Chapter 1

Introduction

Aquaculture is a major industry in the ASEAN region with Thailand being the world's major exporter of shrimp. However, the industry has experienced many economic problems, and the associated socio-economic and environmental impacts of shrimp production give rise for concern (Duraiappah and Israngkura, 2000). Accumulation of dissolved nitrogen, especially ammonium, as a result of food addition and excretion of organisms reared at high density, is one of the main problems in intensive shrimp culture systems, affecting their food ingestion, growth and survival rates (Thompson *et al.*, 2002). Chemolithoautotrophic nitrifying bacteria (nitrifiers) are responsible for ammonia oxidation and its removal from the ecosystem. The mineralizing ability of nitrifying bacteria is well known among aquaculturists, but the understand bacteriology is abstruse (Hagopian and Riley, 1998). Because of the important of these bacteria, understanding of their ecology and physiology has become an importance (Hargreaves, 1998).

The microbial communities catalysing wastewater treatment have long been viewed as "black box". Today, even engineers agree that a thorough knowledge of the structure and function of these complex communities would be a good starting point for future system optimisation (Amann, 1998). Analyses targeting nitrifying bacteria have now provided spectacular detail regarding the identification, numbers, activities, and interaction of different nitrifying populations in a number of different systems. However, it is not yet clear which organisms. Nevertheless, the ability to characterise and quantify nitrifying bacteria populations in such controlled reactor systems is important to better predict and steer the performance of treatment processes (George *et al.*, 2001). The maintenance of specific nitrifying population may be essential to reactor health, and attempts to identify and model these communities are of considerable importance. In the same way, if shrimp farm activities are to be

enhanced by bioaugmentation with nitrifying bacteria, it follows that the inoculum must consist of species that are suited to the shrimp farm environment (George *et al.*, 2001).

The maintenance of water quality conditions favourable for aquaculture, condition that promotes rapid rates of primary production, tends to result in low ammonia concentration. In addition, the interacting roles of the factors that regulate nitrification (temperature, oxygen and substrate concentration) in the water and the sediment of aquaculture ponds required elucidation (Hargreaves, 1998). Biological filter (biofilm) has been applied in aquaculture for elimination of toxic nitrogen compound, ammonia and nitrite (Siriraksophon *et al.*, 1999), by converting highly toxic ammonia to less toxic nitrates (Kaiser and Wheaton, 1983). The appropriate use of nitrifying bacteria could help reduce ammonia and nitrite concentration in both static and recirculation systems (Chen *et al.*, 1991). Since these are intended to control and maintain safe ammonia levels in shrimp culture systems, biological filters are designed to promote the growth of ammonia- and -nitrite oxidizing bacteria (Hovance and Delong, 1996).

Previously, the culture dependent techniques were used to describe the microbial community but there are some limitations with this approach. Investigation of the diversity and ecology of nitrifying bacteria in natural systems by traditional cultivation techniques has been hampered by their slow growth rate and by the biases inherent in all cultured-based studies. Most studies on nitrification were performed ammonia- and nitrite-oxidizing bacteria, which are easy to obtain from international bacteria culture collections but might not represent those nitrifying bacteria dominant in the environments analyzed (Juretschko *et al.*, 1998). Up to date, molecular biology techniques have gained wide spread acceptance for studying the several microbial ecology according to it overcome those problems (Head *el. al.*, 1998; Amann *el. al.*, 1990; Embley and Finlay, 1994; Delong *el. al.*, 1989; Bouvier and Giorgio, 2003).

In this study, the best autotrophic nitrifying bacteria responsible for nitrogen removal from shrimp farming in Songkhla and Nakorn Sri Thammarat province, Southern Thailand was generated and enumerated the microbial communities of them by combination of culture dependent technique and molecular biological techniques.

Hypothesis

Knowledge of nitrifying organisms enables optimization of operating condition for their enhanced growth and nitrification.

Objectives

To obtain the best autotrophic nitrifying bacteria responsible for nitrogen removal from shrimp farming.

To use molecular biological techniques to enumerate the microbial communities of the autotrophic nitrifying bacteria from shrimp farming system.

To use culture dependent techniques to describe and support the presence of nitrifying bacteria in the sequencing batch reactor (SBR).

To generate dominant nitrifying bacteria specific oligonucleotides that can be used for the specific detection of these bacteria in shrimp farm by PCR or FISH technique.

Expected results

Obtain the dominant nitrifying bacteria from shrimp farms.

Know the ecological structure of obtained nitrifying bacteria.

Obtain the pure culture of important nitrifying bacteria responsible for ammonia/ nitrite removal in shrimp farming.

Obtain the dominant nitrifying bacteria specific oligonucleotides.

Literature review

1. Shrimp aquaculture

Shrimp farming has evolved into a multi-billion dollar a year industry over the last two decades (Braaten and Flaherty, 2001). With a result of technological advances, government subsidies, and profitable markets, the culturing of penaeid shrimp has become the leading aquaculture industry in Thailand, as well as other South East Asian Countries (Dierberg and Kiattisimkul, 1996). The black tiger shrimp, *Peanaeus monodon* Fabricius, is the most commonly cultured shrimp in South East Asia. Since 1991, when 162,000 metric tons of cultured marine shrimp were produced, Thailand has maintained its position as the world's leading producers and exporter of black tiger shrimp, by supplying up to 20% of the world trade in shrimp (Patmasiriwat *et al.*, 1999).

1.1. The problem of shrimp culture

1.1.1. The impact of shrimp culture on environment

The production of prawns in aquaculture facilities has caused adverse environmental effects on estuarine ecosystems, regarding to water and sediment qualities containing excessive concentrations of organic matter and nutrients as a direct consequence of intensively and excessively fed prawn stock. A majority of cases, shrimp farms dump their untreated water both during and especially after harvesting directly into the common water canals which eventually flow into the coastal area causing pollution and eutrophication that may result in the deterioration of ecosystem health (Eng *et al.*, 1989; Tookvinas, 1995; Sansanayuth *et al.*, 1996; Thongrak *et al.*, 1997; Naylor *et al.*, 1998).

The environmental impacts of the shrimp aquaculture were grouped into seven major categories: mangrove/wetland destruction; salt water intrusion; land subsidence; water-quality impairments; sediment disposal; abandoned shrimp ponds; and displaced traditional livelihoods (Dierberg and Kiattisimkul,

1996). The abandoned shrimp farms have very little alternative agricultural use due to the high salinity levels in the soils (Duraiappah and Israngkura, 2000). It usually takes about five to seven years before the land can be used again for other agricultural purposes. The problem is worse as the salinisation process from the farms is not localised, it intrudes to other area by surface and sub-surface which force many farmer owners to abandon their farms.

Alongside environmental changes such as eutrophication, salination and land use changes, there are attendant social transformations (Funge-Smith and Briggs, 1998). Policy makers in Thailand have acknowledged the importance of the shrimp aquaculture as a source of revenue, on the other hand, they concerned to the associated environmental and socio-economic problems. Many policies have been drawn up to address the issues but to date they have had limited success (Flaherty and Karnjanakesorn, 1994; Dierberg and Kiattisimkul, 1996; Thongrak *et al.*, 1997).

1.1.2. The toxic of ammonia and nitrite

It is well known that nitrogenous compounds, especially ammonia and nitrite, which accumulate in seawater aquaria, aquaculture ponds and disposal sites, do not only cause eutrophication, but also potentially toxic to aquatic animals at certain concentrations (Miranda-Filho *et al.*, 1995; Marazza *et al.*, 1996; Kawabata *et al.*, 1997; Alcaraz *et al.*, 1999).

Ammonia is excreted as the end product of protein catabolism, and may be toxic if allowed to accumulate. The amount of toxic un-ionized ammonia is a function of pH, temperature, alkalinity and total ammonia concentration (TAN) (Princic *et al.*, 1998). In the case of TAN, the low pH in the ponds (7 to 8) would have resulted in a low proportion of the toxic and unionized form ammonia. The low pH water in these ponds is therefore beneficial to maintain low ammonia concentrations (Burford *et al.*, 2003). The risk of elevated pH and un-ionized ammonia is greater in poorly buffered (low alkalinity) ponds, especially in late afternoon (Anthonisen *et al.*, 1976).

The main sources of ammonia production suggested by Burford and Williams (2001) were gill excretion, leaching from formulated feed, and

leaching from shrimp faeces. They highlighted that leaching from both uneaten feed and shrimp feces results in significant amounts of dissolved organic N (DON) being released into the water. However, Regnault (1987) claimed that the primary source of nitrogenous waste was inorganic and organic nitrogen (urea, uric acid, amino acids) excreted by aquatic animals.

The effects of ammonia on aquatic species are reduction in oxygen consumption, ATP metabolism disorder, lethargy, loss of appetite, laying on the pond bottom with clamped fins, gasping at the water surface if the gills have been affected, growth reduction and death can occur (Miranda-Filho *et al.*, 1995; Marazza *et al.*, 1996; Kawabata *et al.*, 1997; Alcaraz *et al.*, 1999; http://www/ fishdoc.co.uk). At low levels (less than 0.1 mg/l NH_3), it acts as a strong irritant, especially to the gills. Prolonged exposure to sub-lethal levels can lead to skill and gill hyperplasia. Gill hyperplasia is a condition in which the secondary gill lamellae swell and thicken, restricting the water flow over the gill filaments. This can result in respiratory problems and stress as well as creating conditions for opportunistic pathogenic bacteria and parasites to proliferate. Elevated levels are a common precursor to bacteria gill diseases. At higher level (more than 0.1 mg/l NH_3) even relatively short exposure can lead to skin, eyes, and gill damage. Elevated levels can also lead to ammonia poisoning by suppressing normal ammonia excrement from the gills. If fish are unable to excrete this metabolic waste product there is a rise in blood-ammonia levels resulting in damage to internal organs (http://www/ fishdoc.co.uk; http://kungthai.com).

The nitrite toxicity and nitrite uptake are known to be serious in fresh water than marine water (Cheng and Chen, 1998; http://kungthai.com). The low nitrite concentrations are higher toxic to haemoglobin contained aquatic species than haemocyanin contained aquatic species (http://kungthai.com). Due to the major source of toxicity of nitrite is the formation of methaemoglobin (Met-Hb) from oxyhaemoglobin (oxy-Hb). Haemoglobin (Hb) is an iron-containing complex present in erythrocytes with the principal role of oxygen transport. It combines with oxygen to form oxy-Hb, which is red and

dissociates readily to release oxygen to the tissue. A range of oxidizing compounds reacts with oxy-Hb to form Met-Hb (chocolate brown color). Met-Hb has greatly reduced oxygen binding ability; that oxygen present is very strong bound, so that Met-Hb is not available to the essential alternating oxygenation and deoxygenation involved in the transport of oxygen from the lungs to the tissues (Hill, 1991). The acute toxicity of nitrite to shrimp inhibits the combination of haemolymph to oxygen, thus, shrimp is lacked of oxygen even water with saturated dissolved oxygen. In addition, the accumulation of nitrite in water causes the increase in hemolymph nitrite and PO_2 and causes reduction in hemolymph pH, HCO_3^-, oxyhemocyanin and osmolarity of shrimp (Chen and Cheng, 1995, 1996). The safe concentrations for aquatic life are 0.4095 mg/l NO_2-N for nitrite and 0.039 mg/l NH_3 for un-ionized ammonia (Spraque, 1971).

Ammonia is retained in the topsoil by being bound to anionic soil particles, while nitrate is readily leached by rainwater into the unproductive anaerobic layers of soil where it is either lost from the biosphere by denitrification into the underground aquifers (Atlas and Bartha, 1993). The occurrence of nitrite in groundwater is of serious concern, since nitrite can react chemically with amino compounds to form nitrosamines, which are highly carcinogenic. Nitrate in groundwater also constitutes a health hazard. While nitrate itself is not highly toxic, it may be microbially reduced to highly toxic nitrite. The normal stomach acidity of adult humans tends to prevent or minimize such reduction, but infants with lower stomach acidity are highly susceptible (Atlas and Bartha, 1993). The role of nitrate and nitrite in methaemoglobin formation has long been known causing infant methaemoglobin or blue baby syndrome (causing respiratory distress). Clinical symptomatic disease is limited mainly to babies less than 90 days old in populations with an inadequate vitamin C intake, but may be seen in older children when the vitamin C intake is low, and in adult with low gastric acid secretion (Hill, 1991).

If ammonia levels do start to increase they can be reduced by; partial water changed on a daily basis until an acceptable level is obtained; reduction of feeding for 10-40% or stop feeding; maintaining oxygen level not less than 3.5 mg/l and using zeolite or some other form of ion exchanging material. Zeolite acts as a magnet and swap ammonia molecules in the water for another ion, usually sodium. They need to be re-charged, usually by overnight immersion in a strong salt solution. Zeolite can not be used with salted water. It should be remembered that the aims of this management is to reduce the ammonia to an acceptable level-not zero levels, as a continuous supply of ammonia is needed to encourage the growth of nitrifying bacteria (http://www/ fishdoc.co.uk; http://kungthai.com).

1.2. The characteristics of shrimp farm water

1.2.1. A typical composition of shrimp farm water

The quality of wastewater discharged from intensive shrimp ponds was characterized by high, but variable, concentrations of nutrients (BOD), salinity, total suspended solids, chlorophyll a, orthophosphate, nitrite, nitrate and ammonia-nitrogen (Chaiyakam and Songsangjinda, 1992; Dierberg and Kiattisimkul, 1996). A typical composition of shrimp farm effluent is shown in Table 1.

The above values depend on stocking density. Songsangjinda (1994) studied in a total 52 tiger shrimp intensive culture ponds and found that stocking density was the most significant variable which affected water quality. Poor water quality, especially high levels of total ammonia, nitrite, BOD and light transparency, correlated negatively to growth period of shrimp.

Table 1. Average nutrient, oxygen demand, total suspended solids and chlorophyll a concentrations in discharge water of intensively managed shrimp ponds with different stocking densities of *Penaeus monodon* Fabricus (black tiger shrimp)

	Chaiyakam *et al.* (1992)		Tunvilai *et al.* (1993)				
Stocking density(No./ m^2)	30	75	30	40	50	60	70
Ponds (N^*)	22	38	1	1	1	1	1
Sampling period (months)	20	20	3.9	3.9	3.9	3.9	3.9
NO_2-N	‹ 0.01	0.01	0.02	0.01	0.06	0.08	0.08
NO_3-N	0.02	0.03	0.07	0.06	0.15	0.15	0.15
TAN	0.11	0.61	0.98	0.98	6.36	7.87	6.50
Total N	nd	nd	3.55	4.04	14.9	20.9	17.1
Total P	nd	nd	0.18	0.25	0.53	0.49	0.32
COD	27.6	39	nd	nd	nd	nd	nd
BOD_5	5.4	7.1	10	11.4	28.9	33.9	28.8
TSS	184	214	92	114	461	797	498
Chl. a (μg/l)	76	140	70	110	350	460	350

All units in mg/l unless otherwise stated. TAN = total ammoniacal nitrogen. nd = no data. N^* = number of samples

Source: Dierberg and Kiattisimkul (1996)

The high levels of salinity and NH_3-N concentration in the shrimp farm wastewater are the most important characteristic, which impact on the environment of natural water bodies. Normally, the salinity level of shrimp pond water ranges from 5-38 ppt. Natural water contains ammonia in a concentration of 0-0.4 ppm (Boyd, 1989). A 50% marine shrimp died within 48 hours when the unionized ammonia concentrations rose to 1.29 ppm

(Chaiyakam, 1991). Numerous investigations have showed the effect of salinity fluctuations on the ammonia excretion of various crustacean species, including penaeid shrimp. A relatively rapid excretion of free amino acids from muscle into the blood followed by a rapid transfer to the gastric fluid together with an increase in ammonia excretion have been observed in lobster as a mechanism to tolerate the water uptake and to maintain the osmotic pressure (Dall, 1975). Rosas and colleagues (2001) indicated that an increment in blood ammonia and proteins associated in *Litopenaeus vannamei* shrimp exposed to a low salinity (15 ppt), evidencing the way in which protein metabolism is modified when shrimp are exposed to a low-salinity environment.

The acceptable range of physical and chemical parameters during the culture period in shrimp pond were proposed by Boyd and Fast (1992) to be as following; less than 400 µg/l total ammonia nitrogen, less than 100 µg/l unionized ammonia, less than 4000-5000 µg/l nitrite nitrogen, undetectable hydrogen sulphide, 25-30°C, 3.5 mg/l dissolved oxygen, pH 7-9 and 15-30 ppt salinity.

1.2.2. Nitrogen mass balance in shrimp pond

The nitrogen biogeochemistry of aquaculture ponds is affected by feeds and feeding practices, water exchange and circulation, aeration, pond depth and other management procedures (Hargreaves, 1998). Paez-Osuna (1997) presented the mass balance of nitrogen in shrimp farming which can be divided into 2 parts;

1. Input to pond including the inlet water, 17.8%, fertilization, 6.2%, number of shrimp stocked, < 0.1% and shrimp food, 76.0%.

2. Output from pond composed of ammonia vitalization, 27.4%, shrimp harvest, 35.5%, macrofauna associated, 0.4% and outlet water, 36.7% (Percentages are % of total nitrogen).

1.3. The role of nitrogen monitoring and control in aquaculture ponds

1.3.1. Physical methods

There are many reports related to the present study of Cheng *et al.* (2003) indicated that management of environmental parameters like temperature, pH, salinity, dissolved oxygen and ammonia is important in increasing the prawn's resistance to pathogen, and therefore in successful prawn farming. Critical water quality parameters that required engineering systems listed by Ebeling (2000) are as following; dissolved oxygen, un-ionized ammonia-nitrogen, nitrite-nitrogen, carbon dioxide, nitrate-nitrogen, pH, and alkalinity. Engineer controls the system such as water flow, tank level and oxygen concentration, which are essential to prevent catastrophic aquatic animals losses. For example, oxygen level was controlled by solenoid valve that automatically opens an oxygen supply line to oxygenate during a power failure. Engineer designs several types of fixed film biofilters commonly used in intensive recirculating aquaculture systems are: submerged biofilters, trickling biofilters (Ebeling, 2000), rotating biological contractors, RBC (Shankha, 1980; Ebeling, 2000), bead filters and fluidised bed biofilters (Miller and Libey, 1985; Ebeling, 2000). However, the efficiencies of these systems are low and the start-up time is long.

High levels of water exchange are often adopted by farmers to remedy actual or perceived water quality problems as they arise. By contrast, low water exchanges system of moderate intensity are being promoted to reduce the risk of cross-farm pollution and transfer of pathogens, and to reduce the environmental impact of farms (Hopkins *et al.*, 1995; Kongkeo, 1997). The electrochemical method has been successfully employed to deal with various industrial wastewaters, hence, this technique was investigated to remove ammonia and nitrite in aquaculture by Lin and Wu (1996). The result was shown that the electrochemical method is, at least, as effective and promising as the other treatment methods.

1.3.2. Chemical methods

A wide variety of chemical products are used to treat water and sediment from pond in semi-intensive and intensive south-east Asian shrimp farming (Graslund and Bengtsson, 2001). The example of chemicals used to remove ammonia and nitrite are ozone and zeolite. Nitrification by ozonation has received increasing attention because it is efficiently converting the toxic nitrite and ammonia to non-toxic nitrate. However, the limitation of this method is its higher cost than the other methods (Bovendeur *et al.*, 1987; Graslund and Bengtsson, 2001).

1.3.3. Biological methods

Water quality in a pond is improved by enhancing the mineralization process and reducing the accumulation of organic loads. Bioremediation or bioaugmentation is a concept of reducing organic wastes to environmentally safe levels through use of microorganisms (Shariff *et al.*, 2001). Bioaugmentation involves the seeding of water-purifying bacteria into aquaculture systems. Currently, there are several microbial products in the aquacultural market. The report of using commercial seed in aquaculture is both successful and failed. The example of the successful experiment using bioaugmentation is pellet immobilization of indigenous nitrifying bacteria which represents a potentially effective TAN control system for prawn aquaculture in low-cost, but intensive tropical prawn farms (Shan and Obbard, 2001). Kim *et al.* (2000) were successfully evaluated immobilized nitrifiers for the ammonium nitrogen removal from a recirculation aquaculture system. Anyhow, there were also reports on commercial microbial products not being as effective under laboratory as under field conditions (Chiayvareesajja and Boyd 1993; Fungesmith and Howthorn 1996).

The bioaugmentation can be used as a tool to protect the structure and function of the nitrification reaction against a shock load both in industry and aquaculture. Boon *et al.* (2003) added 3-chloroaniline (3-CA)-degrading strain *Comamonas testosterone 12 gfp* in the reactor which pulse by inoculation with

the 3-CA. Two days after the 3-CA shock, ammonia accumulated, and the nitrification activity did not recovered over 12-day period in the nonbioaugmentation, in contrast, nitrification in the bioaugmentation reactors started to recovery on day 4. From the result of DGGE pattern, FISH and real time PCR data can show (immediately after shock load) the microbial community of the bioaugmentation recovered the structure, activity, and abundance of nitrifying bacteria. Therefore, the effective nitrifying bacteria could be applied in the shrimp pond during ammonia and/or nitrite increased quickly at the certain concentration.

Besides much of the research effort in the field of shrimp nutrition has focused on improving feed formulations to minimize the leaching of dissolved products from feeds, and improve nutrient retention by shrimp (Lawrence and Lee, 1997). New feed formulations are generally tried in indoor aquaria rather than ponds. As a result, the nutritionally complete diet most shrimp farmers use in their ponds does not make allowances for the contribution of natural food in pond systems, or consideration given to the effect of the waste feed products on the ecosystem. However, the integration of research on nutrient cycling in ponds and optimization of feed formulations has the potential to significantly improve feed retention and reduce nitrogen wastage (Tacon and Akiyama, 1997; Burford and Williams, 2001).

1.4. Wastewater treatment from aquacultural pond effluent

There are three main options for nitrogen recovery from pond effluent (Lorenzen, 1999; Kumar and Sierp, 2003) firstly, the uptake of dissolved nitrogen (mainly TAN) by aquatic plants (Ellner *et al.*, 1996; Brown, 1999), secondly, the uptake of particulate nitrogen (mainly phytoplankton) by filter feeding animals (Jones and Preston, 1999). The examples of this option are mussels, that could be used in the fish pond with silver perch, rainbow fish. In addition, the aquatic plants such as duckweed is highly efficient in removing ammonia from the wastewater. These could be used as an important plants and animals for nutrient removal purpose. The last option is the uptake of mixed

(particulate and dissolved) nitrogen waste in plant beds; the example is the reuse of highly saline aquaculture effluent to irrigate a potential forage halophyte, *Suaeda estora* (Brown and Glenn, 1999).

2. Biological ammonia and nitrite removal

2.1. The main biological process

Microorganisms play a major role in the nitrogen cycling in the environment (see Figure 1). The microbiology involved in the nitrogen cycling is composed of five steps; nitrogen fixation, assimilation, mineralization, nitrification, and denitrification (Painter, 1970). Ammonia is produced during the composition of organic nitrogen compounds (ammonification) by a wide variety of microorganisms (Painter 1970; Biston, 1994; Medigan, 2000).

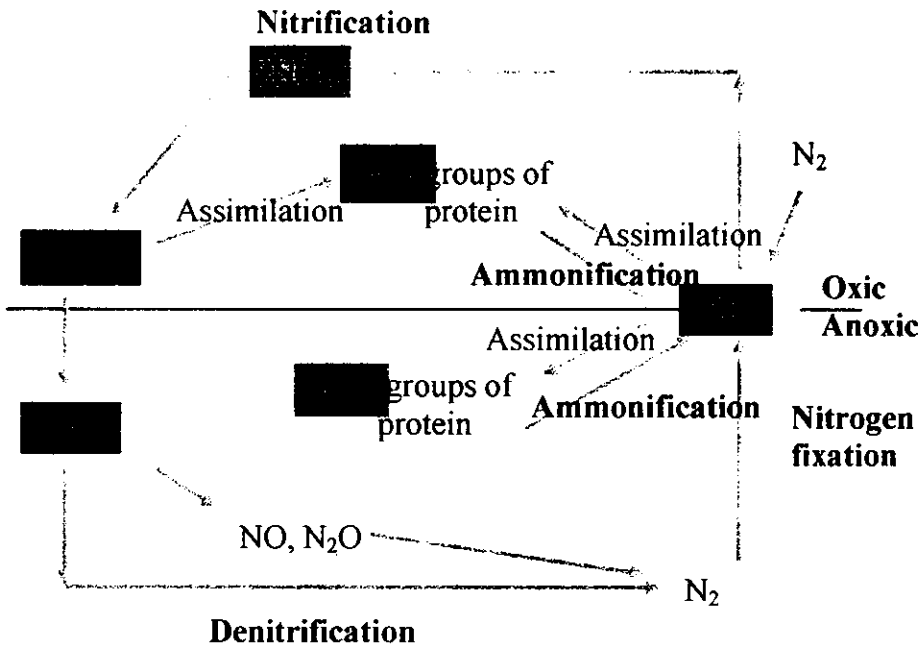

Figure 1. Redox cycle of nitrogen
Source: Medigan *et al.* (2000)

There is no organism described which can directly oxidize ammonia to nitrate. Nitrification process occurs primarily, oxidized ammonia (NH_4^+) to

nitrate (NO_3^-) via nitrite (NO_2^-) under aerobic condition (Belser and Schmidt, 1980; Kuenen and Roberson, 1988; Powell and Prosser, 1991; Randall, 1992). The process of nitrification appeared to be limited for the most part to a restricted number of aerobic chemoautotrophs called the nitrifiers or nitrifying bacteria (Belser and Schmidt, 1980; Keen and Prosser 1987; Hooper *et al.*, 1997). The oxidation of NH_4^+ to NO_2 and then NO_3^- are energy-yielding processes, microorganisms utilize the generated energy to assimilate CO_2 (Biston, 1994).

The general reaction for nitrification reaction is as follows:

$$2NH_4^+ + 3O_2 \longrightarrow 2NO_2^- + 2HO_2 + 4H^+ + \text{biomass}$$

$$2NO_2^- + O_2 \longrightarrow 2NO_3^- + \text{biomass}$$

In the first reaction, molecular oxygen is incorporated into the nitrite molecule. The oxidation is a multiple step process and involves the generation of hydroxylamine (NH_2OH) by ammonia monooxygenase. The substrate for the monooxygenase appears to be NH_3 rather than NH_4^+, so ammonia oxidation proceeds rapidly only at neutral or slightly alkaline pH which favours the formation of the unionized ammonia species (Cole, 1993). The single oxygen atom incorporated into hydroxylamine comes from atmospheric oxygen, half of electrons released during hydroxylamine oxidation to nitrite must therefore be used to regenerate the reduced form of cytochrome P460 (Figure 2). In the next step, hydroxylamine oxidoreductase produces from hydroxylamine and water nitric acid and hydrogen, so the second oxygen atom in nitrite come from water (Atlas and Bartha, 1993; Cole, 1993; Medigan *et al.*, 2000). The production of nitric acid reduce the pH of the environment as a result of ammonia oxidation by ammonia oxidizing bacteria (Painter, 1970; Keen and Prosser, 1987; Atlas *et al.*, 1993).

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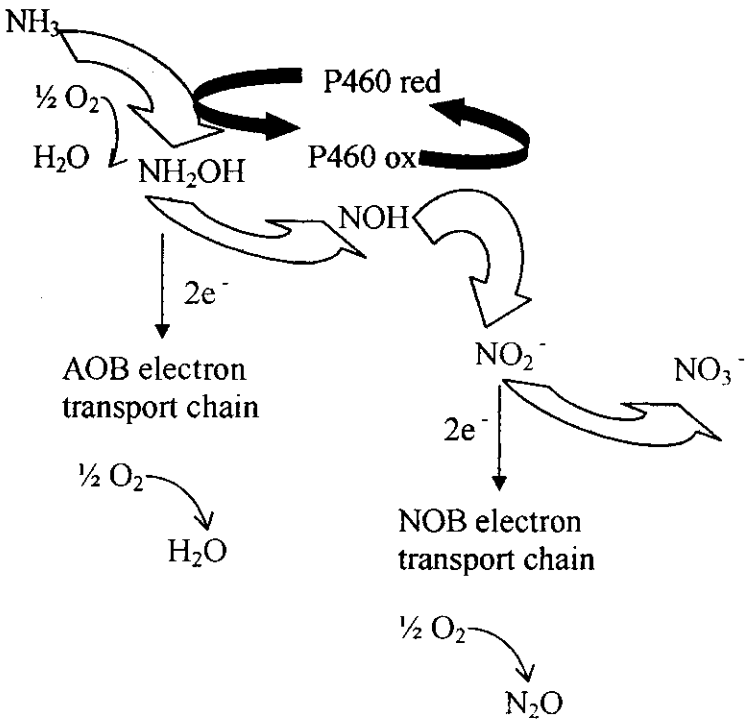

Figure 2. Electron transfer reactions in the nitrification pathway
Source: Cole (1993)

2.2 The characteristics of nitrifying bacteria

2.2.1. The theory of autotroph and heterotroph

The terms that have been used to describe organisms based on their carbon and energy metabolism as concluded in Figure 3. Organisms able to use inorganic chemicals as electron donors are chemolithotroph or autotroph. Examples of inorganic electron donors include hydrogen sulfide (H_2S), hydrogen gas (H_2), ferrous ion (Fe^{2+}), and ammonia (NH_3). Autotroph metabolism usually involves aerobic respiratory processes such as aerobe chemoorganotrophs or heterotroph but using inorganic energy source rather than an organic one. Autotrophs have electron transport components similar to those of heterotroph and form a proton motive force, which drives ATP synthesis. However, one important distinction between autotrophs and heterotrophs is in their source of carbon for biosynthesis. Heterotrophs can generally use compounds such as glucose as carbon sources as well as energy source, but chemolithotroph can not use their inorganic electron donors as

sources of carbon. Most autotrophs use carbon dioxide as a carbon source. Cell carbon is often from CO_2 frequently using the Calvin Cycle. This cycle is the fixation of CO_2 by most phototrophic and other autotrophic organisms in which the enzyme ribulose bisphosphate carboxylase (RubisCo) plays key role. The Calvin cycle is an energy-demanding process in which CO_2 is converted to cell material.

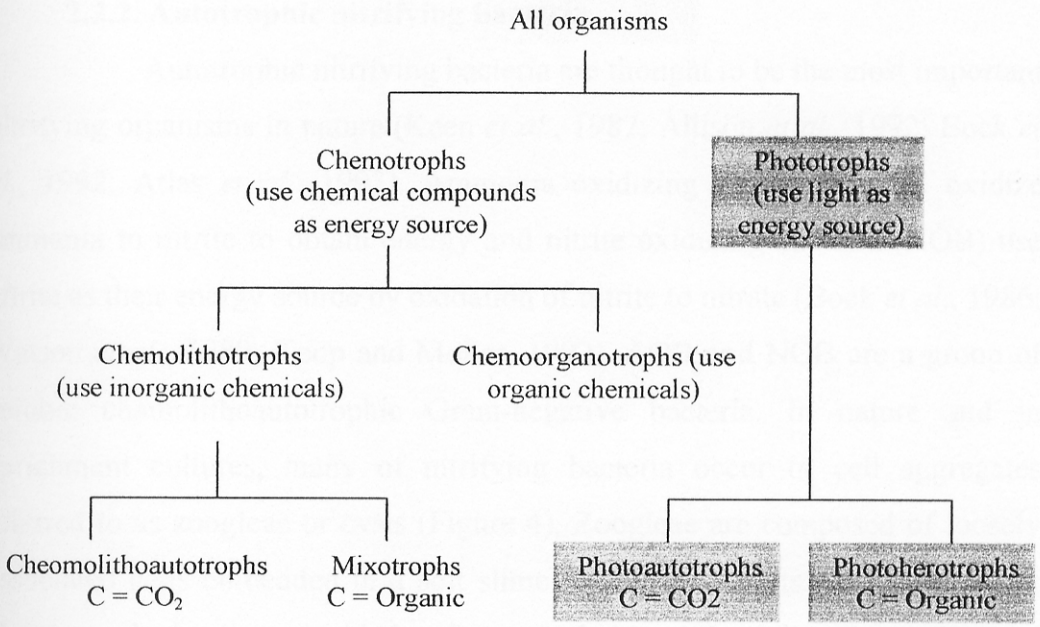

Figure 3. Classification of organisms in terms of energy and carbon source
Source: Medigan *et al.* (2000)

Bacteria need energy and reducing power to grow and to be maintained. The conservation of energy from chemical reactions in living organisms involves oxidation-reduction (also called redox) reaction. An electron donor and terminal electron acceptor are required because cells derive energy from redox reactions. A hydrogen atom (H) consists of an electron plus a proton. When the electron is removed, the hydrogen atom becomes a proton (or hydrogen ion, H^+). In order to understand redox reaction, keep straight the proper half reaction-there must always be one reaction involving an electron donor and another reaction involving electron acceptor. The amount of electrons present in the electron donor, will end up either in the new biomass

formed, or in the terminal electron acceptor. For the case of growth on organic compounds, electrons in substrate are equal to amount of electrons in new biomass plus electrons transferred to terminal electron acceptor. Electron acceptors other than oxygen can function as terminal electron acceptors for energy generation. Because oxygen is absent, this is call anaerobic respiration (all above is concluded from Madigan *et al.*, 2000).

2.2.2. Autotrophic nitrifying bacteria

Autotrophic nitrifying bacteria are thought to be the most important nitrifying organisms in nature (Keen *et al.*, 1987; Allison *et al.*, 1992; Bock *et al.*, 1992; Atlas *et al.*, 1993). Ammonia oxidizing bacteria (AOB) oxidize ammonia to nitrite to obtain energy and nitrite oxidizing bacteria (NOB) use nitrite as their energy source by oxidation of nitrite to nitrate (Bock *et al.*, 1986; Watson *et al.*, 1989; Koop and Moller, 1992). AOB and NOB are a group of aerobic chemolithoautotrophic Gram-negative bacteria. In nature and in enrichment cultures, many of nitrifying bacteria occur in cell aggregates referred to as zoogleae or cysts (Figure 4), Zoogleae are composed of loosely associated cells embedded in a soft slime layer, while cysts are composed of closely packed cells embedded and surrounded by a firm slime layer (Watson *et al.*, 1989).

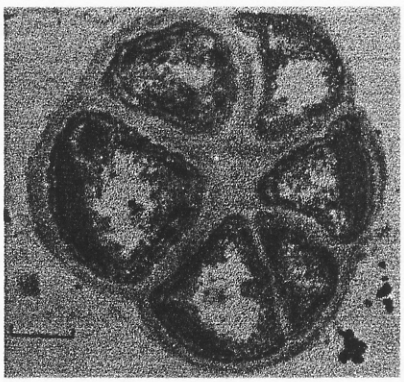

Figure 4. Nitrifying bacteria cyst. Electron micrograph. bar, 0.5 um
Source: Watson *et al.* (1989)

Nitrifying bacteria are chemolithotrophs and utilize the energy derived from nitrification to assimilate CO_2. Carbon requirements for nitrifier are

satisfied by carbon dioxide, bicarbonate, or carbonate (Biston, 1994). Not all of nitrifiers are true chemolithotrophs. Many strains of AOB can also assimilate certain organic carbon compounds while still using ammonia as energy source, and so these grow mixotrophically (Bock *et al.*, 1986).

Neither theoretical considerations nor experimental results indicated that *Nitrobacter* cell should be obligate chemolithotrophs (Watson, 1989; Watson *et al.*, 1989). Most strains of *Nitrobacter* cells grow much slower and are much less efficient when grown heterotrophically than when grown chemolithotrophically (Watson *et al.*, 1989; Bock *et al*, 1992). Why other NOB fail to grow heterotrophically is not understood, but may be because they have lesion in their TCA cycle, lack NADH oxidase system or have poor organic substrate permease transport systems (Watson *et al.*, 1989; Watson, 1989).

These bacteria are widespread in nature and have been isolated from many ecosystems including soil, fresh water, marine environment, brackish water, oceans and wastewater system (Watson *et al.*, 1989; Allison and Prosser, 1993; Koop and Moller, 1992). The fact that in many environments the number of autotrophic nitrite oxidizers is much higher than autotrophic ammonia oxidizer (Kuenen and Robertson, 1987), so it was not surprising to find lithotrophic nitrifiers which always present in natural environments is often produce high amount of nitrate (Bock *et al.*, 1992). In nature, they can be frequently found in suboptimum environments (Table 2). However, obligate psychrophilic or thermophilic nitrifying bacteria have never been isolated (Watson *et al.*, 1989).

2.2.3. Heterotrophic nitrifying bacteria

Many heterotrophs including bacteria (e.g. *Artrobacter*, *Pseudomonas*), fungi (e.g. *Aspergillus*) are capable of reducing organic forms of nitrogen but only a secondary metabolic process, and at low specific rates (Verstraete, 1975; Randall, 1992; Robertson *et al.*, 1992; Verstraete and Alexander, 1972). These organisms obtain no energy from the oxidation of these inorganic nitrogen sources, but from organic carbon compounds and often use organic source of nitrogen rather than ammonia (Prosser, 1989; Bock

et al., 1992; Robertson *et al.*, 1992). Therefore the function of this process is not clear in these microorganisms. The process of heterotrophic nitrification is not coupled to energy generation, the growth of all heterotrophic nitrifying organisms being completely dependent on the oxidation of organic substrates, however, heterotrophic nitrification is much slower than autotrophic nitrification and probably does not have any significant contribution (Kuenen *et al.*, 1988; Bock *et al.*, 1992). The final product of heterotrophic nitrification is often nitrite (Castagnetti and Gunner, 1980), so that heterotrophic nitrification may supply the substrate for autotrophic nitrite oxidizers and heterotrophic denitrifiers. Brierley and Wood (2001) isolated heterotrophic nitrifying bacteria from an acid forest soil in which nitrification occurred via a heterotrophic pathway. This bacterium was Gram-positive, a rod-shaped and identified provisionally as an *Arthrobacter* sp. However, the nitrification rate was very low compare with autotrophic nitrifiers.

Table 2. The example of suboptimum condition environment where nitrifying bacteria was found.

Suboptimum condition	Environment	Reference
- Extremely low oxygen tensions	- sewage disposal systems - marine sediments	Bernet *et al.* (2001); Ravenschlag *et al.* (2001); Dalsgaard and Thamdrup (2002); Freitag and Prosser (2003)
- Extremely low pH	soils having pH of 4	Walker and Wickramasinghe, (1979)
- Extremely low temperature	deep oceans (temperature is less than 5° C)	Bianchi *et al.* (1997)
- Extremely high temperature	deserts and hot springs where the temperature can be 60° C or more	Golovacheva (1976)

2.3. Phylogenetic analysis of nitrifying bacteria

AOB have been divided into two groups of the class *Proteobacteria* based on phylogenetic analysis of 16S rRNA sequences. Most known ammonia-oxidizing autotrophs belong to the β subdivision of the *Proteobacteria* (Figure 5) and are typified by *Nitrosomonas* and *Nitrosospira* and have been found in a broad range of environments; the only exception is *Nitrosococcus oceanus*, which is affiliated with the γ subdivision and restricted to marine environments (Head *et al.* 1993; Hiorn *et al.*, 1995; Hovanec *et al.*, 1996; Purkhold *et al.*, 2000).

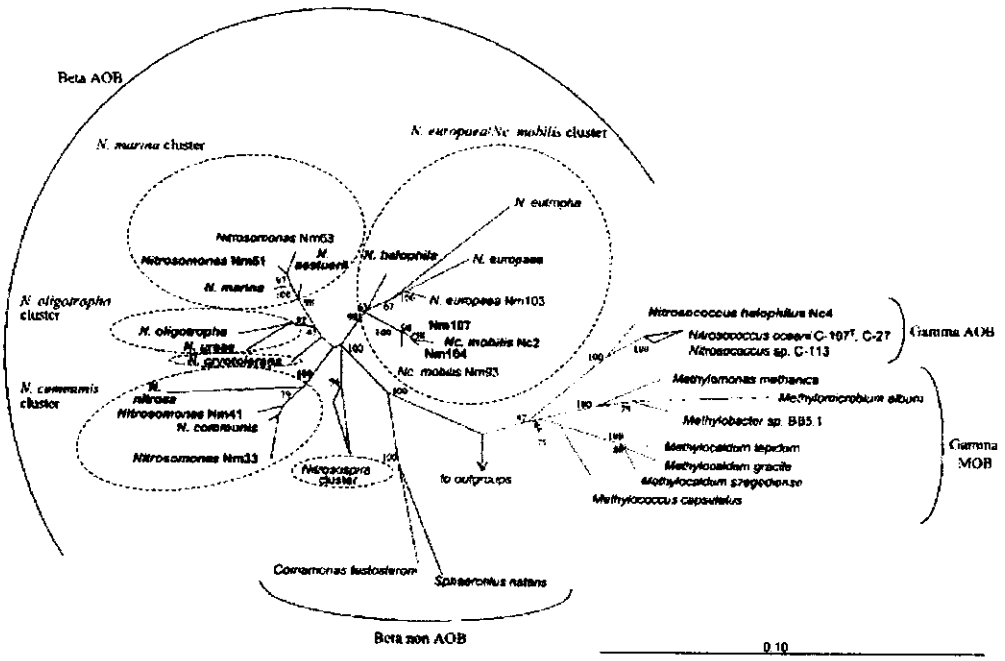

Figure 5. Phylogenetic 16S rRNA tree reflecting the relationships of AOB and several non-AOB reference organisms. The bar indicates 10% estimated sequence divergence.

Source: Purkhold *et al.* (2000)

NOB found more widespread in the *Proteobacteria* division, occurring in the α, δ, γ subdivision. The most commonly studied autotrophic nitrite-oxidizing bacteria belong to the α subdivision of the *Proteobacteria*, of

which *Nitrobacter winogradskyi* is a representative specie (Figure 6). *Nitrobacter* has dominated the studies of nitrite oxidation to nitrate through three other genera are recognized to catalyze the same process. These are *Nitrococcus* (gamma subdivision), *Nitrospina* and *Nitrospira* (Nitrospira phylum); all three grow optimally at high salt concentrations and are found in marine environment (Gibson *et al.*, 1980; Seawaldt *et al.*, 1982; Woese 1987; Orso *et al.*, 1994; Teske *et al.*, 1994; Hovanec *et al.*, 1996; Watanabe and Baker, 2000).

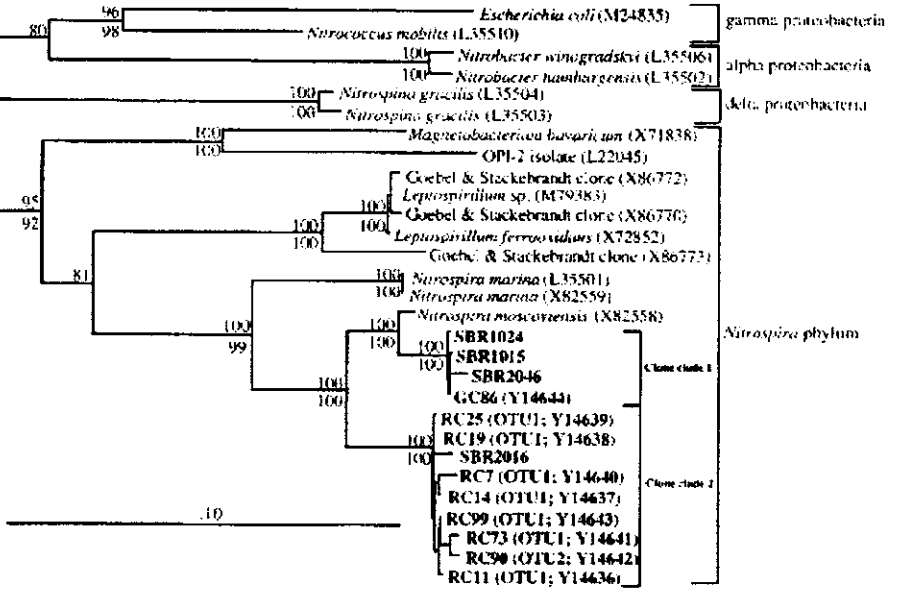

Figure 6. Evolutionary distance tree of the known nitrite oxidizers in the domain *Bacteria* based on a comparative analysis of 1,030 nucleotides. The bar represents 0.1 estimated changes per nucleotide.
Source: Burrell *et al.* (1998)

2.4. Effect of some parameter on nitrifiers

2.4.1. Temperature

Temperature plays an important role in regulating nitrification because nitrifiers are very sensitive to temperature, particularly if it is low, which may lead to decreased growth and nitrification rate (Jones *et al.*, 1980;

Keen *et al.*, 1987; Andreadakis, 1993). The nitrification rates increase follow to the increase of temperature from 5° C to about 35° C (Painter *et al.*, 1963; Halling-Sorensen *et al.*, 1993). The temperature which estuarine isolate and fresh water isolate (40° C and 35° C) observed the maximum activity by Jones and Hood (1980) was slight higher than other isolate (25° to 30° C) by Alexander (1965).

2.4.2. pH

The optimum pH of nitrifying bacteria lies between 7.5 and 8.5 (Painter *et al.*, 1983). Nitrification ceases at or below pH 6.0 (Painter, 1970; Painter *et al.*, 1983; Princic *et al.*, 1998) or above 10.0 (Painter *et al.*, 1983).

2.4.3. Dissolved oxygen (DO)

DO concentrations remain one of the most important factors controlling nitrification. An optimum DO of nitrification rates are 2 and 3 mg/l (Randall, 1992). Limiting amount of DO concentrations (below 2 mg/l) inhibit nitrification and cause nitrite (NO_2) accumulation or nitrous (N_2O) and nitric oxide (NO) production (Goreau *et al.*, 1980; Painter 1986).

2.4.4. Light

The nitrifying bacteria are inhibited by light and killed by direct sunlight (Hill 1991; Alleman, 1987). The exact cause of this negative impact is unknown, but may have some relationship with a superoxide radical produced in conjunction with stagnant membrane oxygen (Alleman, 1987).

2.4.5. Inhibitors

Nitrifiers are subject to various products and substrates inhibition, which the substrate of the other species was found to be much more toxic than the organism's own substrate (Anthonien *et al.*, 1976). Free nitrite depressed both respiration and growth of *Nitrosomonas* while *Nitrobacter* was sensitive to ammonium ions, but even more so to free ammonia (Meiklejohn, 1954). Media containing low substrate concentrations (10 mg/l of NH_4^+) was found to give larger number of AOB than media containing higher NH_4^+ concentrations

(Suwa *et al.*, 1994). The growth rate of *Nitrosomonas* spp. cultures were reduced in the presence of 1,050 to 2,800 mg/l of NH_4^+ (Lozinov and Ermachenko, 1959).

In the biological nitrogen removal system from seawater, the nitrifying and denitrifying activities of bacteria have been reported to be extremely low due to slow growth rate of nitrifying bacteria and the inhibition of free ammonia and nitrite ions, and high salt concentration (Sakairi, *et al.*, 1996). Nitrifiers are also quite sensitive to several toxic compounds found in wastewater. The most toxic compounds to nitrifiers are cyanide, phenol, aniline, and heavy metal (Bitton, 1994).

AOB population shifts did occur at different ammonia concentrations. Low-ammonia (5-10 mg/l NH_3-N) environments will likely produce *Nitrosomonas marina.* As ammonia concentration increases (50-3,000 mg/l of NH_3-N), *Nitrosospira tenuis* and *Nitrosomonas europaea* will become important until at the highest ammonia concentration *Nitrosococcus mobilis* may be predominant (Suwa *et al.*, 1994; Princic *et al.*, 1998; Burrell *et al.*, 2001).

3. Sequencing batch reactor (SBR)

It is well known that SBR processes have a potential to perform biological nutrient removal (BNR) and are able to treat a variety of wastewaters as given in Table 3 (Asher *et al.*, 1992; Shin *et al.*, 1992; Kuba *et al.*, 1993; Belia and Smith, 1997; Carucci *et al.*, 1997; Intrasungkha *et al.*, 1999; Patureau *et al.*, 2001). SBR have been used extensively to evaluate the removal of organic matter such as total nitrogen, carbon, and phosphorus removal activated sludge system (Shin *et al.*, 1992; Tam *et al.*, 1992; Kolarski, 1996; Carucci *et al.*, 1997) as well as phenolic compounds in wastewater (Asher, *et al.*1992; Woolard and Irvine, 1995; Yu and Gu, 1996).

Table 3. Studies on the use of sequencing batch reactor (SBR) in wastewater treatment

Studies	References
- Removal of organic carbon, nitrogen and phosphorus using SBR	Callado and Foresti (2001)
- Study of enhanced biological phosphate removal in SBR	Carucci *et al.* (1994 and 1997)
- Evaluation of the phosphorus removal capability in SBR	Belia and Smith (1997)
- Study of biological nutrient removal in treatment of saline wastewater	Woolard and Irvine (1994 and 1995); Intrasungkha *et al.* (1999)
- The use of SBR to achieve total nitrogen and biological phosphorus removal in a single stage activated sludge system	Kolarski (1996)
- Study of biological phosphorus from wastewater by anaerobic-anoxic sequencing batch reactor	Kuba *et al.* (1993)
- Combined phosphate and nitrogen removal in a sequencing batch reactor using the aerobic denitrifier, Microvirgula aerodenitrificans	Patureau *et al.* (2001)
- Simultaneous removal of phosphorus and nitrogen in SBR	Shin *et al.* (1992)
- Treatment of phenolic wastewater by SBR	Asher *et al.* (1992); Yu and Gu (1996)

SBR with aerated fill and unaerated fill SBR were used to treat synthetic phenolic wastewater under identical conditions of influent phenol concentration and aeration time (Yu and Gu, 1996). It was found that at low influent phenol concentrations (e.g, <400 mg/l), the SBR with unaerated fill

performed better than the SBR with aerated fill, in which there was a tendency for filamentous bacteria to develop. However, when the influent phenol concentration was high (e.g, >800 mg/l), phenol accumulated during the fill period in the SBR with unaerated fill became inhibitory to microorganisms, this led to a reduction in substrate removal efficiency and the growth of dispersed biomass. Woolard and Irvine (1995) isolated moderate halophile from the Great Salt Lake, Utah, U.S.A. and used this organism to develop a halophilic sludge in a SBR operated at 15% salt during a 7 month study period. An average phenol removal of over 99.5% was achieved with this reactor and specific substrate removal rates were similar to those reported for more conventional treatment cultures.

SBR is also used to treat the hypersaline (>3.5% salt) wastewater which generated during industrial activities from chemical manufacturing, oil and gas production and waste minimization practices (Woolard and Irvine, 1994 and 1995; Intrasungkha *et al.*, 1999). In addition, Strous and other (1998) demonstrated that the SBR could be used for the enrichment and quantitative study of a large number of slowly growing microorganisms that are currently out of reach for microbial research.

SBR system has attracted much attention because of its low power consumption, easy operation, short detention time, and low operation and maintenance cost. The SBR is a periodic process utilizing a repetition of fills and draw reactor phases to treat organic wastewater biologically (Lee, 1991).

Confining to simple configuration, the SBR incorporate general concepts of combining bio-oxidation (dissimilatory), biosynthesis (assimilatory) and endogenous respiration (auto-oxidation) together compared to extended biological treatment systems reported (Lee, 1991). The process cycle of the SBR typically consists of five distinct phases, which are named after the major processes taking place during each phase: fill, react, settle, draw, and idle. During the react phase, the system can alter between aerobic, anoxic and anaerobic conditions to obtain organic matter, nitrogen and phosphorus

removal (Katsongiannis *et al.*, 1999). Idle phase may be eliminated in most cases.

4. Culture dependent methods

4.1. A brief introduction to culture dependent methods

Bacteriology became a science only after unique methods were developed, and they are responsible for its continuing influence on and expansion into subsequently developed field such as virology, immunology, and molecular biology. Koch's introduction of pure culture techniques and Pasteur's use of immunological response and chemical analysis remain as influential now as then (Gerhardt, 1981). Traditional culture dependents techniques have been firstly used by Robert Koch in the late eighteenth century (Pelczar *et al.*, 1993). These techniques include viable plate count and most–probable number methods (MPN), and have been, and frequently still are, used for the quantification of active cells in environmental samples (Degrange and Bardin, 1995; Feray *et al.*, 1999; Giuliano *et al.*, 1999).

MPN technique is one mean of determining the potential activity of a microbial population and has been developed for enumeration of the AOB population in soil sample by Rowe *et al.* (1977), called microtechnique based on the MPN method. These developed MPN was done in 96 well microtiter plate (12 dilution, 8 replications per dilution). Higher MPNs were obtained with the microtechnique (than did by the originally methods using the tube techniques) with increased accuracy in endpoint determinations being a possible cause. Considerable saving of time, space, equipment, and reagents are observed using this method. The microtechnique described may be adapted to other microbial population using various types of media and endpoint determinations. These microtechnique based on the MPN method has been used by Verhagen and Lannbroek (1991), Hastings *et al.* (1998), Bruns *et al.* (1999), Deni and Penninckx (1999), McCaig *et al.* (1999), and Jun *et al.* (2000).

Establishing the metabolic properties and potential of diverse organisms in the absence of pure culture presents an immense challenge for

microbial ecologists. The ability to grow and study previously uncultured organisms in pure culture will enhance our understanding of microbial physiology and metabolic adaptation and will provide new sources of microbial metabolites (Zengler *et al.*, 2002). Although molecular techniques have changed our perceptions of bacterial ecology, this does not diminish the need for continued efforts and new techniques in the culturing in the culturing of bacteria (Aakra *et al.*, 1999). The pure culture can be obtained from liquid culture enrichments by end-point dilution and from solid medium enrichments by colony picking and subculture into liquid medium (Prosser *et al.*, 2002). In some studies, end-point dilution has been combined with enumeration by the most probable number (MPN), with isolation and identification of organisms from cultures at the highest dilutions exhibiting growth (Giuliano *et al.*, 1999; Fouratt *et al.*, 2003). The micromanipulation from the dilution method is the other options. Zengler and other (2002) showed the technology that can be applied to samples from several different environments, including seawater and soil. This technique is combines encapsulation of cells in gel microdroplets for massively parallel microbial cultivation under low nutrient flux conditions, followed by flow cytometry to detect microdroplets containing microcolonies.

4.2 The limitation of culture dependent methods

This method is time-consuming and frequently only works for a small percentage of the bacterial species present in a particular sample (Jang *et al.*, 2003; Zengler *et al.*, 2002). Culture dependent techniques are reported to be biased towards organisms able to grow well under the cultural condition provided, but perhaps not significant in the actual system (Amann *et al.*, 1995). Investigation of autotrophic nitrification in natural system has been limited by the difficulties in isolation and culturing ammonia oxidizers (Stephen *et al.*, 1996). The energy yield from ammonia oxidation was low leading to small biomass yield and low maximum specific growth rate. Growth of visible colonies on solid media takes several months and elimination of heterotrophic contaminants is difficult. MPN method have been used for enumeration of

nitrifying bacteria, however, their culturing time is too long. Moreover, enumeration given by MPN method is known to have the possibility of underestimation (Araki *et al.*, 1999) due to cluster formation of AOB (Wagner *et al.*, 1995) and/or difference of culture ability among the species of AOB.

Cultured microbial diversity may reflect only a small proportion of natural microbial diversity, probably less than 1% of bacteria are known (Staley and Konopka, 1985; Amann *et al.*, 1995; Head *et al.*, 1998). Taxonomies which provide a frame work for appreciating diversity, and description of natural communities (even mundane ones) are incomplete. The knowledge of microbial diversity has been limited because of a tradition obstacle to microbiologists as vast majority (more than 99%) of naturally occurring microbes could not be cultured by using standard techniques. If organisms can not be cultured, they can not be available for human use (Hugenholtz and Pace, 1996). For these limitations, non-culture dependent approaches have recently gained more acceptance as methods for studying the microbial ecology of many environmental ranging from natural to man-made systems.

5. Culture independent methods

5.1. A brief introduction to culture independent methods

Previously, microscopic observation and cultivation were the methods widely used for identifying bacteria in the natural environments. However, because the medium used in these methods always selects for certain organisms, the results are always biased toward these organisms (called cultivation bias) (Watanabe and Baker, 2000). Classification of microorganisms based on rRNA analysis has shown that the majority of microbes present in nature have no counterpart among previously cultured organisms (Zengler *et al.*, 2002). Therefore, recently, non- culture dependent approaches have gained more acceptances as methods for studying the microbial ecology of many

environments (Amann *et. al.*, 1990; Embley and Finlay, 1994; Amann *et al.*, 1996; Head *et. al.*, 1998).

Over the last decade there have been major advances in the study of microbes. At the same time many new insights into the composition of uncultivated microbial communities have been gained. Whole groups of organisms that are only known from molecular sequence are now believed to be quantitatively significant in many environments. These investigations have been enhanced through developments in molecular biology that facilitate the detection of populations, cells and functional genes (Head *et al.*, 1998). Pace and other (1986) suggested the use of 16s rRNA sequence information for ecological applications. The16s rRNA sequence is characteristic for nearly every organisms, and the information content of the molecule is high enough to allow statistically valid phylogenetic analysis as well as the identification of a species when the sequences are known.

Molecular studies are now being carried our by appropriate culture-based investigations that will assist in obtaining cultures of organisms that are truly representative of those important in nature (Amann *et al.*, 1990; Head *et al.*, 1998). In addition, where cultivation is not possible or not an appropriate strategy for the study, quantification of organisms in a particular niche, or an appreciation of their spatial distribution are exciting goals made possible by coupling molecular tools with other developing technologies.

5.2. The ribosomal RNA and the classification of organisms

There are three categories of RNA in prokaryote; the short-lived messenger (m)RNA responsible for transmitting information from the chromosome to the ribosome, the stable form, i.e. transfer (t)RNA, which decodes the message, and ribosomal (r)RNA involved in the structure of the ribosome and the reading of the message. Analysis of RNA for taxonomic purposes focuses on the three rRNAs; the 5S, 16S and 23S molecules (Priest and Austin, 1995). It can be argued that the small size of the 5S rRNA molecule detract from its value in measuring relatedness between organisms,

since it can undergo marked mutational change that would be obscured by the long stretches of the conserved sequence present in the larger molecules. Largely for this reason, the 16S molecule, which is somewhat easier to handle than the 23S, has been used extensively for comparative sequencing studies (Woese, 1987).

Due to the ubiquity of ribosomal RNA molecules in all cellular life forms, comparative analysis of their sequence can be universally applied to infer relationships among organisms. The rRNA molecules comprise highly conserved sequence domains interspersed with more variable regions. In general, essential rRNA domains are conserved across all phylogenetic domains, thus universal tracts of sequence can be identified. In addition, it is also possible to identify sequence motifs of increasing phylogenetic resolution (Woese 1987; Amann *et al.*, 1990; Head, 1998).

Classification of the bacteria by the conventional approach based on the conclusion of Priest and Austin (1995) from Bergey's Manual of Systematic Bacteriology, Volume 1 to 4, the bacteria are considered as divided as follows:

Kingdom		Procaryotae
	Division	Gracilicutes (Gram- negative bacteria)
	Class	Scotobacteria
	Class	Anoxyphotobacteria
	Class	Oxyphotobacteria
	Class	Proteobacteria
	Division	Firmicutes (Gram-positive bacteria)
	Class	Firmibacteria
	Class	Thallobacteria
	Division	Tenericutes (Bacteria which lack rigid cell walls)
	Class	Mollicutes
Kingdom		Archaebacteria
	Division	Mendosicutes (Bacteria with unusual cell walls)
	Class	Archaeobacteria

Based on published comparisons of small subunit rRNA (16S or 18S rRNA) sequences from a variety of organisms, all cellular life can be divided into three primary domains comprising the *Bacteria*, the *Archaea* and the *Eucarya*, see Figure 7 (Woese 1987; Woese *et al.*, 1990). At the current stage in the phylogenetic classification of *Bacteria* Domain, divisions are not consistently named or taxonomically ranked. rRNA-defined divisions are identified by classes (or phyla) (e.g. *Proteobacteria*, *Actinobacteria*), order (e.g. *Thermotogales* and *Aquificales*), families (e.g. *Chlorobiaceae*) , generic name such as the *Nitrospira* group (Hugenholtz *et al.*, 1998). From the observation of Hugenholtz (2002), understanding of prokaryote biology from study of pure cultures and genome sequencing has been limited by a pronounced sampling bias towards four bacterial phyla - *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* - out of 35 bacterial and 18 archaeal phylum-level lineages. This bias is beginning to be rectified by the use of phylogenetically directed isolation strategies and by directly accessing microbial genomes from environmental samples.

5.3. The commonly used approaches in molecular microbial ecology

5.3.1. rDNA PCR-cloning of rRNA-sequencing

Watanabe and Baker (2000) proposed a typical scheme for analyzing a microbial consortium using molecular ecological methods. DNA is first extracted from a microbial consortium in the environment and used as a template for PCR to amplify 16S rDNA fragments with a set of universal primers. Thereafter, the PCR products are either separated by denaturing gradient gel electrophoresis (DGGE) or each product is cloned into *E. coli*. 16S rDNA fragments are then sequenced and the determined sequences are compared with the sequences stored in nucleotide databases to phylogenetically identify the detected populations. Moreover, the sequence information can be used to design an oligonucleotide probe for the detection and quantification of a specific bacterial population by fluorescence *in situ* hybridization (FISH). A

typical scheme for analyzing bacterial population in the environment is illustrated in Figure 8.

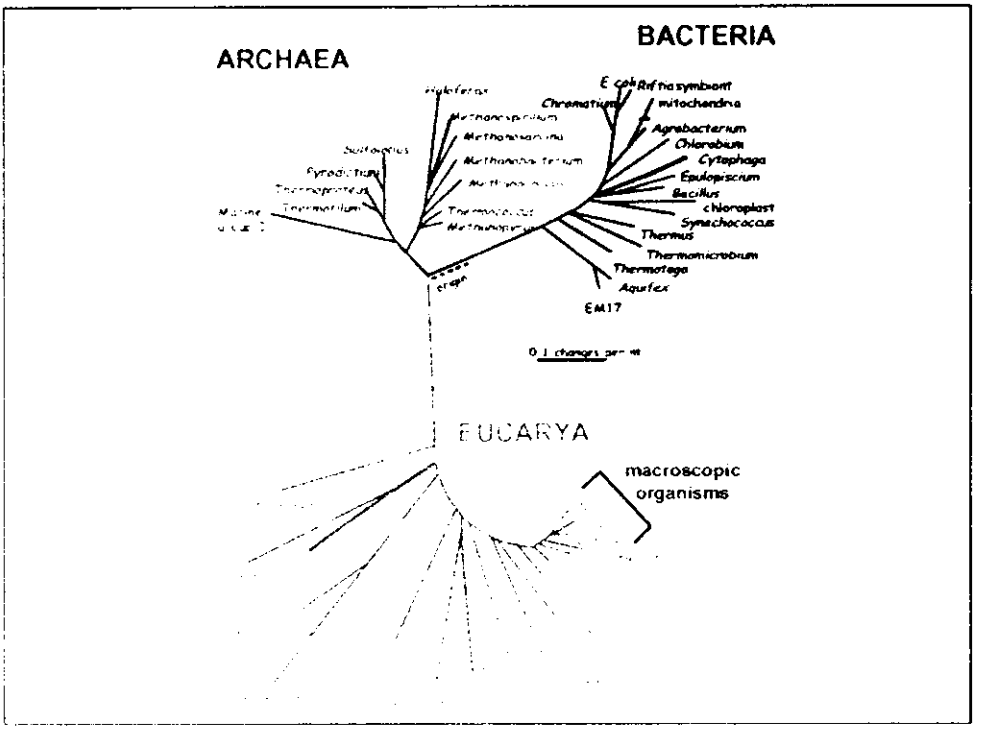

Figure 7. Evolution distance (number of sequence change; see scale bar) is read along line segments about 50,000 rRNA sequences. The sequences used in this tree are represented relatives of major groups. Named lines are from cultivated organisms; line with letter number designation represent natural-population sequence.

Source: Pace (1996)

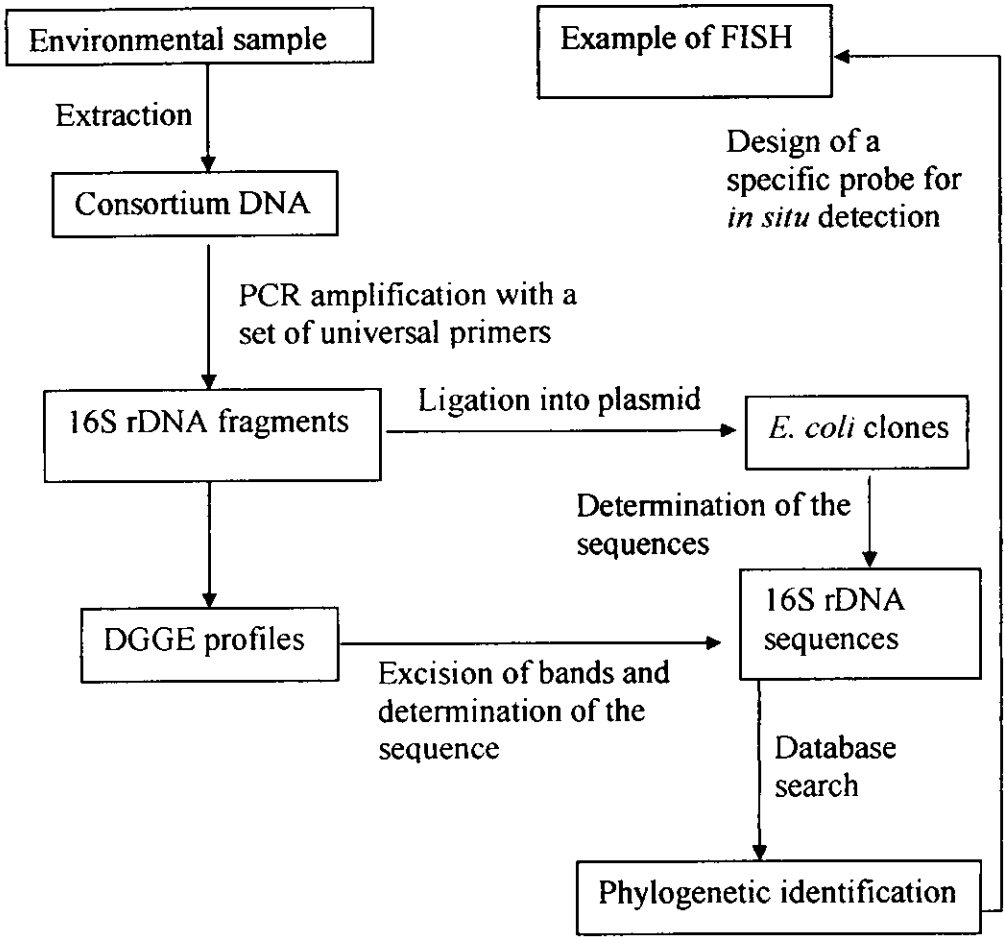

Figure 8. A typical scheme for analyzing a microbial consortium using molecular ecological methods

Source: Watanabe and Baker (2000)

The Polymerase chain reaction (PCR)-clone approach

The starting point for this and related procedures is the extraction of nucleic acids of sufficient quality to permit activity of the enzyme used in subsequent procedure. The extracted DNA is subjected to PCR amplification using universal primer or primer designed to amplify rRNA genes from a particular group of organisms. The PCR product can then be cloned either by filling overhanging 3' deoxyadenosine residues and blunt-end ligation procedures, or by using commercially available kits for the cloning of PCR product (Head *et al.*, 1998).

Screen clone libraries for rRNA genes

Once cloned, the 16s rRNA gene library can be screened by a variety of methods. Colony hybridization procedures using rRNA gene specific oligonucleotide probe of defined phylogenic resolution may be used. However, the specificity of the probe used is important to avoid false positive signals at this stage. Extracted plasmid and restriction digests can be used to confirm the presence of cloned DNA of the correct size, or, alternatively, colony PCR (using, or example, sequencing primers with priming sites that flank the insert DNA) can be used as a rapid screening procedure to detect cloned. PCR product and can also rapidly provide template DNA suitable for sequencing (Head *et al.*, 1998).

Sequencing of specific clones (those containing 16S-rDNA PCR insert)

Automated DNA sequencing system has greatly facilitated the rapid screening and analysis of large gene library. Initial screenings of rRNA gene containing clones were monitored by RFLP (restriction fragments length polymorphism) analysis of the amplified cloned 16S rDNA PCR products. One to three representatives per each RFLP of bacteria were then identified by direct sequencing and analysis of the 16S rRNA (Giuliano *et al.*, 1999). Complete sequencing of the cloned rRNA genesis facilitated by the presence of conserved sequence domains throughout the molecule, allowing primers to be designed that permit clone library sequencing of almost the complete rRNA gene. Once a sequence database has been generated from the clone library, phylogenetic analyses can be carried out, and the diversity of the microbial population can be determined with reference to previously published sequences (Head *et al.*, 1998).

Phylogenetic identification

The rapidly expanding database of rRNA sequence now contains several thousand sequences, and represents an invaluable resource. Largely publicly accessibility database e.g. GeneBank, USA; EMBL, England; DDBL, Japan; Ribosome Database Project (RDP), USA, allow everybody access to this

database. By comparison of the more variable regions of the molecule, it is possible to design oligonucleotides of varying phylogenetic resolutions. These can be utilized in the detection and enumeration of specific group of bacteria. Detection of specific organisms, without cultivation, can be achieved by PCR alone, or combined with the use of diagnostic oligonucleotide probes. Enumeration in PCR-based systems is problematic, and determining relative abundance may be the best that can be confidently achieved within the limit of current technology. For absolute enumeration and spatial localization of specific microorganisms in natural samples, whole cell *in situ* hybridization techniques hold considerable promise (Head *et al.*, 1998). To illustrate the application of phylogenetic group-specific hybridization probe, they have identified and synthesized oligodeoxy- nucleotides that are diagnostic for each of the three primary lines of evolutionary descent: the eubacteria, the archaebacteria, and the eucaryotes.

5.3.2. Denaturing/temperature gradient gel electrophoresis (D/TGGE)

Denaturing/temperature gradient gel electrophoresis (D/TGGE) is widely used in recent years for profiling microbial consortia (Watanabe and Baker, 2000) and determination of the genetic diversity of natural microbial communities (Kurisu *et al.*, 2000). It is a method for separating DNA molecules by sequencing rather than length. In DGGE, DNA fragments are caused to move into an ever greater concentration of chemical denaturing agents while move in temperature gradient in TGGE. Each fragment effectively stop moving when it begin to "fray", and the point at which it does this depends on the proportion and order of the four nucleotide bases it contains (Majerus *et al.*, 1996; Felske *et al.*, 1999). By using DGGE, DNA fragments of the same length but with different base pair sequences, such as PCR fragments obtained from a mixed culture, can be separated. Similarity of microbial communities can be analysed from electrophoresis patterns and bands can further be excised from gels and sequenced to identify the phylogenetic affiliation of the community members. Subsequently, oligonucleotide probes can be designed

based on the sequences of DGGE bands to obtain quantitative information (Kurisu *et al.*, 2000). The limitation of T/DGGE is it require laborious technical optimization including calibration of the linear gradient of DNA denaturants (chemical or physical) and improvement of the PCR primers with the insertion of a GC clamp to obtain better electrophoretic separation of the fragments (Ranjard *et al.*, 2000).

5.3.3. Whole cell probing or FISH (Fluorescent *in situ* hybridization)

FISH of whole cells using 16S rRNA-targeted oligonucleotide probes is a powerful technique with which to evaluate the phylogenetic identity, morphology, number and special arrangements of microorganisms in environmental settings. FISH is useful for many applications in all fields of microbiology. FISH not only allows the detection of culturable microorganisms, but also of yet-to-be cultured (so-called unculturable) organisms, and can therefore help in understanding complex microbial communities (Moter and Gobel, 2000). The FISH method involves application of oligonucleotide probes to permeablized whole microbial cells. The probes enter the cells and specifically hybridize to their complementary target sequence in the ribosomes. If no target sequence is present in the cells ribosomes, probes are unable to hybridize and unbound probes are removed by a subsequent wash step (Hugenholtz *et al.*, 2002). The development of FISH technique over the last decade has had a large impact on the way environmental microbiologists approach their research. This methodology is now widely used by many laboratories worldwide to describe the temporal and spatial distribution of aquatic bacteria, and the specific roles of microbes in biogeochemical cycles and food web dynamics (Bouvier and del Giorgio, 2003). The some applications of a FISH technique for the quantitative analysis of a microbial population are shown in Table 4.

FISH can also be combined with other methods, such as with biosensor for measurement of the in situ activity of microorganisms (Ramsing, 1993; Schramm *et al.*, 1996 and 1999). Recently, microautoradiography has been combined with FISH. With this approach, the uptake of radioactively labelled

substrate by individual cells can be detected, and the cells are subsequently identified by *in situ* hybridization. This combination technique is an elegant approach to evaluate substrate consumption profiles in complex microbial communities such as activated sludge (Lee *et al.*, 1999; Nielsen *et al.*, 1999; Frigon *et al.*, 2002).

Table 4. Examples of a FISH technique application

Applications	References
- Characterized bacterial community of activated sludge	Wagner *et al.* (1993); Mino *et al.* (2000); Mudaly *et al.* (2001)
- Determined the structure and function of nitrification biofilm	Okabe *et al.* (1999 and 2002); Okabe and Watanabe (2000); Nogueira *et al.* (2002)
- Showed spatial distribution of as yet uncultured treponemes in biopsies from digital dermatitis lesions	Moter *et al.* (1998)
- Characterized the biomass in enhanced biological phosphorus removal (EBPR) sludge.	Bond *et al.* (1998)
- Analyzed the microbial community in marine Arctic sediments (Svalbard)	Ravenschlag *et al.* (2001)
- Evidenced the genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation	Schmid *et al.* (2000)
- Showed the interaction and competition between three groups of bacteria in The CANON system (Completely Autotrophic Nitrogen-removal Over Nitrite) under ammonium limitation	Third *et al.* (2001)

5.3.4. Dot blot hybridization

To measure gene abundance in a sample by dot blot or slot blot hybridization, total RNA is extracted from an environmental sample and immobilized (blotted) on a nylon membrane, hybridized with probes and then compared to a standard. DNA oligonucleotides probes are labeled with radioactive or non-radioactive with dioxygenin (DIG), hence the subsequence hybridization signals can be qualified and matched using laser densitometry, radiometry, or image analysis (Stahl *et al.*, 1988; Manz *et al.*, 1992).

5.4. The limitation of culture independent methods

Culture independent methods have the following limitation (Head *et al.*, 1998).

1) The methods limitation involved the extraction of nucleic acid from natural samples, biases and artifacts associated with enzymatic amplification of nucleic acids, cloning of PCR product, sensitivity and target site accessibility in whole cell hybridization techniques.

2) Sequence can provide information about which microorganisms are present in a sample without culture, these approaches often require some prior knowledge of sequences in order to design specific gene probes or PCR primers to a particular 16s rRNA genotype or particular phylogenetic group.

3) Inferring function and activity is more problematic: some phenotype has a restricted distribution (e.g. Methanogenesis, ammonia oxidation) so detecting 16s rRNA gene from one of these groups suggests a particular activity is present but many phenotypes (e.g. Nitrogen fixation) are not limited to particular group. In these cases 16s rRNA provided no clue to phenotypes or role in the environment. Functional gene probes can give information about potential activity e.g. probe to ammonia monooxygenase for ammonia oxidation.

4) Obtaining quantitative information about *in situ* cell activities is still in its infancy.

Despite its limitations, this technology is permitting major advance in understanding of microbial ecology and evolution. Its potential lies not only in the identification of specific organisms in the environment, but also in its ability to complement other methods, to assign them to functional roles and their significance in environmental process.