MOLECULAR DIVERSITY OF AMMONIA AND METHANE OXIDIZING BACTERIA IN DISUSED TIN-MINING PONDS LOCATED WITHIN KAMPAR, PERAK

SWAN SOW LI-SAN

MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCE
FACULTY OF SCIENCE
UNIVERSITI TUNKU ABDUL RAHMAN
DECEMBER 2012

MOLECULAR DIVERSITY OF AMMONIA AND METHANE OXIDIZING BACTERIA IN DISUSED TIN-MINING PONDS LOCATED WITHIN KAMPAR, PERAK

By

SWAN SOW LI-SAN

A thesis submitted to the Department of Biological Science,
Faculty of Science,
Universiti Tunku Abdul Rahman,
in partial fulfilment of the requirements for the degree of
Master of Science
December 2012

Dedication

This piece of work is dedicated to my mom, who made me a stronger person. And my dad, who was always there for me no matter what.

ABSTRACT

MOLECULAR DIVERSITY OF AMMONIA AND METHANE OXIDIZING BACTERIA IN DISUSED TIN-MINING PONDS LOCATED WITHIN KAMPAR, PERAK

Swan Sow Li-San

Disused tin-mining ponds make up a significant amount of water bodies in Malaysia particularly at the Kinta Valley in the state of Perak where tin-mining activities were the most extensive. However, the natural ecology and physicochemical conditions of these ponds, many of which have been altered due to secondary post-mining activities, remains to be explored. As ammonia oxidizing bacteria (AOB) and methane oxidizing bacteria (MOB) are directly related to the nutrient cycles of aquatic environments and are useful bioindicators of environmental variations, the focus of this study was to identify AOBs and MOBs associated with disused tin-mining ponds that have a history of different secondary activities in comparison to ponds which were left untouched and remained as part of the landscape. The *amo*A gene and 16S rDNA as well as the *pmo*A gene were used to detect AOBs and MOBs respectively in the sediment and water sampled from the three types of disused mining ponds (idle, lotus-cultivated and post-aquaculture). When physicochemical properties of the water samples were compared with the sequence and phylogenetic analysis of the AOB clone libraries, both *Nitrosomonas* and *Nitrosospira*-like AOB were detected though *Nitrosospira* spp. was seen to be the most ubiquitous AOB as it was present in all pond types. However, AOBs were not detected in the sediments of idle ponds. A

similar comparison done on MOBs indicated the presence of Type I and Type II MOBs at all study sites although Type Ib MOB affiliated with the *Methylococcus/Methylocaldum* lineage were most ubiquitous and made up to 46.7% of the clones. Based on rarefaction analysis and diversity indices, the disused mining pond with lotus culture was shown to harbour the highest richness of both AOBs and MOBs.

ACKNOWLEDGEMENT

Working on this project for the past three years has certainly not been an easy task. Nonetheless, as I pen down the final statements in this thesis, I realize the abundance in knowledge and experiences that I have gained through the course of this project. Sincere appreciation goes to my project supervisor, Dr. Alan Ong Han Kiat, for giving me this opportunity to pursue to project in the topic of my interest. His patience and constructive advice has greatly assisted me in accomplishing the goals of my project and putting it down in the best words through this thesis. Many thanks as well to my co-supervisor Dr. Gideon Khoo for his assistance in making arrangements during the sampling phase of this project in addition to his helpful opinions and advice. I would also like to credit Professor Dr. Thomas Smith and Mr. Patrick Harrison for their tips and ideas which have aided me in the planning stages of my project.

I express my utmost gratitude to my parents, who have always been the most patient and understanding people in my life. Your unconditional love, comfort, support and trust will be something I will cherish always in the years to come.

Special thanks also goes to Dr. Chong Lee Kim as well as the lab assistants at Malaysia University of Science and Technology for their valuable assistance and helpful guidelines throughout the course of sample processing and analysis in my project. If I had stepped on any toes, I hope my shortcomings will be pardoned.

APPROVAL SHEET

This thesis entitled "**MOLECULAR DIVERSITY OF AMMONIA AND METHANE OXIDIZING BACTERIA IN DISUSED TIN-MINING PONDS LOCATED WITHIN KAMPAR, PERAK"** was prepared by SWAN SOW LI-SAN and submitted as partial fulfilment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Assoc. Prof. Dr. Alan Ong Han Kiat) Date:……..
Professor/Supervisor
Department of Pre-Clinical Sciences
Faculty of Medicine and Health Sciences
Universiti Tunku Abdul Rahman

_____

(Assoc. Prof. Dr. Gideon Khoo) Date:……..
Professor/Co-supervisor
Department of Biological Sciences
Faculty of Science
Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: 6TH DECEMER 2012

SUBMISSION OF THESIS

It is hereby certified that ***SWAN SOW LI-SAN*** (ID No: ***10UEM02100***) has completed this ~~final year project/ dissertation~~/ thesis* entitled "*MOLECULAR DIVERSITY OF AMMONIA AND METHANE OXIDIZING BACTERIA IN DISUSED TIN-MINING PONDS LOCATED WITHIN KAMPAR, PERAK*" under the supervision of *DR. ALAN ONG HAN KIAT* (Supervisor) from the Department of Pre-Clinical Sciences, Faculty of Medicine and Health Sciences, and *DR. GIDEON KHOO* (Co-Supervisor)* from the Department of Biological Science, Faculty of Science.

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

Yours truly,

_____

(*SWAN SOW LI-SAN*)

*Delete whichever not applicable

DECLARATION

I hereby declare that the thesis 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.

Name: _____

Date: _____

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	alpha
β	beta
γ	gamma
λ	lambda
%	percentage
°C	degree Celsius
µg	microgram
µL	microlitre
µm	micrometer
AMO	Ammonia monooxygenase
AOB	Ammonia oxidizing bacteria
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
Ca^{2+}	Calcium ions
cAMP	Cyclic Adenosine Monophosphate
CoA	Coenzyme A
Da	Dalton
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
DO	Dissolved oxygen
EDTA	Ethylenediaminetetraacetic acid
FADH	Formaldehyde dehydrogenase
FDH	Formate dehydrogenase

FMN	Oxidized Flavin Mononucleotide
$FMNH_2$	Reduced Flavin Mononucleotide
H_2O	Water
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kb	kilo base pair
kDa	kilo Dalton
LB	Luria Bertani
M	Molar
MDH	Methanol dehydrogenase
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
mL	millilitre
mM	milimolar
MOB	Methane oxidizing bacteria
NaCl	Sodium chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NO_2^-	Nitrite
NO_3^-	Nitrate
nm	nanometer
O_2	Oxygen
OD_{600}	Optical density at the wavelength of 600 nm
OTUs	Operational Taxonomic Units
PCR	Polymerase Chain Reaction

pMMO	Particulate methane monooxygenase
RCHO	aldehyde
RCOOH	carboxylic acid
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
rpm	revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium dodecyl sulphate
sMMO	Soluble methane monooxygenase
TAN	Total ammonia nitrogen
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
UV	Ultraviolet
V	Volt
X-gal	Bromo-chloro-indolyl-galactopyranoside

CHAPTER 1

INTRODUCTION

Tin is an important metal and tin production was once an important driver of the Malaysian economy (Lau 1999). However, extensive mining has resulted in many abandoned mining pits and excavations, which retain water over time leading to the formation of tin-mine lakes, commonly also known as disused tin-mining pools (Arumugam 1994). Disused tin-mining ponds are considered one of the main inland freshwater ecosystems in Malaysia after rivers, lakes, peat swamps and reservoirs, and host diverse aquatic organisms (Arumugam 1994; Yusoff et al., 2006). While some of the disused tin mining pools have been converted for use as aquaculture ponds, recreational areas and waste disposal, only a fraction have been used for productive secondary purposes. The remainder are left as idle lakes and ponds, where they remain as part of the natural landscape.

Microbial processes of many bacterial species are known to affect the water quality and biogeochemical cycles of aquatic environments (Matias et al., 2002). In particular, the nitrification and methane oxidation processes, which are carried out primarily by the ammonia oxidizing bacteria (AOB) and methane oxidizing bacteria (MOB), respectively, are critically involved in the global nitrogen and carbon cycles (Wahlen 1993; Gruber and Galloway 2008), highly influencing nutrient cycling and biological productivity within aquatic ecosystems (Vitousek et al., 1997; Strauss and Lamberti 2000). The ammonia oxidizing bacteria (AOB) is the bacteria primarily responsible for ammonia oxidation, the first, rate limiting step and driver of the nitrification process,

which is the microbial oxidation of ammonia to nitrate via nitrite (Kowalchuk and Stephen 2001). Similarly, the methane oxidizing bacteria mediates the oxidation of methane via methanol to carbon dioxide and is central to the methane oxidation process (Hanson and Hanson 1996). Essentially, characterizing the composition and diversity of bacteria which are directly involved in the cycling of important nutrients within an aquatic ecosystem is important as these parameters are able to give an indication of how well the ecosystem is functioning (Roose-Amsaleg et al., 2001). Particularly within aquatic ecosystems such as disused tin-mining ponds, activities leading to the degradation and modification of natural habitats have occurred as a result of mining activities and common secondary uses such as aquaculture. Exploring the diversity of key bacteria types such as the AOB and MOB and its relationship with the physicochemical properties and water quality of the pond provides an overview of the ecosystem of disused tin-mining ponds within Malaysia.

Molecular ecology tools and methods, which include the retrieval of the 16S rRNA gene sequences as well as functional gene sequences encoding the key enzymes of ammonia and methane oxidation, have had major advances and is emerging as the routinely used method in the investigation of AOB and MOB composition in the environment as opposed to the traditional cultivation based methods. Culture independent molecular ecology based methods provide a more accurate representation of the widespread natural AOB and MOB diversity present within the environment, and thus far molecular ecology methods have successfully detected AOB and MOB to be present at a wide

variety of environments (Rotthauwe et al., 1997). Cultivation of AOB and MOB are also tedious due to slow growth, particularly when isolated from complex environments such as water, soil and sediments (Pontes et al., 2007). Hence characterizing the composition of AOB and MOB inhabiting disused tin-mining ponds using molecular DNA-based methods seems feasible to obtain and thoroughly study the bacterial diversity present at these sites and form the main aims of this study.

Therefore, the primary objectives of this study are:

- to identify the ammonia oxidizing bacteria and methane oxidising bacteria communities in the water and sediment samples from several disused tin-mining ponds with previous secondary activities by 16S rDNA, *amo*A and *pmo*A DNA marker sequence profile of the bacteria;
- to determine selected physicochemical properties of the disused tin-mining pond water samples which are known to influence ammonia oxidizing bacteria and methane oxidizing bacteria;
- to infer the diversity and richness of ammonia oxidizing bacteria and methane oxidizing bacteria at these disused tin-mining ponds via molecular phylogenetic analyses.

CHAPTER 2

LITERATURE REVIEW

2.1 Tin Mining in Malaysia – A Brief History

Tin mining activities in Malaysia dated back to as early as the ninth century, but took place most actively sometime during the late nineteenth century, shortly after the colonization of the Malay Peninsula by the British (Shamshuddin et al., 1986). The Malayan tin industry was one of the main contributors to the world tin industry and to the Malaysian economy, producing up to more than 40 percent of the global tin production at its peak (Lau 1999).

The discovery of abundant tin deposits in the states of Perak and Selangor led mining activities to be concentrated mostly in Peninsular Malaysia (Awang 1994). The Perak state (Figure 2.1) was the largest producer of tin (63%), followed by Selangor (22%) (Ang 1994). Tin mining was also carried out at other states such as Pahang (Sungai Lembing area), Negeri Sembilan, Terengganu and Johor (Mines 2010). The town of Kampar, where our study site of interest is located, is situated within the Kinta Valley in the state of Perak. It was once a bustling tin-mining town due to the significant source of tin available at this area (Figure 2.1) and was the town with one of the richest deposits of tin within the state of Perak (Foong 2003).

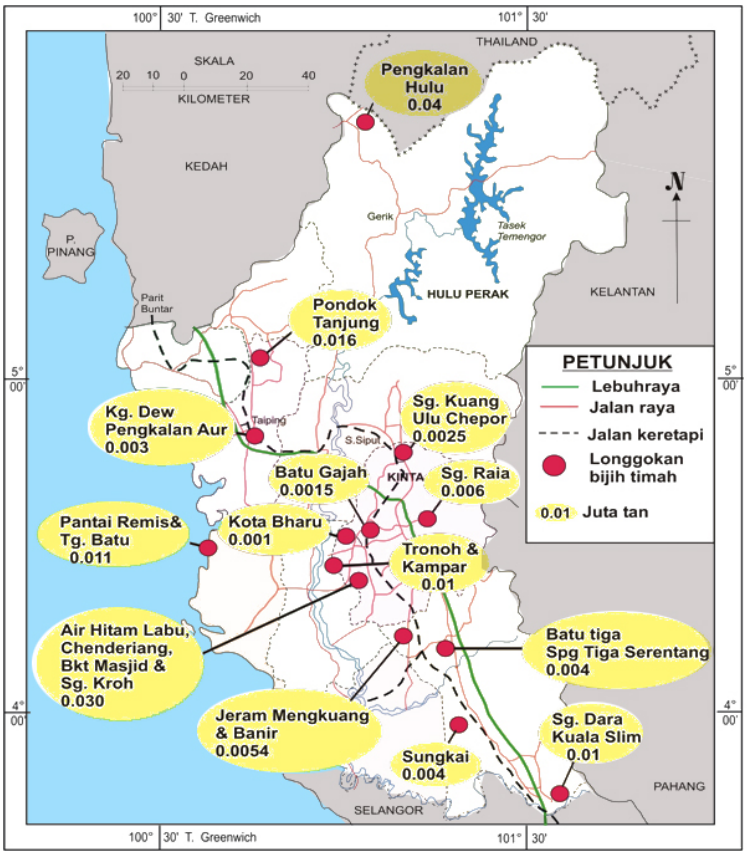


Figure 2.1: Tin mining regions in the state of Perak, Malaysia. Red dots in the map indicate major tin mining areas. Lebuhraya – Expressway; Jalan raya – trunk roads; Jalan keretapi – rail roads; longgokan bijih timah – tin ore mining areas; juta tan – million tonnes (Mines 2008)

2.2 Impact of the Malaysian Tin-Mining Industry on the Ecosystem

Following the downfall of the Malaysian tin-mining industry, today, the country only contributes less than 20% to the world tin industry and hardly exports any tin (Lau 1999) (Table 2.1). However, the active mining activities in the past has resulted in large areas of abandoned tin mines, where the natural habitats, vegetation and geological structures have been severely degraded and polluted by heavy metals such as arsenic (Alshaebi et al., 2009). Ex-mining areas include 113,700 hectors of tin tailings (ex-mining land) (Ang 1994) and 16,440 hectors (164.4 km^2) of disused tin-mining pools/lakes (water bodies) (Yusoff et al., 2006). Tin tailings are defined as tracts of waste land

consisting of washed waste products of alluvial mining (Majid et al., 1994), while disused tin mining pools are formed when rainwater fills and retains in abandoned tin-mine pits, slime retention pools and mining excavations, resulting mostly from the usage of mining techniques such as gravel-pumps and dredges (Arumugam 1994; Lau 1999). There are approximately 4300 disused tin-mining pools in Peninsular Malaysia of varying sizes and depths. Perak has the most abundant number of disused tin-mining pools (2873) followed by Selangor (542), Johor (280) and Pahang (229) (Yusoff et al., 2006).

Table 2.1: The development of the Malaysian tin-mining industry from the years 1970 - 1994 (Lau 1999)

Year	Production (Tonnes)	Import (Tonnes)	Export (Tonnes)	No. of Mines
1970	73,795	13,726	92,631	1083
1975	64,364	18,476	77,940	910
1980	61,404	8,422	69,498	852
1989	32,034	23,857	49,480	255
1990	28,468	21,732	52,703	141
1991	20,710	30,536	42,425	92
1992	14,339	33,264	45,149	63
1993	10,384	27,277	35,545	43
1994	6,458	35,574	35,327	39

While disused tin-mining pools only represent a small fraction of the freshwater aquatic habitats in Peninsula Malaysia (Table 2.2), they can serve as a resource for secondary use. Besides being a source of freshwater for consumption, as many as 271 old tin-mining pools in the Peninsular have been converted for use in aquaculture activities (Arumugam 1994; Majid et al., 1994). Others have been converted into recreational and tourism areas such as

the notable Sunway Lagoon, Mines theme park, Paya Indah Wetlands Sanctuary and Clearwater Sanctuary Park, housing estates, and for use as waste disposal areas (Arumugam 1994; Yusoff et al., 2006). However, only a fraction (9.7%) of the disused tin-mining areas have been used for productive purposes (Ang and Ho 2002). The remainder are left as idle lakes and ponds that are part of the landscape, where the process of natural regeneration and primary succession will occur (Yule et al., 2004).

Table 2.2: Main inland aquatic ecosystems in Peninsular Malaysia (Yusoff et al., 2006)

Habitat	**Area (km^2)**
Rivers (including floodplain areas)	9,111
Peat swamps	4,850
Resevoirs (Man-made lakes)	1,600
Mining pools	164

2.3 Physicochemical Properties & Biota of Disused Tin-Mining Ponds

The physicochemical property and biological content at the disused tin-mining ponds varies depending on the previous and/or current secondary activities that have occurred at the ex-mining ponds. Arumugam (1994) categorized ex-mining lakes into five types: new lakes, acidic lakes, buffered lakes, aquaculture lakes and post-aquaculture lakes, with pH and inhabiting plant communities as the main distinguishing factors of the categories of lakes. His study and those conducted by other researchers found that the physicochemical properties of various disused tin-mining lakes in Malaysia,

particularly the pH value, varied over a wide range from very acidic (pH 3.6) to basic (pH 8.2) (Shamshuddin et al., 1986; Abdul-Rashid and Awang 2004).

Disused tin-mining ponds support a rich diversity of aquatic wildlife and plant species as a result of natural regeneration and secondary activities such as aquaculture, but the species and diversity differ from one pond to another (Ambak and Jalal 2006). The tilapia (*Oreochromis niloticus*), marble goby (*Oxyeleotris marmorata*) and giant freshwater prawn (*Macrobrachium rosenbergii*) are fishes and prawns commonly found in disused tin-mining ponds since they are commonly cultured in these sites for their high commercial value. A previous diversity study on the fish and shrimp community inhabiting disused tin-mining pools of Kampar, Perak found the Eastern Mosquitofish (*Gambusia holbrooki*) and tilapia to be abundant (Ng 2011).

Some of the plants and aquatic macrophytes that have been found in disused tin-mining ponds in Malaysia include kariba weed (*Salvinia molesta*) and the sacred Indian lotus (*Nelumbo nucifera*) (Ashraf et al., 2011). Even though disused mining sites generally present an unfavourable condition for natural vegetation due to low pH, low plant nutrients and elevated levels of toxic metals (such as arsenic, copper, lead, tin, and zinc found mainly in many Malaysian ex-mining ponds), selected plant species are able to tolerate conditions with severe levels of heavy metal contamination through several mechanisms (Baker 1981; Yusof et al., 2001; Ng et al., 2004; Ashraf et al., 2010). In particular, the *Nelumbo nucifera*, which is present at our study sites,

is known to be a hypertolerant plant capable of accumulating metals such as arsenic (commonly found in disused tin-mining pools) (Ashraf et al., 2011).

2.4 Ammonia Oxidizing Microorganisms and Its Relationship With the Global Nitrogen Cycle

2.4.1 The Global Nitrogen Cycle

The global nitrogen cycle is characterized by the maintenance of a small pool of fixed or combined nitrogen in continuous exchange with atmospheric dinitrogen (N_2) (Dalsgaard and Thamdrup 2002). The main processes in the nitrogen cycle include assimilation, ammonification, nitrification, denitrification, nitrogen fixation, and anaerobic ammonia oxidation (annamox), and most of these processes involve microorganisms (You et al., 2009) (Figure 2.2). With the immense importance of nitrogen in plant nutrition (Kowalchuk et al., 1999) and the frequent role of nitrogen as a limiting nutrient for primary production (Vitousek and Howarth 1991), the biological processes which directly and indirectly affect the availability of fixed nitrogen serve as important regulators of ecosystem function and global biogeochemistry. Furthermore, eutrophication, the process by which the nutrient content of an environment is elevated due to the excessive degradation of organic matter or anthropogenic disposal of nitrogen containing waste, is becoming a severe phenomenon observed in many aquatic environments (Smith et al., 1999). The negative effects of eutrophication can often be alleviated when nitrification is coupled with denitrification and/or annamox, as nitrogen will be eliminated to the atmosphere as molecular

nitrogen (Dalsgaard and Thamdrup 2002; Thamdrup and Dalsgaard 2002; Urakawa et al., 2006).

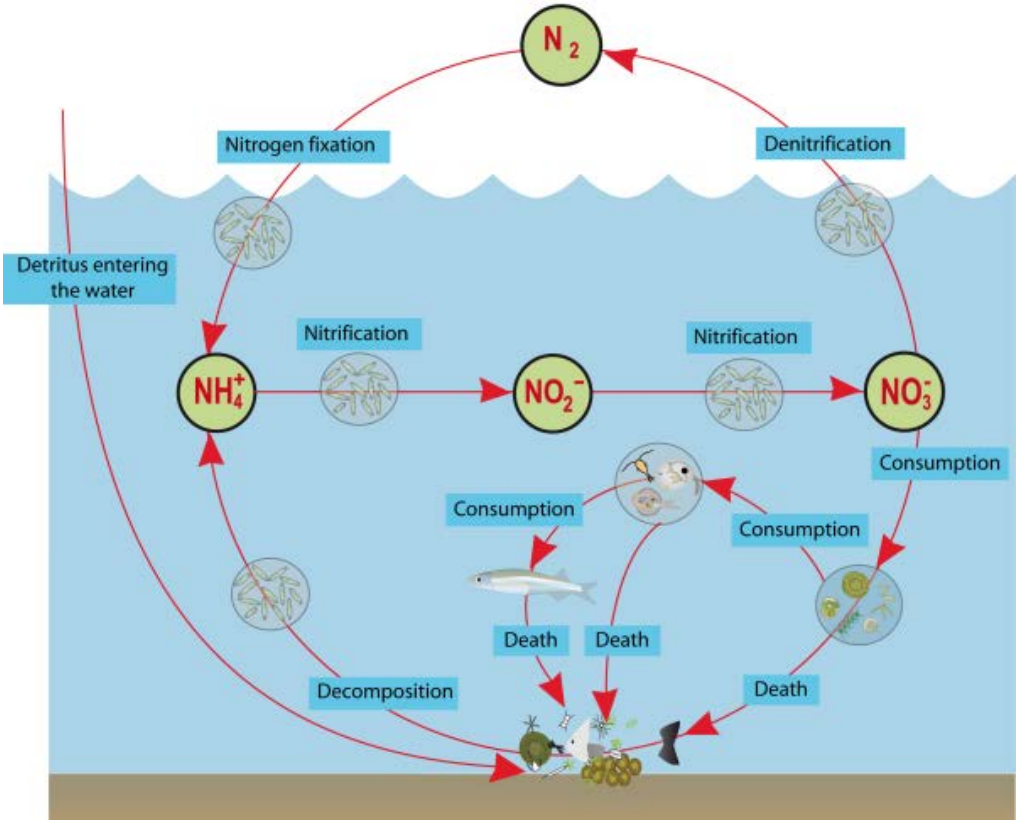


Figure 2.2: Schematic overall diagram of the main processes involved in the nitrogen cycle of an aquatic ecosystem (Department of Environment and Heritage Protection 2013).

2.4.2 Nitrification, their Associated Pathways and Microorganisms

Autotrophic nitrification is the biological transformation process linking the most reduced form of nitrogen (NH_3/NH_4^+) to nitrate (NO_3^-). It is an oxidation process that involves two steps (Kowalchuk and Stephen 2001). The first step is ammonia-oxidation, the conversion of ammonia to nitrite (NO_2^-) through the intermediate hydroxylamine (NH_2OH) by ammonia oxidizing organisms, while the second step involves the conversion of nitrite to nitrate (Figure 2.3) by nitrite-oxidizing organisms (You et al., 2009). As ammonia oxidation is the

first rate limiting step in the nitrification process, ammonia oxidizing organisms drive the process of nitrification in a wide range of environments and thus play important roles in the global cycling of nitrogen (Kowalchuk and Stephen 2001).

$$\textbf{(a)}\ NH_3 + 2H^+ + 2e^- + O_2 \xrightarrow[\text{Ammoniamonooxygenase (AMO)}]{} NH_2OH + H_2O$$

$$\textbf{(b)}\ NH_2OH + \frac{1}{2}O_2 \xrightarrow[\text{HydroxylamineOxidoreductase (HAO)}]{} NO_2^- + 3H^+ + 2e^-$$

$$\textbf{(c)}\ NO_2^- + H_2O \xleftrightarrow[\text{Nitriteoxidoreductase (NOR)}]{} NO_3^- + 2H^+ + 2e^-$$

Figure 2.3: The 2 step nitrification pathway. In the first step, (a) Ammonia is first converted to the intermediate hydroxylamine in a reaction catalyzed by the ammonia monooxygenase (AMO) enzyme. (b) The intermediate hydroxylamine is then converted to nitrite, aided by the hydroxylamine oxidoreductase (HAO) enzyme. In the second step (c) nitrite, catalyzed by nitrite oxidoreductase (NOR), is finally converted to the end product of nitrification, nitrate (You et al., 2009).

Traditionally, autotrophic ammonia-oxidation has been known to be an obligatory aerobic process undertaken by two groups of *Proteobacteria* known as the ammonia-oxidizing bacteria (explained in further detail in section 2.6.1). Ammonia-oxidizing bacteria are obligate chemolithoautotrophs, as they obtain energy for survival solely from the nitrification process (Teske et al., 1994). Recently, however, another type of microorganism from the archaeal domain was also found to be capable of aerobic ammonia oxidation (Konneke et al., 2005). These ammonia oxidizing archaea (AOA) are from the *Crenarchaeota* kingdom and were found to possess the genes encoding the AMO enzyme (Konneke et al., 2005). Though the AOA were found in abundance in certain environments, the significance of their role in ammonia-

oxidation remains a subject of debate (Mosier and Francis 2008; Herrmann et al., 2008; Jiang et al., 2009). The AOA may be the important ammonia oxidizers in conditions unfavourable for ammonia oxidation, such as lack of substrate or low pH (Herrmann et al., 2009) .

The established views of ammonia oxidation by aerobic bacteria have also been challenged by the discovery of anaerobic ammonia oxidation (annamox). The annamox process is an alternative N_2 producing process where nitrite is combined with ammonium and converted to dinitrogen gas via the intermediate hydrazine, aided by the hydrazine oxidoreductase (HZO) enzyme (Strous et al., 1999; Jetten et al., 2009) (Figure 2.4). The process occurs under strictly anoxic conditions and without the need for carbon (Jetten et al., 2009). Known annamox bacteria fall under *Brocadiaceae* family in the *Planctomycetales* phylum and five *Candidatus* genera have been described so far (Dang et al., 2010a). A similar process, the oxygen-limited autotrophic nitrification/denitrification (OLAND) system, has also been discovered in autotrophic ammonia-oxidizers. OLAND does not require strictly anoxic conditions and can proceed under microaerophilic conditions (Kuai and Verstraete 1998).

$$\mathbf{NH_4^+ + NO_2^- \xrightarrow[hydrazineoxidoreductase\ (HZO)]{} N_2 + 2H_2O}$$

Figure 2.4: A simplified representation of the annamox process. Ammonia, coupled together with nitrite, are directly converted to dinitrogen (N_2) in the absence of oxygen via the intermediate hydrazine, catalyzed by the enzyme hydrazine oxidoreductase.

2.4.3 The AMO Gene Cluster

The ammonia monooxygenase enzyme, which functions to convert ammonia to hydroxylamine in AOB, is a multi-subunit membrane bound enzyme. The first subunit, AmoA, is a 27 – 30 kDa membrane associated subunit containing the active binding site of the protein and is encoded by the gene *amo*A (Calvó and Garcia-Gil 2004) belonging to an operon with the structure *amo*CAB. The operon is shown to be conserved in all investigated genomes of AOB (Junier et al., 2009). The second subunit of the AMO protein, AmoB, is a 38 – 43 kDa iron – copper subunit (Klotz et al., 1997) encoded by the *amo*B gene. The *amo*C gene encodes for the AmoC subunit, which was found to be approximately 31 kDa in size in the AOB *Nitrosospira* sp. NpAV (Norton et al., 2002). The functions of the *amo*B and *amo*C gene products are unknown (Stein et al., 2000), but it has been shown that the two genes are a part of the functional operon and are required for AMO synthesis (Norton et al., 2002).

There are three nearly identical copies of the *amo*CAB operon in β-subgroup AOB, but only one copy has been detected in γ-subgroup AOB (Calvó and Garcia-Gil 2004). Recently, two new genes, the *amo*R and *amo*D genes have been discovered in the γ-subgroup AOB *Nitrosococcus oceani* ATCC 19707, hence deeming the *amo* operon of this AOB to be a five gene operon with the genes arranged in the sequence *amo*RCABD. The *amo*D genes were found to be homologues of genes enconding copper enzymes in MOB, while the AmoR protein was alleged to function as a regulator of ammonia catabolism (El Sheikh et al., 2008).

2.5 Methane and the Global Carbon Cycle

Methane is the most abundant reactive and organic trace gas and the third most abundant greenhouse gas in the atmosphere (Wuebbles and Hayhoe 2002). It is a very stable carbon compound that functions as a crucial intermediate leading to the mineralization of organic matter (Hanson and Hanson 1996). Out of the many atmospheric methane sources, natural and cultivated wetlands are one of the major sources, contributing to approximately 40%, which further contribute to roughly 15% of the greenhouse effect (Cao et al., 1998). The illustration in Figure 2.5 below gives an overview of the methane cycle:

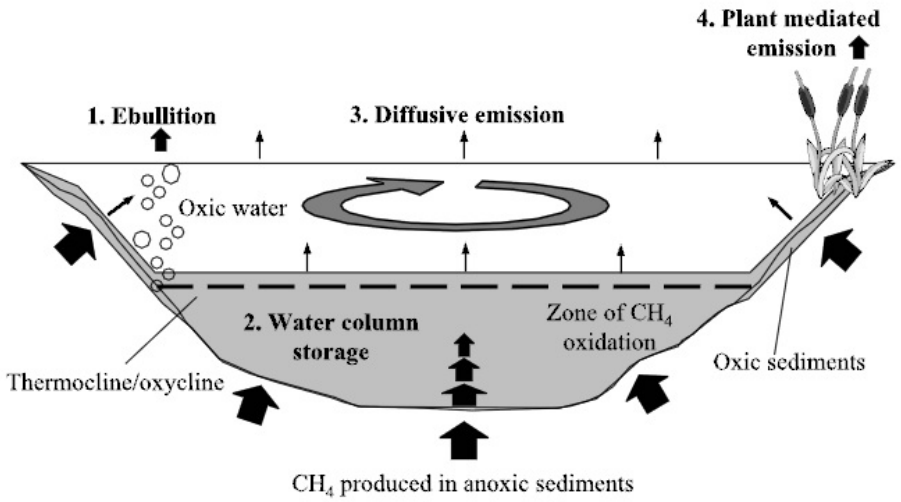


Figure 2.5: Overview of the methane cycle within a stratified lake (Bastviken et al., 2004).

Complex physiological processes involving plants and other microorganisms influence the production and emission of methane in wetlands, beginning with plant deposits and root exudates into the soil, then followed by a fermentation

of soil organic matter into a methanogenic substrate and subsequent methanogenesis, and re-oxidation (Cao et al., 1998).

2.5.1 Methane Oxidation and the Methanotrophs

The most commonly known process of methane oxidation is an aerobic process. This involves the conversion of methane, the most reduced form of carbon, into a more oxidized form carbon dioxide via a four step process (Hanson and Hanson 1996; Bodelier and Frenzel 1999) (Figure 2.6). However, the first step, the conversion of methane to methanol catalysed by the enzyme methane monooxygenase (MMO) is the defining characteristic of methane oxidizing bacteria, and has been the most studied division of the aerobic methane oxidation process (Hanson and Hanson 1996).

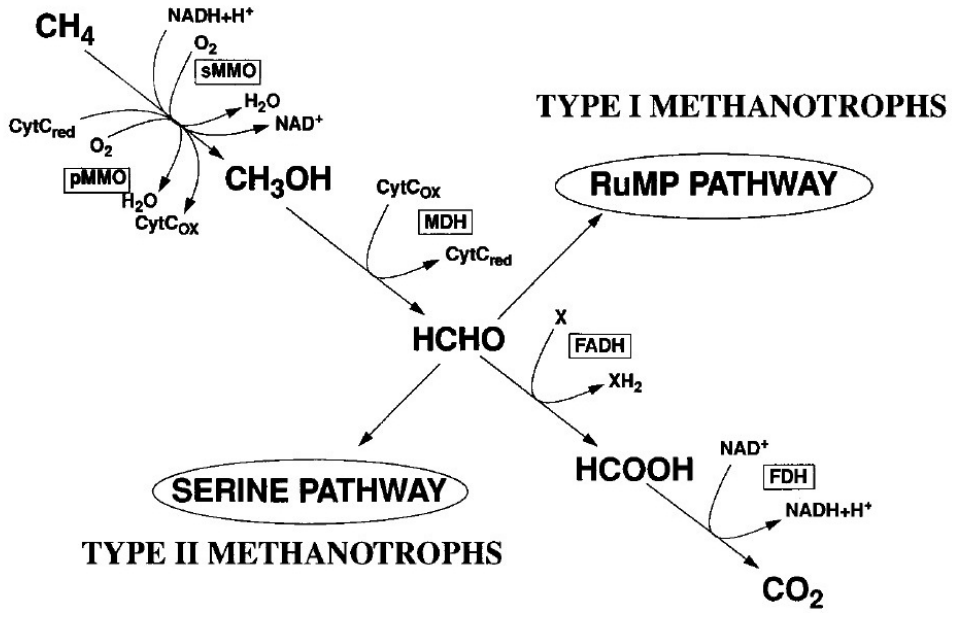


Figure 2.6: Illustration of the aerobic methane oxidation pathway (Hanson and Hanson 1996). Enzymes catalysing each reaction are shown.

The methane oxidation process is also known to occur anaerobically, most commonly at marine sediments (Eller et al., 2005; Caldwell et al., 2008). The exact mechanism of anaerobic methane oxidation is not known, but sulfate and nitrate are used as electron donors during the process (Valentine 2002) (Figure 2.7). Anaerobic methane oxidation is thought to be conducted by a group of methane oxidizing archaea and sulfate reducing bacteria (Valentine 2002). However studies relating to anaerobic methane oxidation have been much more limited compared to aerobic methane oxidation due to the lack of suitable culture dependent and independent (molecular) techniques (Caldwell et al., 2008).

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$$

Figure 2.7: Reaction equation of the anaerobic methane oxidation process (Valentine 2002)**.**

2.5.2 Methane Oxidizing Bacteria

Methane oxidizing bacteria (MOB), also commonly known as methanotrophic bacteria or methanotrophs, are a division of the methylotrophs, aerobic bacteria that utilize one carbon compounds more reduced than formic acid as their primary sources of energy (Hanson and Hanson 1996). Methanotrophs are uniquely recognized to utilize only methane and/or methanol as their carbon and energy source, oxidizing the substrate they consume to formaldehyde and further assimilating the formaldehyde to the end product of carbon dioxide. Energy is also derived from the complete oxidation of formaldehyde to carbon dioxide (Hanson and Hanson 1996; Martin 2002).

2.5.3 The Methane Monooxygenase Enzymes

As mentioned above, the aerobic methane oxidation process is catalysed by methane monooxygenase (MMO) enzymes. There are two currently known variations of the MMO enzymes. The first is a membrane bound, or particulate MMO (pMMO), while the other is a cytoplasmic, or soluble MMO (sMMO). pMMO have been found to be present in all methanotrophs except for the *Methylocella* genus (Theisen et al., 2005). On the other hand, sMMO are only found to be present in a subset of methanotrophs, namely the Type II methanotrophs, Type X (*Methylococcus capsulatus*) and Type I (*Methylomonas methanica* & marine *Methylomicrobium*) methanotrophs (Koh et al., 1993). sMMO is usually expressed only under copper-limiting conditions (Koh et al., 1993), and hence the type of MMO present is commonly correlated with copper availability in an environment (Stanley et al., 1983; Buchholz et al., 1995).

2.5.3.1 The Particulate Methane Monooxygenase (pMMO) Gene Cluster

The genes in the *pmo* gene cluster encode for the particulate methane monooxygenase enzyme, a membrane bound, copper and iron containing enzyme which is one of the two main enzymes responsible for the conversion of methane to methanol in the methane oxidation pathway (Hakemian and Rosenzweig 2007). From the sequencing of structural genes of this enzyme from two Type II MOB (*Methylocystis* sp. strain M, and *Methylosinus trichosporium* OB3b) and one Type X MOB (*Methylococcus capsulatus* Bath), the genes encoding this enzyme have been shown to lie in a three-gene

operon, *pmo*CAB, of which each gene encodes for the three integral membrane polypeptides of approximately 23, 27, and 45 kDa, respectively (Zahn and DiSpirito 1996; McDonald et al., 2007).

The *pmo*A gene has been shown to encode the subunit harbouring the active site of the pMMO enzyme, and as a gene that is highly conserved among methanotrophs, it is the commonly utilized gene in the detection of MOB from diverse environments (Gilbert et al., 2000). Most recently, the pmoB subunit of the pMMO enzyme which is encoded by the *pmo*B gene has been shown to harbour a dinuclear copper site (Miyaji 2011) which apparently serves as an active centre for the oxidation of methane to methanol (Balasubramanian et al., 2010). The toxicity of certain parts of the *pmo* genes to *Escherichia coli* have proven it difficult to clone for further and more detailed study (Nguyen et al., 1998). In particular, an over-expression of *pmo*C seemed to be lethal to *E. coli* possibly due the reason that the expression of the *pmo*C gene is controlled by a promoter that is active in *E. coli* as well (Gilbert et al., 2000). This might be the contributing reason that there has been no further study so far on the function of the *pmo*C gene.

2.5.3.2 The Soluble Methane Monooxygenase (sMMO) Gene Cluster

For the soluble, cytoplasmic methane monooxygenase, the enzyme is encoded by a six gene operon (Stainthorpe et al., 1990; Cardy et al., 1991). A dimer of three subunits (αβγ) forms the first methane hydroxylase-dioxygen activation component (MMOH), which is the active site of the enzyme encoded by the genes *mmo*XYZ, respectively. This active site harbours a di-iron center (Kopp

and Lippard 2002). The second regulatory protein B component (MMOB) is a coupling protein encoded by the *mmo*B gene and required for efficient catalysis, while the third component, protein C (MMOR), is an iron sulphur flavoprotein that functions as a reductase and is encoded by the *mmo*C gene. The final component, MMOD is encoded by an open reading frame (*orf*Y), and is suspected to function in the assembly of the MMOH diiron center (Merkx and Lippard 2002).

2.6 Phylogeny and Molecular Diversity of the Ammonia Oxidizers and Methanotrophs

2.6.1 Phylogeny of the Autotrophic Ammonia Oxidizing Bacteria

AOB have been grouped under two phylogenetically distinct subdivisions of *Proteobacteria*. Two species of *Nitrosococcus* (*N. oceani* and *N. halophilus*) are grouped into the γ-subdivision of *Proteobacteria*, while the other main group, the *β*-subdivision *Proteobacteria*, includes the genera *Nitrosomonas* and *Nitrosospira* (Lipponen et al., 2004; Coci et al., 2008).

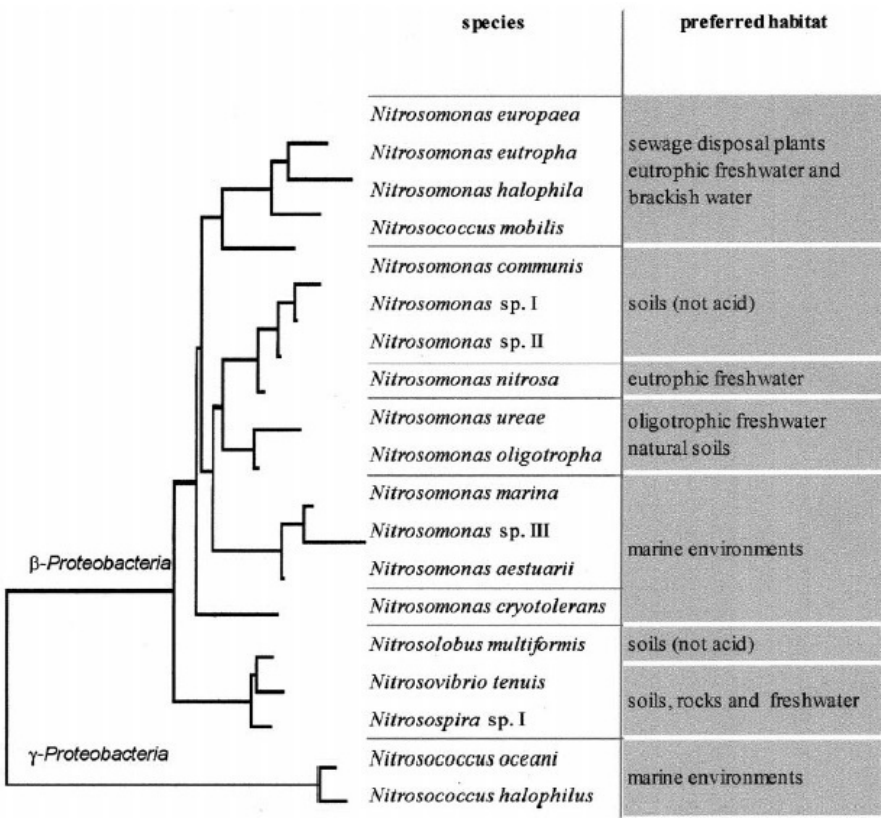
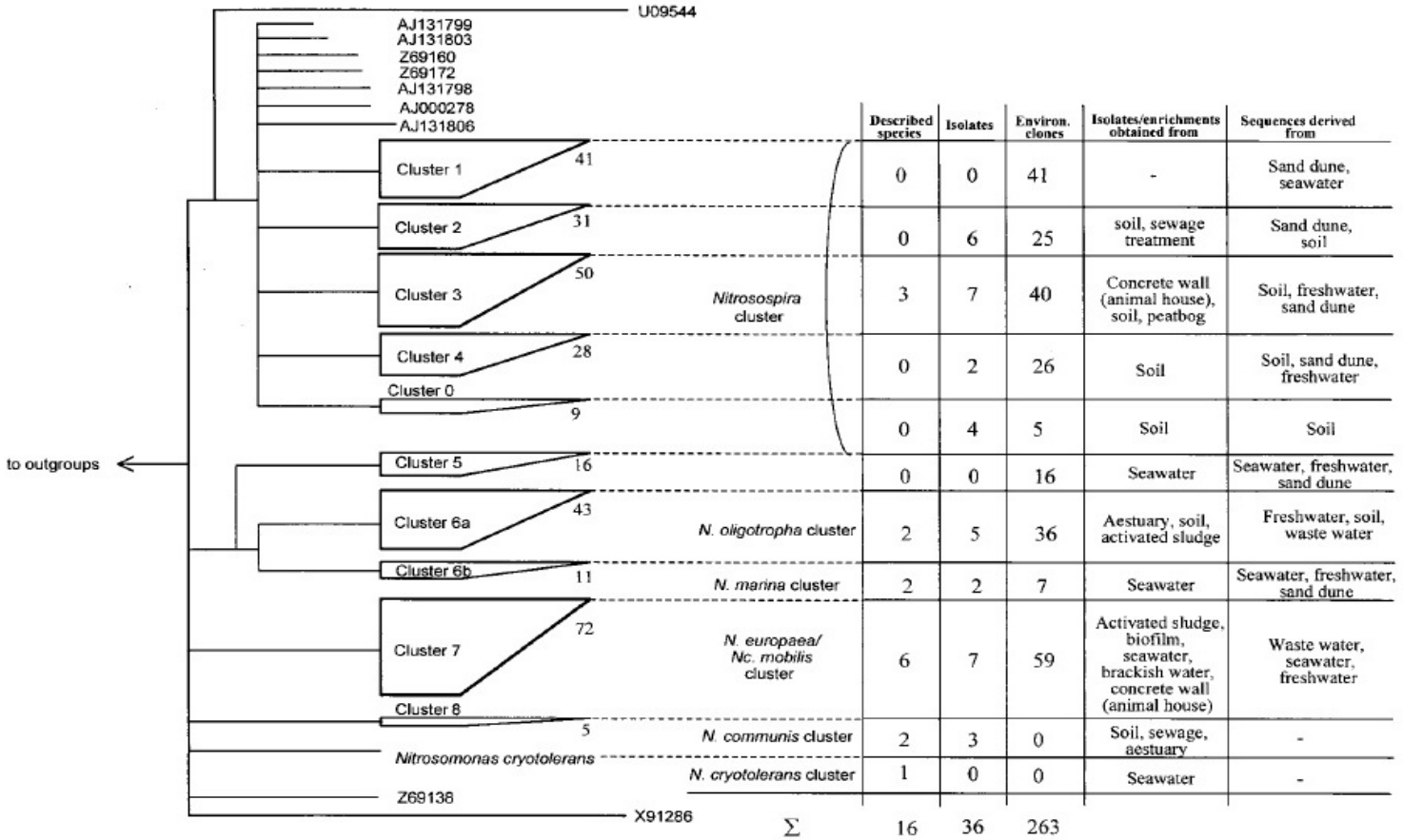


Figure 2.8: Phylogenetic tree depicting the relationship among the two subdivisions of cultured ammonia-oxidizing *Proteobacteria*. Diagram adapted from Koops and Pommerening-Röser (2001).

While the γ-Proteobacteria AOB is only represented by two species in a single genus (Figure 2.8), *Nitrosococcus*, β-*Proteobacteria* AOB are much more diverse. The *Nitrosospira* genus can be further subdivided into five clusters, while the *Nitrosomonas* genus can be divided into six distinct lineages (Figure 2.9). *Nitrosococcus mobilis* clustered together with the *Nitrosomonas* genus and has been renamed *Nitrosomonas mobilis* (Koops and Pommerening-Röser 2001). Similarly, *Nitrosolobus multiformis* and *Nitrosovibrio tenuis* are now also classified as belonging to the *Nitrosospira* lineage (Purkhold et al., 2003). Recently, Dang et al. (2010b) proposed three new clusters belonging to the *Nitrosospira* lineage and one new cluster of the *Nitrosomonas* lineage based

on their culture independent study of beta subdivision AOB from a coastal bay site.

To date, the most easily isolated and cultured ammonia-oxidizing species is *Nitrosomonas europea* (Kowalchuk et al., 1997), but numerous strains of *Nitrosospira* spp. have also been cultured (Shaw et al., 2006). While AOBs are known to possibly inhabit almost all aerobic environments in which organic matter is mineralized (Purkhold et al., 2003), they have been refractory to conventional culture and isolation techniques due to the extremely slow growth rate and long generation time (8 hours to a few days) of the organism (Kowalchuk et al., 1997; Urakawa et al., 2006). Hence, cultivation-dependent analysis have been deemed to be too time consuming (Rotthauwe et al., 1997) and often lead to significantly underestimated cell counts (Cebron et al., 2003).



	Described species	Isolates	Environ. clones	Isolates/enrichments obtained from	Sequences derived from
Cluster 1	0	0	41	-	Sand dune, seawater
Cluster 2	0	6	25	soil, sewage treatment	Sand dune, soil
Cluster 3	3	7	40	Concrete wall (animal house), soil, peatbog	Soil, freshwater, sand dune
Cluster 4	0	2	26	Soil	Soil, sand dune, freshwater
Cluster 0	0	4	5	Soil	Soil
Cluster 5	0	0	16	Seawater	Seawater, freshwater, sand dune
Cluster 6a	2	5	36	Aestuary, soil, activated sludge	Freshwater, soil, waste water
Cluster 6b	2	2	7	Seawater	Seawater, freshwater, sand dune
Cluster 7	6	7	59	Activated sludge, biofilm, seawater, brackish water, concrete wall (animal house)	Waste water, seawater, freshwater
Cluster 8	2	3	0	Soil, sewage, aestuary	-
Nitrosomonas cryotolerans	1	0	0	Seawater	-
Z69138					
X91286					
Σ	16	36	263		

Figure 2.9: Schematic classification of the β-subdivision AOB and their main isolation sites (Purkhold et al., 2000)

2.6.2 Phylogeny of Methane Oxidizing Bacteria

The methanotrophs have been categorized into two main groups, Type I and Type II (Lüke et al., 2010). Type I methanotrophs can be further sub-grouped into Type Ia and Type Ib (Type X) methanotrophs. All Type I methanotrophs fall under the γ-subdivision *Proteobacteria*, where Type Ia methanotrophs comprise the genera: *Methylomonas*, *Methylobacter*, *Methylosoma*, Methylosarcina and *Methylomicrobium*, while the Type Ib methanotrophs consist of the genera *Methylococcus* and *Methylocaldum* (Lüke et al., 2010). Type II methanotrophs fall under the α-subdivision of *Proteobacteria* and consist of only 4 genera: *Methylocystis*, *Methylosinus*, *Methylocella*, and *Methylocapsa* (McDonald et al., 2007) (Figure 2.10).

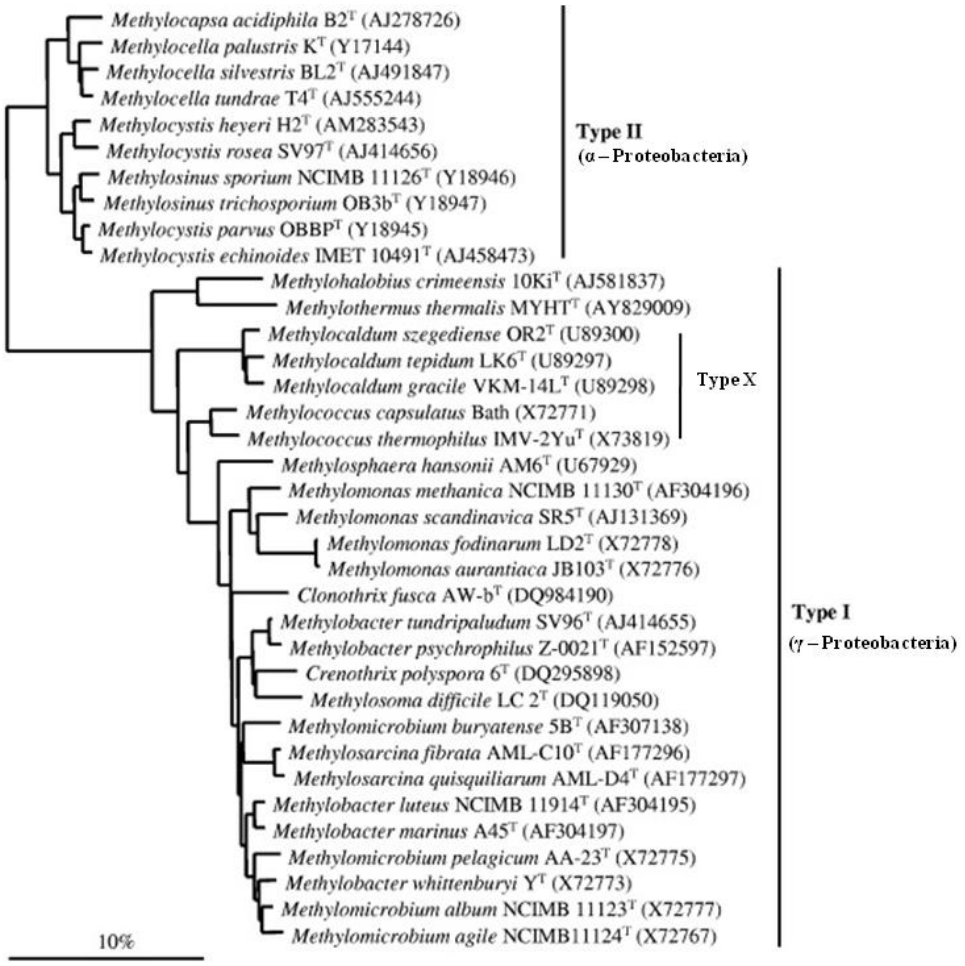


Figure 2.10: 16S rDNA phylogenetic tree of the type strains of methanotrophs. Diagram modified from McDonald et al. (2007).

Besides their phylogenetic groupings, Type I and type II methanotrophs also defer in characteristics such as intracytoplasmic membrane ultrastructure, enzymatic characteristics, fatty acid carbon chain length, and G + C values (moles percentage) (Table 2.3). Additionally, Type 1b (formerly known as Type X) methanotrophs have been further classified as a separate subgroup of Type I methanotrophs due to their unique ability of being able to withstand and grow at temperatures as high as 45°C (Hanson and Hanson 1996).

Table 2.3: Characteristics of the three Methanotroph Groups. Adapted from Hanson and Hanson (1996).

Characteristics	Type I Methanotrophs	Type X Methanotrophs	Type II Methanotrophs
Family	*Methylococcaceae*		*Methylocystaceae*
Phylogenetic group	Gamma	Gamma	Alpha
Member genera	*Methylobacter* *Methylomicrobium* *Methylosphaera* *Methylothermus* *Methylosarcina* *Methylohalobius* *Methylosoma*	*Methylococcus* *Methylocaldum*	*Methylocystis* *Methylosinus* *Methylocella* *Methylocapsa*
Resting stages	*Azotobacter*-type cysts (or none)	*Azotobacter*-type cysts	Exospores or lipoidal cysts
Intracytoplasmic membranes	Type I	Type I	Type II
Soluble methane monooxygenase (sMMO)	None (except some strains of *Methylococcus* and *Methylomonas*)		Present
Carbon assimilation pathway	RuMP	RuMP	Serine
Benson-Calvin cycle enzymes	Absent	Present	Absent
Major fatty acid carbon chain length	16	16	18
Major quinone	Q-8 or MQ-8	MQ-8	Q-10
Mol% G+C (Tm)	43–60	56–65	60–67
Growth at 45°C	No	Yes	No

2.7 Factors Influencing the Diversity of Ammonia Oxidizers and Methanotrophs

In non-marine environments, ammonia oxidizers have been detected from diverse environments ranging from terrestrial soils, sewage and activated sludge (Purkhold et al., 2000), freshwater bodies and sediments (Kowalchuk and Stephen 2001), aquaculture filter material (Itoi et al., 2006), paddy fields (Rotthauwe et al., 1997; Briones et al., 2002; Wang et al., 2009; Fujii et al., 2010), mangrove sediments (Li et al., 2011; Cao et al., 2011c) and concrete walls (Kowalchuk and Stephen 2001). The diversity and community structure of ammonia-oxidizers are affected by environmental physicochemical parameters such as pH, temperature, substrate and oxygen concentration, and salinity (Coci et al., 2008). As mentioned above, since pH affects the availability of ionized and non-ionized nitrogen present, it in turn affects substrate availability to the ammonia oxidizers. In laboratory conditions, ammonia oxidizers grow optimally at a pH range of 5.8 to 8.5 (Prinčič et al., 1998). Low environmental temperature seems to decrease the diversity of the ammonia-oxidizing community (Belser 1979).

Oxygen concentration and substrate (ammonia) concentration are thought to play major roles towards rate of nitrification and hence also influence the community structure of ammonia-oxidizers (Prinčič et al., 1998). While several studies have shown that environmental oxygen concentration do not select towards a particular group of ammonia oxidizers (Kowalchuk et al., 1998), environments with increased oxygen levels seem to have a higher diversity (Briones et al., 2002) and number (Bodelier et al., 1996) of

ammonia-oxidizers. On the other hand, substrate concentration is seen to directly affect the distribution of ammonia-oxidation in an environment due to the physiological differences between different clades of ammonia oxidizers (Bollmann et al., 2002). Enzyme-substrate affinity (K_m value) vary between different genera of ammonia-oxidizers, which in turn affect their competition for limiting amount of growth resources in the natural environment. With the exception of lineage 6a of the *Nitrosomonas* genus (including *Nitrosomonas oligotropha* and *Nitrosomonas ureae*), most of the *Nitrosomonas*-like ammonia oxidizers particularly *Nitrosomonas europaea*, have low substrate affinities and are poor ammonium competitors (Bollmann and Laanbroek 2001); hence they are more commonly isolated from environments rich in nitrogen such as wastewater (Bollmann et al., 2002). *Nitrosospira* sp. and cluster 6a *Nitrosomonas* sp. have higher growth substrate affinities (low K_m values), and are therefore more competitive in low nitrogen environments such as freshwater lakes (Cebron et al., 2003). Essentially, substrate concentration variations will usually cause a shift in ammonia-oxidizer community to take advantage of the in-situ substrate concentration (Chen et al., 2009).

Due to their obligately halophilic nature, the γ-subdivision ammonia-oxidizers (*Nitrosococcus oceani* and *Nitrosococcus halophilus*) have only been detected from marine environments. The *Nitrosomonas marina* and *Nitrosomonas cryotolerans* lineage β-subdivision ammonia oxidizers are also obligately halophilic and therefore also only found in oceans and marine habitats (Koops and Pommerening-Röser 2001).

Similarly, a wide variety of factors seem to influence the diversity & abundance of methanotrophs, including but not limited to key factors such as pH (Dunfield 2003), temperature, soil nutrient content, oxygen and combined nitrogen availability (Bodelier and Laanbroek 2004; Han et al., 2009; Nyerges et al., 2010). Methanotrophs are a considerably well studied microorganism that has been detected and isolated in diverse environments. Since methanotrophs play an integral role in oxidizing methane naturally produced in the environment, it would naturally thrive in environments rich in methane, such as natural wetlands (a major source of atmospheric methane), vegetated wetlands, paddy fields, coal mines, soils, sediments and sewage treatment plants (Hanson and Hanson 1996; Heyer et al., 2002; McDonald et al., 2007).

In soils, sediments, and many natural environments, type II methanotrophs seem to be more abundantly and frequently detected (Henckel et al., 1999; Heyer et al., 2002), particularly using molecular methods, as compared to type I methanotrophs although type I methanotrophs are more phylogenetically diverse. It has been proposed that the growth of Type I methanotrophs is generally more favoured in environments with low methane, high oxygen, high nitrogen and copper conditions (Amaral and Knowles 1995). This is probable because type II and type X methanotrophs are capable of nitrogen fixation (Hanson 1980), while most type I methanotrophs only harbour pMMO which require higher levels of copper for its expression while lacking the genetic ability for sMMO synthesis (Graham et al., 1993).

2.8 Ammonia-Oxidizers & Methanotrophs in Environments with Aquatic Macrophytes

In freshwater bodies and lakes, the presence of aquatic macrophytes presents a huge impact on the trophic status and nutrient content of the water body. They are a common part of the ecosystem and natural regeneration processes. As AOB, MOB and many bacteria are known to grow both free-living and attached to surfaces, aquatic macrophytes provide a suitable niche for the propagation of AOB and MOB (Coci et al., 2008). The association between aquatic plants and microbes occur mainly at the rhizosphere, the narrow region of water body/aquatic sediments surrounding the plant roots (Stout and Nusslein 2010). Organic matter is released from plant exudates or root decomposition, and oxygen transported through the aerenchyma tissues of many wetland plants seeps out from respiring roots. Both factors above make the environment highly suitable to be inhabited by diverse types of microbes, considering many microbial processes, including ammonia and methane oxidation, are aerobic processes (Christensen et al., 1994; Bodelier et al., 1996) while the region of soil or sediment under water are usually anoxic (Bodelier et al., 1996). In addition, varying plant species have different level of oxygen release and nitrogen requirements which will select for and affect the community composition of AOB and MOB (Briones et al., 2002).

2.8.1 Diversity of the AOB Community in the Paddy Fields and Ponds with Aquatic Macrophytes

In paddy fields, ammonia-oxidizers play a crucial role in the regulation of nitrogen supply, which in turn influences crop yield (Briones et al., 2002; Nicolaisen et al., 2004). As mentioned above, the oxygen supply from wetland plants create a suitable environment for ammonia oxidation as bulk wetland sediments are usually anoxic (Briones et al., 2002). Molecular ecological approaches have shown that both ammonia oxidizers from the *Nitrosomonas* sp. and *Nitrosospira* sp. genus were detected from rice field environments (Briones et al., 2002; Fujii et al., 2010), though *Nitrosospira* sp. seems to be the predominant ammonia-oxidizer present at root regions of rice plants (Rotthauwe et al., 1997; Briones et al., 2002; Ikenaga et al., 2003). Investigations of the ammonia-oxidizer community inhabiting the bulk soil of paddy field, however, showed that it was dependent mainly on the trophic status (Nitrogen load) of the pond and soil depth (oxygen availability). Both the studies conducted by Nicolaisen et al. (2004) and Wang et al. (2009) found that in the bulk soil of nitrogen fertilized paddy fields, *Nitrosomonas communis* affiliated ammonia oxidizers were dominant, though Wang et al. (2009) also detected cluster 3 *Nitrosospira* sp., with numbers that correlated inversely with nitrogen (fertilizer) load. *Nitrosomonas* sp. affiliated ammonia-oxidizers were more abundant at the oxic layers of the sediment (Wang et al., 2009). Bowatte et al. (2006), on the other hand, detected only *Nitrosospira* spp. ammonia-oxidizers at the surface soil of rice fields.

Ponds vegetated with other aquatic macrophytes were also found to support the nitrification process through root oxygen release (Bodelier et al., 1996; Ottosen et al., 1999). In eutrophic ponds and artificial wetland systems, aquatic macrophytes (and their associated microorganisms) remove significant amounts of nitrogen and reduce algal bloom (Wei et al., 2011), while in oligotrophic ponds, plant-AOB interaction restored nitrogen loss from rhizosphere denitrification and promote plant succession. Both Coci et al. (2008) and Herrmann et al. (2009) found that *Nitrosomonas* spp. and *Nitrosospira* spp. associated ammonia oxidizers inhabited freshwater (oligotrophic to mesotrophic) ponds with varying species of submerged macrophytes. However, Herrmann et al. (2009) found no variation in the AOB diversity between lake compartments (benthic/pelagic regions) or between vegetated and unvegetated sediments. Further, the rhizosphere of floating macrophytes, such as those of *Eichhornia crassipes* (Common Water Hyacinth), and the one involved in our study, *Nelumbo nucifera* (Indian lotus) could be capable of increased oxygen transport rate which might promote ammonia oxidation (Moorhead and Reddy 1988).

2.8.2 Diversity of the MOB Community in the Paddy Fields and Ponds with Aquatic Macrophytes

Much study have been focused on the methanotrophs in paddy fields (Bodelier and Frenzel 1999; Henckel et al., 1999; Henckel et al., 2000; Niswati et al., 2004; Takeda et al., 2008). It is an economically important crop, and also a source of the greenhouse gas methane that present a complex ecosystem and microenvironment with a variation of methane and oxygen concentration at different parts of the plant or during varying crop seasons (van Bodegom et al., 2001; Asakawa and Kimura 2008).

Both type I and type II methanotrophs have been detected in paddy fields (Henckel et al., 1999; Jia et al., 2007), but type I methanotrophs seem to be more abundant at the rice roots (Horz et al., 2001; Wu et al., 2009), while type II methanotrophs are more frequently detected in bulk soil and regions nearing the root compartment (rhizosphere) (Horz et al., 2001). Eller and Frenzel (2001) detected both type I and type II methanotrophs at the bulk soil and rhizosphere, though type II methanotrophs were dominant and stably detected throughout the study period and type I methanotrophs were only present at high numbers at the rhizosphere. The study of methanotrophs in aquatic environments associated with several other aquatic macrophytes (*Pontederia cordata, Sparganium eurycarpum, Sagittaria latifolia*) also found both group I and group II methanotrophs, but similarly, group II methane-oxidizers were more frequently detected (King 1994; Calhoun and King 1998).

2.9 Ammonia Oxidizers in Aquaculture Environments

Idle freshwater ponds and water bodies such as abandoned mining pools are commonly known to be used for aquaculture activity. In a similar case to aquatic macrophytes, activities such as aquaculture will also alter the trophic and nutrient status of ponds. Aquaculture system environments generally have a higher nitrogen load due to ammonia directly excreted by fish and crustaceans in their faeces and from the decomposition of unconsumed feed and dead organisms (Carman 1994). The accumulation of ammonia and ammonium is toxic to fishes, and hence the presence of nitrifying bacteria is crucial to oxidize the excess ammonium to nitrite (Crab et al., 2007).

Itoi et al. (2006), in their molecular study of microbial communities inhabiting a freshwater recirculating aquaculture tank, detected *Nitrosomonas oligotropha* and *Nitrosomonas* Nm143 affiliated ammonia oxidizers. Both *Nitrosospira* sp. and *Nitrosomonas* sp. related AOB were detected at a marine fish farm sediment (McCaig et al., 1999).

2.10 Use of AOB & MOB in Bioremediation and the Industry

Since ammonia oxidizing bacteria is directly involved in the rate limiting step of autotrophic nitrification, it is traditionally used in the process of biological nitrogen removal at environments such as bioreactors (You and Chen 2008), aquaculture wastes (Chavez-Crooker and Obreque-Contreras 2010), and wastewater treatment plants (Abd El Haleem et al., 2000). Additionally, the ammonia monooxygenase enzyme complex is known to have broad enzyme specificity, and was found to have the ability to co-oxidize a wide variety of

hydrocarbon substrates including alkanes, alkenes, and aromatic, aliphatic and halogenated compounds (Hyman et al., 1988; Keener and Arp 1994; Hooper et al., 1997). As such, waste treatment has been one of the major biotechnological applications of AOB (Kowalchuk and Stephen 2001).

Methanotrophs have gained the attention of many researchers due to the fact that they play a major role in controlling the emission of methane, an important greenhouse gas, in the environment (Watanabe 2001). Nonetheless, the broad specificity of MMO and its ability to co-metabolise many other substrates has made it a promising tool in biotechnological and commercial applications. Both pMMO and sMMO have a wide specificity of substrates, but sMMO seem to have a broader substrate specificity (Lee et al., 2006) and higher rate of substrate oxidation (Tsien et al., 1989; DiSpirito et al., 1992). Numerous research works has focused on the biodegradation of trichloroethylene (TCE) by methanotrophs (Tsien et al., 1989; Koh et al., 1993; McDonald et al., 1997 ; Shukla et al., 2009; Shukla et al., 2010), due to the detrimental effects and frequent presence of TCE in groundwaters, but methanotrophs are also known to be able to degrade other harmful and toxic pollutants such as alkanes, alkenes, ethers, and alicyclic, aromatic and heterocyclic compounds (Colby et al., 1977). However, methanotrophs harbouring only pMMO did not seem to co-oxidize aromatic and alicyclic compounds (Burrows et al., 1984). Most recently, methanotrophs were also found to bioremediate chromium (VI) pollution (Al Hasin et al., 2010). Methanotrophs also have the commercial potential in the production of low volume and bulk chemicals such as propylene oxide (Cardy et al., 1991).

Clearly, both AOB and methanotrophs have an immense potential in many commercial and biotechnological applications, particularly for bioremediation and environmental restoration (Jiang et al., 2010).

2.11 Molecular Methods for the Environmental Detection of Microbes

Microbial diversity is a field of study that has been garnering increased interest due to its impact on global nutrient, biogeochemical cycles and climatic changes (Dorigo et al., 2005), and also the significant role it plays in the removal of pollutants and bioremediation (Watanabe 2001). Soil environments, in particular, harbour a large community of bacteria, many of which play major roles in the cycling of organic and inorganic compounds sustaining an ecosystem (Nehl and Knox 2006). Yet, methods previously used for detection and biodiversity studies of microorganism such as culture dependent microbiological methods have only enabled a very small fraction of microbial diversity to be assessed, due mainly to the relatively small size of microorganism, lack of discriminating (phenotypic) characteristics and particularly their inability to be cultured (Torsvik and Øvreås 2002). The development of molecular, non-cultivation methods such as polymerase chain reaction (PCR), fluorescence in-situ hybridization (FISH) and several other PCR based methods has enabled a more thorough and accurate study of bacterial diversity and have certainly aided in the detection and analysis of bacteria in natural samples from many complex environments such as soil, sediments and water (Voytek and Ward 1995; Amann et al., 1995).

2.11.1 16S rRNA Gene in the Phylogenetic Investigation of Bacteria

Ribosomal RNAs are at present the most useful and commonly used molecular chronometers (Woese 1987). They are ubiquitous in all cellular life forms (Head et al., 1998) since they are the primordial participants of the cell protein production machinery (Pontes et al., 2007), besides having sequence domains that are highly conserved between organisms which are phylogenetically distant (Head et al., 1998; Pontes et al., 2007). More specifically, rRNAs are mosaics of sequence stretches which range from conserved to more variable, but yet are functionally highly constrained (Acinas et al., 2004). The rRNA gene (rDNA) is also hardly affected by horizontal gene transfer (HGT) as HGT has been found to less likely affect informational genes (e.g. rDNAs) (Jain et al., 1999). A combination of these aspects make rRNAs (and rDNAs) uniquely suited for varying applications.

The 5S, 16S and 23S rDNA are typically arranged in one common operon, containing internal transcribed spacers that vary widely in length and sequence. Spacer regions located between the 16S and 23S rDNA are known to have more genetic variation due to differences in length and number of tRNA genes available in the sequences (Acinas et al., 2004; Pontes et al., 2007). For bacteria and prokaryotes in general, the small subunit 16S rRNA gene have become the standard of determination to infer relationships between organisms, gauging the diversity in an environment and the detection of a specific population of interest in an environment (Head et al., 1998). This can be attributed to the suitable size of the 16S rDNA (which is approximately 1500 bp) and the ease of manipulation of this gene. The 5S rDNA have been

excluded in most studies due to its relatively small size (approximately 120 bp) (Amann et al., 1995). Most importantly, the rDNA mosaic sequence range allows distinguishing phylogenies in the varied segment of the sequence while universal or near universal sequence for PCR primer binding can be achieved in the conserved segment (Frank et al., 2008).

To date, bacterial 16S rRNA genes have been identified to having nine hypervariable regions, termed V1 – V9, respectively (Figure 2.11). The hypervariable regions are not continuous, but instead interspersed in between by conserved stretches in most bacteria species (Van de Peer et al., 1996). The V2 (nucleotides 137 – 242 in the *Escherichia coli* rRNA coordinates), V3 (nucleotides 433 – 497) and V6 regions (nucleotides 986–1043) were found to contain the highest amount of nucleotide heterogeneity, and hence provides maximal discrimitory power amongst bacterial species. Region V5 contained a higher degree of sequence conservation and was thus deemed less suitable for species identification (Chakravorty et al., 2007) .

There are 2 main approaches commonly used to narrow down on the identification of AOBs. The first targets the 16S rRNA of betaproteobacterial ammonia oxidizers. This approach is possible due to the fact that most ammonia oxidizers, with the exception of a few marine gammaproteobacterial AOBs, fall within the monophyletic group of the *Betaproteobacteria* (Mahmood et al., 2006). The primers CTO189f (forward) and CTO654r (reverse) (Kowalchuk et al., 1997) have been specially designed to enable the amplification of a 465 bp fragment of the 16S rDNA region spanning the V-2

and V-3 variable domains from beta subdivision AOBs, while still being specific enough to exclude other non-ammonia oxidizers (Mahmood et al., 2006). It has been used widely in studies of different environments such as freshwater lakes (Coci et al., 2008), hydrocarbon polluted soil (Deni and Penninckx 1999), dune soil (Kowalchuk et al., 1997) and compost (Kowalchuk et al., 1999).

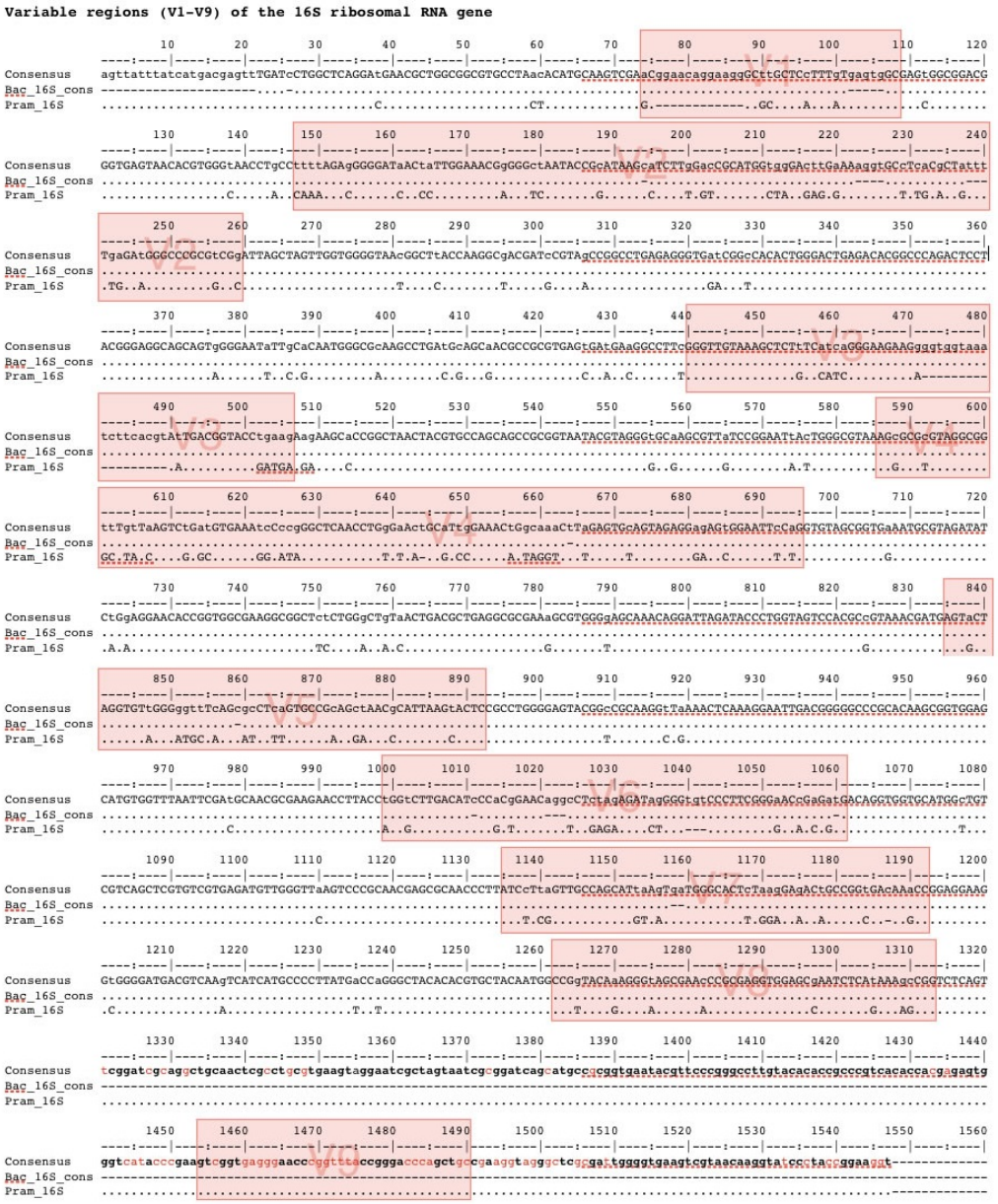


Figure 2.11: The nine hyper-variable regions of the bacteria 16S rRNA gene spanned nucleotides 69 – 99, 137 – 242, 433 – 497, 576 – 682, 822 – 879, 986 – 1043, 1117 – 1173, 1243 – 1294, and 1435 – 1465 for V1 through V9, respectively. Numbering is based on the *E. coli* system of nomenclature (Brosius et al., 1978; TheWalserGroup 2012).

2.11.2 The *amo*A Gene and its Significance as a Function Specific Marker

The second approach commonly used in the identification of AOBs target the *amo*A gene, which is a function specific marker gene that can only be found in AOBs. As mentioned above, the enzyme ammonia monooxygenase (AMO), synthesized by AOBs, is composed of 3 subunits: AmoA, a membrane bound protein containing the active site of the enzyme and encoded by the *amo*A gene, AmoB and AmoC (encoded by the *amo*B and *amo*C genes, respectively). Primers targeting the *amo*A gene has the advantage of confirming the presence of species undetected by the primers targeting the 16S rRNA gene due to the reduced specificity of 16S rDNA primers (Chen et al., 2009), and guarantees the enclosure of the whole physiological group of ammonia oxidizing bacteria (Calvó and Garcia-Gil 2004). Many studies have successfully detected AOBs in varying environments such as rice fields (Jia et al., 2007), seawater (Sinigalliano et al., 1995), lakes (Chen et al., 2009), and activated sludge (Juretschko et al., 1998) by targeting the *amo*A gene. The commonly used primers targeting the *amo*A gene would be the forward primer Amo-1F, targeting a stretch corresponding to positions 332 to 349, and the reverse primer Amo-2R, targeting a stretch corresponding to positions 802 to 822 of the open reading frame published previously for the *amo*A gene sequence of *Nitrosomonas europaea* (Rotthauwe et al., 1997). Both primers together generate a 491 bp fragment in PCR amplification.

2.11.3 The *pmo*A Gene as a Function Specific Marker to detect MOB

Similar to the detection of AOB, MOB can also be detected by using primers targeting housekeeping genes (16S rDNA) and function specific marker genes.

Using function specific genes for diversity studies enables higher detection sensitivity in complex ecosystems, and also has the advantage over the 16S rRNA gene which provides little information on organism physiology when novel sequences are detected (McDonald et al., 2007). The two unique function specific genes most commonly used in ecological diversity studies of MOB are the *pmo*A and *mmo*X genes, which, as mentioned above, are part of the gene operons encoding the pMMO and sMMO enzymes. Primers targeting the *pmo*A gene are more frequently used since the *pmo*A gene is found in almost all methanotrophs with the exception of the *Methylocella* genus (Theisen et al., 2005). On the contrary, the *mmo*X gene is limited to only a subset of methanotrophs, and the primers designed from the limited *mmo*X database would usually causes a bias in the detected sequences towards known existing types of methanotrophs. Given that the phylogenetic trees constructed based on function specific genes such as the *pmo*A have been shown to be reasonably congruent to 16S rDNA gene based phylogenetic trees, they have been widely used in studying the diversity of methanotrophs (Heyer et al., 2002; McDonald et al., 2007).

The original *pmo*A targeting primers that were extensively used in phylogenetic studies were the A189f /A682r primer pair. However, given the high level of similarity of the *amo*A and *pmo*A genes, the primer designed were non-specific and detected both MOB as well as AOB (Holmes et al., 1995). The mb661r (Costello and Lidstrom 1999) and A650r (Bourne et al., 2001) reverse primers were developed later on and used in conjunction with

the A189f forward primer, but further studies revealed the mb661 primer to give a better coverage in diversity studies (Bourne et al., 2001).

2.12 Quantitative Analyses of Microbial Diversity

The analysis of microbial diversity through the use of housekeeping (16S rRNA) and functional gene libraries has aided in a more thorough and accurate insight into microbial ecology from many complex environments as well as to address many key questions about the environmental factors controlling microbial diversity, distribution, and function of the ecosystem (Amann et al., 1995; Lozupone et al., 2007). However, obtaining a vast inventory of sequences from varying microbial communities would remain insignificant and impractical unless appropriate analytical methods are used in the measurement and assessment of diversity within the microbial communities in these environments (Hughes et al., 2001). Measuring microbial diversity within (α-diversity) and between (β-diversity) communities using species as the fundamental unit of analysis are two important critical parameters that contribute towards better understanding the microbial community structure and dynamics (Cohan 2002; Lozupone and Knight 2008), and the significance of diversity is often inferred to by the comparison of communities identified from varying environments (Martin 2002).

Microbial α-diversity is usually expressed by statistical indices in terms of species richness, the total number of species; species evenness, the relative abundance of the species, or statistical indices that are a combination of both species richness and evenness (Lozupone and Knight 2008). Indices that

encompass only species richness are considered to be a qualitative α-diversity, where only the presence or absence of the species is of concern, and some of popular indices are the Chao1 (Chao 1984), ACE (Chazdon et al., 1998) and rarefaction analysis (Heck et al., 1975). When the α-diversity measurements jointly considers the presence and frequency of observed species, and characterizes the species evenness component or a combination of both evenness and richness, it is categorized as a quantitative α-diversity. The Shannon-Weaver's index (Shannon and Weaver 1949) and the Simpson's diversity index (Simpson 1949) are two popular measurements of quantitative α-diversity.

More recently, the measurement of β-diversity amongst the microbial community has also been receiving more attention (Francis et al., 2003; Dang et al., 2009; Herrmann et al., 2009; Dang et al., 2010b; Cao et al., 2011c) as it is capable of evaluating differences between two or more local assemblages or between local and regional assemblages (Koleff et al., 2003). the microbial β-diversity can also be broadly categorized into qualitative and quantitative, where species-based qualitative β-diversity indices include the Sörensen index and Jaccard Index (Magurran 2004), while the species-based quantitative β-diversity include the Sörensen quantitative index and the Morista-Horn measure (Horn 1966). Newer divergence-based β-diversity, such as UniFrac (Lozupone and Knight 2005) and the Dissimilarity indexes (F_{ST}) (Reynolds et al., 1983), rely on phylogenetic distance measures which considers the degree of divergence between different sequence, and have been argued to be more suited to the evaluation of microbial communities due to the wider

evolutionary divergence between microbial populations (Lozupone and Knight 2008).

2.13 Past Researches at Malaysian Disused Tin-Mining Sites

Studies pertaining to the disused tin-mining ponds in Malaysia are scarce despite the abundance of these sites within our country. To date, most of the research only focus on the physicochemical and mineralogical aspects of disused mining land and tin tailings within Malaysia (Majid et al., 1994; Ang 1994; Awang 1994; Ang and Ho 2002; Alshaebi et al., 2009). A few studies have been done on the physicochemical properties, water quality as well as floral and aquatic organism community inhabiting selected disused tin-mining pools within Malaysia (Arumugam 1994; Abdul-Rashid and Awang 2004; Ashraf et al., 2010; Ashraf et al., 2011). Ashraf et al. (2011) focused on the heavy metal absorption and accumulation by plants inhabiting disused tin-mining sites and pools. For the disused tin-mining ponds located within the vicinity of UTAR campus, Kampar, only two previous studies have been conducted, focusing on the fish and shrimp species inhabiting these sites (Ng 2011), as well as antibiotic, antimicrobial and enzymatic activities of bacteria isolated via non-molecular methods (Ong et al., 2011). Given the importance of the role played by ammonia oxidizing bacteria and methane oxidizing bacteria towards the nutrient cycling and biological productivity within aquatic ecosystems (Strauss and Lamberti 2000), the characterization of these two bacteria within disused tin-mining pools would provide further indications on the functioning and interaction of the ecosystem within disused tin-mining pools, which might lead towards the better development, use or preservation

of these aquatic sites which possess much potential economic, conservatory and research value.

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of Apparatus and Materials Used

3.1.1 Preparation of Glassware and Plasticware

Prior to commencement of bench work and preparation of reagents and media, all glassware and plasticware used in media and reagent preparation, and apparatus used for subsequent extraction and cloning procedures were cleaned and rinsed with distilled water (with the exception of new, disposable apparatus), then sterilized by autoclaving at 15 psi (1.05 kg/cm^2) on a liquid cycle for 15 – 20 minutes and dried in an incubator set to 70°C before use. The complete list of apparatus and machinery used, and their respective manufacturers are listed in Appendix A.

3.1.2 Preparation of Buffers and Chemical Reagents

After proper mixing of the appropriate components of a buffer or reagent, the prepared reagents/buffers (autoclavable) were autoclaved at 15 psi (1.05 kg/cm^2) on a liquid cycle for 15 – 20 minutes before use in subsequent experimental procedures. Listing of chemicals together with their respective manufacturing companies, and compositions of buffers and other miscellaneous reagents or solutions used can be found in Appendix A.

3.1.3 Preparation of Media for Bacterial Cultivation

Cultivation of *E. coli* cells for the cloning of the *amo*A and *pmo*A gene was achieved using Luria-Bertani (LB) agar and broth (prepared), of which the composition is listed in Appendix B.

All prepared media was autoclaved for 15 – 20 minutes at 15 psi (1.05 kg/cm^2) on liquid cycle prior to use. Autoclaved LB agar was cooled to approximately 60°C before being divided accordingly to Petri plates. Preparation of agar plates and solidification of agar was done in a laminar air flow to ensure sterility of the prepared media. Solidified media were stored at 4°C prior to further use.

3.2 Sampling Site Description

The Universiti Tunku Abdul Rahman (Kampar, Perak campus) was built on a 1300-acre land surrounded by approximately 14 disused tin-mining ponds with depths of 10 meters or more, northeast of the town of Kampar, Perak, Malaysia. Among the ponds surrounding the university, two are post aquaculture disused tin-mining ponds, one has been cultured with the Indian lotus (*Nelumbo nucifera*) (lotus pond), and the rest have been left idle as oligotrophic ponds with low primary productivity (idle pond). The sites were L1 & L2 from the ponds with lotus, F1, F2 and F3 from the post aquaculture ponds, and I1 and I2 from the idle pond, as shown in the Figure 3.1. Illustrations of the actual fish pond, lotus pond, and idle pond are shown in Figure 3.2, Figure 3.3, and Figure 3.4 respectively.

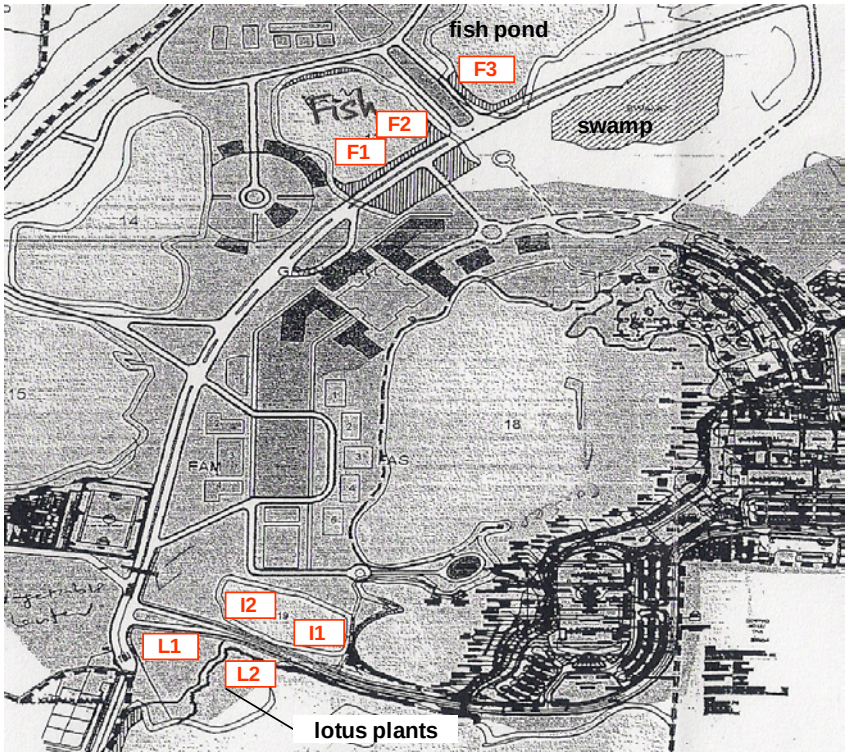


Figure 3.1: Map of UTAR, Kampar, Perak and the surrounding Disused Tin Mining Ponds, showing the location of the sampling sites

Figure 3.2: Sampling Site One – Disused tin-mining pond with post aquaculture activity (Referred to as Post Aquaculture Pond in this thesis)

Figure 3.3: Sampling Site Two – Disused tin-mining pond with cultivation of lotus (Referred to as Lotus Pond in this thesis)

Figure 3.4: Sampling Site Three – Untouched, mesotrophic disused tin-mining pond with low primary productivity (Referred to as Idle Pond in this thesis).

3.3 Sample Collection

Plastic containers and bottles used for the collection of the pond sediment and water samples were rinsed three times with 70% ethanol to sterilized, and then further rinsed three times with distilled deionised water to remove any traces of alcohol.

Samples were collected on the 23rd of July, 2010 at approximately 9.00 to 10.00 a.m. in the morning. Before the water samples were collected, temperature, pH, salinity and dissolved oxygen was measured *in situ* using a pre-calibrated HI 9828 Multiparameter Water Quality Portable Meter (Hanna Instruments, United States).

Two litres of water samples were collected in a sterile sampling container from the surface layer (0.5 m – 1 m depth) within the littoral zone of each pond type, and water samples were collected prior to the sediment samples to reduce the resuspension of sediment particles. Surface sediments (1 – 3 cm) were collected from the littoral zone of each pond type. All samples were transported to the laboratory on ice within one hour of collection. Approximately 1.5 litres of the water samples were filtered through pre-washed 0.2 µm mixed cellulose ester membrane filters (Advantec, Japan). Sediment samples and the filter membranes containing suspended residual water particles were each stored separately at -80°C until DNA extraction procedures were carried out. The remaining 500 mL of water samples were used in the physicochemical analysis of nitrate (NO_3^-), nitrite (NO_2^-), total

ammonia nitrogen (NH_3), sulfate (SO_4^{2-}), suspended solids and turbidity of the water samples.

3.4 Physicochemical Analysis

Ten parameters were taken into consideration when testing for the physicochemical properties of the water sampled from the disused tin-mining ponds, including temperature, pH, dissolved oxygen (DO), salinity, suspended solids, turbidity, nitrate (NO_3^-), nitrite (NO_2^-), total ammonia nitrogen (TAN, NH_3), and sulphate (SO_4^{2-}). pH, DO and salinity were measured *in situ* as mentioned in Section 3.3. The remaining parameters were measured using the DR/890 Colorimeter (Hach, United States) and methods used in the measurement of each physicochemical parameter can be summarized in table below:

Table 3.1: List of Physicochemical Parameters Used And the Respective Methods

No.	Parameter	Name of Method	Method No.
1	Nitrate, NO_3^-	Cadmium reduction method	8171
2	Nitrite, NO_2^-	Diazotization method	8507
3	TAN, NH_3	Salicylate method	8155
4	Phosphate , PO_4^{3-}	Ascorbic Acid method	8048
5	Sulphate, SO_4^{2-}	SulfaVer 4 method	8051
6	Suspended Solids	Photometric method	8006
7	Turbidity	Absorptometric method	8237

Detailed protocols for the methods used in the measurement of the various physicochemical parameters can be found in DR/890 Colorimeter Procedures Manual (Hach 2009).

3.5 Extraction and Analysis of Genomic DNA

3.5.1 Extraction of Sediment Samples via Bead Beating Method

The sediment samples were thoroughly mixed using a clean, sterile spatula to ensure an even sample mix. The beads and solution in the PowerBead Tubes provided in the PowerSoil® DNA Isolation Kit (Mo-Bio Laboratories Inc., United States) were first transferred into sterile 1.5 mL microcentrifuge tubes. Approximately 0.5 g of sediment samples were then added to the empty PowerBead tubes and centrifuged at room temperature for 1 minute at 10,000 ×g (approximately 13,000 rpm). Excessive liquid was removed with a pipette tip and the PowerBead tube was re-weighed to check for the weight of soil sample remaining in the tube. The centrifugation process was repeated to accumulate approximately 0.3 g of sediment samples with the lowest possible liquid content. Subsequent genomic DNA extraction and purification steps were then performed according to the PowerSoil® DNA Isolation Kit Instruction Manual (Mo-Bio 2010). Extracted genomic DNA was contained in labelled 1.5 µL microcentrifuge tubes and stored at -20°C until further analysis.

3.5.2 Extraction of Water Samples via Bead Beating Method

Genomic DNA was also extracted from the water samples via a bead beating method using the PowerSoil® DNA Isolation Kit (Mo-Bio Laboratories Inc., United States), with minor modifications to the original protocol provided by the manufacturer. After 1.5 L of water samples from each site were filtered through a 0.2 µm membrane filter, the filter membranes were cut into small squares measuring approximately 4 mm × 4 mm each with a sterile disposable

scalpel on a sterile Petri dish. The filter membrane pieces were placed into the PowerBead tubes provided and DNA extraction proceeded according to step 3 of the PowerSoil® DNA Isolation Kit Instruction Manual (Mo-Bio 2010). After labelling, the tubes of genomic DNA were stored at -20°C until further use.

3.5.3 Analysis of Extracted Genomic DNA Samples via Agarose Gel Electrophoresis

Prior to amplification, presence of the extracted genomic DNA was detected by running agarose gel electrophoresis of the DNA samples using a 1% (w/v) agarose gel, prepared using 1X TBE buffer according to recommended standard protocols (Sambrook and Russell 2003). Approximately 1 uL of genomic DNA sample was mixed with 1 uL of 6X DNA loading dye and was loaded into the wells of the prepared agarose gel. The gel was subjected to a voltage of 80V for approximately 45 minutes, then stained with 3X GelRed™ nucleic acid stain (Biotium Inc., United States) viewed under a UV transilluminator and the gel image captured using the BioSpectrum® Imaging System (UVP LLC, United States).

3.5.4 Quantification of Extracted Genomic DNA Samples

The samples were then further quantified by reading the absorbance of 1 uL of the extracted genomic DNA sample at 260 nm using a nanophotometer (Implen GmbH, Germany). Concentration of the extracted DNA was

calculated by assuming the samples with an absorbance value of 1.0 at 260 nm will have approximately 50 µg/mL of double stranded DNA (dsDNA), while the purity of the extracted sample was estimated based on the ratio of DNA absorbance at 260 nm to its absorbance at 280 nm.

3.6 *In Vitro* Amplification by the Polymerase Chain Reaction (PCR)

3.6.1 PCR Primers

3.6.1.1 PCR Primers Targeting the Detection of AOB

Two different sets of primers were used for the detection of AOBs from the sampling sites. The first primer set, AmoA-1F and AmoA-2R generates a 491bp fragment and specifically amplifies AOB belonging to the beta subclass of the Proteobacteria (Rotthauwe et al., 1997). The second primer set, CTO189f and CTO654r, generates a 465bp fragment and were used to amplify partial rDNA sequences from the beta subdivision ammonia-oxidizing bacteria while excluding other taxa for which sequences are available (Kowalchuk et al., 1997) (Table 3.2).

Table 3.2: PCR Primers used in the detection of AOBs, their respective sequences and target position

No.	Primer Name	Sequence (5' to 3')	Target Position (Reference Organism)
1	AmoA-1F	GGG GTT TCT ACT GGT GGT	332 to 349 (*N. europaea*)
2	AmoA-2R	CCC CTC KGS AAA GCC TTC TTC	802 to 822 (*N. europaea*)
3	CTO189f	GGA GRA AAG CAG GGG ATC G	189 to 207 (*E. coli*)
4	CTO654r	CTA GCY TTG TAG TTT CAA ACG C	654 to 675 (*E. coli*)

3.6.1.2 PCR Primers Targeting the Detection of MOB

MOB was detected using primers targeting the *pmo*A gene. The primers A189 (Holmes et al., 1995) and MB661 (Costello and Lidstrom 1999) generates a 510 bp DNA fragment (McDonald et al., 2007), targeting the *pmo*A gene of all known methanotrophs and excludes the detection of the *amo*A gene (Table 3.3).

Table 3.3: List of PCR Primers used in the detection of MOBs, their respective sequences and target position

No.	Primer Name	Sequence (5' to 3')	Target Position
1	A189f	GAA SGC NGA GAA GAA SGC	189 to 206 (*M. capsulatus*)
2	MB661	CCG GMG CAA CGT CYT TAC C	661 to 679 (*M. capsulatus*)

3.6.2 PCR Amplification Conditions

The concentration of reagents used for the PCR reaction was the same for all 3 sets of primers. The final concentrations of the reagents used were 1× i-*Taq* $MgCl_2$-free PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2.5 mM magnesium chloride ($MgCl_2$), 0.25 mM dNTP, 1.5 units i-*Taq* DNA polymerase (Intron Biotechnology, Korea), 0.5 µM each of forward and reverse primers (Bioneer Inc., Korea), and 10 ng of template DNA. The total volume of each PCR reaction was topped up to 50 uL with nuclease-free water.

Amplification cycles were performed in the PTC-200 thermal cycler (MJ Research, United States). For the AmoA-1F and AmoA-2R primer pair, the thermocycling conditions consisted of an initial denaturation of 5 minutes at 94°C, followed by 40 repetitive cycles of 1 minute denaturation at 94°C, 1.5 minutes of primer annealing at 60°C, and 1.5 minutes of elongation at 72°C. Upon completion of the 40 cycles, the reaction was allowed a final elongation period of 7 minutes at 72°C before bringing down the temperature to 4°C to put the reaction on hold. The PCR products were stored at -20°C until further use.

For the CTO189f and CTO654r primer pair, thermocycling conditions consisted of an initial denaturation 5 minutes at 94°C, followed by 30 repetitive cycles of 30 seconds denaturation at 94°C, 30 seconds of primer annealing at 55°C, and 70 seconds of elongation at 72°C. This was followed

by a final elongation of 2 minutes at 72°C, and the PCR reaction was held at 4°C.

Thermocycling conditions for the detection of MOB using the A189 and MB661 primer pair consisted of an initial denaturation of 5 minutes at 95°C, followed by 30 repetitive cycles of 1 minute denaturation at 95°C, 1.5 minutes of primer annealing at 60°C, and 1.5 minutes of elongation at 72°C. Final elongation was for 5 minutes at 72°C. For all PCR products, 1 uL aliquots of the PCR products were electrophoresed and visualized with 2% agarose gels using standard electrophoresis procedures as described in section 3.5.3, to check for the suitable size of PCR product generated and PCR product quality. PCR products were stored at -20°C until further use.

3.6.3 Purification of PCR Products

Triplicates of all PCR products were pooled and agarose gel purified before subsequent cloning and further manipulation. Purification of the resulting PCR product was done by with the aid of the MEGAquick-spin™ PCR and Agarose Gel DNA Extraction Kit (Intron Biotechnology, Korea) after the PCR products were electrophoresed using a 2% agarose gel and the desired band size excised.Purification was done according to the MEGAquick-spin™ PCR and Agarose Gel DNA Extraction Kit manual (Intron Biotechnology, Korea), with minor modifications to the protocol.Samples were eluted to a final volume of 40 µL using sterile Mili-Q™ ultrapure water (Milipore, United States)

3.7 Cloning of Amplified PCR Products in *E. Coli*

3.7.1 Preparation of Ligation Reaction Mixture

The purified PCR products were ligated into a suitable general purpose cloning vector, pST Blue™-1 **A**ccep**T**or™ vector (Novagen®, United States) with dual opposed T7 and SP6 promoters, and ampicillin resistance. The pST Blue™-1 **A**ccep**T**or™ cloning vector map, complete with illustrations of the multiple cloning site, restriction enzyme sites and promoter sites can be found at Appendix C. Ligation mixture was prepared based on the following compositions (as listed in Table 3.4) in a PCR tube.

Table 3.4: Components of the Ligation Reaction Mixture

Component	**Volume per Reaction (µL)**
pST Blue™-1 **A**ccep**T**or™ vector (50 ng/µL)	1.0
Purified PCR Product	2.0
Clonables™ 2X Ligation Premix	5.0
Nuclease free water	2.0
Total Volume	**10.0**

The ligation reaction mixture was lightly mixed by tapping the sides of the PCR tube before proceeding with incubation according to instructions provided by the manufacturer (Novagen 2008). After the ligation process was complete, the ligation mixture was stored at -20°C until used for transformation procedures.

3.8 Transformation of Competent *E. Coli* Cells and Screening of Bacterial Colonies via α-Complementation

Transformation was done using NovaBlue Singles™ Competent *E. Coli* Cells (Novagen, United States), which have a high transformation efficiency (> 1.5 x 10^8 cfu/μg) and is blue/white screening enabled.

Transformation procedures were conducted using a heat shock method as recommended in the user protocol of the AccepTor™ Vector Kit (Novagen 2008) with minor modifications. 35 μL each of the transformation mixtures was plated on LB agar plates, each containing 50 μg/mL of ampicillin and pre-spread with 40 μL of X-gal (2% w/v) and 7 μL IPTG (0.8 M). A positive control was also prepared by adding 1 μL of the provided test plasmid DNA and proceeding with the above mentioned transformation and plating procedures, while negative control plates were prepared by plating only untransformed competent cells, to check for contamination. Plates were sealed and incubated at an inverted position for 15 – 18 hours at 37°C.

Following incubation, the plates were stored at 4°C for several hours to allow for the blue colour of blue colonies formed via α-complementation to develop to its fullest extent for a more accurate observation visibility. Formation of blue and white bacterial colonies was then observed for the identification and selection of colonies carrying the recombinant plasmids. White and eggshell white colonies were selected for a further second round of screening by sub-cloning. White and eggshell white positive sub-clones were then selected for further confirmation of recombinant clones via colony PCR.

3.9 Screening of Recombinant Clones via Colony PCR

The presence and size of the insert in the recombinant clones were determined by colony PCR using vector-specific primers flanking the cloning site. Details of the primers used and expected size of the PCR product are summarized in the following table:

Table 3.5: Primers used in Colony PCR and Expected Product Size

Primer Name	**Sequence (5' to 3')**	**Expected Size of Colony PCR Product**
T7 Promoter U-19mer	TAA TAC GAC TCA CTA TAG GG GTT TTC CCA GTC ACG ACG T	231 bp + insert size

For each of the positive sub-clones, the colony was lightly touched using a sterile toothpick and transferred to a sterile PCR tube containing 10 µL of nuclease free water by lightly washing the tip of the toothpick in the water. The tubes were then transferred to the heating block of a thermal cycler set at 100°C and incubated for 5 minutes to lyse the bacterial cells and denature any DNAase present. PCR master mix with the final concentration of 1X i-*Taq* $MgCl_2$-free PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2.5 mM magnesium chloride ($MgCl_2$), 0.20 mM dNTP, 1.5 units i-*Taq* DNA polymerase (Intron Biotechnology, Korea), 0.5 µM each of forward and reverse primers (Bioneer Inc., Korea), was added to the PCR tubes to a final volume of 50 µL. Amplification was performed in the PTC-200 thermal cycler (MJ Research, United States), with thermocycling conditions consisting of a 12 minutes initial denaturation at 95°C, followed by 30 repetitive cycles of 60 seconds denaturation at 94°C, 60 seconds of primer annealing at 50°C, and 60

seconds of elongation at 72°C. This was followed by a final elongation of 5minutes at 72°C, and the PCR reaction was held at 4°C. Following amplification, the products were resolved on a 2% agarose gel using standard electrophoresis procedures, and then visualized as described in section 3.5.3. The presence of insert and insert orientation was checked; only plasmids with correctly oriented insert will yield PCR products with the expected size.

3.10 Restriction Fragment Length Polymorphism (RFLP) of Clone Libraries

Clones were screened and selected via restriction digests and agarose gel electrophoresis before DNA sequencing to reduce repetitive and redundant sequencing of clones carrying similar inserts. The positive colony PCR products from both the *amo*A and *pmo*A clone libraries were digested using the *Hae*III (*Bsu*RI) restriction endonuclease (Fermentas, United States)(Costello and Lidstrom 1999; You and Chen 2008), while the colony PCR products from the CTO (16S rDNA) clone library was digested using the *Mse*I (*Tru*1I) restriction endonuclease (Fermentas, United States)(Tan 2010). Restriction digest was done following the recommended protocol provided by the manufacturer, which consisted of the following components:

Table 3.6: Composition of the Restriction Enzyme Digestion Mix

Reagent	**Volume (µL)**
Buffer R (10X)*	1.0
Colony PCR Product	5.0
Restriction Endonuclease	0.5
Nuclease-free water	3.5
Total	**10.0**

*Supplied by manufacturer for optimum reaction condition of the restriction endonuclease.

The tubes containing all restriction digestion mixtures were flicked to gently mix the contents. Restriction digests containing the *Hae*III endonuclease was incubated at 37°C for 2 hours, while restriction digests containing the *Mse*I enzyme was incubated at 65°C for 2 hours. Following incubation, the restriction enzymes were inactivated by adding 5 µL of 6X DNA gel electrophoresis loading dye (New England Biolabs Inc., United States). The digested colony PCR products were then analyzed by agarose gel electrophoresis, using a 3% TBE agarose gel at 70V. Patterns were grouped manually and 1-2 clone representatives of each restriction pattern at each sampling site were randomly chosen for plasmid extraction.

3.11 Plasmid DNA Extraction of Recombinant *E. Coli* Cells

RFLP selected plasmids from successfully transformed recombinant *E. Coli* cells carrying the desired insert was extracted and purified using the GeneMATRIX Plasmid Miniprep DNA Purification Kit (EURx, Poland). Selected clones were each inoculated separately into 5 mL of LB medium supplemented with 50 µg/mL of ampicillin. The inoculated medium was incubated at 37°C with vigorous agitation at 200 rpm for 15 – 18 hours. Subsequent extraction and purification steps were then performed according to the GeneMATRIX Plasmid Miniprep DNA Purification Kit protocol (EURx 2008). Presence of extracted plasmid was determined by electrophoresis of the extracted plasmid samples using a 1% agarose gel using standard electrophoresis procedures.

3.12 Sequencing of Extracted Recombinant Plasmid DNA

Extracted recombinant plasmid DNA was sequenced and analyzed by Bioneer, Inc. (Korea) using a 3730XL DNA sequencer (Applied Biosystems, United States). Prior to sending the purified plasmids for sequencing, concentration of the extracted plasmid DNA was checked to ensure it fell within the suitable range for analysis as required by the sequencing company (150-200 ng/µl). The T7 promoter primer, provided by the sequencing company, was used for sequencing of the recombinant plasmid DNA. Characteristics of the pST Blue™-1 **A**ccep**T**or™ vector T7 promoter priming site can be found in Appendix C.

3.13 Sequence Alignment and Phylogenetic Analysis

Sequences were then aligned with DNA databases currently available at GenBank, National Centre for Biotechnology Information using the Basic Local Alignment Search Tool (blastn). Multiple alignment of sequences were performed using Clustal W (Thompson et al., 1994). Three phylogenetic trees were constructed from a total of 112 AOB *amo*A sequences (38 sequences from this study), 182 AOB 16S rDNA sequences (107 sequences from this study) and 309 *pmo*A sequences (165 from this study) by the maximum likelihood (ML), neighbor-joining (NJ) and maximum parsimony (MP) approach using MEGA5 (Tamura et al., 2011). ML and NJ trees were constructed using the best-fit substitution model determined based on the program jModelTest (Posada 2008). Bootstrap analysis was performed with 1000 replicates and included an outgroup for all trees.

3.14 Quantitative Analyses

To compare the diversity between clone libraries, the *amo*A, CTO and *pmo*A sequences were analysed using the DOTUR program (Schloss and Handelsman 2005) using the furthest neighbor assignment. For the *amo*A clone libraries, a 5% nucleotide sequence difference cutoff value (Francis et al., 2003) was used in defining the number of operational taxonomic units (OTUs), while the OTUs for the CTO and *pmo*A clone libraries were defined using 3% (Hughes et al., 2001) and 13% (Degelmann et al., 2010) nucleotide sequence differences, respectively (detailed explanation in section 5.2). Diversity indices (Shannon and Simpson) and rarefaction analysis were also determined on DOTUR. Coverage of each clone library was calculated using the formula $C = [1- (n1/N)] \times 100$, in which n1 is the number of OTUs and N is the total number of clones in each library (Mullins et al., 1995).

Weighted principle coordinates analyses (PCoA) and Jackknife Environment Clusters were also conducted using the online software UniFrac (http://bmf2.colorado.edu/unifrac/index.psp) to compare between microbial communities based upon phylogenetic information (Lozupone and Knight 2005; Lozupone et al., 2007). The UniFrac software measures the distance between microbial communities based on the lineages they contain both qualitatively (unweighted UniFrac) and quantitatively (weighted UniFrac)(Lozupone and Knight 2005). A single rooted phylogenetic tree containing all the sequences to be considered, together with an accompanying environment file linking each sequence to its originating environment was used as an input(Lozupone et al., 2006). For the weighted UniFrac analyses,

the number of times an OTU/clone was observed (determined based on DOTUR analyses) was considered and included in the input environment file.

3.15 Nucleotide Sequence Accession Numbers

The nucleotide sequences from this study have been submitted to the GenBank database under the accession numbers JX157920 to JX157957 (*amo*A sequences) and JX184132 to JX184403 (16S rDNA and *pmo*A sequences). The full inventory of sequences submitted to GenBank and their respective accession numbers can be found in Appendix D.

CHAPTER 4

RESULTS

4.1 Physicochemical Properties Analysis

The physicochemical properties of the water sampled from the sites of the three different types of ponds are summarized in Table 4.1. Comparisons of selected physicochemical property values between the three ponds with different environmental conditions are graphically represented in Figure 4.1 to Figure 4.6.

The post-aquaculture pond recorded higher total ammonia nitrogen (TAN) reading among the ponds (Figure 4.2). On the other hand, the lotus pond recorded lower dissolved oxygen (Figure 4.1), TAN (Figure 4.2) and suspended solid content (Figure 4.5) and was least turbid (Figure 4.6), but had the highest content of nitrate (Figure 4.4). Other parameters, including pH, temperature, nitrite and salinity differed only nominally amongst the sampling sites. pH fell within the narrow range of 7.21 - 8.22, with the lowest at the post-aquaculture pond and highest at the idle pond. Temperature was within the range of 30.73 - 31.62°C, while salinity was between 0.06 - 0.07 PSU and fell within the range of oligohaline water bodies (Oertli 1958). Sulfate was below detectable levels at all sampling sites (Table 4.1).

Table 4.1: Physicochemical properties of water sampled from disused tin-mining ponds under study

Parameter	Value at Various Sampling Sites*		
	Aquaculture Pond	**Lotus Pond**	**Idle Pond**
Temperature (°C)	31.62 ± 0.54	30.73 ± 0.57	31.35 ± 1.35
pH	7.21 ± 0.71	7.56 ± 0.34	8.22 ± 0.04
Dissolved Oxygen (mg/L)	7.26 ± 1.26	3.75 ± 0.32	6.01 ± 0.01
TAN, NH_3 (mg/L)	0.76 ± 0.51	0.25 ± 0.21	0.26 ± 0.29
Nitrite, NO_2 (mg/L)	0.027 ± 0.017	0.014 ± 0.002	0.014 ± 0.011
Nitrate, NO_3 (mg/L)	1.37 ± 0.64	2.20 ± 0.99	0.30 ± 0.00
Sulfate (mg/L)	0	0	0
Suspended Solids (mg/L)	13.7 ± 3.21	4.5 ± 0.71	25 ± 2.82
Turbidity (FAU)	16.70 ± 5.50	6.00 ± 4.24	46.50 ± 19.09
Salinity(PSU)	0.06 ± 0.01	0.07 ± 0.00	0.06 ± 0.02

***** Values are given as mean ± standard error of the mean. Water was sampled from 3 sites for the aquaculture pond, and 2 sites each for the lotus and idle pond.

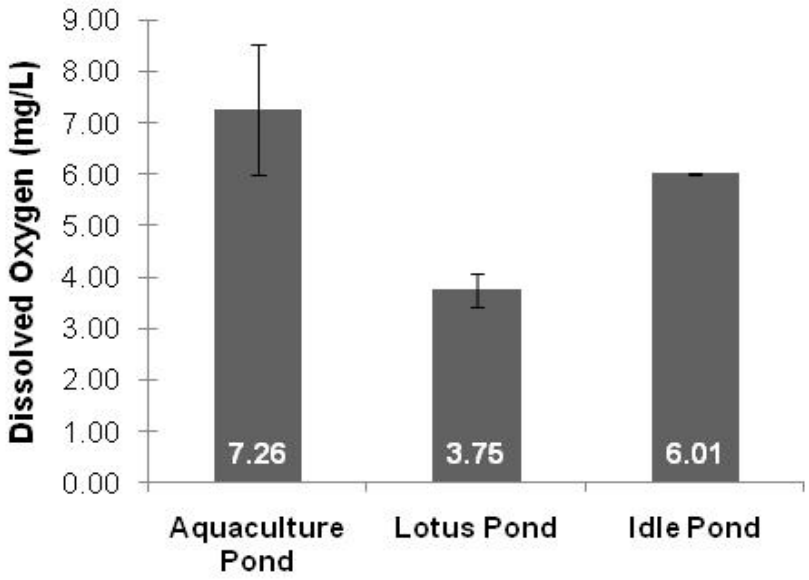


Figure 4.1: Comparison of dissolved oxygen values of water samples between the three disused tin-mining pond sites. Average values of each site with their standard errors are shown.

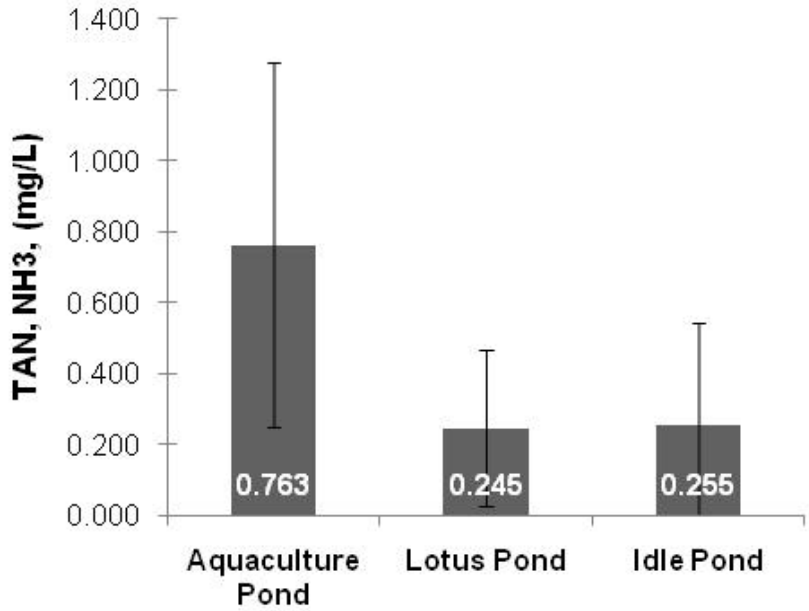


Figure 4.2: Comparison of TAN values between the water samples of the three disused tin-mining pond sites. Average values of each site with their standard errors are shown.

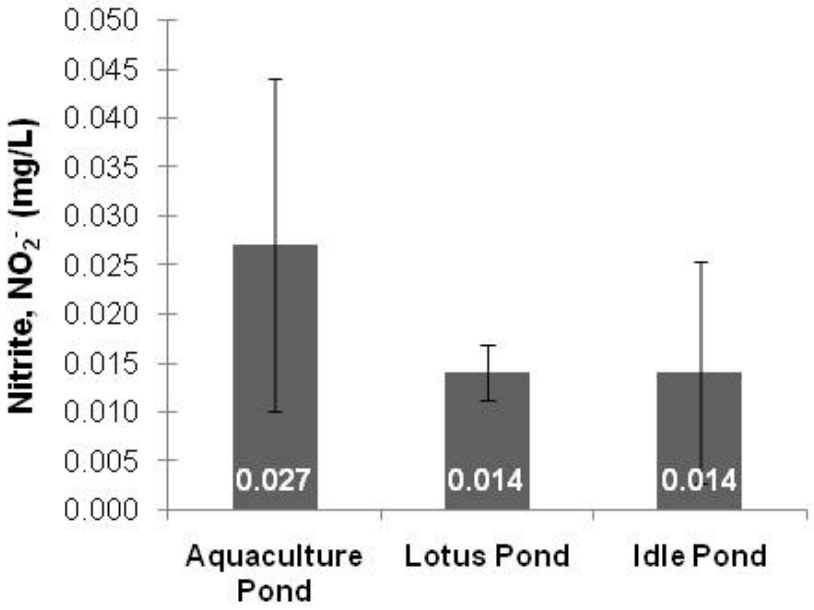


Figure 4.3: Comparison of nitrite (NO_2^-) values between the water samples of the three disused tin-mining pond sites. Average values of each site with their standard errors are shown.

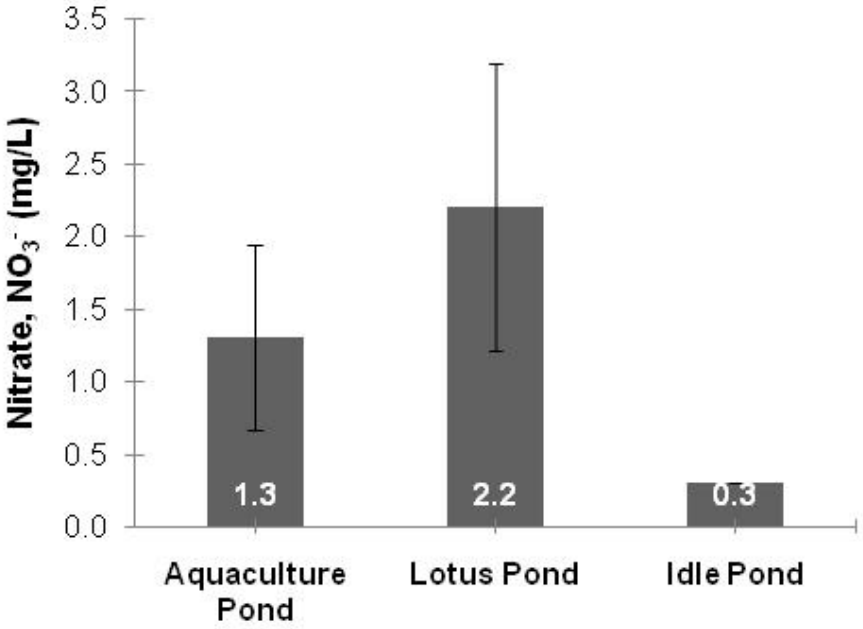


Figure 4.4: Comparison of Nitrate (NO_3^-) values between the water samples of the three disused tin-mining pond sites. Average values of each site with their standard errors are shown.

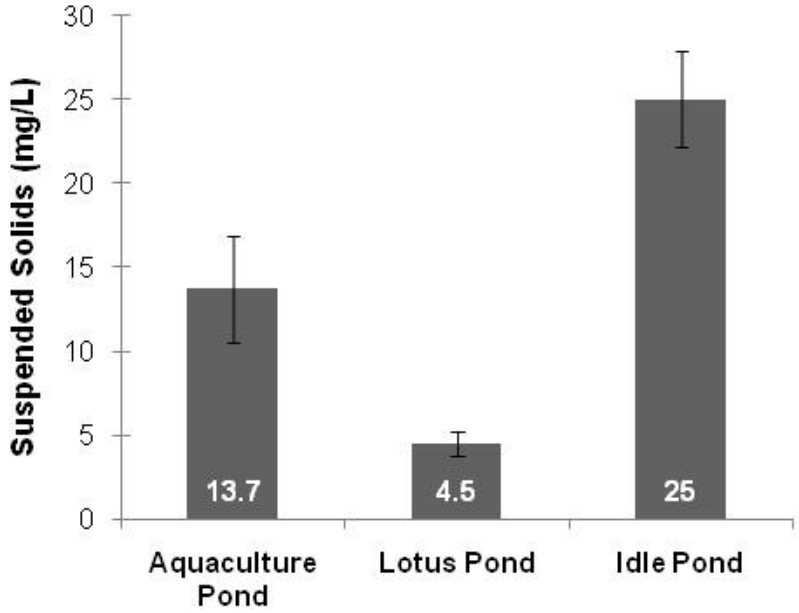


Figure 4.5: Comparison of suspended solids values between the water samples of the three disused tin-mining pond sites. Average values of each site with their standard errors are shown.

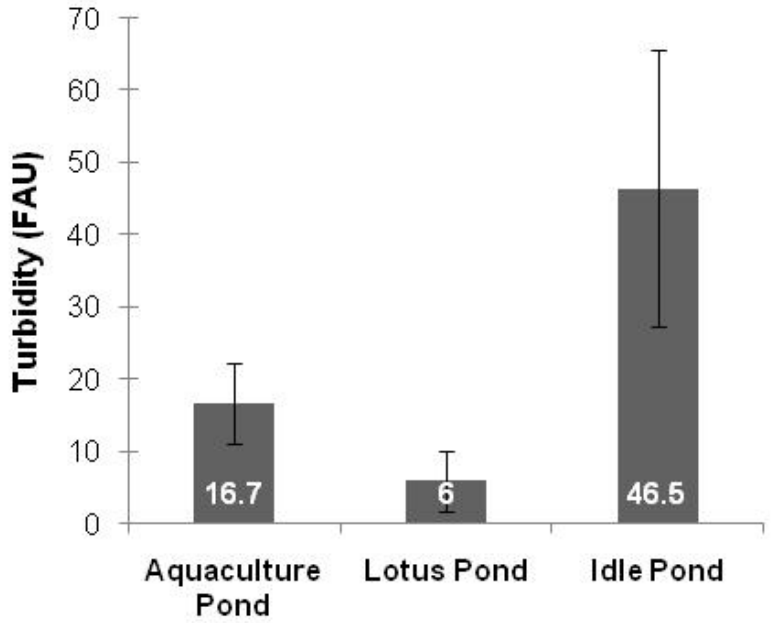


Figure 4.6: Comparison of turbidity values between the water samples of the three disused tin-mining pond sites. Average values of each site with their standard errors are shown.

4.2 Analysis of Genomic DNA Extraction

4.2.1 Agarose Gel Electrophoresis of Extracted DNA Samples

After extraction of the genomic DNA from the sediment and water samples of the ponds, the presence, quality and estimated size of the extracted DNA were analysed using agarose gel electrophoresis with a concentration of 1%. The agarose gel image is shown in Figure 4.7 below:

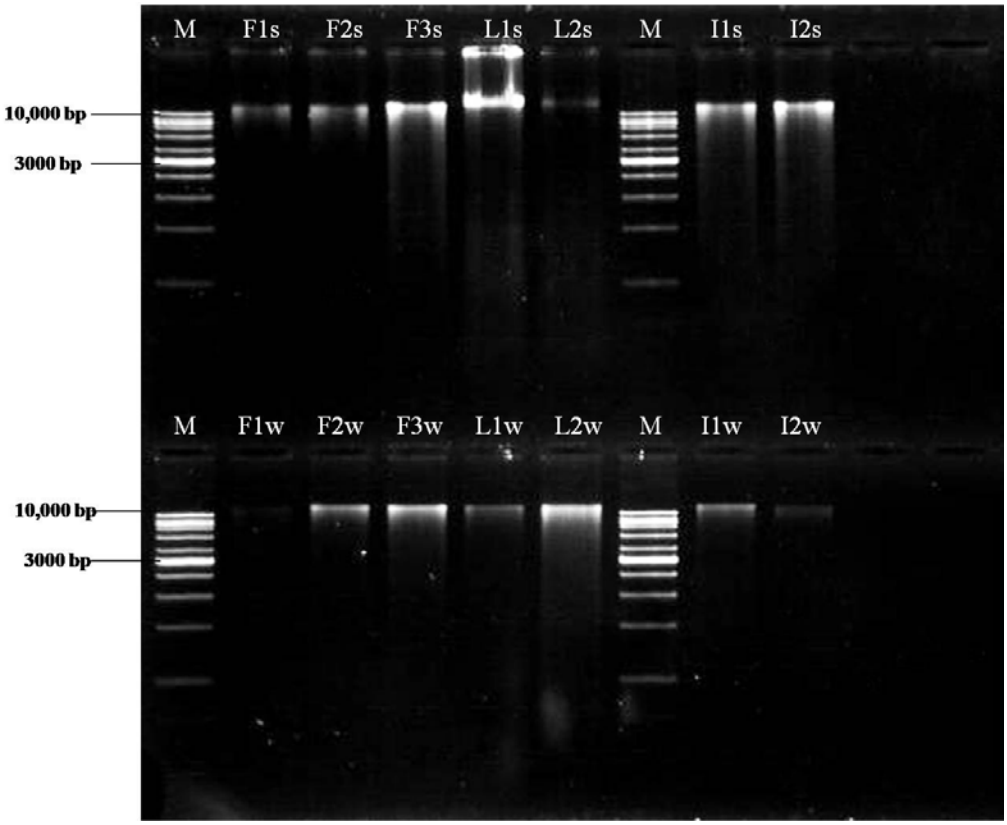


Figure 4.7: Agarose Gel Image of Extracted Genomic DNA. Lanes M: 1kb DNA ladder; Lanes F1s – F3s: Fish pond sites 1 – 3 sediment samples; Lanes L1s – L2s: Lotus pond sites 1 – 2 sediment samples; Lanes I1s – I2s: Idle pond sites 1 – 2 sediment samples; Lanes F1w–F3w: Fish pond sites 1 – 3 water samples; Lanes L1w – L2w: Lotus pond sites 1 – 2 water samples; Lanes I1w – I2w: Idle pond sites 1 – 2 water samples. Electrophoresis was conducted on a 1% agarose gel at 85V.

4.2.2 Quantification of Extracted Genomic DNA

The concentration of the genomic DNA samples (ng/µL) was auto-calculated by the nanophotometer software from the absorbance of the DNA samples at 260 nm, while the purity of the genomic DNA samples over protein was determined using the software by taking the absorbance ratios for 260 nm/280 nm. Concentration of genomic DNA extracts in our study fell within the range of 746.91 - 1056.37. The concentrations proved to be sufficient for PCR since the recommended concentration of DNA template to be used for PCR reactions using the iTaq DNA polymerase (Intron Biotechnology, Korea) is from 1 ng - 1 µg. Higher concentrations of DNA might decrease amplicon specificity.

Eluted genomic DNA extracts were observed to be clear, with no visible brown coloured humic substance contamination, known to inhibit PCR reactions. The purity of the genomic DNA extracts over protein (260 nm/280 nm) fell within the range of 1.834 - 1.922 which was within the acceptable DNA purity range. The ideal value for DNA purity should fall within the range of 1.74 - 2.00 (Sambrook and Russell 2003). This suggests that a very low level of possible protein contamination was detected which should not significantly interfere with subsequent processes.

4.3 Amplification of the genes of interest and Analysis of PCR Products

4.3.1 Amplification of the *amo*A Gene of Ammonia Oxidizing Bacteria

Amplification of the *amo*A gene at an optimized annealing temperature of 60°C according to the PCR reagent profiles described in Section 3.6.2 and the

subsequent agarose gel electrophoresis of the PCR products obtained is shown in Figure 4.8 below:

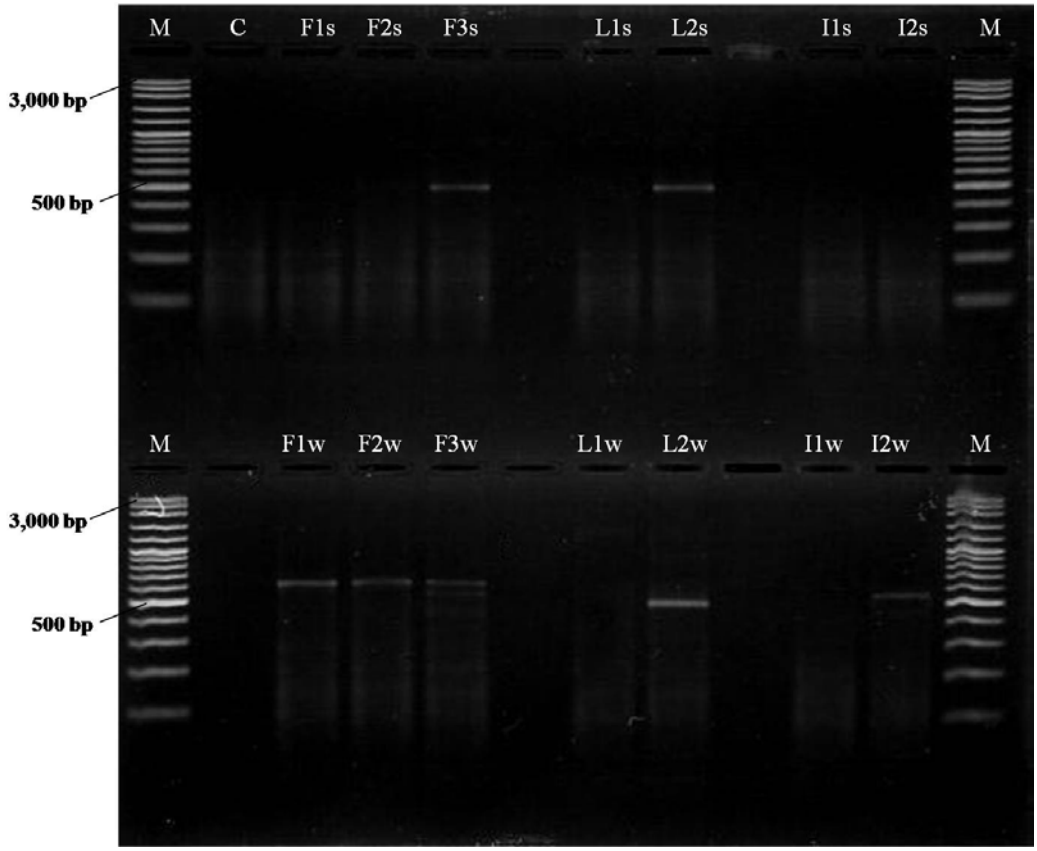


Figure 4.8: Agarose gel image of the resulting PCR products from the amplification of the *amo*A gene using the AMO-1F and AMO-2R primer pair. Lanes M: 100 bp plus DNA marker; Lane C: negative control; Lanes F1s – F3s: PCR amplification of Fish pond sites 1 – 3 sediment samples; Lanes L1s – L2s: PCR amplification of Lotus pond sites 1 – 2 sediment samples; Lanes I1s – I2s: PCR amplification of Idle pond sites 1 – 2 sediment samples; Lanes F1w – F3w: PCR amplification of Fish pond sites 1 – 3 water samples; Lanes L1w – L2w: PCR amplification of Lotus pond sites 1 – 2 water samples; Lanes I1w – I2w: PCR amplification of Idle pond sites 1 – 2 water samples. Electrophoresis was conducted on a 2% agarose gel at 75V.

The expected band size of *amo*A gene was successfully amplified using the AMO-1F and AMO-2R primer for samples F3s, L2s and L3w only, where a clear band of approximately 500 bp could be seen (Figure 4.8). Unspecific priming was observed for samples F1w, F2w and F3w, and no PCR product was observed to be amplified in the rest of the samples. No band was observed at the negative control lane.

4.3.2 Amplification of the 16S rRNA Gene of Ammonia Oxidizing Bacteria

Amplification of the 16S rRNA gene at an optimized annealing temperature of 55°C according to the PCR reagent profiles described in Section 3.6.2 and the subsequent agarose gel electrophoresis of the PCR products obtained are shown in Figure 4.9.

The 16S rRNA gene of ammonia oxidizing bacteria was successfully amplified using the CTO189f and CTO654r primers for all samples except for I1s, I2s and I1w, where clear bands of approximately 465 bp were observed. No bands and hence no PCR product was observed to be amplified in samples I1s, I2s and I1w, indicating an undetectable level of ammonia oxidizing bacteria in the sediment samples of the Idle pond and water sample of site 1 in the idle pond. No band was observed at the control lane, indicating that there was no contamination to the PCR reaction.

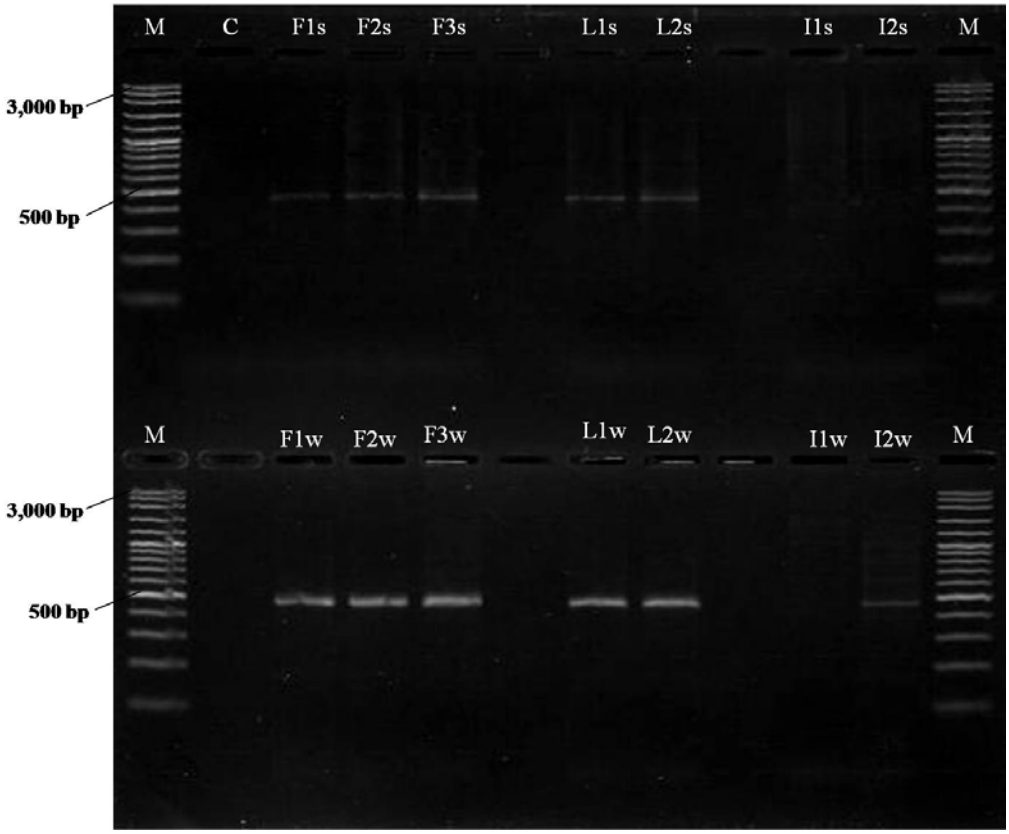


Figure 4.9: Agarose gel image of the resulting PCR products from the amplification of the AOB 16SrRNA gene using the primers CTO189f and CTO654r. Lanes M: 100 bp plus DNA marker; Lane C: negative control; Lanes F1s – F3s: PCR amplification of Fish pond sites 1 – 3 sediment samples; Lanes L1s – L2s: PCR amplification of Lotus pond sites 1 – 2 sediment samples; Lanes I1s – I2s: PCR amplification of Idle pond sites 1 – 2 sediment samples; Lanes F1w – F3w: PCR amplification of Fish pond sites 1 – 3 water samples; Lanes L1w – L2w: PCR amplification of Lotus pond sites 1 – 2 water samples; Lanes I1w – I2w: PCR amplification of Idle pond sites 1 – 2 water samples. Electrophoresis was conducted on a 2% agarose gel at 75V.

4.3.3 Amplification of the *pmo*A Gene of Methane Oxidizing Bacteria

Amplification of the *pmo*A gene at an optimized annealing temperature of 55.0°C according to the PCR reagent profiles described in Section 3.6.2 and the subsequent agarose gel electrophoresis of the PCR products obtained are shown in Figure 4.10.

From Figure 4.10, it is observed that the *pmo*A gene of methane oxidizing bacteria was successfully amplified using the A189 and MB661 primers for all samples, indicating the presence of methane oxidizing bacteria at all sites.

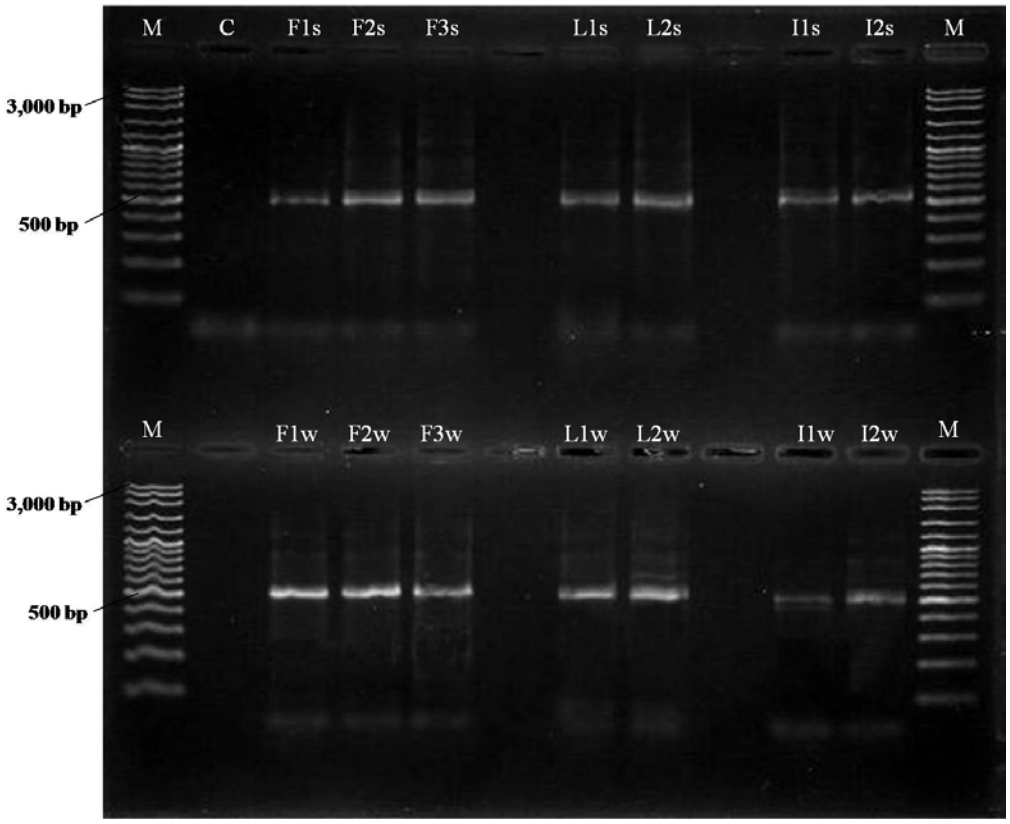


Figure 4.10: Agarose gel image of the resulting PCR product from the amplification of the MOB *pmo*A gene using the primers A189 and MB661. Lanes M: 100 bp plus DNA marker; Lane C: negative control; Lanes F1s – F3s: PCR amplification of Fish pond sites 1 – 3 sediment samples; Lanes L1s – L2s: PCR amplification of Lotus pond sites 1 – 2 sediment samples; Lanes I1s – I2s: PCR amplification of Idle pond sites 1 – 2 sediment samples; Lanes F1w – F3w: PCR amplification of Fish pond sites 1 – 3 water samples; Lanes L1w – L2w: PCR amplification of Lotus pond sites 1 – 2 water samples; Lanes I1w – I2w: PCR amplification of Idle pond sites 1 – 2 water samples. Electrophoresis was conducted on a 2% agarose gel at 75V.

4.4 Analysis of Purified PCR Products

All PCR products obtained were purified using the GeneJET™ Gel Extraction Kit (Fermentas, United States). Presence of the purified PCR product after purification was confirmed by running agarose gel electrophoresis of the purified PCR product. Based on the agarose gel images (images not shown), clear distinct bands of the correct band size were obtained for all the amplified products. The purified products were then used in cloning procedures.

4.5 Cloning, Colony PCR & Identification of Positive Clones

The cloning reactions generally produced plates with 80 - 150 white colonies and less than 10 blue colonies per plate. Following subcloning, about 25% of the white colonies from the subclones carried unstable inserts were observed as blue colonies in the subclone plates. White colonies from each sub-cloned plates were randomly chosen for colony PCR to further confirm the presence of inserts of the correct size and orientation (Figure 4.11). A total of 50 clones showing the correct insert size based on the colony PCR products from each sample were further screened by restriction digestion using the suitable restriction enzymes.

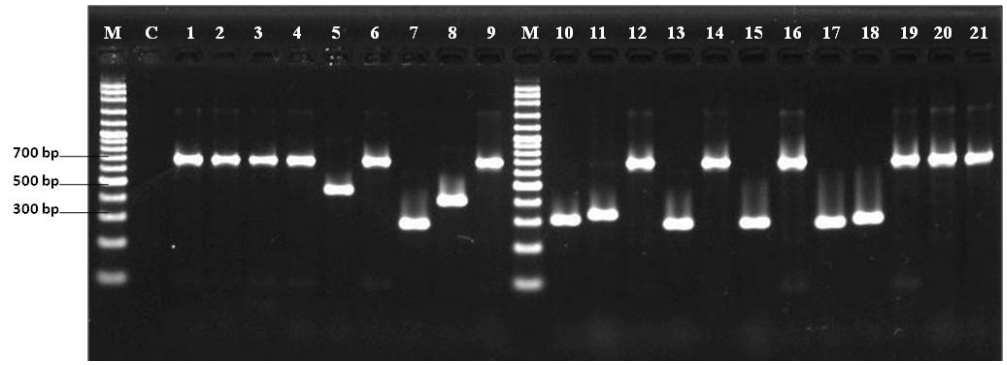


Figure 4.11: Example of an electrophoresis gel image for the colony PCR products of clones carrying the CTO insert (approximate insert size: 465 bp). Colonies carrying no insert should produce 231 bp PCR products (Novagen 2008). Lanes 1, 2, 3, 4, 6, 9, 12, 14, 16, 19, 20 and 21 are example of PCR products of the clones carrying correct insert size (positive colony PCR products). The PCR products at lanes 7, 10, 11, 13, 15, 17 and 18 are those of clones with no insert, while those at lanes 5 and 8 carry truncated/fragmented inserts.Lane C: Negative control; Lanes M: 100 bp plus DNA marker. Electrophoresis was conducted using a 1.5% agarose gel at 80 V.

4.6 Restriction Digests

A total of 16 unique RFLP patterns were observed from the agarose gel image of *Hae*III restriction enzyme digested colony PCR products from the *amo*A clone library, while 22 unique patterns were found from the CTO (16S rRNA) clone library (all samples). The *pmo*A clone library colony PCR products, also digested using the *Hae*III enzyme, had a total of 35 unique restriction patterns. Examples of the restriction patterns obtained from the digestion of the *amo*A, CTO and *pmo*A clone libraries are shown in Figure 4.12, Figure 4.13, and Figure 4.14, respectively.

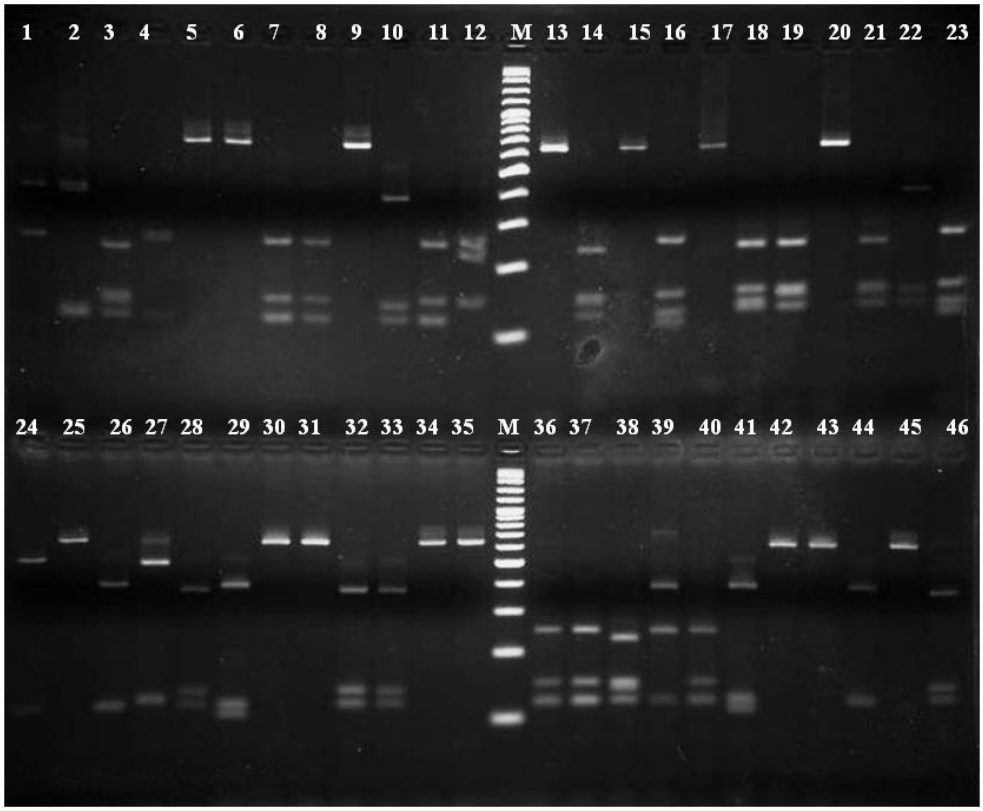


Figure 4.12: Example of an electrophoresis gel image for the colony PCR products of clones carrying the *amo*A insert digested using the *Hae*III restriction endonuclease. Lane M: 100 bp plus DNA marker. Electrophoresis was carried out using a 3% agarose gel at 70V.

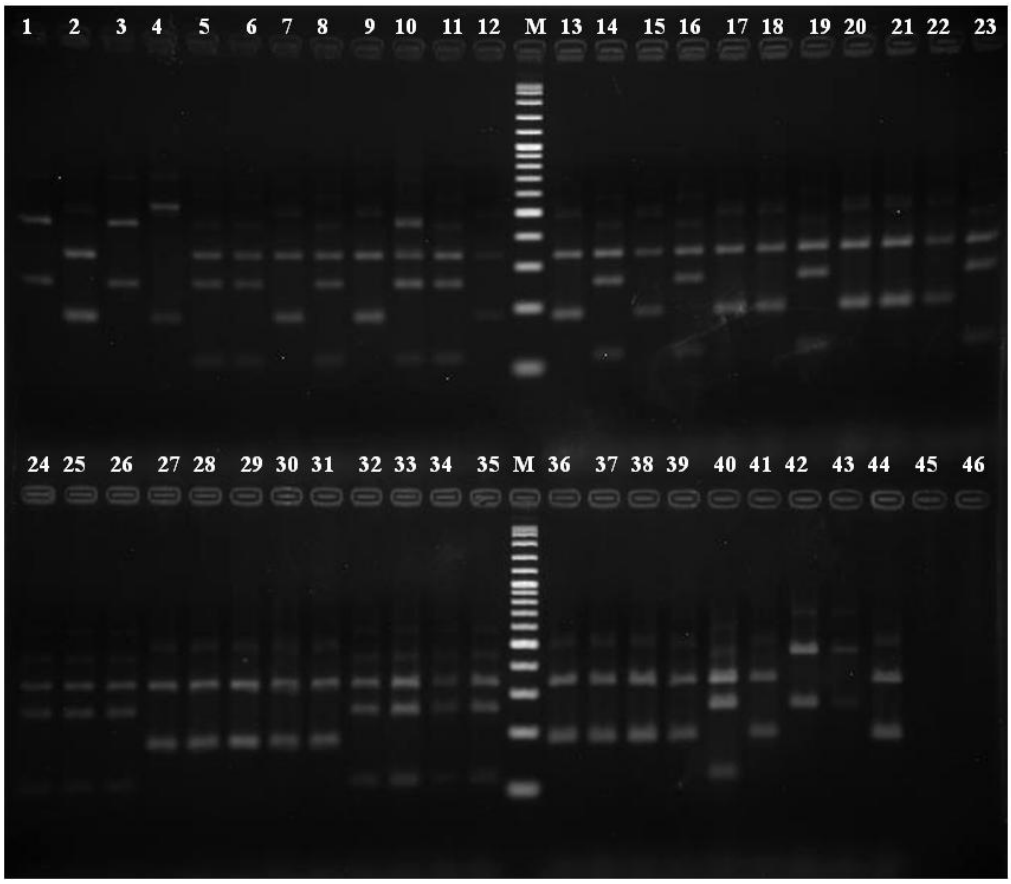


Figure 4.13: Example of an electrophoresis gel image for the colony PCR products of clones carrying the CTO (16s rDNA) insert digested using the *Tru*1I (*Mse*I) restriction endonuclease**.** Lane M: 100 bp plus DNA marker. Electrophoresis was carried out using a 3% agarose gel at 70 V.

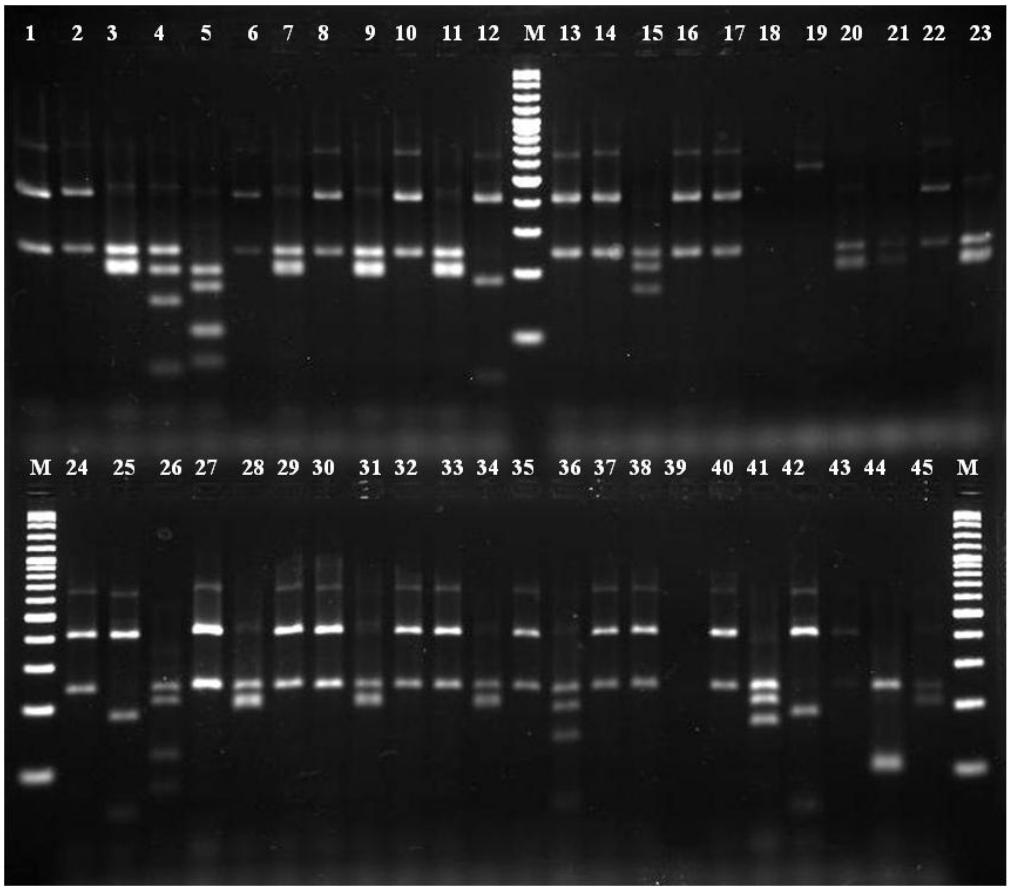


Figure 4.14: Example of an electrophoresis gel image for the colony PCR products of clones carrying the *pmo*A inserts digested using the *Hae*III restriction endonuclease. Lane M: 100 bp plus DNA marker. Electrophoresis was carried out using a 3% agarose gel at 70 V.

4.7 BLAST Alignment & Sequence Analysis

Table 4.2 summarizes the closest relatives for the *amo*A, 16S rDNA and *pmo*A clone sequences, as obtained from BLAST-N analysis. BLAST-N analysis for *amo*A clones indicated that out of a total 37 successful clone sequences, the majority of the clones (76.3%) showed 97 – 100% similarity to uncultured bacterium and uncultured ammonia-oxidizing bacteria, whereas only 1 clone showed close affiliation to *Nitrosomonas europaea* (99%). Similarly, the BLAST-N analysis for CTO clones resulted in a large number of clones (80.4%) showing to be closely related (95 – 99%) to uncultured

bacterium. A total of 9 clone sequences were closely related to uncultured *Nitrosospira* sp. DGGE bands (95 – 99%), whereas only 3 clones were 97% related to *Nitrosomonas* sp. clones.

For the *pmo*A clones, BLAST-N analysis showed that out of the 165 successful clone sequences from all the study sites, a majority of the clones (152 clones) showed 88–99% similarity to uncultured bacterium and methanotrophs. Another 9 (5.5%) of the clones were affiliated to Type I methanotroph sequences in a similarity range of 90–96% (Table 4.2). A total of 20 clones distributed across the various sites showed low similarity (87–90%) to uncultured bacterium or uncultured methanotrophic bacterium, suggesting that these sequences might be unique to this environment or had not been described previously.

Table 4.2: Closest relatives for the *amo*A, 16S rDNA and *pmo*A clone sequences as determined from the GenBank database using the BLAST-N search tool. Complete list of best hits for BLAST searches, their respective accession numbers and percentage similarities can be found in Appendix F.

Sequence Type	Category of Closest Relative in Genbank (Accession Number)[ϱ]	Percentage Similarity Range	No. of Clones	No. of Clones according to Sampling Site[ǂ]					
				Fs	Fw	Ls	Lw	Is	Iw
*amo*A	Uncultured Bacterium	97 – 100%	29/38 (76.3%)	11	-	9	9	-	-
	Uncultured ammonia-oxidizing bacteria	96 – 100%	8/38 (21.0%)	-	-	-	8	-	-
	Nitrosomonas europaea ATCC 19718 (AL954747.1)	99%	1/38 (2.7%)	-	-	-	1	-	-
16S rDNA (CTO Sequences)	Uncultured *Nitrosospira sp*. DGGE Bands	95 – 99%	9/107 (8.4%)	6	-	2	1	-	-
	Uncultured Bacterium	95 – 99%	86/107 (80.4%)	13	27	10	13	-	23
	Uncultured β-*proteobacterium*	98 – 99%	9/107 (8.4%)	4	-	2	1	-	2
	Uncultured *Nitrosomonas sp*. clones	97%	3/107 (2.8%)	-	-	3	-	-	-
*pmo*A	Uncultured Bacterium & methane-oxidizing bacteria	88 - 99%	152/165 (92.1%)	36	24	27	19	24	22
	Uncultured γ-subdivision MOB	90 – 96%	9/165 (5.5 %)	-	5	1	2	-	1
	Methylococcaceae bacterium OS501 (AB636304.1)	91 – 99%	3/165 (1.8%)	-	2	-	1	-	-
	Methylomonas methanica MC09 (CP002738.1)	92%	1/165 (0.1%)	-	1	-	-	-	-

[ϱ] Where the closest relatives were listed without the respective accession numbers, these were the closest relatives of similar types of clones grouped into a common category for categories with number of clones too numerous to list (i.e. numerous different clones under the uncultured bacterium category).

[ǂ] Fs – fish pond sediment; Fw – fish pond water; Ls – lotus pond sediment; Lw – lotus pond water; Is – idle pond sediment; Iw – Idle pond water

4.8 Quantitative Analyses

4.8.1 Diversity and Richness of the Ammonia Oxidizing Bacteria and Methane Oxidizing Bacteria

A total of 38 *amo*A, 107 CTO and 165 *pmo*A sequences were obtained from the corresponding 3 *amo*A, 5 CTO and 6 *pmo*A clone libraries constructed. On the basis of the RFLP analysis, a total of 16 unique RFLP patterns were observed from the *amo*A clone libraries, while 22 and 35 unique RFLP patterns were observed from the CTO and *pmo*A clone libraries, respectively. On the other hand, the DOTUR program reported a total of 15, 26 and 39 unique operational taxonomic units (OTUs) for the *amo*A, CTO and *pmo*A clone libraries, respectively (Table 4.3). For each sample, 5 - 9 *amo*A OTUs, 5 - 14 CTO OTUs and 7 - 16 *pmo*A OTUs were observed.

Table 4.3: Biodiversity of AOB (predicted from the *amo*A and CTO clone libraries) and MOB (predicted from the *pmo*A clone libraries) of the sampling sites with varying ecological conditions.

Sequence Type	Sampling Site	No. of Clones	No of Unique Sequence[a]	No. of OTUs[b]	Library Coverage, C[c] (%)	Shannon Index, H	Simpson Index, $\frac{1}{D}$
*amo*A	Fish Pond Sediment (Fs)	11	5	5	54.55	1.12	3.13
	Lotus Pond Sediment (Ls)	9	4	5	44.44	1.21	4.00
	Lotus Pond Water (Lw)	18	11	9	50.00	1.61	4.76
	Total	38	16	15	60.53		
CTO	Fish Pond Sediment (Fs)	24	16	14	41.67	2.29	10.00
	Fish Pond Water (Fw)	27	8	4	85.19	0.47	2.00
	Lotus Pond Sediment (Ls)	17	12	12	29.41	2.20	16.76
	Lotus Pond Water (Lw)	15	7	7	53.33	1.59	4.55
	Idle Pond Water (Iw)	25	5	5	80.00	0.60	1.43
	Total	107	22	26	75.70		
*pmo*A	Fish Pond Sediment (Fs)	36	17	13	63.89	2.42	14.28
	Fish Pond Water (Fw)	32	18	12	62.50	2.20	9.09
	Lotus Pond Sediment (Ls)	28	18	16	42.86	2.55	16.67
	Lotus Pond Water (Lw)	22	11	10	54.54	2.17	12.50
	Idle Pond Sediment (Is)	24	13	11	54.17	2.14	11.11
	Idle Pond Water (Iw)	23	18	7	69.56	1.58	4.17
	Total	165	35	39	76.36		

[a] Number of unique sequences determined based on RFLP analyses

[b] Number of unique OTUs were determined using DOTUR, based on a 5% sequence difference cut-off for *amo*A sequences, 3% sequence cutoff for CTO sequences, and 13% sequence cutoff for *pmo*A sequences. Library coverage, Shannon and Simpson indices were derived from OTU data based on the same sequence difference cut-Foff values.

[c] $C = [1- (n_1/N)] \times 100$, where n_1 is the number of OTUs and N is the total number of clones in each library.

4.8.2 Rarefaction Analysis

Rarefaction analysis of the CTO clone libraries indicated that the post aquaculture and lotus pond sediments had the highest number of OTUs (richness) recovered as observed from the steeper gradient of the rarefaction curve (Figure 4.15). This was consistent with the Shannon and Simpson diversity indices obtained in Table 4.3. The overlapping rarefaction curves of the post-aquaculture and lotus pond sediment clone sequences suggest that both have very similar levels of richness. Post-aquaculture pond water was observed to harbour the lowest richness, whether according to the rarefaction curve or the Simpson/Shannon diversity indices. Rarefaction curves for the *amo*A clones were not included here since clones from the idle ponds and water samples of the post-aquaculture pond were not detected. As for the *pmo*A clones, the rarefaction curve (Figure 4.16), as well as the Shannon and Simpson diversity indices (Table 4.3) indicated the highest richness/diversity at the lotus pond sediment and lowest richness/diversity at the idle pond water. Post-aquaculture and lotus pond water samples, and idle pond sediment samples appear to show similar levels of richness (Figure 4.16).

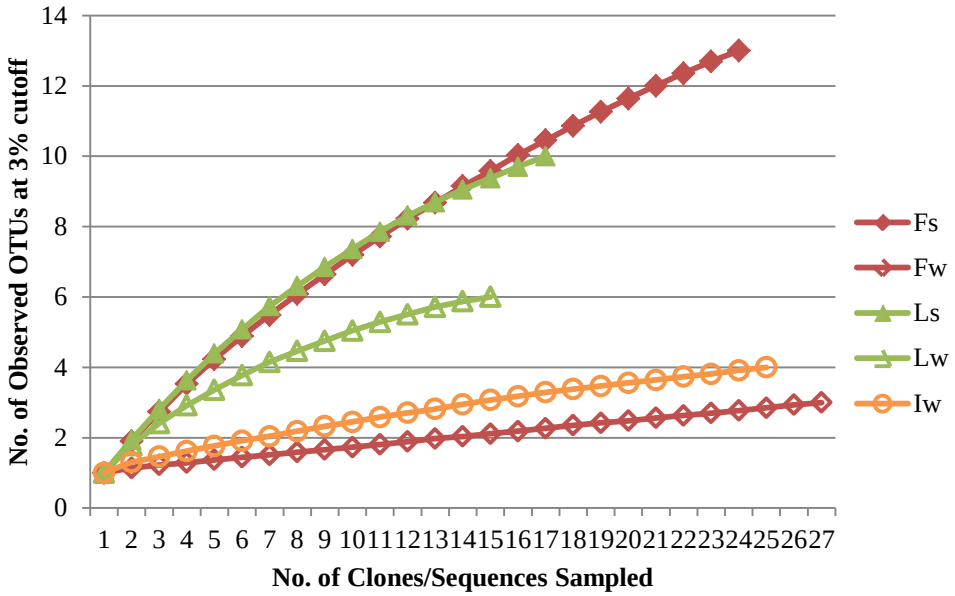


Figure 4.15: Rarefaction analysis curves for the CTO (16S rDNA) clone sequences. OTUs were defined as groups of sequences which differed by ≤ 3% at the DNA level.

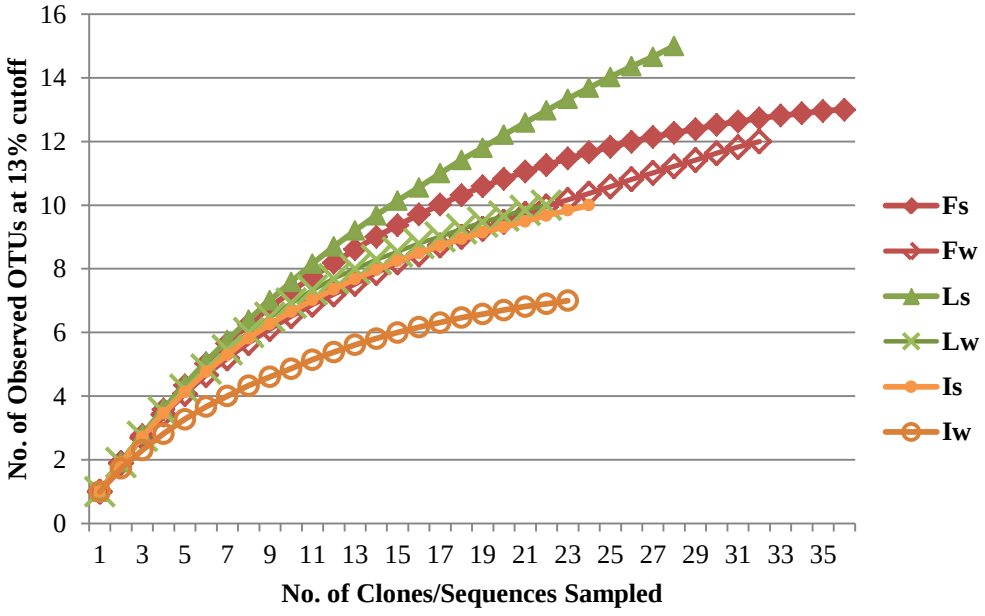


Figure 4.16: Rarefaction analysis curves for the *pmo*A clone sequences. OTUs were defined as groups of sequences which differed by ≤ 13% at the DNA level.

4.9 Phylogenetic Analysis

Phylogenetic trees generated with all three methods (maximum-likelihood, neighbour-joining and maximum parsimony) were congruent. Therefore, only maximum-likelihood phylogenetic trees constructed for the *amo*A, AOB 16S rRNA and *pmo*A clone sequences are presented here. Branch nodes supported by trees constructed using both the maximum parsimony and neighbour joining methods are indicated by closed white circles.

4.9.1 Phylogenetic Analysis of the Ammonia Oxidizing Bacteria Community

Two phylogenetic trees were constructed, where one was based on the sequence analysis of 419 bp *amo*A sequences (Figure 4.17) and the other based on the sequence analysis of 465 bp 16S rDNA (CTO) sequences (Figure 4.18). The AOB detected based on the *amo*A gene fell into five distinct clusters within the *Nitrosomonas* genus but none clustered with the *Nitrosospira* genus (Figure 4.17). A majority of the *amo*A sequences from the post aquaculture pond (10 sequences) fell into cluster amo1a and were closely affiliated with environmental sequences isolated from pre-treated water biofilms and river confluences. On the other hand, sequences from the lotus pond grouped mostly in cluster amo 3 (12 sequences) and amo 4 (7 sequences) that were most closely affiliated to the *Nitrosomonas nitrosa* lineage. Only 2 other sequences from the post aquaculture sediment and lotus pond water grouped closely with *Nitrosomonas oligotropha* and *Nitrosomonas europaea* like sequences.

The phylogenetic tree based on the CTO sequences showed that the 107 clones were distributed throughout six clusters, three of which fell within the *Nitrosospira* lineage (Clusters CTO 4, 5, and 6), and the remaining three under the *Nitrosomonas* lineage (Clusters CTO 1, 2, and 3) (Figure 4.18). Nonetheless, a large number of the clones (44 out of 107) clustered together in CTO Cluster 6. CTO cluster 6 consisted of sequences isolated from all the types of sites in our study except the post aquaculture sediment samples and had a high number of sequences isolated from the water samples of the post aquaculture and idle ponds (16 and 19 sequences, respectively). A total of 30 sequences from CTO cluster 5 and 22 sequences in CTO cluster 4 originated mainly from the post aquaculture pond, and grouped closely with uncultured *Nitrosospira* sequences isolated from paddy soils, activated sludge, polluted mangrove sediments and eutrophic lakes. All the CTO sequences under the *Nitrosospira* lineage in this study fell into distinct clusters and did not group with any of the currently known main lineages/pure culture sequences of the *Nitrosospira* division, suggesting that they are new groups of AOB that are found in disused mining-sites or similar environments. The remaining three clusters falling under the *Nitrosomonas* division contained sequences that originated only from the lotus and post aquaculture pond. These sequences affiliated closely with the *Nitrosomonas oligotropha* and *Nitrosomonas* lineage, and one cluster, CTO2 was a distinct cluster comprising seven sequences mainly isolated from the lotus pond, and grouped with other uncultured *Nitrosomonas* sequences from activated sludge and wastewater treatment sludges.

Taking both the *amo*A and CTO phylogenetic trees into consideration (Figure 3 and Figure 4) it is evident that both *Nitrosomonas* and *Nitrosospira*-like sequences are present in our study sites. We observed that the lotus pond seemed to show the largest richness of AOB, with clone sequences phylogenetically related to *N. europaea*-like, *N. oligotropha*-like, *N. communis*-like, *N. nitrosa*-like, and uncultured *Nitrosospira* lineages (Table 4.4). Clone sequences from the post aquaculture ponds were mostly affiliated to uncultured *Nitrosospira* sequences, but also consisted of a small number of *Nitrosomonas*-like affiliated clones. These *Nitrosomonas*-like clones originated from the sediment samples and were not closely affiliated with currently known *Nitrosomonas* lineages (Cluster amo1a). In contrast, clone sequences from the idle pond which harboured the least richness, grouped mainly with the uncultured *Nitrosospira* sequences. A summary of the AOB community detected from the different disused tin-mining ponds is presented in Table 4.4.

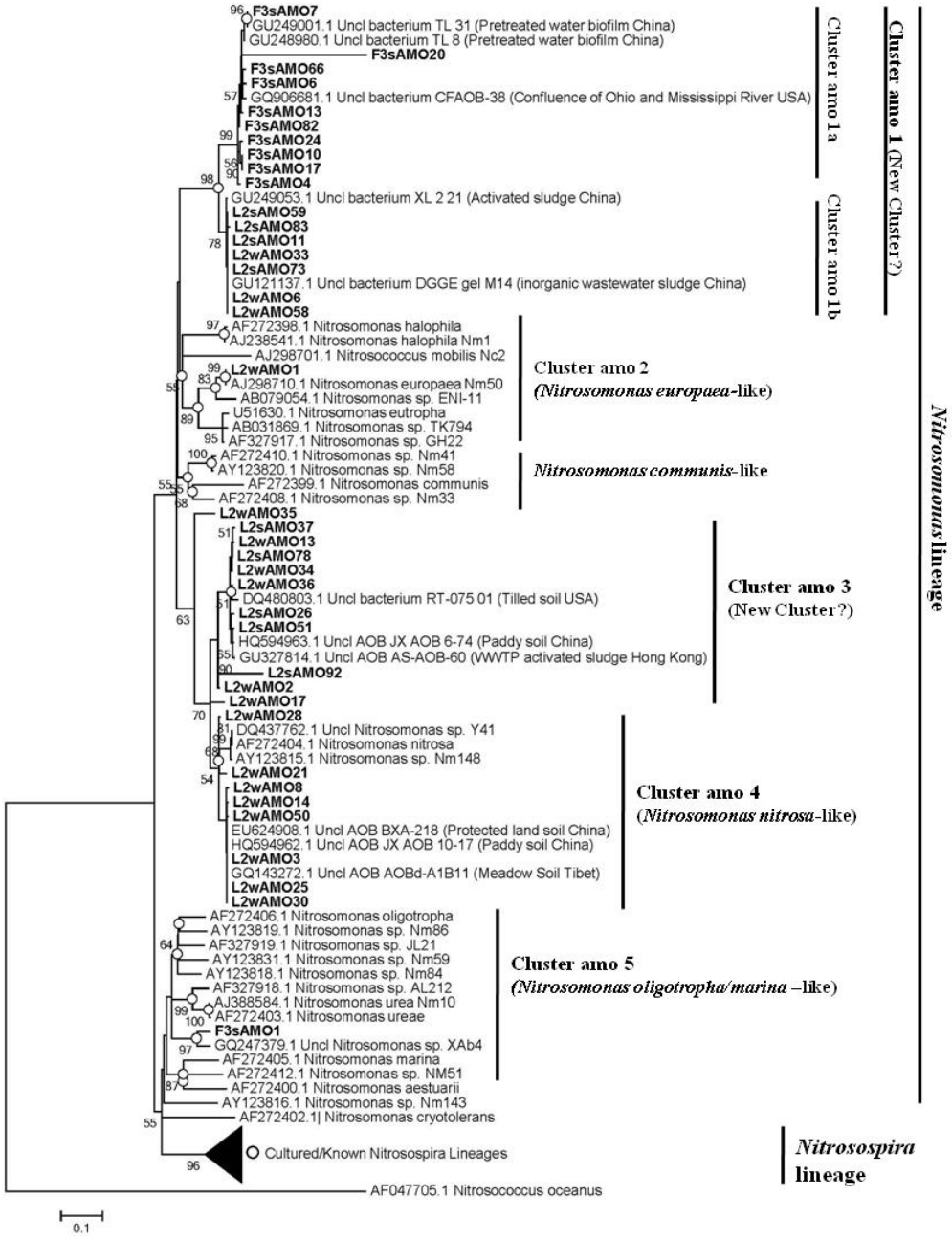


Figure 4.17: Maximum Likelihood (ML) phylogenetic tree constructed based on *amo*A nucleotide sequences of β-subdivision *Proteobacteria*. Clone sequences from this study are depicted in bold, where 'F', 'L' and 'I' represent sequences from ponds with previous aquaculture, lotus and idle ponds, respectively. The ensuing 'w' or 's' represent water or sediment samples.. The tree was rooted with *amo*A sequences of γ-subdivision *Proteobacteria*. Branch nodes supported by maximum parsimony (MP) and NJ analyses are indicated as closed white circles. Numbers at the branch are bootstrap values from the NJ analysis. Values below 50% are not shown. Reference *amo*A sequences from both cultured and environmental *pmo*A clones obtained from GenBank are shown here preceded by their respective accession numbers. from both cultured and environmental *pmo*A clones obtained from GenBank are shown here preceded by their respective accession numbers.

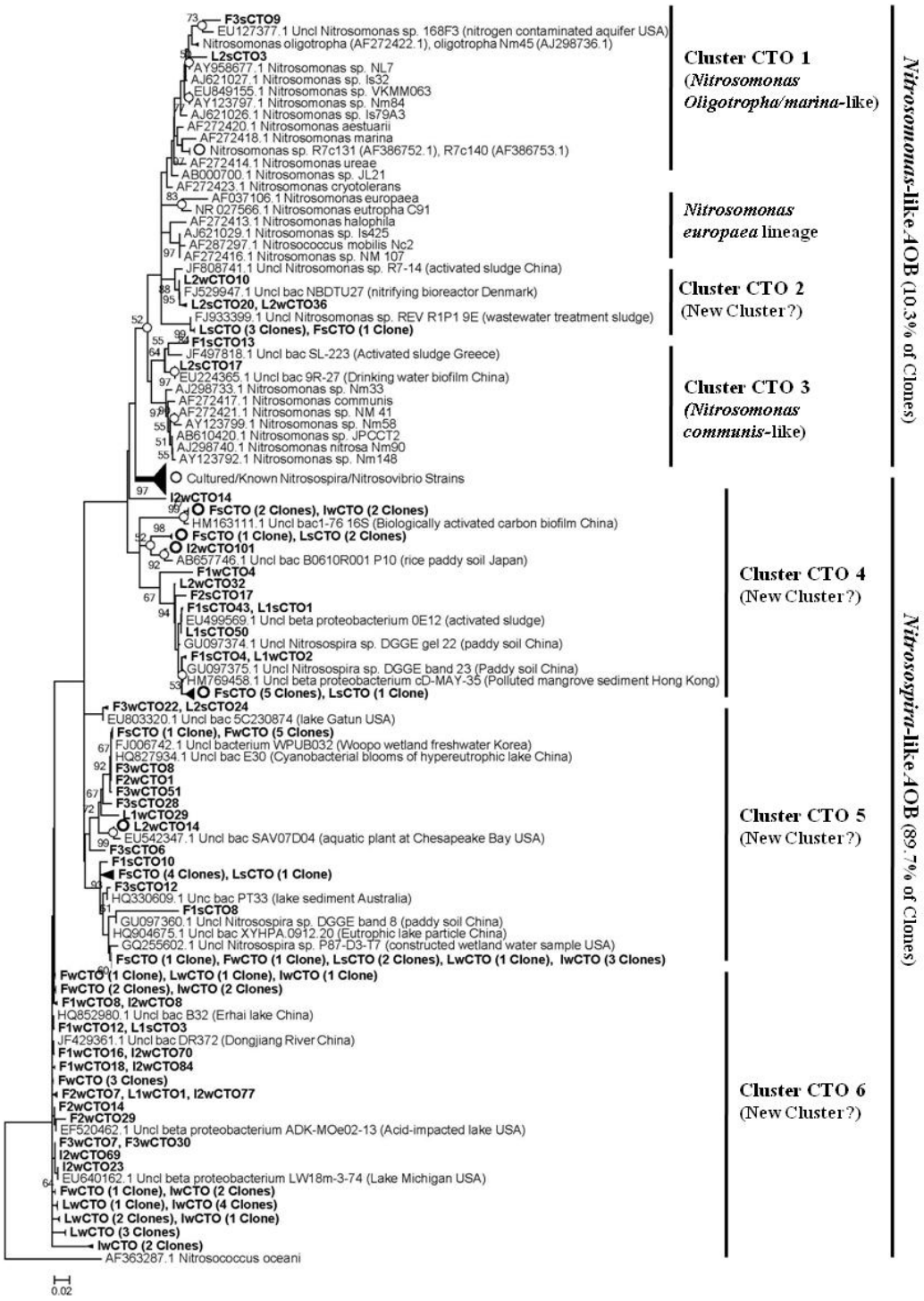


Figure 4.18: Maximum-likelihood (ML) phylogenetic tree constructed based on 16S rRNA nucleotide sequences of β-subdivision *Proteobacteria*. Clone sequences from this study are depicted in bold, where 'F', 'L' and 'I' represent sequences from ponds with previous aquaculture, lotus, and idle ponds, respectively. The ensuing 'w' or 's' represent water or sediment samples. The tree was rooted with 16S rRNA gene sequences of γ-subdivision *Proteobacteria*. Branch nodes supported by maximum parsimony (MP) and NJ analyses are indicated as closed white circles. Numbers at the branch are bootstrap values from the NJ analysis. Values below 50% are not shown. Reference 16S rDNA sequences from both cultured and environmental *pmo*A clones obtained from GenBank are shown here preceded by their respective accession numbers.

Table 4.4: Summary of AOB population found in the different disused tin-mining ponds based on phylogenetic analysis.

Sampling Site	Sample Type	Closest Relative (Based on Phylogenetic Analysis)	Type of Clone (No. of Clones/Total No. of Clones)
Ponds with Previous aquaculture activity	*Sediment*	*Nitrosomonas oligotropha*	*amo*A (1/38), CTO (1/107)
		Nitrosomonas communis	CTO (1/107)
		Uncultured *Nitrosospira* sp.	CTO (20/107)
		Uncultured *Nitrosomonas*-like (Cluster amo 1a)	*amo*A (10/107)
	Water	Uncultured *Nitrosospira sp.*	CTO (27/107)
Ponds with lotus plants	*Sediment*	*Nitrosomonas oligotropha*	CTO (1/107)
		Nitrosomonas communis	*CTO* (1/107)
		Uncultured *Nitrosomonas*-like (Cluster amo 1b, amo 3, CTO 2)	*amo*A (l6/38), CTO (4/107)
		Uncultured *Nitrosospira* sp.	CTO (10/107)
	Water	*Nitrosomonas nitrosa*	*amo*A (9/38)
		Uncultured *Nitrosomonas*-like (Cluster amo 1b, amo 3, CTO 2)	*amo*A (8/38), CTO (3/107)
		Nitrosomonas europaea	*amo*A (1/38)
		Uncultured *Nitrosospira* sp.	CTO (13/107)
Ponds with no significant activity (Idle)	*Sediment*	No AOBs detected	-
	Water	Uncultured *Nitrosospira* sp.	CTO (26/107)

4.9.2 Phylogenetic Analysis of the Methane Oxidizing Bacteria Community

Altogether, a total of six clone clusters could be identified from the 165 *pmo*A sequences obtained from the various sites in this study (Figure 4.19). Four (clusters MOB 1 - MOB 4), with a total of 132 clones, belonged to the γ-*Proteobacteria Methylococcaceae* family (Type I MOB), while the other 2 grouped with the α-*Protoebacteria Methylocystaceae* family (Type II MOB). 45% of the clones fell within the MOB 1 cluster (75 clone sequences), which were closely affiliated to the *Methylococcus*-like lineage. Some uncultured methanotoph *pmo*A sequences that grouped closely within this cluster include those isolated from lakes, lake sediments and wetlands (Lake Constance, Lake Kinneret) and 2 sequences from coal mine and oil field soils (Han et al., 2009). Of the 77 clone sequences in the MOB 1 cluster, the majority were sequences isolated from water samples (49 sequences), with the most being water samples isolated from the idle pond (21 sequences). Cluster MOB 2 were closely related to sequences isolated from rice fields in India and rice roots (Lüke et al., 2010) but was a distinct cluster not closely related to any pure culture sequences or known lineages. The MOB 2 cluster consisted of 34 sequences from all sites of study but had a high number of sequences from the post aquaculture pond sediments (16 sequences). Cluster MOB 3, with 8 sequences only from the sediments of the post aquaculture and lotus ponds, were closely related to cultured *Methylobacter*-like sequences. On the other hand, cluster MOB 4 had 11 sequences originating from the water and sediment samples of the post aquaculture and lotus ponds, and were closely affiliated to *Methylomonas* spp. and *Methylomicrobium pelagicum* sequences.

From the remaining clones clustered belonging to the *Methylocystaceae* family, Cluster MOB 5 consisted of only 3 clones (1 lotus pond sediment, 2 idle pond sediment) which related closely to *Methylosinus sporium* H1b. In contrast, cluster MOB6 harboured 29 clones from all sites in this study, a high number being from the water samples of the post-aquaculture pond. Most of the sequences in this cluster were closely related to *Methylocystis* strains and pure cultures isolated from other studies, but three sequences were closely related to *Methylosinus* sp. D28 and *Methylosinus* sp. LW2. Uncultured sequences from other studies that clustered within this group include sequences that were isolated from Lake Kinneret, rhizosheric soils of flooded rice fields (Shrestha et al., 2010), river aquifers (Erwin et al., 2005) and one trichloroethylene-degrading methanotroph isolated from rice rhizosphere (Shukla et al., 2010). A summary of the MOB community detected from the different disused tin-mining ponds are presented in Table 4.5.

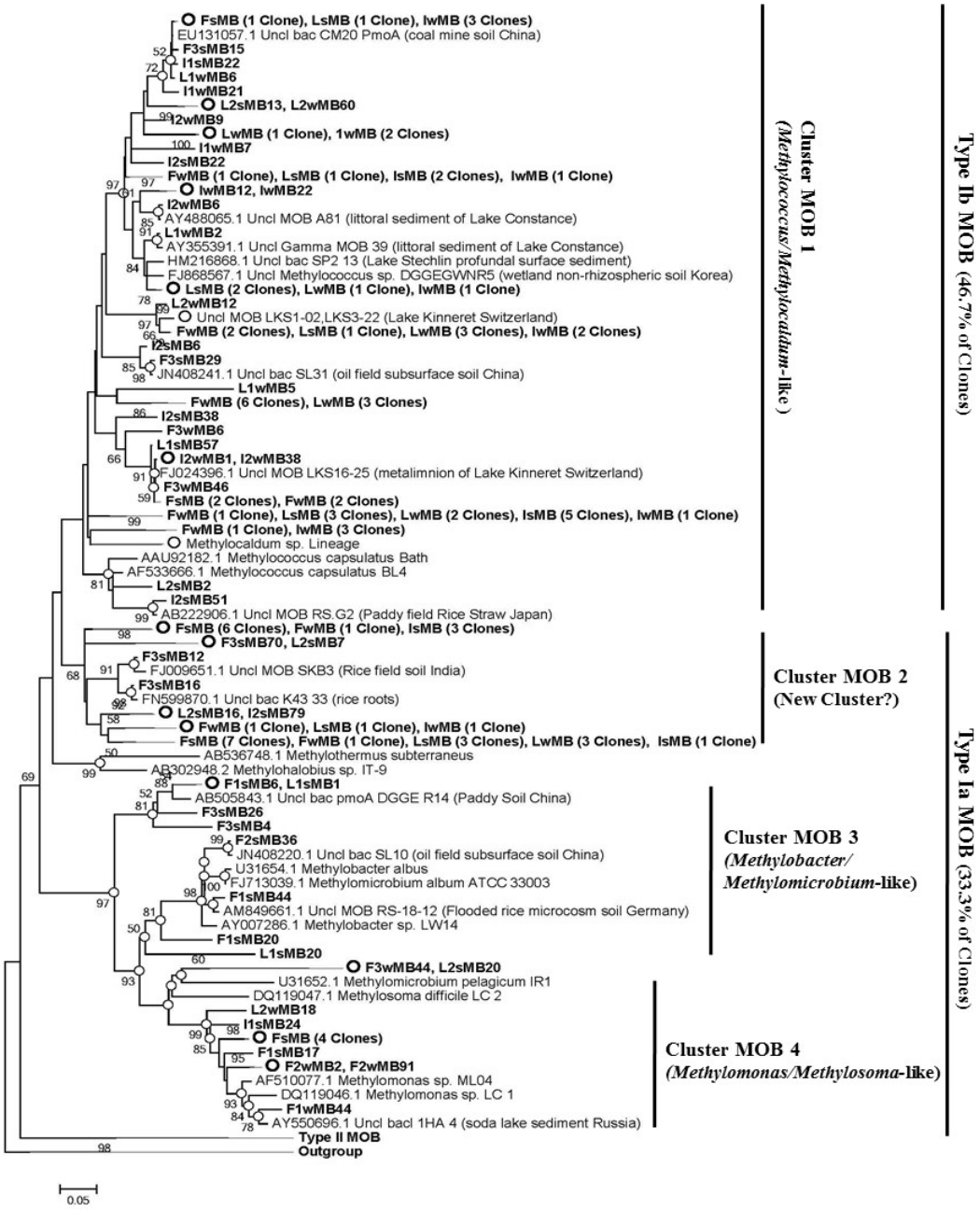


Figure 4.19 (a): Maximum-likelihood (ML) phylogenetic tree constructed based on *pmo*A clone library nucleotide sequences, indicating the Type I MOBs. Clone sequences from this study are depicted in bold, where 'F', 'L' and 'I' represent sequences from ponds with previous aquaculture, lotus, and idle ponds, respectively. The ensuing 'w' or 's' represent water or sediment samples. The tree was rooted with *amo*A sequences of the β-subdivision *Proteobacteria*. Numbers at the branch are bootstrap values from the ML analysis. Branch nodes supported by maximum parsimony (MP) and NJ analyses are indicated as closed white circles.Values below 50% are not shown. Reference *pmo*A sequences from both cultured and environmental *pmo*A clones obtained from GenBank are shown here preceded by their respective accession numbers.

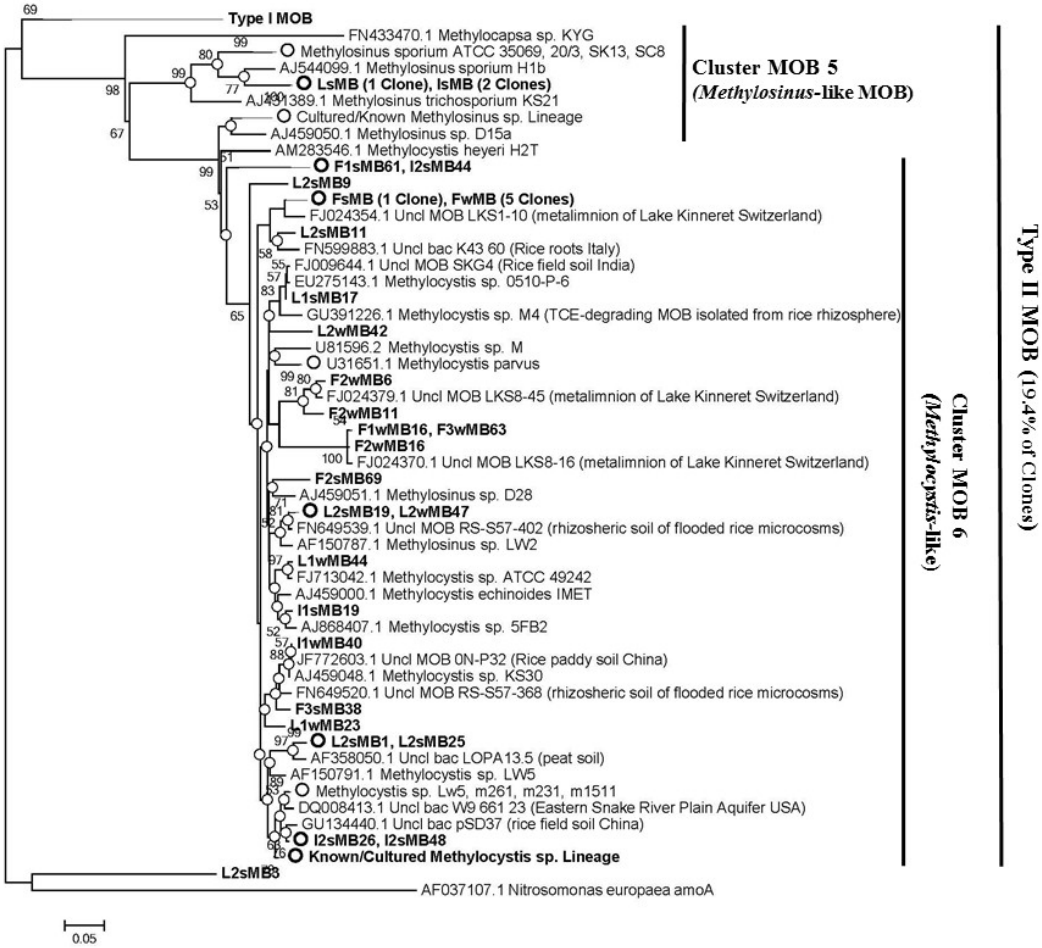


Figure 4.19 (b): Maximum-likelihood (ML) phylogenetic tree constructed based on *pmo*A clone library nucleotide sequences, indicating the Type II MOBs. Clone sequences from this study are depicted in bold, where 'F', 'L' and 'I' represent sequences from ponds with previous aquaculture, lotus, and idle ponds, respectively. The ensuing 'w' or 's' represent water or sediment samples. The tree was rooted with *amo*A sequences eof the β-subdivision *Proteobacteria*. Numbers at the branch are bootstrap values from the ML analysis. Branch nodes supported by maximum parsimony (MP) and NJ analyses are indicated as closed white circles. Values below 50% are not shown. Reference *pmo*A sequences from both cultured and environmental *pmo*A clones obtained from GenBank are shown here preceded by their respective accession numbers.

Table 4.5: Summary of MOB population found in the different disused tin-mining ponds based on phylogenetic analysis of *pmo*A clone sequences.

Sampling Site	Sample Type	Closest Relative (Based on Phylogenetic Analysis)	No. Of Clones
Ponds with Previous aquaculture activity 6	*Sediment*	*Methylococcus*-like	5/165
		Methylobacter-like	6/165
		Methylomonas-like	5/165
		Uncultured Gamma MOB (Cluster MOB 2)	16/165
		Methylocystis-like	3/165
		Methylosinus-like	1/165
	Water	*Methylococcus*-like	15/165
		Methylomonas-like	4/165
		Uncultured Gamma MOB (Cluster MOB 2)	3/165
		Methylocystis-like	10/165
Ponds with lotus plants	*Sediment*	*Methylococcus*-like	10/165
		Methylobacter-like	2/165
		Methylomonas-like	1/165
		Uncultured Gamma MOB (Cluster MOB 2)	8/165
		Methylocystis-like	6/165
		Methylosinus-like	1/165
	Water	*Methylococcus*-like	12/165
		Methylomonas-like	1/165
		Uncultured Gamma MOB (Cluster MOB 2)	3/165
		Methylocystis-like	5/165
		Methylosinus-like	1/165
Ponds with no significant activity (Idle)	*Sediment*	*Methylococcus*-like	14/165
		Uncultured Gamma MOB (Cluster MOB 2)	5/165
		Methylocystis-like	4/165
		Methylosinus-like	1/165
	Water	*Methylococcus*-like	21/165
		Uncultured Gamma MOB (Cluster MOB 2)	1/165
		Methylocystis-like	1/165

4.10 Multiple Sequence Alignment of Nucleic Acid and Deduced Amino Acid Sequences

The 38 AmoA deduced amino acid sequences obtained from this study were aligned together with several sequences of the main cultured, known lineages of ammonia-oxidizing bacteria (Figure 4.20). Variable regions are observed to be widely dispersed across the 138 amino acid residues and constitute approximately 9.4% (13/138) of the residues, but showed similar positions for the region of variation in comparison to the AmoA amino acid alignments by Cao et al. (2011a). One sequence, F3sAMO20, contained substantially more hypervariable sites (39/138 residues, 28.3%) compared to the other sequences.

In contrast, nucleic acid alignments for the 16S rRNA gene of the CTO clones showed a region of hypervariation that concentrated at the position 254 - 303 of the 424 bp sequence (Figure 4.21). The hypervariable region observed was made up of 11.6% (49/424) of the sequence and corresponded to similar positions that were seen when only 16S rRNA gene sequences of pure culture and established lineages of ammonia-oxidizing bacteria were aligned.

Similar to the AmoA, multiple sequence alignment of the deduced PmoA amino acid sequences from this study with reference sequences of known methane oxidizing bacteria lineages also showed widely dispersed variable regions across the 154 long sequence (Figure 4.22). Approximately 20% (31/154) of the residues were hypervariable sites and a comparison to the alignment shown by Fjellbirkeland et al. (2001) also showed the hypervariable regions to be present at similar positions/sites.

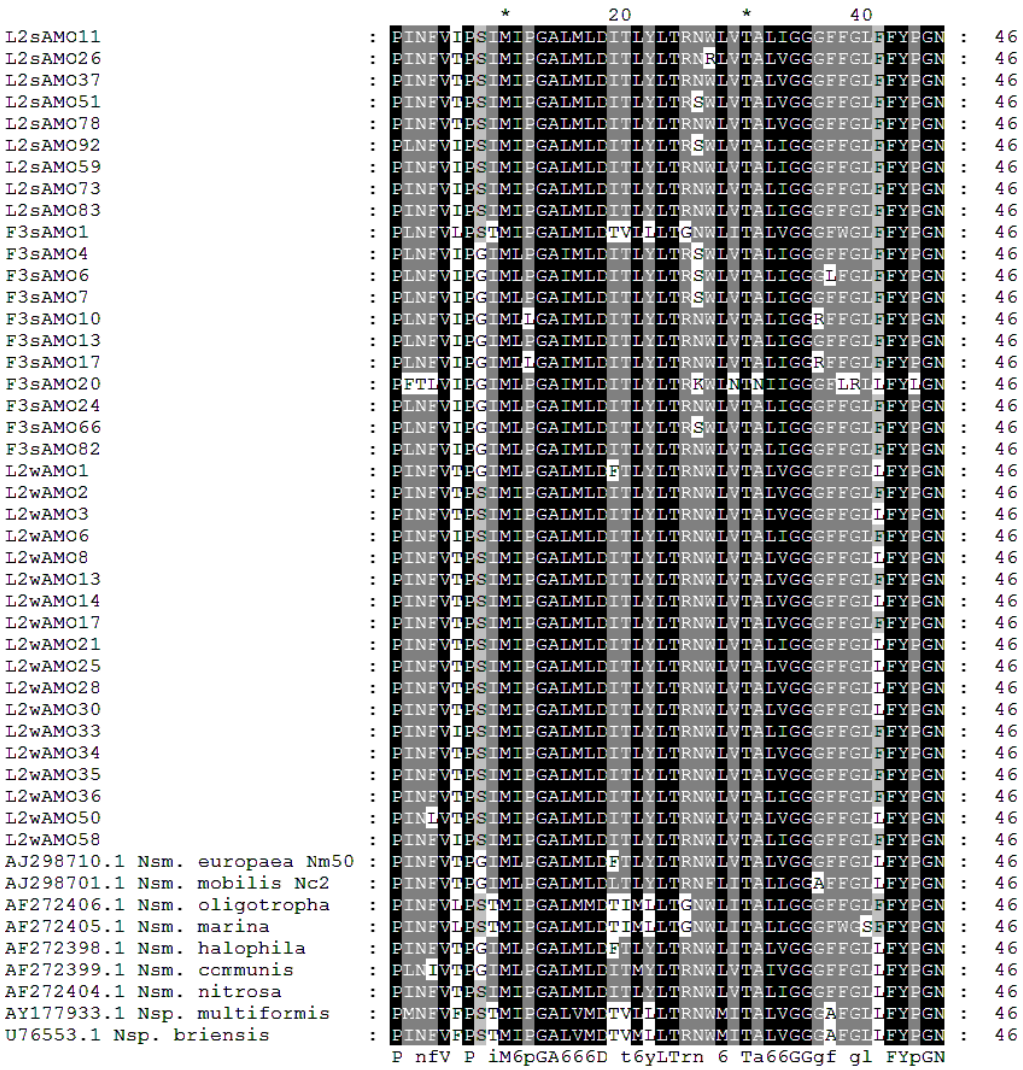


Figure 4.20: Multiple sequence alignment of deduced AmoA amino acid sequence alignments of AOBs detected in this study and closely related lineages. Residues boxed in black are conserved in all the sequences. Residues in dark and light grey are conserved in more than 80% or 60% of the sequences respectively. Nsm. - *Nitrosomonas*; Nsp. - *Nitrosospira*; Nsc. – *Nitrosococcus*.

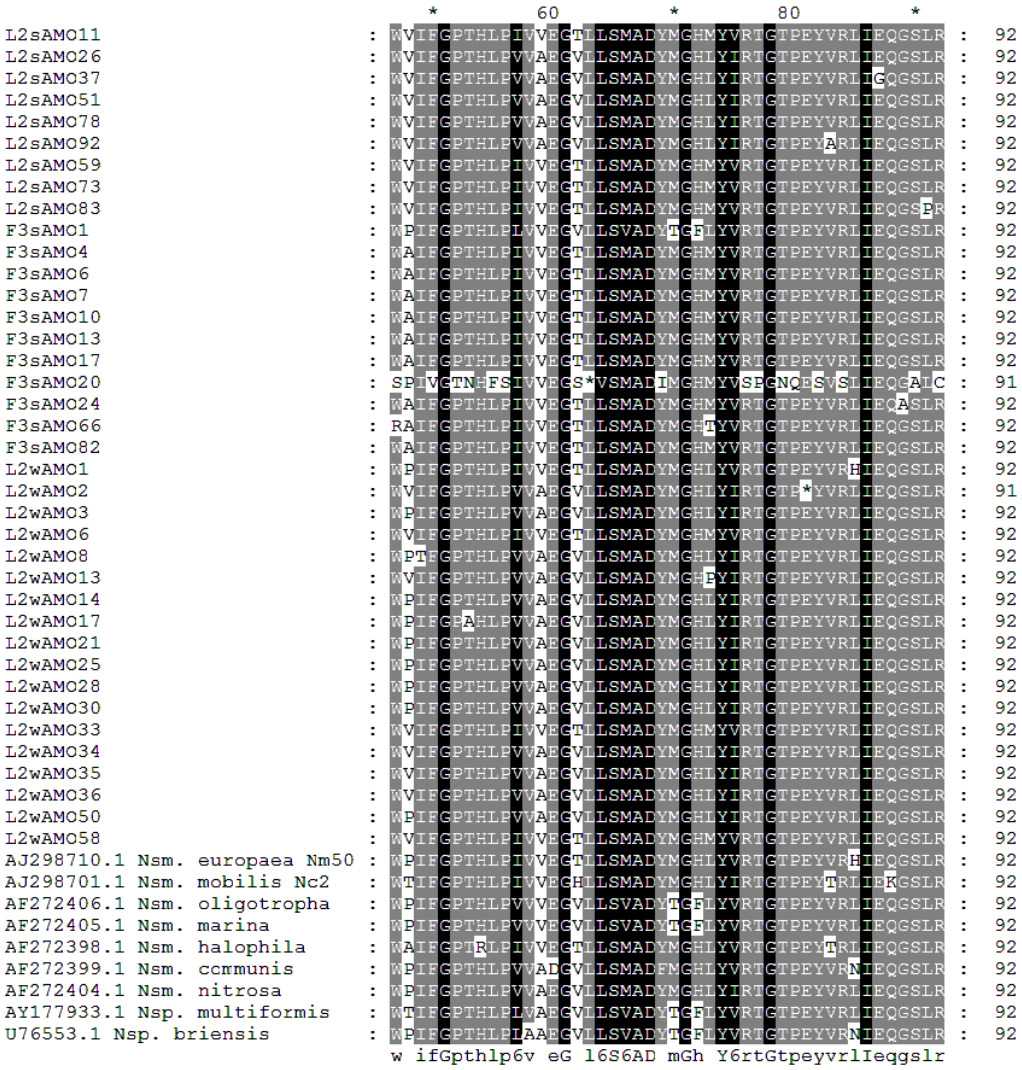


Figure 4.20 (continued): Multiple sequence alignment of deduced AmoA amino acid sequence alignments of AOBs detected in this study and closely related lineages.

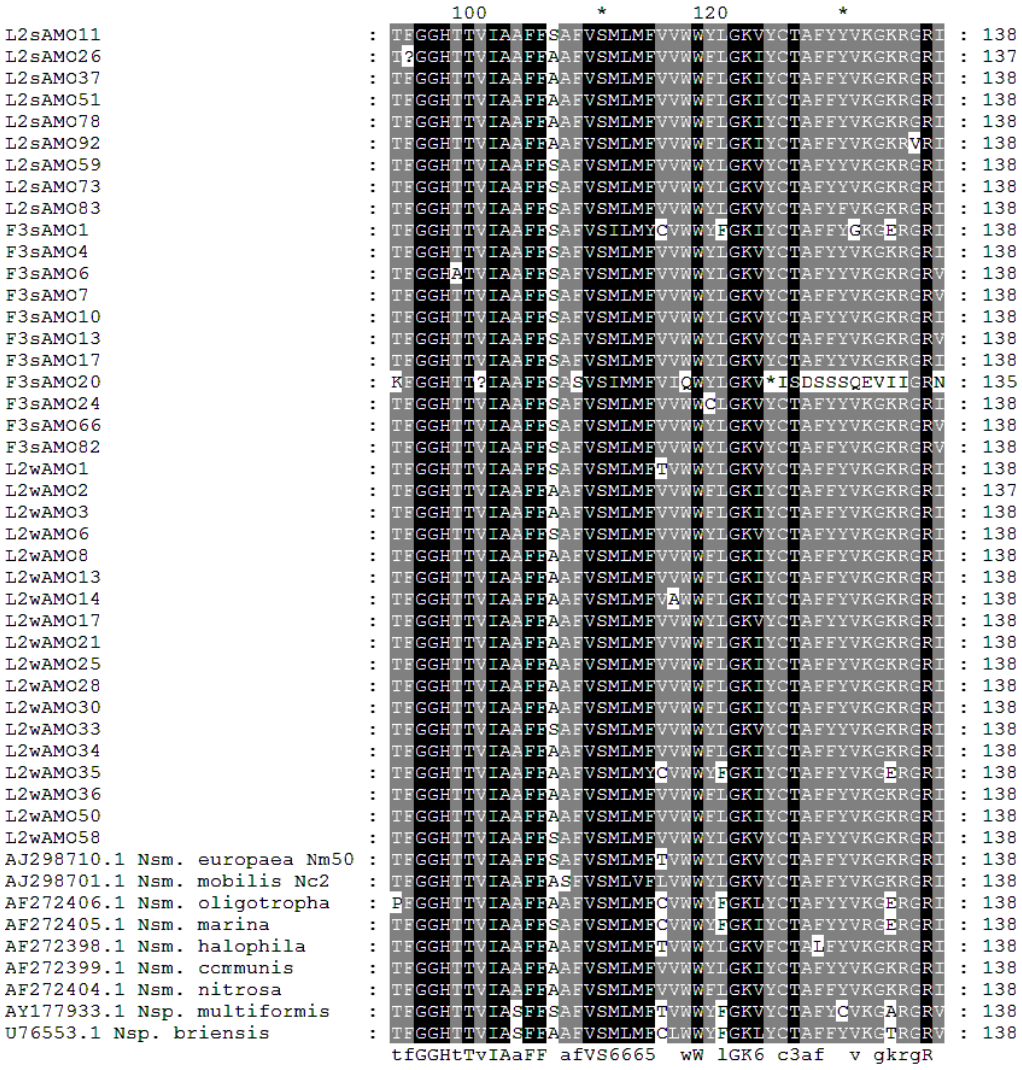


Figure 4.20 (continued): Multiple sequence alignment of deduced AmoA amino acid sequence alignments of AOBs detected in this study and closely related lineages.

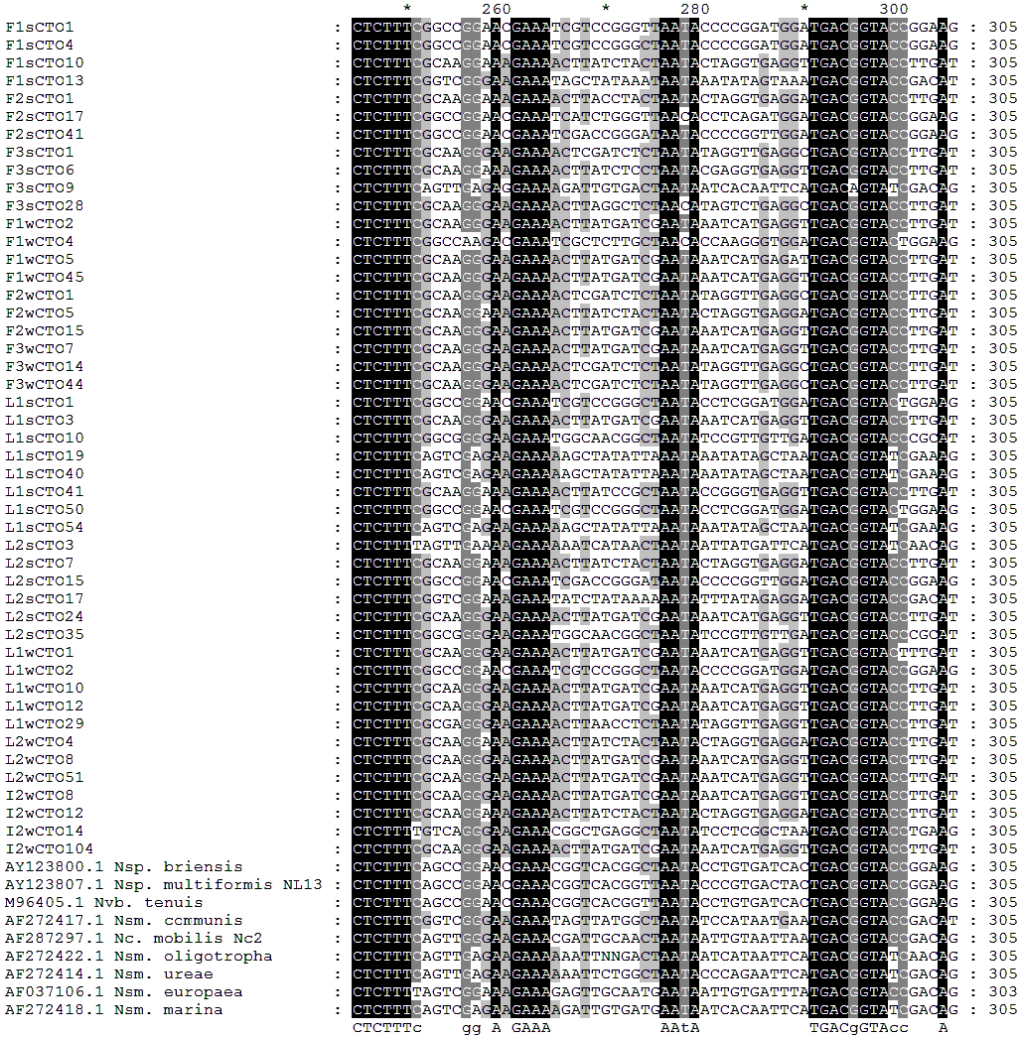


Figure 4.21: Partial multiple sequence alignment of 16S rDNA nucleic acid alignments of selected AOBs detected in this study and closely related lineages, indicating the region of hypervariation that is concentrated at the position 254 - 303 of the 424 bp sequence. Residues boxed in black are conserved in all the sequences. Residues in dark and light grey are conserved in more than 80% or 60% of the sequences respectively. Nsm. - *Nitrosomonas*; Nsp. - *Nitrosospira*; Nsc. - *Nitrosococcus*. The full multiple sequence alignment is shown in Appendix E.

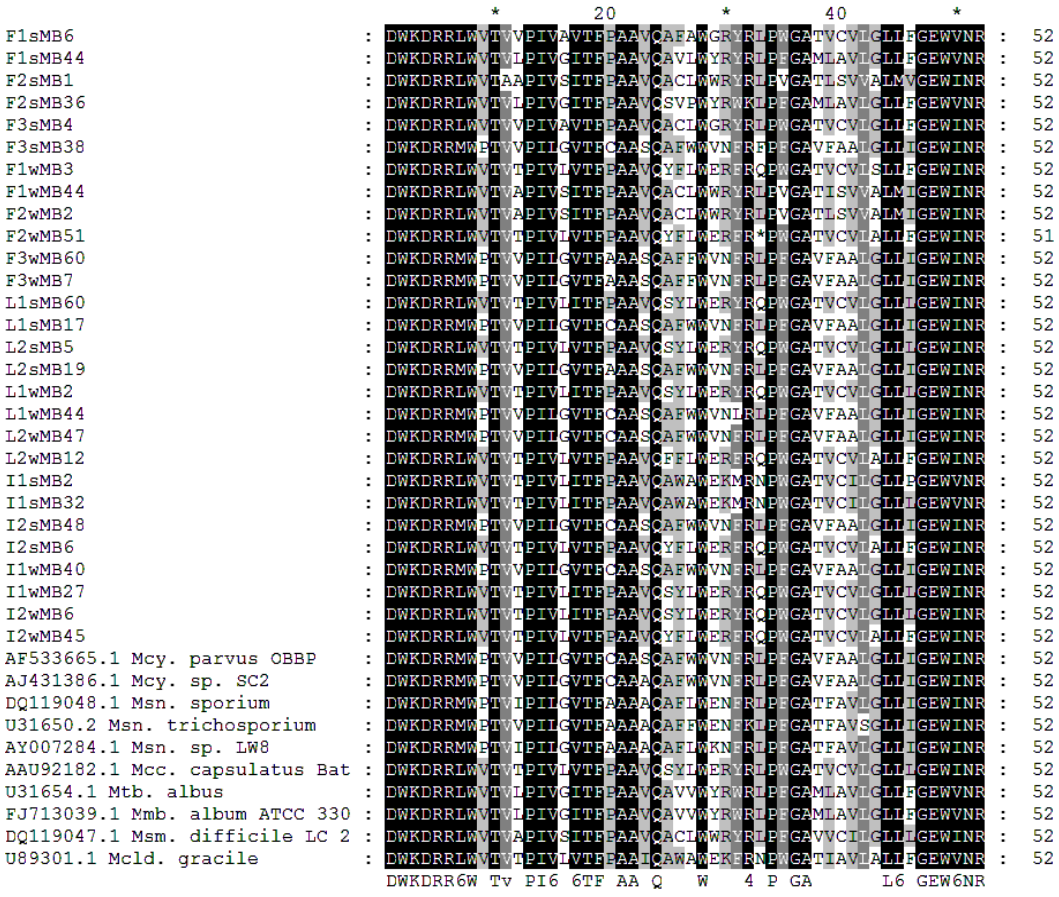


Figure 4.22: Multiple sequence alignment of deduced PmoA amino acid sequence alignments of selected MOBs detected in this study and closely related lineages. Residues boxed in black are conserved in all the sequences. Residues in dark and light grey are conserved in more than 80% or 60% of the sequences respectively Mcy. - *Methylocystis*; Msn - *Methylosinus*; Mcc. - *Methylococcus*; Mtb. - *Methylobacter*; Mmb. - *Methylomicrobium*; Msm. - *Methylosoma*; Mcld. –*Methylocaldum*

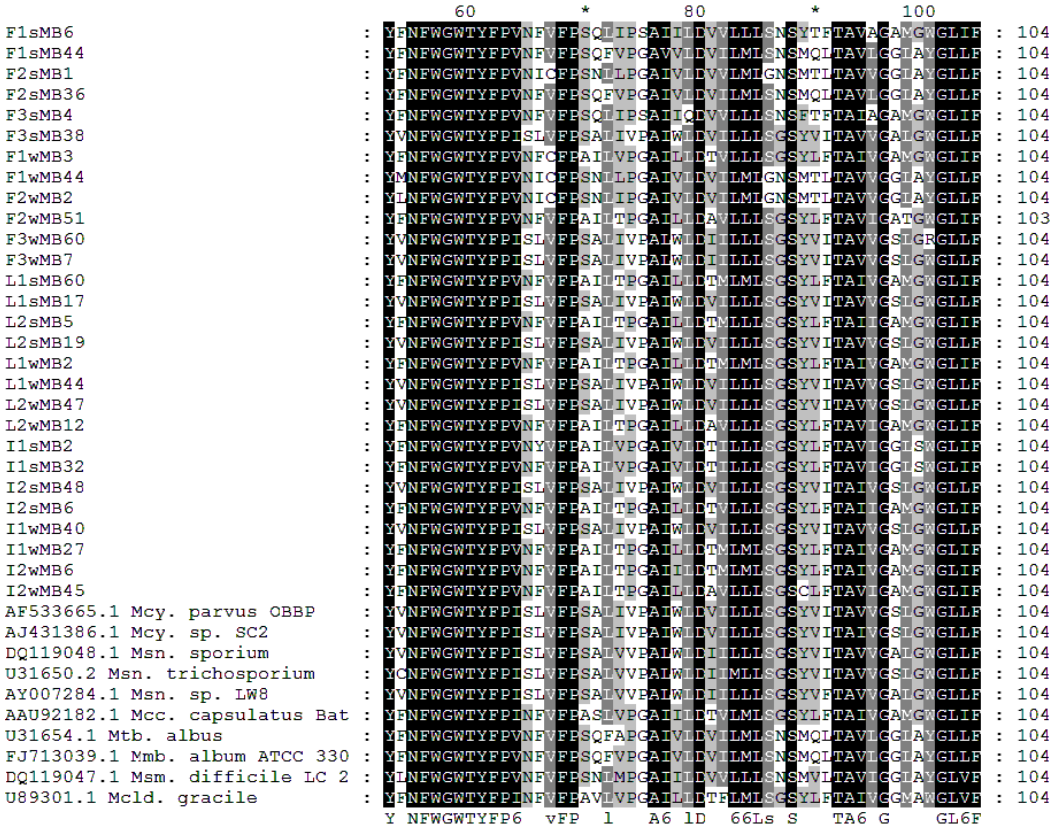


Figure 4.22 (continued): Multiple sequence alignment of deduced PmoA amino acid sequence alignments of selected MOBs detected in this study and closely related lineages.

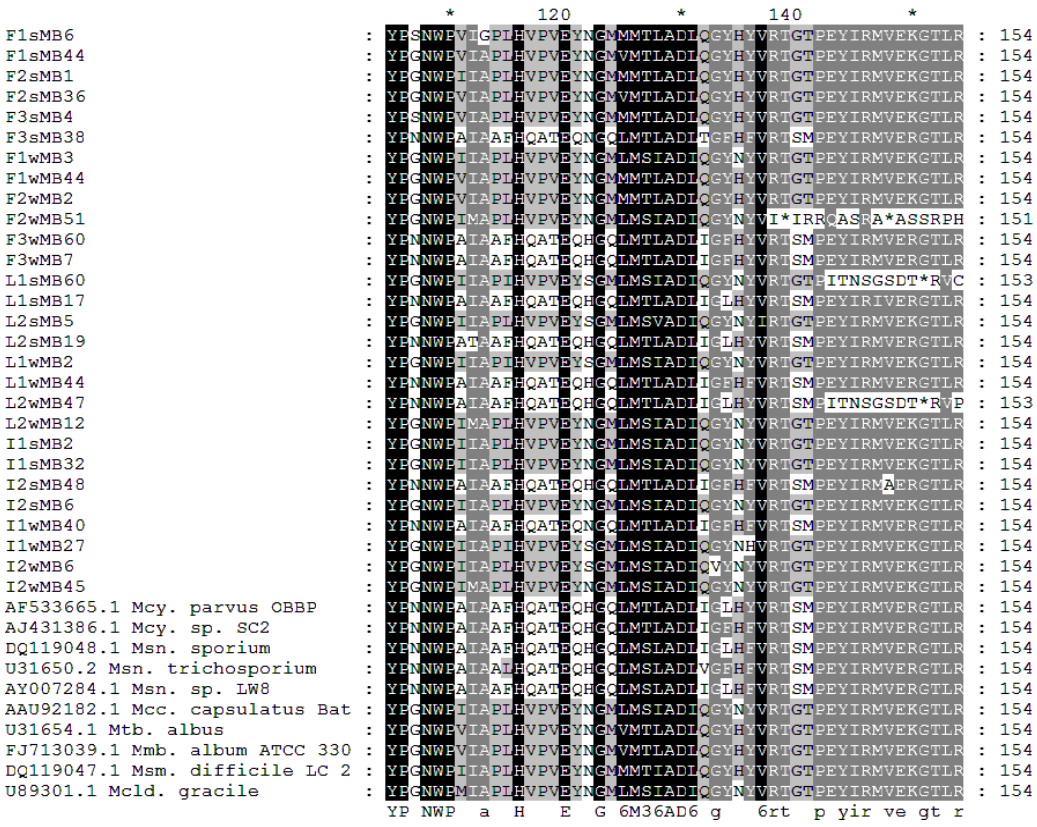


Figure 4.22 (continued): Multiple sequence alignment of deduced PmoA amino acid sequence alignments of selected MOBs detected in this study and closely related lineages.

4.11 Community Structure and Classification of the AOB and MOB

For the AOBs, two main groups could be observed for the weighted UniFrac Jackknife environmental clustering analysis of the *amo*A sequences (Figure 4.24), and three main groups for clustering analysis of the CTO sequences (Figure 4.26). The post-aquaculture sediment AOB community seems to be consistently separated from the rest, whether based on clustering analysis of the *amo*A or CTO sequences. On the other hand, the AOB community of the water samples from the three pond types clustered together (Figure 4.26). These observations were further supported by the weighted UniFrac Principle Coordinate analysis (PCoA) of the CTO sequences (Figure 4.25). The first principle coordinate (P1), explaining 86.85% of the total community variability, also clearly indicated a close clustering of AOB communities from the water samples and showed the post aquaculture sediment community to be isolated from the rest. The all-environment UniFrac significance test indicated a marginally significant difference ($P = 0.05$) among the AOB communities (from the different environments) deduced from the CTO sequences, and a significant difference ($P \leq 0.01$) amongst the communities deduced from the *amoA* sequences.

Three main groups were observed for the Jackknife environmental clustering analysis of the *pmo*A sequences (Figure 4.28). MOB community of the post-aquaculture and idle pond sediment samples, and lotus pond water samples formed a cluster, while the post aquaculture water and lotus sediment MOB community formed another cluster. The idle pond water community separated from the rest, as similarly observed in the P1 of the *pmo*A PCoA analysis

where 50.01% of the total community variability is explained (Figure 4.27). A significant difference ($P \leq 0.01$) among the MOB communities of the varying environments was obtained from the all environment significance test.

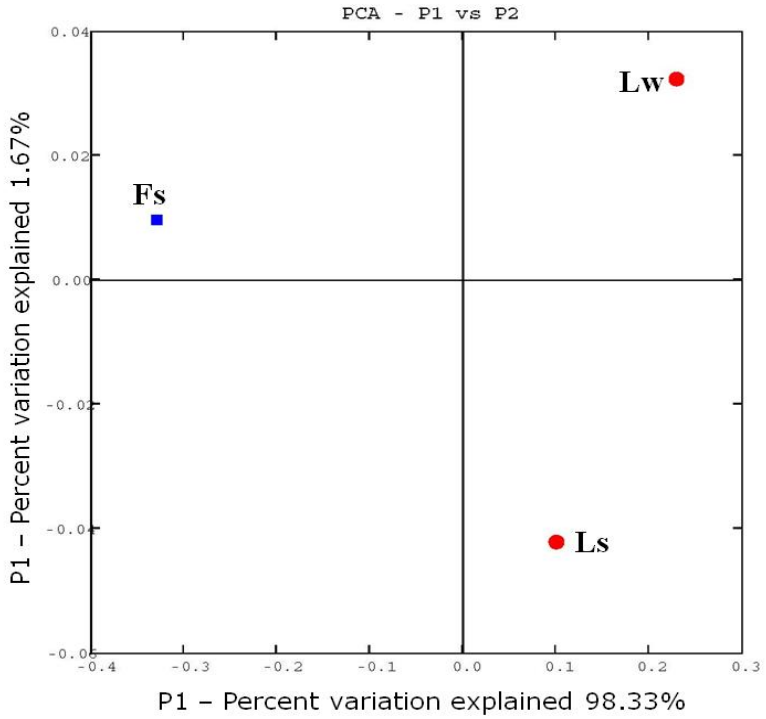


Figure 4.23: Weighted UniFrac Principle Coordinate Analyses (PCoA) for ammonia oxidizing bacteria based on the *amo*A clones. Fs - fish pond/post-aquaculture pond sediment; Ls - lotus pond sediment; Lw - lotus pond water.

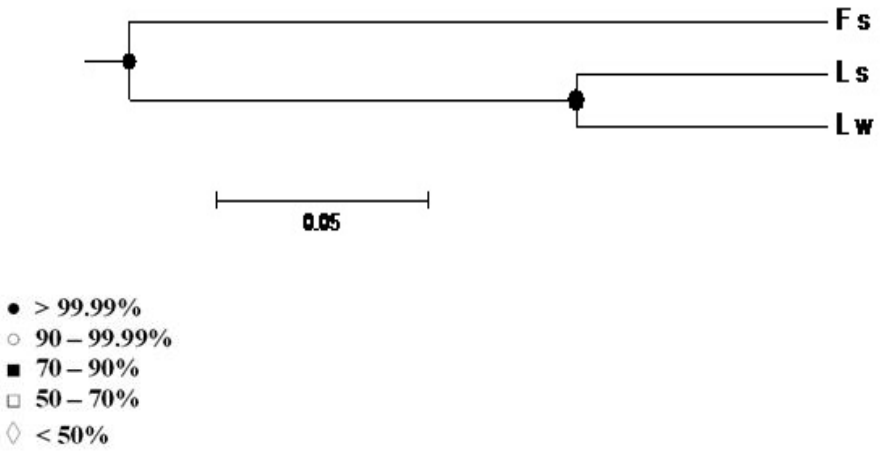


Figure 4.24: Weighted UniFrac Jackknife Environment Clusters for ammonia oxidizing bacteria based on the *amoA* clones. Fs - fish pond/post-aquaculture pond sediment; Ls - lotus pond sediment; Lw - lotus pond water.

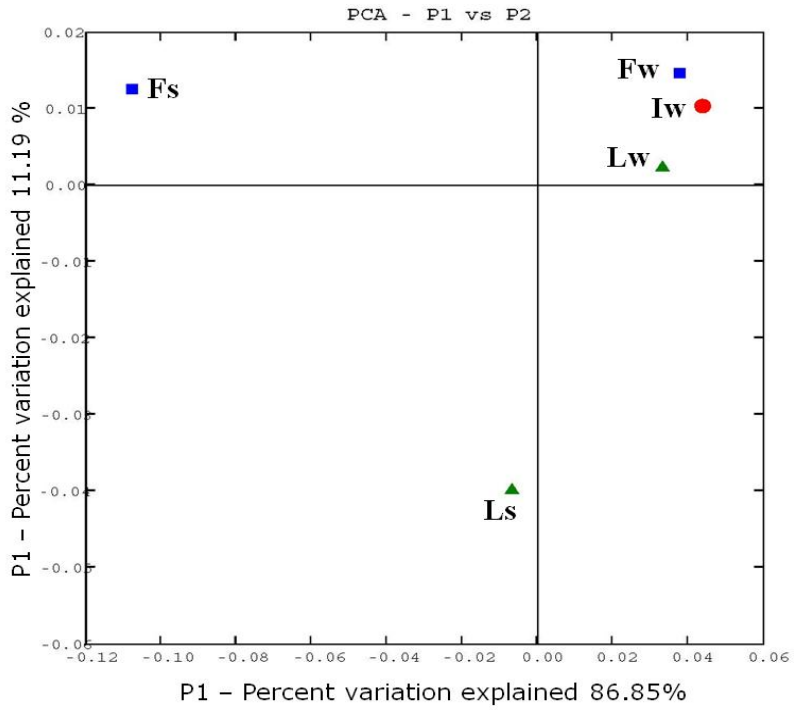


Figure 4.25: Weighted UniFrac Principle Coordinate Analyses (PCoA) for ammonia oxidizing bacteria based on the CTOclones. Fs - fish pond/post-aquaculture pond sediment; Fw - fish pond water; Ls - lotus pond sediment; Lw - lotus pond water; Iw - Idle pond water.

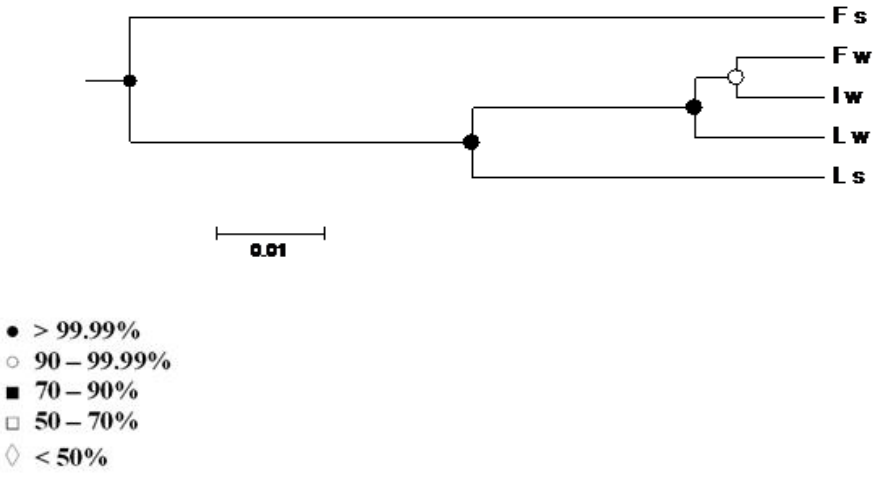


Figure 4.26: Weighted UniFrac Jackknife Environment Clusters for ammonia oxidizing bacteria based on the CTO clones. Fs - fish pond/post-aquaculture pond sediment; Fw - fish pond water; Ls - lotus pond sediment; Lw - lotus pond water; Iw - Idle pond water.

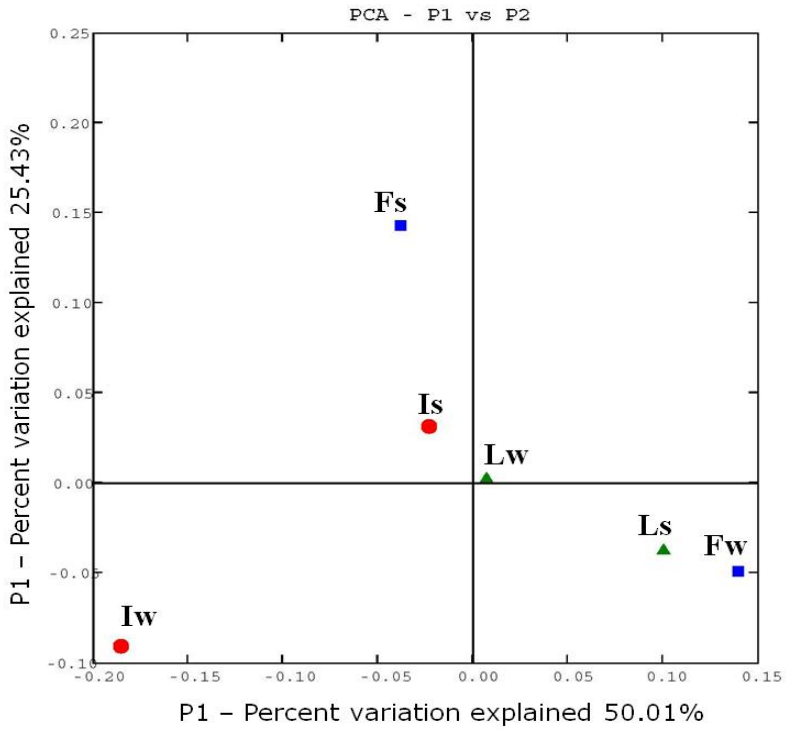


Figure 4.27: Weighted UniFrac Principle Coordinate Analyses (PCoA) for methane oxidizing bacteria based on the *pmo*Aclones. Fs - fish pond/post-aquaculture pond sediment; Fw - fish pond water; Ls - lotus pond sediment; Lw - lotus pond water; Is - Idle pond sediment; Iw - Idle pond water.

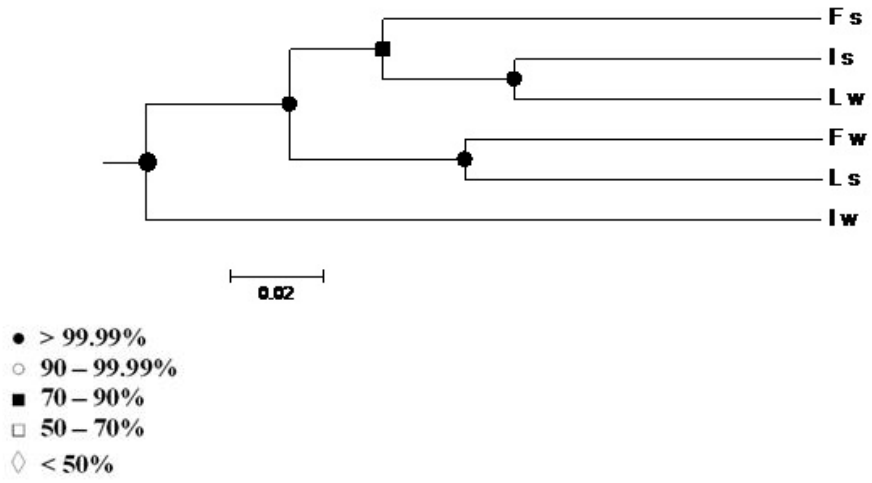


Figure 4.28: Weighted UniFrac Jackknife Environment Clusters for methane oxidizing bacteria based on the *pmo*A clones. Fs - fish pond/post-aquaculture pond sediment; Fw - fish pond water; Ls - lotus pond sediment; Lw - lotus pond water; Is - Idle pond sediment; Iw - Idle pond water.

CHAPTER 5

DISCUSSION

5.1 Physicochemical Property Variation of the Ponds

Aquatic ecosystems (i.e. lakes, ponds, rivers, streams) are complex and consolidated ecosystems that are regulated by a variety of factors, both physical (e.g. temperature, turbidity, suspended solids) and chemical (e.g. pH, dissolved oxygen, nitrate, nitrite, ammonium). As the composition, distribution, and abundance of aquatic organisms are significantly affected by the physicochemical parameters of the aquatic environment, assessing the physicochemical properties will give an idea of the interaction and relationship between organism and environment (Mustapha and Omotosho 2005). More specifically, physicochemical parameters such as pH, dissolved oxygen, ammonium (Hanson and Hanson 1996; Prinčič et al., 1998; Bodelier and Laanbroek 2004), temperature (Urakawa et al., 2008) and suspended solid concentration (Xia et al., 2009) are key factors within an environment known to affect ammonia oxidizers and methanotrophs. This study was initiated to assess the physical and chemical properties of several disused tin-mining ponds located within Kampar, Perak, Malaysia, as well as to analyse the ammonia oxidizing and methane oxidizing bacteria within the same study site in an effort to understand the relation between these bacteria and the physicochemical condition within its inhabiting site.

The physicochemical characteristics of the sampling sites, which were disused tin-mining ponds with varying past or present secondary activities, clearly showed a distinct trend of variation. The TAN concentration was three fold

higher in water sampled from the post aquaculture pond as compared to the lotus and idle pond (Figure 4.2), which might possibly be due to nitrogenous waste discharges from previous aquaculture activities. Nevertheless, the highest TAN value recorded in our study was still significantly lower than the ammonium values recorded at another marine aquaculture system (McCaig et al., 1999) but were comparable to the range of ammonium values detected at other oligotrophic freshwater basins (Calhoun and King 1998; Chen et al., 2009). On the other hand, highest nitrate values were recorded from the pond with lotus plants and the values fell within the range similar to those observed by Calhoun and King (1998) in their study on several mesotrophic and oligotrophic lakes harbouring aquatic plants. In contrast to terrestrial plants, many aquatic plants are known to favour the uptake of ammonium over nitrates as their source of nitrogen (Wahlen 1993; Gruber and Galloway 2008) since the nitrate uptake process and metabolism in aquatic environments is known to require more energy (Gruber and Galloway 2008). Aquatic macrophytes provide a suitable colonization site for the attachment of nitrifying bacteria which proceeds with bacterial nitrification (Coci et al., 2008). Hence, both the plants and nitrifying bacteria (AOB) will be in direct competition for ammonium content in the pond, which most probably explains the lower TAN and highest nitrate content in the lotus pond as a result of the accumulation of nitrate converted by the nitrifying bacteria.

The dissolved oxygen concentrations (Figure 4.1), suspended solid contents (Figure 4.5) and turbidity (Figure 4.6) were recorded to be the lowest at the lotus pond in comparison with the other two study sites and dissolved oxygen

levels were slightly lower than those measured at other known mesotrophic/oligotrophic freshwater lakes (Coci et al., 2008). Floating macrophytes such as the lotus with dense, large floating leaves are known to impede the exchange of gas between the open atmosphere and water body, and block penetration of sunlight which limits the photosynthetic processes of submerged plants, altogether reducing dissolved oxygen concentrations (Erwin et al., 2005). Nonetheless, the oxygen levels at the lotus pond were still above those which inhibit ammonia and methane oxidation (Prinčič et al., 1998). Aquatic plants are further known to stabilize sediments, restrict water movements and reduce turbulent mixing, which might attribute to the significantly lower suspended solids and turbidity readings recorded at the lotus ponds (Erwin et al., 2005).

In comparison to our study site, other disused tin-mining ponds have recorded wider ranges of pH values from acidic (pH 3.6) to neutral (pH 7.2) (Abdul-Rashid and Awang 2004; Ashraf et al., 2010). On the other hand, freshwater lakes from another study have recorded higher pH values (pH 9.5) (Coci et al., 2008) as compared to our study site. Nonetheless, the narrow range of pH values recorded at all our sites (Table 4.1) fell within the optimal pH growth range for cultured ammonia oxidizers (5.8 - 8.5) (Prinčič et al., 1998).

5.2 Quantitative & Qualitative Analyses of Clone Sequences

5.2.1 Selection of Sequence Difference Cut-off Points in Operational Taxonomic Units (OTU) determination

The conventional and established methods of restriction fragment length polymorphism (RFLP) (Massol-Deya et al., 1995) has been quite widely used to detect variation at the DNA sequence level and OTU determination for characterizing AOB and MOB communities as well as a screening method for clone redundancy at numerous different environments (Costello and Lidstrom 1999; Auman et al., 2000; You and Chen 2008; Chen et al., 2009; Dang et al., 2010b). Nevertheless, there have been reported limitations of RFLP (Muyzer 1999; Schloss and Handelsman 2005). Hence, in our study, we have included a complementary computational approach using the DOTUR program, which assigns clone sequences to OTU groups based on genetic distance between DNA sequences (Schloss and Handelsman 2005). However, the comparison of OTU-based relative richness and diversities of the *amo*A, 16S rRNA and *pmo*A genes in the environment where the OTU is determined based on genetic distance relies highly on the definition of an OTU, through the selection of an appropriate cut-off point that distinguishes between two sequence groups (Martin 2002). Depending on the target gene of interest used, the cut-off points might be different due to the varying divergence rate, phylogenetic redundancy, and evolutionary history of the gene of interest (Martin 2002; Francis et al., 2003). A cut-off point of 1 to 3% sequence difference (or 97% sequence similarity) has been most commonly used in the OTU definition of 16S rRNA genes (Hughes et al., 2001). For the 16S rRNA gene (CTO) sequences employed in the investigation of AOBs in our study, a

3% threshold was used since it is the typical point used to define OTUs at the species level (Stackebranct and Goebel 1994; Schloss and Handelsman 2005).

In the case of the functional *amo*A gene, varying divergence rates and evolutionary history (as compared to the 16S rRNA gene) warrants the careful implementation of slightly different sequence difference thresholds. In our study, we used a 5% cutoff point at the DNA level as suggested by Francis et al. (2003), who explained that this threshold was more appropriate to maintain functional relevance of more divergent functional genes. Furthermore, the 5% cut-off point has been widely used in many studies (Dang et al., 2010b; Cao et al., 2011c; Cao et al., 2011a; Cao et al., 2011b; Li et al., 2011; Wei et al., 2011), and hence the use of an equal threshold in our study would facilitate easier and a less biased comparison between studies. On the other hand, a 13% sequence difference cut-off point at the DNA level was used to define an OTU based on the functional *pmo*A gene of the methanotrophs in this study, as suggested by Degelmann et al. (2010). In reference to the linearly correlated sequence similarities between *pmo*A and 16S rRNA genes of 22 methanotrophic isolates previously mentioned by Heyer et al. (2002), Degelmann et al., (2010) concluded that a 13% difference cut-off value for methanotrophic *pmo*A-based OTUs at the species level corresponded to the 3% threshold employed for 16S rRNA gene of methanotrophs. This was based on a *pmo*A percentage sequence similarity versus 16S rRNA percentage sequence similarity plot of the gene sequence pairs of the 22 methanotrophic isolates.

5.2.2 Quantitative & Qualitative Measurement of Diversity Across Varying Communities

Both α-diversity and β-diversity are known to be important measurements of diversity in which species is assumed to be the fundamental unit of analysis (Lozupone and Knight 2008). Assigning sequences to OTUs and estimating the total number of species (species richness) within a community satisfies α-diversity, the diversity within a given community. We also address β-diversity, the measurement of diversity which is shared among communities or environments (i.e. the sediment or water samples from ponds with varying conditions within our research) (Whittaker 1972; Lozupone and Knight 2008). β-diversity is further classified into qualitative β-diversity, which is evaluated or measured only from the presence or absence of a particular sequence data, and quantitative β-diversity, which also takes into consideration the relative abundance of the present sequence data. The β-diversity comparison of AOB and MOB communities between varying environments within our study was achieved using the UniFrac phylogenetic method which computes the variation between microbial communities using a lineage based phylogenetic distance measurement (Lozupone and Knight 2005). Phylogenetic based methods are advantageous because the evolutionary divergence between taxa are accounted for during the analysis, which may vary widely amongst diverse microbial populations (Lozupone et al., 2006). We chose to analyse our data based on quantitative measures using the quantitative UniFrac method, termed 'weighted UniFrac', which takes into account the OTUs present within the communities as well as the number of times each of the OTUs were observed present. Weighted UniFrac was chosen primarily because it was capable of

illustrating community structural differences of the AOB and MOB microbial community hypothesized to be influenced by the transient physicochemical properties present at the disused tin-mining pond which have undergone various post-mining secondary activities, as opposed to qualitative (unweighted) UniFrac which only indicates the variation of bacterial composition between communities (Dang et al., 2009).

5.3 The Community Composition and Diversity of the Ammonia Oxidizing Bacteria

Compared to the AOBs sampled from other sites in this study, the conditions at the lotus pond sediment seem to be favourable for a higher level of AOB richness as evidenced by the substantially steeper gradient of the rarefaction curve (Figure 4.15), Shannon and Simpson index (Table 4.3), as well as better distribution of the lotus sediment AOB clone sequences amongst the *Nitrosomonas* and *Nitrosospira*-like clusters on the phylogenetic tree (Figure 4.18). Periodic root oxygen release into the anoxic pond sediments (Bodelier et al., 1996; Ottosen et al., 1999), exudation of organic and nitrogenous compounds (Herrmann et al., 2009), as well as the preferential growth of ammonia oxidizers on sediment particle surfaces (Aakra et al., 2000; Xia et al., 2009) and root biofilms (Briones et al., 2002) can potentially promote the relative abundance and a higher AOB richness at the lotus sediment. Higher OTUs and more diverse communities of AOB have also previously been recorded at two other study sites with vegetated sediments/soils as compared to those that were unvegetated (Briones et al., 2002; Herrmann et al., 2009).

The lack or absence of aquatic vegetation at the idle pond sediments may also be the contributing factor leading to the undetected AOBs within the idle pond sediment samples.

AOBs affiliated with both the *Nitrosomonas* and *Nitrosospira* lineage was detected though *Nitrosospira* affiliated AOBs seemed to be more prevalent at our study site. In many environments, particularly lake water and sediments, the *Nitrosospira* lineage is known to be ubiquitous owing to its high growth substrate affinity and adaptability to low ammonia content environments (Hiorns et al., 1995; Cebron et al., 2003). The overall phylogenetic analysis showed that the majority of our AOB sequences (74% *amo*A sequences, 96% CTO sequences) fell into distinct phylogenetic clusters that were closely affiliated to uncultured environmental AOB sequences, while only a very marginal portion of our AOB sequences were found to be phylogenetically related to currently known *Nitrosomonas* or *Nitrosospira* pure culture lineages available in public databases. This has been a similarly observed phenomenon in AOB studies at several other aquatic sites (Francis et al., 2003; Herrmann et al., 2009; Dang et al., 2010b; Cao et al., 2011c), suggesting that these distinct clusters of sequences might represent novel groups or lineages of AOBs that are found primarily within the study sites of disused tin mining pond.

The AOBs also sub-clustered according to sample type (i.e. sediment or water samples). *Nitrosomonas* and *Nitrosospira* affiliated AOB from the sediments of post-aquaculture and lotus ponds clustered closely with AOBs isolated from other study sites with higher levels of attachment surfaces (paddy fields,

activated sludges, polluted mangrove sediments) (Kraigher et al., 2008; Cao et al., 2011c; Wang et al., 2012). Conversely, the *Nitrosospira*-like AOBs from the water samples of all three pond types grouped closely together (Figure 4.25) and were closely affiliated with other AOBs from sites such as freshwater lakes and rivers (Mueller-Spitz et al., 2009; Hu et al., 2011; Liu et al., 2012). Next to the overall conditions of the lakes sampled, the compartments of the lake (i.e. water to sediment) has been recognized to harbour different niches and select for varying AOB types whether within or between the two main lineages of AOB currently known (Coci 2007). The varying nutrient content as well as attachment surface present at the sediment compartment in comparison to the surrounding water column would allow bacteria activity and interactions not possible at the water column (Phillips et al., 1999). Furthermore, as AOBs are known to differ in substrate affinity and requirements from species to species despite their common basic metabolism, this subsequently affects their adaptability to varying environments (Stehr et al., 1995; Koops and Pommerening-Röser 2001), resulting in AOB communities that vary in the sediment and water columns .

5.4 The Community Composition and Diversity of the Methane Oxidizing Bacteria

Similar to the richness of the AOBs, the sediment samples from the lotus pond also harboured the highest richness of methane oxidizers (Figure 4.16, Table 4.3), which was reflected on the *pmo*A phylogenetic tree in which the lotus pond sediment sequences were more evenly distributed between all the clusters (Figure 4.19). In contrast, the idle pond water samples harboured the least richness of methane oxidizers, with most of the *pmo*A sequences from this site forming a cluster at MOB 1. Previous studies on wetlands (Siljanen et al., 2011) and paddy field soils (Wu et al., 2009) have also reported higher diversities of methanotrophs in the vegetated sediments of these water bodies. As for the AOBs, the MOBs would also benefit from the oxygen release by the plant roots into the otherwise anoxic bulk sediment (King 1994) which most likely can promote the increase in methanotroph diversity at vegetated sites.

Phylogenetic analysis of the *pmo*A sequences revealed the presence of a wide diversity of both type I and type II methanotrophs at our study sites but Type I methanotrophs are clearly prevalent, making up 80% of the clone sequences. Similar observations of dominant type I methanotrophs were also recorded from the study of freshwater sediments of Lake Constance, Germany (Pester et al., 2004; Rahalkar and Schink 2007) and Lake Washington, United States (Costello and Lidstrom 1999; Costello et al., 2002; Nercessian et al., 2005), in which the same *pmo*A gene targeting PCR primers were used. Type I methanotrophs are known to be dominant within both the water and surface

sediment of aquatic environments (Henckel et al., 1999). They compete better in wet environments (Siljanen et al., 2011) because they are r-life strategists, described as competitors who possess the ability to respond quickly to the presence of environments favourable for growth and continuously changing environments, such as those occurring at the surface sediments of lakes, where sediment resuspension is common. Sediment resuspension also increases oxygen availability (Weyhenmeyer 1998; Bussmann et al., 2004) which is preferred by Type I methanotrophs (Amaral and Knowles 1995) as compared to anoxic bottom sediment columns (Hanson and Hanson 1996). However, within the dominant type I methanotrophs in our study, Type Ib *Methylococcus*/*Methylocaldum*-like cluster (Cluster MOB 1) were the most numerous (77 clone sequences), in contrast with the type Ia methanotrophs of the *Methylomonas* and *Methylobacter* lineage found at many lake sediments. Type Ib methanotrophs were previously found to be prevalent at rice roots (Lüke et al., 2010), and the wettest littoral area of a boreal lake (Siljanen et al., 2011).

Approximately 15% of the *pmo*A clone sequences showed less than 90% sequence similarity to the currently known databases (Table 4.2). These sequences fell into a distinct phylogenetic cluster that did not affiliate with the currently recognized lineages (Cluster MOB 2) (Figure 4.19) and only clustered with sequences within our study and two other *pmo*A sequences isolated from rice field soil and rice roots (Lüke et al., 2010). As such, these sequences might be novel and unique strains of methanotrophs forming a new lineage within the Type I methanotroph, which might be methanotrophs that

have adapted and evolved to the environmental conditions present at disused tin-mining sites.

Compared to the ammonia oxidizers, the community composition of the methanotrophs among sampling sites and sample type seem to be less varied. The presence of wetland plants, previous aquaculture activities (higher N content), or the variation in level of suspended solids did not seem to select for a particular type of methanotroph. There was no clear clustering pattern between sites or sample type among the *pmo*A sequences, whether based on the principle coordinate analysis (Figure 4.27) or the phylogenetic tree (Figure 4.19). Although lotus plants seem to promote a higher relative diversity of methanotrophs, the relative diversity/richness of the methanotrophs between the sampling sites varied within a smaller range compared to that of ammonia oxidizers (Table 4.3)**.** From our findings, it seems that MOBs are more tolerant to environmental variations as compared to AOBs, and the physicochemical property variation amongst our sampling sites were not sufficient to cause an observable clustering trend of the MOB community composition.

5.5 Other Factors Potentially Affecting the Richness & Diversity of AOB and MOB at Disused Tin-Mining Ponds

It is recognized that the community structure and diversity of microbes, including AOBs (Horz et al., 2004) and MOBs (Horz et al., 2005), are affected by and respond to multifactorial and multiple co-occurring changes,

particularly in complex environments such as soils and sediments. Within the context of disused tin-mining lakes/ponds as a type of freshwater aquatic ecosystem, multiple factors including but not limited to the physicochemical properties of the sediment and water body of the pond (e.g. oxygen, nitrogen content, pH, salinity), and presence of varying plant species are able to directly or indirectly exert its effect on the AOB and MOB community within the system. Heavy metals have also been found to significantly inhibit the ammonia-oxidizing rates of beta-Proteobacterium AOB (Stephen et al., 1999), as well as methane oxidation (Mishra et al., 1999; Mohanty et al., 2000).

Plants are known for the uptake of toxic metals as micronutrients (Lasat 2000). In particular, the Indian Lotus (*Nelumbo nucifera*) was found to be a heavy metal hypertolerant plant species that adopted a heavy metal accumulation strategy in environments with elevated amounts of toxic metals (Ashraf et al., 2011). Our study indicates that lotus plant colonized disused tin-mining ponds harbour a richer diversity of both ammonia oxidizers and methanotrophs, which suggests that the lotus plants might have the potential to improve the overall condition (i.e. increased nutrients & reduced toxicity levels) of disused tin-mining ponds in our study. This observation is similar to that of another study on copper mine tailings where the diversity of free-living nitrogen fixing microbes there increased with the age of plant colonization and the improvement of wasteland properties (Zhan and Sun 2011). Further research would be necessary to verify the influence of heavy metals on the ammonia oxidizer and methanotroph community as well as to monitor the

effects of heavy metal tolerant plant species presence on the communities of both the above mentioned bacteria.

5.6 Future Prospects

The critical role played by AOB and MOB that have been discovered in many freshwater aquatic ecosystems, as well as the diverse AOB and MOB community found in our study here at disused tin-mining ponds, indicate that both AOB and MOB might also play important role(s) contributing to nutrient cycling within ecology of disused tin-mining ponds. While molecular methods have been explored for the identification of AOB and MOB community composition inhabiting disused tin-mining ponds in this study, this has only enabled the determination of the relative diversity and abundance of the AOB and MOB community of our study site. Perhaps a suitable method could be improvised based on quantitative real-time PCR protocols (Arya et al., 2005) in future studies to determine the gene copy numbers of *amo*A and *pmo*A, which would enable a more accurate quantification of the AOB and MOB and hence the degree of its contribution to the disused tin-mining pond ecosystem.

Disused tin-mining pond sediments, particularly those sampled from the disused tin-mining ponds cultured with lotus as well as those previously used for aquaculture activities, seem to harbour a richer diversity of both AOB and MOB. Hence, analysing the physicochemical properties of the sediment samples, including important attributes such as oxygen content, total organic carbon (TOC), total nitrogen (TN) and pH would be beneficial for subsequent studies as these factors would provide a clearer perspective in the correlation

with the AOB and MOB community composition within the disused tin-mining pond sediments. Additional physicochemical parameters such as heavy metal content of the pond sediments could be considered as well. Critical ones such as copper and iron, for example, affect trace metal availability and hence the regulation of nitrogen transformation as the enzymes involved in catalyzing microbial carbon and nitrogen cycles are metalloenzymes (enzymes containing metal ion cofactors) (Morel and Price 2003). Both the ammonia monooxygenase and methane monooxygenase enzymes involved in ammonia and methane oxidation are known to have copper (Ensign et al., 1993; Murrell et al., 2000) and possibly iron as a part of the active sites (Zahn et al., 1996). The levels of other heavy metals and pollutants such as arsenic and tin, which are the main elements found in some disused tin-mining ponds within Malaysia (Yusof et al., 2001; Ashraf et al., 2010) might also be worthy of investigation. A comparison between the levels of the critical heavy metals in disused tin-mining pools with abundant natural regeneration or cultivation of aquatic plants and those without could also be done to gauge the effects of wetland plants on heavy metal toxicity and subsequently its effects on the ammonia oxidizing and methane oxidizing bacteria composition. Furthermore, while studies on the fish and shrimp species present in the disused tin-mining pools within the Kampar UTAR campus has been conducted, the aquatic plant species present at these sites, whether through natural regeneration or cultivation, have not been characterized. As varying plant species are known to be capable of tolerating different levels of heavy metal toxicity and possess different metal toleration strategies, characterizing the plant species at our

current study site will possibly provide additional supporting clues to the currently observed AOB and MOB community.

Finally, another organism that has been more recently found to be capable and possibly play a major role in ammonia oxidation besides the ammonia oxidizing bacteria is the ammonia oxidizing archaea (You et al., 2009). These organisms were first discovered to be abundant in the ocean (Francis et al., 2005), but have lately also been found to dominate some soils (Leininger et al., 2006) and aquatic environments (Jiang et al., 2009). Additionally, ammonia oxidizing archaea have also been found to be more tolerant of heavy metal contamination as compared to ammonia oxidizing bacteria (Li et al., 2009). Hence, theoretically, ammonia oxidizing archaea could be the dominating ammonia oxidizer in disused tin-mining pond environments and would be a strongly viable topic for further research.

CHAPTER 6
CONCLUSIONS

As a whole, this study gave an insight into the ammonia oxidizing and methanotroph communites inhabiting disused tin-mining ponds with varying physicochemical properties. The ammonia oxidizing bacteria community comprising of both the *Nitrosomonas* sp. and *Nitrosospira* sp. clade were present at all sites with the exception of the idle pond sediments and clustered according to the water and sediment columns of the lakes. *Nitrosospira*-like ammonia oxidizers were dominantly detected at our study sites. On the other hand, both Type I and Type II methanotrophs were present at all sites but displayed a less distinct variation among pond type or column and a majority of the community could be assigned to Type Ib methanotrophs which are most closely affiliated to the *Methylococcus* sp. and *Methylocaldum* sp. clade.

The highest richness was observed for both the ammonia oxidizing bacteria and methanotroph communities from the lotus pond sediments while substantially lower richness of ammonia oxidizers and methanotrophs were detected from water sampled at the idle pond. The presence of aquatic vegetation, particularly the dominant plant community *Nelumbo nucifera* is seen to house an environment suitable for a higher relative diversity/richness of both the ammonia oxidizers and methanotrophs, particularly at the surface sediment column of the lake. As reported in other similar studies, the presence of aquatic plants as well as secondary activity (i.e. aquaculture) can clearly cause alterations towards the ecological conditions of aquatic environments.

This might explain the observable trend of variation between the physicochemical characteristics as well as the AOB and MOB communities inhabiting the respective disused tin-mining ponds in our study. Several distinct clusters of AOBs and MOBs, which did not group with currently recognized lineages were also detected and were unique to our study sites.

REFERENCES

Aakra, A., Hesselsøe, M. and Bakken, L.R., 2000. Surface Attachment of Ammonia-Oxidizing Bacteria in Soil. *Microbial Ecology,* 39**,** pp. 222–235.

Abd El Haleem, D. et al., 2000. Phylogenetic analysis of rhizosphere-associated β-subclass proteobacterial ammonia oxidizers in a municipal wastewater treatment plant based on rhizoremediation technology. *Letters in Applied Microbiology,* 31**,** pp. 34 - 38.

Abdul-Rashid, Z. and Awang, Z., 2004. Fish and Water Quality Survey in Paya Indah Wetlands Sanctuary. *Tropical peat swamps: safe guarding a global natural resource.* Selangor: Universiti Putra Malaysia**,** pp. 211-218.

Acinas, S.G. et al., 2004. Divergence and Redundancy of 16S rRNA Sequences in Genomes with Multiple rrn Operons. *Journal of Bacteriology,* 186 (9)**,** pp. 2629–2635.

Al Hasin, A. et al., 2010. Remediation of chromium (VI) by a methane-oxidizing bacterium. *Environmental Science & Technology,* 44 (1)**,** pp. 400 - 405

Alshaebi, F.Y. et al., 2009. Risk Assessment at Abandoned Tin Mine In Sungai Lembing, Pahang, Malaysia. *Electronic Journal of Geotechnical Engineering,* Volume 14 (Bundle D)**,** pp. 1 - 9.

Amann, R.I., Ludwig, W. and Schleifer, K.H., 1995. Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. *Microbiological Reviews,* 59 (1)**,** pp. 143 - 169.

Amaral, J.A. and Knowles, R., 1995. Growth of methanotrophs in methane and oxygen counter gradients. *FEMS Microbiology Letters* 126**,** pp. 215 - 220.

Ambak, M.A. and Jalal, K.C.A., 2006. Sustainability issues of reservoir fisheries in Malaysia. *Aquatic Ecosystem Health & Management,* 9 (2)**,** pp. 165 - 173.

Ang, L.H., 1994. Problems and Prospects of Afforestation On Sandy Tin Tailings in Peninsular Malaysia. *Journal of Tropical Forest Science,* 7 (1)**,** pp. 87 - 105.

Ang, L.H. and Ho, W.M., Afforestation of Tin Tailings in Malaysia. 12th ISCO Conference, 2002 Beijing. 440 - 445.

Arumugam, P.T., 1994. Present Utilization and Recommendations for Management of Tin-Mine Lakes in Malaysia. *International Association of Theretical And Applied Limnology Communications,* 24**,** pp. 265-272.

Arya, M. et al., 2005. Basic Principles of Real-time Quantitative PCR. *Expert Review of Molecular Diagnostics,* 5 (2)**,** pp. 209-219.

Asakawa, S. and Kimura, M., 2008. Comparison of Bacterial Community Structures at Main Habitats in Paddy Field Based Ecosystem Based on DGGE. *Soil Biology & Biochemistry,* 40**,** pp. 1322 – 1329.

Ashraf, M.A., Maah, M.J. and Yusoff, I., 2011. Heavy metals accumulation in plants growing in ex tin mining catchment. *International Journal of Environmental Science and Technology,* 8 (2)**,** pp. 401 - 416.

Ashraf, M.A., Maah, M.J. and Yusoff, I.B., 2010. Study of Water Quality and Heavy Metals in Soil & Water of Ex-Mining Area Bestari Jaya. *International Journal of Basic and Applied Sciences,* 10 (3)**,** pp. 7 - 27.

Auman, A.J. et al., 2000. Molecular Characterization of Methanotrophic Isolates from Freshwater Lake Sediment. *Applied and Environmental Microbiology,* 66 (12)**,** pp. 5259–5266.

Awang, K., 1994. Growth of Three Multipurpose Tree Species on Tin Tailings in Malaysia. *Journal of Tropical Forest Science,* 7 (1)**,** pp. 106 - 112.

Baker, A.J.M., 1981. Accumulators and excluders strategies in the response of plants to heavy metals. *Journal of Plant Nutrition,* 3 (1 - 4)**,** pp. 643 - 654.

Balasubramanian, R. et al., 2010. Oxidation of methane by a biological dicopper centre. *Nature,* 465**,** pp. 115–119.

Bastviken, D. et al., 2004. Methane emissions from lakes: Dependence of lake characteristics, two regional assessments, and a global estimate. *Global Biogeochemical Cycles,* 18 (GB4009)**,** pp. 1-12.

Belser, L.W., 1979. Population Ecology of Nitrifying Bacteria. *Annual Review of Microbiology,* 33**,** pp. 309 - 333.

Bodelier, P.L.E. and Frenzel, P., 1999. Contribution of Methanotrophic and Nitrifying Bacteria to CH_4 and NH_4^+ Oxidation in the Rhizosphere of Rice Plants as Determined by New Methods of Discrimination. *Applied and Environmental Microbiology,* 65 (5)**,** pp. 1826–1833.

Bodelier, P.L.E. and Laanbroek, H.J., 2004. Nitrogen as a regulatory factor of methane oxidation in soils and sediments. *FEMS Microbiology Ecology,* 47 (3)**,** pp. 265-277.

Bodelier, P.L.E. et al., 1996. Dynamics of Nitrification and Denitrification in Root-Oxygenated Sediments and Adaptation of Ammonia-Oxidizing Bacteria to Low-Oxygen or Anoxic Habitats. *Applied and Environmental Microbiology,* 62 (11)**,** pp. 4100 - 4107.

Bollmann, A., Bar-Gilissen, M.J. and Laanbroek, H.J., 2002. Growth at Low Ammonium Concentrations and Starvation Response as Potential Factors Involved in Niche Differentiation among Ammonia-Oxidizing Bacteria. *Applied and Environmental Microbiology,* 68 (10)**,** pp. 4751-4757.

Bollmann, A. and Laanbroek, H.J., 2001. Continuous culture enrichments of ammonia-oxidizing bacteria at low ammonium concentrations. *FEMS Microbiology Ecology,* 1279**,** pp. 1 - 11.

Bourne, D.G., McDonald, I.R. and Murrell, J.C., 2001. Comparison of *pmo*A PCR Primer Sets as Tools for Investigating Methanotroph Diversity in Three Danish Soils. *Applied and Environmental Microbiology,* 67 (9)**,** pp. 3802–3809.

Bowatte, S. et al., 2006. Molecular analysis of the ammonia oxidizing bacterial community in the surface soil layer of a Japanese paddy field. *Soil Science & Plant Nutrition,* 52 (4)**,** pp. 427 - 431.

Briones, A.M. et al., 2002. Influence of different cultivars on populations of ammonia-oxidizing bacteria in the root environment of rice. *Applied and Environmental Microbiology,* 68**,** pp. 3067–3075.

Brosius, J. et al., 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proceedings from the National Academy of Sciences of the United States of America,* 75 (10)**,** pp. 4801-4805.

Buchholz, L.A. et al., 1995. Activity of methanotrophic bacteria in Green Bay sediments. *FEMS Microbiology Ecology,* 16**,** pp. 1 - 8.

Burrows, K.J. et al., 1984. Substrate Specificities of the Soluble and Particulate Methane Mono-oxygenases of *Methylosinus trichosporium* OB3b. *Journal of General Microbiology,* 130**,** pp. 3327 - 3333.

Bussmann, I. et al., 2004. Preferential cultivation of type II methanotrophic bacteria from littoral sediments (Lake Constance). *FEMS Microbiology Ecology,* 47**,** pp. 179 - 189.

Caldwell, S. et al., 2008. Anaerobic Oxidation of Methane: Mechanisms, Bioenergetics, and the Ecology of Associated Microorganisms. *Environmental Science & Technology,* 42 (18)**,** pp. 6791-6799.

Calhoun, A. and King, G.M., 1998. Characterization of Root-Associated Methanotrophs from Three Freshwater Macrophytes: *Pontederia cordata, Sparganium eurycarpum,* and *Sagittaria latifolia*. *Applied and Environmental Microbiology,* 64 (3)**,** pp. 1099–1105.

Calvó, L. and Garcia-Gil, J., 2004. Use of *amo*B as a new molecular marker for ammonia-oxidizing bacteria. *Journal of Microbiological Methods,* 57**,** pp. 69– 78.

Cao, H. et al., 2011a. Community shift of ammonia-oxidizing bacteria along an anthropogenic pollution gradient from the Pearl River Delta to the South China Sea. *Applied Microbiology and Biotechnology***,** pp.

Cao, H. et al., 2011b. Diversity and abundance of ammonia-oxidizing prokaryotes in sediments from the coastal Pearl River estuary to the South China Sea. *Antonie van Leeuwenhoek,* 100 (4)**,** pp. 545-556.

Cao, H. et al., 2011c. Diversity and abundance of ammonia-oxidizing archaea and bacteria in polluted mangrove sediment. *Systematic and Applied Microbiology,* 34 (7)**,** pp. 513 - 523

Cao, M.K., Gregson, K. and Marshall, S., 1998. Global Methane Emissions from Wetlands and its Sensitivity to Climate Change. *Atmospheric Environment,* 32 (19)**,** pp. 3293 - 3299.

Cardy, D.L.N. et al., 1991. Molecular analysis of the methane monooxygenase (MMO) gene cluster of *Methylsinus trichosporium* 0B3b. *Molecular Microbiology,* 5 (2)**,** pp. 335 - 342.

Carman, K.R., 1994. Stimulation of marine free-living and epibiotic bacterial activity by copepod excretions. *FEMS Microbiology Ecology,* 14**,** pp. 255 - 265.

Cebron, A., Berthe, T. and Garnier, J., 2003. Nitrification and Nitrifying Bacteria in the Lower Seine River and Estuary (France). *Applied and Environmental Microbiology,* 69 (12)**,** pp. 7091–7100.

Chakravorty, S. et al., 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *Journal of Microbiological Methods,* 69**,** pp. 330–339.

Chao, A., 1984. Non-parametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics,* 11 (4)**,** pp. 265-270.

Chavez-Crooker, P. and Obreque-Contreras, J., 2010. Bioremediation of aquaculture wastes. *Current Opinion in Biotechnology,* 21 (3)**,** pp. 313-317.

Chazdon, R.L. et al., 1998. Statistical Methods for Estimating Species Richness of Woody Regeneration in Primary and Secondary Rain Forests of northeastern Costa Rica. In*:* Dallmeier, F. & Comiskey, J. A. (eds.) *Forest Biodiversity Research, Monitoring and Modelling: Conceptual Background and Old World Case Studies.* Paris: Parthenon Publishing**,** pp. 285-309.

Chen, G.Y., Qiu, S.L. and Zhou, Y.Y., 2009. Diversity and abundance of ammonia-oxidizing bacteria in eutrophic and oligotrophic basins of a shallow chinese lake (Lake Donghu). *Research in Microbiology,* 160**,** pp. 173 - 178.

Christensen, P.B., Revsbech, N.P. and Sand-Jensen, K., 1994. Microsensor Analysis of Oxygen in the Rhizosphere of the Aquatic Macrophyte *Littorella uniflora* (L.) Ascherson. *Plant Physiology,* 105**,** pp. 847 - 852.

Coci, M., 2007. *Niche differentiation between ammonia-oxidizing bacteria in aquatic environments.* pHD Thesis, Universiteit Utrecht.

Coci, M., Bodelier, P.L.E. and Laanbroek, H.J., 2008. Epiphyton as a Niche for Ammonia-Oxidizing Bacteria: Detailed Comparison with Benthic and Pelagic Compartments in Shallow Freshwater Lakes. *Applied and Environmental Microbiology,* 74 (7)**,** pp. 1963 - 1971.

Cohan, F.M., 2002. What Are Bacterial Species? *Annual Review of Microbiology,* 56**,** pp. 457-487.

Colby, J., Stirling, D.I. and Dalton, H., 1977. The Soluble Methane Mono-oxygenase of *Methylococcus capsulatus* (Bath) - Its Ability to Oxygenate n-Alkanes, n-Alkenes, Ethers, And Alicyclic, Aromatic And Heterocyclic Compounds. *Biochemical Journal,* 165**,** pp. 395 - 402.

Costello, A.M. et al., 2002. Estimation of methanotroph abundance in a freshwater lake sediment. *Environmental Microbiology,* 4 (8)**,** pp. 443–450.

Costello, A.M. and Lidstrom, M.E., 1999. Molecular Characterization of Functional and Phylogenetic Genes from Natural Populations of Methanotrophs in Lake Sediments. *Applied and Environmental Microbiology,* 65 (11)**,** pp. 5066–5074.

Crab, R. et al., 2007. Nitrogen removal techniques in aquaculture for a sustainable production. *Aquaculture,* 270**,** pp. 1 - 14.

Dalsgaard, T. and Thamdrup, B., 2002. Factors controlling anaerobic ammonium oxidation with nitrite in marine sediments. *Applied and Environmental Microbiology,* 68 (8)**,** pp.

Dang, H. et al., 2010a. Environmental factors shape sediment anammox bacterial communities in hypernutrified Jiaozhou Bay, China. *Applied and Environmental Microbiology,* 76 (21)**,** pp. 7036-7047.

Dang, H. et al., 2010b. Diversity, abundance, and spatial distribution of sediment ammonia-oxidizing Betaproteobacteria in response to environmental gradients and coastal eutrophication in Jiaozhou Bay, China. *Applied and Environmental Microbiology,* 76 (14)**,** pp. 4691-4702.

Dang, H. et al., 2009. Diversity and spatial distribution of *amo*A-encoding archaea in the deep-sea sediments of the tropical West Pacific Continental Margin. *Journal of Applied Microbiology,* 106 (5)**,** pp. 1482-1493.

Degelmann, D.M. et al., 2010. Different atmospheric methane-oxidizing communities in European beech and Norway spruce soils. *Applied and Environmental Microbiology,* 76 (10)**,** pp. 3228-3235.

Deni, J. and Penninckx, M.J., 1999. Nitrification and Autotrophic Nitrifying Bacteria in a Hydrocarbon-Polluted Soil. *Applied and Environmental Microbiology,* 65 (9)**,** pp. 4008 - 4013.

Department of Environment and Heritage Protection, Q., 2013, *Coastal and subcoastal non-floodplain tree swamp—Melaleuca spp. and Eucalyptus spp. – Nutrient dynamics, WetlandInfo* [Online]. Available at: http://wetlandinfo.ehp.qld.gov.au/wetlands/ecology/aquatic-ecosystems-natural/palustrine/non-floodplain-tree-swamp/nutrients.html [Accessed: 6 May 2013].

DiSpirito, A.A. et al., 1992. Trichloroethylene oxidation by the membrane-associated methane monooxygenase in type I, type II and type X methanotrophs *Biodegradation,* 2 (3)**,** pp. 151 - 164.

Dorigo, U., Volatier, L. and Humbert, J.F., 2005. Molecular approaches to the assessment of biodiversity in aquatic microbial communities. *Water Research,* 39**,** pp. 2207 - 2218.

Dunfield, P.F., 2003. Methylocella silvestris sp. nov., a novel methanotroph isolated from an acidic forest cambisol. *International Journal of Systematic and Evolutionary Microbiology,* 53 (5)**,** pp. 1231-1239.

El Sheikh, A.F., Poret-Peterson, A.T. and Klotz, M.G., 2008. Characterization of two new genes, *amo*R and *amo*D, in the *amo* operon of the marine ammonia oxidizer *Nitrosococcus oceani* ATCC 19707. *Applied and Environmental Microbiology,* 74 (1)**,** pp. 312-318.

Eller, G. and Frenzel, P., 2001. Changes in activity and community structure of methane-oxidizing bacteria over the growth period of rice. *Applied and Environmental Microbiology,* 67 (6)**,** pp. 2395 - 2403.

Eller, G., Kanel, L. and Kruger, M., 2005. Cooccurrence of aerobic and anaerobic methane oxidation in the water column of Lake Plusssee. *Applied and Environmental Microbiology,* 71 (12)**,** pp. 8925-8.

Ensign, S.A., Hyman, M.R. and Arp, D.J., 1993. In vitro activation of ammonia monooxygenase from *Nitrosomonas europaea* by copper. *Journal of Bacteriology,* 175 (5)**,** pp. 1971-1980

Erwin, D.P. et al., 2005. Diversity of oxygenase genes from methane- and ammonia-oxidizing bacteria in the Eastern Snake River Plain aquifer. *Applied and Environmental Microbiology,* 71 (4)**,** pp. 2016-2025.

EURx, 2008, GeneMATRIX Plasmid Miniprep DNA Purification Kit. Available at: http://www.eurx.com.pl/csComp-6-154-19-0.html.

Fjellbirkeland, A., Torsvik, V. and Øvreås, L., 2001. Methanotrophic diversity in an agricultural soil as evaluated by denaturing gradient gel electrophoresis profiles of *pmo*A, *mxa*F and 16S rDNA sequences. *Antonie van Leeuwenhoek* 79**,** pp. 209–217.

Foong, T.H., 2003, Tin mining town strikes gold. Available at: http://www.reocities.com/alfix7/news0005.htm.

Francis, C.A., O'Mullan, G.D. and Ward, B.B., 2003. Diversity of ammonia monooxygenase (*amoA*) genes across environmental gradients in Chesapeake Bay sediments. *Geobiology,* 1**,** pp. 129 - 140.

Francis, C.A. et al., 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proceedings from the National Academy of Sciences of the United States of America,* 102 (41)**,** pp. 14683-14688.

Frank, J.A. et al., 2008. Critical Evaluation of Two Primers Commonly Used for Amplification of Bacterial 16S rRNA Genes. *Applied and Environmental Microbiology,* 74 (8)**,** pp. 2461 - 2470.

Fujii, C. et al., 2010. Succession and community composition of ammonia-oxidizing archaea and bacteria in bulk soil of a Japanese paddy field. *Soil Science & Plant Nutrition,* 56 (2)**,** pp. 212 - 219.

Gilbert, B. et al., 2000. Molecular Analysis of the *pmo* (Particulate Methane Monooxygenase) Operons from Two Type II Methanotrophs. *Applied and Environmental Microbiology,* 66 (3)**,** pp. 966–975.

Graham, D.W. et al., 1993. Factors affecting competition between type I and type II methanotrophs in two-organism, continuous-flow reactors *Microbial Ecology,* 25 (1)**,** pp. 1 - 17.

Gruber, N. and Galloway, J.N., 2008. An Earth-system perspective of the global nitrogen cycle. *Nature,* 451 (7176)**,** pp. 293-296.

Hach, 2009, DR/890 Colorimeter Procedures Manual. Available at: http://www.hach.com/hc/view.file.invoker/4847000/View=FILCAT_MAN_PHOTOMETERS-DR800/NewLinkLabel=DR%26frasl%3B800+Series+Colorimeter+Manuals/SESSIONID%7CBmt6TkRVeU56TXdKbWQxWlhOMFIwOVNVa1V4TXc9PUJ6QXdOeg==%7C.

Hakemian, A.S. and Rosenzweig, A.C., 2007. The Biochemistry of Methane Oxidation. *Annual Review of Biochemistry,* 76 (1)**,** pp. 223-241.

Han, B. et al., 2009. Diversity and activity of methanotrophs in alkaline soil from a Chinese coal mine. *FEMS Microbiology Ecology,* 70 (2)**,** pp. 40-51.

Hanson, R.S., 1980. Ecology and diversity of methanotrophic organisms. *Advances in Applied Microbiology,* 26**,** pp. 3 - 39.

Hanson, R.S. and Hanson, T.E., 1996. Methanotrophic Bacteria. *Microbiological Reviews,* 60 (2)**,** pp. 439–471.

Head, I.M., Saunders, J.R. and Pickup, R.W., 1998. Microbial Evolution, Diversity, and Ecology: A Decade of Ribosomal RNA Analysis of Uncultivated Microorganisms. *Microbial Ecology,* 35**,** pp. 1 - 21.

Heck, K.L.J., van Belle, G. and Simberloff, D., 1975. Explicit Calculation of the Rarefaction Diversity Measurement and the Determination of Sufficient Sample Size. *Ecology,* 56 (6)**,** pp. 1459-1461.

Henckel, T., Friedrich, M. and Conrad, R., 1999. Molecular Analyses of the Methane-Oxidizing Microbial Community in Rice Field Soil by Targeting the Genes of the 16S rRNA, Particulate Methane Monooxygenase and Methanol Dehydrogenase. *Applied and Environmental Microbiology,* 65 (5)**,** pp. 1980 - 1990.

Henckel, T., Roslev, P. and Conrad, R., 2000. Effects of O_2 and CH_4 on presence and activity of the indigenous methanotrophic community in rice field soil. *Environmental Microbiology,* 2 (6)**,** pp. 666 - 679.

Herrmann, M., Saunders, A.M. and Schramm, A., 2008. Archaea dominate the ammonia-oxidizing community in the rhizosphere of the freshwater macrophyte *Littorella uniflora*. *Applied and Environmental Microbiology,* 74 (10)**,** pp. 3279-83.

Herrmann, M., Saunders, A.M. and Schramm, A., 2009. Effect of lake trophic status and rooted macrophytes on community composition and abundance of ammonia-oxidizing prokaryotes in freshwater sediments. *Applied and Environmental Microbiology,* 75 (10)**,** pp. 3127 - 3136.

Heyer, J., Galchenko, V.F. and Dunfield, P.F., 2002. Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments. *Microbiology,* 148**,** pp. 2831 - 2846.

Hiorns, W.D. et al., 1995. Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of nitrosospiras in the environment. *Microbiology,* 141**,** pp. 2793 - 2800.

Holmes, A.J. et al., 1995. Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. *FEMS Microbiology Reviews,* 132**,** pp. 203-208.

Hooper, A.B. et al., 1997. Enzymology of the oxidation of ammonia to nitrite by bacteria. *Antonie van Leeuwenhoek,* 71**,** pp. 59 - 67.

Horn, H.S., 1966. Measurement of "Overlap" in comparative ecological studies. *The American Naturalist,* 100**,** pp. 419-424.

Horz, H.P. et al., 2004. Ammonia-oxidizing bacteria respond to multifactorial global change. *Proceedings from the National Academy of Sciences of the United States of America,* 101 (42)**,** pp. 15136-15141.

Horz, H.P. et al., 2005. Methane-oxidizing bacteria in a California upland grassland soil: diversity and response to simulated global change. *Applied and Environmental Microbiology,* 71 (5)**,** pp. 2642-2652.

Horz, H.P., Yimga, M.T. and Liesack, W., 2001. Detection of Methanotroph Diversity on Roots of Submerged Rice Plants by Molecular Retrieval of *pmo*A, *mmo*X, *mxa*F, and 16S rRNA and Ribosomal DNA, Including pmoA-Based Terminal Restriction Fragment Length Polymorphism Profiling. *Applied and Environmental Microbiology,* 67 (9)**,** pp. 4177-4185.

Hu, M.M. et al. 2011. Phylogenetic analysis of the composition and seasonal changes of bacteria community in Erhai Lake. Chinese Academy of Science, .

Hughes, J.B. et al., 2001. Counting the Uncountable: Statistical Approaches to Estimating Microbial Diversity. *Applied and Environmental Microbiology,* 67 (10)**,** pp. 4399–4406.

Hyman, M.R., Murton, I.B. and Arp, D.J., 1988. Interaction of Ammonia Monooxygenase from *Nitrosomonas europaea* with Alkanes, Alkenes & Alkynes. *Applied and Environmental Microbiology,* 54 (12)**,** pp. 3187 - 3190.

Ikenaga, M. et al., 2003. Phylogenetic Study on CTO Primer-Amplified Ammonia-Oxidizing Bacteria and Beta-Proteobacteria Associated with Rice Roots Grown in a Flooded Paddy Soil. *Soil Science & Plant Nutrition,* 49 (5)**,** pp. 719 - 727.

Itoi, S., Niki, A. and Sugita, H., 2006. Changes in microbial communities associated with the conditioning of filter material in recirculating aquaculture systems of the pufferfish Takifugu rubripes. *Aquaculture,* 256**,** pp. 287 - 295.

Jain, R., Rivera, M.C. and Lake, J.A., 1999. Horizontal gene transfer among genomes: The complexity hypothesis. *Proceedings from the National Academy of Sciences of the United States of America,* 96**,** pp. 3801–3806.

Jetten, M.S. et al., 2009. Biochemistry and molecular biology of anammox bacteria. *Critical Reviews in Biochemistry and Molecular Biology,* 44 (2-3)**,** pp. 65-84.

Jia, Z.J. et al., 2007. Molecular Identification of Methane Oxidizing Bacteria in a Japanese Rice Field Soil. *Biology and Fetility of Soils* 44**,** pp. 121 - 130.

Jiang, H. et al., 2010. Methanotrophs: Multifunctional bacteria with promising applications in environmental bioengineering. *Biochemical Engineering Journal* 49**,** pp. 277 – 288.

Jiang, H.C. et al., 2009. Diversity and Abundance of Ammonia-Oxidizing Archaea and Bacteria in Qinghai Lake, Northwestern China. *Geomicrobiology Journal,* 26**,** pp. 199–211.

Junier, P. et al., 2009. Phylogenetic and functional marker genes to study ammonia-oxidizing microorganisms (AOM) in the environment. *Applied Microbiology and Biotechnology,* 85 (3)**,** pp. 425-440.

Juretschko, S. et al., 1998. Combined Molecular and Conventional Analyses of Nitrifying Bacterium Diversity in Activated Sludge: *Nitrosococcus mobilis* and *Nitrospira*-Like Bacteria as Dominant Populations *Applied and Environmental Microbiology,* 64 (8)**,** pp. 3042 - 3051.

Keener, W.K. and Arp, D.J., 1994. Transformations of Aromatic Compounds by *Nitrosomonas europaea*. *Applied and Environmental Microbiology,* 60 (6)**,** pp. 1914 - 1920.

King, G.M., 1994. Associations of Methanotrophs with the Roots and Rhizomes of Aquatic Vegetation. *Applied and Environmental Microbiology,* 60 (9)**,** pp. 3220-3227.

Klotz, M.G., Alzerreca, J. and Norton, J.M., 1997. A gene encoding a membrane protein exists upstream of the *amo*A/*amo*B genes in ammonia oxidizing bacteria: a third member of the *amo* operon? *FEMS Microbiology Letters,* 150**,** pp. 65 - 73.

Koh, S.C., Bowman, J.P. and Sayler, G.S., 1993. Soluble Methane Monooxygenase Production and Trichloroethylene Degradation by Type I Methanotroph *Methylomonas methanica*. *Applied and Environmental Microbiology,* 59 (4)**,** pp. 960 - 967.

Koleff, P., Gaston, K.J. and Lennon, J.J., 2003. Measuring beta diversity for presence–absence data. *Journal of Animal Ecology,* 72**,** pp. 367–382.

Konneke, M. et al., 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature,* 437 (7058)**,** pp. 543-546.

Koops, H.P. and Pommerening-Röser, A., 2001. Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. *FEMS Microbiology Ecology* 37**,** pp. 1 - 9.

Kopp, D.A. and Lippard, S.J., 2002. Soluble methane monooxygenase: activation of dioxygen and methane. *Current Opinion in Chemical Biology,* 6**,** pp. 568 - 576.

Kowalchuk, G.A. et al., 1998. Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. *FEMS Microbiology Ecology,* 27 (339 - 350)**,** pp.

Kowalchuk, G.A. et al., 1999. Molecular Analysis of Ammonia-Oxidizing Bacteria of the Beta Subdivision of the Class Proteobacteria in Compost and Composted Materials. *Applied and Environmental Microbiology,* 65 (2)**,** pp. 396 - 403.

Kowalchuk, G.A. and Stephen, J.R., 2001. Ammonia Oxidizing Bacteria - A Model for Molecular Microbial Ecology. *Annual Reviews of Microbiology,* 55**,** pp. 485 - 529.

Kowalchuk, G.A. et al., 1997. Analysis of Ammonia-Oxidizing Bacteria of the Beta Subdivision of the Class Proteobacteria in Coastal Sandy Dunes by Denaturing Gradient Gel Electrophoresis and Sequencing of PCR-Amplified 16S Ribosomal DNA Fragments. *Applied and Environmental Microbiology,* 63 (4)**,** pp. 1489 - 1497.

Kraigher, B. et al., 2008. Influence of pharmaceutical residues on the structure of activated sludge bacterial communities in wastewater treatment bioreactors. *Water Research,* 42 (17)**,** pp. 4578–4588.

Kuai, L.P. and Verstraete, W., 1998. Ammonium Removal by the Oxygen-Limited Autotrophic Nitrification-Denitrification System. *Applied and Environmental Microbiology,* 64 (11)**,** pp. 4500–4506.

Lasat, M.M., 2000, The Use of Plants for the Removal of Toxic Metals from Contaminated Soil. Available at: http://nepis.epa.gov.

Lau, R., 1999. TED Case Studies: Tin Mining in Malaysia - Present and Future. *Trade and Environment Database Case Studies,* 9 (3)**,** pp. 576.

Lee, S.W. et al., 2006. Mixed pollutant degradation by *Methylosinus trichosporium* OB3b expressing either soluble or particulate methane monooxygenase: can the tortoise beat the hare? *Appl Environ Microbiol,* 72 (12)**,** pp. 7503-9.

Leininger, S. et al., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature,* 442**,** pp. 806-809.

Li, M. et al., 2011. Spatial distribution and abundances of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in mangrove sediments. *Applied Microbiology and Biotechnology,* 89 (4)**,** pp. 1243-54.

Li, X.F. et al., 2009. Do ammonia-oxidizing archaea respond to soil Cu contamination similarly as ammonia-oxidizing bacteria? *Plant and Soil,* 324 (1-2)**,** pp. 209-217.

Lipponen, M.T.T. et al., 2004. Occurence of nitrifiers and diversity of ammonia-oxidizing bacteria in developing drinking water biofilms. *Water Research,* 38**,** pp. 4424 - 4434.

Liu, Z. et al., 2012. Phylogenetic diversity, composition and distribution of bacterioplankton community in the Dongjiang River, China. *FEMS Microbiology Ecology,* 80 (1)**,** pp. 30-44.

Lozupone, C. et al., 2007. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology,* 73 (5)**,** pp. 1576-1785.

Lozupone, C., Hamady, M. and Knight, R., 2006. UniFrac - an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics,* 7**,** pp. 371.

Lozupone, C. and Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology,* 71 (12)**,** pp. 8228-8235.

Lozupone, C. and Knight, R., 2008. Species divergence and the measurement of microbial diversity. *FEMS Microbiology Reviews,* 32 (4)**,** pp. 557-578.

Lüke, C. et al., 2010. Biogeography of wetland rice methanotrophs. *Environmental Microbiology,* 12 (4)**,** pp. 862-872.

Magurran, A.E., 2004. *Measuring Biological Diversity.* Oxford: Blackwell Publishing.

Mahmood, S., Freitag, T.E. and Prosser, J.I., 2006. Comparison of PCR primer-based strategies for characterization of ammonia oxidizer communities in environmental samples. *FEMS Microbiology Ecology,* 56**,** pp. 482–493.

Majid, N.M., Hashim, A. and Abdol, I., 1994. Rehabilitation of Ex-Tin Mining Land by Agroforestry Practice. *Journal of Tropical Forestry Science,* 7 (1)**,** pp. 113 - 127.

Martin, A.P., 2002. Phylogenetic Approaches for Describing and Comparing the Diversity of Microbial Communities. *Applied and Environmental Microbiology,* 68 (8)**,** pp. 3673 - 3682.

Massol-Deya, A.A. et al., 1995. Bacterial community fingerprinting of amplified 16S and 16S-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). *Molecular Microbial Ecology Manual,* 3.3.2**,** pp. 1-18.

Matias, H.B. et al., 2002. Effects of Commercial Microbial Products on Water Quality in Tropical Shrimp Culture Ponds. *Asian Fisheries Science,* 15**,** pp. 239 - 248.

McCaig, A.E. et al., 1999. Nitrogen Cycling and Community Structure of Proteobacterial β-Subgroup Ammonia-Oxidizing Bacteria within Polluted Marine Fish Farm Sediments. *Applied and Environmental Microbiology,* 65 (1)**,** pp. 213 - 220.

McDonald, I.R. et al., 2007. Molecular Ecology Techniques for the Study of Aerobic Methanotrophs. *Applied and Environmental Microbiology,* 74 (5)**,** pp. 1305-1315.

McDonald, I.R. et al., 1997 The Soluble Methane Monooxygenase Gene Cluster of the Trichloroethylene-Degrading Methanotroph *Methylocytis* sp. Strain M. *Applied and Environmental Microbiology,* 63 (5)**,** pp. 1898–1904.

Merkx, M. and Lippard, S.J., 2002. Why OrfY? Characterization of MMOD, a long overlooked component of the soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). *Journal of Biological Chemistry,* 277 (8)**,** pp. 5858 - 5865.

Mines, M.C.o. 2008. Perak Mineral Map.

Mines, M.C.o., 2010. *Tin Story: Heritage of Malaysia.*

Mishra, S.R. et al., 1999. Effects of Heavy Metals on Methane Production in Tropical Rice Soils. *Ecotoxicology and Environmental Safety* 44**,** pp. 129-136.

Miyaji, A., 2011. Particulate Methane Monooxygenase from *Methylosinus trichosporium* OB3b. *Methods in Enzymology,* 495**,** pp. 211 - 225.

Mo-Bio, 2010, PowerSoil®DNA Isolation Kit Instruction Manual. Available at: http://www.mobio.com/images/custom/file/protocol/12888.pdf.

Mohanty, S.R. et al., 2000. Influence of heavy metals on methane oxidation in tropical rice soils. *Ecotoxicology and Environmental Safety,* 47 (3)**,** pp. 277 - 284.

Moorhead, K.K. and Reddy, K.R., 1988. Oxygen transport through selected aquatic macrophytes. *Journal of Environmental Quality,* 17 (1)**,** pp. 138 - 142.

Morel, F.M.M. and Price, N.M., 2003. The Biogeochemical Cycles of Trace Metals in the Oceans. *Science,* 300 (5621)**,** pp. 944-947

Mosier, A.C. and Francis, C.A., 2008. Relative abundance and diversity of ammonia-oxidizing archaea and bacteria in the San Francisco Bay estuary. *Environmental Microbiology,* 10 (11)**,** pp. 3002-3016.

Mueller-Spitz, S.R., Goetz, G.W. and McLellan, S.L., 2009. Temporal and spatial variability in nearshore bacterioplankton communities of Lake Michigan. *FEMS Microbiology Ecology,* 67 (3)**,** pp. 511-522.

Mullins, T.D. et al., 1995. Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. *Limnology and Oceanography,* 40 (1)**,** pp. 148 - 158.

Murrell, J.C., McDonald, I.R. and Gilbert, B., 2000. Regulation of expression of methane monooxygenases by copper ions. *Trends in Microbiology,* 8 (5)**,** pp. 221-225.

Mustapha, M.K. and Omotosho, J.S., 2005. An Assessment of the Physico-chemical Properties of Moro Lake, Kwara State, Nigeria. *African Journal of Applied Zoology & Environmental Biology,* 7**,** pp. 73 - 77.

Muyzer, G., Genetic fingerprinting of microbial communities - present status and future perspectives. *In:* Bell, C. R., Brylinsky, M. & Johnson-Green, P., eds. 8th International Symposium on Microbial Ecology, 1999. Atlantic Canada Society for Microbial Ecology.

Nehl, D.B. and Knox, O.G.G., 2006. Significance of Bacteria in the Rhizosphere. *Soil Biology,* 7**,** pp. 89-119.

Nercessian, O. et al., 2005. Bacterial populations active in metabolism of C_1 compounds in the sediment of Lake Washington, a freshwater lake. *Applied and Environmental Microbiology,* 71 (11)**,** pp. 6885-6899.

Ng, K.S., Ujang, Z. and Le-Clech, P., 2004. Arsenic removal technologies for drinking water treatment. *Reviews in Environmental Science and BioTechnology,* 3**,** pp. 43-53.

Ng, W.L., 2011. *Diversity Studies Of Fish And Shrimp Species In Disused Tin-Mining Ponds of Kampar.* Bachelors Degree Thesis, Universiti Tunku Abdul Rahman.

Nguyen, H.H.T. et al., 1998. The Particulate Methane Monooxygenase from *Methylococcus capsulatus* Bath Is a Novel Copper-containing Three-subunit Enzyme: Isolation and Characterization. *Journal of Biological Chemistry,* 273 (14)**,** pp. 7957–7966.

Nicolaisen, M.H. et al., 2004. Nitrification-denitrification dynamics and community structure of ammonia oxidizing bacteria in a high yield irrigated Philippine rice field. *FEMS Microbiology Ecology,* 49 (3)**,** pp. 359 - 369.

Niswati, A. et al., 2004. Analysis of Communities of Ammonia Oxidizers, Methanotrophs, and Methanogens Associated with Microcrustaceans in the Floodwater of a Rice Field Microcosm. *Soil Science & Plant Nutrition,* 50 (3)**,** pp. 447 - 455.

Norton , J.M. et al., 2002. Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. *Archives of Microbiology,* 177**,** pp. 139–149.

Novagen, 2008, AccepTor™ Vector Kits User Protocol Available at: http://www.ebiotrade.com/buyf/productsf/Novagen/C133-000.pdf.

Nyerges, G., Han, S.K. and Stein, L.Y., 2010. Effects of Ammonium and Nitrite on Growth and Competitive Fitness of Cultivated Methanotrophic Bacteria. *Applied and Environmental Microbiology,* 76 (16)**,** pp. 5648-5651.

Oertli, H.J., 1958. The Venice System for the Classification of Marine Waters According to Salinity. *Association for the Sciences of Limnology and Oceanography,* 3 (3)**,** pp. 346 - 347.

Ong, K.S., Chin, H.S. and Teo, K.C., 2011. Screening of antibiotic sensitivity, antibacterial and enzymatic activities of microbes isolated from ex-tin mining lake. *African Journal of Microbiology Research,* 5 (17)**,** pp. 2460-2466.

Ottosen, L.D.M., Risgaard-Petersen, N. and Nielsen, L.P., 1999. Direct and indirect measurements of nitrification and denitrification in the rhizosphere of aquatic macrophytes. *Aquatic Microbial Ecology,* 19 (1)**,** pp. 81 - 91.

Pester, M. et al., 2004. *pmo*A-Based Analysis of Methanotrophs in a Littoral Lake Sediment Reveals a Diverse and Stable Community in a Dynamic Environment. *Applied and Environmental Microbiology,* 70 (5)**,** pp. 3138 - 3142.

Phillips, C.J. et al., 1999. Phylogenetic Differences between Particle-Associated and Planktonic Ammonia-Oxidizing Bacteria of the β Subdivision of the Class Proteobacteria in the Northwestern Mediterranean Sea. *Applied and Environmental Microbiology,* 65 (2)**,** pp. 779-786.

Pontes, D.S. et al., 2007. Molecular approaches: advantages and artifacts in assesing bacterial diversity. *Journal of Industrial Microbiology and Biotechnology,* 34**,** pp. 463 - 473.

Posada, D., 2008. jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution,* 25 (7)**,** pp. 1253-1256.

Prinčič, A. et al., 1998. Effects of pH and Oxygen and Ammonium Concentrations on the Community Structure of Nitrifying Bacteria from Wastewater. *Applied and Environmental Microbiology,* 64 (10)**,** pp. 3584–3590.

Purkhold, U. et al., 2000. Phylogeny of All Recognized Species of Ammonia Oxidizers Based on Comparative 16S rRNA and amoA Sequence Analysis: Implications for Molecular Diversity Surveys. *Applied and Environmental Microbiology,* 66 (12)**,** pp. 5368 - 5382.

Purkhold, U. et al., 2003. 16S rRNA and amoA-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a newlineage within the nitrosomonads. *International Journal of Systematic and Evolutionary Microbiology,* 53**,** pp. 1485–1494.

Rahalkar, M. and Schink, B., 2007. Comparison of aerobic methanotrophic communities in littoral and profundal sediments of Lake Constance by a molecular approach. *Applied and Environmental Microbiology,* 73 (13)**,** pp. 4389-4394.

Reynolds, J., Weir, B.S. and Cockerham, C.C., 1983. Estimation for the coancestry coefficient: basis for a short-term genetic distance. *Genetics,* 105**,** pp. 767-779.

Roose-Amsaleg, C.L., Garnier-Sillam, E. and Harry, M., 2001. Extraction and purification of microbial DNA from soil and sediment samples. *Applied Soil Ecology,* 18**,** pp. 47–60.

Rotthauwe, J.H., Witzel, K.P. and Liesack, W., 1997. The Ammonia Monooxygenase Structural Gene amoA as a Functional Marker: Molecular Fine-Scale Analysis of Natural Ammonia-Oxidizing Populations. *Applied and Environmental Microbiology,* 63 (12)**,** pp. 4704–4712.

Sambrook, J. and Russell, D.W., 2003. *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Harbor Laboratory Press.

Schloss, P.D. and Handelsman, J., 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Applied and Environmental Microbiology,* 71 (3)**,** pp. 1501-1506.

Shamshuddin, J., Mokhtar, N. and Paramananthan, S., 1986. Morphology, Mineralogy and Chemistry of an Ex-Mining Land in Ipoh, Perak. *Pertanika,* 9 (1)**,** pp. 89 - 97.

Shannon, C.E. and Weaver, W., 1949. *The Mathematical Theory of Communication.* Urbana, Illinois: University of Illinois Press.

Shaw, L.J. et al., 2006. *Nitrosospira* spp. can produce nitrous oxide via a nitrifier denitrifier pathway. *Environmental Microbiology,* 8 (2)**,** pp. 214 - 222.

Shrestha, M. et al., 2010. Effect of nitrogen fertilization on methane oxidation, abundance, community structure, and gene expression of methanotrophs in the rice rhizosphere. *The ISME Journal*, 4 (12)**,** pp. 1545-1556.

Shukla, A.K. et al., 2010. Kinetics of bio-filtration of trichloroethylene by methanotrophs in presence of methanol. *Bioresour Technol,* 101 (21)**,** pp. 8119-26.

Shukla, A.K. et al., 2009. Biodegradation of trichloroethylene (TCE) by methanotrophic community. *Bioresour Technol,* 100 (9)**,** pp. 2469-74.

Siljanen, H.M. et al., 2011. Hydrology is reflected in the functioning and community composition of methanotrophs in the littoral wetland of a boreal lake. *FEMS Microbiology Ecology,* 75 (3)**,** pp. 430-445.

Simpson, E.H., 1949. Measurement of Diversity. *Nature,* 163**,** pp. 688.

Sinigalliano, C.D., Kuhn, D.N. and Jones, R.D., 1995. Amplification of the *amo*A Gene from Diverse Species of Ammonium-Oxidizing Bacteria and from an Indigenous Bacterial Population from Seawater. *Applied and Environmental Microbiology,* 61 (7)**,** pp. 2702–2706.

Smith, V.H., Tilman, G.D. and Nekola, J.C., 1999. Eutrophication: impacts of excess nutrient inputs on freshwater,marine, and terrestrial ecosystems. *Environmental Pollution* 100**,** pp. 179-196.

Stackebranct, E. and Goebel, B.M., 1994. Taxonomic Note: A Place for DNA-DNA Reassociation and 16s rRNA Sequence Analysis in the Present Species Definition in Bacteriology. *International Journal of Systematic Bacteriology,* 44 (4)**,** pp. 846-849.

Stainthorpe, A.C. et al., 1990. The methane monooxygenase gene cluster of *Methylococcus capsulatus* (Bath). *Gene,* 91 (27 - 34)**,** pp.

Stanley, S.H. et al., 1983. Copper stress underlies the fundamental change in intracellular location of methane mono-oxygenase in methane-oxidizing organisms: Studies in batch and continuous cultures. *Biotechnology Letters,* 5 (7)**,** pp. 487-492.

Stehr, G. et al., 1995. The ammonia-oxidizing nitrifying population of the River Elbe estuary. *FEMS Microbiology Ecology,* 17**,** pp. 177-186.

Stein, L.Y. et al., 2000. Differential regulation of *amo*A and *amo*B gene copies in *Nitrosomonas europaea*. *FEMS Microbiology Letters,* 192**,** pp. 163 - 168.

Stephen, J.R. et al., 1999. Effect of Toxic Metals on Indigenous Soil Beta-Subgroup Proteobacterium Ammonia Oxidizer Community Structure and Protection against Toxicity by Inoculated Metal-Resistant Bacteria. *Applied and Environmental Microbiology,* 65 (1)**,** pp. 95–101.

Stout, L. and Nusslein, K., 2010. Biotechnological potential of aquatic plant-microbe interactions. *Current Opinion in Biotechnology,* 21 (3)**,** pp. 339-45.

Strauss, E.A. and Lamberti, G.A., 2000. Regulation of nitrification in aquatic sediments by organic carbon. *Limnology and Oceanography,* 45 (8)**,** pp. 1854–1859.

Strous, M., Kuenen, J.G. and Jetten, M., 1999. Key Physiology of Anaerobic Ammonium Oxidation. *Applied and Environmental Microbiology,* 65 (7)**,** pp. 3248–3250.

Takeda, K. et al., 2008. Characterization of cultivable methanotrophs from paddy soils and rice roots. *Soil Science and Plant Nutrition,* 54**,** pp. 876 – 885.

Tamura, K. et al., 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* pp. In Press.

Tan, C.S., 2010. *Molecular Characterization of Bacteria Diversity in a Recirculating Aquaculture System (RAS) For Nile Tilapia.* Bachelor of Science (Hons) Biotechnology, Universiti Tunku Abdul Rahman.

Teske, A. et al., 1994. Evolutionary Relationships among Ammonia and Nitrite Oxidizing Bacteria. *Journal of Bacteriology,* 176 (21)**,** pp. 6623 - 6630.

Thamdrup, B. and Dalsgaard, T., 2002. Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. *Applied and Environmental Microbiology,* 68 (3)**,** pp. 1312 - 1318.

Theisen, A.R. et al., 2005. Regulation of methane oxidation in the facultative methanotroph *Methylocella silvestris* BL2. *Molecular Microbiology,* 58 (3)**,** pp. 682 - 692.

TheWalserGroup, 2012, *Draft Genome of Pasteuria ramosa* [Online]. Available at: http://evolution.unibas.ch/walser/pasteuria.htm [Accessed: 18 May 2013].

Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research,* 22 (22)**,** pp. 4673-4680.

Torsvik, V. and Øvreås, L., 2002. Microbial diversity and function in soil: from genes to ecosystems. *Current Opinion in Microbiology* 5**,** pp. 240–245.

Tsien, H.C. et al., 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. *Applied and Environmental Microbiology,* 55 (12)**,** pp. 3155 - 3161.

Urakawa, H. et al., 2006. Abundance and Population Structure of Ammonia-Oxidizing Bacteria That Inhabit Canal Sediments Receiving Effluents from Municipal Wastewater Treatment Plants. *Applied and Environmental Microbiology,* 72 (10)**,** pp. 6845 - 6850.

Urakawa, H. et al., 2008. Low temperature decreases the phylogenetic diversity of ammonia-oxidizing archaea and bacteria in aquarium biofiltration systems. *Applied and Environmental Microbiology,* 74 (3)**,** pp. 894-900.

Valentine, D., 2002. Biogeochemistry and microbial ecology of methane oxidation in anoxic environments: a review. *Antonie van Leeuwenhoek,* 81**,** pp. 271-282.

van Bodegom, P. et al., 2001. Methane oxidation and the competition for oxygen in the rice rhizosphere. *Appl Environ Microbiol,* 67 (8)**,** pp. 3586-97.

Van de Peer, Y., Chapelle, S. and Watcher, R.D., 1996. A quantitative map of nucleotide substitution rates in bacterial rRNA. *Nucleic Acids Research,* 24 (17)**,** pp. 3381 - 3391.

Vitousek, P.M. et al., 1997. Human Alteration of Global Nitrogen Cycle: Source and Consequences. *Ecological Applications,* 7 (3)**,** pp. 737–750.

Vitousek, P.M. and Howarth, R.W., 1991. Nitrogen Limitation on Land and in the Sea: How Can It Occur? *Biogeochemistry,* 13 (2)**,** pp. 87-115.

Voytek, M.A. and Ward, B.B., 1995. Detection Of Ammonium-Oxidizing Bacterial Of The Beta-Subclass Of The Class Proteobacteria In Aquatic Samples With The PCR. *Applied and Environmental Microbiology,* 61 (4)**,** pp. 1444-1450.

Wahlen, M., 1993. The Global Methane Cycle. *Annual Review of Earth and Planetary Sciences,* 21**,** pp. 407-426.

Wang, S. et al., 2012. Abundance and diversity of nitrogen-fixing bacteria in rhizosphere and bulk paddy soil under different duration of organic management. *World Journal of Microbiology and Biotechnology,* 28 (2)**,** pp. 493-503

Wang, Y. et al., 2009. Community composition of ammonia-oxidizing bacteria and archaea in rice field soil as affected by nitrogen fertilization. *Systematic and Applied Microbiology,* 32 (1)**,** pp. 27-36.

Watanabe, K., 2001. Microorganisms relevant to bioremediation. *Current Opinion in Biotechnology,* 12**,** pp. 237 - 240.

Wei, B. et al., 2011. Comparison of the community structures of ammonia-oxidizing bacteria and archaea in rhizoplanes of floating aquatic macrophytes. *Microbiological Research,* 166 (6)**,** pp. 468-74.

Weyhenmeyer, G.A., 1998. Resuspension in lakes and its ecological impact - a review. *Archiv für Hydrobiologie (Fundamental and Applied Limnology),* 51**,** pp. 185 - 200.

Whittaker, R.H., 1972. Evolution and Measurement of Species Diversity. *Taxon,* 21**,** pp. 213 - 251.

Woese, C., 1987. Bacterial Evolution. *Microbiological Reviews* 51 (2)**,** pp. 221 - 271.

Wu, L., Ma, K. and Lu, Y., 2009. Rice roots select for type I methanotrophs in rice field soil. *Systematic and Applied Microbiology,* 32 (6)**,** pp. 421-428.

Wuebbles, D.J. and Hayhoe, K., 2002. Atmospheric methane and global change. *Earth-Science Reviews,* 57**,** pp. 177–210.

Xia, X.H., Yang, Z.F. and Zhang, X.Q., 2009. Effect of Suspended-Sediment Concentration on Nitrification in River Water: Importance of Suspended Sediment-Water Interface. *Environmental Science & Technology,* 43 (10)**,** pp. 3681–3687.

You, J. et al., 2009. Ammonia-oxidizing archaea involved in nitrogen removal. *Water Research,* 43 (7)**,** pp. 1801-1809.

You, S.J. and Chen, W.Y., 2008. Ammonia oxidizing bacteria in a nitrite-accumulating membrane bioreactor. *International Biodeterioration & Biodegradation,* 62 (3)**,** pp. 244-249.

Yule, C.M. et al., 2004, The Ecology of Malaysian Tin Mine Lakes. Available at: http://www.bio.uu.nl/intecol/programme/cnt_abstract.php?frm=T9_cs7_7.pdf [Accessed: March 15, 2011].

Yusof, A.M. et al., 2001. Water quality studies in an aquatic environment of disused tin-mining pools and in drinking water. *Ecological Engineering,* 16**,** pp. 405–414.

Yusoff, F.M., Shariff, M. and Gopinath, N., 2006. Diversity of Malaysian aquatic ecosystems and resources. *Aquatic Ecosystem Health & Management,* 9 (2)**,** pp. 119-135.

Zahn, J.A. et al., 1996. Evidence for an iron center in the ammonia monooxygenase from *Nitrosomonas europaea*. *FEBS Letters,* 397 (1)**,** pp. 35–38.

Zahn, J.A. and DiSpirito, A.A., 1996. Membrane-Associated Methane Monooxygenase from *Methylococcus Capsulatus* (Bath). *Journal of Bacteriology,* 178 (4)**,** pp. 1018–1029.

Zhan, J. and Sun, Q., 2011. Diversity of free-living nitrogen-fixing microorganisms in the rhizosphere and non-rhizosphere of pioneer plants growing on wastelands of copper mine tailings. *Microbiological Research***,** pp.

APPENDICES

APPENDIX A

Sources of Equipment and Materials

Table A.1: Apparatus and Machinery with their respective manufacturers

No.	Apparatus/Machinery	Manufacturing Company
1	Autoclave	Hirayama
2	Balance (2 decimals)	AdventurerPro Ohaus
3	Balance (4 decimals)	Sartorius
4	Centrifuge tubes (15 mL)	Greiner
5	Electrophoresis Gel Tank	Thermo Fisher Scientific
6	Freezer	Acson; Hestar
7	Incubator	Memmert
8	Laminar Air Flow	ESCO
9	Centrifuge	Beckman Coulter
10	Microcentrifuge	Sigma
11	Microcentrifuge tubes (1.5 mL)	Axygen
12	Micropipettes	HTL
13	Micropipette tips	Axygen, Gilson
14	Microwave oven	Sanyo
15	Nanophotometer	Implen GmbH
16	PCR tubes (0.2 mL)	JetBioFil
17	pH Meter	Mettler Toledo
18	Petri dishes	Greiner
19	Power Supply (DC, for electrophoresis)	Thermo Fisher Scientific
20	Reagent bottles	Schott Duran; Kimax
21	Refrigerator	Panasonic; Toshiba
22	Shaking Incubator	Labtech
23	Thermal Cycler (Gradient)	MJ Research
24	UV Transilluminator	Alpha Innotech
25	Vortex Machine	Harmony

Table A.1: Apparatus and Machinery with their respective manufacturers

No.	Apparatus/Machinery	Manufacturing Company
26	Water bath	Memmert

Table A.2: Chemicals, reagents and media (prepared) with their respective manufacturers

No.	Chemical/Reagent	Manufacturer
1	Agar Powder	BD
2	Agarose Powder	Promega
3	Ampicillin	Amresco
4	Boric Acid (Molecular Biology Grade)	SYSTERM
5	Bromo-chloro-indolyl-galactopyranoside (X-gal)	Eppendorf AG
6	Dimethylformamide	Amresco
7	DNA Ladder (100 bp Plus, ready to use)	Fermentas
8	DNA Ladder (1 kb, ready to use)	Fermentas
9	dNTP Mix	Intron Biotechnology, Inc.
10	Ethylenediaminetetraacetic acid (EDTA) Sodium Salt	SYSTERM
11	Ethyl Alcohol (Ethanol)	SYSTERM
12	Taq Polymerase	Intron Biotechnology, Inc.
13	Isopropylthio-β-D-galactoside (IPTG)	Eppendorf AG
14	Loading Dye (6X)	New England Biolabs
15	Luria-Bertani (LB) Broth (Lennox), ready mix	Laboratorios CONDA
16	PCR Primers	Bioneer, Inc.
17	Peptone	R & M Chemicals
18	Sodium Chloride	R & M Chemicals
19	Sodium Dodecyl Sulfate	Biobasic Inc.
20	Sodium Hydroxide	R & M Chemicals
21	Tris(hydroxymethyl)aminomethane Base	Calbiochem

Table A.3: Extraction/Molecular Cloning Kits and their Respective Manufacturers

No.	Extraction/Molecular Cloning Kits	Manufacturer
1	Agarose Gel Extraction Kit	Intron Biotechnology, Inc.
2	PCR Cloning Kit& Competent Cells	Novagen
3	Plasmid Extraction Kit	EuRx, Ltd.
4	Soil DNA Extraction Kit	MoBio, Inc.

APPENDIX B

Composition of Media, Buffers & Other Solutions

Table B.1: Compositions of media used for bacterial cultivation

Type of Media	Ingredient	Amount Per Litre (g)
Luria Bertani (LB) Agar	LB Broth	20.0
	Difco™ Bacto Agar	15.0
Luria Bertani Broth	LB Broth	20.0

Table B.2: Composition of Buffers and Solutions

No.	Buffer/Solution	Composition
3	EDTA (0.5M, pH 8.0)	181.1 g disodium EDTA·$2H_2O$ dissolved in 800 mL distilled water, adjust to pH 8.0 with ~20 g NaOH pellets
4	Ethanol (70% v/v)	35 mL Ethanol, top up to 50 mL with distilled water
5	GelRed™ Nucleic Acid Stain (3X)	75 µL GelRed™ Nucleic Acid Stain (10,000X) and 5 mL NaCl (5M), top up to 225 mL with distilled water
6	IPTG (20% w/v, 0.8M)	Dissolve 2 g IPTG in 8 mL distilled water, adjust final volume to 10 mL with distilled water
9	NaCl (5M)	29.22 g NaCl, top up to 100 mL with distilled water
13	TBE Buffer (5X)	54 g Tris base, 27.5 g boric acid, 20 mL 0.5M EDTA (pH 8.0), top up to 1 L with distilled deionised water
15	X-gal (2% w/v)	20 mg X-gal dissolved in 1 mL dimethylformamide, store in microcentrifuge tube wrapped with aluminium foil at -20°C

APPENDIX C

Clonining Vector Map & Cloning Site Sequences

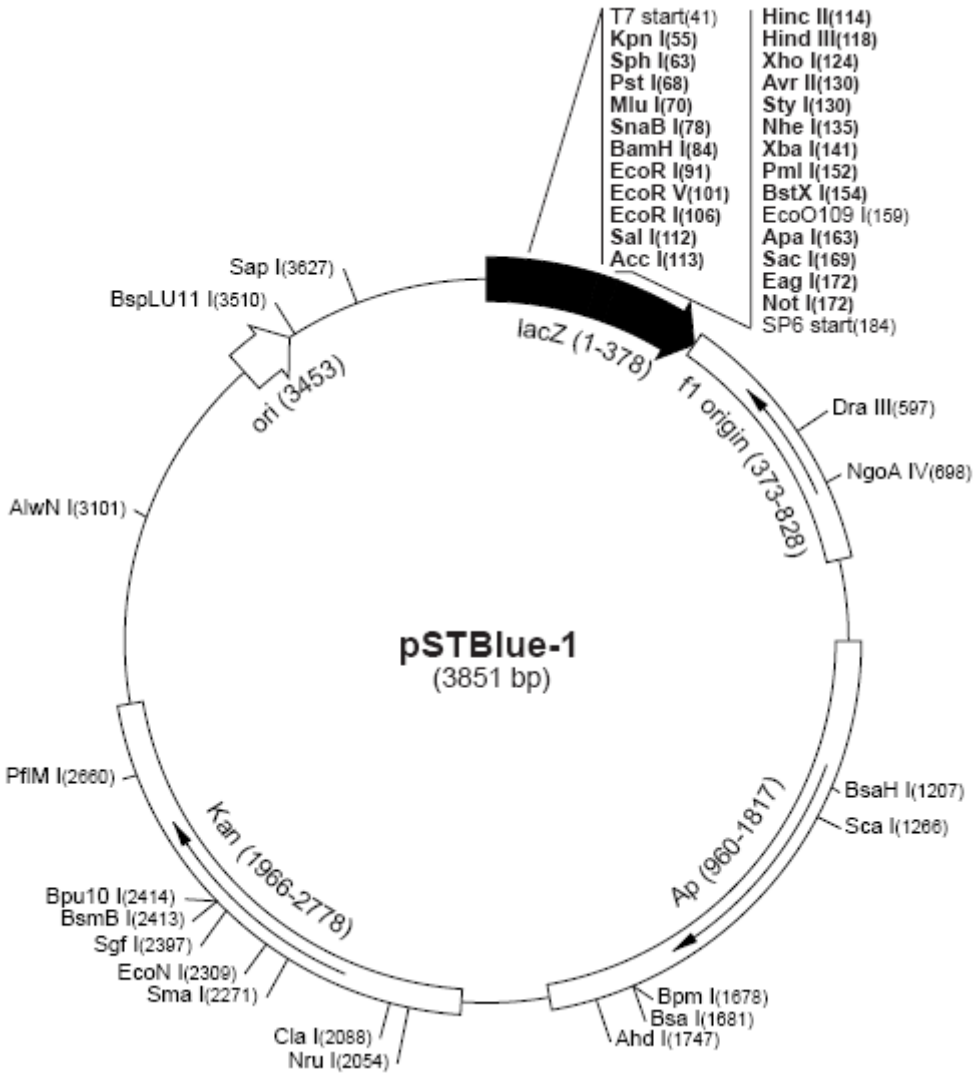


Figure C.1: pST Blue-1 Vector Map(Novagen 2008). Illustrated are the multiple cloning site, restriction enzyme, and promoter sites. Location for the origin of replication, phage f1 origin, antibiotic resistant genes, and LacZ α-peptide are also indicated.

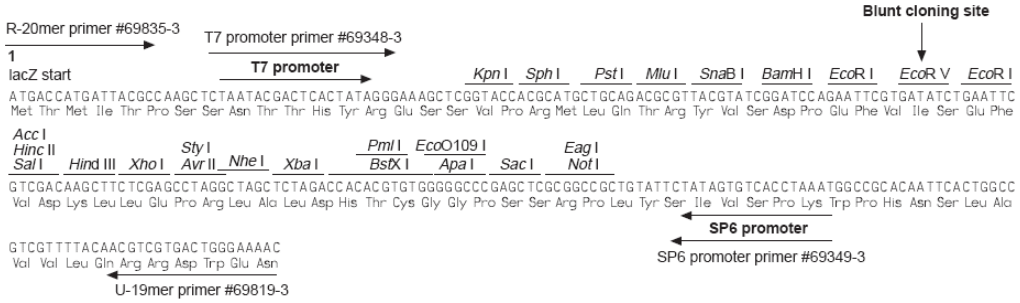


Figure C.2: Regions surrounding the cloning site of the pSTBlue-1 Vector. Illustrated are the LacZ α-peptide sequence, promoters, primer sites and unique restriction sites (Novagen 2008).

APPENDIX D

Accession Numbers of Sequences Submitted to GenBank

Table D.1: List of *amo*A sequences and their respective accession numbers

No.	Name of Sequence	GenBank Accession Number
1	L2sAMO11	JX157920
2	L2sAMO26	JX157921
3	L2sAMO37	JX157922
4	L2sAMO51	JX157923
5	L2sAMO78	JX157924
6	L2sAMO92	JX157925
7	L2sAMO59	JX157926
8	L2sAMO73	JX157927
9	L2sAMO83	JX157928
10	F3sAMO1	JX157929
11	F3sAMO4	JX157930
12	F3sAMO6	JX157931
13	F3sAMO7	JX157932
14	F3sAMO10	JX157933
15	F3sAMO13	JX157934
16	F3sAMO17	JX157935
17	F3sAMO20	JX157936
18	F3sAMO24	JX157937
19	F3sAMO66	JX157938
20	F3sAMO82	JX157939
21	L2wAMO1	JX157940
22	L2wAMO2	JX157941
23	L2wAMO3	JX157942
24	L2wAMO6	JX157943
25	L2wAMO8	JX157944
26	L2wAMO13	JX157945
27	L2wAMO14	JX157946
28	L2wAMO17	JX157947
29	L2wAMO21	JX157948
30	L2wAMO25	JX157949
31	L2wAMO28	JX157950
32	L2wAMO30	JX157951
33	L2wAMO33	JX157952
34	L2wAMO34	JX157953
35	L2wAMO35	JX157954
36	L2wAMO36	JX157955
37	L2wAMO50	JX157956
38	L2wAMO58	JX157957

Table D.2: List of 16S rDNA (CTO) sequences and their respective accession numbers

No.	Name of Sequence	GenBank Accession Number
1	F1sCTO1	JX184132
2	F1sCTO3	JX184133
3	F1sCTO4	JX184134
4	F1sCTO10	JX184135
5	F1sCTO13	JX184136
6	F1sCTO43	JX184137
7	F2sCTO1	JX184138
8	F2sCTO17	JX184139
9	F2sCTO32	JX184140
10	F2sCTO34	JX184141
11	F2sCTO35	JX184142
12	F2sCTO38	JX184143
13	F2sCTO41	JX184144
14	F3sCTO1	JX184145
15	F3sCTO6	JX184146
16	F3sCTO8	JX184147
17	F3sCTO9	JX184148
18	F3sCTO10	JX184149
19	F3sCTO12	JX184150
20	F3sCTO14	JX184151
21	F3sCTO16	JX184152
22	F3sCTO25	JX184153
23	F3sCTO28	JX184154
24	F1wCTO2	JX184155
25	F1wCTO4	JX184156
26	F1wCTO5	JX184157
27	F1wCTO8	JX184158
28	F1wCTO12	JX184159
29	F1wCTO16	JX184160
30	F1wCTO18	JX184161
31	F1wCTO45	JX184162
32	F2wCTO1	JX184163
33	F2wCTO2	JX184164
34	F2wCTO5	JX184165
35	F2wCTO7	JX184166
36	F2wCTO8	JX184167
37	F2wCTO14	JX184168
38	F2wCTO15	JX184169
39	F2wCTO29	JX184170
40	F2wCTO48	JX184171
41	F3wCTO1	JX184172
42	F3wCTO7	JX184173
43	F3wCTO8	JX184174
44	F3wCTO14	JX184175
45	F3wCTO16	JX184176
46	F3wCTO22	JX184177

Table D.2 (continued): List of 16S rDNA (CTO) sequences and their respective accession numbers

No.	Name of Sequence	GenBank Accession Number
47	F3wCTO30	JX184178
48	F3wCTO44	JX184179
49	F3wCTO48	JX184180
50	F3wCTO51	JX184181
51	L1sCTO1	JX184182
52	L1sCTO2	JX184183
53	L1sCTO3	JX184184
54	L1sCTO10	JX184185
55	L1sCTO19	JX184186
56	L1sCTO40	JX184187
57	L1sCTO41	JX184188
58	L1sCTO50	JX184189
59	L1sCTO54	JX184190
60	L1sCTO58	JX184191
61	L2sCTO3	JX184192
62	L2sCTO7	JX184193
63	L2sCTO15	JX184194
64	L2sCTO17	JX184195
65	L2sCTO20	JX184196
66	L2sCTO24	JX184197
67	L2sCTO35	JX184198
68	L1wCTO1	JX184199
69	L1wCTO2	JX184200
70	L1wCTO10	JX184201
71	L1wCTO12	JX184202
72	L1wCTO29	JX184203
73	L2wCTO1	JX184204
74	L2wCTO4	JX184205
75	L2wCTO10	JX184206
76	L2wCTO12	JX184207
77	L2wCTO14	JX184208
78	L2wCTO32	JX184209
79	L2wCTO36	JX184210
80	L2wCTO46	JX184211
81	L2wCTO51	JX184212
82	L2wCTO8	JX184214
83	I2wCTO8	JX184213
84	I2wCTO10	JX184215
85	I2wCTO12	JX184216
86	I2wCTO14	JX184217
87	I2wCTO20	JX184218
88	I2wCTO23	JX184219
89	I2wCTO28	JX184220
90	I2wCTO33	JX184221
91	I2wCTO36	JX184222
92	I2wCTO38	JX184223

Table D.2 (continued): List of 16S rDNA (CTO) sequences and their respective accession numbers

No.	Name of Sequence	GenBank Accession Number
93	I2wCTO45	JX184224
94	I2wCTO69	JX184225
95	I2wCTO70	JX184226
96	I2wCTO71	JX184227
97	I2wCTO72	JX184228
98	I2wCTO77	JX184229
99	I2wCTO81	JX184230
100	I2wCTO84	JX184231
101	I2wCTO97	JX184232
102	I2wCTO99	JX184233
103	I2wCTO101	JX184234
104	I2wCTO104	JX184235
105	I2wCTO105	JX184236
106	I2wCTO106	JX184237
107	I2wCTO108	JX184238

Table D.3: List of *pmo*A sequences and their respective accession numbers

No.	Name of Sequence	GenBank Accession Number
1	F1sMB6	JX184239
2	F1sMB10	JX184240
3	F1sMB17	JX184241
4	F1sMB20	JX184242
5	F1sMB26	JX184243
6	F1sMB44	JX184244
7	F1sMB50	JX184245
8	F1sMB61	JX184246
9	F1sMB66	JX184247
10	F1sMB67	JX184248
11	F2sMB1	JX184249
12	F2sMB3	JX184250
13	F2sMB5	JX184251
14	F2sMB42	JX184252
15	F2sMB67	JX184253
16	F2sMB69	JX184254
17	F2sMB58	JX184255
18	F2sMB22	JX184256
19	F2sMB36	JX184257
20	F2sMB37	JX184258
21	F2sMB44	JX184259
22	F2sMB50	JX184260
23	F2sMB66	JX184261
24	F3sMB4	JX184262
25	F3sMB10	JX184263
26	F3sMB12	JX184264

Table D.3 (continued): List of *pmo*A sequences and their respective accession numbers

No.	Name of Sequence	GenBank Accession Number
27	F3sMB15	JX184265
28	F3sMB16	JX184266
29	F3sMB26	JX184267
30	F3sMB27	JX184268
31	F3sMB29	JX184269
32	F3sMB38	JX184270
33	F3sMB58	JX184271
34	F3sMB62	JX184272
35	F3sMB70	JX184273
36	F3sMB74	JX184274
37	F1wMB3	JX184275
38	F1wMB6	JX184276
39	F1wMB8	JX184277
40	F1wMB10	JX184278
41	F1wMB12	JX184279
42	F1wMB16	JX184280
43	F1wMB23	JX184281
44	F1wMB41	JX184282
45	F1wMB44	JX184283
46	F2wMB2	JX184284
47	F2wMB5	JX184285
48	F2wMB6	JX184286
49	F2wMB11	JX184287
50	F2wMB16	JX184288
51	F2wMB30	JX184289
52	F2wMB34	JX184290
53	F2wMB44	JX184291
54	F2wMB51	JX184292
55	F2wMB71	JX184293
56	F2wMB91	JX184294
57	F3wMB1	JX184295
58	F3wMB2	JX184296
59	F3wMB6	JX184297
60	F3wMB7	JX184298
61	F3wMB16	JX184299
62	F3wMB18	JX184300
63	F3wMB20	JX184301
64	F3wMB44	JX184302
65	F3wMB45	JX184303
66	F3wMB46	JX184304
67	F3wMB60	JX184305
68	F3wMB63	JX184306
69	L1sMB1	JX184307
70	L1sMB4	JX184308
71	L1sMB9	JX184309
72	L1sMB17	JX184310

Table D.3 (continued): List of *pmo*A sequences and their respective accession numbers

No.	Name of Sequence	GenBank Accession Number
73	L1sMB20	JX184311
74	L1sMB29	JX184312
75	L1sMB38	JX184313
76	L1sMB41	JX184314
77	L1sMB51	JX184315
78	L1sMB57	JX184316
79	L1sMB60	JX184317
80	L2sMB1	JX184318
81	L2sMB2	JX184319
82	L2sMB3	JX184320
83	L2sMB4	JX184321
84	L2sMB5	JX184322
85	L2sMB7	JX184323
86	L2sMB9	JX184324
87	L2sMB11	JX184325
88	L2sMB12	JX184326
89	L2sMB13	JX184327
90	L2sMB15	JX184328
91	L2sMB16	JX184329
92	L2sMB19	JX184330
93	L2sMB21	JX184331
94	L2sMB20	JX184332
95	L2sMB24	JX184333
96	L2sMB25	JX184334
97	L1wMB2	JX184335
98	L1wMB5	JX184336
99	L1wMB6	JX184337
100	L1wMB8	JX184338
101	L1wMB9	JX184339
102	L1wMB12	JX184340
103	L1wMB23	JX184341
104	L1wMB28	JX184342
105	L1wMB42	JX184343
106	L1wMB43	JX184344
107	L1wMB44	JX184345
108	L2wMB2	JX184346
109	L2wMB12	JX184347
110	L2wMB14	JX184348
111	L2wMB18	JX184349
112	L2wMB41	JX184350
113	L2wMB42	JX184351
114	L2wMB47	JX184352
115	L2wMB51	JX184353
116	L2wMB45	JX184354

Table D.3 (continued): List of *pmo*A sequences and their respective accession numbers

No.	Name of Sequence	GenBank Accession Number
117	L2wMB57	JX184355
118	L2wMB60	JX184356
119	I1sMB1	JX184357
120	I1sMB2	JX184358
121	I1sMB3	JX184359
122	I1sMB13	JX184360
123	I1sMB16	JX184361
124	I1sMB19	JX184362
125	I1sMB22	JX184363
126	I1sMB24	JX184364
127	I1sMB28	JX184365
128	I1sMB32	JX184366
129	I1sMB38	JX184367
130	I1sMB44	JX184368
131	I2sMB6	JX184369
132	I2sMB10	JX184370
133	I2sMB16	JX184371
134	I2sMB22	JX184372
135	I2sMB26	JX184373
136	I2sMB38	JX184374
137	I2sMB44	JX184375
138	I2sMB48	JX184376
139	I2sMB51	JX184377
140	I2sMB70	JX184378
141	I2sMB79	JX184379
142	I2sMB94	JX184380
143	I1wMB7	JX184381
144	I1wMB8	JX184382
145	I1wMB10	JX184383
146	I1wMB12	JX184384
147	I1wMB16	JX184385
148	I1wMB21	JX184386
149	I1wMB22	JX184387
150	I1wMB24	JX184388
151	I1wMB27	JX184389
152	I1wMB39	JX184390
153	I1wMB40	JX184391
154	I2wMB1	JX184392
155	I2wMB3	JX184393
156	I2wMB6	JX184394
157	I2wMB9	JX184395
159	I2wMB38	JX184397
160	I2wMB43	JX184398
158	I2wMB16	JX184396

Table D.3 (continued): List of *pmo*A sequences and their respective accession numbers

No.	Name of Sequence	GenBank Accession Number
161	I2wMB45	JX184399
162	I2wMB53	JX184400
163	I2wMB57	JX184401
164	I2wMB60	JX184402
165	I2wMB64	JX184403

APPENDIX E

Full Multiple Sequence Alignments

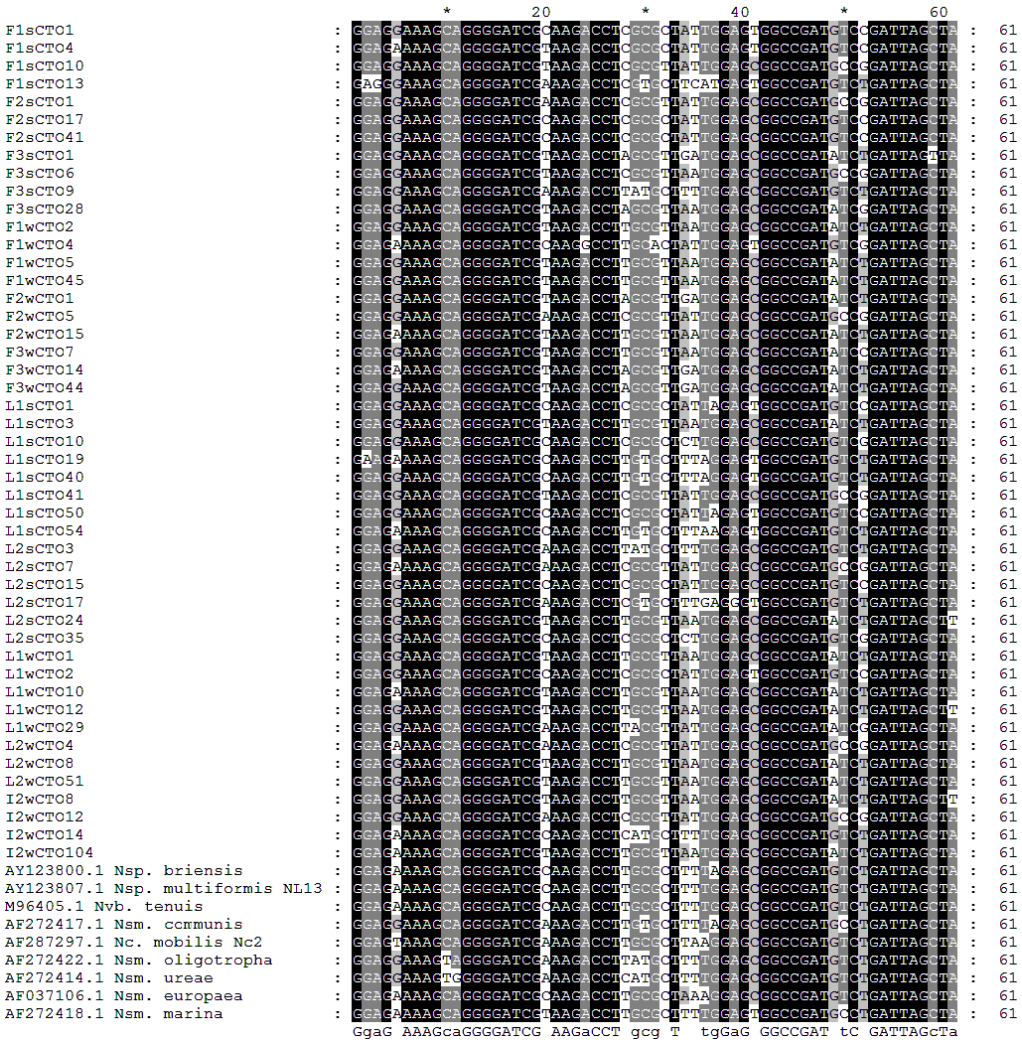


Figure E.1: Multiple sequence alignment of 16S rDNA Nucleic Acid Alignments of selected AOBs detected in this study and closely related lineages. Residues boxed in black are conserved in all the sequences. Residues in dark and light grey are conserved in more than 80% or 60% of the sequences respectively. Nsm. - *Nitrosomonas*; Nsp. - *Nitrosospira*; Nsc. - *Nitrosococcus*.

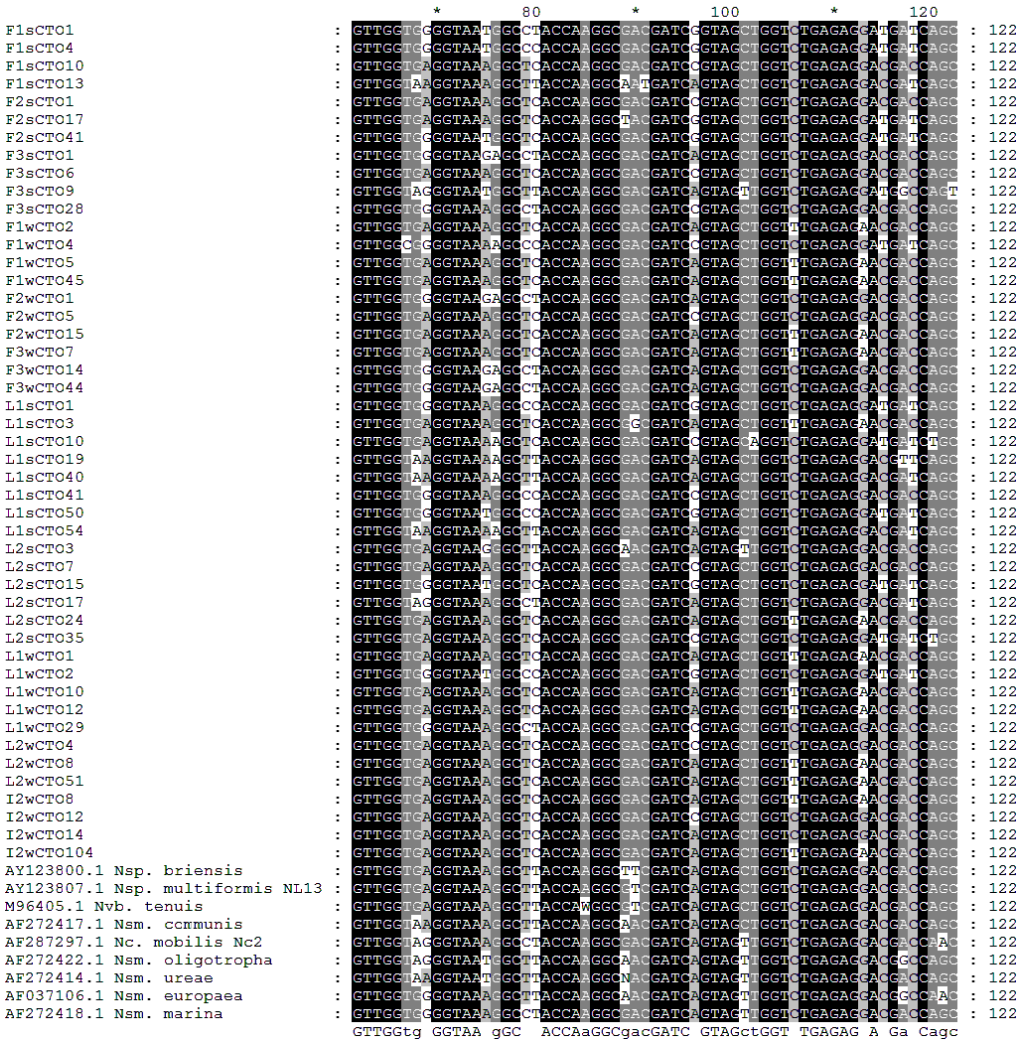


Figure E.1 (continued): Multiple sequence alignment of 16S rDNA Nucleic Acid Alignments of selected AOBs detected in this study and closely related lineages.

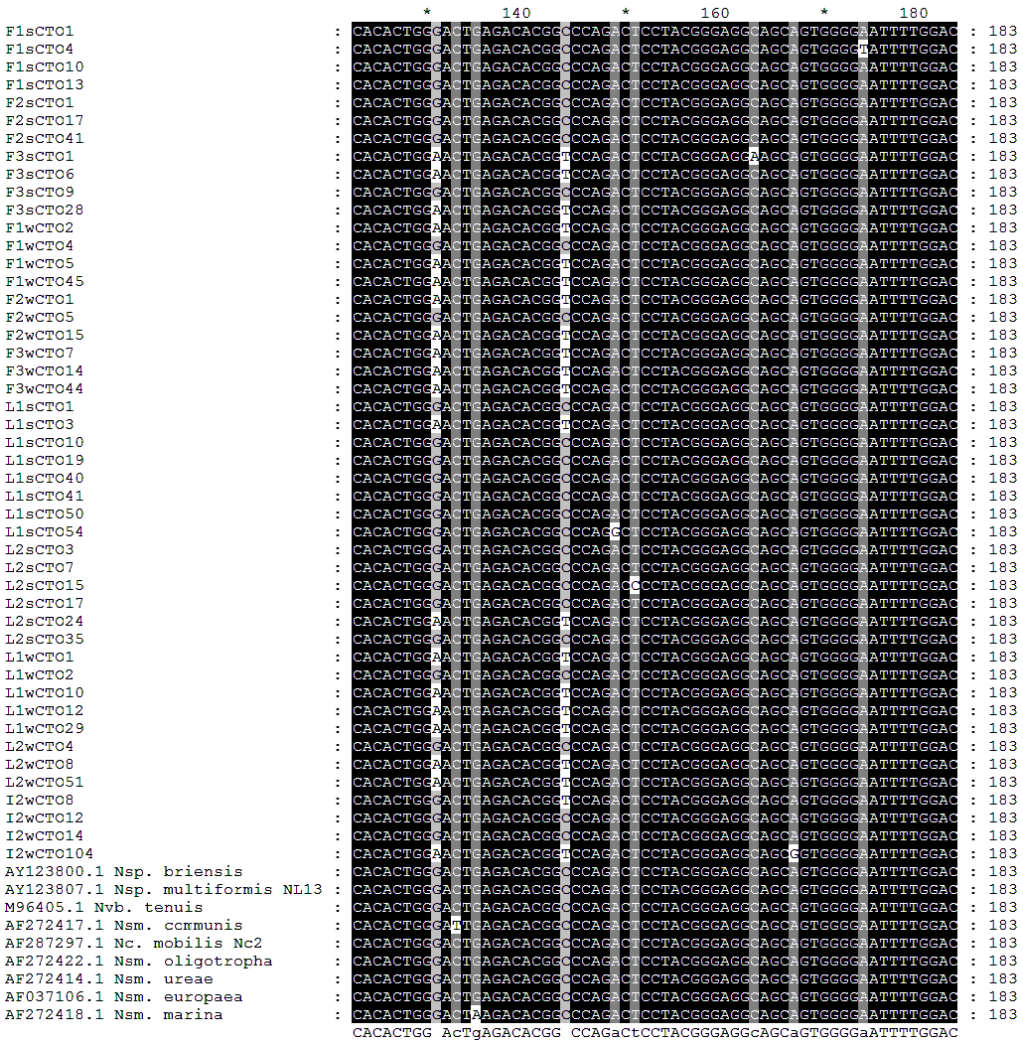


Figure E.1 (continued): Multiple sequence alignment of 16S rDNA Nucleic Acid Alignments of selected AOBs detected in this study and closely related lineages.

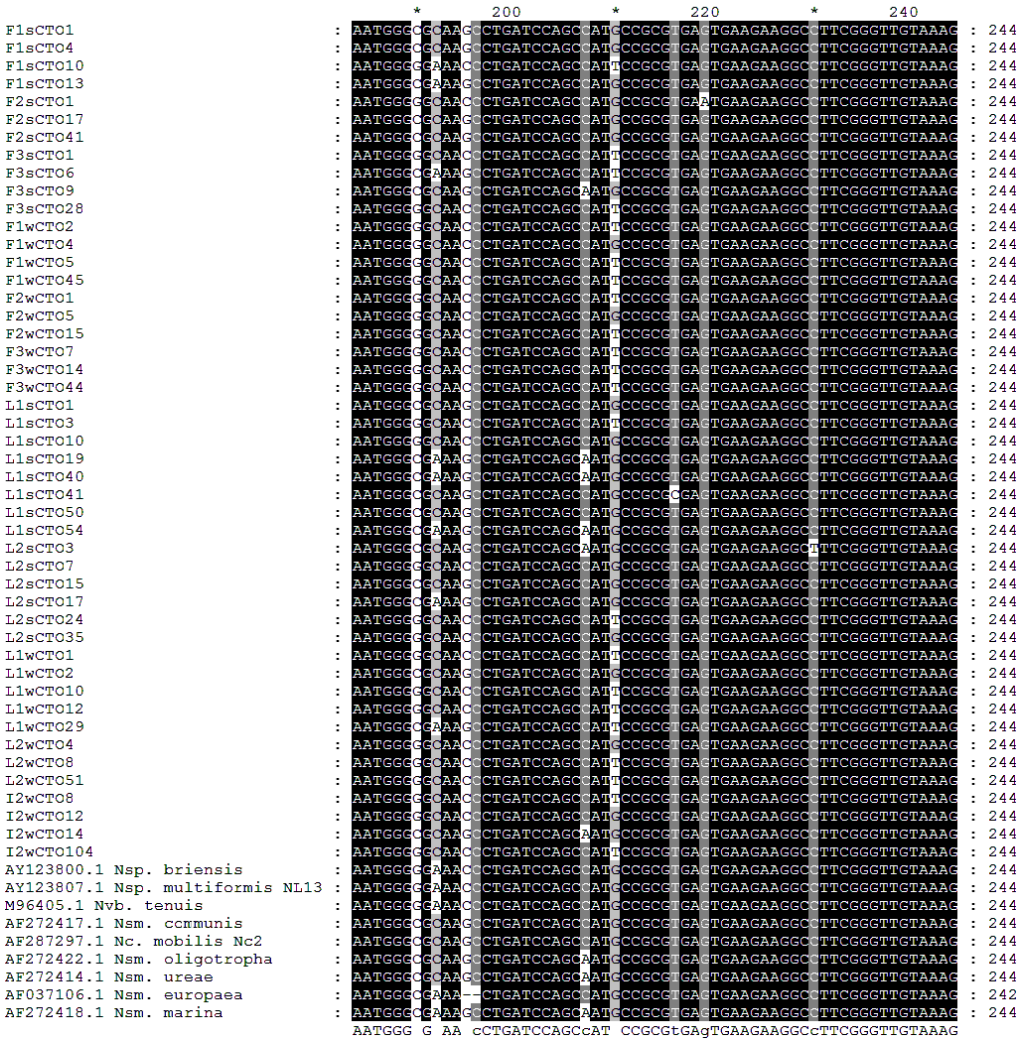


Figure E.1 (continued): Multiple sequence alignment of 16S rDNA Nucleic Acid Alignments of selected AOBs detected in this study and closely related lineages.

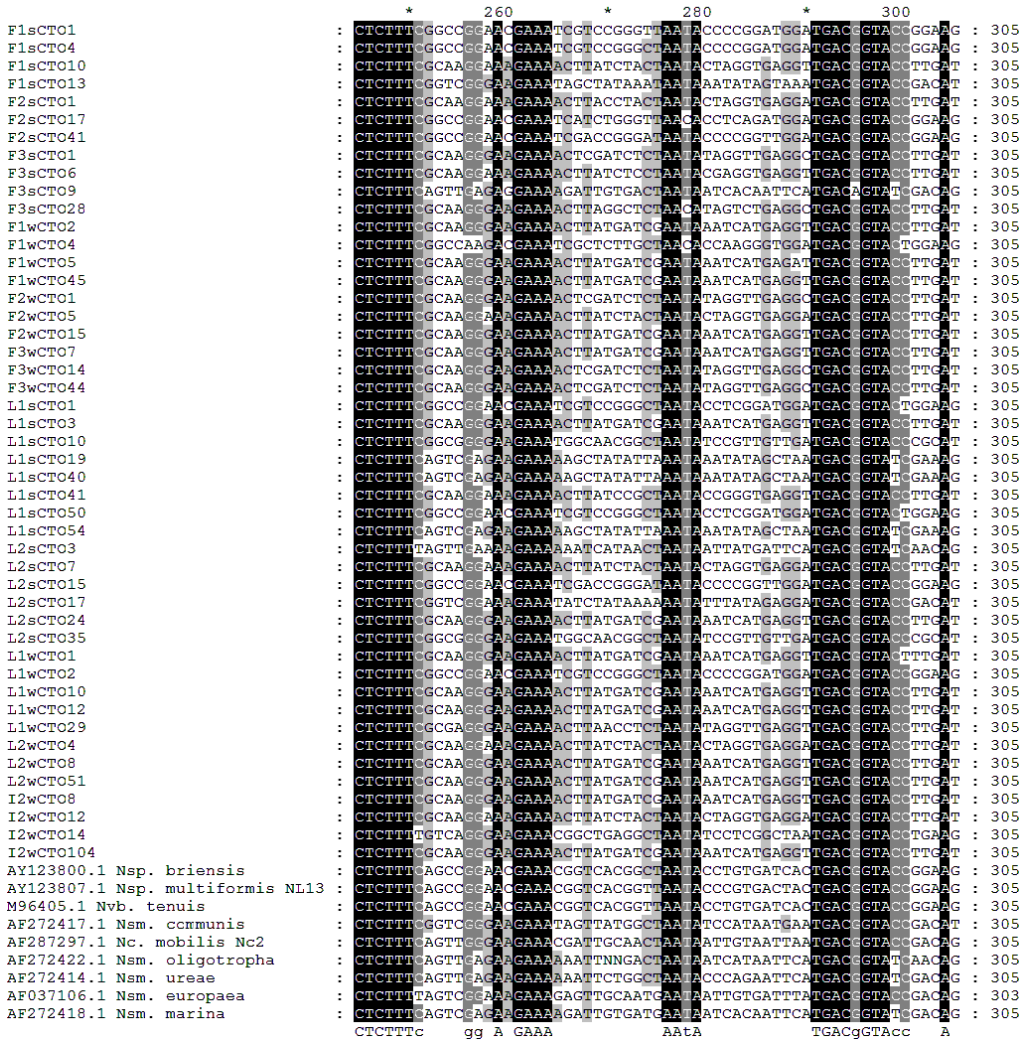


Figure E.1 (continued): Multiple sequence alignment of 16S rDNA Nucleic Acid Alignments of selected AOBs detected in this study and closely related lineages.

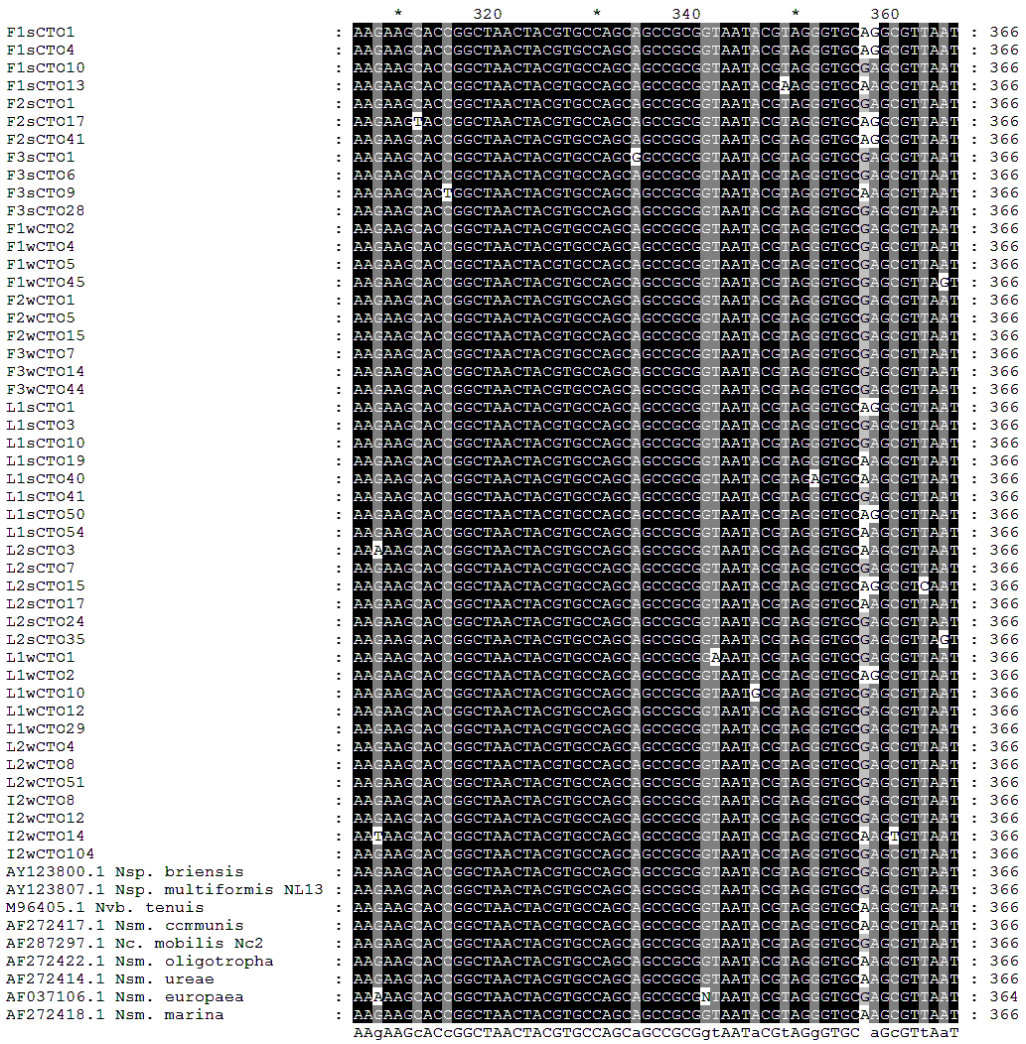


Figure E.1 (continued): Multiple sequence alignment of 16S rDNA Nucleic Acid Alignments of selected AOBs detected in this study and closely related lineages.

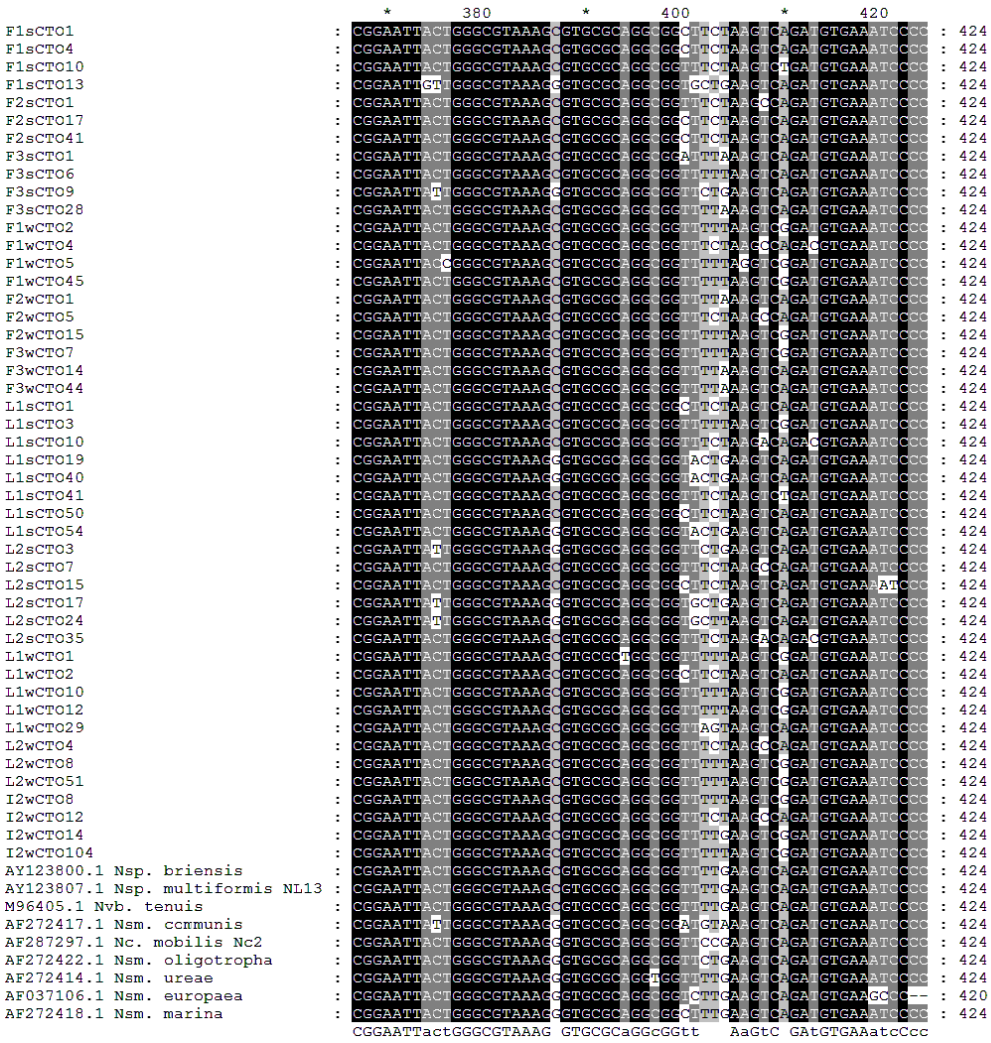


Figure E.1 (continued): Multiple sequence alignment of 16S rDNA Nucleic Acid Alignments of selected AOBs detected in this study and closely related lineages.

APPENDIX F

Complete List of Best Hits Identified from BLAST Searches and their Respective Accession Numbers

Table F.1: List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the *amo*A clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
L2sAMO11	GU249053.1 Uncultured bacterium clone XL_2_21	99
L2sAMO26	HQ190103.1 Uncultured bacterium clone BNamo29	99
L2sAMO37	JF743019.1 Uncultured bacterium clone HX3_41	99
L2sAMO51	HQ190103.1 Uncultured bacterium clone BNamo29	99
L2sAMO78	HQ190114.1 Uncultured bacterium clone BRamo29	99
L2sAMO92	HQ190103.1 Uncultured bacterium clone BNamo29	93
L2sAMO59	GU121137.1 Uncultured bacterium isolate DGGE gel band M14	100
L2sAMO73	GU249053.1 Uncultured bacterium clone XL_2_21	99
L2sAMO83	GU249053.1 Uncultured bacterium clone XL_2_21	99
F3sAMO1	GU249030.1 Uncultured bacterium clone XL1_29	97
F3sAMO4	GQ906681.1 Uncultured bacterium clone CFAOB-38	99
F3sAMO6	GQ906681.1 Uncultured bacterium clone CFAOB-38	99
F3sAMO7	GU249001.1 Uncultured bacterium clone TL_31	100
F3sAMO10	GQ906681.1 Uncultured bacterium clone CFAOB-38	98
F3sAMO13	GU249001.1 Uncultured bacterium clone TL_31	99
F3sAMO17	GQ906681.1 Uncultured bacterium clone CFAOB-38	98
F3sAMO20	GQ906681.1 Uncultured bacterium clone CFAOB-38	83
F3sAMO24	GQ906681.1 Uncultured bacterium clone CFAOB-38	98
F3sAMO66	GU248980.1 Uncultured bacterium clone TL_8	99
F3sAMO82	GU248980.1 Uncultured bacterium clone TL_8	99
L2wAMO1	AL954747.1 *Nitrosomonas europaea* ATCC 19718, complete genome	100
L2wAMO2	JF743019.1 Uncultured bacterium clone HX3_41	99
L2wAMO3	HQ594962.1 Uncultured ammonia oxidising bacterium clone JX_AOB_10-17	99
L2wAMO6	GU249053.1 Uncultured bacterium clone XL_2_21	99
L2wAMO8	EU624908.1 Uncultured ammonia-oxidizing bacterium clone BXA-218	99
L2wAMO13	HQ190103.1 Uncultured bacterium clone BNamo29	99
L2wAMO14	FJ812514.1 Uncultured bacterium clone F1h1	99
L2wAMO17	GQ143272.1 Uncultured ammonia-oxidizing bacterium clone AOBd-A1B11	96

Table F.1 (continued): List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the *amo*A clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
L2wAMO21	HQ594962.1 Uncultured ammonia oxidising bacterium clone JX_AOB_10-17	99
L2wAMO25	HQ594962.1 Uncultured ammonia oxidising bacterium clone JX_AOB_10-17	100
L2wAMO28	EU624908.1 Uncultured ammonia-oxidizing bacterium clone BXA-218	99
L2wAMO30	HQ594962.1 Uncultured ammonia oxidising bacterium clone JX_AOB_10-17	100
L2wAMO33	GU249053.1 Uncultured bacterium clone XL_2_21	99
L2wAMO34	HQ190103.1 Uncultured bacterium clone BNamo29	99
L2wAMO35	HQ190114.1 Uncultured bacterium clone BRamo29	91
L2wAMO36	HQ190103.1 Uncultured bacterium clone BNamo29	98
L2wAMO50	GQ143272.1 Uncultured ammonia-oxidizing bacterium clone AOBd-A1B11	99
L2wAMO58	GU249053.1 Uncultured bacterium clone XL_2_21	99

Table F.2: List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the CTO clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
F1sCTO1	GU097375.1 Uncultured *Nitrosospira* sp. isolate DGGE gel band 23	99
F1sCTO3	GU097375.1 Uncultured *Nitrosospira* sp. isolate DGGE gel band 23	99
F1sCTO4	HM769458.1 Uncultured beta *proteobacterium* clone D-MAY-35	99
F1sCTO10	HQ904675.1 Uncultured bacterium clone XYHPA.0912.20	99
F1sCTO13	JF497818.1 Uncultured bacterium clone SL-223	96
F1sCTO43	GU097375.1 Uncultured *Nitrosospira* sp. isolate DGGE gel band 23	98
F2sCTO1	HQ904675.1 Uncultured bacterium clone XYHPA.0912.20	98
F2sCTO17	GU097374.1 Uncultured *Nitrosospira* sp. isolate DGGE gel band 22	97
F2sCTO32	AM900186.1 Uncultured bacterium partial 16S rRNA gene, clone library NP, clone 02_B05	98
F2sCTO34	HM163111.1 Uncultured bacterium clone 1-76	98
F2sCTO35	HM769458.1 Uncultured beta *proteobacterium* clone D-MAY-35	99
F2sCTO38	HQ904675.1 Uncultured bacterium clone XYHPA.0912.20	97
F2sCTO41	GU097375.1 Uncultured *Nitrosospira* sp. isolate DGGE gel band 23	98

Table F.2 (continued): List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the CTO clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
F3sCTO1	HQ827934.1 Uncultured bacterium clone E30	99
F3sCTO6	GU097360.1 Uncultured *Nitrosospira* sp. isolate DGGE gel band 8	99
F3sCTO8	FJ393084.1 Uncultured *Azospira* sp. clone MFC-B162-C02	99
F3sCTO9	EU127377.1 Uncultured *Nitrosomonas* sp. clone 168F3	98
F3sCTO10	HQ904675.1 Uncultured bacterium clone XYHPA.0912.20	97
F3sCTO12	HQ330609.1 Uncultured bacterium clone PT33	99
F3sCTO14	HQ904675.1 Uncultured bacterium clone XYHPA.0912.20	96
F3sCTO16	GU097375.1 Uncultured *Nitrosospira* sp. isolate DGGE gel band 23	98
F3sCTO25	HM163111.1 Uncultured bacterium clone 1-76	98
F3sCTO28	FJ006742.1 Uncultured bacterium clone WPUB032	97
F1wCTO2	JF429361.1 Uncultured bacterium clone DR372	99
F1wCTO4	JN004288.1 Uncultured bacterium clone JJ105	95
F1wCTO5	JF429361.1 Uncultured bacterium clone DR372	99
F1wCTO8	JF429361.1 Uncultured bacterium clone DR372	99
F1wCTO12	JF429361.1 Uncultured bacterium clone DR372	99
F1wCTO16	JF429361.1 Uncultured bacterium clone DR372	99
F1wCTO18	JF429361.1 Uncultured bacterium clone DR372	99
F1wCTO45	JF429361.1 Uncultured bacterium clone DR372	99
F2wCTO1	HQ827934.1 Uncultured bacterium clone E30	99
F2wCTO2	JF429361.1 Uncultured bacterium clone DR372	99
F2wCTO5	HQ904675.1 Uncultured bacterium clone XYHPA.0912.20	99
F2wCTO7	JF429361.1 Uncultured bacterium clone DR372	99
F2wCTO8	JF429361.1 Uncultured bacterium clone DR372	99
F2wCTO14	JF429361.1 Uncultured bacterium clone DR372	99
F2wCTO15	JF429361.1 Uncultured bacterium clone DR372	99
F2wCTO29	JF429361.1 Uncultured bacterium clone DR372	99
F2wCTO48	HQ827934.1 Uncultured bacterium clone E30	99
F3wCTO1	HQ827934.1 Uncultured bacterium clone E30	99
F3wCTO7	JF429361.1 Uncultured bacterium clone DR372	99
F3wCTO8	HQ827934.1 Uncultured bacterium clone E30	99
F3wCTO14	HQ827934.1 Uncultured bacterium clone E30	99
F3wCTO16	JF429361.1 Uncultured bacterium clone DR372	99
F3wCTO22	EU803320.1 Uncultured bacterium clone 5C230874	99
F3wCTO30	JF429361.1 Uncultured bacterium clone DR372	99
F3wCTO44	HQ827934.1 Uncultured bacterium clone E30	99
F3wCTO48	HQ827934.1 Uncultured bacterium clone E30	99
F3wCTO51	HQ827934.1 Uncultured bacterium clone E30	99

Table F.2 (continued): List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the CTO clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
L1sCTO1	EU499569.1 Uncultured beta *proteobacterium* clone 0E12	99
L1sCTO2	HQ904675.1 Uncultured bacterium clone XYHPA.0912.20	99
L1sCTO3	JF429361.1 Uncultured bacterium clone DR372	99
L1sCTO10	FJ393084.1 Uncultured *Azospira* sp. clone MFC-B162-C02	99
L1sCTO19	FJ933399.1 Uncultured *Nitrosomonas* sp. clone REV_R1P1_9E	97
L1sCTO40	FJ933399.1 Uncultured *Nitrosomonas* sp. clone REV_R1P1_9E	97
L1sCTO41	GU097360.1 Uncultured *Nitrosospira* sp. isolate DGGE gel band 8	98
L1sCTO50	GU097375.1 Uncultured *Nitrosospira* sp. isolate DGGE gel band 23	99
L1sCTO54	FJ933399.1 Uncultured *Nitrosomonas* sp. clone REV_R1P1_9E	97
L1sCTO58	GU097375.1 Uncultured *Nitrosospira* sp. isolate DGGE gel band 23	99
L2sCTO3	AY958677.1 *Nitrosomonas* sp. NL7 16S ribosomal RNA gene, partial sequence	97
L2sCTO7	GU097360.1 Uncultured *Nitrosospira* sp. isolate DGGE gel band 8	99
L2sCTO15	GU097375.1 Uncultured *Nitrosospira* sp. isolate DGGE gel band 23	98
L2sCTO17	EU224365.1 Uncultured bacterium clone 9R-27	99
L2sCTO20	JF808741.1 Uncultured *Nitrosomonas* sp. clone R7-14	99
L2sCTO24	EU803320.1 Uncultured bacterium clone 5C230874	98
L2sCTO35	HM066465.1 Uncultured bacterium clone EDW07B003_61	98
L1wCTO1	JF429361.1 Uncultured bacterium clone DR372	99
L1wCTO2	GU097375.1 Uncultured *Nitrosospir*a sp. isolate DGGE gel band 23	99
L1wCTO10	JF429361.1 Uncultured bacterium clone DR372	99
L1wCTO12	HQ852980.1 Uncultured bacterium clone B32	99
L1wCTO29	JF922402.1 Uncultured bacterium clone B1-18	96
L2wCTO1	HQ852980.1 Uncultured bacterium clone B32	99
L2wCTO4	HQ904675.1 Uncultured bacterium clone XYHPA.0912.20	99
L2wCTO8	JF429361.1 Uncultured bacterium clone DR372	99
L2wCTO10	FJ529947.1 Uncultured bacterium clone NBDTU27	99
L2wCTO12	JF429361.1 Uncultured bacterium clone DR372	98
L2wCTO14	EU542347.1 Uncultured bacterium clone SAV07D04	99
L2wCTO32	HM769432.1 Uncultured beta *proteobacterium* clone D-MAY-9	99

Table F.2 (continued): List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the CTO clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
L2wCTO36	FJ529947.1 Uncultured bacterium clone NBDTU27	98
L2wCTO46	HQ852980.1 Uncultured bacterium clone B32	99
L2wCTO51	JF429361.1 Uncultured bacterium clone DR372	99
I2wCTO8	HQ852980.1 Uncultured bacterium clone B32	99
I2wCTO10	JN389733.1 Uncultured bacterium clone D18	98
I2wCTO12	HQ904675.1 Uncultured bacterium clone XYHPA.0912.20	99
I2wCTO14	HM811387.1 Uncultured bacterium clone nby232f05c1	98
I2wCTO20	JN389733.1 Uncultured bacterium clone D18	98
I2wCTO23	EU640162.1 Uncultured beta *proteobacterium* clone LW18m-3-74	99
I2wCTO28	HQ852980.1 Uncultured bacterium clone B32	99
I2wCTO33	HQ852980.1 Uncultured bacterium clone B32	99
I2wCTO36	EF520462.1 Uncultured beta *proteobacterium* clone ADK-MOe02-13	99
I2wCTO38	HQ852980.1 Uncultured bacterium clone B32	99
I2wCTO45	HQ904675.1 Uncultured bacterium clone XYHPA.0912.20	98
I2wCTO69	HQ852980.1 Uncultured bacterium clone B32	99
I2wCTO70	HQ852980.1 Uncultured bacterium clone B32	99
I2wCTO71	HQ852980.1 Uncultured bacterium clone B32	99
I2wCTO72	JF429361.1 Uncultured bacterium clone DR372	99
I2wCTO77	JF429361.1 Uncultured bacterium clone DR372	99
I2wCTO81	JF429361.1 Uncultured bacterium clone DR372	99
I2wCTO84	JF429361.1 Uncultured bacterium clone DR372	99
I2wCTO97	JF429361.1 Uncultured bacterium clone DR372	99
I2wCTO99	JF429361.1 Uncultured bacterium clone DR372	99
I2wCTO101	AB657746.1 Uncultured bacterium RNA for 16S rRNA, partial sequence, clone: B0610R001_P10	98
I2wCTO104	JF429361.1 Uncultured bacterium clone DR372	99
I2wCTO105	HQ852980.1 Uncultured bacterium clone B32	99
I2wCTO106	HQ904675.1 Uncultured bacterium clone XYHPA.0912.20	99
I2wCTO108	HQ852980.1 Uncultured bacterium clone B32	99

Table F.3: List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the *pmo*A clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
F1sMB6	AB505843.1 Uncultured bacterium *pmo*A gene for particulate methane mono-oxygenase, partial cds, isolate: DGGE band:R14	96
F1sMB10	AB500821.1 Uncultured bacterium *pmo*A gene for particulate methane monooxygenase subunit A, partial cds, clone: FL28pmo	93
F1sMB17	AB280415.1 Uncultured bacterium *pmo*A gene for particulate methane monooxygenase, partial cds, clone: R1.PmoA-1	95
F1sMB20	HE617955.1 Uncultured bacterium partial *pmo*A gene for particulate methane monooxygenase	94
F1sMB26	AB500807.1 Uncultured *Methylocystis* sp. *pmo*A gene	95
F1sMB44	AM849661.1 Uncultured methanotrophic bacterium partial *pmo*A	97
F1sMB61	DQ008409.1 Uncultured bacterium clone W9_661_14	91
F1sMB66	FN597118.1 Uncultured bacterium partial *pmo*A	92
F1sMB67	AY488076.1 Uncultured methanotrophic *proteobacterium* clone A19	92
F2sMB1	AB280415.1 Uncultured bacterium *pmo*A gene	94
F2sMB3	AB500821.1 Uncultured bacterium *pmo*A gene	89
F2sMB5	AY488076.1 Uncultured methanotrophic *proteobacterium* clone A19	94
F2sMB42	FJ024393.1 Uncultured methane-oxidizing bacterium clone LKS16-21-*pmo*A	98
F2sMB67	EU131057.1 Uncultured bacterium clone CM20 PmoA (alkaline coal mine soil China)	99
F2sMB69	FN649651.1 Uncultured methanotrophic bacterium partial mRNA for methane	95
F2sMB58	AB500821.1 Uncultured bacterium *pmo*A gene Flpmo	89
F2sMB22	AB280415.1 Uncultured bacterium *pmo*A gene Clone R1. PMOA-1	94
F2sMB36	JN408220.1 Uncultured bacterium clone SL10	98
F2sMB37	AB280415.1 Uncultured bacterium *pmo*A gene Clone R1. PMOA-1	94
F2sMB44	EU193294.1 Uncultured bacterium clone JH-TY29	89
F2sMB50	AB280415.1 Uncultured bacterium *pmo*A gene	94
F2sMB66	FN597118.1 Uncultured bacterium partial *pmo*A	92
F3sMB4	HE617817.1 Uncultured bacterium partial *pmo*A gene	92
F3sMB10	AB500821.1 Uncultured bacterium *pmo*A gene for particulate methane monooxygenase subunit A, partial cds, clone: FL28pmo	89

Table F.3 (continued): List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the *pmo*A clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
F3sMB12	FJ009651.1 Uncultured methanotrophic bacterium clone SKB3 PmoA	98
F3sMB15	EU131057.1 Uncultured bacterium clone CM20 PmoA	99
F3sMB16	FN599870.1 Uncultured bacterium partial *pmo*A gene	99
F3sMB26	EU358979.1 Uncultured bacterium clone IB1	92
F3sMB27	AY488076.1 Uncultured methanotrophic *proteobacterium* clone A19	94
F3sMB29	JN408241.1 Uncultured bacterium clone SL31	99
F3sMB38	HM216859.1 Uncultured bacterium clone D1_12	98
F3sMB58	HE617680.1 Uncultured bacterium partial *pmo*A gene LL_HA_A04	95
F3sMB62	GQ477176.1 Uncultured methanotrophic *proteobacterium* clone 189	89
F3sMB70	Uncultured bacterium gene clone FW-G	83
F3sMB74	FN597143.1 Uncultured bacterium partial *pmo*A gene I09_43A	94
F1wMB3	FJ024393.1 Uncultured methane-oxidizing bacterium clone LKS16-21	99
F1wMB6	AB636304.1 Methylococcaceae bacterium OS501 *pmo*A gene	91
F1wMB8	JF706210.2 NC10 bacterium enrichment culture clone 'WWTP a Lieshout clone	92
F1wMB10	GU134446.1 Uncultured bacterium clone pSD43	88
F1wMB12	AB636304.1 Methylococcaceae bacterium OS501	90
F1wMB16	FJ024370.1 Uncultured methane-oxidizing bacterium clone LKS8-16-*pmo*A	99
F1wMB23	EF623801.1 Uncultured gamma *proteobacterium* clone Ssedi-26	90
F1wMB41	FJ024393.1 Uncultured methane-oxidizing bacterium clone LKS16-21	99
F1wMB44	AY550696.1 Uncultured bacterium clone 1HA_4	96
F2wMB2	CP002738.1 *Methylomonas methanica* MC09, complete genome	92
F2wMB5	FJ024349.1 Uncultured methane-oxidizing bacterium clone LKS1-02-*pmo*A	96
F2wMB6	FJ024379.1 Uncultured methane-oxidizing bacterium clone LKS8-45	98
F2wMB11	FJ024379.1 Uncultured methane-oxidizing bacterium clone LKS8-45-*pmo*A	96
F2wMB16	FJ024370.1 Uncultured methane-oxidizing bacterium clone LKS8-16-*pmo*A	99
F2wMB30	EF623759.1 Uncultured gamma *proteobacterium* clone S14m-40	91

Table F.3 (continued): List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the *pmo*A clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
F2wMB44	AY488078.1 Uncultured methanotrophic *proteobacterium* clone B41	92
F2wMB51	FJ024364.1 Uncultured methane-oxidizing bacterium clone LKS3-22-pmoA	96
F2wMB71	EF623704.1 Uncultured gamma *proteobacterium* clone Psedi-42	90
F2wMB91	AB280417.1 Uncultured bacterium *pmoA* gene	92
F3wMB1	FJ024354.1 Uncultured methane-oxidizing bacterium clone LKS1-10-*pmo*A	95
F3wMB6	FJ024396.1 Uncultured methane-oxidizing bacterium clone LKS16-25-*pmo*A	93
F3wMB7	FJ024354.1 Uncultured methane-oxidizing bacterium clone LKS1-10-*pmo*A	96
F3wMB16	FJ009640.1 Uncultured methanotrophic bacterium clone SKBR9 PmoA	91
F3wMB18	AY488073.1 Uncultured methanotrophic *proteobacterium* clone B33	91
F3wMB20	FJ024354.1 Uncultured methane-oxidizing bacterium clone LKS1-10-*pmo*A	96
F3wMB44	HQ883355.1 Uncultured *Methylobacter* sp. clone C21	88
F3wMB45	AB500821.1 Uncultured bacterium *pmo*A gene	89
F3wMB46	FJ024396.1 Uncultured methane-oxidizing bacterium clone LKS16-25	99
F3wMB60	FJ024354.1 Uncultured methane-oxidizing bacterium clone LKS1-10-*pmo*A	95
F3wMB63	FJ024370.1 Uncultured methane-oxidizing bacterium clone LKS8-16-*pmo*A	99
L1sMB1	EU358979.1 Uncultured bacterium clone IB1	94
L1sMB4	GQ906792.1 Uncultured bacterium clone S-7	92
L1sMB9	FJ024349.1 Uncultured methane-oxidizing bacterium clone LKS1-02-*pmo*A	97
L1sMB17	FJ009644.1 Uncultured methanotrophic bacterium clone SKG4 PmoA	99
L1sMB20	EF587725.1 Uncultured methanotrophic *proteobacterium* clone Littoral-site2-29	92
L1sMB29	AY488076.1 Uncultured methanotrophic *proteobacterium* clone A19	93
L1sMB38	AY488071.1 Uncultured methanotrophic *proteobacterium* clone B63	94
L1sMB41	AY781163.1 Uncultured bacterium isolate DGGE band L4dol	93
L1sMB51	GU056150.1 Uncultured methanotrophic bacterium clone DG1.5-11	92

Table F.3 (continued): List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the *pmo*A clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
L1sMB57	FJ024396.1 Uncultured methane-oxidizing bacterium clone LKS16-25-*pmo*A	98
L1sMB60	HM216868.1 Uncultured bacterium clone SP2_13 (Lake Stechlin profundal surface sediment)	97
L2sMB1	AF358050.1 Uncultured bacterium clone LOPA13.5	97
L2sMB2	AM910129.1 Uncultured bacterium partial *pmo*A gene	95
L2sMB3	AY355388.1 Uncultured methanotrophic gamma *proteobacterium* clone 15 (littoral sediment of central European Lake Constance)	78
L2sMB4	AY424845.1 Uncultured bacterium clone mvpa13.7	92
L2sMB5	EU131057.1 Uncultured bacterium clone CM20 PmoA (*pmo*A)	99
L2sMB7	EU193290.1 Uncultured bacterium clone JH-TY25 (paddy field soil)	85
L2sMB9	AB280427.1 Uncultured bacterium *pmo*A gene	95
L2sMB11	FN599883.1 Uncultured bacterium partial *pmo*A	96
L2sMB12	GU056131.1 Uncultured methanotrophic bacterium clone DG0.5-11	92
L2sMB13	EU131057.1 Uncultured bacterium clone CM20 PmoA (alkaline coal mine soil China)	87
L2sMB15	HM216868.1 Uncultured bacterium clone SP2_13	97
L2sMB16	AM910106.1 Uncultured bacterium partial *pmo*A gene	92
L2sMB19	FN649539.1 Uncultured methanotrophic bacterium partial *pmo*A gene RS-S57-402	98
L2sMB21	GU056147.1 Uncultured methanotrophic bacterium clone DG1.5-8 (soil sample above gas and oil field)	89
L2sMB20	HE617819.1 Uncultured bacterium partial *pmo*A gene (landfill-cover soil)	82
L2sMB24	FN562554.1 Uncultured bacterium partial *pmo*A gene	94
L2sMB25	AF358050.1 Uncultured bacterium clone LOPA13.5	97
L1wMB2	AY355391.1 Uncultured methanotrophic gamma *proteobacterium* clone 39	99
L1wMB5	FJ009647.1 Uncultured methanotrophic bacterium clone SKG7 PmoA (rice field soil India)	86
L1wMB6	EU131057.1 Uncultured bacterium clone CM20 PmoA (*pmo*A)	99
L1wMB8	FN597118.1 Uncultured bacterium partial pmoA gene	93
L1wMB9	FJ024358.1 Uncultured methane-oxidizing bacterium clone LKS1-29-*pmo*A	91
L1wMB12	AY488076.1 Uncultured methanotrophic *proteobacterium* clone A19	93

Table F.3 (continued): List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the *pmo*A clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
L1wMB23	FN649520.1 Uncultured methanotrophic bacterium partial *pmo*A gene	97
L1wMB28	FM986005.1 Uncultured methanotrophic bacterium partial *pmo*A	91
L1wMB42	GU056147.1 Uncultured methanotrophic bacterium clone DG1.5-8	92
L1wMB43	AB636304.1 Methylococcaceae bacterium OS501 *pmo*A	90
L1wMB44	JF772627.1 Uncultured methanotrophic bacterium clone 1000N-P59	99
L2wMB2	FJ024349.1 Uncultured methane-oxidizing bacterium clone LKS1-02-*pmo*A	95
L2wMB12	FJ024349.1 Uncultured methane-oxidizing bacterium clone LKS1-02-*pmo*A	95
L2wMB14	EF623801.1 Uncultured gamma *proteobacterium* clone Ssedi-26	90
L2wMB18	AB280415.1 Uncultured bacterium *pmo*A gene	92
L2wMB41	FJ024349.1 Uncultured methane-oxidizing bacterium clone LKS1-02-*pmo*A	96
L2wMB42	FN600113.1 Uncultured bacterium partial *pmo*A gene	98
L2wMB47	FM986121.1 Uncultured methanotrophic bacterium partial *pmo*A	99
L2wMB51	AF150764.1 Uncultured eubacterium pAMC512	93
L2wMB45	FJ024349.1 Uncultured methane-oxidizing bacterium clone LKS1-02-*pmo*A	96
L2wMB57	FJ024349.1 Uncultured methane-oxidizing bacterium clone LKS1-02-*pmo*A	97
L2wMB60	DQ142748.1 Uncultured bacterium clone EPMF1-3 PmoA	94
I1sMB1	AB500821.1 Uncultured bacterium *pmo*A gene (rice field soil)	89
I1sMB2	DQ142762.1 Uncultured bacterium clone EPMU3-2 PmoA (*pmo*A)	96
I1sMB3	JF706210.2 NC10 bacterium enrichment culture clone 'WWTP a Lieshout clone	95
I1sMB13	GQ477176.1 Uncultured methanotrophic *proteobacterium* clone 189	93
I1sMB16	AB500821.1 Uncultured bacterium *pmo*A gene (rice field soil)	88
I1sMB19	FM986083.1 Uncultured methanotrophic bacterium partial *pmo*A	99
I1sMB22	EU131057.1 Uncultured bacterium clone CM20 PmoA	99
I1sMB24	JN408235.1 Uncultured bacterium clone SL25	94

Table F.3 (continued): List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the *pmo*A clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
I1sMB28	FJ009640.1 Uncultured methanotrophic bacterium clone SKBR9 PmoA	91
I1sMB32	DQ142762.1 Uncultured bacterium clone EPMU3-2 PmoA (*pmo*A)	96
I1sMB38	JF706210.2 NC10 bacterium enrichment culture clone 'WWTP a Lieshout clone	95
I1sMB44	GU056126.1 Uncultured methanotrophic bacterium clone DG0.5- (gas and oil field in China)	89
I2sMB6	JN408241.1 Uncultured bacterium clone SL31	97
I2sMB10	AY424845.1 Uncultured bacterium clone mvpa13.7	92
I2sMB16	AY488071.1 Uncultured methanotrophic *proteobacterium* clone B63	93
I2sMB22	AM910108.1 Uncultured bacterium partial *pmo*A gene	94
I2sMB26	GU134440.1 Uncultured bacterium clone pSD37	98
I2sMB38	JN408241.1 Uncultured bacterium clone SL31	92
I2sMB44	AY662380.1 Uncultured bacterium clone LIW-21	91
I2sMB48	DQ008413.1 Uncultured bacterium clone W9_661_23	98
I2sMB51	AB222906.1 Uncultured methanotrophic bacterium PmoA	97
I2sMB70	HM216868.1 Uncultured bacterium clone SP2_13	97
I2sMB79	AM910106.1 Uncultured bacterium partial *pmo*A gene	93
I2sMB94	AY488071.1 Uncultured methanotrophic *proteobacterium* clone B63	93
I1wMB7	AY355389.1 Uncultured methanotrophic gamma *proteobacterium* clone 66	92
I1wMB8	GU134446.1 Uncultured bacterium clone pSD43 (Rice field soil)	88
I1wMB10	EU131057.1 Uncultured bacterium clone CM20 PmoA (*pmo*A)	99
I1wMB12	AY424853.1 Uncultured bacterium clone mvpb13.7	93
I1wMB16	GU134446.1 Uncultured bacterium clone pSD43 (Rice field soil)	87
I1wMB21	EU131057.1 Uncultured bacterium clone CM20 PmoA	96
I1wMB22	FN597129.1 Uncultured bacterium partial *pmo*A gene	93
I1wMB24	GU134446.1 Uncultured bacterium clone pSD43	88
I1wMB27	HM216868.1 Uncultured bacterium clone SP2_13	97
I1wMB39	EU131057.1 Uncultured bacterium clone CM20 PmoA	99
I1wMB40	JF772603.1 Uncultured methanotrophic bacterium clone 0N-P32	99

Table F.3 (continued): List of best hits identified from BLAST searches, their respective accession numbers and percentage similarity for the *pmo*A clones

Clone	Accession Number & Description of Best Hit	Percentage Similarity
I2wMB1	FJ024396.1 Uncultured methane-oxidizing bacterium clone LKS16-25-*pmo*A	99
I2wMB3	AY488071.1 Uncultured methanotrophic *proteobacterium* clone B63	93
I2wMB6	AY488065.1 Uncultured methanotrophic *proteobacterium* clone A81	98
I2wMB9	EU131057.1 Uncultured bacterium clone CM20 PmoA (*pmo*A)	94
I2wMB16	EU131057.1 Uncultured bacterium clone CM20 PmoA (*pmo*A) gene	99
I2wMB38	FJ024396.1 Uncultured methane-oxidizing bacterium clone LKS16-25-*pmo*A	99
I2wMB43	FJ024349.1 Uncultured methane-oxidizing bacterium clone LKS1-02-*pmo*A	97
I2wMB45	FJ024349.1 Uncultured methane-oxidizing bacterium clone LKS1-02-*pmo*A	97
I2wMB53	FN597140.1 Uncultured bacterium partial *pmo*A gene	92
I2wMB57	GU735544.1 Uncultured methanotrophic bacterium clone F478	94
I2wMB60	JF706210.2 NC10 bacterium enrichment culture clone 'WWTP a Lieshout clone	92
I2wMB64	AY488078.1 Uncultured methanotrophic *proteobacterium* clone B41	94