CHAPTER II

LITTERATURE REVIEW

2.1 Shrimp Culturing in Thailand

Shrimp culture in Thailand has been developed for many years, especially over the last two decade, in response to the increasing world market demand. The production system evolved from extensive toward intensive with increasing input of high quality feed and water supply (Thakur and Lin 2003). The management used in marine shrimp farming can be separated in to four types *i.e.* extensive, semi- intensive, intensive and super-intensive shrimp farming. Each culturing type is difference in both pond size and pond management.

2.1.1 Extensive culture

The extensive shrimp farming system is primarily used by farmers in rural areas with inexpensive land price and limited infrastructure. The ponds are located along bays and tidal rivers and are constructed by the elevated mud walls around the bottom of the ponds. The extensive shrimp farms are often found in mangrove swamps or in salt flats, because the environment is not suitable for more intensive culture. In some cases, fertilizers or manure are added to promote algal growth. The shrimp larvae that migrated into the pond during high tide are captured by retaining water in the pond and let grow naturally to market size (Tobey *et al.*, 1998). Disease outbreak is rare because of the low shrimp densities (Dierberg and Kiattisimkul, 1996). Although the survival and yield of

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extensive shrimp culture are the lowest among all culture systems, production cost and risk are relatively low and therefore make it profitable.

2.1.2 Semi-intensive culture

Semi-intensive shrimp farms usually located above the high tide area. The stocking densities are higher (5 – 10 shrimp per m^2) than the extensive culture so an additional feeding input is needed (Dierberg and Kiattisimkul, 1996). The ponds are smaller (1-6 ha) than that used in extensive culture and are more regular in shape. This leads to better control over the grow-out environment. The pond is designed with dikes that make it is easier to harvest than the extensive ponds. Nursery ponds are often used. In semi-intensive shrimp farming, pumping systems and sometimes aerators driven by diesel or electrical energy are used to regulate the water exchange and to bring oxygen to the bottom of the pond. The daily water exchange rate is 0 – 25% (Rosenberry, 2001).

2.1.3 Intensive culture

In intensive shrimp farming the ponds varies in size from 0.16 to 1 ha. The intensive system operates using around the clock management. There are greater inputs of operating capital, equipments, skilled labor, feeding, fertilizer and other chemicals including drugs and antibiotics. The shrimp are fed up to five times daily supplemented with vitamins and minerals. The ponds are aerated with paddle wheels or submersible aerator. The stocking densities are high (30 – 60 shrimp per m^2). In intensive shrimp culture, 2 - 3 cultivation crops per year could be expected and water exchange rates can be as high as 40% daily. The risk of disease, caused especially by bacteria and viruses, is

high and a whole crop can be lost in a short time following infection. There are different types of intensive cultivation systems: A) open, C) semi-closed, B) closed and super-intensive systems (Dierberg and Kiattisimkul, 1996).

2.1.3.1 Open intensive systems

Traditionally, water quality in shrimp pond is maintained by high rate of water exchange to flush excess plankton, nutrients and waste out of the pond. The open intensive ponds require large amounts of daily water exchange to maintain suitable water quality (Dierberg and Kiattisimkul, 1996).

2.1.3.2 Semi-closed intensive systems

Due to environmental problem in shrimp farm areas and the outbreak of shrimp viral diseases, many farmers as driven by the "Good Aquaculture Practice" regulation of the governmental agencies such as the Department of Fisheries in Thailand have been encouraged to recycle water in their farm. In semi-closed farming systems there is no water exchange during the first month of production (except for compensation of evaporative loss) due to the high risk of disease during this time. A limited water exchange is then commence during the second month of production (Dierberg and Kiattisimkul, 1996).

2.1.3.4 Closed intensive systems

Fully closed intensive shrimp farming operates with no water exchange to the outside during the whole production period. mWater is only added for compensation of evaporative loss and seepage. In most closed systems, water is discharged to the environment at harvest. Some closed intensive farms, however, treat and recycle the water after harvesting. This is known as the zero water exchange system (Rosenberry, 2001).

2.1.3.5 Super-intensive systems

Super-intensive shrimp farming is still in its experimental stages. It requires huge amounts of water, around 50% daily water exchange, and can yield up to 10-100 ton/ha/year. Super-intensive production requires huge investments in technology, equipments, staff expertise and overall management. Generally, these farms still have problems with management, diseases, crop failures, water quality, finances and the environment (Rosenberry, 2001).

2.2 Sediment development

During pond construction, the pond bottom usually is scraped off and used as earth fill for embankments. The newly finished pond bottom is normally low in organic matter content and nutrients. In tropical and subtropical areas with highly leached soils, pond bottom is often high in clay content and of low pH. After filling with water, various processes begin to transform the bottom of a new pond into a pond soil. Erosion of the

watershed results in suspended particles of mineral soil and organic matter in the pond. Wave action, rainfall, and water currents from mechanical aeration also erode embankments and shallow edges to suspend soil particles. In addition, nutrients input to the pond in whatever fertilizer, manure, and feed causes phytoplankton blooms that increase the concentration of suspended organic particles. Suspended particles settle in ponds with large sand particles settling first, following by silt-sized particles, and finally clay particles and fine-divided organic matter. Water currents and activity of fish and other organisms continually re-suspend particles from the bottom and re-settle subsequently (Boyd and Bowman, 1997).

The continuous input, deposition, resuspension, and redeposition of particles in a pond result in a sorting of particles with the fine clay and organic matter particles settling in the deeper water and the coarser particles settling in shallower water. Deeper areas gradually fill in, and ponds may decrease in volume. Sediment thickness in deep areas of aquaculture ponds usually increases at 0.5 to 1 cm per year (Munsiri *et al.*, 1995).

Organic matter settled at the bottom is decomposed by microorganisms. Easily decomposable organic matter, such as simple carbohydrates, protein, and other cellular constituents, is rapidly degraded. More resistant material, such as complex carbohydrates and other cell wall components, usually accumulates in the pond because it is degraded slowly. Since organic matter input accumulates at the bottom, benthic microorganisms are continually decomposing both fresh, easily degradable (labile) organic matter and older, resistant (refractory) organic matter. Because there is a more or less continuous resuspension and redeposition of particles and stirring of the surface sediment by fish and other organisms, the organic matter becomes rather uniformly mixed at the upper layer of

sediment. Nevertheless, there is a layer of fresh organic matter usually found at the sediment surface that has not been completely mixed into the sediment. Hence, organic matter concentration is therefore greatest near the sediment surface (Munsiri *et al.*, 1995). The ratio of labile organic matter to refractory organic matter also is greatest near the sediment surface (Sonnenholzner and Boyd, 2000). Organic matter concentrations in pond bottom do not continue to increase indefinitely. If aquaculture practices, *e.g.* species, stocking, fertilization, feeding rate, water exchange, amount of mechanical aeration, and treatment of fallow bottom between crops, is operated with the traditional manner, the annual input of organic matter and the rate of organic matter decomposition will remain in constant rate (Munsiri *et al.*, 1995).

New ponds usually have low organic matter content in bottom sediment, and the labile organic matter added each year will largely decompose, while a considerable proportion of refractory organic matter will accumulate (Boyd, 1995). Over a fairly short period, often only four or five crops, organic matter in the soil will reach a level that the annual rate of organic matter decomposition is equal the annual input of organic matter. At this time, equilibrium will occur and soil organic matter content will remain constant from year to year. Dissolved oxygen cannot enter rapidly in bottom soil because it must diffuse through the tiny water-filled pore spaces among soil particles. At a depth of only a few millimeters below the soil surface, the demand for dissolved oxygen by microorganisms can exceed the dissolved oxygen diffusion rate to that particular depth. This is the condition that induces the formation of anaerobic environment. The oxidized (aerobic) top layer of the sediment usually has lighter color than the deeper, reduced (anaerobic) sediment that is usually gray or black in color. This color is a result of ferrous iron substance. Processes described above consequently affect the bulk density

(weight of soil per unit volume, usually given in grams dry soil per cubic centimeter) of the distinct sediment layer.

A core sampling taken through the sediment and extending into the original bottom soil is used to study profile of the pond bottom. Layers in the profile are known as horizons (Munsiri *et al.*, 1995). Boyd (1995) described these horizons and assigned letters to them to facilitate discussion (Figure 2.1). The F and S horizons are among the most important in aquaculture pond because the exchanging activity between substances in sediment and overlaying water can influence water quality in the pond (Munsiri *et al.*, 1995; Boyd, 1995).

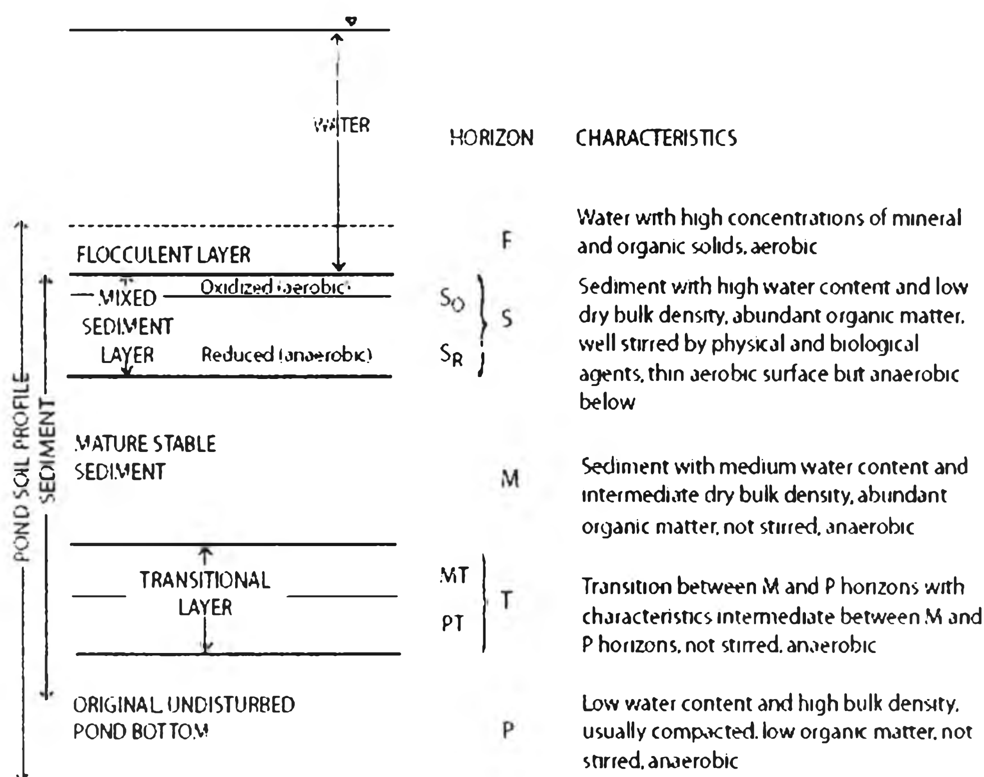

figure 2-1: Typically profile of aquaculture pond (Boyd, 1995)

Typical profile of aquaculture ponds is shown in Figure 2-1. Sediment is referred to geological material that being suspended and transported by water movement and being settled or deposited in still water. In many places, the soil was formed by sedimentation. All aquaculture pond bottoms become covered with sediment, and this sediment can be considered as an aquaculture pond soil. In describing various physical, chemical, and biological processes occurring in the pond bottom, it is convenient to refer the bottom deposits as sediment. However, in the discussion of management of pond bottoms, the sediment layers in the bottom of an aquaculture pond is usually referred to pond soil (Sonnenholzner and Boyd, 2000).

2.2.1 The oxidized layer in sediment

The oxidized layer at the sediment surface is highly beneficial and should be maintained throughout the aquaculture crop. Metabolic products of aerobic decomposition are carbon dioxide, water, ammonia, and other nutrients. In anaerobic sediment, some microorganisms decompose organic matter by fermentation processs that produce alcohols, ketones, aldehydes, and other organic compounds as their metabolites. Other anaerobic microorganisms are able to use oxygen from nitrate, nitrite, iron and manganese oxides, sulfate, and carbon dioxide to decompose organic matter, but they release nitrogen gas, ammonia, ferrous iron, manganese, hydrogen sulfide, and methane as their metabolites (Boyd *et al.*, 2002). Some of these metabolites, especially hydrogen sulfide, nitrite, and certain organic compounds, can enter the water and be potentially toxic to fish or shrimp.

The oxidized layer at the sediment surface prevents diffusion of most toxic metabolites into pond water because they are oxidized to non-toxic forms by chemical and biological activity while passing through aerobic surface layer. Nitrite will be oxidized to nitrate, ferrous iron converted to ferric iron, and hydrogen sulfide will be transformed to sulfate (Boyd and Tucker, 1998). Thus, it is extremely important to maintain the oxidized layer at the sediment surface in aquaculture ponds. Methane and nitrogen gas pass through the layer and diffuse from the pond water to the atmosphere. These two gases do not cause toxicity to aquatic organisms under normal circumstances. Loss of the oxidized layer can result when soils accumulate large amounts of organic matter and dissolved oxygen is used up within the flocculent layer before it can penetrate the soil surface (Gross *et al*, 2000).

2.2.2 Organic matter content in sediment

It is difficult to assess organic matter content in soil because there are different kinds of organic matter. Organic soil has high concentrations of often recognizable plant remains that are quite resistant to be decomposed by bacteria and other microorganisms. Organic soils with 30 to 40% organic matter have approximately 15 to 20% organic carbon content. Such soil is unsuitable for pond aquaculture and should be avoided. Soil with less organic matter is known as mineral soils, and as previously discussed, organic matter in mineral soil is considered to be labile if microorganisms can decompose it readily or refractory if it decays slowly (Gross *et al*, 2000). Thus, a soil with 10% organic matter may be perfectly acceptable for pond aquaculture if most of the organic matter is refractory but unacceptable if the organic matter is mostly labile. Organic carbon concentrations for pond aquaculture are shown in Table2-1.

Table 2-1: A classification of soil organic carbon concentrations for pond aquaculture

Organic Carbon (%)	Comment
> 15	Organic soil
3.1 to 15	Mineral soil, high organic matter content
1.0 to 3.0	Mineral soil, moderate organic matter content, best range for aquaculture
< 1	Mineral soil, low organic matter content

(Boyd *et al.*, 2002)

Munsiri *et al.* (1996) reported that concentration of nutrients in bottom soil of older ponds was higher than new ponds. Ritvo *et al.* (1998) found that concentrations of several elements in the soil increased during one shrimp crop. Ritvo *et al.* (1997a) found that the concentration of C, N, Mg, K and Na increased rapidly in new ponds and reaching maximal levels after five production cycles. On the other hand, concentration of S, P and Zn in the sediment continued to increase while soil pH decreased markedly after one growing cycle, but then stabilized.

Organic carbon (OC), to a large extent, was consumed for respiration and released as CO_2. Accumulation of organic carbon in the sediments was about 25% of the organic carbon input from feed. Similar studies were conducted in shrimp ponds. Lin and Nash (1996) estimated that 26 % of the nitrogen and 24 % of the phosphorus applied as feed accumulated in the sediments of intensive shrimp ponds. Funge-Smith and Briggs (1998) found that the sediment accumulated 24% of nitrogen and 84% of phosphorus from feed. Peaez-Osuna *et al.* (1997) reported that sediment in semi-intensive shrimp ponds accumulated up to 63.5% of phosphorus input. Paez-Osuna *et al.* (1999) estimated that

47.2% of phosphorus input in shrimp pond was adsorbed by the sediment, while Martin *et al.* (1998) found that up to 38% of total nitrogen input accumulated in the sediment.

2.2.3 Sediment pH

The pH is the negative logarithm of the hydrogen ion concentration, and the pH range is given as between 1 to 14. A pH of 7 indicates neutral condition (neither acidic nor basic), where hydrogen ions equal to hydroxide ions in concentration. A pH below 7 indicates more hydrogen ions than hydroxide ions or acidic condition. At pH above 7, there are more hydroxide ions than hydrogen ions and basic or alkaline conditions exist. The pH in pond bottom soil can range from less than 4 to more than 9, but the suitable pH for aquaculture pond soils is usually neutral (Boyd, 1995).

Most soil microorganisms, especially soil bacteria, function best at pH 7 to 8. The source of acidity in most pond soils is aluminum ion. Clay and organic matter particles in soil are negatively charged and attract cations to their surfaces. Aluminum ions at cation exchange sites in soil are at equilibrium with aluminum ions in the pore water surrounding soil particles. Aluminum ion hydrolyzes to aluminum hydroxide, releasing hydrogen ions (Figure 2-2). The greater the proportion of aluminum ions on cation exchange sites in soil, the greater the acidity. Limestone neutralizes the acidity and calcium ions replace aluminum ions of cation exchange sites to make soil less acidic in reaction. Waters in ponds with acidic soils typically have low concentrations of bicarbonate, carbonate, calcium, and magnesium (Funge-Smith and Briggs, 1998).

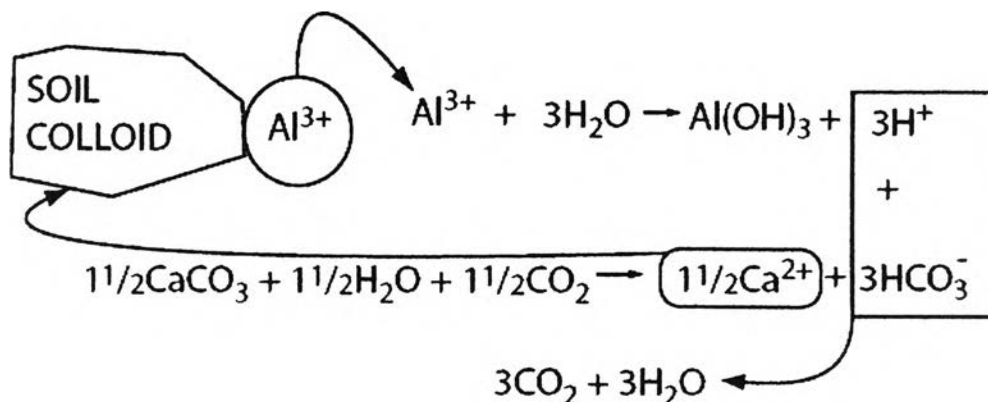

Figure 2-2: Model for neutralization of exchangeable acidity in soil by calcium carbonate (Martin *et al.*, 1998).

Waters with low concentration of bicarbonate and carbonate will have low total alkalinity, and those with low concentrations of calcium and magnesium have low concentrations of total hardness. Usually, water with low alkalinity also is low in hardness. Such waters are not well buffered against pH change, and they do not have large reserves of inorganic carbon to support phytoplankton photosynthesis. Application of agricultural limestone to acidic ponds can increase soil pH, increase concentrations of total alkalinity and total hardness in waters, increase the availability of inorganic carbon for photosynthesis, and buffer waters against wide diurnal changes in pH.

Soils in humid areas tend to be highly leached of exchangeable bases and therefore acidic. However, if soils in humid areas contain a source of carbonate, such as limestone, they will not be acidic. In semi-arid and arid regions, evaporation exceeds precipitation and salts tend to accumulate in soils. Such soils will be neutral or basic in reaction. In coastal aquaculture, ponds are often filled with seawater or water from estuaries that usually have moderate total alkalinity (75 to 120 mg/L) and high total

hardness (1,000 to 6,000 mg/L). Nevertheless, soils in coastal ponds may still be acidic and respond positively to liming (Munsiri *et al.*, 1995).

2.3 Nutrient in Shrimp pond

At the start of the growing season, earthen ponds are stocked with P-15 to P-25 shrimp post-larva measuring about one centimeter (figure2-3). Some of the pellet feed that is added to the pond is converted into shrimp flesh, but a major proportion (about 80% of the feed nitrogen) enters the pond ecosystem as input to nutrient cycling. These processes involve with wide variety of microorganisms including phytoplankton and common marine or estuarine bacteria. Living microorganisms take up nitrogen, in the form of ammonia, but release it when they die.

Figure2-3: Early growth stage shrimp or prawn pond (Trott, 2000)

After seasons of shrimp cultivation, more nutrients are added to the pond. This made the farmer a hard work to maintain optimal water quality (figure 2-4). One of the main problems is that phytoplankton biomass can increase too quickly and, at night time,

reduce oxygen in the water to critically low levels. In order to avoid low oxygen levels in the ponds, and also in the water discharge, farmers periodically replenish a small percentage of the total pond volume. This practice generally occurs during the last three months of the growing season, with about 7% of pond water replenished daily from creeks, rivers or estuaries. Water discharged from shrimp ponds contains higher sediment and nutrient loads than the intake water. Until recently, the most common global practice in shrimp farming is still discharge this pond water directly into receiving waters.

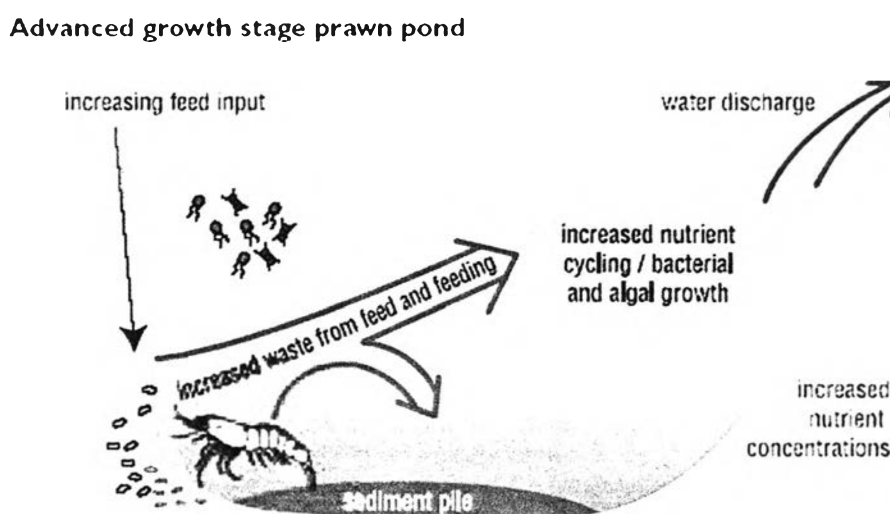

Figure2-4: Advanced growth stage shrimp or prawn pond (Trott, 2000)

2.3.1 Transformation of nitrogen compounds in aquaculture

The nutrients nitrogen (N) and phosphorus (P) are elements, and are essential building blocks for plant and animal growth. Nitrogen is an integral component of organic compounds such as amino acids, proteins, DNA and RNA. Phosphorus is found in nucleic acids and certain fats (phospholipids). Chemical and biological processes transfer nitrogen and phosphorus through the lithosphere, atmosphere, hydrosphere and biosphere.

This is called nitrogen and phosphorus cycling (Seitzinger *et al* 1984). Nitrogen-fixing bacteria convert di-nitrogen gas into organic nitrogen species that can enter the hydrological cycle and food webs.

Nitrogen exists in water both as inorganic and organic species, and in dissolved and particulate forms. Inorganic nitrogen is found both as oxidized species (*e.g.* nitrate (NO_3^-) and nitrite (NO_2^-)) and reduced species (*e.g* ammonia (NH_4^+) and di-nitrogen gas (N_2)). The occurrence of the different forms of ammonia depends on pH. At the pH of average seawater, ~95% of ammonia is in the cationic form which is called ammonium (NH_4^+). However, roughly half the ammonia will be in the (toxic) gaseous form (NH_3), if the pH rises to ~9.5 in seawater (Christensen *et al* 2000). Total dissolved nitrogen (TDN) consists of dissolved inorganic nitrogen (DIN) and dissolved organic nitrogen (DON), and is readily available for plant uptake. DIN comprises NO_2^-, NO_3^+, NH_4^+. Dissolved organic nitrogen is found in a wide range of complex chemical forms such as amino acids, proteins, urea and humic acids. Ammonium is the form of nitrogen taken up most readily by phytoplankton because nitrate must first be reduced to ammonia before it is assimilated into amino acids in organisms (Ward, 2000). The particulate nitrogen pool consists of plants and animals, and their remains, as well as ammonia adsorbed onto mineral particles. Particulate nitrogen can be found in suspension or in the sediment. Some portion of the particulate nitrogen pool is subject to rapid mineralization, and is biologically available. Total nitrogen (abbreviated TN) is a measure of all forms of dissolved and particulate nitrogen present in a water sample (McCarthy, 1997).

2.3.2 Transformation of phosphorus in aquaculture

Phosphorus in natural waters is divided into three components: soluble reactive phosphorus (SRP), soluble unreactive or soluble organic phosphorus (SUP) and particulate phosphorus (PP) (Rigler 1973). The sum of SRP and SUP is called soluble phosphorus (SP), and the sum of all phosphorus components is termed total phosphorus (TP). Soluble and particulate phosphorus are differentiated by whether or not they pass through a 0.45 micron membrane filter.

The phosphate ion (PO_4^{3-}) is a highly particle-reactive anion and thus the sorption properties of sediment are crucial for P-retention capacity. Generally, aerobic conditions are considered to promote P sorption and anoxic conditions to favoring P release. After the dissolution of particulate P into inorganic form in sediment, PO_4^{3-} ion is easily absorbed into inorganic particulate form. The storage of surplus P by microorganisms can, however, compete with the chemical immobilization of P (Lucas, 1999). Normal bacteria have low P content in living cells but some bacteria accumulate intracellular polyphosphate (poly-P) in very large amounts, up to *ca.* 20% of their dry weight. These bacteria store P under aerobic conditions and release intracellular P via enzymatic hydrolysis when conditions turn anaerobic (Deinema, 1985).

In organic-rich lake sediment, microorganisms are able to take up and release P, depending on redox conditions, and sterilization of oxic sediments can reduce the microbial take up of P (Gächter, 1988). Both non-biological and biological binding mechanisms of P are able to keep the pore water P concentration low and reduce the release of P from sediment to water.

Due to surface area effects, the sorption reactions of P caused by inorganic particles are enhanced as a function of decreasing sediment grain size. Fine-grained (<2 μm) constituents of sediments are silicates, clays, carbonates, Fe and Al oxides, and humic acids. The surface of fine-grained minerals such as Fe and Al oxides, clay minerals with sufficient Fe and Al, and possibly also Mn adsorb P efficiently. Humic compounds containing Fe and Al can also absorb P. Silicates and carbonates in larger particles such as sand poorly adsorb P (Gächter, 1988).

It is widely accepted that sedimentary P cycling is linked mainly to the Fe cycle in lake and marine systems. In marine sediment, however, most of the total Fe (60-80%) is bound to silicates (e.g. chlorite). Adsorption of P by silicate-bound Fe is insignificant compared to that by Fe(III) oxides, which are considered to be the main Fe compounds in P binding. Sediment contains a multitude of Fe(III) oxides, often in complex mixtures, range widely in degree of crystallization, particle size, available surface area, reactivity and oxidation state (Lovley, 1991). The P sorption capacity of amorphous, *i.e.* poorly crystallized, $Fe(OH)_3$ and lepidocrocite (-FeOOH), is about 20 times that of crystalline Fe(III) oxides such as goethite (%-FeOOH) and hematite (%-Fe_2O_3).

Other negatively charged anions can also compete with the PO_4^{3-} adsorption sites in sediment. For example, the effect of pH can be related to competition for adsorption sites, because desorption of P from clay minerals and Fe and Al (hydr) oxides is based on ligand exchange, in which P is substituted by OH^-. An increase in pH can also increase the negative charge of the sorbing oxides (Hartikainen, 1996). A significant decrease in P sorption to Fe (III) oxides occurs at pH >6.5. Silicon (Si) may

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also compete with P for adsorption sites (Tallberg, 2000). Silicate (SiO_4) and P are absorbed onto the surfaces of hydrated Al and Fe oxides by the same specific mechanism and, thus, SiO_4 may chemically compete with PO_4^{3-} for adsorption sites (Lovley, 1991).

The main transport mechanism of P to sediments is clearly the settling of particulate matter, although influx of dissolved P to sediment may also occured. Surface of the sediment receives a mixture of particulate inorganic and organic compounds containing P. In sediment, the settled particulate inorganic P can be divided into Fe-, Mn-, Al- and Ca-bound P, whereas the particulate organic P consists of living and dead algae, plant debris, zooplankton, bacteria and detritus. Part of the settled particulate P behaves as inert material and is buried in its original form, whereas part of the P (*i.e.* mobile P) is involved in various physico-chemical and biological reactions before final burial as inert material in sediments. Mobile pools of loosely sorbed P, Fe-bound P and fresh organic P may constitute over 50% of the surface TP but are largely depleted below a depth of couple of centimeters. The non-mobilized, buried P consists mostly of stable minerals such as apatite and refractory organic P, whereas the Fe-bound P constitutes are only a minor proportion of the burial flux of P.

Particulate organic P is considered to be the main compound transporting P to sediments. In marine regions with marked seasonality such as at northern temperate latitudes, the settling of the spring bloom with its high P content carries a large portion of the organic particulate P to the surface of the sediment. In the Baltic Sea, for example, seasonal short-term changes in the phytoplankton biomass in the water mass are due to the rapid sedimentation of phytoplankton (Heiskanen, 1998). Large part of dissolved phosphorus may be stripped from the entire water column by this event. Although

organic matter is mineralized during sinking, a substantial portion of the settled particulate organic matter decomposes within the sediments in relatively shallow environments. In estuaries, however, the sedimentation of particulate matter may be accentuated by salt-induced flocculation of colloidal and particulate matter containing P (Edzwald, 1974); further, the dissolved P may precipitate with Fe (III) oxides (Gunnars, 2002). The precipitation on Fe (III) oxides in water may be major binding process of P in water depths, where biological uptake is minor.

Labile organic matter rich in P tends to increase the release of P from sediment to water within a period of days after settling. The settling and subsequent mineralization of labile organic matter does not, however, necessarily lead to the immediate release of P from sediment to water owing to the sorption capacity of P in sediments. A major portion of the organic P in sedimentation flux can be mineralized in sediments, but the mineralized P is partitioned between the pore water and adsorption sites of the sediment. The proportion of organic P may then decrease but that of Fe(III) oxide-bound P increase in surface sediment (Jensen, 1995).

Therefore, although the settling of particulate organic P is an important transport mechanism of P into sediment, the P is partly bound to inorganic compounds after mineralization in sediments. However, neither fresh organic P nor Fe (III) oxide bound P is efficiently buried, because organic P is mineralized and Fe-bound P is dissolved in the reduction of Fe (III) oxides in sediments (Hupfer, 1991).

2.4 Microbial in shrimp pond

Ruangpan, *et al.* (1996) investigated bacterial community in shrimp pond sediment throughout the culture period. Eight species of *Vibrio* were identified from a total of 262 isolates. They were *V. harveyi, V. alginolylicus, V. parahaemolyticus, V. vulnificus, V.fluvialis* type I, *V anguillarum, V. damsela,* and *V. cholerae.* The other species of bacteria that were found in the sediment were *Acinetobacter, Flavobacterium, Escherichia coli, Salmonella, Pseudomonas, Bacillus,* gram positive bacteria, and others. This investigation revealed that *Pseudomonas* were the most common bacteria in the sediment of shrimp ponds. The number of bacteria increased through the rearing period and the highest number were found at 90-120 days of the production crop.

2.4.1 Microbial related to nitrogen cycle

In shrimp pond organic matter content is generally high compared with natural environment. This due to extraneous inputs during shrimp culture such as feed, excreta, fertilizer, etc. The microorganisms such as bacteria, fungi, protozoa, carry out active decomposition of left over feed and metabolite to inorganic forms such as ammonia, hydrogen sulphide, carbondioxide, through the process of mineralization (Moriarty, 1997). Microorganisms help not only in the production and breakdown of organic matter, but also in the nutrient recycling in aquatic ecosystem. The nitrogen cycle occupies an important place in organic matter recycling and involves nitrogen-fixation, ammonification, nitrification and denitrification processes carried out by different microorganisms.

Nitrogen-fixing bacteria are able to fix atmospheric nitrogen and to use it as the nitrogen source for growth and biosynthesis. The key groups of nitrogen-fixing microorganisms are Cyanobacteria, specialised bacteria from the family of Azotobacteriaceae and anaerobic bacteria from the genus *Clostridium*.

Ammonification bacteria carry out the catabolic decomposition of nitrogen-containing substances, which results in the formation of ammonia. They are represented in water by numerous taxa, including aerobic saprophyte bacteria, actinomycetes and moulds, as well as anaerobic bacteria.

Nitrification, the oxidation of ammonia to nitrate via nitrite, occupies a central position within the global nitrogen cycle. Nitrifying bacteria are the only organisms capable of converting the most reduced form of nitrogen, ammonia, to the most oxidised form, nitrate and also carry out a range of other processes within the nitrogen cycle. Nitrification influences nitrogen supply to plants, increases loss of soil nitrogen through leaching and volatilisation, limits the rate of throughput in aerobic sewage treatment processes, increases nitrate pollution of groundwater and generates reactive gases involved in the production of atmospheric ozone and acid rain, the destruction of stratospheric ozone and global warming. Despite the ubiquity of abundant and active populations of nitrifying bacteria in natural environments, the organisms are difficult to isolate, grow and maintain in laboratory culture (Alleman, 1991). *Nitrosomonas europaea*, *Nitrospira briensis*, *Nitrosococcus nitrosus*, *Nitrosococcus oceanus* and *Nitrosolobus multiformis* are among the member of nitrifying bacteria (Wood, 1986).

Denitrification is the microbial reduction of nitrate to di-nitrogen gas (N_2). Denitrification is sometimes referred to as 'dissimilatory' nitrate reduction because it occurs in association with the dissimilation (decomposition) of organic matter (Postgate, 1998). The denitrification steps are as follows (Froelich *et al,* 1979):

NO3 (Nitrate)—> NO2 (Nitrite)—> NO (Nitric Oxide)—> N2O (Nitrous Oxide)—> N2 (Nitrogen)

The nitrate (NO_3^-) is used in the respiratory process of the microbes, and can be derived from the water column or from nitrification occurring during the mineralization of organic matter in sediment. When nitrate for denitrification is derived from nitrification, the process is called coupled nitrification-denitrification. Denitrification in sediment appears highly sensitive to carbon loading (trophic status) (Eyre and Ferguson, 2002; Seitzinger and Nixon, 1985; Heggie *et al,* 1999b).

Denitrifying bacteria are anaerobic but they require an oxidized form of nitrogen (*e.g.* nitrate). Denitrification can be enhanced by the presence of benthic fauna which increase sediment surface area and enhance irrigation (oxidation) of deeper sediments. Benthic invertebrates thus cause localized increases in concentration of organic matter and solutes (*i.e.* ammonium) and ultimately enhance microbial activity and oxic/anoxic microenvironment (and therefore coupled nitrification / denitrification) in their burrow linings, excreta and organic particles (Nixon, 1988, cited in Harris 1999., Haese, 2002.).

2.4.2 Microbial related to phosphorus cycle

Plants and bacteria require phosphorus in the dissolved form, generally as orthophosphate, for their nutrition (Henry and Heinke, 1996). Phosphates are not toxic

unless they are present at very high levels (Murphy, 2000b), however, excessive amounts of orthophosphate in the aquatic environment increase algae and aquatic plant growth. As before, this change results in decreased dissolved oxygen concentration as bacteria decompose algal debris also consume oxygen in the decomposition process. When dissolved oxygen concentrations fall below the levels required for metabolic requirements of aquatic biota, both lethal (*e.g.*, fish kills) and sublethal effects can occur. Oxygen loss in bottom waters can also free phosphorus previously trapped in the sediment, increasing the amount of available phosphorus and continuing the process of decreasing dissolved oxygen (Murphy, 2000b).

Nitrogen and phosphorus are the primary causes of eutrophication. The most recognizable evidence of eutrophication is algal blooms that occur during the summer. Symptoms of nutrient over enrichment include murky water, low dissolved oxygen and fish killed. Excessive amounts of nutrients can also stimulate the activity of microbes, such as *Pfiesteria piscicida* that may be harmful to human health (Grubbs, 2001).

The phosphate ion (PO_4^{3-}) is a highly particle-reactive anion and thus the sorption properties of sediment are crucial for P-retention capacity. Generally, aerobic condition is considered to promote P sorption and anoxic condition to favor phosphate release. After the dissolution of particulate phosphate into inorganic form in sediments, PO_4^{3-} ion is easily adsorbed into inorganic particulate form. The storage of surplus phosphorus by microorganisms can, however, compete with the chemical immobilization of phosphorus (Hupfer, 1991). Normal bacteria have low phosphate content in living cells but some bacteria accumulate polyphosphate (poly-P) intra-cellular in very large amounts, up to *ca.* 20% of their dry weight. These bacteria store phosphorus under aerobic conditions and

release intracellular phosphate via enzymatic hydrolysis when conditions turn anaerobic (Deinema, 1985). In organic-rich lake sediments, microorganisms are able to take up and release phosphorus, depending on redox conditions, and sterilization of oxic sediments can reduce the microbial take up of phosphorus (Gächter *et al*, 1988). Both nonbiological and biological binding mechanisms of phosphorus are able to keep the pore water phosphorus concentration low and reduce the release of phosphorus from sediment to water.

Meizoaki *et al*, (1999) reported that phosphate-accumulating bacteria can use nitrate as terminal electron acceptor instead of oxygen. Identification of isolates revealed that the most active denitrifiers were *Ochrobactrum*, *Pseudomonas*, *Corynebacterium*, *Agrobacterium*, *Aquaspirillum*, *Haemophilus*, *Xanthomonas*, *Aeromonas*, and *Shewanella*. The most active phosphate accumulating and denitrifying bacteria were identified as *Agrobacterium tumefaciens*, *Aquaspirillum dispar*, and *Agrobacterium radiobacter*. The results from their study showed that the active phosphate accumulating-bacteria were also the most efficient denitrifying bacteria.

Under aerobic conditions (presence of oxygen), the natural cycles may be more or less in balance until an excess of nitrate (nitrogen) and/or phosphate enters the system. At this time the water plants and algae begin to grow more rapidly than normal. As this happens there is also an excess die off of the plants and algae as sunlight is blocked at lower levels. Bacteria try to decompose the organic waste, consuming the oxygen, and releasing more phosphate which is known as "recycling or internal cycling". Some of the phosphate may be precipitated as iron phosphate and stored in the sediment where it can then be released if anoxic conditions developed (Deinema, 1985).

Under anaerobic conditions (absence of oxygen), with high phosphate and nitrate in the water, all of the oxygen may be used up by bacteria that trying to decompose all of the waste. At this condition, various bacteria continue to carry on decomposition, however, the products are drastically different. The carbon is converted to methane gas instead of carbon dioxide while sulfur is converted to hydrogen sulfide gas. Some of the sulfide may be precipitated as iron sulfide. Under anaerobic condition the iron phosphate precipitated in the sediments may be released from the sediment making the phosphate bioavailability. This is a key component of the growth and decay cycle. The pond, stream, or lake may gradually fill with decaying and partially decomposed plant materials to make a swamp, which is the natural aging process. The problem is that this process has been significantly accelerated (Hupfer, 1991).

2.5 Method for monitoring microbial diversity in environment

The most useful definition of biodiversity is given by the International Union for Conservation of Nature and Natural Resources as: biodiversity encompasses all life forms, ecosystems and ecological processes and acknowledges the hierarchy at genetic, taxon and ecosystem levels. Microbial diversity, on the other hand, includes the diversity of bacteria, protozoa, fungi, unicellular algae and constitutes the most extraordinary reservoir of life in the biosphere, and one which we have only begun to explore (Amann *et al*, 1995).

Diversity is composed of two elements: *richness* and *evenness*, so that the highest diversity occurs in communities with many different species present (richness) in relatively equal abundance (evenness) (Huston, 1994). The richness and evenness of bacterial communities reflect selective pressure that shape diversity within communities.

Measuring these parameters is most useful when assessing treatment effects (*e.g.*, physical disturbances, pollution, nutrient addition, predation, climate change, etc.) on community diversity.

Ward *et al*, (1992) reported that more than 90% of the microorganisms in nature are thought to have escaped traditional cultivation techniques. Use of the 16S rDNA approach to assess prokaryotic biodiversity in different ecosystems has, in part, alleviated the limitations of traditional cultivation, and has facilitated microbiological studies. The application of molecular techniques, which allows identification of microorganisms without prior use of standard cultivation and isolation techniques, has revealed much of the microbial diversity that previously escaped identification (Delong 1992; Massana *et al*, 1997; Dojka *et al*, 1998; Hugenholtz *et al*, 1998a) and has indicated that the actual number of bacterial species exceeds the number of cultivated ones by at least two orders of magnitude (Amann *et al*, 1995). It has been repeatedly demonstrated that only a small fraction of bacteria existing in various environments has been identified, primarily due to the presence of non-culturable organisms. Specifically, in seawater it has been estimated that less than 0.1% of the bacterial population can be cultured (Kogure *et al*, 1980). In comparison, soil has an equally low percentage of 0.3% (Torsvik *et al*, 1990), whereas in activated sludge the percentage is anywhere from 1-15% (Wagner *et al*, 1993).

2.5.1 Culture dependent methods

In culture dependent methods, bacteria are isolated from environmental samples with culture medium. Nucleic acid is then extracted from the bacterial culture. The biggest drawback in exploring bacterial biodiversity is the issue of viable but non-

cultivable organisms. Diversity in bacterial communities is normally determined by phenotypic characterization of isolated strains. A problem is that phenotypic methods can be used only on bacteria which can be isolated and cultured. While many advances have been made in microbiological culture techniques, it is still not possible to grow a majority of bacterial species using the standard laboratory culturing techniques. Conventional characterization of microbial strains therefore has been subjected to debate, as it is dependent on the ability of the strains to grow under specific environmental conditions (Bakonyi *et al.*, 2003).

These types of classic microbiological methods are indirect and produce artificial changes in the microbial community structure. So, most bacteria would be excluded when phenotypic diversity is estimated. The isolated bacteria may account for only a minor proportion of the total bacterial diversity in soil, while knowledge about the dominant part is still unexplored.

2.5.2 Biochemical techniques

Various biochemical methods are used to monitoring microbial communities in environment such as Fatty acid methyl ester analysis (FAME), phospholipids fatty acid (PFLA) and dehydrogenase enzyme assay.

FAME is a chemical analysis of phospholipids involves: extraction of total lipids from the cell material using organic solvents; chromatographic separation from other lipids not associated with cell membranes; de-esterification to release the fatty acids; methlyation of the acids to form the fatty acid methyl esters (FAME); and identification

and quantification of the FAME by gas chromatography (GC). Gas chromatography-mass spectrometry (GC-MS) is also used to confirm FAME identifications. The distribution of FAME derived from the phospholipid fraction can give strong indications of the identity of a bacterial species. For example, a commercially available software package, mainly aimed at the pathology and agriculture industries, makes use of these differences to rapidly and reasonably accurately obtain identities for over 1000 species of bacteria. Some fatty acids are sufficiently limited in their distribution amongst the bacteria that they can be used as stand-alone biomarkers, that is strong indicators of the presence of a particular genus or families of bacteria (Guckert *et al.*, 1985; White *et al.*, 1979).

The amount of FAME per bacterial cell is reasonably constant. Furthermore, phospholipid rapidly breaks down on death of the cell. By measuring the amount of phospholipid-derived FAME (PLFA) in a sample, it is possible to estimate the abundance of viable bacterial cells. This is particularly useful in environmental studies, in which the number of bacteria in, for example, a soil or mineral sample, can be determined without having to count the cells, which is difficult in such heterogenous samples (Vestal and White, 1989; Tunlid and White, 1992).

Microbioassays using bacteria or enzymes are increasingly applied to measure chemical toxicity in the environment. Attractive features of these assays may include low cost, rapid response to toxicants, high sample throughput, modest laboratory equipment and space requirements, low sample volume, portability, and reproducible responses. Enzymatic tests rely on measurement of either enzyme activity or enzyme biosynthesis. Dehydrogenases are the enzymes most used in toxicity testing. Assay of dehydrogenase

activity is conveniently carried out using oxidoreduction dyes such as tetrazolium salts (Ferguson and Krzycki, 1997; González and Moran, 1999). Other enzyme activity tests utilize ATPases, esterases, phosphatases, urease, luciferase, beta-galactosidase, protease, amylase, or beta-glucosidase. Recently, the inhibition of enzyme (beta-galactosidase, tryptophanase, alpha-glucosidase) biosynthesis has been explored as a basis for toxicity testing. Enzyme biosynthesis was found to be generally more sensitive to organic chemicals than enzyme activity. Bacterial toxicity tests are based on bioluminescence, motility, growth, viability, ATP, oxygen uptake, nitrification, or heat production. An important aspect of bacterial tests is the permeability of cells to environmental toxicants, particularly organic chemicals of hydrophobic nature (Finster *et al*, 1990).

2.5.3 Molecular Techniques

In the wake of "the great plate count anomaly" (Staley and Konopka 1985) and the discovery that the majority of environmental microorganisms are effectively "unculturable", a variety of culture-independent methods have been developed and routinely used to analyze microbial community composition. Culture independent methods employ direct extraction of nucleic acids from environmental samples. It often involves the amplification of DNA or cDNA from RNA extracted from environmental samples by PCR and the subsequent analysis of the diversity of the amplified molecules (community fingerprinting) (Ludwig *et al*, 1994). Alternatively, the amplified products may be cloned and sequenced to identify and enumerate bacterial species present in the sample. Also, the direct extraction of nucleic acids from environmental samples accounts for the very large proportion of microorganisms that are not readily cultured in the laboratory, but that may be responsible for the majority of the biodegradation activity of

The ribosomal RNA (rRNA) approach is becoming a widely used method for studying the microbial community structure of natural and man-made environments in a truly cultivation-independent way. Ribosomal RNA genes have particular advantages as a molecular marker in molecular methods. Firstly, all living cells contain ribosomes, which are part of the cells apparatus for translating deoxyribonucleic acid (DNA) into protein. This rRNA is a dominant cellular macromolecule. Most bacterial cells have somewhere between 103 and 105 ribosomes (Rheims *et al*, 1996). This natural amplification results in excellent sensitivities of hybridization assays. Secondly, the cellular RNA content varies depending on the general metabolic activity or growth rate of a given species (Olsen *et al*,1986). Thirdly, rRNA are excellent molecules for discerning evolutionary relationships among bacteria because RNA molecules contain conserved and variable regions which make it possible to find general as well as specific target sites for probes. These regions are used for identification purposes. A practical reason for using rRNA is the public availability of large databases. They have enough sequence information to be used as a phylogenetic marker (Ludwig and Schleifer, 1994).

DNA based techniques have been developed, which allow the identification of single bacterial species in sample material without the cultivation of the organisms (Amann *et al*, 1995; Ludwig *et al*, 1994; Muyzer *et al*, 1993; Ward *et al*, 1990). Most of the experiments which have been carried out in this field so far are based on ribosomal sequences, which are used as phylogenetic markers (Woese, 1987). The ribosomal sequences are presented in all organisms and contained variable and highly conserved regions which allow distinguish between organisms on all phylogenetic levels. In addition, a lot of data exist in the databases (Maidak *et al,* 1999), which can be used to

compare the DNA-sequences of unknown microorganisms and allow a phylogenetic identification.

To identify bacteria in sample material, ribosomal sequences are analyzed by transcribing ribosomal RNA into cDNA, which can then be cloned (Ward *et al*, 1990). Alternatively, extracted DNA can be used as a template to amplify ribosomal gene fragments with primers for universal sequences by PCR. The PCR amplified fragments can be cloned as well. The result of both strategies is a clone library, containing ribosomal sequences as inserts. By sequencing individual inserts and comparing the obtained sequences with sequences present in databases, it is possible to identify the phylogenetic position of the corresponding bacteria without their cultivation (Woese, 1987; Ludwig and Schleifer, 1994).

Genetic fingerprinting techniques provide a pattern or profile of the genetic diversity in a microbial community. Recently, several fingerprinting techniques have been developed and used in microbial ecology studies such as bioremediation. The separation of or detection of small differences in specific DNA sequences can give important information about the community structure and diversity of microbes containing a critical gene. These techniques are important in separating and identifying PCR-amplified products that might have the same size but slightly different nucleotide sequences (Ward *et al*, 1990).

2.6.3.1 POLYMERASE CHAIN REACTION (PCR)

2.5.3.1 DNA AMPLIFICATION FINGERPRINTING (DAF) and RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

DAF and RAPD are amplification-based nucleic acid fingerprinting techniques (concurrent detection of multiple loci without assignment of a genotype) that use an *in vitro* enzymatic reaction to specifically amplify a multiplicity of target sites in one or more nucleic acid molecules (Micheli et al., 1994). The amplification reaction is generally driven by short synthetic oligonucleotides of arbitrary or semi-arbitrary sequence, which produce a collection of amplified products of largely non-allelic nature. DAF uses a single primer (5-10 bp) to amplify genomic DNA at random.

2.5.3.2 RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLPs)

The procedures involve isolation of DNA, digestion of DNA with restriction endonucleases, size fractionation of the resulting DNA fragments by electrophoresis, DNA transfer from electrophoresis gel matrix to membrane, preparation of radiolabelled and chemiluminiscent probes, and hybridization to membrane-bound DNA. RFLP fingerprinting technique is regarded as the most sensitive method for strain identification and several bacterial strains have been widely studied using this technique Kabadjova *et al.* (2002)

2.5.3.3 AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS (AFLPs)

AFLP is a variation of RAPD, able to detect restriction site polymorphisms without prior sequence knowledge using PCR amplification for detection of restriction

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fragment (Blears et al., 1998; Mueller and Wolfenbarger, 1999; Vos et al., 1995; Zabeau and Vos, 1993). Here, the template for a PCR reaction is a restriction enzyme digested genomic DNA. The primers contain the restriction enzyme recognition site as well as additional 'arbitrary' nucleotides that extend beyond the restriction site. The fixed portion gives the primer stability and the random portion allows it to detect many loci. Amplified products are resolved by polyacrylamide gel electrophoresis. AFLP analysis is one of the robust multiple-locus fingerprinting techniques among genetic marker techniques that have been evaluated for genotypic characterization (Koeleman *et al.*, 1997).

2.5.3.4 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is the method if choice for is a gel electrophoresis method that separates genes of the same size that differ in base sequence. The technique employs a gradient of a DNA denaturant, such as a mixture of urea and formamide. When a double-stranded DNA fragment moving through the gel reaches a region containing sufficient denaturant, the strands begin to "melt," at which point migration stops. Differences in melting properties are to a large degree controlled by differences in base sequence. Thus, the different bands observed in a DGGE gel are different forms of a given gene that vary, sometimes only very slightly, in their sequences (Muyzer *et al,* 1993, Lapara *et al,* 2000).

The denaturant concentration at which a DNA duplex melts is influenced by two factors: a) hydrogen bonds between complementary base pairs GC rich regions melts at higher concentration than AT rich regions; and b) attraction between neighboring bases of the same strand ["stacking"]. Thus, a DNA molecule may have several melting

domains with characteristic melting denaturant concentrations determined by the nucleotide sequence (Muyzer *et al,* 1993).

DGGE exploits the fact that identical DNA molecules which differing only one nucleotide, require different melting denaturant concentrations. When separated by electrophoresis through a gradient of increasing denaturant concentrations, the mobility of the molecule is retarded at the concentration at which the DNA strands of low melt domain dissociate. The branched structure of the single stranded of the molecule becomes entangled in the gel matrix and no further movement occurs. Complete strand separation is prevented by the presence of a high melting domain, which is artificially created at one end of the molecule by incorporation of a GC clamp. This is accomplished during PCR amplification using a PCR primer with a 5' tail consisting of 40 GC (Sheffeld *et al,* 1989).

Once DGGE has been preformed, individual bands can be excised and sequenced. Using 16S rRNA genes, for example, DGGE analysis yield a detailed picture of the number of phylotypes (distinct 16S rRNA genes) present in a habitat. By sequencing these bands, the actual species present in the community can be determined by comparison of the sequences with those of known species available from appropriate database. Using other genes, such as metabolic genes, information can be obtained in the same way according to the number of different types of organisms present in the community that contain the specific gene (Ferris and Ward, 1997; Kowalchuk *et al,* 1997; Øvreas *et al,* 1997).

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