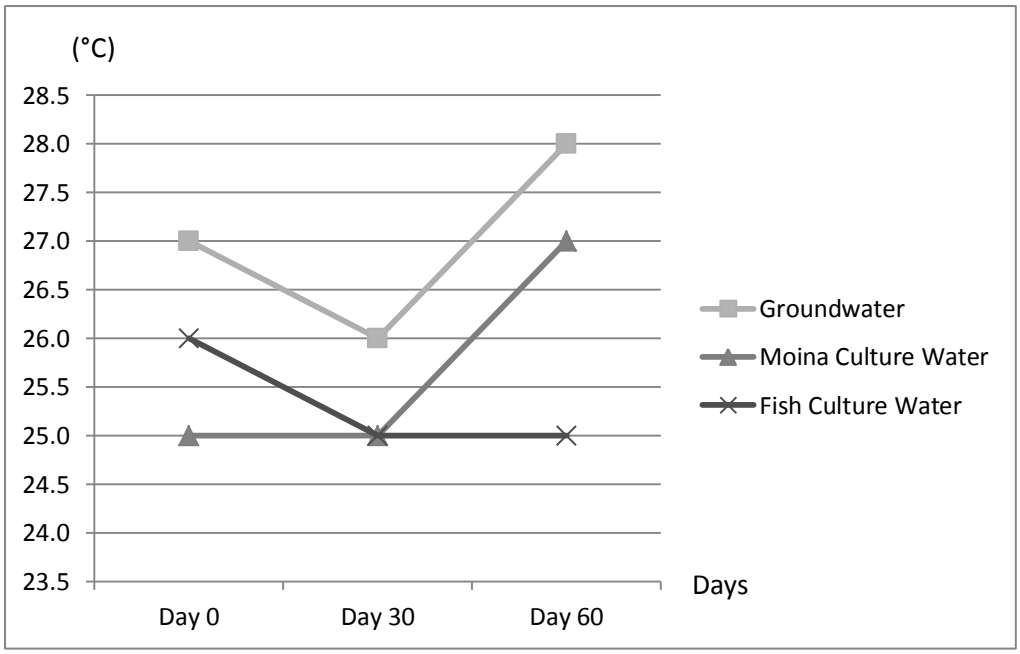


Figure 4.1: Comparison of Temperature (°C) of water samples from Groundwater, *Moina* Culture Water and Fish Culture Water.

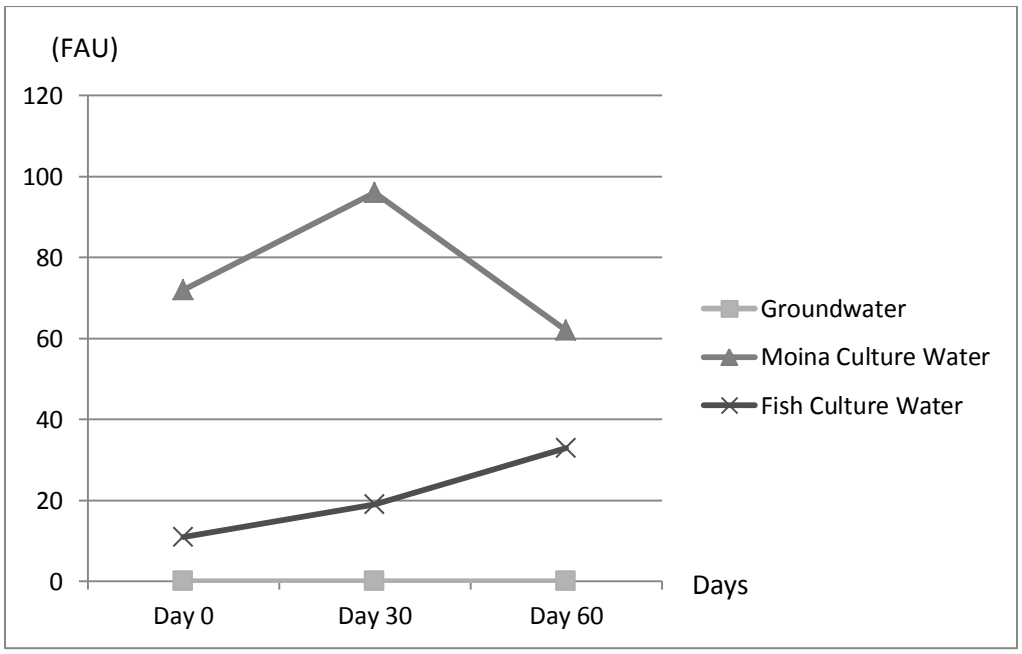


Figure 4.2: Comparison of Turbidity (FAU) of water samples from Groundwater, *Moina* Culture Water and Fish Culture Water.

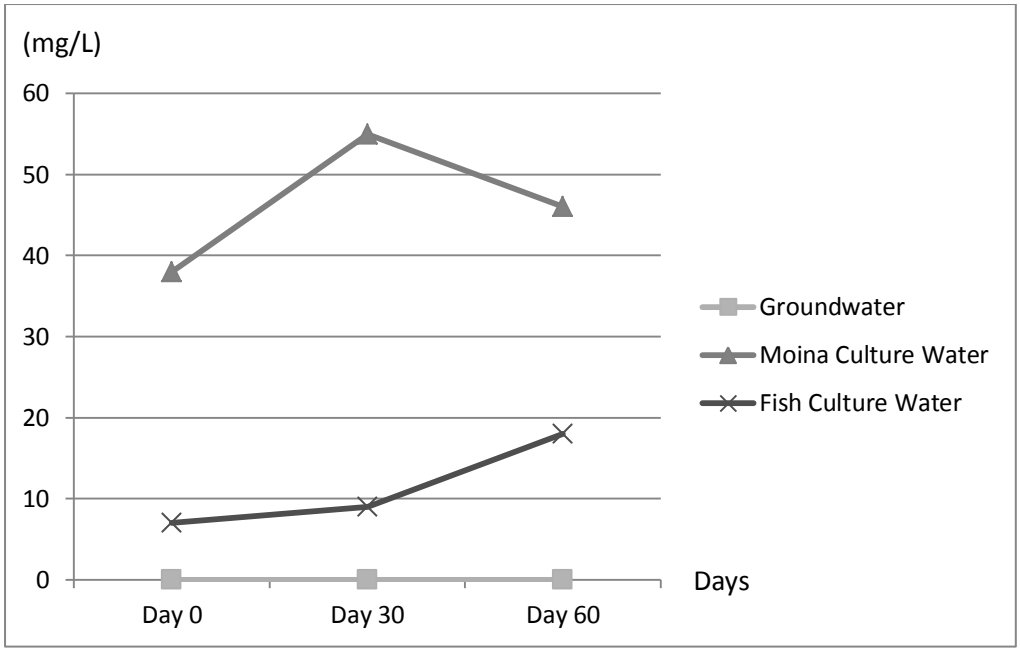


Figure 4.3: Comparison of Suspended solid (mg/L) of water samples from Groundwater, *Moina* Culture Water and Fish Culture Water.

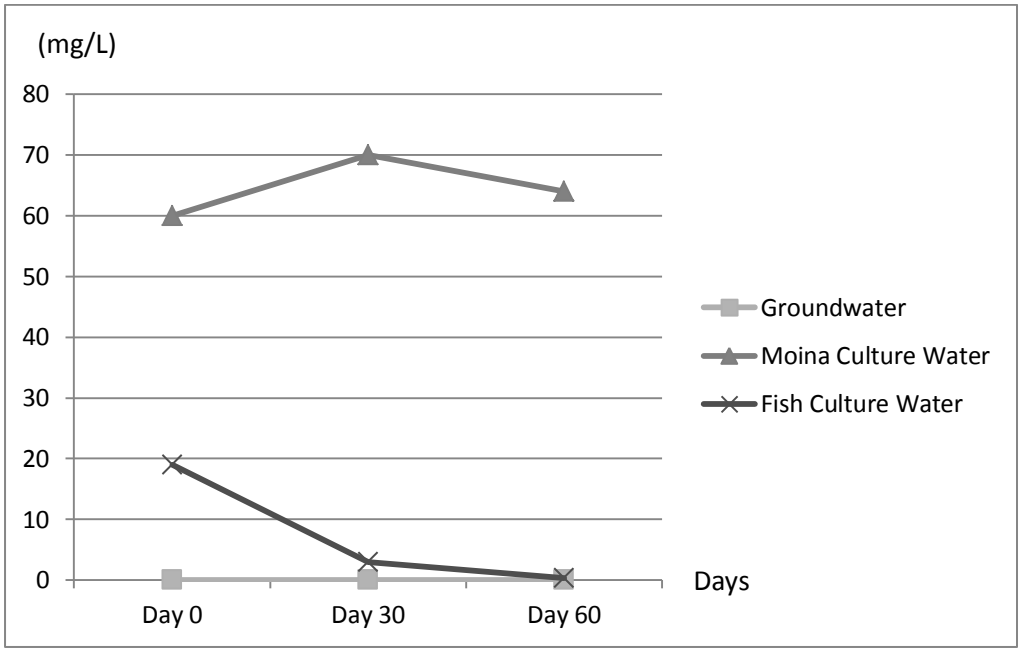


Figure 4.4: Comparison of Ammonia (NH_3) (mg/L) of water samples from Groundwater, *Moina* Culture Water and Fish Culture Water.

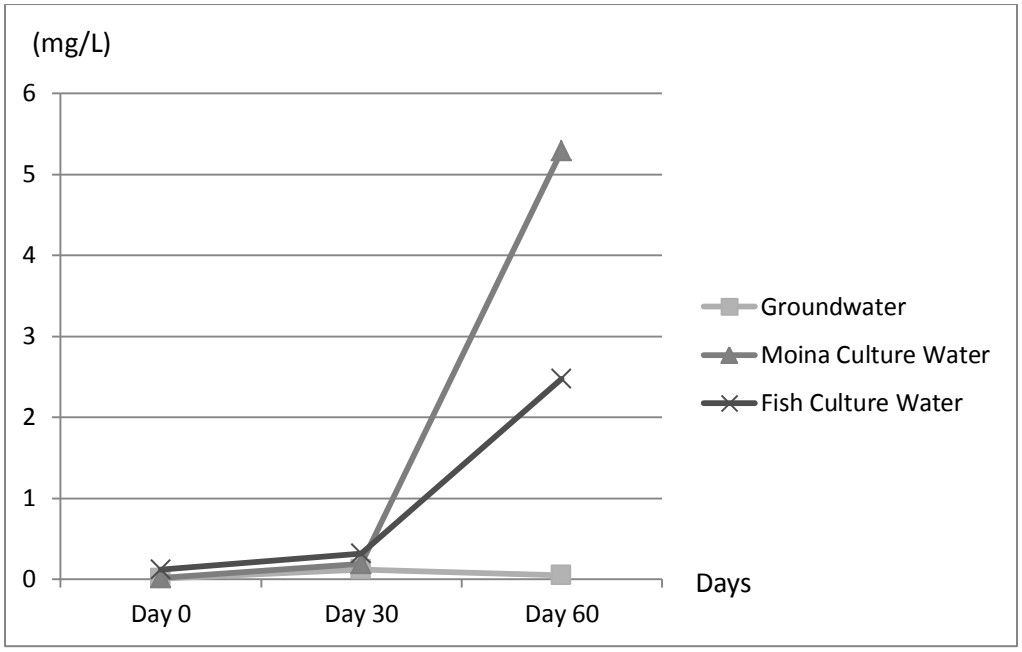


Figure 4.5: Comparison of Nitrite (NO_2) (mg/L) of water samples from Groundwater, *Moina* Culture Water and Fish Culture Water.

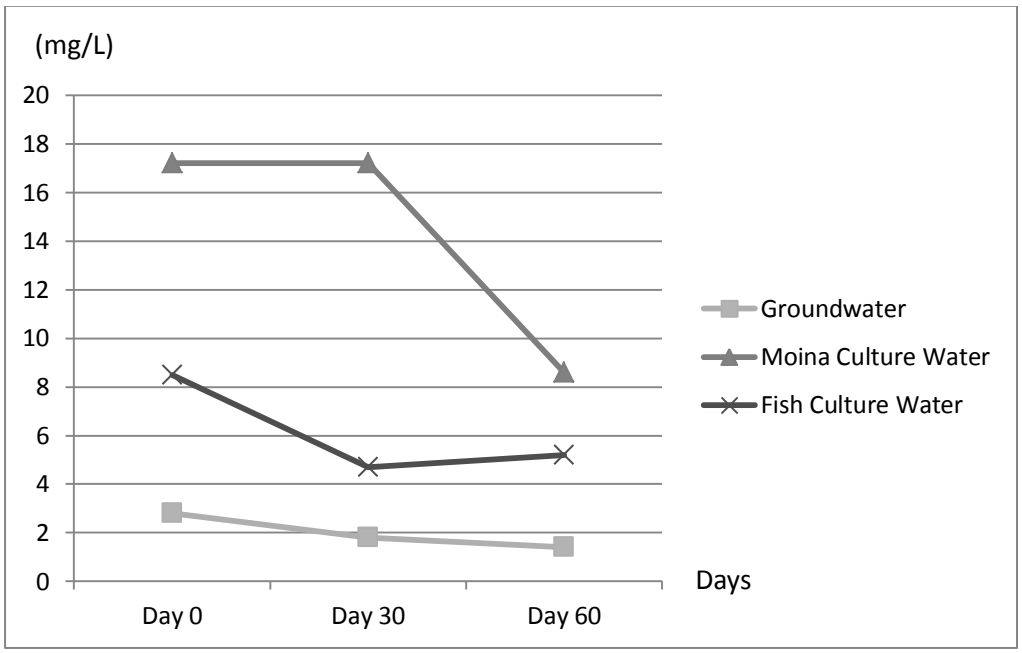


Figure 4.6: Comparison of Nitrate (NO_3) (mg/L) of water samples from Groundwater, *Moina* Culture Water and Fish Culture Water.

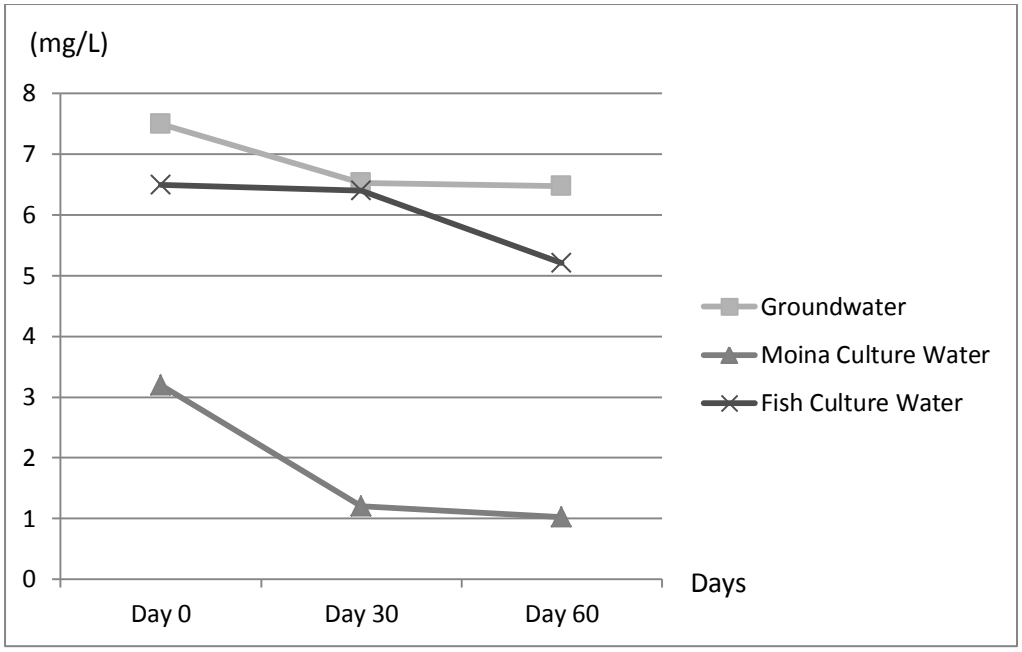


Figure 4.7: Comparison of Oxygen (O_2) (mg/L) of water samples from Groundwater, *Moina* Culture Water and Fish Culture Water.

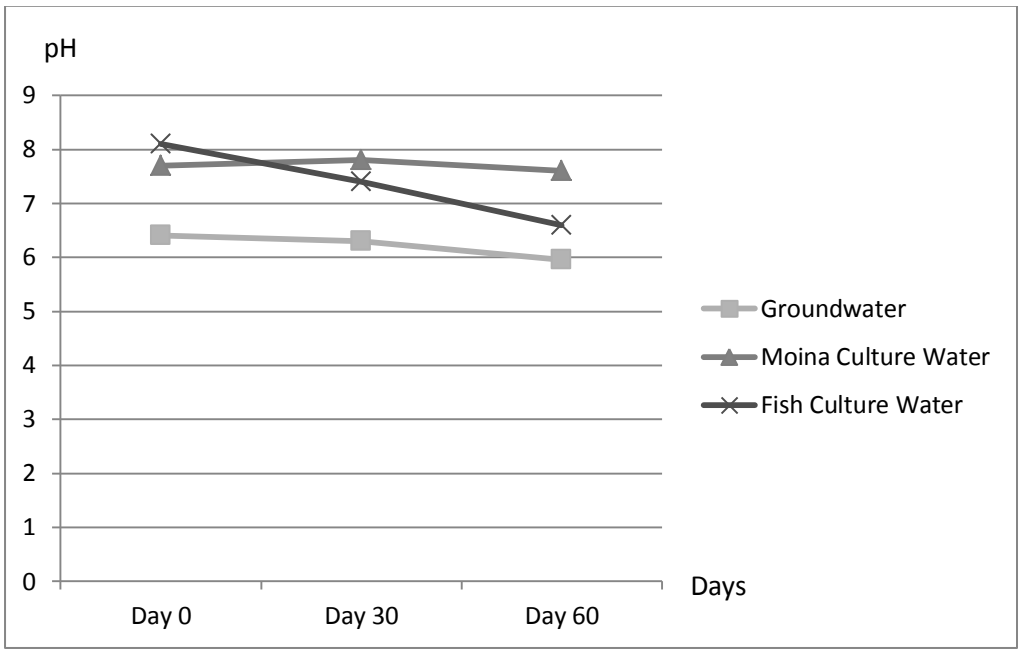


Figure 4.8: Comparison of pH of water samples from Groundwater, *Moina* Culture Water and Fish Culture Water.

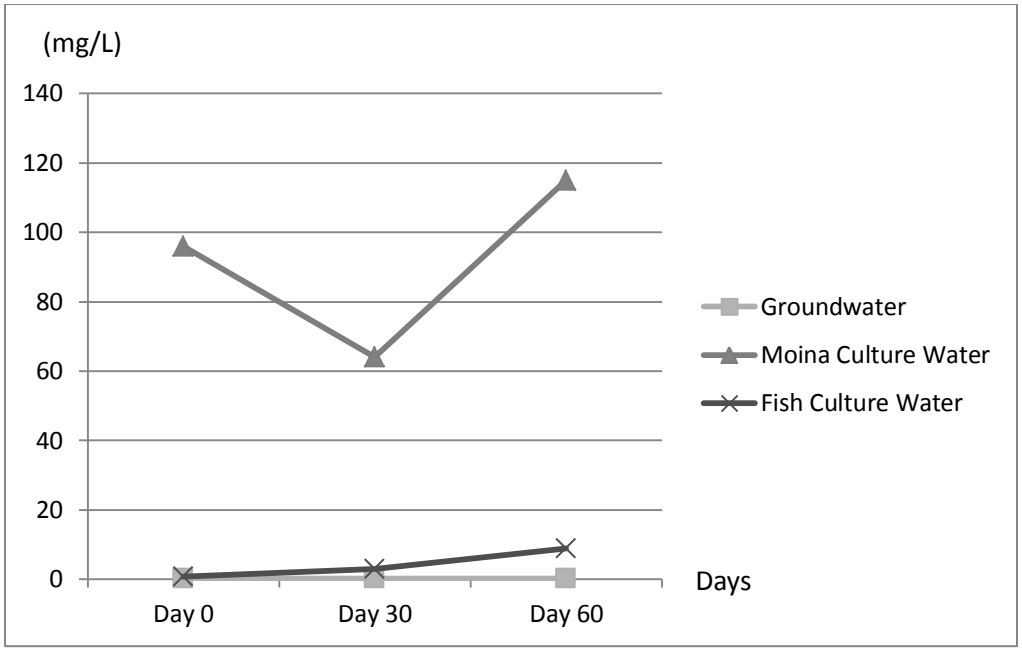


Figure 4.9: Comparison of Phosphorus (mg/L) of water samples from Groundwater, *Moina* Culture Water and Fish Culture Water.

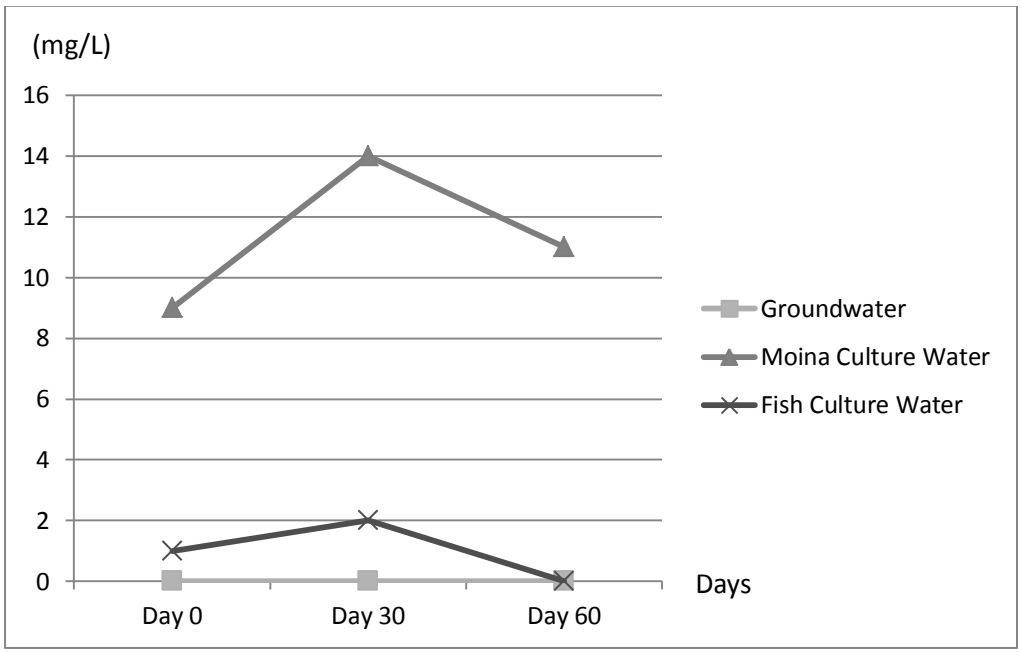


Figure 4.10: Comparison of Sulfate (mg/L) of water samples from Groundwater, *Moina* Culture Water and Fish Culture Water.

4.2 Quantification of gDNA

Extracted genomic DNA (gDNA) was subjected to quantification by absorbance for all samples. Table 4.2 is the tabulation of optical density (OD) readings using SmartSpec 3000 UV/Vis Spectrophotometer (Biorad, USA) for each sample namely Groundwater (GW), *Moina* Culture Water (MCW), Fish Culture Water (FCW), *Moina* sp. (M), Fish Larvae (F) and Broodstock Culture Water (BCW) with their average values and standard error.

Table 4.1: Tabulation of optical density (OD) readings for gDNA samples with their average values and standard errors.

Sample	A_{260}	A_{280}	Concentration (ng/µL)	A_{260}/A_{280}
GW	0.1703 ± 0.0096	0.1046 ± 0.0042	425.85 ± 21.5114	1.6283 ± 0.0318
MCW	2.1416 ± 0.0917	1.2240 ± 0.0526	5354.00 ± 229.3556	1.7498 ± 0.0225
FCW	2.1478 ± 0.0016	1.2110 ± 0.0051	5369.50 ± 4.1079	1.7736 ± 0.0069
M	1.6214 ± 0.0320	0.9932 ± 0.0194	4053.50 ± 80.0703	1.6325 ± 0.0101
F	2.6574 ± 0.0200	1.5214 ± 0.0108	6643.50 ± 49.9875	1.7467 ± 0.0124
BCW	1.8774 ± 0.0066	1.1238 ± 0.0090	4693.50 ± 16.5454	1.6706 ± 0.0083

*Groundwater (GW), *Moina* Culture Water (MCW), 1kb Ladder (L), Fish Culture Water (FCW), *Moina* sp. (M), Fish Larvae (F), Broodstock Culture Water (BCW).

Agarose gel quantification was also carried out in order to check for the quality of the extracted gDNA. Figure 4.11 refers to the gel image obtained for all samples. Genomic DNA was expected to be bigger than 1 kb. The agarose gel image complies with the expected band size. The ladder was obtained from Promega 1 kb DNA ladder.

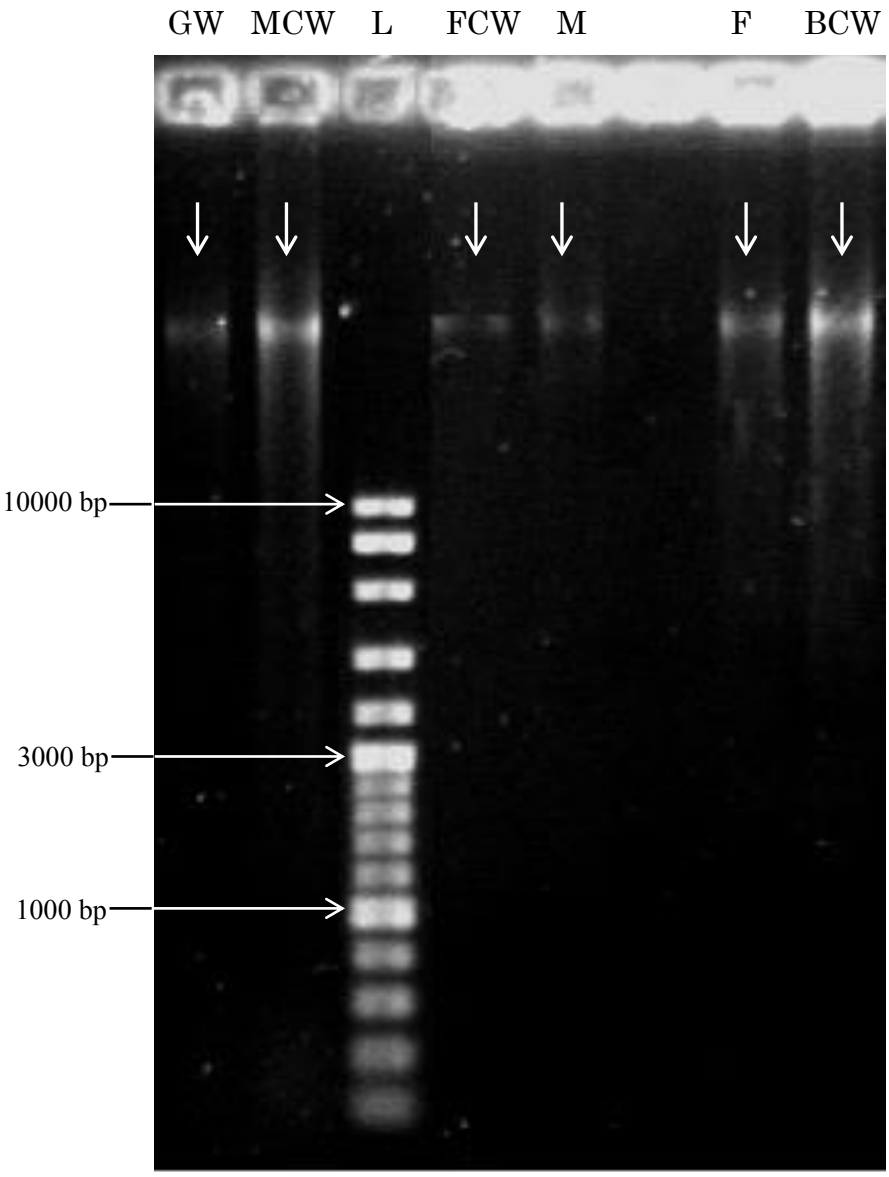


Figure 4.11: 1% Agarose Gel running at 80V for 45 mins

*Groundwater (GW), *Moina* Culture Water (MCW), 1kb Ladder (L), Fish Culture Water (FCW), *Moina* sp. (M), Fish Larvae (F), Broodstock Culture Water (BCW).

4.3 Polymerase Chain Reaction (PCR)

PCR was carried out using primer pair 27F/1492R and was quantitated using agarose gel electrophoresis using 2% agarose gel running at 80V for 1 hour using 1X TBE buffer. Primer pair 27F/1492R gave an expected band size of about 1.5 kb. All the PCR products were about 1.5 kb. Figure 4.12 is the gel image for all the PCR products.

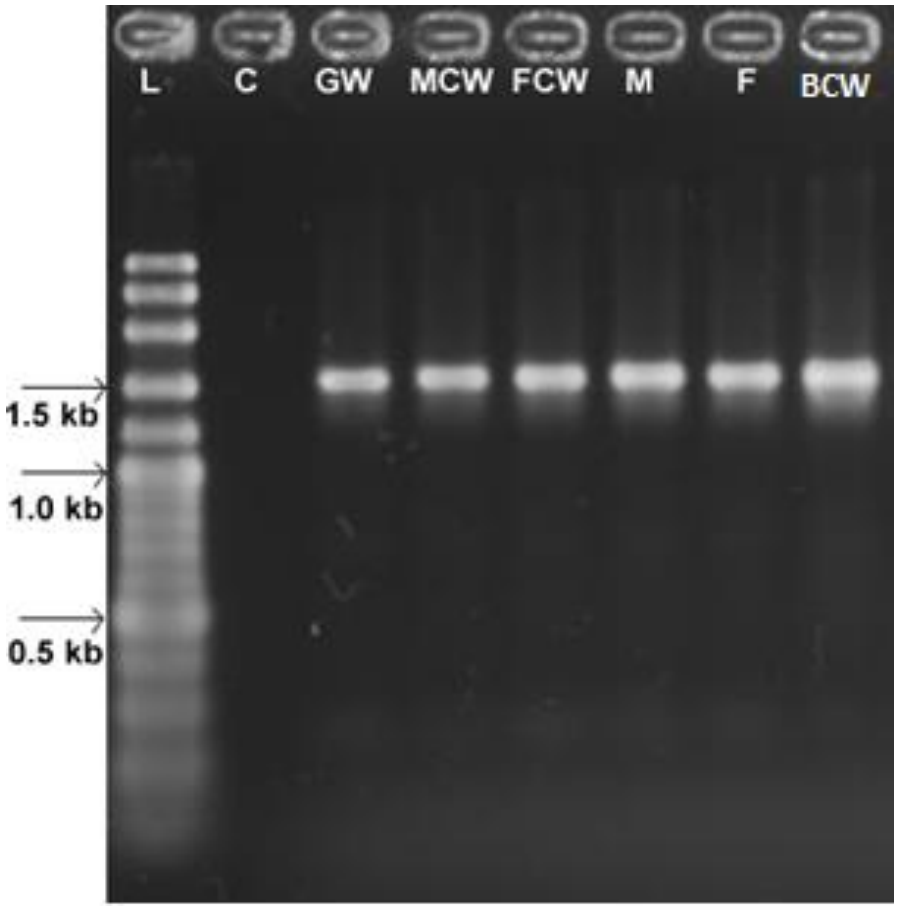


Figure 4.12: 2% Agarose Gel 80V, 1 hour using primer set 27F/1492R

*100bp Ladder (L), Control (C), Groundwater (GW), *Moina* Culture Water (MCW), Fish Culture Water (FCW), *Moina* sp. (M), Fish Larvae (F), Broodstock Culture Water (BCW).

Another primer set was also used in this study which was 27F/704R. Figure 4.13 is the gel image for all the PCR products using primer set 27F/704R running at 80V for 1 hour using 1X TBE buffer with an expected band size of about 700 bp. All samples were about 700 bp which indicates successful amplification.

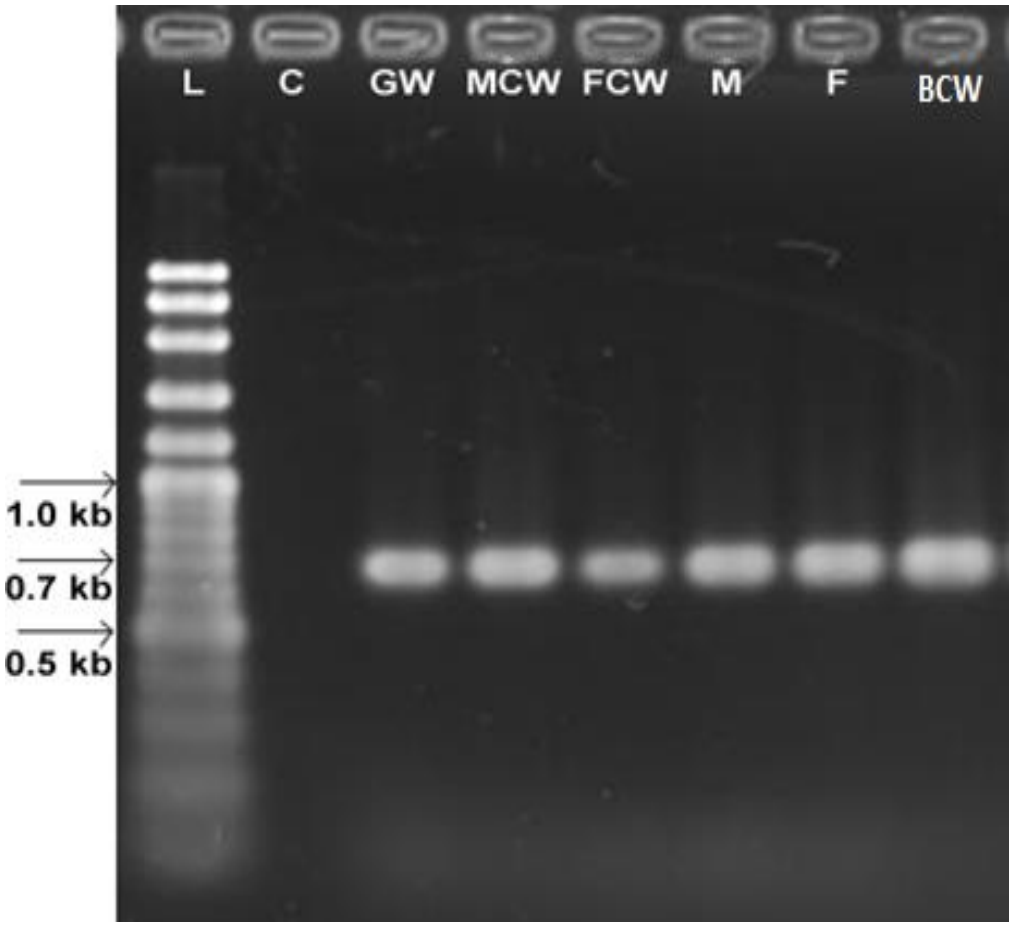


Figure 4.13: 2% Agarose Gel 80V, 1 hour using primer set 27F/704R

*100bp Ladder (L), Control (C), Groundwater (GW), *Moina* Culture Water (MCW), Fish Culture Water (FCW), *Moina* sp. (M), Fish Larvae (F), Broodstock Culture Water (BCW).

4.4 Cloning

Cloning was carried out using Novagen pSTBlue-1 cloning kit in accordance with manufacturer's recommendations. Blue-white screening was carried out to determine successful clones before colony PCR. Figure 4.14 is an image for Groundwater (GW) sample. The same process was repeated for all samples to screen for successful clones.

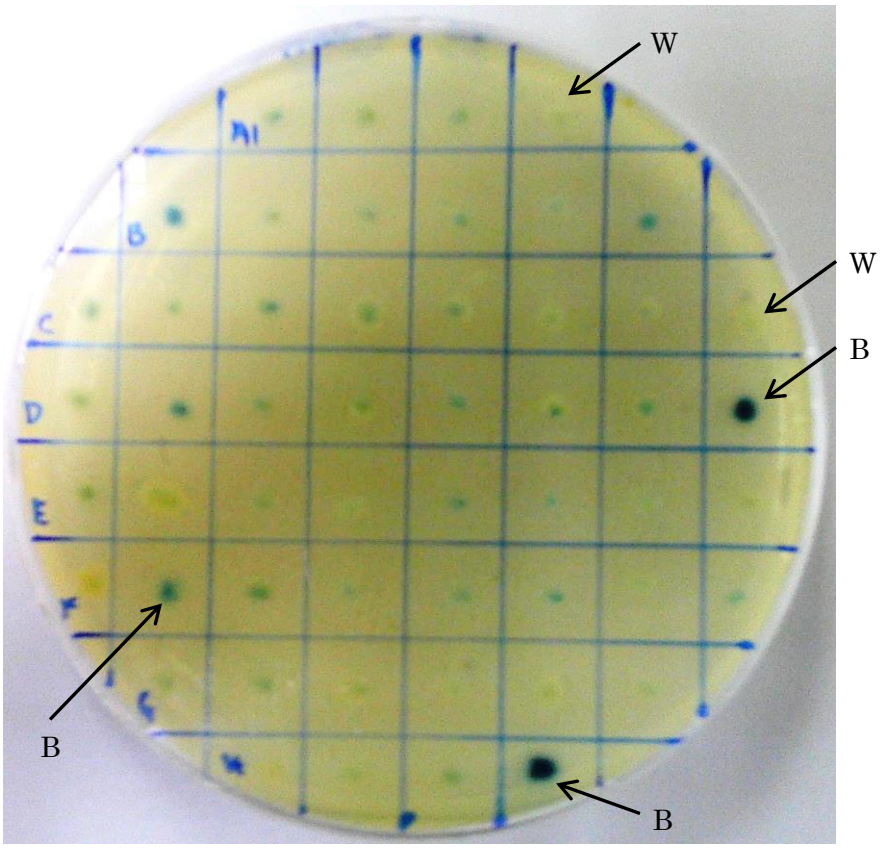


Figure 4.14: Image of Blue-White Screening, Groundwater (GW) Sample

*Blue (B), White (W) colonies. Arrows highlighting some of the colonies.

4.5 Colony PCR

Colony PCR was carried out on white clones to screen for clones with gene of interest using primer set T7-promoter and U19-mer. 2% agarose gel was used for this purpose and was operated for 1 hour with 80V. Figure 4.15 is a gel image of colony PCR for Groundwater sample transformed using PCR products from primer pair 27F/1492R. The total band size for colony PCR is about 1.7 kb due to the vector which has 229 bp in addition to the insert with 1.5 kb. Vivantis 100 bp plus DNA ladder was used for subsequent gel images.

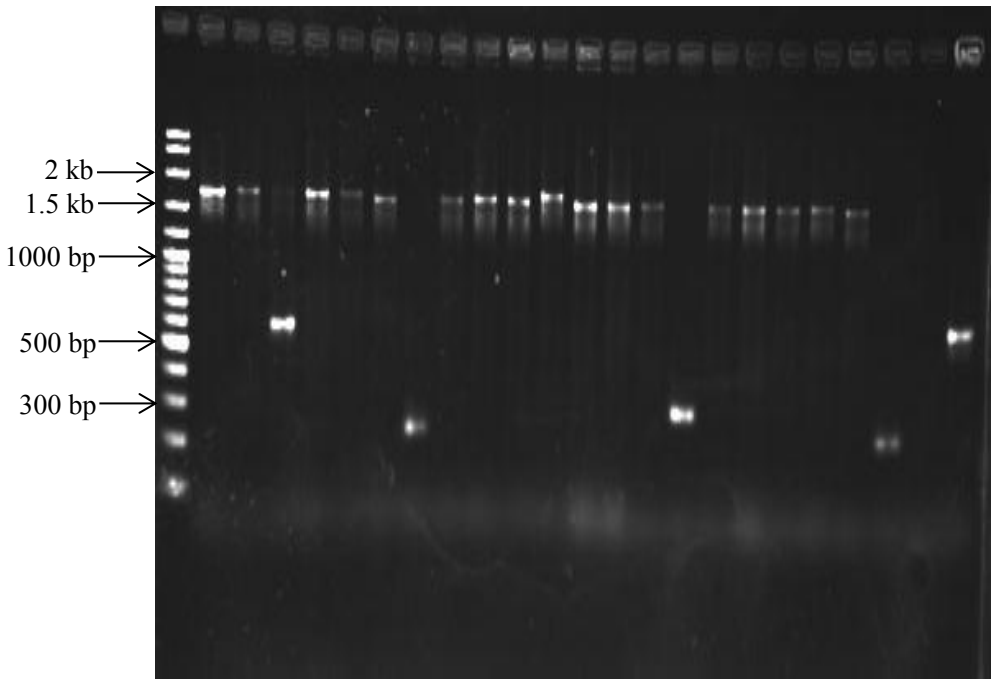


Figure 4.15: Agarose gel electrophoresis image for white colonies from Groundwater sample using 27F/1492R PCR product as insert.

Subsequently, colony PCR was carried out for another clone using PCR products from primer set 27F/704R as insert. Figure 4.16 is the gel image of colony PCR using primer set T7-promoter/U19-mer from white colonies running at 2% agarose gel, 80V for 1 hour.

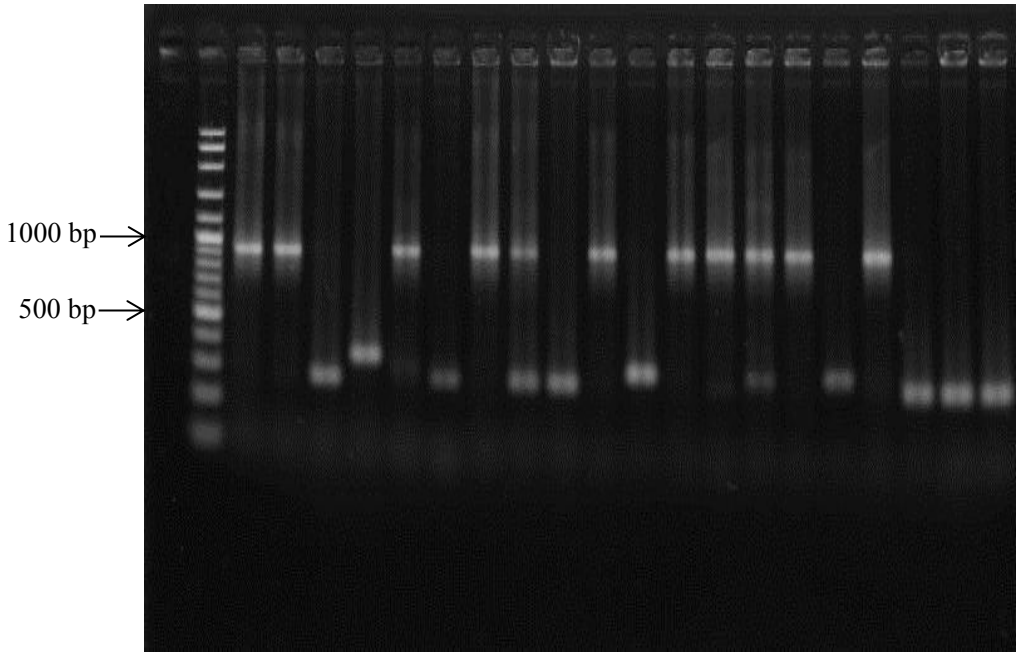


Figure 4.16: Agarose gel electrophoresis image for white colonies from Groundwater sample using 27F/704R PCR product as insert.

4.6 Plasmid Extraction

Successful amplification of clones were then subjected to culturing in nutrient broth for plasmid extraction. Plasmids were then purified and quality check was carried out before sending for sequencing. Figure 4.17 shows the extracted plasmid on 1% agarose gel run for 45 minutes at 75V. Extracted plasmid which showed high intensity and a minimum of two conformations were sent for sequencing for subsequent analysis.

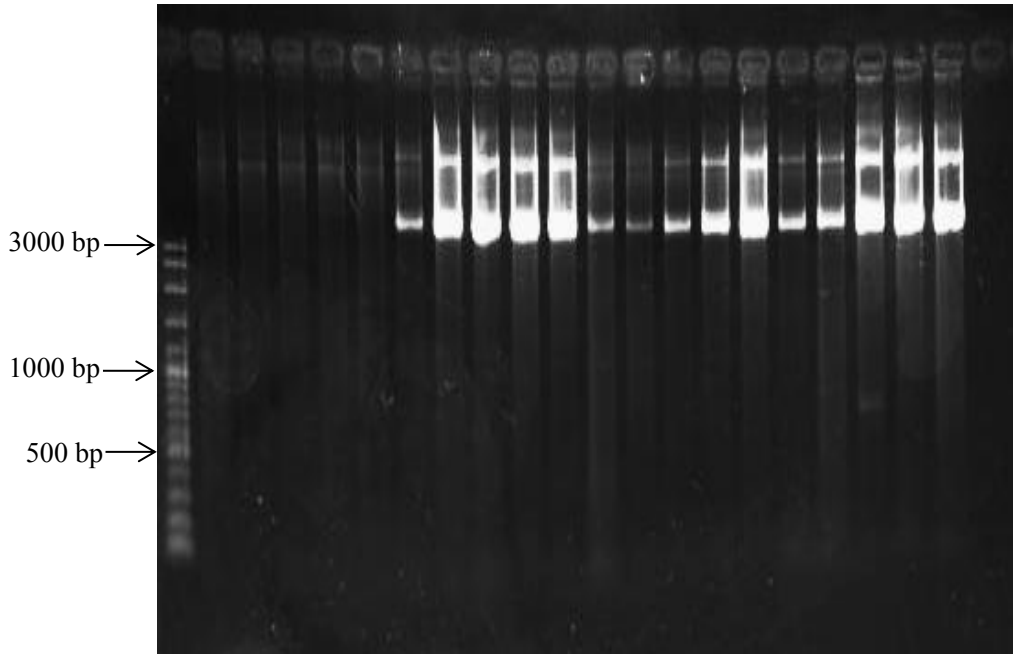


Figure 4.17: Agarose gel electrophoresis image of plasmids extracted from Groundwater sample with 100 bp ladder.

4.7 Sequencing, Alignment and Analysis

A total of 177 clones were sequenced out of which, 22 clones were obtained from GW samples, 43 clones from MCW samples, 28 clones from FCW samples, 30 clones from M samples, 25 clones from F samples and 29 clones from BCW samples. Sequences obtained were submitted to NCBI pending accession number assignment.

Alignment was carried out using Mega 5.1 (Appendix E) and SINA Alignment Services which can be accessed at http://www.arb-silva.de/aligner/. Table 4.2 summarises the closest relatives for 16S rDNA sequences as obtained by SINA Alignment Services via ARB-SILVA website and its comparison with all samples.

Sequences obtained from SINA were also analysed using MEGAN 4 to generate a taxonomic tree for samples from this study (Figure 4.18).

Figure 4.18 summarises the taxonomy of all 177 sequences whereby 33 sequences were grouped as cellular organisms, 142 sequences as Bacteria where 17 sequences were only available in the *Bacteria* domain level, seven sequences in *Cyanobacteria* phylum, 22 sequence in *Bacteroidetes* phylum where 21 sequences in *Bacteroidetes* class, five sequences in *Actinobacteria* class, five sequences in *Planctomycetaceae* family. There were 86 sequences grouped in the *Proteobacteria* phylum where six sequences were within *α-Proteobacteria* class, 41 sequences were within *β-Proteobacteria* class out of which 16 sequences grouped in *Burkholderiale*s order. Twenty-three sequences were associated with the *γ-Proteobacteria* class out of which six sequences grouped in *Legionellales* order. Out of the total 177 sequences, two sequences were unassigned.

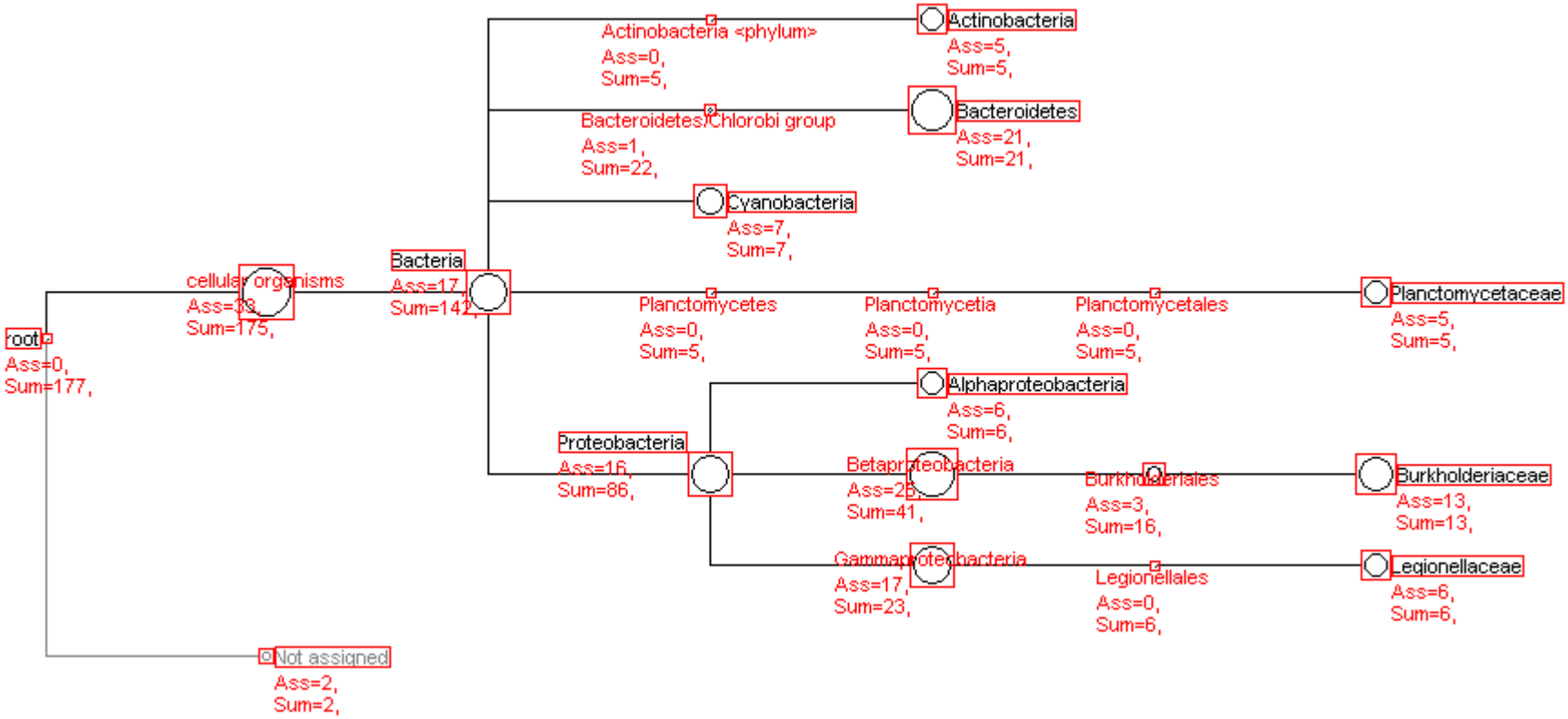


Figure 4.18: Taxanomic tree of all 177 samples from GW, MCW, FCW, M, F and BCW generated by MEGAN 4.

* GW, Groundwater; MCW, *Moina* Culture Water; FCW, Fish Culture Water; M, *Moina* sp.; F, Fish Larvae and BCW, Broodstock Culture Water

Table 4.2: Closest Relative for 16S rDNA Clone Sequences Determine by ARB-SILVA Database using SINA Alignment and MEGAN 4.

Grouping (Percentage Similarity)	Source	*GW	MCW	FCW	M	F	BCW	Total
Bacteria								
Actinobacteria								
Actinobacteria								
Actinomycetales (94%)	Freshwater pond		1					
Armatimonadetes (88% - 89%)	Hydrothermal oxides/ Human Skin	2						
Bacteroidetes/ Chlorobi group								
Chlorobi								
Clorobia								
Chlorobiales (92%)	Lake reservoir water						1	
Cytophagia								
Cytophagales								
Cytophagaceae								
Emticicia ginsengsoli (89%)	Bay water		1					

Grouping (Percentage Similarity)	Source	GW	MCW	FCW	M	F	BCW	Total
Flavobacteria								
Flavobacteriales								
Flavobacteriaceae								
Candidatues Chryseobacterium massiliae (95%)	Water sample				1			
Chryseobacterium sp. (96%)	Saltwater lake water				1		1	
Flavobacterium succinicans (93%)	Fresh water			1				
Cryomorphaceae								
Fluviicola (92%)	Freshwater lake			1				
Sphingobacteria								
Sphingobacteriales (87% - 96%)	Sewage sludge/ Wastewater sludge/ Spring water		4					
Chitinophagaceae (90%)	Crab intestine		1					
Ferruginibacter alkalilentus (95%)	N/A			1				
Runella slithyformis (93%)	Activated sludge			2				
Sediminibacterium (93%)	Activated sludge						1	
Sediminibacterium salmoneum (93% - 97%)	Subsurface aquifer sediment/ Freshwater seep		1				2	

Grouping (Percentage Similarity)	Source	GW	MCW	FCW	M	F	BCW	Total
Flexibacteraceae								
Flexibacter flexilis (93%)	Collection		1					
Flexibacter sp. (93%)	Lake water			1				
Saprospiraceae (88%)	Waste water			1				
Sphingobacteriaceae (81%)	Lake sediment		1					
Nubsella zeaxanthinifaciens (92%)	Activated sludge				1			
Candidate division OD1 (84% - 86%)	Lake sediment/ Soil		1	2				
Chloroflexi								
Anaerolineae (93%)	Activated sludge			1				
Cyanobacteria (82%)	N/A		1					
Oscillatoriophycideae								
Oscillatoriales								
Pseudanabaena								
Pseudanabaena sp. (92% - 95%)	Microcystis bloom & surface sediment	7						

Grouping (Percentage Similarity)	Source	GW	MCW	FCW	M	F	BCW	Total
Fibrobacteres								
Fibrobacteria								
Fibrobacterales								
Fibrobacteraceae (94%)	Water			1				
Firmicutes								
Erysipelotrichi								
Erysipelotrichales								
Erysipelotrichidae								
Erysipelothrix (61% - 96%)	Wastewater sludge		2	1				
Planctomycetes								
Planctomycetia								
Planctomycetales								
Planctomycetaceae								
Blastopirellula (89%)	Grass carp		1					
Gemmata (90%)	Waste sludge	1						
Planctomyces sp. (86% - 91%)	Hypereutrophic lake/ Soil	1					1	
Schlesneria (94%)	Sedimentary rock	1						

Grouping (Percentage Similarity)	Source	GW	MCW	FCW	M	F	BCW	Total
Proteobacteria								
Alpha Proteobacteria (90%)	Activated sludge		1					
Rhodospirillales (95%)	Zebrafish intestine						1	
Rhodospirillaceae								
Novispirillum (84%)	Sea water/ Lake water		2					
Unclassified Rhodospirillales								
Reyranella massiliensis (94%)	Weathered crop						1	
Rhodobacterales								
Rhodobacteraceae								
Rhodobacter (95%)	Biologically activated carbon			1				
Hyphomonadaceae (91%)	Membrane bioreactor			1				
Rhizobiales								
Hyphomicrobiaceae								
Ancalomicrobium (93%)	Human stomach biopsy	1						

Grouping (Percentage Similarity)	Source	GW	MCW	FCW	M	F	BCW	Total
Rickettsiales								
Candidatus Midichloriaceae								
Candidatus Midichloria (81% - 90%)	Glacier ice/ Ixodes holocyclus (Paralysis tick)			2				
Holosporaceae								
Candidatus Captivus (88%)	Forest soil						1	
Rickettsiales genera incertae sedis								
Candidatus Odyssella thessalonicensis (91%)	Forest soil						1	
Beta Proteobacteria (92% - 95%)	Freshwater/ Riverwater		4					
Burkholderiales								
Burkholderiaceae								
Pandoraea sp. (95% - 98%)	Anaerobic sludge			1	7		3	
Polynucleobacter (92%)	Estuary water						1	
Ralstonia (98%)	Biological degreasing systems				1			
Comamonadaceae (85% - 96%)	River water/ Waste water/ Lake water		4					

Grouping (Percentage Similarity)	Source	GW	MCW	FCW	M	F	BCW	Total
Delftia acidovorans (95% - 99%)	Tricholoma matsutake (Pine mushroom)/ Aircraft cabin/ Epithelium				8		1	
Oxalobacteraceae (91% - 96%)	Freshwater pond water/ River water		1				1	
Neisseriales								
Neisseriaceae (88%)	Epithelium		1					
Chitinibacter tainanensis (99%)	Mud flat				1			
Vogesella perlucida (98% - 99%)	Slightly alkaline water				2			
Methylophilales								
Methylophilaceae (95%)	Lake water		4					
Methylophilus (94%)	Sewage sludge			1				
Unclassified Burkholderiales								
Methylibium (95%)	Lake sediment						1	
Sphaerotilus sp. (95% - 97%)	Lake water/sludge			2				

Grouping (Percentage Similarity)	Source	GW	MCW	FCW	M	F	BCW	Total
Gamma Proteobacteria								
Aeromonadales								
Aeromonadaceae								
Aeromonas jandaei (93% - 99%)	Intestine of silver carp				2			
Aeromonas veronii (99%)	Deep seawater				2			
Chromatiales								
Chromatiacea								
Thiodictyon bacillosum (92%)	Wastewater		1					
Enterobacteriales								
Enterobacteriaceae (96%)							1	
Citrobacter werkmanii (96%)	Lake water					2		
Morganella morganii (94% - 96%)	Soil/ River water/ Digestive tract of ground beetle				1	17		
Serratia grimesii (99%)	Bombyx mori (silk moth)				1			
Serratia marcescens (99%)	Stream water				1			

Grouping **(Percentage Similarity)**	**Source**	**GW**	**MCW**	**FCW**	**M**	**F**	**BCW**	**Total**
Legionellales								
Legionellaceae								
Legionella birminghamensis (92%)	Loamy sand						1	
Legionella impletisoli (94%)	Paddy field soil						1	
Legionella taurinensis (91% - 92%)	Epithelium of hydra						5	
Pseudomonadales								
Moraxellaceae								
Acinetobacter sp. (79% - 98%)	Ocean water/ Amphibian skin	4	3	2		4		
Pseudomonadaceae								
Pseudomonas aeruginosa (99%)	Activated sewage sludge				1			
Delta Proteobacteria								
Myxococcales (82%)	Spring water		1					
Kofleriaceae								
Haliangium (87%)	Zebrafish gut			1				

Grouping (Percentage Similarity)	Source	GW	MCW	FCW	M	F	BCW	Total
Spirochaetes								
Spirochaetales								
Spirochaetaceae (92% - 93%)	Freshwater pond water/ Lake water						4	
Verrucomicrobia (93%)	Tar pond sediment			1				
Verrucomicrobiae								
Verrucomicrobiales								
Verrucomicrobiaceae								
Prosthecobacter vanneervenii (95% - 97%)	N/A			3				
Viridiplantae								
Chlorophyta								
Trebouxiophyceae								
Chlorellales								
Chlorellaceae								
Closteriopsis acicularis (92% - 94%)	N/A	3						
Parachlorella kessleri (92%)	N/A	1						

Grouping (Percentage Similarity)	Source	GW	MCW	FCW	M	F	BCW	Total
Eukaryota								
Euglenozoa								
Euglenida								
Euglenales								
Phacaceae								
Phacus orbicularis (74% - 94%)	N/A		6					
Metazoa								
Coleoptera								
Carabidae								
Paussinae								
Arthropterus sp. (73% - 75%)	N/A			1		1		
Unclassified		1				1		
	Total	22	43	28	30	25	29	177

* GW, Groundwater; MCW, *Moina* Culture Water; FCW, Fish Culture Water; M, *Moina* sp.; F, Fish Larvae and BCW, Broodstock Culture Water

A more comprehensive tabulation of closest relative organisms is presented in Appendix C.

4.8 Quantitative Analysis of Samples

Analysis for all samples were carried out to determine whether the clone library was sufficient using methods described by Kemp and Aller (2004b), whereby S_{Chao1} has been recommended to estimate phylotypes richness from prokaryotic 16S rDNA libraries and Coverage (Good's C) of the clone libraries. S_{Chao1} estimator is non-parametric (non-dependent on parameter) which is suitable for data where the phylotypes are comparatively infrequent and Kemp and Aller (2004b) concluded that the asymptotic values of S_{Chao1} are unbiased or minimally biased estimates of phylotypes richness.

Figure 4.19 to Figure 4.30 depicts the charts obtained through an input form to evaluate library size developed by Kemp and Aller (2004b). The input form is available at www.aslo.org/lomethods/free/2004//0114a.html (Appendix C). Based on the charts from S_{Chao1} and Coverage (Good's C), all samples, Groundwater, *Moina* Culture Water, Fish Culture Water, *Moina* sp., Fish Larvae and Broodstock Culture Water achieved asymptotic maximum which indicates the sample have reached a suitable level of richness and sufficient for the clone library size.

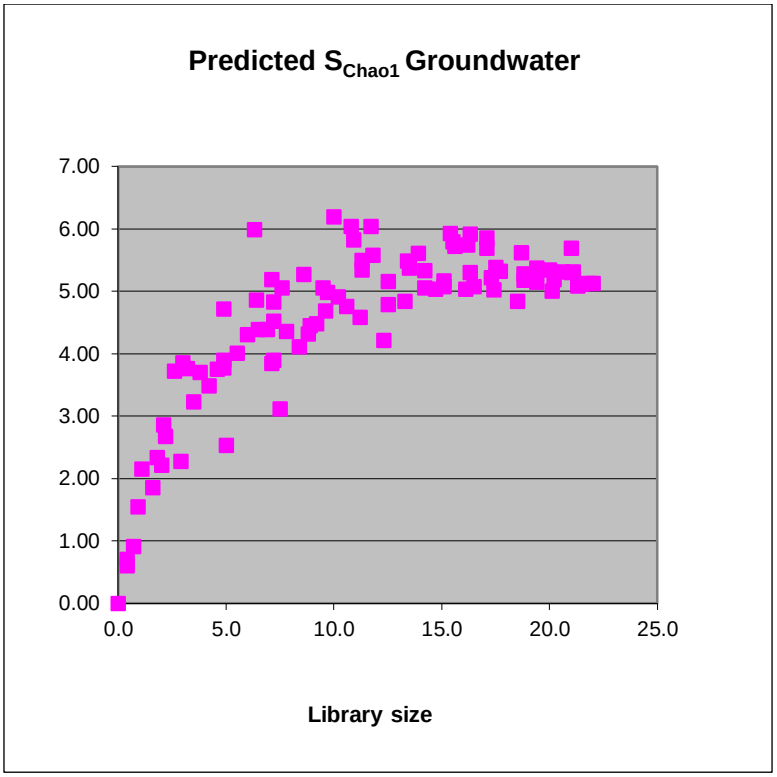


Figure 4.19: Predicted S_{Chao1} for Groundwater samples

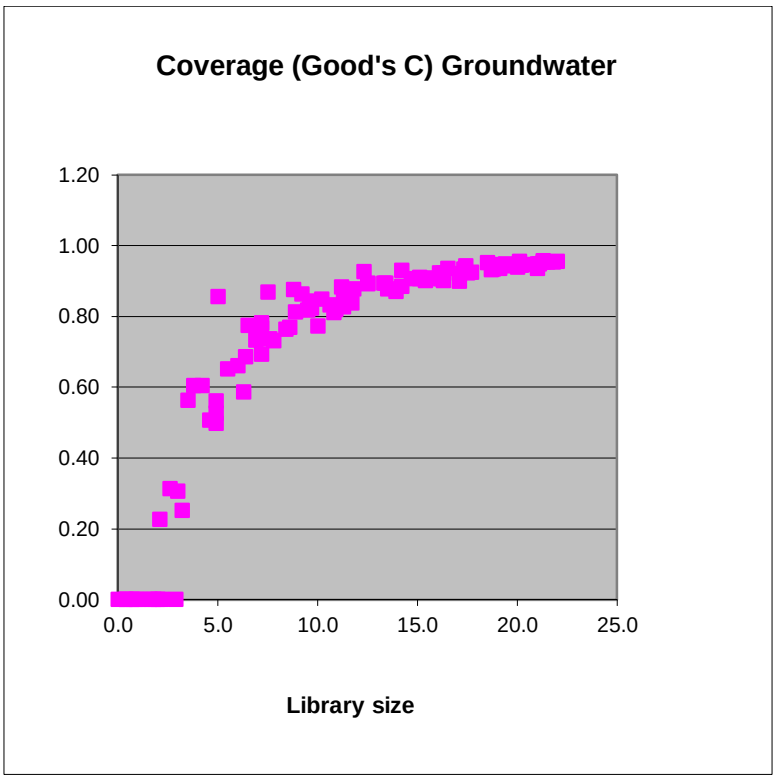


Figure 4.20: Coverage (Good's C) for Groundwater samples

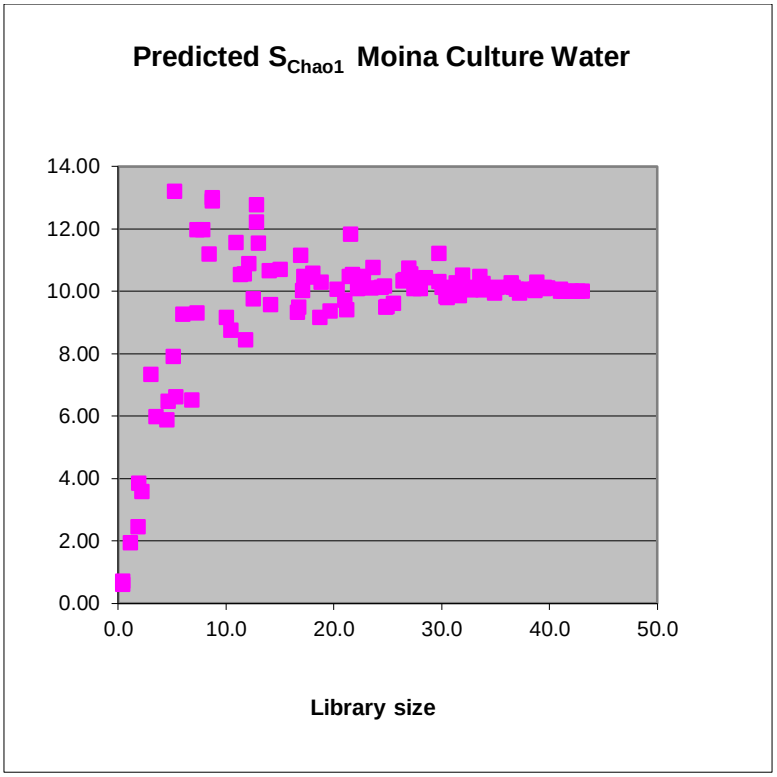


Figure 4.21: Predicted S_{Chao1} for *Moina* Culture Water samples

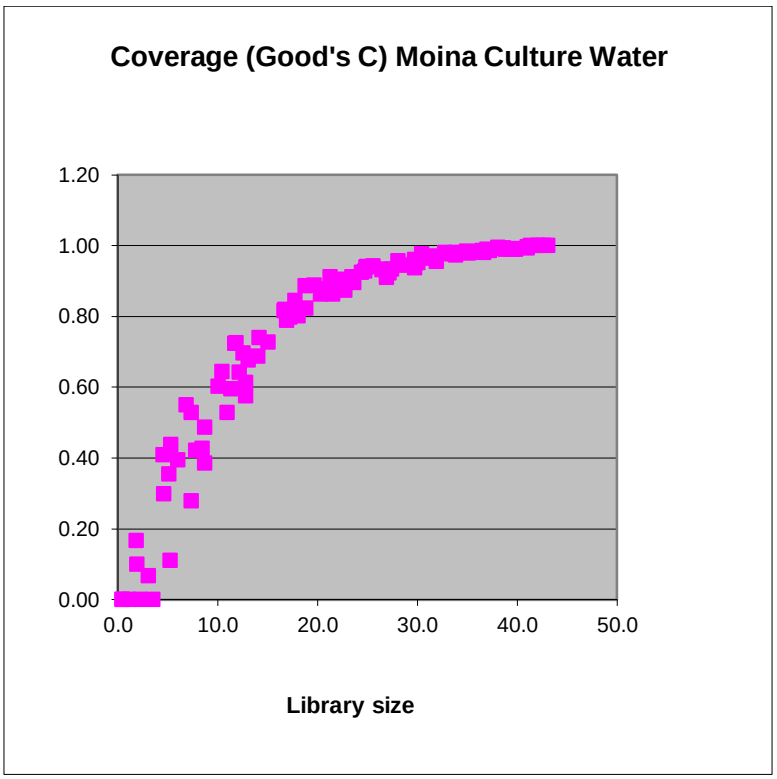


Figure 4.22: Coverage (Good's C) for *Moina* Culture Water samples

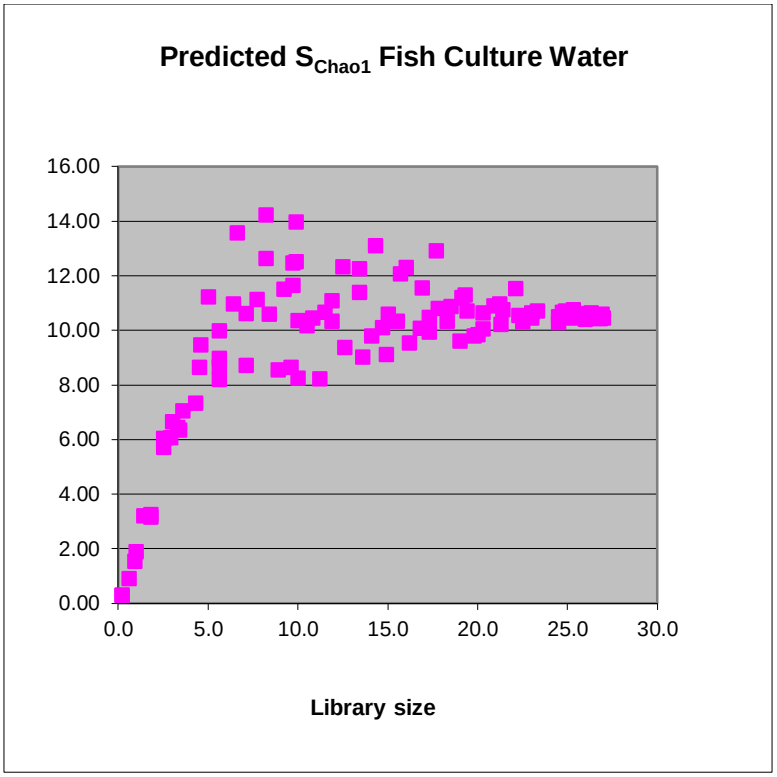


Figure 4.23: Predicted S_{Chao1} for Fish Culture Water samples

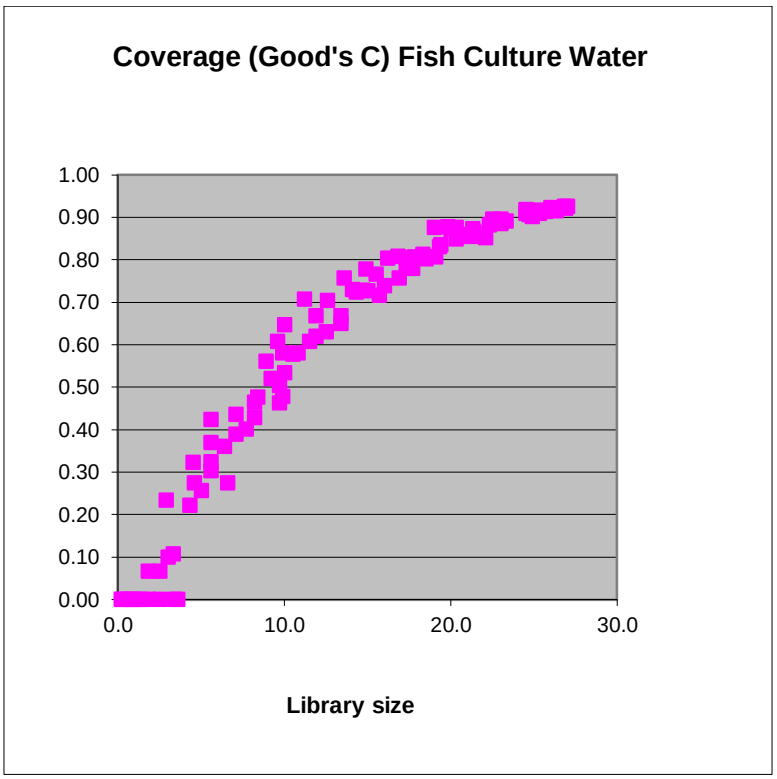


Figure 4.24: Coverage (Good's C) for Fish Culture Water samples

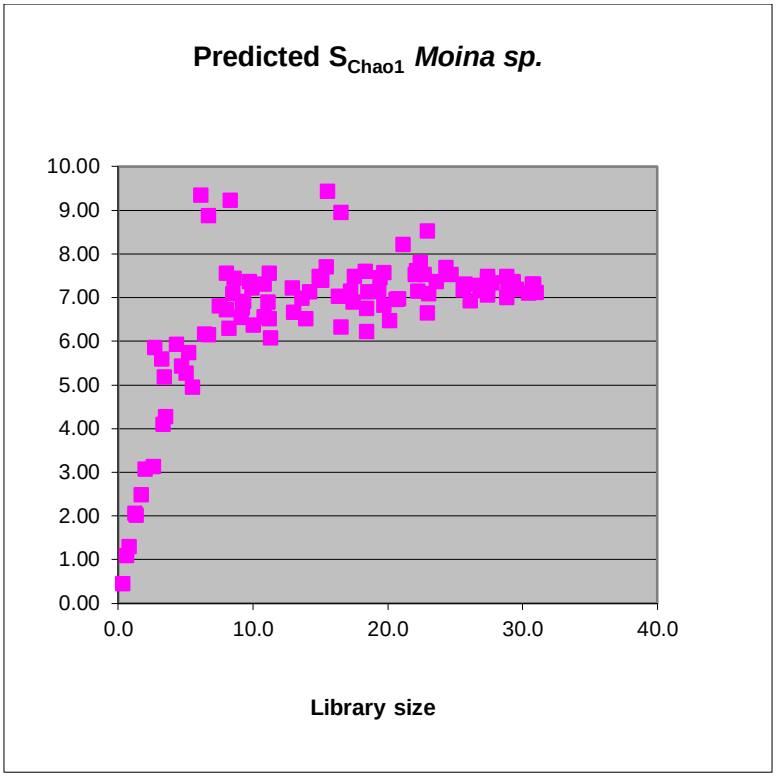


Figure 4.25: Predicted S_{Chao1} for *Moina sp.* samples

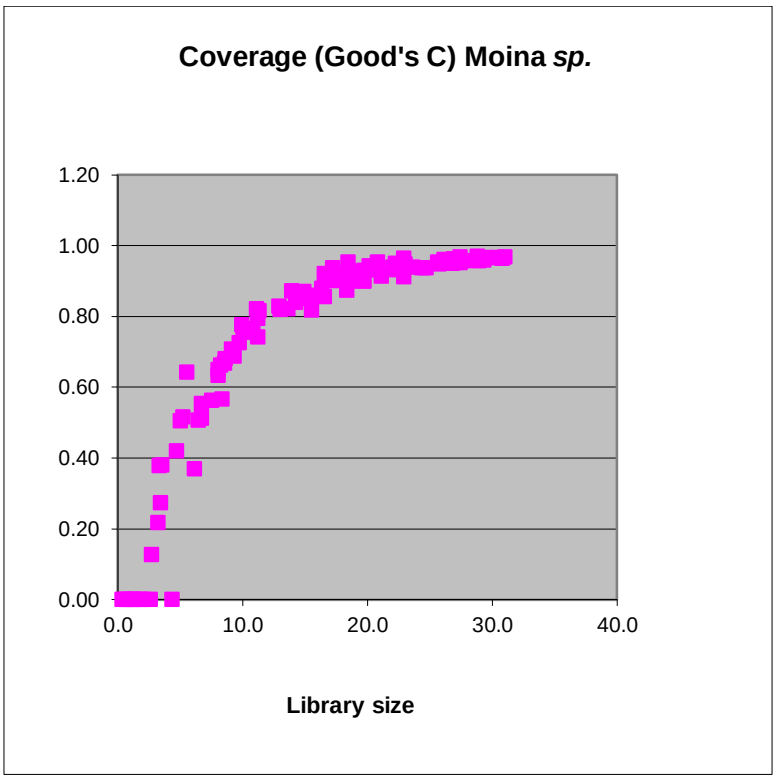


Figure 4.26: Coverage (Good's C) for *Moina sp.* samples

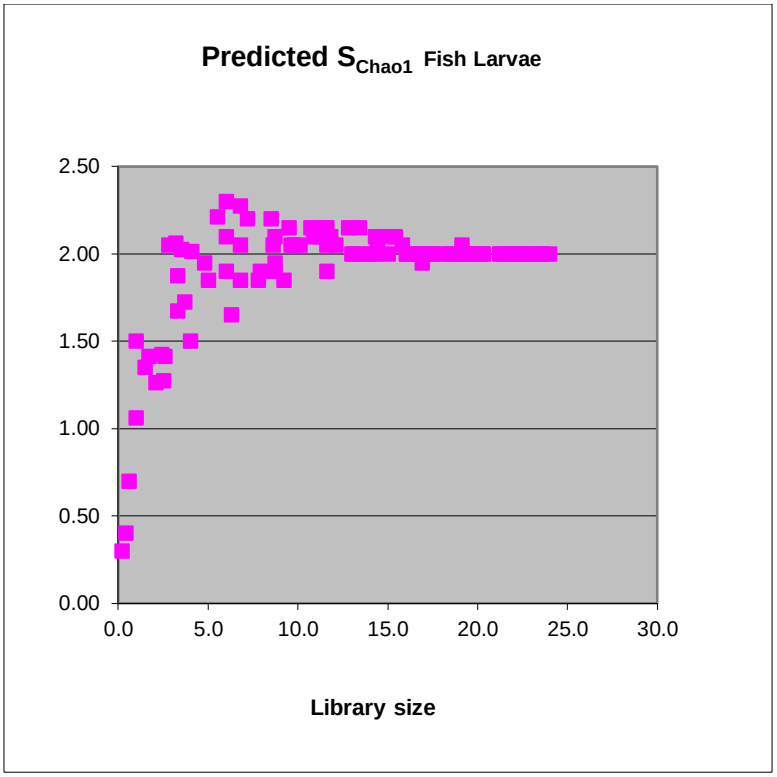


Figure 4.27: Predicted S_{Chao1} for Fish Larvae samples

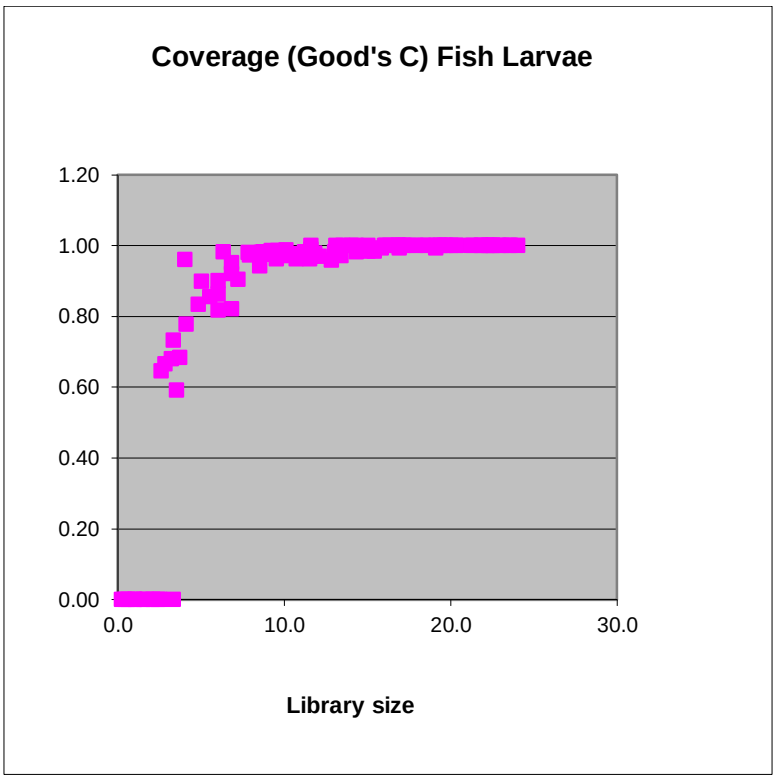


Figure 4.28: Coverage (Good's C) for Fish Larvae samples

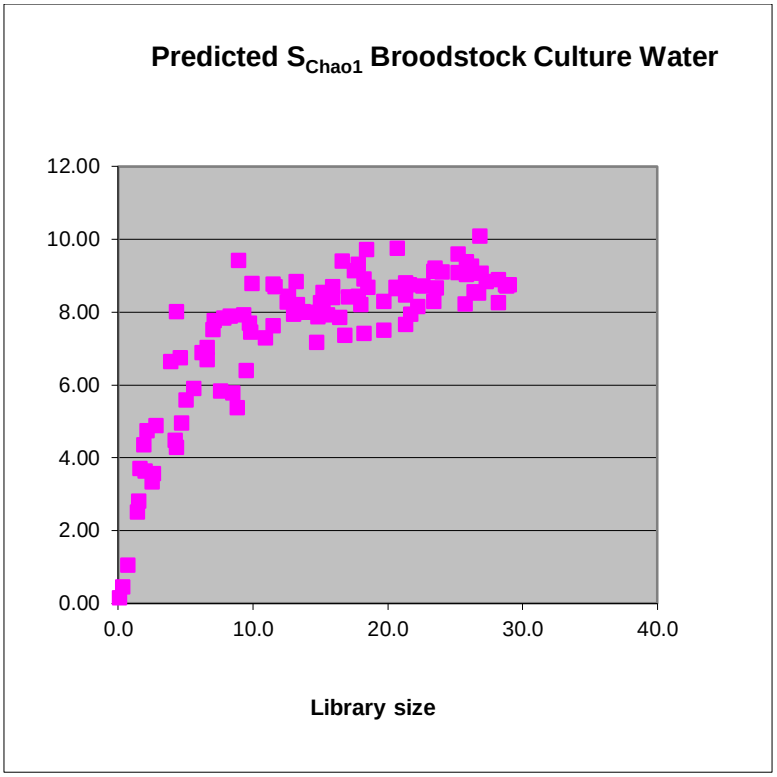


Figure 4.29: Predicted S_{Chao1} for Broodstock Culture Water samples

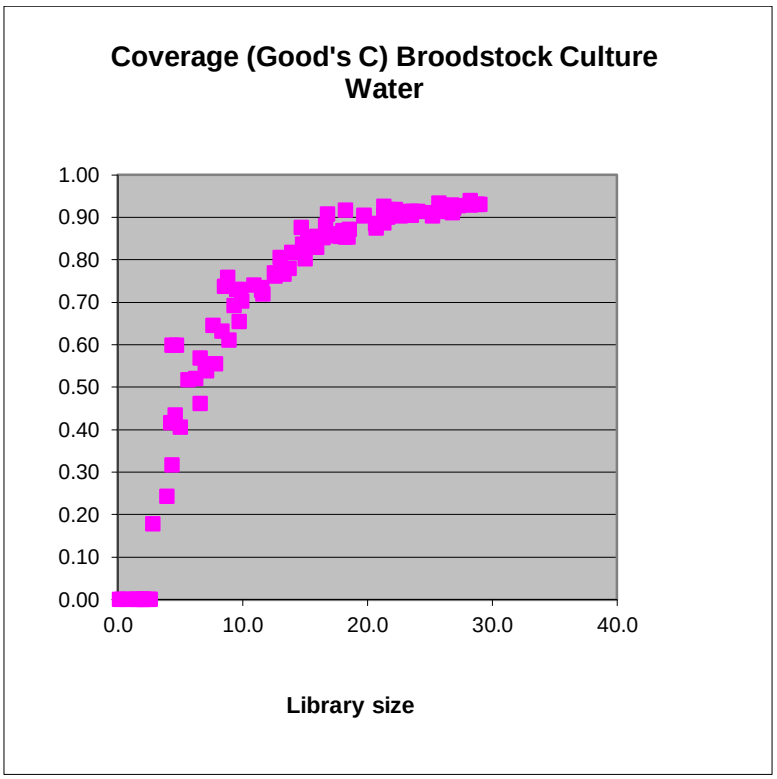


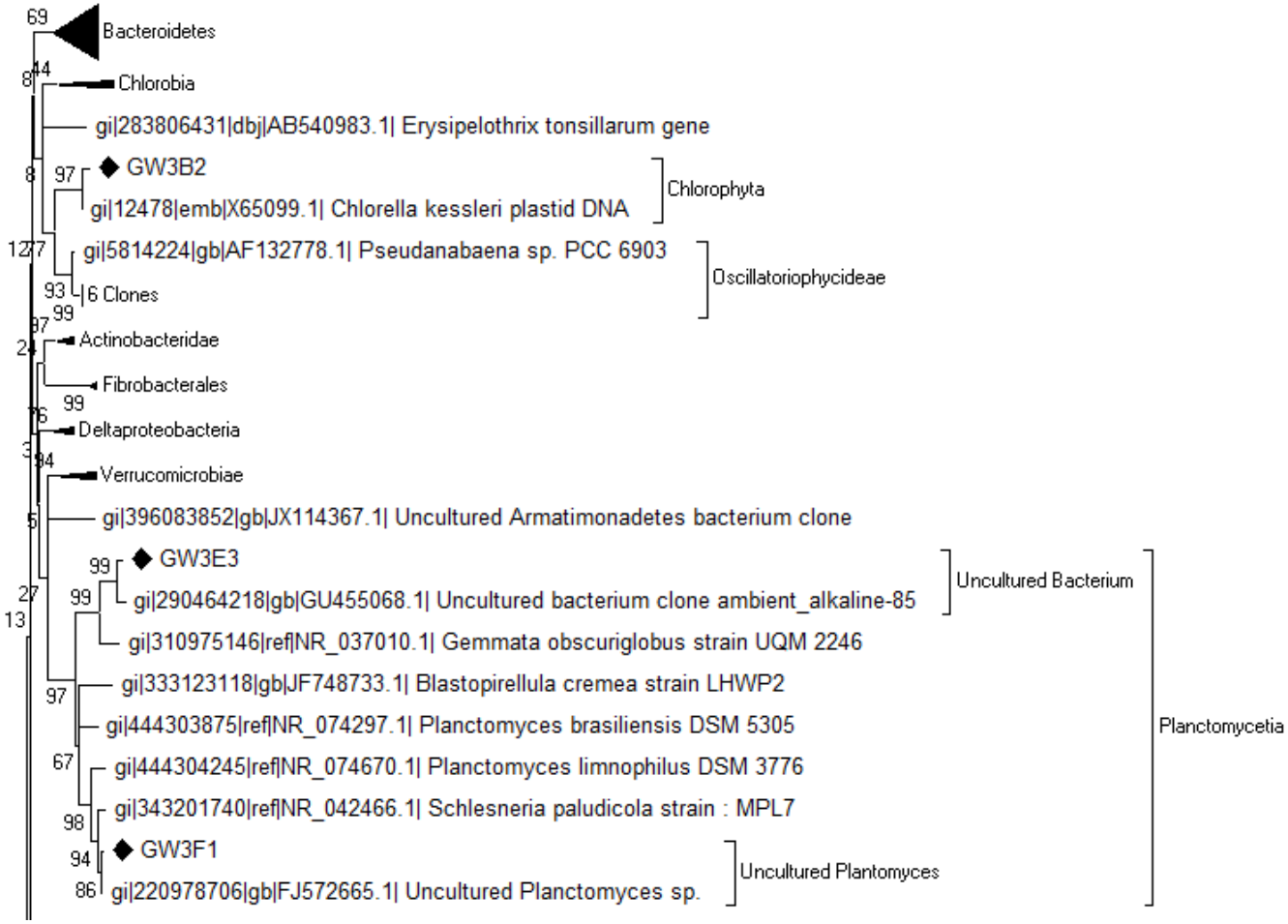
Figure 4.30: Coverage (Good's C) for Broodstock Culture Water samples

4.9 Phylogenetic Analysis

Phylogenetic trees were constructed using Neighbour-Joining Method with Bootstrap Test of Phylogeny and Maximum Composite Likelihood model was congruent with phylogenetic trees constructed using Maximum Likelihood (ML) Method with Bootstrap Test of Phylogeny for GW, MCW, FCW, *Moina* M, F and BCW samples. Therefore, only Maximum Likelihood phylogenetic trees are presented here.

4.9.1 Phylogenetic Analysis of Groundwater Microflora Communities

A phylogenetic tree was constructed based on the sequence analysis of 1.5kb 16S rDNA sequence (Figure 4.31) for Groundwater samples. Sequences from this study are marked with (♦). One sample was clustered to *Chlorophyta* division while six samples were clustered to *Pseudanabaena* sp. Two samples clustered with phylum *Planctomycetia*. One sample each was clustered to *Ancalomicrobium* and *Nostocales* order. There were 11 samples from this study that did not cluster with any sequences obtained from National Centre for Biotechnology Information (NCBI) in the inferred tree.


Bacteroidetes
Chlorobia
gi|283806431|dbj|AB540983.1| Erysipelothrix tonsillarum gene
GW3B2
gi|12478|emb|X65099.1| Chlorella kessleri plastid DNA
Chlorophyta
gi|5814224|gb|AF132778.1| Pseudanabaena sp. PCC 6903
6 Clones
Oscillatoriophycideae
Actinobacteridae
Fibrobacterales
Deltaproteobacteria
Verrucomicrobiae
gi|396083852|gb|JX114367.1| Uncultured Armatimonadetes bacterium clone
GW3E3
gi|290464218|gb|GU455068.1| Uncultured bacterium clone ambient_alkaline-85
Uncultured Bacterium
gi|310975146|ref|NR_037010.1| Gemmata obscuriglobus strain UQM 2246
gi|333123118|gb|JF748733.1| Blastopirellula cremea strain LHWP2
gi|444303875|ref|NR_074297.1| Planctomyces brasiliensis DSM 5305
gi|444304245|ref|NR_074670.1| Planctomyces limnophilus DSM 3776
gi|343201740|ref|NR_042466.1| Schlesneria paludicola strain : MPL7
GW3F1
gi|220978706|gb|FJ572665.1| Uncultured Planctomyces sp.
Uncultured Plantomyces
Planctomycetia

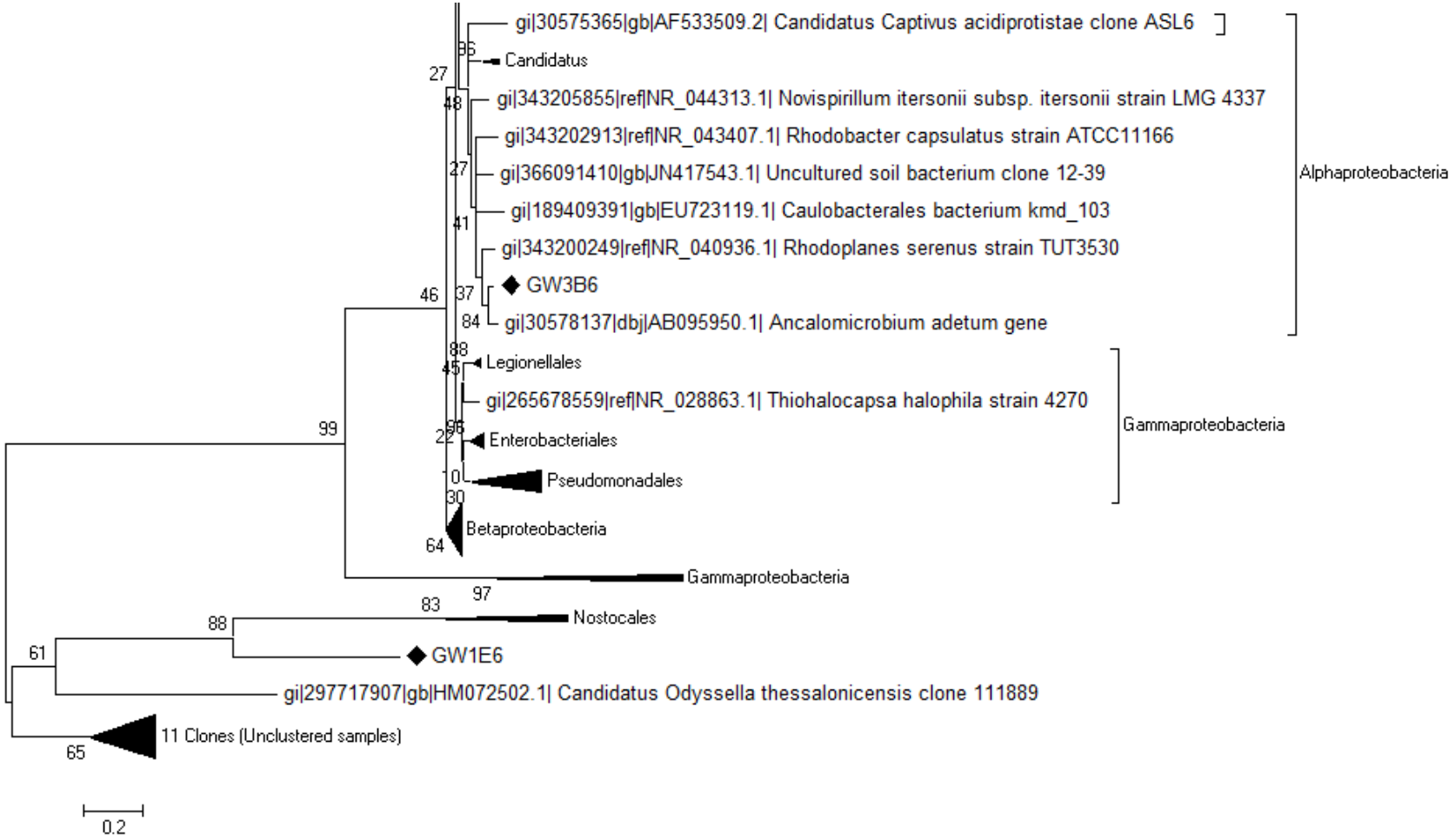
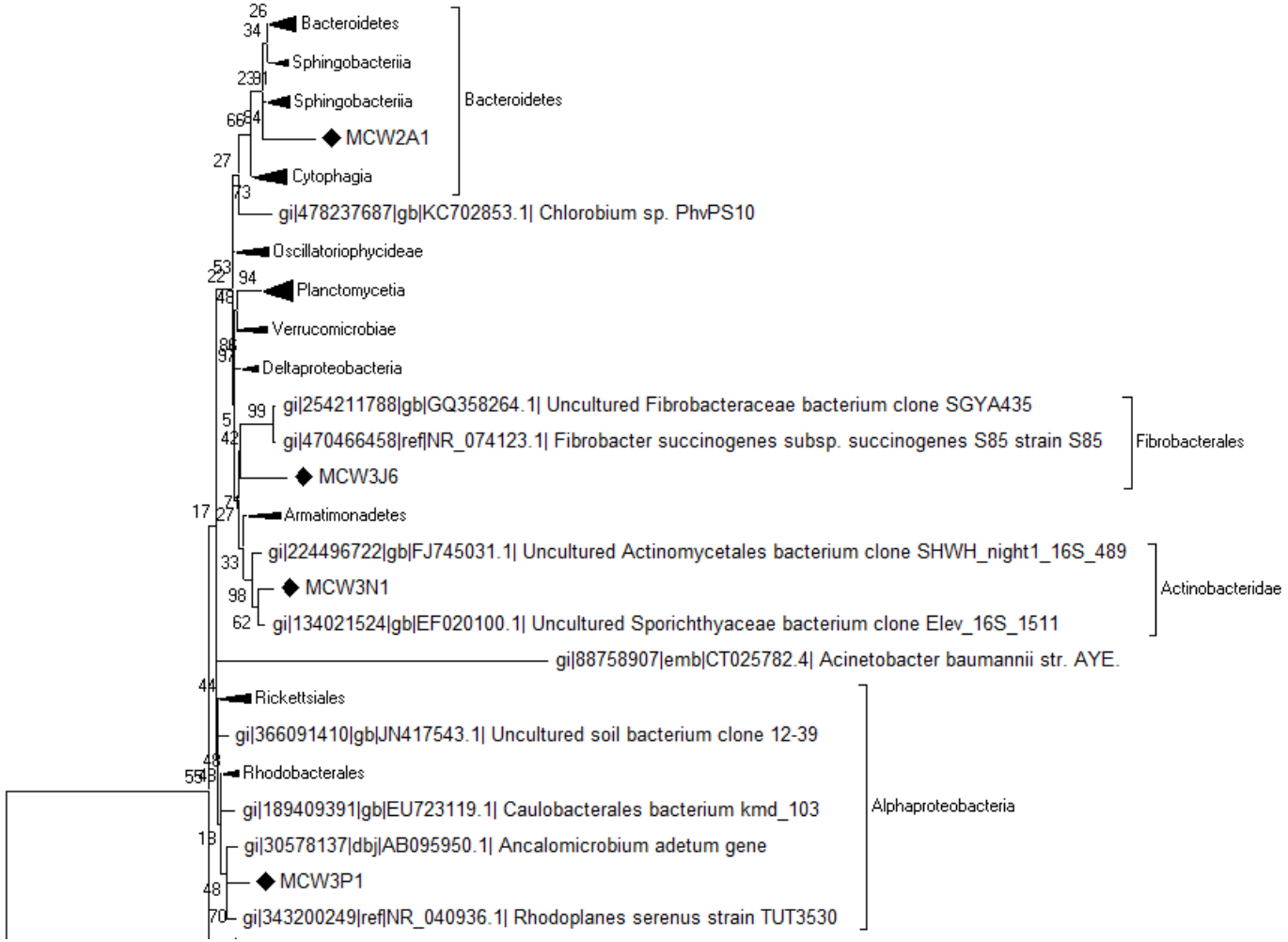

gi|30575365|gb|AF533509.2| Candidatus Captivus acidiprotistae clone ASL6
Candidatus
gi|343205855|ref|NR_044313.1| Novispirillum itersonii subsp. itersonii strain LMG 4337
gi|343202913|ref|NR_043407.1| Rhodobacter capsulatus strain ATCC11166
gi|366091410|gb|JN417543.1| Uncultured soil bacterium clone 12-39
gi|189409391|gb|EU723119.1| Caulobacterales bacterium kmd_103
gi|343200249|ref|NR_040936.1| Rhodoplanes serenus strain TUT3530
GW3B6
gi|30578137|dbj|AB095950.1| Ancalomicrobium adetum gene
Alphaproteobacteria
Legionellales
gi|265678559|ref|NR_028863.1| Thiohalocapsa halophila strain 4270
Enterobacteriales
Pseudomonadales
Gammaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Nostocales
GW1E6
gi|297717907|gb|HM072502.1| Candidatus Odyssella thessalonicensis clone 111889
11 Clones (Unclustered samples)
0.2

Figure 4.31: Molecular Phylogenetic analysis by Maximum Likelihood method of Groundwater Samples

Maximum Likelihood method based on Tamura-Nei model was used to infer the evolutionary history. Bootstrap consensus tree was inferred from 500 replicates. The analysis involved 104 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. Fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position.

4.9.2 Phylogenetic Analysis of *Moina Culture Water* Microflora Communities

A phylogenetic tree was constructed based on the sequence analysis of 1.5kb 16S rDNA sequence (Figure 4.32) for *Moina Culture Water* samples. Sequences from this study are marked with (♦). There were 35 samples from this study that did not cluster with the sequences obtained from NCBI. Four samples were clustered to *Chloroflexi* while one sample each has been closely associated with *Bacteroidetes*, *Fibrobacterales*, *Actinobacteridae* and *Ancalomicrobium*.


Bacteroidetes
Sphingobacteriia
Sphingobacteriia
MCW2A1
Cytophagia
Bacteroidetes
gi|478237687|gb|KC702853.1| Chlorobium sp. PhvPS10
Oscillatoriophycideae
Planctomycetia
Verrucomicrobiae
Deltaproteobacteria
gi|254211788|gb|GQ358264.1| Uncultured Fibrobacteraceae bacterium clone SGYA435
gi|470466458|ref|NR_074123.1| Fibrobacter succinogenes subsp. succinogenes S85 strain S85
MCW3J6
Fibrobacterales
Armatimonadetes
gi|224496722|gb|FJ745031.1| Uncultured Actinomycetales bacterium clone SHWH_night1_16S_489
MCW3N1
gi|134021524|gb|EF020100.1| Uncultured Sporichthyaceae bacterium clone Elev_16S_1511
Actinobacteridae
gi|88758907|emb|CT025782.4| Acinetobacter baumannii str. AYE.
Rickettsiales
gi|366091410|gb|JN417543.1| Uncultured soil bacterium clone 12-39
Rhodobacterales
gi|189409391|gb|EU723119.1| Caulobacterales bacterium kmd_103
gi|30578137|dbj|AB095950.1| Ancalomicrobium adetum gene
MCW3P1
gi|343200249|ref|NR_040936.1| Rhodoplanes serenus strain TUT3530
Alphaproteobacteria

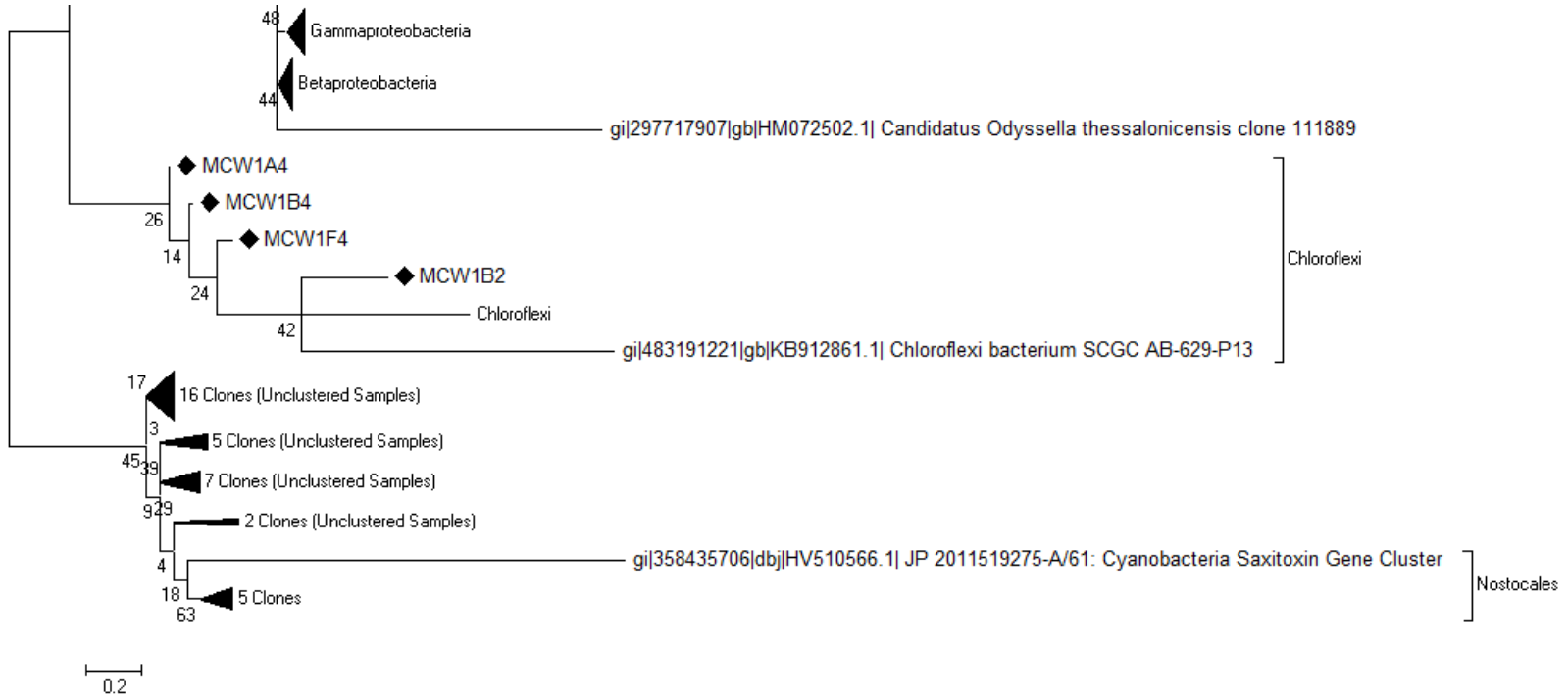


Figure 4.32: Molecular Phylogenetic analysis by Maximum Likelihood method of *Moina* Culture Water Samples

The analysis involved 125 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. Fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position.

4.9.3 Phylogenetic Analysis of Fish Culture Water Microflora Communities

A phylogenetic tree was constructed based on the sequence analysis of 1.5kb 16S rDNA sequence (Figure 4.33) for Fish Culture Water samples. Sequences from this study are marked with (♦). Two samples were clustered with *Planctomycetia*, while one sample each was clustered to *Actinobacteridae*, *δ-Proteobacteria* and *Bacteroidetes*. There were five samples that were clustered to *Erysipelotrichia*, two clustered to *Sphingobacteriia*, one clustered to *β-Proteobacteria* and one sample clustered to *Verrucomicrobiae*. Based on the phylogenetic tree inferred, 14 samples were unclustered.

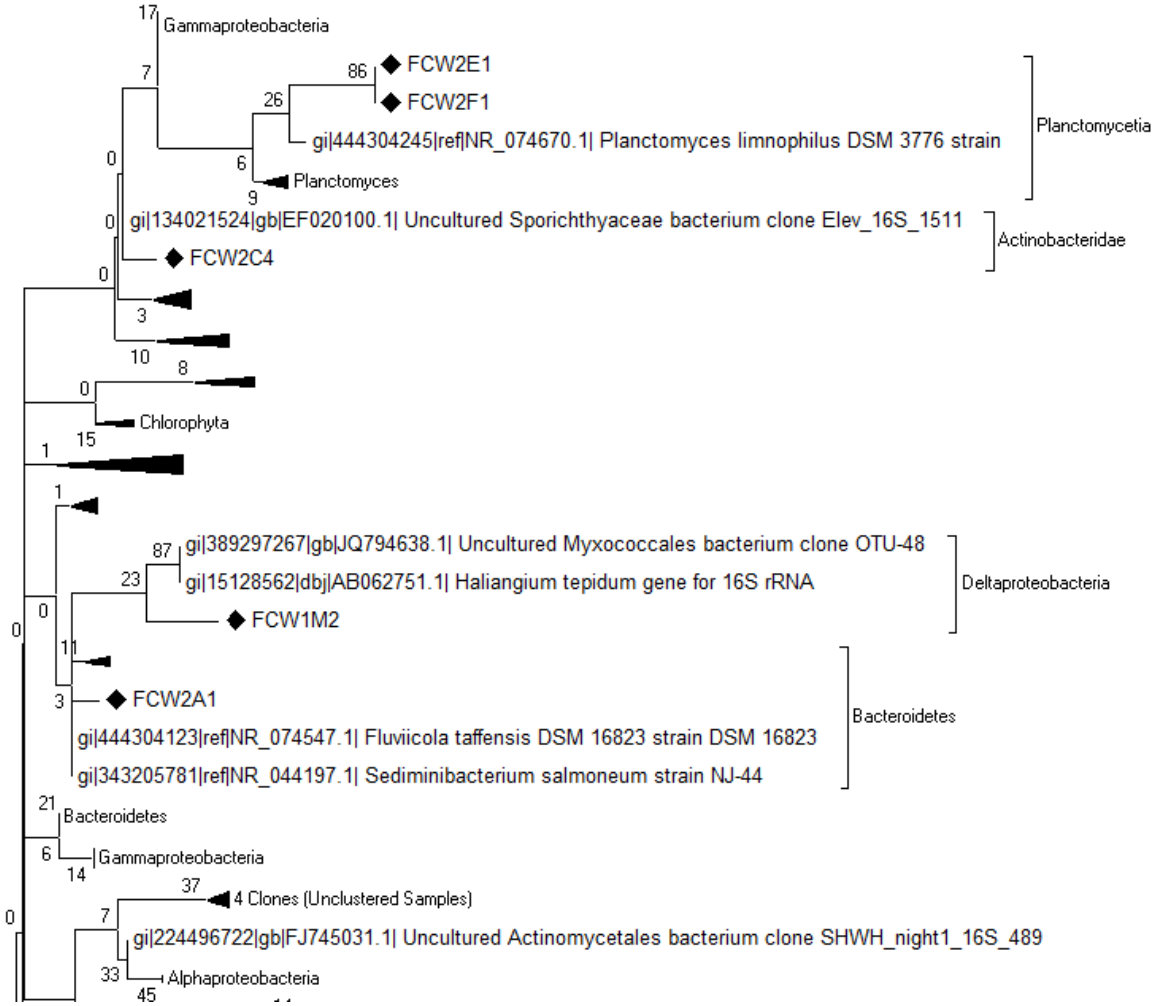
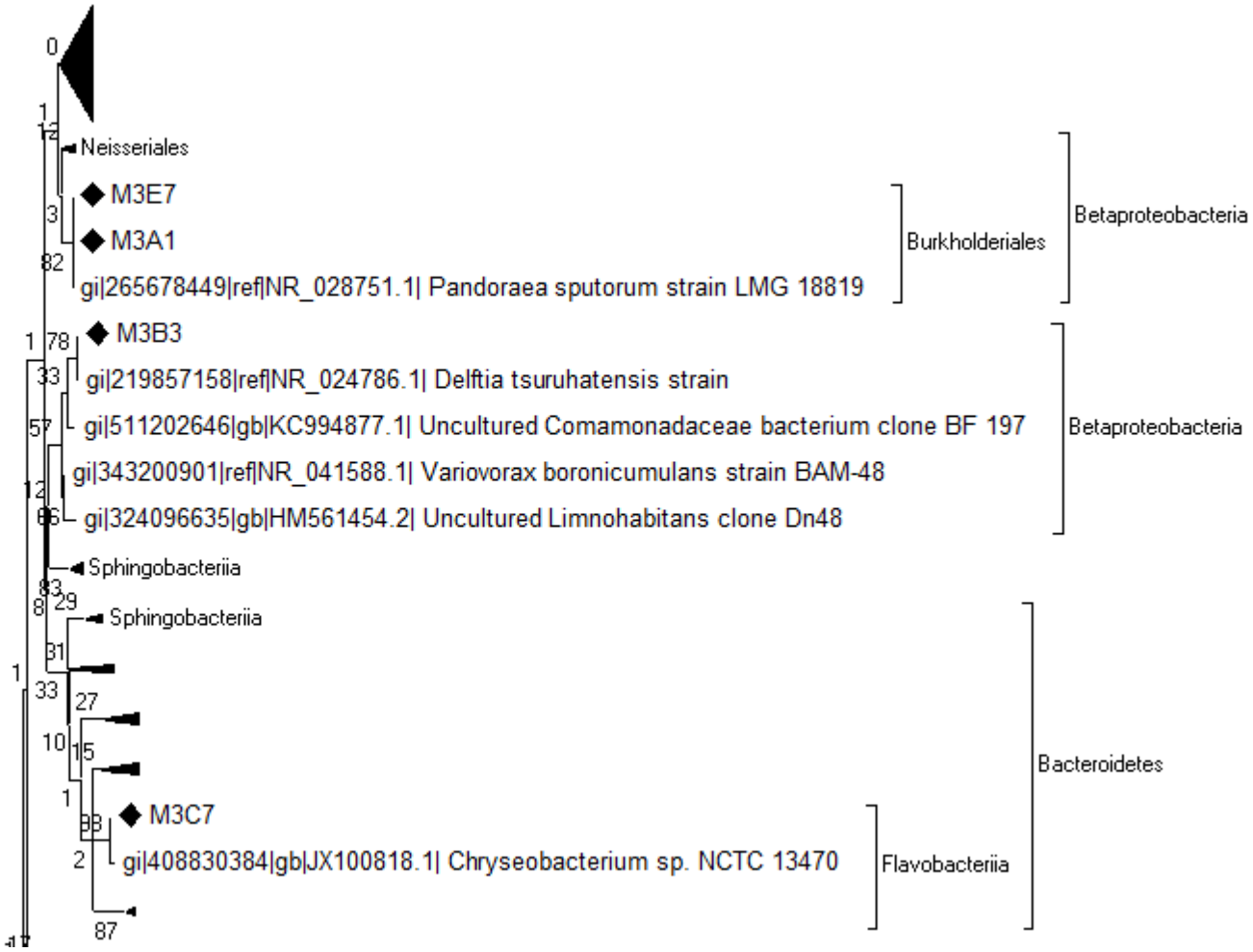

Gammaproteobacteria
FCW2E1
FCW2F1
gi|444304245|ref|NR_074670.1| Planctomyces limnophilus DSM 3776 strain
Planctomycetia
Planctomyces
gi|134021524|gb|EF020100.1| Uncultured Sporichthyaceae bacterium clone Elev_16S_1511
Actinobacteridae
FCW2C4
Chlorophyta
gi|389297267|gb|JQ794638.1| Uncultured Myxococcales bacterium clone OTU-48
gi|15128562|dbj|AB062751.1| Haliangium tepidum gene for 16S rRNA
Deltaproteobacteria
FCW1M2
FCW2A1
gi|444304123|ref|NR_074547.1| Fluviicola taffensis DSM 16823 strain DSM 16823
gi|343205781|ref|NR_044197.1| Sediminibacterium salmoneum strain NJ-44
Bacteroidetes
Bacteroidetes
Gammaproteobacteria
4 Clones (Unclustered Samples)
gi|224496722|gb|FJ745031.1| Uncultured Actinomycetales bacterium clone SHWH_night1_16S_489
Alphaproteobacteria

Figure 4.33: Molecular Phylogenetic analysis by Maximum Likelihood method of Fish Culture Water

The analysis involved 114 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. Fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position.

4.9.4 Phylogenetic Analysis of *Moina sp.* Microflora Communities

A phylogenetic tree was constructed based on the sequence analysis of 1.5kb 16S rDNA sequence (Figure 4.34) for *Moina sp.* samples. Sequences from this study are marked with (♦). Two samples have been associated with *Pandoraea* sp., one sample each was clustered with *β-Proteobacteria*, *Bacteroidetes* and Candidatus *Odysella*. Another two samples were clustered with *Aeromonas* sp. A total of 21 samples were not clustered to sequences obtained from NCBI.


Neisseriales
M3E7
M3A1
gi|265678449|ref|NR_028751.1| Pandoraea sputorum strain LMG 18819
Burkholderiales
Betaproteobacteria
M3B3
gi|219857158|ref|NR_024786.1| Delftia tsuruhatensis strain
gi|511202646|gb|KC994877.1| Uncultured Comamonadaceae bacterium clone BF 197
gi|343200901|ref|NR_041588.1| Variovorax boronicumulans strain BAM-48
gi|324096635|gb|HM561454.2| Uncultured Limnohabitans clone Dn48
Betaproteobacteria
Sphingobacteriia
Sphingobacteriia
M3C7
gi|408830384|gb|JX100818.1| Chryseobacterium sp. NCTC 13470
Flavobacteriia
Bacteroidetes

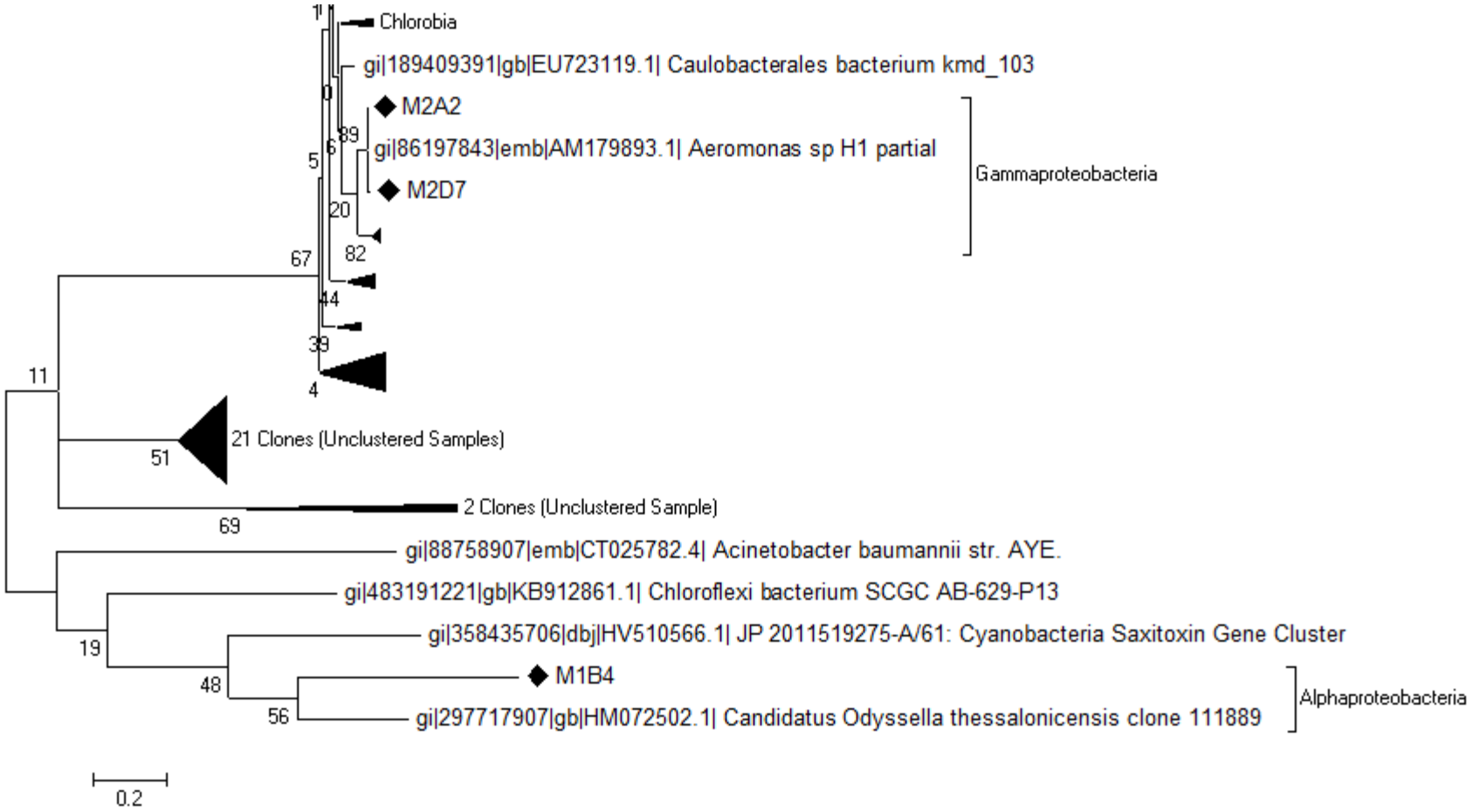

Chlorobia
gi|189409391|gb|EU723119.1| Caulobacterales bacterium kmd_103
M2A2
gi|86197843|emb|AM179893.1| Aeromonas sp H1 partial
M2D7
Gammaproteobacteria
21 Clones (Unclustered Samples)
2 Clones (Unclustered Sample)
gi|88758907|emb|CT025782.4| Acinetobacter baumannii str. AYE.
gi|483191221|gb|KB912861.1| Chloroflexi bacterium SCGC AB-629-P13
gi|358435706|dbj|HV510566.1| JP 2011519275-A/61: Cyanobacteria Saxitoxin Gene Cluster
M1B4
gi|297717907|gb|HM072502.1| Candidatus Odyssella thessalonicensis clone 111889
Alphaproteobacteria
0.2

Figure 4.34: Molecular Phylogenetic analysis by Maximum Likelihood method of *Moina sp.*

The analysis involved 112 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. Fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position.

4.9.5 Phylogenetic Analysis of Fish Larvae Microflora Communities

A phylogenetic tree was constructed based on the sequence analysis of 1.5kb 16S rDNA sequence (Figure 4.35) for Fish Larvae samples. Sequences from this study are marked with (♦). Most of the samples (22 clones) from Fish Larvae did not cluster with any sequences obtained from NCBI. Only one sequence each that was clustered to *Morganella morganii*, *Cyanobacteria* and *Candidatus Odysella*.

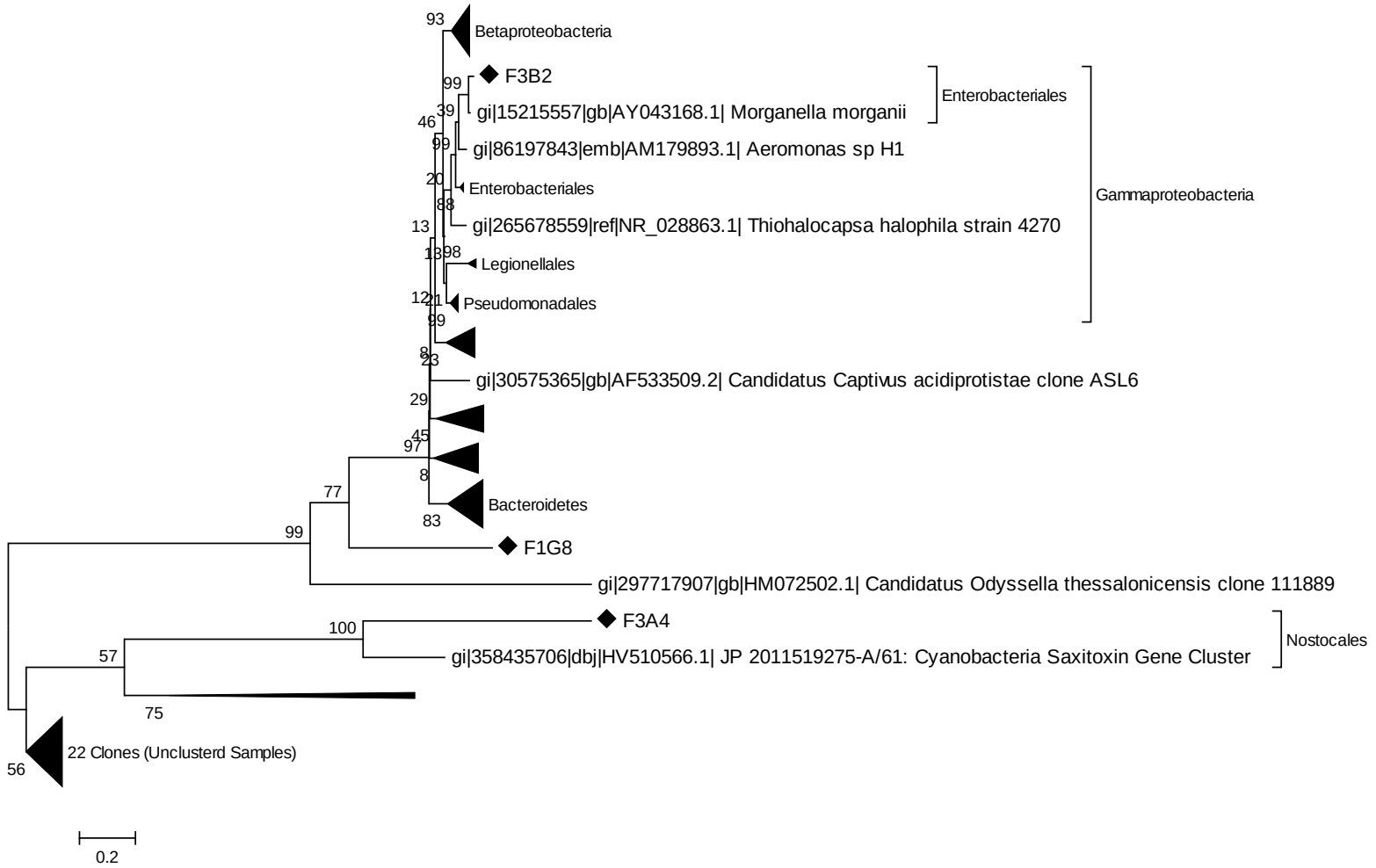
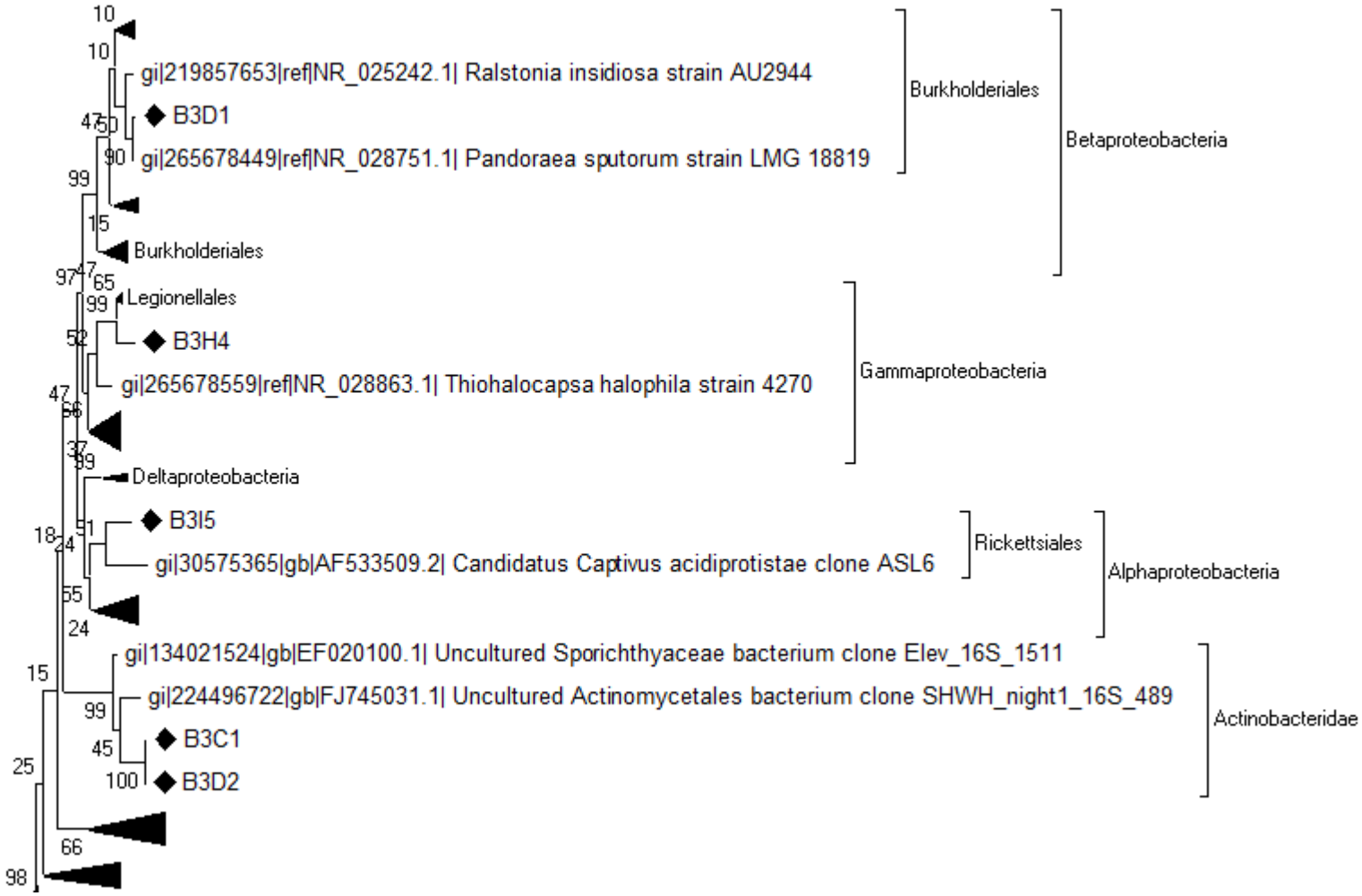

93
Betaproteobacteria
F3B2
99
Enterobacteriales
gi|15215557|gb|AY043168.1| Morganella morganii
39
46
99
gi|86197843|emb|AM179893.1| Aeromonas sp H1
20
Enterobacteriales
88
gi|265678559|ref|NR_028863.1| Thiohalocapsa halophila strain 4270
13
Gammaproteobacteria
13
98
Legionellales
12
21
Pseudomonadales
99
8
23
gi|30575365|gb|AF533509.2| Candidatus Captivus acidiprotistae clone ASL6
29
45
97
8
77
Bacteroidetes
83
99
F1G8
gi|297717907|gb|HM072502.1| Candidatus Odyssella thessalonicensis clone 111889
F3A4
100
Nostocales
57
gi|358435706|dbj|HV510566.1| JP 2011519275-A/61: Cyanobacteria Saxitoxin Gene Cluster
75
22 Clones (Unclusterd Samples)
56
0.2

Figure 4.35: Molecular Phylogenetic analysis by Maximum Likelihood method of Fish Larvae Samples

The analysis involved 107 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. Fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position.

4.9.6 Phylogenetic Analysis of Broodstock Culture Water Microflora Communities

A phylogenetic tree was constructed based on the sequence analysis of 1.5kb 16S rDNA sequence (Figure 4.36) for Broodstock Culture Water samples. Sequences from this study are marked with (♦). Out of 29 samples, 21 samples were not clustered to the sequences obtained from NCBI. Only one sample from this study has been clustered within the *Pandoraea* sp. while one sample each clustered to *Legionallales*, Candidatus *Captivus* and *Chryseobacterium*. Two samples were clustered with uncultured *Actinomycetales* and another two samples clustered with *Sediminibacterium*.


gi|219857653|ref|NR_025242.1| Ralstonia insidiosa strain AU2944
B3D1
gi|265678449|ref|NR_028751.1| Pandoraea sputorum strain LMG 18819
Burkholderiales
Betaproteobacteria
Legionellales
B3H4
gi|265678559|ref|NR_028863.1| Thiohalocapsa halophila strain 4270
Gammaproteobacteria
Deltaproteobacteria
B3I5
gi|30575365|gb|AF533509.2| Candidatus Captivus acidiprotistae clone ASL6
Rickettsiales
Alphaproteobacteria
gi|134021524|gb|EF020100.1| Uncultured Sporichthyaceae bacterium clone Elev_16S_1511
gi|224496722|gb|FJ745031.1| Uncultured Actinomycetales bacterium clone SHWH_night1_16S_489
B3C1
B3D2
Actinobacteridae

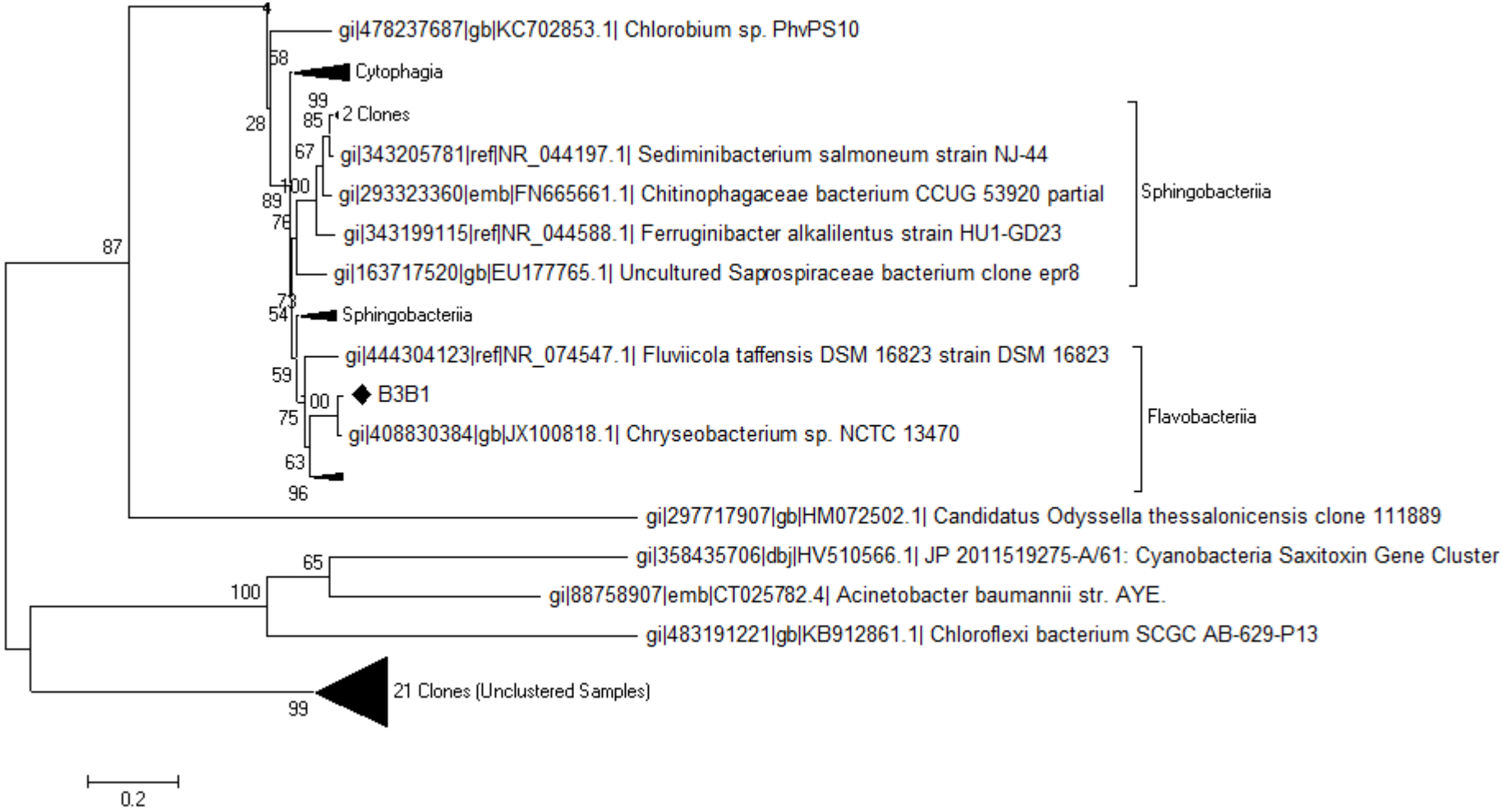

gi|478237687|gb|KC702853.1| Chlorobium sp. PhvPS10
Cytophagia
2 Clones
gi|343205781|ref|NR_044197.1| Sediminibacterium salmoneum strain NJ-44
gi|293323360|emb|FN665661.1| Chitinophagaceae bacterium CCUG 53920 partial
gi|343199115|ref|NR_044588.1| Ferruginibacter alkalilentus strain HU1-GD23
gi|163717520|gb|EU177765.1| Uncultured Saprospiraceae bacterium clone epr8
Sphingobacteriia
Sphingobacteriia
gi|444304123|ref|NR_074547.1| Fluviicola taffensis DSM 16823 strain DSM 16823
B3B1
gi|408830384|gb|JX100818.1| Chryseobacterium sp. NCTC 13470
Flavobacteriia
gi|297717907|gb|HM072502.1| Candidatus Odyssella thessalonicensis clone 111889
gi|358435706|dbj|HV510566.1| JP 2011519275-A/61: Cyanobacteria Saxitoxin Gene Cluster
gi|88758907|emb|CT025782.4| Acinetobacter baumannii str. AYE.
gi|483191221|gb|KB912861.1| Chloroflexi bacterium SCGC AB-629-P13
21 Clones (Unclustered Samples)
0.2

Figure 4.36: Molecular Phylogenetic analysis by Maximum Likelihood method of Broodstock Culture Water

The analysis involved 111 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. Fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position.

4.10 Environmental Data and Physiochemical Properties Analysis

Principal Coordinates Analysis (PCA) was carried out for all samples in six different environments namely GW, MCW, FCW, M, F and BCW. Figure 4.37 denotes PCA plot. The PCA plot indicates that the bacterial community from *Moina* sp. is significantly different from the rest of the samples in this study. Bacterial community in MCW and FCW were relatively similar where they are plotted in the same quadrant. Bacterial community in F and BCW shows some degree of similarities while bacterial community in Groundwater is relatively different from the other three water sources (MCW, FCW and BCW).

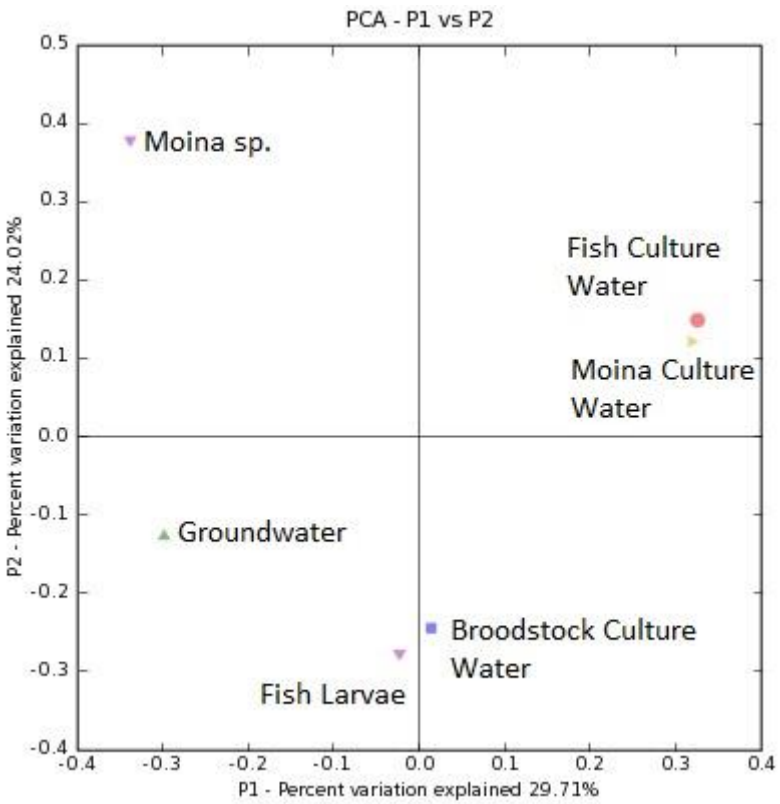


Figure 4.37: PCA analysis of environment P1 versus P2.

Canonical correspondence analysis (CCA) has been carried out using Canoco 4.5. Figure 4.38 refers to a graph obtained by supplying samples environment (GW, MCW, FCW, M, F and BCW) and physiochemical properties data. The plot denotes the samples obtained from different culture environment which thrives in the physiochemical conditions as portrayed.

Based on the plot, GW's physiochemical properties is related to lower content of nitrite, nitrate, turbidity and suspended solids. As for MCW and M, the culture condition affects concentration of ammonia, turbidity, suspended solids, sulfate and phosphorus. FCW and F are more dependent on oxygen as compared to other physiochemical properties. BCW is significantly away from the physiochemical properties.

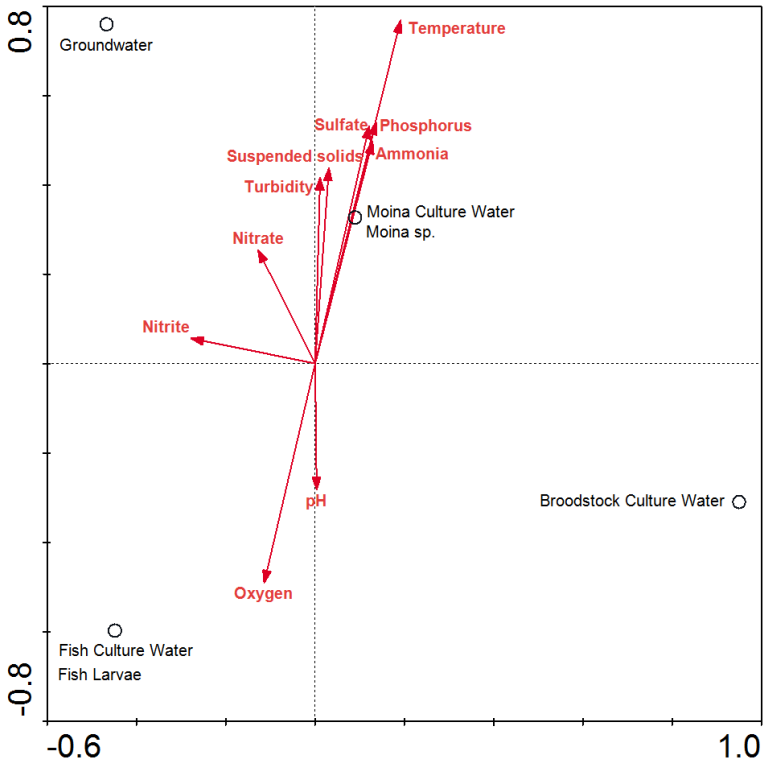


Figure 4.38: Simple ordination plot of samples and physiochemical properties using Canoco 4.5 CCA method.

CHAPTER 5

DISCUSSION

5.1 Water Quality Analysis

Monitoring was carried out on the water sample to check the suitability of the environment for the fish larvae and for environmental monitoring purposes. Valuable information can be obtained from monitoring the water conditions as it is associated to the growth of the fishes and bacterial diversity. Based on Urakawa et al. (2006) certain bacteria (ammonia-oxidising) are affected by the physical condition (salinity) which causes population shift. Differences in oxygen level, ammonia concentrations may also change the population of the bacteria and affect the livelihood of fish larvae.

Throughout the sampling period, it was carried out at about the same time which was 9.30 am in the morning. This was to ensure consistency in reading and sampling. Sampling was not carried out on the day that rain was observed on the previous night which is to avoid any fluctuations on temperature or disparity in readings. Temperature plays a major role in all biological and chemical processes in aquaculture followed by dissolved oxygen which is also dependent on temperature whereby it has to be maintained as not to stress the fishes (Buttner et al., 1993).

The physiochemical conditions of GW were in the normal range of culture conditions whereby it recorded low levels of turbidity and suspended solids. It was noted that the pH level of GW was slightly acidic at pH 6.04 ±

0.41 but within the normal range.

As for MCW, turbidity, suspended solids, ammonia, nitrite, nitrate, phosphorus and sulfate were high, while oxygen level was low. This is caused by the feeding of fish faeces to M. High turbidity in a culturing tank usually indicates lower level of oxygen in the system and higher suspended solids. Furthermore, the system was not disturbed during the sampling period and was protected with netting to avoid mosquitoes from breeding.

There was a spike on the nitrite content for MCW which was 5.290. This spike could be due to introduction of fresh fish waste into the culture tank on the day before sample was taken. An increased of nitrate levels in MCW indirectly indicates the presence of nitrifying bacteria in the system as it is the end product from the conversion of nitrites.

In FCW, turbidity and suspended solids were relatively high as the sediments in the water sample were the main cause of suspended solids which mainly consist of residues from organic matter. High ammonia content was recorded in the earlier stages and reduces as time passes by. This could be due to the population increase of ammonia-oxidising bacteria. According to Purkhold et al. (2000, 2003), ammonia-oxidising bacteria converts ammonia to nitrite (NO_2) and gain energy at the same time. Based on the data obtained for nitrite (NO_2) during the period of time, it increases gradually as ammonia decreases. This suggests presence of ammonia-oxidising bacteria and its activity.

It has been reported by Svobodova et al. (2005) that imbalances of nitrification process due to a number of factors, often lead to an increase in nitrite concentrations. Nitrite is dangerous to fishes as it binds to the haemoglobin in the blood preventing it from carrying oxygen. This indirectly suffocates the fishes which causes mortality. Hargreaves and Tucker (2004) mentioned nitrification process which is carried out by bacteria that oxidises ammonia and the main factors that affects it is ammonia concentration, temperature and oxygen concentration. With the optimum conditions, nitrification process is able to reduce the amount of ammonia in the system to a non-toxic level.

Artificial aeration was introduced to the system for FCW which allows the oxygen level to maintain at a constant. The amount of dissolved oxygen in the tank must be maintained through artificial aeration either with atmospheric oxygen (air) or pure gaseous oxygen injection (Losordo, Masser & Rakocy, 1992). This is because as the dissolved oxygen (DO) is depressed by the solid faecal waste, carbon dioxide (CO_2) and ammonia will be produced (Rakocy et al., 2006).

Ammonia being the main nitrogenous waste in most fishes was analysed as it is crucial to test for the ionised and un-ionised (toxic) form influenced by temperature and pH (Swann, 1997; Buttner et al., 1993). Increase in nitrite and nitrate level indicates biological activity where microorganisms convert toxic ammonia to less toxic form. Hargreaves and

Tucker (2004) highlighted the relationship between ammonia and pH, whereby the toxic form of ammonia which is un-ionised ammonia (ammonium ion, NH_4^+) predominates or increases when the pH is high and vice versa. Usually, the main source of ammonia in a fish pond is fish excretion and it is directly related to the feeding rate and protein that is being supplied (Hargreaves and Tucker, 2004).

At a lower level of pH, it was observed that the ammonia level was low and at a higher pH, the ammonia level increased too as observed in MCW and FCW. This is due to the fact that at lower pH, ammonium ion (NH_4^+) is being ionised as compared to higher pH where ammonia is not ionised. The equation below explains the relationship of unionised pH and ionised pH. As reported by Sawyer (2008), at low temperatures the activity of aqueous ammonia is much lower and higher in warm temperatures. This indicates that at lower pH and temperature, NH_3 is also low while NH_4^+ is higher. It has been observed in this study that there is a proportional relationship between pH and ammonia level.

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^-$$

Suspended solids and turbidity is often associated with plankton, fish wastes, uneaten fish feed suspended in water (Swann, 1997). High level of phosphates has been linked with *Cyanobacteria* booming. *Cyanobacteria* are also known as blue-green bacteria which obtains energy through photosynthesis. Very high amount of phosphorus was observed in MCW

which suggests an increased population of *Cyanobacteria*. Sulphate (SO_4^{2-}) concentration is taken into consideration because of the odour and due to the release of hydrogen sulphide.

The high content of ammonia and sulphate contributes to the unpleasant smell of MCW. Higher level of phosphorus was linked to higher level of algal growth (Stone & Thomforde, 2004) previously but is limited to space of the pond and prevailing conditions. Algal growth is thought to reduce ammonia content as they are being taken up by algae (Hargreaves & Tucker, 2004).

5.2 Quantitative and Qualitative Analyses

5.2.1 Molecular Analysis

Molecular analysis was first carried out by determining the quality of genomic DNA prior to PCR followed by cloning, colony PCR and sequencing enables the characterisation of bacterial population (Hovdaa et al., 2007) in environmental samples. Molecular analyses of environmental communities revealed that <1% of the total number of prokaryotic species present in any given sample are cultivable (Rastogi and Sani, 2011).

Schloss and Handelsman (2004) pointed out that based on 16S rRNA genes using molecular methods discloses candidate bacterial divisions such as BRC1, OP10, OP11, SC3, TM7, WS2, and WS3 have no cultured representation and they are known only by their molecular sequences.

Hugenholtz (2002) mentioned that PCR amplification of 16S rRNA conserved genes from environmental sample are being used extensively in microbial ecology studies because the genes are ubiquitous structurally, functionally conserved and contains variable and highly conserved regions. The suitable gene size (~1,500 bp) and growing number of 16S rRNA sequences available in the database has been dubbed by Rastogi and Sani (2011) as the "gold standard" choice in microbial ecology. This suitable gene size of 1.5 kb provides a wealth of information and allows further analysis.

PCR products were then subjected to cloning whereby six clone libraries from six different samples were prepared. The cloning and sequencing method was employed to decipher the microbial diversity of the study undertaken.

5.2.2 Multiple Sequence Align

Sequences obtained were matched against SILVA database through its SILVA Incremental Aligner (SINA). Comprehensive, quality checked and regularly updated datasets were provided by SILVA of aligned small (16S/18S, SSU) and large subunit (23S/28S, LSU) ribosomal RNA (rRNA) sequences for all three domains of life (*Bacteria*, *Archaea* and *Eukarya*) (http://www.arb-silva.de/). SINA has been evaluated comparatively with other high throughput MSA programs such as PyNAST and mothur whereby SINA achieved higher accuracy than the PyNAST and mothur in the performed benchmark (Pruesse, Peplies, and Glöckner, 2012). Castelle et al. (2013) noted that SILVA managed to classify additional 14 sequences as compared with NCBI database.

Comparison between BLASTN to reference database SILVA allowed Galand et al. (2009) to divide sequences into abundant and rare phylotypes whereby SILVA provided higher accuracy for 16S rDNA and 16s rRNA sequences.

The SILVA database is considered one of most preferred database for 16s rDNA and rRNA sequences due to its extensive range of data and accuracy. It is based on the International Nucleotide Sequence Database Collaboration (INSDC) which is being maintained by the SILVA project (Quast et al., 2013).

5.2.3 Taxonomic Analysis

Taxonomic analysis was performed on all samples under this study. Each sequence read onto a node of the NCBI taxonomy based on the gene content and for each read that matches, the program places the read on to the lowest common ancestor (LCA) node to the species that are known to the gene (Huson et al., 2011). In this study, SILVA database MSA results were used in MEGAN4 and a taxonomic tree (Figure 4.18) was built to determine which classification the sequences belong to. According to Huson et al. (2011), data integration is a major issue in analysing environmental sequences to address both taxonomic and functional analysis.

Thomsen et al. (2012) managed to identify the taxon of fish species based on the environmental DNA obtained from sea sample using MEGAN4 and subsequently classifying them. In the study of Steward et al. (2013), they have presumed that 41% - 43% of sequences that has no match to GenBank

database are viruses as they were found to assembled with virus-like sequences and MEGAN's annotations were more comprehensive with the larger percentages were accepted as correct.

5.2.4 Quantitative Analysis

Quantitative analysis carried out in this study has determined that the samples obtained from each environment under study were sufficient. Phylotype richness in small clone libraries are usually underestimated (Hugenholtz, 2002; Stach et al., 2003) and rely on species or operational taxonomic unit (OTU), where OTU are based on 16S rDNA gene similarity (Stach et al., 2003). Kemp and Aller (2004a) described that most techniques in microbial estimation are bias as true richness of the community has not been exhaustively sampled. The richness estimator S_{Chao1} based on Kemp and Aller (2004a) gave greater confidence as it is the best compromise and thus chosen for this study. Biers, Sun and Howard (2009) made the same conclusion with Kemp and Aller (2004a) that S_{Chao1} when used as a relative measure is the best estimate of lower bound species richness whereby in their study, they managed to determine the domination of *Alphaproteobacteria* on the surface ocean water.

5.3 Microbial Communities

In this study, not all the bacteria sequenced matched NCBI databases and most of them returned as uncultured bacterium clone while SILVA database was able to give a more general view of the bacterial communities whereby certain clones were able to match either at or from domain to species level.

5.3.1 Groundwater Microbial Community

Groundwater has been widely used in many countries as a source of water for drinking, aquaculture and other purposes. It has been known that groundwater has significant microbial content that are pathogenic in nature (Tanaka et al., 2012). Phylum *Armatimonadetes* has been formally known as candidate phylum OP10 was found in GW samples and has been reported by Tamaki et al. (2011) to have found a novel gene species *Armatimonas rosea* has been isolated from rhizoplane of aquatic plant where the exact function has yet to be established.

Pseudanabaena sp. was found at 31.82% out of total clones from Groundwater samples which belongs to *Cyanobacteria* phylum and causes *Cyanobacteria* bloom. Ernst (2008) mentioned that *Cyanobacteria* has made the most impact on aquatic organism because it can affect them via multiple routes and *Planktothrix rubescens* is one of the most toxic *Cyanobacteria* which causes impair fitness in coregonids (whitefish). In this study, the clones did not match for *Planktothrix rubescens*. *Planctomycetes* was detected in Groundwater samples which have been described by Jogler, Glöckner and Kolter (2011) as important participant in nitrogen and carbon cycle which possesses anaerobic oxidation of ammonium.

Acinetobacter is an opportunistic bacterium which many strains of

Acinetobacter are known to be multi-drug resistance microbes (Tanaka et al., 2012). There was no specific report which shows the pathogenicity of *Acinetobacter* on fishes. Sarioglu, Suluyayla and Tekinay (2012) proposed a novel *Acinetobacter calcoaceticus* as a nitrogen balancer as this strain is efficient in removing ammonium at various concentrations.

5.3.2 *Moina* Culture Water Microbial Community

MCW has the highest bacterial clones amounting to 24.29% out of 177 clones for all samples. This is attributed to the recycling of adult fish faeces used as enrichment culture for live feed (*Moina* sp.). Samples obtained from MCW have detected the presence of *Cyanobacteria* and *Acinetobacter* which was previously found in Groundwater samples. This is not surprising as the water that was used to culture *Moina* comes from groundwater. Despite the culture condition of *Moina*, *Acinetobacter* seems to be able to grow well in the tank. Besides *Acinetobacter* and uncultured bacterium, *Actinomycetales* and *Sphingobacteriales* which includes *Chitinophagaceae, Flexibacteraceae*, and *Sphingobacteriaceae* were also detected in the samples.

Actinobacteria are common in water samples. It has been suggested by Kumar and Rao (2012) that marine *Actinobacteria* from salt pan environment is able to produce new drug molecule against multi drug resistance *Staphylococcus aureus*. Polti et al. (2011) reported that *Actinobacteria* and *Zea mays* have a cooperative action in soil chromium bioremediation. *Shingobacteria* are ubiquitous in the environment which are capable of producing shingolipids. *Flexibacteria* causes columnaris (cotton wool disease)

in fishes as reported by Hawke and Khoo (2004). While MCW has the highest diversity of phylotypes, no pathogens has been detected in the tank.

5.3.3 Fish Culture Water Microbial Community

Acinetobacter has been detected in FCW samples based on the phylogenetic tree obtained from MEGA5. Souza and Silva-Souza (2001) conducted a bacteriological study on fish and water from Congonhas River and detected *Pseudomonas*, *Acinetobacter*, *Aeromonas*, *Enterobacteriaceae*, *Bacillus* and *Flavobacterium* whereby *Acinetobacter* and *Flavobacterium* were the most abundant.

In this study, *Flavobacterium* was also found to be available in the water samples followed by uncultured bacterium and *Pandorea*. During fish stress, several bacteria such as *Enterobacteriaceae*, *Bacillus* and *Aeromonas* were found to have higher chances of mortality as they are opportunistic pathogens (Sousa et al., 1996) and have also observed these bacteria in both aquatic environment and internal fish organs (Souza and Silva-Souza, 2001). It has been reported by Coenye et al. (2001) that *Pandoraea* are normally associated with cystic fibrosis (CF) patients and can be found in blood, soil, water and food.

5.3.4 *Moina sp.* Microbial Community

Aeromonas, *Enterobacteriacaea*, *Flavobacteriaceae*, *Pandorea* and *Ralstonia*

were detected in *Moina sp*. samples in this study. Most of these bacteria detected are opportunistic pathogens and it is not uncommon to find these bacteria in *Moina sp*. samples due to the nature of the culture environment. Fish faeces were used in the culturing of *Moina sp*. as described by Loh et al. (2009) as a potential food source. Zaky et al. (2011) reported that *Aeromonas* were commonly found in faecal contaminated water samples. Ryan et al. (2007) reported that *Ralstonia* sp. may exist in ultrapure water and has bioremediation properties due to its ability to breakdown toxins. Significant amount of *Delftia acidovorans* has been detected in *Moina* sp. but it has not been reported as a pathogen in fish.

5.3.5 Fish Larvae Microbial Community

The bacterial community detected in this study for F samples were mostly *Morganella morganii* which was at 68% out of 25 clones. The others mostly consist of *Acinetobacter*. *Morganella* is grouped in the *Enterobacteriaceae* family. It is a commonly found bacterium in the environment and in the intestinal tract of humans, mammals and reptiles as normal flora (Shenoy et al., 2003). Rajasekaran (2008) found it to be unexceptional for *Enterobacteriaceae* group of organisms or bacteria to be present in sewage-fed water fishes.

5.3.6 Brood Culture Water Microbial Community

As in all the water culture samples in this study, bacteria such as *Pandorea*, *Actinobacterium*, *Flavobacterium* and uncultured bacterium were detected.

Legionella were detected in this sample and is of a significant number whereby 24.13% of the sample was of *Legionella* genus. It is common to find *Legionella* in many environments including water but it was not detected in other samples. *Legionella* causes Legionellosis which comes in two forms which are pontiac fever and Legionnarires' Disease to humans but do not pose hazards to animals (EPA, 2000).

5.4 Carry-over of Microbial Communities

It is essential to understand the microbial communities in the aquaculture system in order to maintain a low mortality rate of fish larvae or indirectly fishes. This study which aims to track the microbial communities in each sample and the effect of environment or culture conditions towards the diversity of the communities itself. There have been connections between each sample taken and the microbial communities that represent it.

Table 5.1: Summary of Microbes based on Taxonomic Analysis

MCW	FCW	M	F
Acinetobacter sp.	*Acinetobacter sp.*	*Aeromonas jandaei*	*Acinetobacter sp.*
Actinomycetales	*Anaerolineae*	*Aeromonas veronii*	*Artropterus sp.*
Alpha-proteobacterium	*Candidate Division OD1*	*Candidatus chryseobacterium*	*Citrobacter werkmanii*
Blastopirellula	*Candidatus midichloria*	*Chitinibacter tainanensis*	*Morganella morganii*
Beta proteobacterium	*Erysipelothrix*	*Chryseobacterium*	
Chitinophagaceae	*Ferruginibacter alkalilentus*	*Delftia acidovorans*	
Candidate Division OD1	*Fibrobacteraceae*	*Lachnospiraceae*	
Comamonadaceae	*Flavobacterium succinicans*	*Morganella morganii*	
Cyanobacteria	*Flexibacter*	*Nubsella zeaxanthinifaciens*	
Emticicia ginsengsioli	*Fluviicola*	*Pandoraea sp.*	
Erysipelothrix	*Hyphomonadaceae*	*Ralstonia*	
Flexibacter flexilis	*Pandoraea sp.*	*Serratia grimesii*	
Methylophilaceae	*Prosthecobacter vanneervenii*	*Serratia marcescens*	
Myxococcales	*Rhodobacter*	*Vogesella perlucida*	
Neisseriaceae	*Saprospiraceae*		
Novispirullum	*Sphaerotilus*		
Oxalobacteraceae			
Phacus orbicularis			
Sediminibacterium			
Sphingobacteriales			
Thiodictyon bacillosum			

As observed from the taxonomic analysis summary (Table 5.1) and phylogenetic analyses, there were overlapping microbes that were detected in nearly all samples. Uncultured soil bacterium was found in both MCW and FCW but was not reflected in GW where both samples share the same source of water. This could be due to the culture environment in both MCW and FCW which was suitable for the growth of uncultured soil bacterium. *Cyanobacteria* were observed in GW and MCW which is not surprising as it is the same source of water.

Acinetobacter exist in all samples except *Moina sp*. samples. This suggests that the *Acinetobacter* may not survive in *Moina* sp. or were outgrown by other bacteria. *Pandoraea* sp. was detected in *Moina* sp. and FCW but was not observed in fish larvae. It is being suggested that other genus of bacteria populates fish larvae. Some pathogens that were found in *Moina sp*. was not entirely found in fish larvae samples but *Morganella morganii* was detected in both samples. *Morganella morganii* has been reported in cod digestive tract by McEwan and Treasurer (2008).

This would suggest that *Moina* sp. ingest *Morganella morganii* from the adult fish faeces (food source for *Moina* sp.) and it was carried over to fish larvae. Wong and Rawls (2012) describes the intestinal microbiota of fishes is influenced by the host ecology and environment which is associated with the host diet, anatomy and phylogeny. The observation of microbial communities in this study is similar to other studies conducted even though of different culture conditions, environment and samples (Souza and Silva-Souza, 2001; Sugita, Nakamura, Shimada, 2005). It is not clear at this moment whether other bacteria was carried over from live feed to fish larvae but could not be detected due to survivability in fish larvae.

However, based on the results obtained in this study, there was possible carry-over of microbial community from one source to another in a commercial fish farming system where they are linked. Several families of bacteria have been detected in both MCW and FCW such as *Chitinophagaceae, Flexibacteraceae, Sphingobacteriaceae,*

Erysipelotrichidae, Methylophilaceae and *Moraxellaceae.*

5.5 Environmental Data and Physiochemical Properties Relationship

Principal Coordinates Analysis (PCA) shows that bacterial community in *Moina* sp. is significantly different from all other samples in this study. As *Moina* sp. is a live feed which has been cultured through fish faeces enrichment, it is believed that the culture environment greatly differs as observed from the physiochemical properties. Skjermo and Vadstein (1993) studied the bacterial density and composition associated with cultivated rotifers found out that the enrichment culture influenced a shift in dominant bacterial composition from *Cytophaga/ Flavobacterium* to *Pseudomonas/ Alcaligenes.*

FCW and MCW shared the same quadrant as both of the samples were from the same source even though they were cultured under different conditions, the physiochemical properties did not show significant differences in the plot as opposed to GW. GW consists of *α-Proteobacteria* with a lesser presence of *β-Proteobacteria* and *Actinobacteria* (Nishimaru and Nagata, 2007).

BCW and F were close to each other and shared the same quadrant suggested that they were similar. According to Blancheton et al. (2013) a commercial fish farming system may include heterotrophic bacteria which consisted of α-*Proteobacteria* and β-*Proteobacteria*, nitrifiers, pathogens, opportunistic bacteria and probionts.

Through the Canonical Correspondence Analysis (CCA), it is observed that the culture environments were dependent on certain physiochemical conditions such as temperature, phosphorus, sulfate, ammonia, suspended solids, turbidity, nitrate, nitrite, oxygen and pH. Microflora community in GW was affected by the presence of nitrite and nitrate while microbial communities of BCW were not closely related to the culture conditions in the fish farm as the broodstock was sourced from a supplier. As for *Moina* sp. and MCW, the bacterial communities that thrive were inclined to high amount of ammonia, suspended solids, phosphorus, sulfate and turbidity this could be due to the recycled fish faeces that was being used as food source and culture medium. While F bacterial communities seem to populate during higher oxygen level.

CHAPTER 6

CONCLUSION

The study of characterising microbial communities using molecular techniques gave an insight on microbial diversity in an aquaculture environment on a local context. Not many studies have been carried locally to understand the microbial communities which might affect the livelihood of aquatic animals and indirectly the breeders. It is an economic viable option to study on the carry-over effect of such microbial communities to the benefit of commercial fish farming community.

Through this study, there have been no reports on pathogens for fishes but possibility of diseases that were caused by opportunistic pathogens such as *Acinetobacter*. Diseases can be avoided when care and preventive measures are taken into considerations during culturing. A diverse group of bacteria were detected in the study which sees the class of *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria* and *δ-Proteobacteria*. Other phylum of bacteria such as *Actinobacteria, Armatimonadetes, Bacteroidetes,* Candidate division OP10 which was closely related to *Armatimonadetes, Chloroflexi, Cyanobacteria, Fibrobacteres, Firmicutes, Planctomycetes, Spirochates* and *Verrumicrobia* were also detected in the study.

Several uncultured bacterium and candidate division were detected did not cluster to any currently recognised lineages were unique to this study and requires further studies on the subject matter. It has been indicated that possible carry-over of *Morganella morganii* from adult fish faeces to *Moina* sp. and finally to Fish Larvae was seen in this study. *Acinetobacter* was found in almost all the samples except for *Moina* sp. and Broodstock Culture Water. There were also several overlapping family of bacteria observed.

Even though only one bacterium was found to have this carry-over effect from live feed to fish larvae, it does not conclude that other bacteria were not carried over from water source to live feed and to fish larvae. Instead, this study has managed to highlight the co-relation between water samples and their cultures. A sustainable recycled food source (fish faeces) has shown to be a possible to culture live feed (*Moina* sp.) as no pathogens were reportedly being transmitted through this culture method. However, it is still not clear at this point how the bacteria interact and populate in their culture environment. Further studies can be carried out to determine the characteristics and growth of specific bacteria. The objectives of this study have been achieved with the successful detection of microflora in water sources, live feed and fish larvae.

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Appendix A

Table A.1: Water Quality Monitoring for Groundwater, *Moina* Culture Water and Fish Culture Water.

Condition/Date	Groundwater			Moina Culture Water			Fish Culture Water		
	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
Temperature (°C)	27.000	26.000	28.000	25.000	25.000	27.000	26.000	25.000	25.000
Turbidity (FAU)	0.000	0.000	0.000	72.000	96.000	62.000	11.000	19.000	33.000
Suspended solid (mg/L)	0.000	0.000	0.000	38.000	55.000	46.000	7.000	9.000	18.000
Ammonia (NH_3) (mg/L)	0.000	0.000	0.000	60.000	70.000	64.000	19.000	3.000	0.320
Nitrite (NO_2) (mg/L)	0.007	0.120	0.053	0.021	0.190	5.290	0.120	0.318	2.480
Nitrate (NO_3) (mg/L)	2.800	1.800	1.400	17.200	17.200	8.600	8.500	4.700	5.200
Oxygen (O_2) (mg/L)	7.500	6.530	6.470	3.200	1.200	1.030	6.500	6.400	5.210
pH	6.400	6.300	5.950	7.700	7.800	7.600	8.100	7.400	6.600
Phosphorus (mg/L)	0.170	0.200	0.310	96.000	64.000	115.000	0.860	3.000	8.900
Sulfate (mg/L)	0.000	0.000	0.000	9.000	14.000	11.000	1.000	2.000	0.000

Appendix B

Table A.2: Physiochemical Properties of BCW

Condition/Date	BCW
	18-Nov
Temperature (°C)	25.000
Turbidity (FAU)	4.000
Suspended solid (mg/L)	1.000
Ammonia (NH_3) (mg/L)	11.000
Nitrite (NO_2) (mg/L)	0.080
Nitrate (NO_3) (mg/L)	10.100
Oxygen (O_2) (mg/L)	8.200
pH	8.000
Phosphorus (mg/L)	0.640
Sulfate (mg/L)	1.000

Appendix C

Table A.3: Closest Relative for 16S rDNA Clone Sequences Determine by ARB-SILVA Database and NCBI BLASTN.

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
1	GW1B3	Acinetobacter score=79.0	Uncultured Acinetobacter sp. clone C146Chl360 16S ribosomal RNA gene, partial sequence	63%	89%	JX530115.1	Ocean water
2	GW1B4	Acinetobacter sp. PAMU-1.11 score=96.0	Acinetobacter sp. Ac6 16S ribosomal RNA gene, partial sequence	98%	96%	KC853110.1	Amphibian skin
3	GW1D6	Acinetobacter sp. PAMU-1.11 score=98.0	Acinetobacter sp. TD5 16S ribosomal RNA gene, partial sequence	94%	99%	EF468657.1	N/A
4	GW1E5	Acinetobacter sp. PAMU-1.11 score=96.0	Uncultured Acinetobacter sp. clone M1-5 16S ribosomal RNA gene, partial sequence	99%	97%	JQ885539.1	N/A
5	GW1E6	Unclassified	Unclassified				
6	GW3A1	Pseudanabaena sp. PCC 6903 score=93.0	Uncultured bacterium clone sa0.37 16S ribosomal RNA gene, partial sequence	89%	97%	HQ904133.1	Microcystis bloom & surface sediment
7	GW3A2	Planctomyces sp. score=91.0	Uncultured bacterium clone E34 16S ribosomal RNA gene, partial sequence	85%	98%	HQ827938.1	Hypereutrophic lake

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
8	GW3A4	Armatimonadetes score=89.0	Uncultured Armatimonadetes bacterium clone T13M-B31 16S ribosomal RNA gene, partial sequence	90%	93%	JN860416.1	Hydrothermal oxides
9	GW3B1	Pseudanabaena sp. PCC 6903 score=94.0	Pseudanabaena sp. 0tu30s18 partial 16S rRNA gene, strain 0tu30s18	90%	96%	AM259268.1	Eutrophic Lake
10	GW3B2	Parachlorella kessleri score=92.0	Chlorella kessleri plastid DNA for 16S-like rRNA small subunit, strain SAG 211-11g	81%	98%	X65099.1	N/A
11	GW3B6	Ancalomicrobium score=93.0	Uncultured Rhizobiales bacterium Sto3-1 16S ribosomal RNA gene, complete sequence	88%	94%	AY138237.1	Human stomach biopsy
12	GW3C3	Closteriopsis acicularis score=92.0	Closteriopsis acicularis plastid 16S rRNA gene	92%	91%	Y17632.1	N/A
13	GW3C6	Pseudanabaena sp. PCC 6903 score=92.0	Uncultured bacterium clone sa0.37 16S ribosomal RNA gene, partial sequence	84%	97%	HQ904133.1	Microcystis bloom & surface sediment
14	GW3C7	Armatimonadetes score=88.0	Uncultured bacterium clone ncd222h11c1 16S ribosomal RNA gene, partial sequence	83%	95%	HM266973.1	Human Skin
15	GW3D2	Closteriopsis acicularis score=93.0	Closteriopsis acicularis plastid 16S rRNA gene	94%	90%	Y17632.1	N/A
16	GW3D4	Pseudanabaena sp. PCC 6903 score=95.0	Uncultured bacterium clone sa0.45 16S ribosomal RNA gene, partial sequence	95%	98%	HQ904134.1	Microcystis bloom & surface sediment

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
17	GW3D7	Pseudanabaena sp. PCC 6903 score=95.0	Uncultured bacterium clone sa0.45 16S ribosomal RNA gene, partial sequence	95%	98%	HQ904134.1	Microcystis bloom & surface sediment
18	GW3E3	Gemmata score=90.0	Uncultured bacterium clone ambient_alkaline-85 16S ribosomal RNA gene, partial sequence	95%	91%	GU455068.1	Waste sludge
19	GW3E6	Pseudanabaena sp. PCC 6903 score=94.0	Uncultured bacterium clone A-90 16S ribosomal RNA gene, partial sequence	94%	98%	HQ860462.1	Lake water
20	GW3F1	Schlesneria score=94.0	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: MIZ48	95%	98%	AB179539.1	Sedimentary rock
21	GW3F2	Closteriopsis acicularis score=94.0	Closteriopsis acicularis plastid 16S rRNA gene	94%	90%	Y17632.1	N/A
22	GW3F3	Pseudanabaena sp. PCC 6903 score=93.0	Uncultured bacterium clone sa0.37 16S ribosomal RNA gene, partial sequence	91%	97%	HQ904133.1	Microcystis bloom & surface sediment
23	MCW1A4	Acinetobacter sp. PAMU-1.11 score=92.0	Acinetobacter sp. AKB-2008-JO101 partial 16S rRNA gene, strain AKB-2008-JO101	61%	99%	AM989148.1	Lake water
24	MCW1B4	Acinetobacter sp. PAMU-1.11 score=95.0	Acinetobacter sp. AKB-2008-JO101 partial 16S rRNA gene, strain AKB-2008-JO101	75%	99%	AM989148.1	Lake water
25	MCW1F4	Acinetobacter sp. PAMU-1.11 score=93.0	Acinetobacter sp. AKB-2008-JO101 partial 16S rRNA gene, strain AKB-2008-JO101	71%	99%	AM989148.1	Lake water

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
26	MCW2A1	Sphingobacteriales score=87.0	Uncultured Sphingobacterium sp. clone JXSH1-1 16S ribosomal RNA gene, partial sequence	95%	95%	JX535192.1	Sewage sludge
27	MCW2A3	Novispirillum score=84.0	Uncultured alpha proteobacterium clone D8W_124 16S ribosomal RNA gene, partial sequence	89%	84%	HM057728.1	Sea water
28	MCW2A4	Novispirillum score=84.0	Uncultured bacterium clone Nit2A0620_10 16S ribosomal RNA gene, partial sequence	88%	85%	EU265937.1	Lake water
29	MCW2B1	Comamonadaceae score=94.0	Uncultured bacterium clone DR158 16S ribosomal RNA gene, partial sequence	85%	97%	JF429199.1	River water
30	MCW2B2	Erysipelothrix score=96.0	Uncultured bacterium partial 16S rRNA gene, clone SHA-44	91%	97%	AJ306753.1	Environment
31	MCW2B7	Sediminibacterium salmoneum score=97.0	Uncultured bacterium clone EMIRGE_OTU_s1t2b_2437 16S ribosomal RNA gene, partial sequence	92%	98%	JX221906.1	Subsurface aquifer sediment
32	MCW2C5	Beta proteobacterium MWH-UniP1 score=94.0	Beta proteobacterium MWH-UniP4 partial 16S rRNA gene, isolate MWH-UniP4	93%	92%	AJ565422.1	Freshwater
33	MCW2D1	beta proteobacterium F06002 score=92.0	Uncultured bacterium clone DR360 16S ribosomal RNA gene, partial sequence	95%	92%	JF429353.1	River water
34	MCW2D7	Sphingobacteriales score=89.0	Uncultured Sphingobacterium sp. clone JXSH1-1 16S ribosomal RNA gene, partial sequence	96%	98%	JX535192.1	Sewage sludge

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
35	MCW2E1	Neisseriaceae score=88.0	Uncultured beta proteobacterium clone AEP-eGFP-peri_16 16S ribosomal RNA gene, partial sequence	94%	92%	FJ517739.1	Epithelium
36	MCW2E8	Emticicia ginsengisoli score=89.0	Uncultured bacterium clone 3C002852 16S ribosomal RNA gene, partial sequence	93%	87%	EU801536.1	Bay water
37	MCW2F2	Betaproteobacteria score=95.0	Uncultured bacterium clone C39 16S ribosomal RNA gene, partial sequence	94%	94%	EU234274.1	River water
38	MCW2F3	Erysipelothrix score=97.0	Bacterium enrichment culture clone EtOH-24 16S ribosomal RNA gene, partial sequence	95%	94%	FJ799140.1	Wastewater sludge
39	MCW2F5	Thiodictyon bacillosum score=92.0	Uncultured bacterium clone 0-4-101 16S ribosomal RNA gene, partial sequence	95%	93%	JN609322.1	Wastewater
40	MCW2G3	Comamonadaceae score=85.0	Uncultured bacterium clone NP6D72 16S ribosomal RNA gene, partial sequence	94%	84%	GU222245.1	Wastewater
41	MCW2G7	Beta proteobacterium F1021 score=93.0	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone: PW92	91%	94%	AB745655.1	Hotspring water
42	MCW2H1	Candidate division OD1 score=85.0	Uncultured bacterium clone ZY-30 16S ribosomal RNA gene, partial sequence	91%	94%	KC424740.1	Lake sediment
43	MCW2H5	Comamonadaceae score=95.0	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone: PW16	95%	95%	AB745413.1	Hotspring water

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
44	MCW3A2	Oxalobacteraceae score=96.0	Uncultured bacterium clone DR378 16S ribosomal RNA gene, partial sequence	95%	98%	JF429366.1	River water
45	MCW3A4	Blastopirellula score=89.0	Uncultured bacterium partial 16S rRNA gene, clone 738	95%	98%	FR853628.1	Ctenopharyngodon idellus (Grass Carp)
46	MCW3C4	Sphingobacteriales score=96.0	Uncultured bacterium clone AA235 16S ribosomal RNA gene, partial sequence	95%	98%	JX271985.1	Wastewater sludge
47	MCW3C6	Phacus orbicularis score=94.0	Phacus orbicularis strain NJ04 16S ribosomal RNA gene, partial sequence; chloroplast	94%	97%	FJ719687.1	N/A
48	MCW3C8	Chitinophagaceae score=90.0	Uncultured bacterium clone C1Q 16S ribosomal RNA gene, partial sequence	94%	91%	DQ856516.1	Intestinal microflora in Chinese mitten crab (Eriocheir sinensis
49	MCW3D1	Comamonadaceae score=96.0	Uncultured bacterium clone S2_081 16S ribosomal RNA gene, partial sequence	95%	98%	JX406264.1	Lake water
50	MCW3E2	Phacus orbicularis score=76.0	Phacus hamatus strain ASW 08032 16S ribosomal RNA gene, partial sequence; chloroplast	95%	87%	JQ398649.1	N/A
51	MCW3E4	Cyanobacteria score=82.0	Lepocinclis acus var. major strain UTEX1316 16S ribosomal RNA gene, partial sequence; chloroplast	94%	84%	FJ719673.1	N/A

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
52	MCW3F1	Phacus orbicularis score=75.0	Phacus hamatus strain ASW 08032 16S ribosomal RNA gene, partial sequence; chloroplast	91%	86%	JQ398649.1	N/A
53	MCW3G3	Phacus orbicularis score=81.0	Phacus orbicularis strain NJ04 16S ribosomal RNA gene, partial sequence; chloroplast	93%	87%	FJ719687.1	N/A
54	MCW3G4	Sphingobacteriales score=96.0	Uncultured bacterium clone EG67 16S ribosomal RNA gene, partial sequence	94%	97%	KC189646.1	Spring water
55	MCW3H1	Phacus orbicularis score=74.0	Phacus hamatus strain ASW 08032 16S ribosomal RNA gene, partial sequence; chloroplast	93%	86%	JQ398649.1	N/A
56	MCW3I1	Phacus orbicularis score=75.0	Phacus hamatus strain ASW 08032 16S ribosomal RNA gene, partial sequence; chloroplast	92%	87%	JQ398649.1	N/A
57	MCW3J1	Methylophilaceae score=95.0	Uncultured bacterium clone E153 16S ribosomal RNA gene, partial sequence	96%	97%	HQ828043.1	Lake water
58	MCW3J6	Myxococcales score=82.0	Uncultured bacterium clone EG110 16S ribosomal RNA gene, partial sequence	95%	92%	KC189665.1	Spring water
59	MCW3K8	Sphingobacteriaceae score=81.0	Uncultured Bacteroidetes bacterium clone KWK12S.44 16S ribosomal RNA gene, partial sequence	90%	89%	KC424694.1	Lake sediment

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
60	MCW3L1	Flexibacter flexilis score=93.0	Flexibacter flexilis gene for 16S rRNA, partial sequence, strain: NBRC 16027	88%	94%	AB681038.1	Collection
61	MCW3N1	Actinomycetales score=94.0	Uncultured bacterium clone C14 16S ribosomal RNA gene, partial sequence	95%	99%	KC253333.1	Freshwater pond
62	MCW3N8	Methylophilaceae score=95.0	Uncultured bacterium clone E153 16S ribosomal RNA gene, partial sequence	93%	97%	HQ828043.1	Lake water
63	MCW3O2	Methylophilaceae score=95.0	Uncultured bacterium clone E153 16S ribosomal RNA gene, partial sequence	96%	97%	HQ828043.1	Lake water
64	MCW3P1	Alpha proteobacterium 34619 score=90.0	Uncultured alpha proteobacterium clone OTU-B6-13 16S ribosomal RNA gene, partial sequence	89%	91%	JQ996709.1	Activated sludge
65	MCW3P3	Methylophilaceae score=95.0	Uncultured bacterium clone E153 16S ribosomal RNA gene, partial sequence	95%	97%	HQ828043.1	Lake water
66	FCW1C7	Acinetobacter sp. PAMU-1.11 score=98.0	Acinetobacter sp. TD5 16S ribosomal RNA gene, partial sequence	95%	99%	EF468657.1	Collection
67	FCW1G2	Acinetobacter sp. PAMU-1.11 score=98.0	Acinetobacter sp. TD5 16S ribosomal RNA gene, partial sequence	96%	99%	EF468657.1	Collection
68	FCW1M2	Arthropterus sp. KAO-1997 score=75.0	Uncultured Acinetobacter sp. clone S2P2093 16S ribosomal RNA gene, partial sequence	74%	96%	KF145733.1	Soil

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
69	FCW2A1	Flexibacter score=93.0	Uncultured bacterium clone WS-34 16S ribosomal RNA gene, partial sequence	95%	93%	KC424708.1	Lake water
70	FCW2A2	Pandoraea sp. G5084 score=98.0	Pandoraea sp. LB-7 16S ribosomal RNA gene, partial sequence	92%	99%	DQ831002.1	N/A
71	FCW2A4	Prosthecobacter vanneervenii score=97.0	Prosthecobacter vanneervenii 16S rRNA gene (partial)	94%	99%	AJ966883.1	N/A
72	FCW2B1	Prosthecobacter vanneervenii score=95.0	Prosthecobacter vanneervenii 16S rRNA gene (partial)	94%	96%	AJ966883.1	N/A
73	FCW2B2	Candidatus Midichloria score=81.0	Uncultured bacterium clone glb335c 16S ribosomal RNA gene, partial sequence	95%	93%	EU978823.1	Glacier ice
74	FCW2B4	Saprospiraceae score=88.0	Uncultured bacterium clone a-89 16S ribosomal RNA gene, partial sequence	95%	90%	JX040387.1	Waste water
75	FCW2B6	Sphaerotilus montanus score=97.0	Uncultured bacterium clone Q7565-HYSO 16S ribosomal RNA gene, partial sequence	95%	98%	JN391978.1	Sludge
76	FCW2C1	Erysipelotrichaceae score=61.0	Unclassified				
77	FCW2C3	Candidatus Midichloria mitochondrii score=90.0	Candidatus Midichloria sp. Ixholo1 partial 16S rRNA gene, isolate Ixholo1	94%	91%	FM992372.1	Ixodes holocyclus

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
78	FCW2C4	Fibrobacteraceae score=94.0	Uncultured bacterium clone 258ds10 16S ribosomal RNA gene, partial sequence	96%	94%	AY212707.1	Water
79	FCW2C6	Flavobacterium succinicans score=93.0	Flavobacterium sp. JS2(2011) 16S ribosomal RNA gene, partial sequence	90%	97%	JF922307.1	Fresh water
80	FCW2D1	Fluviicola score=92.0	Uncultured bacterium clone H0017 16S ribosomal RNA gene, partial sequence	95%	93%	FJ820380.1	Freshwater lake
81	FCW2D2	Rhodobacter score=95.0	Uncultured bacterium clone M40 16S ribosomal RNA gene, partial sequence	95%	96%	HQ697517.1	Biologically activated carbon
82	FCW2D4	Prosthecobacter vanneervenii score=96.0	Prosthecobacter vanneervenii 16S rRNA gene (partial)	95%	96%	AJ966883.1	N/A
83	FCW2D8	Ferruginibacter alkalilentus score=95.0	Ferruginibacter alkalilentus strain HU1-GD23 16S ribosomal RNA, partial sequence	89%	96%	NR_044588.1	N/A
84	FCW2E1	Candidate division OD1 score=84.0	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: RH2155	81%	82%	AB510989.1	Soil
85	FCW2E5	Hyphomonadaceae score=91.0	Uncultured bacterium clone M3B31 16S ribosomal RNA gene, partial sequence	95%	97%	FJ439862.1	Membrane bioreactor biofilm
86	FCW2E8	Anaerolineae score=93.0	Uncultured bacterium clone BXHB7 16S ribosomal RNA gene, partial sequence	94%	99%	GQ480058.1	Activated sludge
87	FCW2F1	Candidate division OD1 score=86.0	Uncultured soil bacterium clone S03 16S ribosomal RNA gene, partial sequence	94%	84%	AF507684.1	Forest soil

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
88	FCW2F2	Runella slithyformis score=93.0	Uncultured bacterium clone BXHB50 16S ribosomal RNA gene, partial sequence	95%	97%	GQ480089.1	Activated sludge
89	FCW2F7	Verrucomicrobia score=93.0	Uncultured bacterium clone STPCL-17 16S ribosomal RNA gene, partial sequence	93%	94%	HM124404.1	Tar pond sediment
90	FCW2F8	Methylophilus score=94.0	Uncultured bacterium clone DB51 16S ribosomal RNA gene, partial sequence	95%	94%	JX542565.1	Sewage sludge
91	FCW2G1	Haliangium score=87.0	Uncultured bacterium clone JFR0702_jaa38g08 16S ribosomal RNA gene, partial sequence	94%	86%	HM780039.1	Zebrafish gut
92	FCW2G2	Sphaerotilus score=95.0	Uncultured bacterium clone reservoir-145 16S ribosomal RNA gene, partial sequence	95%	96%	JF697526.1	Lake water
93	FCW2G4	Runella slithyformis score=93.0	Uncultured bacterium clone BXHB50 16S ribosomal RNA gene, partial sequence	95%	97%	GQ480089.1	Activated sludge
94	M1B4	Lachnospiraceae score=56.0	Pseudomonas aeruginosa partial 16S rRNA gene, strain TBA12	84%	99%	FR745414.1	Activated sewage sludge
95	M1B6	Ralstonia score=98.0	Uncultured Ralstonia sp. clone F3Baug.5 16S ribosomal RNA gene, partial sequence	99%	98%	GQ417828.1	Biological degreasing systems
96	M2A2	Aeromonas jandaei score=99.0	Aeromonas jandaei strain LNC206 16S ribosomal RNA gene, partial sequence	95%	99%	FJ940821.1	Intestine of silver carp
97	M2A3	Aeromonas veronii score=99.0	Uncultured bacterium clone JdFBBkgd41 16S ribosomal RNA gene, partial sequence	95%	99%	JQ678456.1	Deep seawater

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
98	M2B1	Vogesella perlucida score=99.0	Freshwater bacterium LH6-8 16S ribosomal RNA gene, partial sequence	94%	100%	EU626190.1	Slightly alkaline (pH 8.8) lake water
99	M2B6	Morganella morganii score=96.0	Morganella morganii strain DAHM1 16S ribosomal RNA gene, partial sequence	89%	99%	KC465904.1	Soil
100	M2C4	Vogesella perlucida score=98.0	Freshwater bacterium LH6-8 16S ribosomal RNA gene, partial sequence	94%	99%	EU626190.1	Slightly alkaline (pH 8.8) lake water
101	M2C6	Chitinibacter tainanensis score=99.0	Chitinibacter sp. SK16 16S ribosomal RNA gene, partial sequence	95%	98%	JN981166.1	Mud flat
102	M2D3	Aeromonas veronii score=99.0	Uncultured bacterium clone B10 16S ribosomal RNA gene, partial sequence	95%	99%	EF655645.1	Dye containing wastewater treatment
103	M2D7	Aeromonas jandaei score=93.0	Uncultured bacterium clone JFR0702_jaa49a03 16S ribosomal RNA gene, partial sequence	88%	95%	HM780274.1	Zebrafish intestine
104	M3A1	Pandoraea sp. G5084 score=98.0	Pandoraea sp. G5084 16S ribosomal RNA gene, partial sequence	94%	99%	AF247693.1	N/A
105	M3A2	Serratia grimesii score=99.0	Serratia sp. DT-1 16S ribosomal RNA gene, partial sequence	95%	99%	JQ954965.1	Bombyx mori
106	M3A3	Delftia acidovorans score=99.0	Uncultured Delftia sp. clone AV_7N-G06 16S ribosomal RNA gene, partial sequence	95%	99%	EU341254.1	Commercial aircraft cabin air

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
107	M3A4	Pandoraea sp. G3307 score=98.0	Pandoraea sp. G3307 16S ribosomal RNA gene, partial sequence	95%	99%	AF247699.1	N/A
108	M3B2	Delftia acidovorans score=98.0	Delftia sp. Hg4-10 16S ribosomal RNA gene, partial sequence	95%	99%	EU304256.1	Hepialus gonggaensis larva guts
109	M3B3	Delftia acidovorans score=99.0	Uncultured bacterium clone rRNA361 16S ribosomal RNA gene, partial sequence	95%	99%	AY959134.1	Human vaginal epithelium
110	M3B6	Delftia acidovorans score=98.0	Uncultured Delftia sp. clone AV_7N-G06 16S ribosomal RNA gene, partial sequence	95%	99%	EU341254.1	Commercial aircraft cabin air
111	M3C1	Pandoraea sp. G3307 score=97.0	Pandoraea sp. G3307 16S ribosomal RNA gene, partial sequence	95%	97%	AF247699.1	N/A
112	M3C3	Delftia acidovorans score=97.0	Uncultured Delftia sp. clone AV_7N-G06 16S ribosomal RNA gene, partial sequence	91%	97%	EU341254.1	Commercial aircraft cabin air
113	M3C4	Candidatus Chryseobacterium massiliae score=95.0	Uncultured Flavobacteriaceae bacterium clone sh-xj127 16S ribosomal RNA gene, partial sequence	95%	97%	JQ328000.1	Water samples obtained from a microsaltwater lake
114	M3C6	Delftia acidovorans score=96.0	Uncultured Delftia sp. clone AV_7N-G06 16S ribosomal RNA gene, partial sequence	91%	97%	EU341254.1	Commercial aircraft cabin air
115	M3C7	Chryseobacterium sp. ACP12 score=96.0	Chryseobacterium sp. ACP12 small subunit ribosomal RNA gene, partial sequence	95%	96%	AY464462.1	N/A

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
116	M3D1	Pandoraea sp. G3307 score=97.0	Pandoraea sp. G3307 16S ribosomal RNA gene, partial sequence	94%	96%	AF247699.1	N/A
117	M3D4	Nubsella zeaxanthinifaciens score=92.0	Nubsella sp. EsD18 16S ribosomal RNA gene, partial sequence	90%	95%	JX879739.1	Activated sludge
118	M3D6	Delftia acidovorans score=97.0	Uncultured Delftia sp. clone AV_7N-G06 16S ribosomal RNA gene, partial sequence	94%	97%	EU341254.1	Commercial aircraft cabin air
119	M3D8	Pandoraea sp. G3307 score=96.0	Pandoraea sp. G3307 16S ribosomal RNA gene, partial sequence	94%	96%	AF247699.1	N/A
120	M3E1	Pandoraea sp. G3307 score=99.0	Pandoraea sp. G3307 16S ribosomal RNA gene, partial sequence	95%	99%	AF247699.1	N/A
121	M3E2	Serratia marcescens score=99.0	Serratia sp. DCM0915 16S ribosomal RNA gene, partial sequence	95%	100%	KC007128.1	Stream water
122	M3E6	Delftia acidovorans score=99.0	Uncultured Delftia sp. clone AV_7N-G06 16S ribosomal RNA gene, partial sequence	95%	99%	EU341254.1	Commercial aircraft cabin air
123	M3E7	Pandoraea sp. G5084 score=98.0	Pandoraea sp. G5084 16S ribosomal RNA gene, partial sequence	94%	99%	AF247693.1	N/A
124	F1F2	Acinetobacter sp. PAMU-1.11 score=97.0	Acinetobacter sp. TD5 16S ribosomal RNA gene, partial sequence	96%	99%	EF468657.1	N/A
125	F1F8	Acinetobacter sp. PAMU-1.11 score=85.0	Uncultured Acinetobacter sp. clone M1-5 16S ribosomal RNA gene, partial sequence	94%	91%	JQ885539.1	N/A

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
126	F1G8	Arthropterus sp. KAO-1997 score=73.0	Uncultured bacterium clone nby584g08c1 16S ribosomal RNA gene, partial sequence	41%	77%	HM841007.1	Skin of mouse
127	F1H4	Acinetobacter sp. PAMU-1.11 score=97.0	Acinetobacter sp. TD5 16S ribosomal RNA gene, partial sequence	96%	99%	EF468657.1	N/A
128	F1J2	Acinetobacter sp. PAMU-1.11 score=97.0	Acinetobacter sp. Tol 5 gene for 16S rRNA, partial sequence	100%	99%	AB542909.1	N/A
129	F3A1	Morganella morganii score=96.0	Morganella morganii subsp. morganii KT, complete genome	96%	97%	CP004345.1	Blood sample
130	F3A2	Morganella morganii score=96.0	Morganella morganii strain DAHM1 16S ribosomal RNA gene, partial sequence	94%	98%	KC465904.1	Soil
131	F3A4	Unclassified	Unclassified				
132	F3A5	Morganella morganii score=95.0	Morganella morganii strain DAHM1 16S ribosomal RNA gene, partial sequence	94%	97%	KC465904.1	Soil
133	F3B2	Morganella morganii score=98.0	Morganella sp. TR 90 16S rRNA gene, isolate TR 90	95%	98%	AM156948.1	River water
134	F3B4	Morganella morganii score=96.0	Uncultured bacterium clone PCA-27 16S ribosomal RNA gene, partial sequence	95%	95%	EF608514.1	Digestive tract of ground beetle
135	F3B5	Morganella morganii score=96.0	Morganella sp. TR 90 16S rRNA gene, isolate TR 90	95%	97%	AM156948.1	River water
136	F3C1	Morganella morganii score=94.0	Morganella sp. TR 90 16S rRNA gene, isolate TR 90	90%	96%	AM156948.1	River water

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
137	F3C2	Morganella morganii score=96.0	Morganella sp. TR 90 16S rRNA gene, isolate TR 90	90%	97%	AM156948.1	River water
138	F3C3	Morganella morganii score=96.0	Morganella morganii strain VAR-06-2076 16S ribosomal RNA gene, partial sequence	92%	97%	DQ513315.1	Domesticated rabbit with pneumonia
139	F3C4	Morganella morganii score=96.0	Morganella sp. TR 90 16S rRNA gene, isolate TR 90	91%	97%	AM156948.1	River water
140	F3C5	Morganella morganii score=95.0	Morganella sp. TR 90 16S rRNA gene, isolate TR 90	95%	95%	AM156948.1	River water
141	F3C6	Morganella morganii score=95.0	Morganella sp. TR 90 16S rRNA gene, isolate TR 90	94%	96%	AM156948.1	River water
142	F3C8	Morganella morganii score=95.0	Morganella sp. TR 90 16S rRNA gene, isolate TR 90	93%	96%	AM156948.1	River water
143	F3D1	Morganella morganii score=94.0	Morganella sp. TR 90 16S rRNA gene, isolate TR 90	94%	95%	AM156948.1	River water
144	F3D2	Morganella morganii score=96.0	Morganella sp. TR 90 16S rRNA gene, isolate TR 90	93%	96%	AM156948.1	River water
145	F3D4	Citrobacter werkmanii score=96.0	Uncultured bacterium clone ZY-12 16S ribosomal RNA gene, partial sequence	95%	96%	KC424723.1	Lake water
146	F3D5	Morganella morganii score=95.0	Morganella sp. TR 90 16S rRNA gene, isolate TR 90	96%	96%	AM156948.1	River water

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
147	F3D7	Citrobacter werkmanii score=96.0	Uncultured bacterium clone ZY-12 16S ribosomal RNA gene, partial sequence	93%	96%	KC424723.1	Lake water
148	F3D8	Morganella morganii score=95.0	Morganella sp. TR 90 16S rRNA gene, isolate TR 90	95%	95%	AM156948.1	River water
149	B3A4	Chlorobiales score=92.0	Uncultured bacterium clone MYS22 16S ribosomal RNA gene, partial sequence	81%	89%	GU305742.1	Lake reservoir water
150	B3A5	Sediminibacterium score=93.0	Uncultured Tolumonas sp. clone R40-93 16S ribosomal RNA gene, partial sequence	96%	94%	JF808982.1	Activated sludge
151	B3B1	Chryseobacterium sp. AU939 score=96.0	Uncultured Flavobacteriaceae bacterium clone sh-xj127 16S ribosomal RNA gene, partial sequence	95%	98%	JQ328000.1	Saltwater lake water
152	B3B2	Legionella taurinensis score=91.0	Uncultured Legionellales bacterium clone 14-1_15 16S ribosomal RNA gene, partial sequence	91%	93%	FJ517691.1	Epithelium of Hydra
153	B3B3	Sporichthyaceae score=92.0	Uncultured bacterium clone CB25 16S ribosomal RNA gene, partial sequence	95%	95%	KC253296.1	Freshwater pond water
154	B3B4	Legionella impletisoli score=94.0	Uncultured soil bacterium clone 1_H3 16S ribosomal RNA gene, partial sequence	95%	92%	EU589279.1	Rice paddy field soil
155	B3B6	Candidatus Captivus score=88. 0	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: GJ16S1_H11	92%	87%	AB821129.1	Soil of Gotjawal forest

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
156	B3C1	Sporichthyaceae score=93.0	Uncultured bacterium clone E43 16S ribosomal RNA gene, partial sequence	95%	98%	HQ827947.1	Lake water
157	B3C6	Sediminibacterium salmoneum score=94.0	Uncultured bacterium clone IS-88 16S ribosomal RNA gene, partial sequence	93%	94%	GQ339178.1	Freshwater seep
158	B3C7	Pandoraea sp. G9805 score=95.0	Uncultured Pandoraea sp. clone EUB64 16S ribosomal RNA gene, partial sequence	93%	92%	AY693821.1	Anaerobic sludge
159	B3C8	Legionella taurinensis score=91.0	Uncultured bacterium clone F20 16S ribosomal RNA gene, partial sequence	95%	91%	FJ230896.1	River water
160	B3D1	Pandoraea sp. G5084 score=96.0	Pandoraea sp. LB-7 16S ribosomal RNA gene, partial sequence	96%	96%	DQ831002.1	N/A
161	B3D2	Sporichthyaceae score=92.0	Uncultured bacterium clone E43 16S ribosomal RNA gene, partial sequence	95%	97%	HQ827947.1	Lake water
162	B3D3	Sediminibacterium salmoneum score=93.0	Uncultured Tolumonas sp. clone R40-93 16S ribosomal RNA gene, partial sequence	96%	92%	JF808982.1	Activated sludge
163	B3D7	Reyranella massiliensis score=94.0	Uncultured Acetobacteraceae bacterium clone LWM2-71 16S ribosomal RNA gene, partial sequence	96%	94%	HQ674815.1	Weathered crop
164	B3D8	Planctomyces score=86.0	Uncultured bacterium clone HWGB-4 16S ribosomal RNA gene, partial sequence	94%	92%	JQ684262.1	Soil
165	B3E3	Pandoraea sp. G9805 score=97.0	Pandoraea sp. G3307 16S ribosomal RNA gene, partial sequence	95%	96%	AF247699.1	N/A

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
166	B3E4	Legionella taurinensis score=91.0	Uncultured Legionellales bacterium clone 14-1_15 16S ribosomal RNA gene, partial sequence	94%	93%	FJ517691.1	Epithelium of Hydra
167	B3F2	Methylibium score=95.0	Uncultured bacterium clone NY-15 16S ribosomal RNA gene, partial sequence	94%	95%	KC290415.1	Lake sediment
168	B3F5	Enterobacteriaceae score=96.0	Serratia sp. 1136 16S ribosomal RNA gene, partial sequence	92%	97%	JX566540.1	Purple paddy soil
169	B3F7	Legionella taurinensis score=92.0	Uncultured bacterium clone GOUTB2 16S ribosomal RNA gene, partial sequence	95%	92%	AY050590.1	Reactor system
170	B3G1	Legionella taurinensis score=91.0	Uncultured Legionellales bacterium clone 14-1_15 16S ribosomal RNA gene, partial sequence	95%	92%	FJ517691.1	Epithelium of Hydra
171	B3G4	Polynucleobacter score=92.0	Uncultured bacterium clone 2C228828 16S ribosomal RNA gene, partial sequence	94%	92%	EU800665.1	Estuary water
172	B3G5	Oxalobacteraceae score=91.0	Uncultured bacterium clone C39 16S ribosomal RNA gene, partial sequence	94%	88%	KC253356.1	Freshwater pond water
173	B3H1	Sporichthyaceae score=93.0	Uncultured bacterium clone CB25 16S ribosomal RNA gene, partial sequence	94%	95%	KC253296.1	Freshwater pond water
174	B3H4	Legionella birminghamensis score=92.0	Uncultured bacterium isolate 1112865261661 16S ribosomal RNA gene, partial sequence	95%	92%	HQ120655.1	Loamy sand

No.	Sample Name	Closest Relative from SILVA	Closest Relative from NCBI	Query Coverage	% Identity	Genbank Ref. No.	Origin
175	B3I2	Delftia acidovorans score=95.0	Uncultured bacterium clone TM06 16S ribosomal RNA gene, partial sequence	95%	94%	AY838513.1	Fruiting body of Tricholoma matsutake
176	B3I5	Candidatus Odyssella thessalonicensis score=91.0	Uncultured bacterium clone HF_NC_25 16S ribosomal RNA gene gene, partial sequence	95%	92%	FJ625364.1	Boreal pine forest soil
177	B3I7	Rhodospirillales score=95.0	Uncultured bacterium clone JFR0702_jaa40b08 16S ribosomal RNA gene, partial sequence	95%	93%	HM780088.1	Zebrafish intestines

Appendix D

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Input Form to Evaluate Library Size

From: P.F. Kemp, J.Y. Aller. Limnol. Oceanogr. Methods (2004) 2:114-125

Instructions for Data Input

Use the following to enter your library information. Enter the number of phylotypes that appeared 1-150 times each; e.g. if you observed ten phylotypes once each, enter "10" in the box adjacent to frequency = 1.

Example: A library consisting of 10 phylotypes (A,B,C,D,E,F,G,H,I,J) distributed among 20 clones as follows. A A A A A A A B B C D E F F F G G G H I J J. The frequencies to be entered into the form are:
Number of phylotypes that appear 1 time: 5 phylotypes
Number of phylotypes that appear 2 times: 2 phylotypes
Number of phylotypes that appear 3 times: 2 phylotypes
Number of phylotypes that appear 4 times: 0 phylotypes (or leave blank)
Number of phylotypes that appear 5 times: 0 phylotypes (or leave blank)
Number of phylotypes that appear 6 times: 0 phylotypes (or leave blank)
Number of phylotypes that appear 7 times: 1 phylotype

After entering the frequency data, click on the submit button and wait until the Results Screen is displayed. Please be patient: the calculations may take 10-20 seconds. Further instructions will be provided on the Results Screen to evaluate your results.

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41	42	43	44	45	46	47	48	49	50
51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70
71	72	73	74	75	76	77	78	79	80
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121	122	123	124	125	126	127	128	129	130
131	132	133	134	135	136	137	138	139	140
141	142	143	144	145	146	147	148	149	150

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Figure 4.39: Input form for Evaluation of Phylotype Richness.

Appendix E

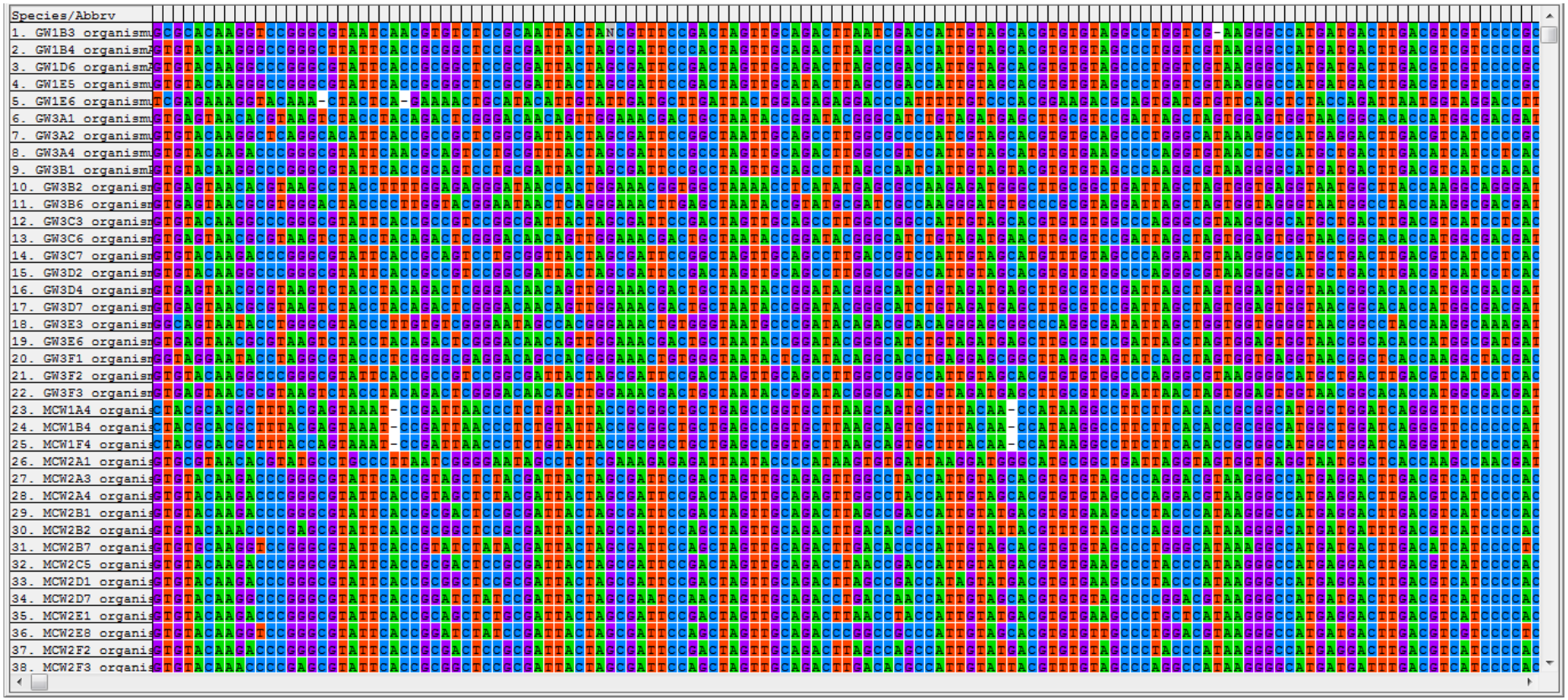

Figure 4.40: Multiple Sequence Alignment (MSA) using MEGA5 for 177 sequences including one sequence of out-group.

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5. GW1E6 organism
6. GW3A1 organism
7. GW3A2 organism
8. GW3A4 organism
9. GW3B1 organism
10. GW3B2 organism
11. GW3B6 organism
12. GW3C3 organism
13. GW3C6 organism
14. GW3C7 organism
15. GW3D2 organism
16. GW3D4 organism
17. GW3D7 organism
18. GW3E3 organism
19. GW3E6 organism
20. GW3F1 organism
21. GW3F2 organism
22. GW3F3 organism
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24. MCW1B4 organism
25. MCW1F4 organism
26. MCW2A1 organism
27. MCW2A3 organism
28. MCW2A4 organism
29. MCW2B1 organism
30. MCW2B2 organism
31. MCW2B7 organism
32. MCW2C5 organism
33. MCW2D1 organism
34. MCW2D7 organism
35. MCW2E1 organism
36. MCW2E8 organism
37. MCW2F2 organism
38. MCW2F3 organism

Species/Abbrv
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3. GW1D6 organism
4. GW1E5 organism
5. GW1E6 organism
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7. GW3A2 organism
8. GW3A4 organism
9. GW3B1 organism
10. GW3B2 organism
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13. GW3C6 organism
14. GW3C7 organism
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38. MCW2F3 organism

Species/Abbrv
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3. GW1D6 organismA
4. GW1E5 organismu
5. GW1E6 organismu
6. GW3A1 organismu
7. GW3A2 organismu
8. GW3A4 organismu
9. GW3B1 organismA
10. GW3B2 organism
11. GW3B6 organism
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14. GW3C7 organism
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19. GW3E6 organism
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30. MCW2B2 organis
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Species/Abbrv
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Species/Abbrv
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47. MCW3C6 organis
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64. MCW3P1 organis
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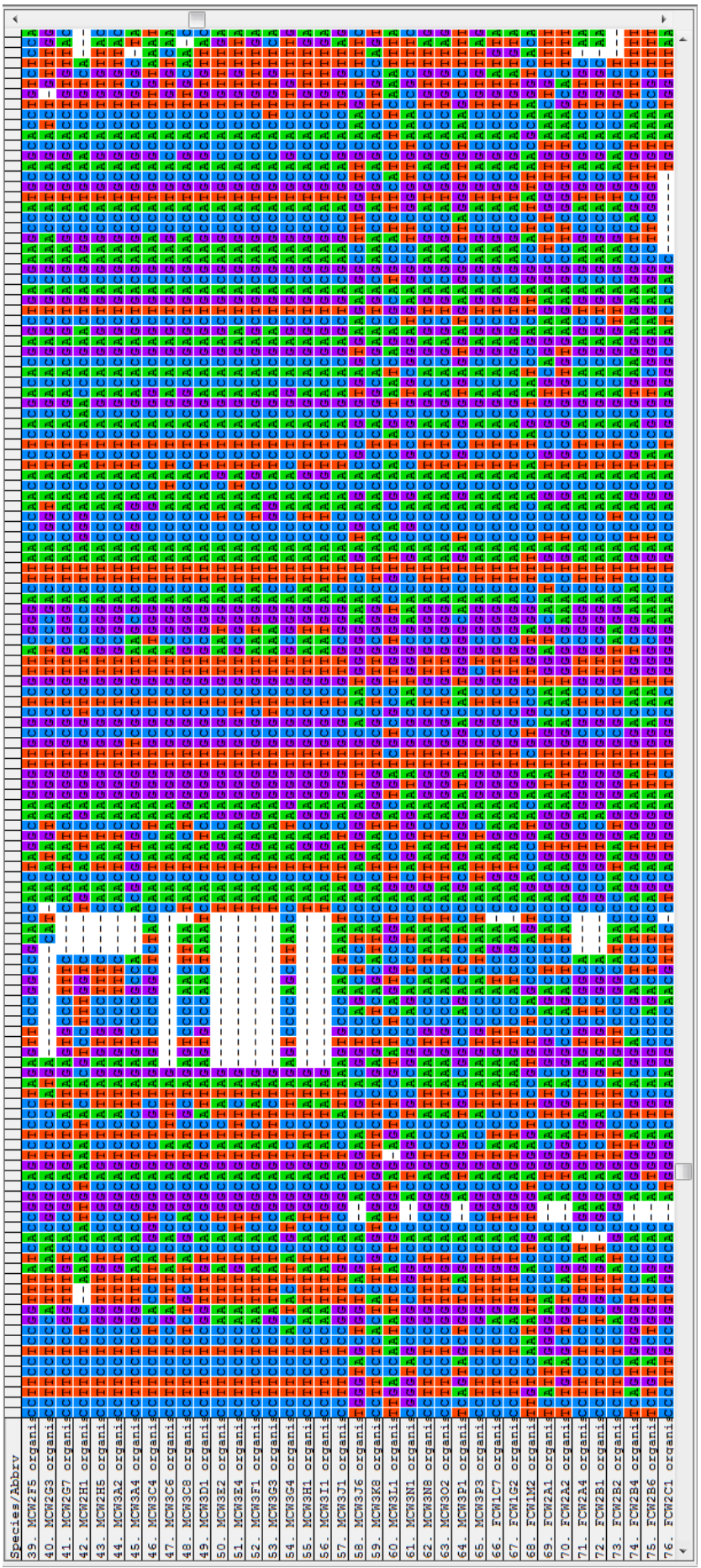
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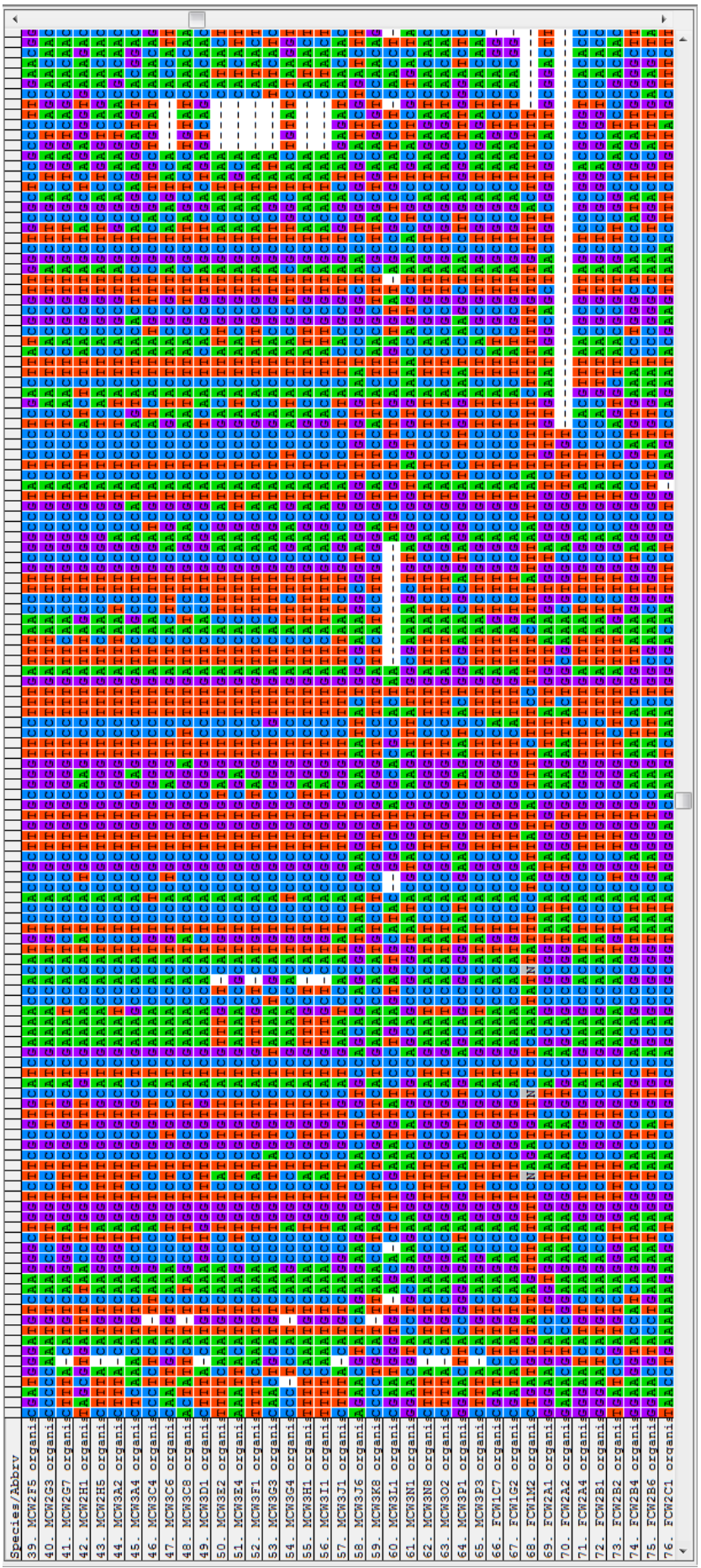
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76. FCW2C1 organs


Species/Abbrv
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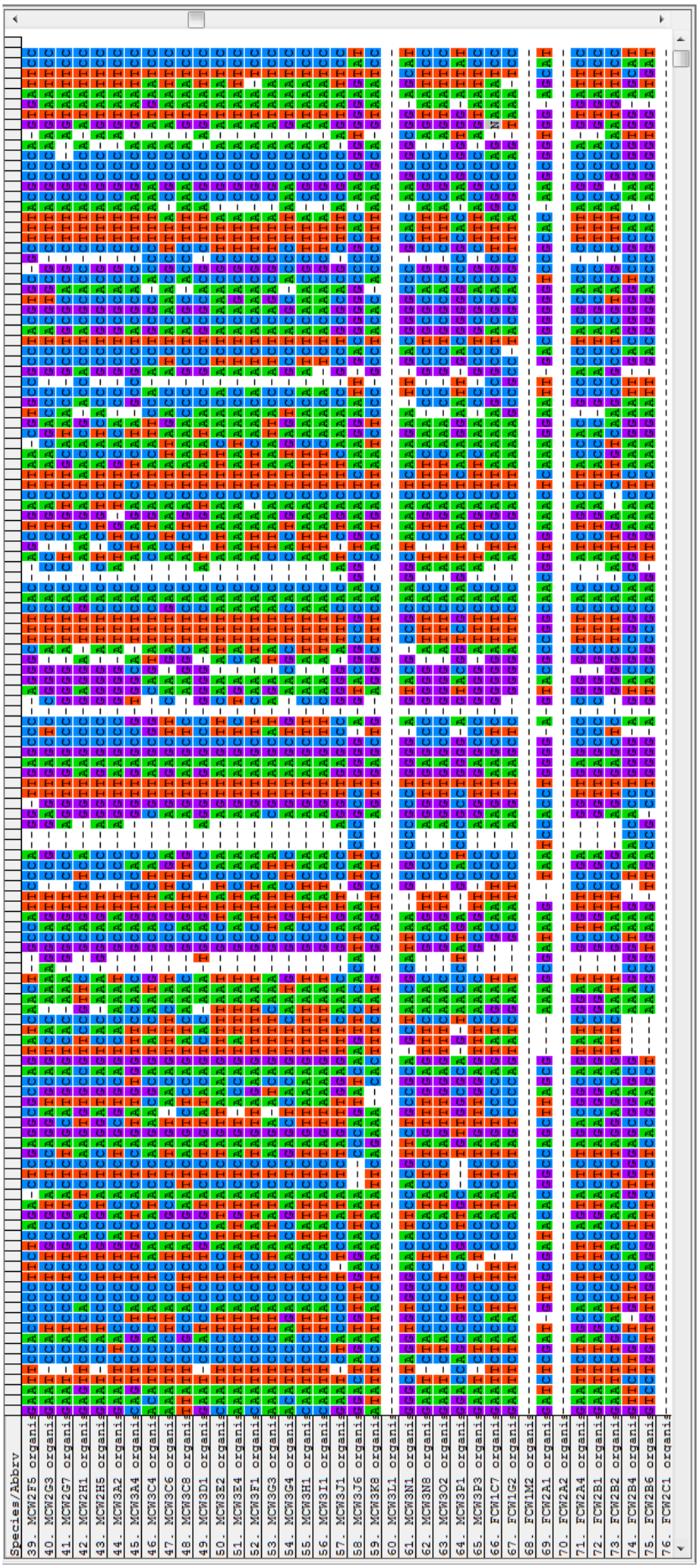
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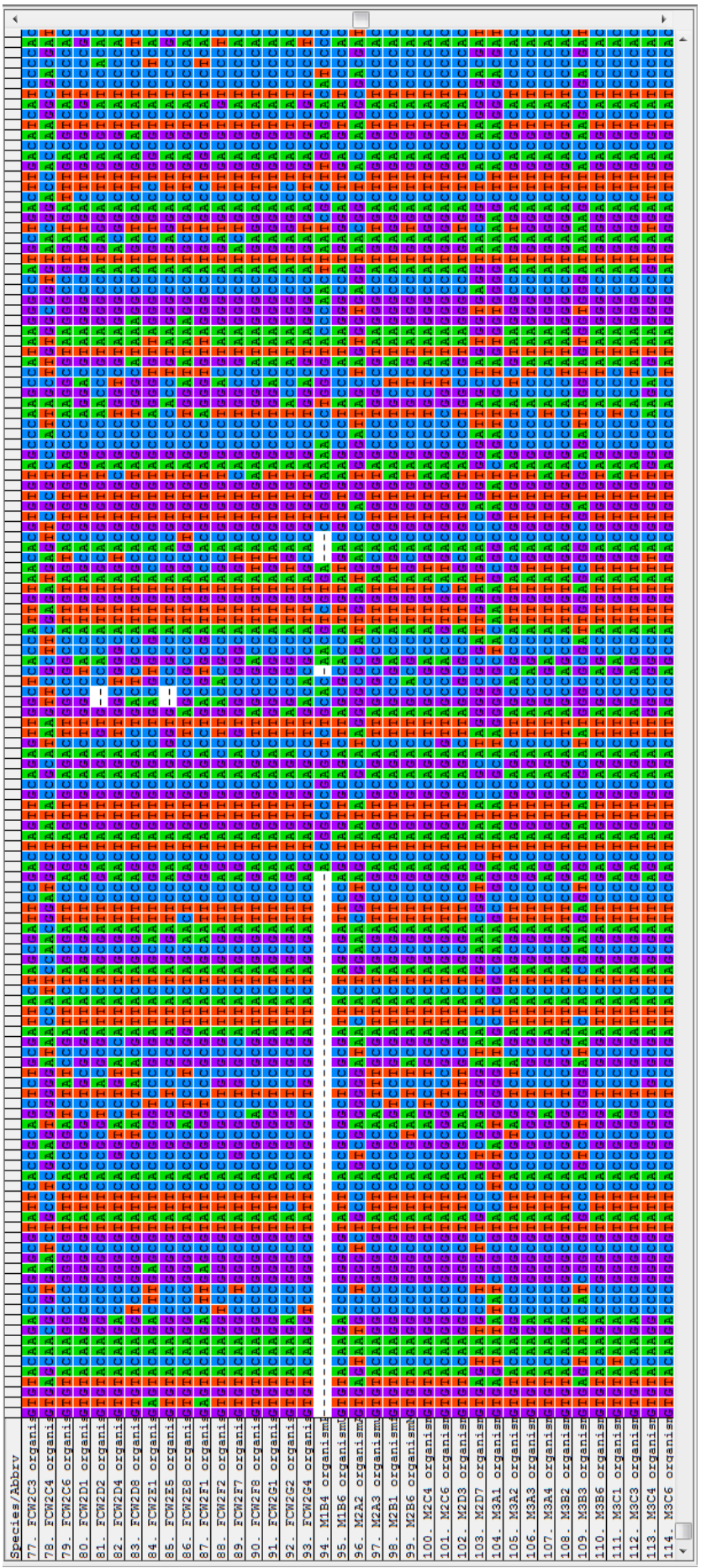
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Species/Abbrv
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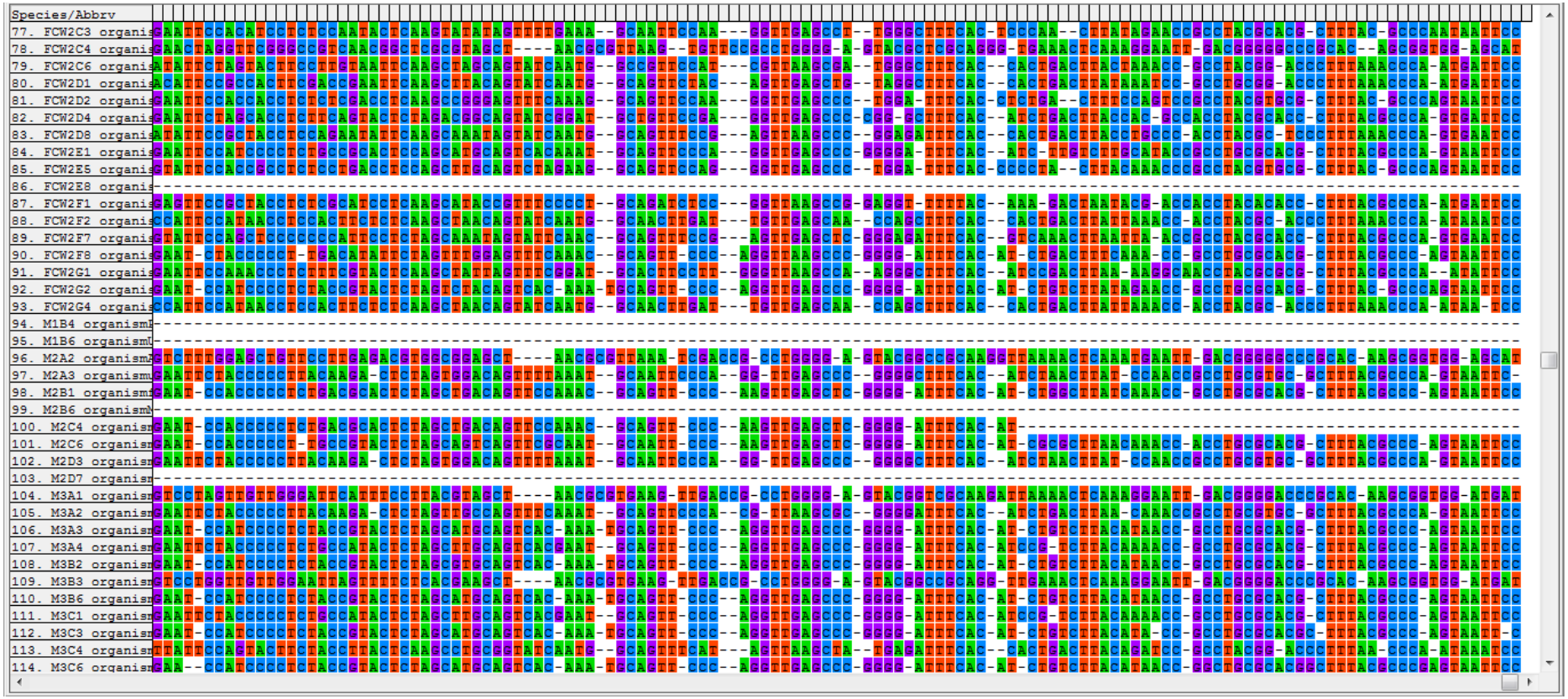
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Species/Abbrv
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Species/Abbrv
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150. B3A5 organism
151. B3B1 organism
152. B3B2 organism

Species/Abbrv
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116. M3D1 organism
117. M3D4 organism
118. M3D6 organism
119. M3D8 organism
120. M3E1 organism
121. M3E2 organism
122. M3E6 organism
123. M3E7 organism
124. F1F2 organism
125. F1F8 organism
126. F1G8 organism
127. F1H4 organism
128. F1J2 organism
129. F3A1 organism
130. F3A2 organism
131. F3A4 organism
132. F3A5 organism
133. F3B2 organism
134. F3B4 organism
135. F3B5 organism
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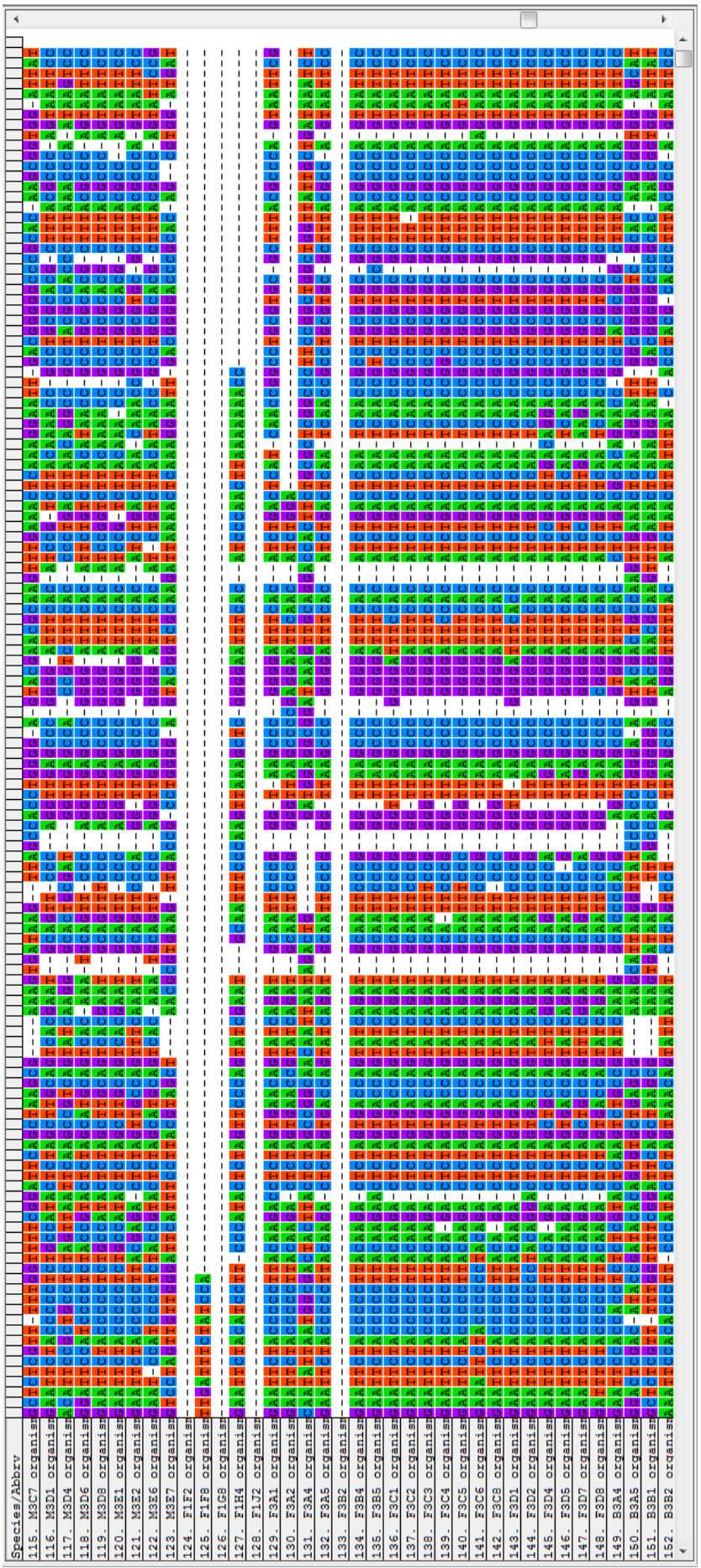
Species/Abbrv
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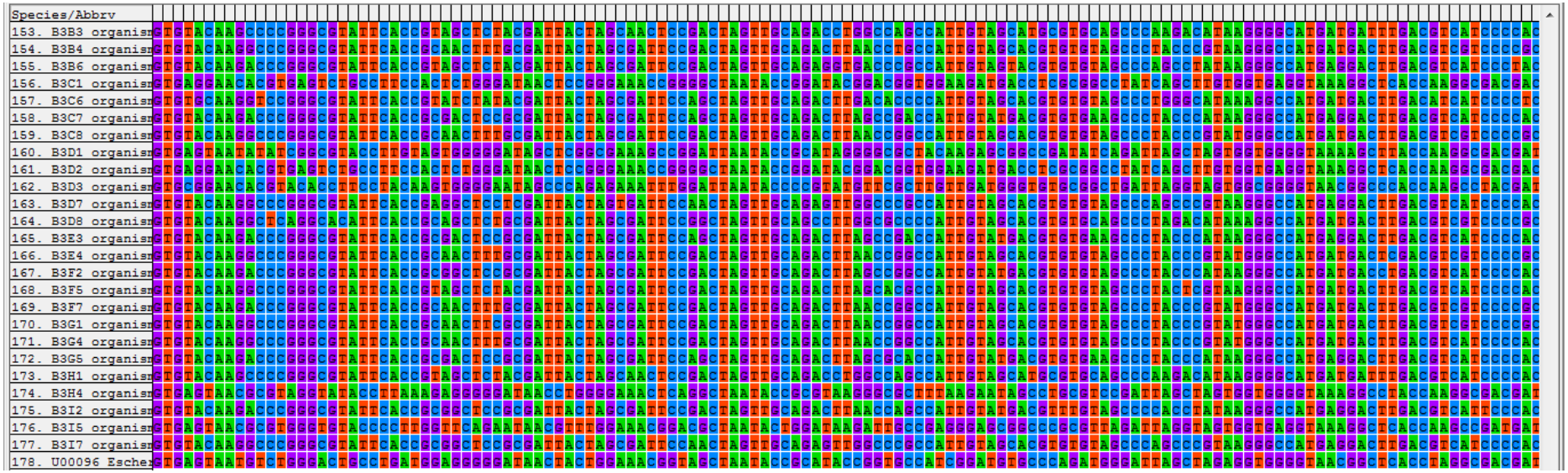
Species/Abbrv
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165. B3E3 organism
166. B3E4 organism
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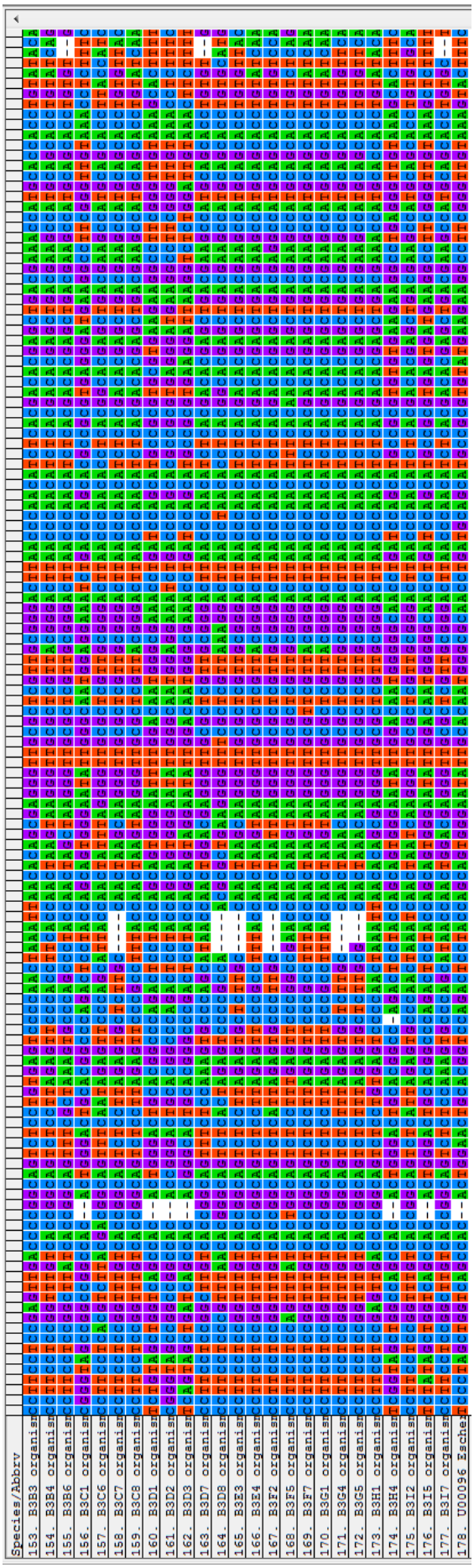
Species/Abbrv
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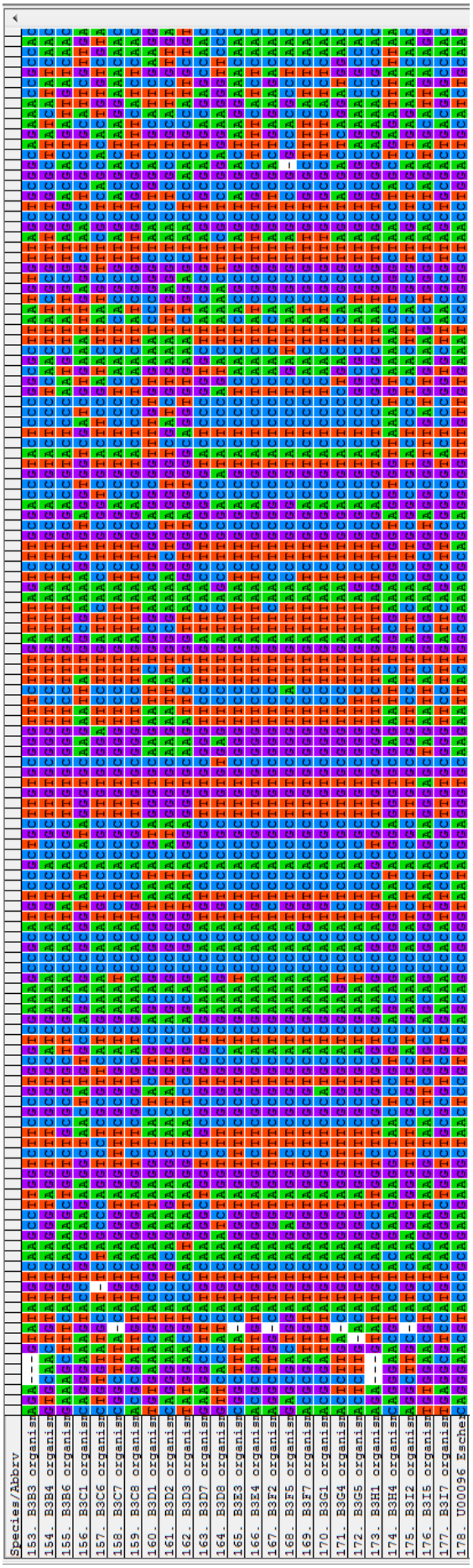
Species/Abbrv
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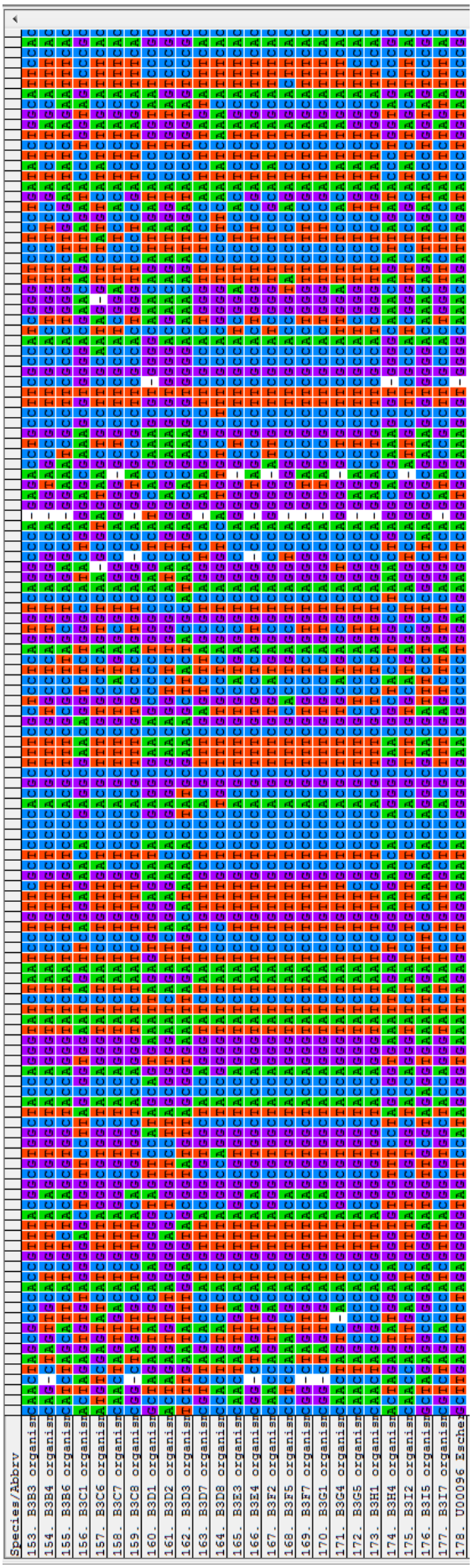
Species/Abbrv
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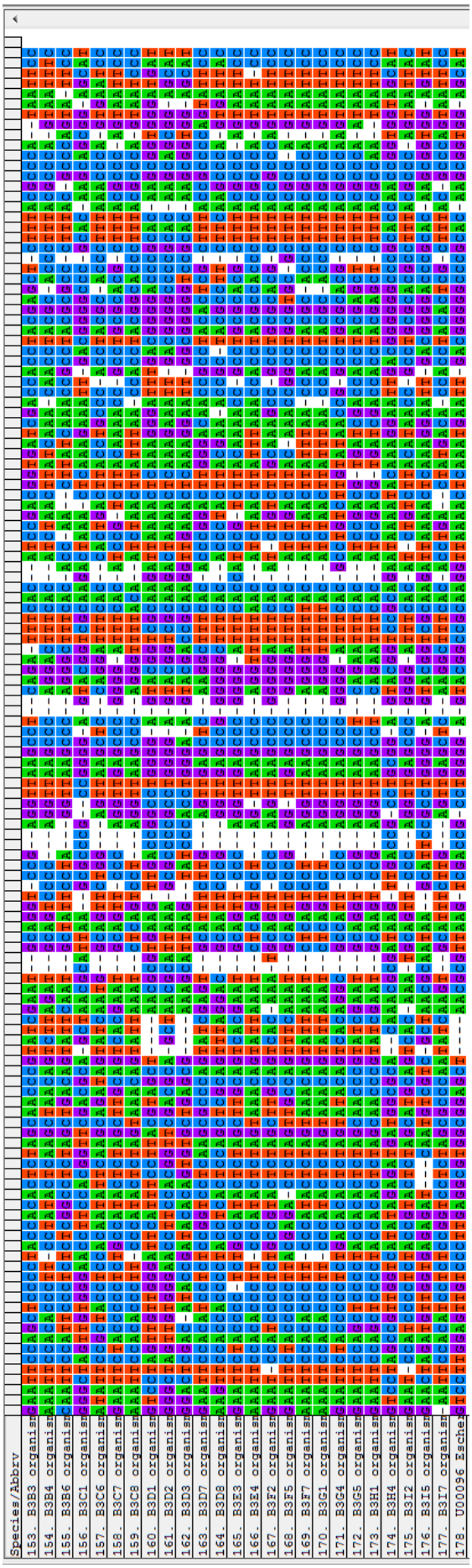
Species/Abbrv
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178. U00096 Eschez