CHAPTER 1

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1.1. Nitrification

Nitrification is the biological oxidation of reduced forms of nitrogen, usually ammonium, to nitrate. The group of bacteria of the family Nitrobacteriaceae (Buchanan, 1917), collectively known as nitrifiers, which undertake the nitrification reactions, include two discrete microbial partners tied faithfully to a life of biochemical harmony, namely ammonia oxidizers (nitritifiers) and nitrite oxidizers (nitratifiers). Each partner critically depends on the other. The two steps in nitrification can be summarized as follows:

\[
\begin{align*}
\text{NH}_4^+ + 1.5 \text{O}_2 & \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + 2\text{H}^+ \quad G = -65 \text{ kCal mol}^{-1} \text{N} \\
\text{NO}_2^- + 0.5 \text{O}_2 & \rightarrow \text{NO}_3^- \quad G = -18 \text{ kCal mol}^{-1} \text{N}
\end{align*}
\]

The overall nitrification reaction is as follows:

\[
\text{NH}_4^+ + 2\text{O}_2 \rightarrow \text{NO}_3^- + 2\text{H}^+ + \text{H}_2\text{O}
\]

Schlöesing and Muntz (1877) were the first to demonstrate the biological nature of nitrification by preventing nitrification in soil percolation columns by the addition of chloroform.

In 1890, Frankland and Frankland published a paper describing the isolation of a pure culture of an ammonia oxidizing, nitrite producing bacteria, in inorganic media by a process of terminal dilution. Winogradsky (1890) isolated a pure culture of nitrifying bacterium by ‘inverse gelatine method’. Mineral based enrichment media was prepared whose pH was stabilized using sediment
magnesium carbonate, in which ammonia oxidizers grew in zoogloeas, which entrapped magnesium carbonate and form recognizable clots in the sediment. Some clots were washed with sterile water and placed on gelatin plates. Those containing heterotrophic contaminants produced colonies on the plates and autotrophic nitrifiers did not which produced tiny or no colonies in gelatin. He described the application of Von Kuhne silica gel medium (Von Kuhne, 1890), to nitrification. Winogradsky initiated isolation studies of nitrifiers on a series of soils from all over the world. (Winogradsky, 1892) His daughter Helene also joined hands and described more genera of nitrifying bacteria. Marine and terrestrial nitrifiers have been later described by other workers like Watson (1971 a, 1974). Soriano and Walker (1968), Belser and Schmidt (1978), Mac Donald (1979), Walker and Wickramasinghe (1979), etc. The current taxonomy is based on the classical work of Sergei and Helene Winogradsky from 1890 to 1937 and the more recent investigations by Watson and co workers from 1965 to 1986.

The classification of the nitrifying bacteria is primarily based on morphological characteristics. Recent studies on the phylogeny of the nitrifying bacteria indicate that the family Nitrobacteriaceae include atleast three different groups of organisms which are not closely related to phylogenetically (Woese et al., 1984 a, 1984 b, 1985). The nitrite oxidizing species of the ammonia oxidizing cells are usually Gram negative, ellipsoidal, or short rods and nitrite oxidizers are short wedge or pear shaped cells with flagellum and are Gram negative. The presence of cytochromes result in yellow or red suspensions. Both are obligate chemolithotrophs, with the exception of Nitrobacter which can grow mixotrophically. Additional species of nitrifying bacteria probably exist that have not been either cultured, isolated or described, since relatively few investigators have isolated nitrifying bacteria.
1.1.1 Cell biology of nitrifying bacteria

1.1.1.a Ammonia oxidizing bacteria

All species of these groups are Gram negative organisms, which are obligatory lithotrophic using ammonia as the sole energy source. They grow autotrophically with CO₂ as the main carbon source, but can also grow mixotrophically, assimilating organic compounds. Most species grow optimally at 25-30°C, at pH 7.5-8 and ammonia concentration of 2-10 mM. The generation time varies from 8 hours to several days.

The cells of the species of *Nitrosomonas* are ellipsoidal or rod shaped with rounded or pointed ends. Most stains are motile with polar flagella and intracytoplasmic memembranes are arranged as flat ended vescicles in the peripheral regions of the cytoplasm. All strains of marine and brackish water genus *Nitrooccus* were members of α subdivision of the purple bacteria, the ammonia oxidizing species of *Nitrosomonas europaea, Nitrosococcus mobilis, Nitrosospira briensis, Nitrosolobus multiformis* and *Nitrosovibrio tenius* were in β subdivision of purple bacteria and *Nitrosococcus oceanus* in the γ subdivision (Woese et al., 1985). It has been suggested that the genetic make up of their sub sets do not show evidence of a common genetic structure between ammonia oxidizers and nitrite oxidizers and each might have evolved from a distinctly different ancestral linkage.

The marine species are obligately halophilic. The marine strains have an additional cell wall layer outside the typical Gram negative cell envelope (Watson and Remsen 1969), strains of some species causes carboxysomes (Wullenweber et al., 1977) while strains of other species do not possess polyhedral inclusion bodies.
Genus *Nitrosococcus* includes at least three species *N. nitrosus*, *N. oceanus* and *N. mobilis*. *N. nitrosus* cells were spherical with a diameter of 1.5-1.7 \( \mu \text{m} \) and non motile while *N. oceanus* were spherical to ellipsoidal, with diameter of 1.8 to 2.2 \( \mu \text{m} \) and occur singly or in pairs with cysts observed in mixed cultures (Watson, 1965), and can be distinguished by centrally located stack of parallel, flattened membrane vesicles Watson and Remsen (1969), observed two additional cell wall layers outside the Gram negative cell envelope. Cells were generally motile possessing 1-20 polar flagella. *N. mobilis* cells were variable in size (1.2-1.9 \( \mu \text{m} \) in diameter) when grown in diluted (2:3) natural seawater, occur singly, in pairs or as short chains. Intracytoplasmic membranes are present with an additional cell wall layer and cells are motile with a tuft of 1-22 flagella. All strains isolated so far are from brackish water and are obligately halophilic.

The genus *Nitrosospira* includes at least five species whose cells are tightly wound spirals with 3-20 turns and when motile, possess peritrichous flagella. Intracytoplasmic membranes are not observed and are distributed in soils and fresh water and not in marine habitats.

Genus *Nitrosolobus* has two species isolated from soil and from sewage. *N. multiformis* are pleomorphic motile with 1-20 peritrichous flagella, lobate in appearance, divided by constriction. Cells are partially compartmentalized by invaginations of the plasma membrane and other segments of cell envelope with 1-4 central compartments surrounded by 5-20 peripheral compartments. The second species isolated from sewage are smaller, 1-1.5 \( \mu \text{m} \) wide and 1-2.5 \( \mu \text{m} \) long.

Cells of *Nitrosovibrio* are slender, curved rods 0.3-0.4 \( \mu \text{m} \) by 1.1-3.0 \( \mu \text{m} \), motile by 1-4 subpolar to lateral flagella and lack an extensive intracytoplasmic membrane system. All strains were isolated from soil and a strain from an acidic
tea soil with pH of 4-4.5 was isolated by Walker and Wickramasinghe (1979). A second species has also been detected by DNA-DNA hybridization techniques.

1.1.1.b Nitrite oxidizing bacteria

These Gram negative organisms grow lithoautotrophically with nitrite as energy source and CO$_2$ as the main carbon source. Species of the genus *Nitrobacter* are able to grow heterotrophically on simple carbon compounds like pyruvate, acetate and glycerol. Most species grow optimally at 25-30°C, at pH 7.5-8 and at nitrite concentrations from 2-30 mM. Generation time varies from 10 hours to several days.

Genus *Nitrobacter* consist of two species, *N. winogradskyi* and *N. hamburgensis*. *N. winogradskyi* are pleomorphic with a size of 0.6-0.8 x 1.2-2 μm. and motile with a single polar or lateral flagellum. The outer cell membrane is asymmetric the inner layer being more electron dense than the outer layer and peptidoglycan layer is not detectable. Intracytoplasmic membranes are arranged as a polar cap composed of 4-6 layers of paired membranes. The cytoplasmic side is stacked with 7-9 nm particles, which are reduced in number in resting cells and absent in heterotrophically grown cells. Cytoplasmic inclusions are carboxysomes, poly-β-hydroxybutyrate, glycogen and polyphosphates. *N. hamburgensis* are similar in shape, size and ultrastructure to *N. winogradskyi* and contain three different plasmids 117, 191 and 281 kb (Kraft and Bock, 1984). A third species with biphasic mixotrophic growth where first nitrite is oxidized, then, after a lag phase, organic substrates are metabolized is also there in the genus *Nitrobacter*.

Genus *Nitrobacter* consists of a single species, *N. mobilis* isolated from marine environment, which are 1.5-1.8 μm. in diameter and are generally motile with one or two flagella.
Clumps of hundred or more cells embedded in slime matrix have been observed. Intracytoplasmic membranes arranged as tubular invaginations of cytoplasmic membranes distributed randomly throughout the cytoplasm, poly-β-hydroxybutyrate granules and electron dense bodies believed to be glycogen storage products are observed.

Genus *Nitrospira* has one species isolated from marine environment, *N. gracilis* whose cells are long, slender rods of 0.3-0.4 \( \times \) 1.7-6.0 \( \mu \)m. which in older cultures has spherical form of 1.35-1.45 \( \mu \)m in diameter. Cells lack extensive intracytoplasmic membrane systems and when stained with glycogen specific stains, darkly stained bodies of 30-40 nm in diameter are seen.

In the genus *Nitrospira*, only species described, *N. marina* was isolated from marine environment is obligately halophilic and grows best mixotrophically on nitrite and pyruvate or glycerol. They are curved rods, which occur as tightly to loosely wound spirals with up to 20 turns when grown lithoautotrophically and with one turn when grown mixotrophically. Cell width is 0.3-0.4 \( \mu \)m. and amplitude of spirals is 0.8-1.0 \( \mu \)m. Cyst formation is frequently observed in enrichment cultures. Another strain has been cultured from soil sample from Namibia.

1.1.1.c Growth of nitrifiers

Growth of nitrifying bacteria is slow even under optimal conditions. Lithoautotrophic growth is maximum at 28-30\(^{\circ}\)C, at pH 7.6-7.8 and at a specific partial pressure of oxygen. Generation time vary from 8 hour for *Nitrosomonas* and 10 hour for *Nitrobacter* to 60 hour for *Nitrosospira*. The oxidation products, nitrite and nitrate are inhibitory for ammonia oxidizers and nitrite oxidizers respectively, the concentration of inhibition being different in different species. In lithoautotrophic cells of *Nitrobacter*, only 2-11% of the energy generated by
nitrite oxidation is used for the cell growth. Due to the low cell concentrations, estimation of biomass by absorbance technique is not normally possible.

Cell number increases in the presence of organic material and cultural filtrates of heterotrophic bacteria stimulate nitrite oxidation and increase cell yield (Steinmuller and Bock, 1976), and nitrification is often faster than in the pure cultures. Mixotrophic growth of different species of *Nitrobacter* exhibits different patterns. *N. hamburgenensis* uses nitrite and organic material simultaneously and the cell yield is high. Growth of *N. winogradskyi* is similar but less efficient while *Nitrobacter* sp shows diphasic growth, where nitrite and then organic materials are oxidised. *Nitrobacter* is also capable of anaerobic growth, reducing nitrate to nitrite with pyruvate, acetate or glycerol as electron donors. Visible, blue and long wavelength UV light are lethal to nitrifying organisms. (Muller-Neugluck and Engel, 1961; Schon and Engel, 1962). *Nitrobacter* is more sensitive than *Nitrosomonas* (Boch, 1965; Olson, 1981). Photoinhibition is due to photo oxidation of cytochrome C (Bock, 1965).

### 1.1.2 Biochemistry of nitrification

Ammonia oxidizing bacteria are obligate chemolithotrophs, oxidizing ammonia to nitrite, fixing carbon dioxide to fulfil their carbon and energy needs. The first step, the oxidation of ammonia to hydroxylamine is catalysed by a hydroxylamine to nitrite is catalysed by the hydroxylamine oxidoreductase. Carbon dioxide is fixed via the Calvin cycle and serves as the main carbon source. 18 mol ATP and 12 mol reduced NAD(P) are required to fix 6 mol carbon dioxide. Although ammonia-oxidizing bacteria can grow mixotrophically on several organic compounds, (Krummel and Harms, 1982), heterotrophic growth has never been observed.
Nitrobacter species, when grown lithoautotrophically oxidize nitrite to nitrate thus producing ATP and NADH for CO₂ fixation via the Calvin cycle. The source of oxygen for nitrite oxidation has been shown to be water (Aleem et al., 1965). The nitrite oxidizing system is membrane bound and the membranes possess a brownish colour, which is typical for all nitrite oxidizers. The enzyme responsible for nitrite oxidation is nitrite oxidoreductase.

1.1.3 Inhibition of nitrification

Hauck (1980), listed 17 pesticides known to inhibit nitrification and 13 chemicals that have been developed commercially as specific inhibitors of nitrification for inhibiting nitrification in soils to minimize losses of nitrate by leaching or denitrification. Nitrpyrin, allylthiourea, diethyldithiocarbamate acetylene and ethyl xanthanate are specific inhibitors of nitrification which inhibit the cytochrome oxidase in turn inhibits the nitrification reaction. There are also non-specific inhibitors of nitrification, which are chemicals that affect the growth and proliferation of bacteria. Nitrifiers are notoriously sensitive to S-containing aminoacids. Carbon disulfide and other reduced sulfur compounds, thiocarbamates and thiosulfates are inhibitory. Ammonium oxidizers are partially affected by dithiocarbamates that release CS₂. Some plant products like karanjin, a flavonoid from the karanjin seed (Sahrawat, 1981), and Margosa seed cake (Mishra et al., 1975), also inhibit nitrification.

1.1.4 Heterotrophic nitrification

In addition to autotrophic nitrification many heterotrophic microorganisms can convert both organic and inorganic reduced nitrogen compounds to nitrate. But chemoautotrophic process is the dominant one. Heterotrophic nitrifiers include fungi (Odu and Adeoye, 1970), heterotrophic bacteria (Nelson, 1929; Cutler and Mukerji, 1931) and actinomycetes (Remacle, 1974). Among fungi, Aspergillus flavus shows significant nitrification. Numerous fungi isolated from
acid coniferous forest soils have the ability to nitrify like a species of *Penicillium* isolated from Germany (Remacle, 1977). Nitrification products appear to accumulate in heterotrophic nitrification when active growth ceases (Schmidt, 1960) and the energy gain by the process is very low. Certain of the heterotrophic nitrification products are known to be toxins and mutagens. A key role of heterotrophic nitrification lie in the production of compounds such as hydroxamic factors (Verstraete, 1975) and have been implicated in the uptake of iron by microorganisms.

### 1.1.5 Adhesion and biofilm formation of nitrifying bacteria

The adhesive properties of nitrifying bacteria have traditionally found application in biological filters. Ardakani *et al.* (1974) has observed that nitrifying bacteria are located to a large extent at the surface of soil and suspended particles. Underhill and Prosser (1987), have shown that ion exchange resins or glass surface enhanced the specific growth of *Nitrosomonas europaea* and *Nitrobacter* sp.cells and attributed this to attachment of bacteria on surfaces. Diab and Shilo (1988), found enhancement in nitrifying activity as a result of attachment *Nitrosomonas* and *Nitrobacter* cells incubated under aerobic conditions in the absence of ammonia or nitrite was considerably longer than that of free cells. Physiological processes upon attachment changes based on nutrient concentration and/or nutrient availability at the attachment site, modification of cell membrane associated process and formation of a polymeric matrix, which may affect the interactions between cells and protect against access of toxic substances, phages and even predators.

Kholdebarin and Oertli (1977), concluded in their study that nitrification is enhanced in the presence of particles which increases the area for physical attachment of bacteria. Nitrifiers are found to embed their cells within zoogloea and most of the bacteriological activity in filters occurs inside living films attached to solid surfaces. Thus they gets attached to the substratum and act upon
the nutrients which come into contact with the cells. Alleman and Preston (1991) has opined that by getting attached, nitrifiers may be conserving their energy.

1.2 Major nitrogen removal processes

Major processes for nitrogen removal are air stripping (ammonia stripping), breakpoint chlorination (superchlorination) selective ion exchange and biological nitrification

1.2.1 Air stripping

Ammonia is a gas, which dissolves in water, and the dissolution controlled by the partial pressure of ammonia in the air adjacent to water. The molecular state of ammonia is highly pH dependent and in alkaline pH, it exists in the unionized form (NH$_3$). In alkaline pH (10-11) when partial pressure is reduced ammonia leaves the liquid phase and enters the gaseous phase. Ammonia can be stripped from wastewater by bringing small drops of water in contact with a large amount of ammonia free air. This process does not affect nitrite, nitrate and organic nitrogen. The disadvantages are its inefficiency in cold weather and formation of calcium carbonate scales in air stripping towers.

1.2.2 Breakpoint Chlorination

This is a highly efficient method of ammonia removal with absolute removal efficiencies. Chlorine is added to the wastewater in an amount to oxidize ammonia to nitrogen gas. After sufficient chlorine is added to oxidize the organic matter and other oxidizable substances, a stepwise reaction of chlorine with NH$_4^+$ takes place as follows:

\[ 3 \text{Cl}_2 + 2 \text{NH}_4^+ \rightarrow 6 \text{HCl} + 2 \text{H}^+ + \text{N}_2 \]
In practice, approximately, 10 mg L\(^{-1}\) of chlorine is required for every 1 mg L\(^{-1}\) of ammonia-nitrogen. The acidity produced has to be neutralized by the addition of caustic soda or lime which adds to the total dissolved solids and increases the operating expense. Also the health effects of breakpoint chlorination or superchlorination products are highly controversial and this process does not remove nitrate.

1.2.3 Selective ion exchange

Ammonia removal of 90-97% is accomplished by this method. Here the wastewater is passed through a column of clinoptilolite, a naturally occurring zeolite, which has a high selectivity for ammonium ion at a pH of 6.5. When all the exchange sites are utilized, regeneration can be done. Nitrite and nitrate cannot be removed by this process.

1.2.4 Biological nitrification

Removal of ammonia in biological nitrification is by transformation of ammonia to nitrate via nitrite undertaken by bacterial cells. It can be carried out in conjunction with secondary or tertiary treatment using either suspended growth reactors (activated sludge) or attached growth reactors (trickling filters etc)

1.2.4.a Activated sludge process

In activated sludge process, wastewater is brought into contact with a mixed microbial population in the form of a flocculant suspension with sufficient aeration. Suspended and colloidal matters are removed rapidly by adsorption and agglomeration onto the microbial flocs. This and the nutrients are broken down slowly by microbial metabolism and oxidized to simpler products such as carbon dioxide. Endogenous respiration also plays a part. Then the sludge is removed in clarifier or settling tanks. A part of sludge is reused as microbial inoculum and
the rest is disposed off. This is the most widely used process for treatment of organic and industrial wastewater and a number of modification on the basic process has been done to make it move versatile.

1.2.4.b Trickling filter

The wastewater is allowed to trickle or percolate through a stationary bed of stones, gravels etc., which serve as an attachment surface for microbes. The water comes through a nozzle, the sprinkle rotates and the water is sprinkled all over the bed. The surface in contact with the nutrient rich wastewater containing microbes will develop a biologically active slime layer. The dissolved oxygen is transferred to the microbial film and the oxygen and nutrients from the water diffuse into the microbes in slime. Trickling filters are stable, simple and fairly fool proof in design.

1.3 Application of nitrification in hatcheries

1.3.1 Ammonia and nitrite formation in hatcheries

Nitrogen, as ammonia enters hatchery seawater through diffusion from the atmosphere and subsurface air bubbles, excretion by prawns, oxidation process of heterotrophic bacteria and lysis of bacterial cells. Nitrite occurs due to ammonia oxidation by ammonia oxidizers.

The balance between unionized and ionized ammonia is highly pH dependent. As pH increases by one unit, the amount of toxic unionized ammonia increases about 10 times. A decrease in dissolved oxygen increases the toxicity of un-ionized ammonia.
1.3.2 Factors affecting nitrification

1.3.2.a Oxygen tension

Every milligram of nitrogen passed through their full nitrification pathway from ammonia to nitrite requires approximately 4.5 mg of dissolved oxygen (Alleman and Preston, 1991), to scavenge electrons drawn from their nitrogenous substrates. If dissolved oxygen drops below a few mg per litre, nitrifier metabolism will markedly slow down.

1.3.2.b pH and alkalinity

Nitrifying bacteria prefer an alkaline pH range between 7 and 8. Alkalinity levels adequate to stop pH from dropping below the preferred alkaline range is required for nitrification to proceed smoothly.

1.3.2.c Temperature

Nitrifiers prefer moderate to warm temperatures, ranging from 28-38°C. The growth constants of nitrifying bacteria were affected greatly by temperature (Sharma and Ahlert, 1977)

1.3.2.d Salinity

Nitrifiers have a sizeable range of tolerable osmotic pressures, ranging from fresh to saline, depending on the particular genus form. Many nitrifiers are able to rapidly switch from one salt level to another with little impact on their activity.
1.3.2.e Light

Nitrifiers are sensitive to visible, blue and long wavelength U.V. light. Photoinhibition has been demonstrated to be due to photooxidation of cytochrome C (Bock, 1965).

1.3.3 Ammonia and nitrite toxicity and management practices in hatcheries

In general, the concentration of ammonia (as total $\text{NH}_4^+$) should not exceed 0.1 ppm. Sublethal toxicity results in reduction in growth rate. Exposure to ammonia causes gill hyperplasia, which is a precursor to bacterial gill diseases, and in extreme cases, causes death. Concentration of nitrite should not be greater than 1 ppm in prawn rearing ponds. Nitrite toxicity causes brown blood disease, growth retardation and death.

More and more hatchery operators are now-a-days preferring closed system hatchery, management to avoid the risks of disease outbreaks and predator invasions, so the management of toxic metabolites like ammonia is a major problem faced by aquaculturists. Daily specific excretion of ammonia by post-larvae is about five folds higher than excretion by adults. In hatcheries, there should be a proper technology for removal of ammonia, which could other wise lead to a catastrophe.

The usual practice in hatcheries to remove ammonia and nitrite is by using bacteriological filters. In a bacteriological filter, solid particulate media is held in a container over which water circulates. The nitrifying bacteria attached to the media acts upon ammonia or nitrite and oxidizes them to $\text{NO}_3^-$. But the main drawback with filters is the long conditioning period required for establishing good nitrifying potential or spiking the water with nutrients. Inoculates of nitrifying bacteria for addition to newly established filter beds can be obtained from pure laboratory cultures, commercial suppliers, in a freeze-dried state or
from conditioned filter beds of similar temperature and salinity. Nitrifying bacteria are difficult to be cultured. Also, laboratory cultures require time to adapt to the hatchery conditions. With freeze dried products, the results have proved inconsistent (Spotte, 1992). The easiest way now available is to add a handful of old gravel from a conditioned filter bed. But this works only when again, the chances of transferring disease causing organism along with the sand is there. When a biological filter is attached to a hatchery system it becomes difficult to subject it to disinfection programmes as the nitrifiers will be totally washed out and it would take yet another couple of months for the conditioning of the filter. More over the conventional biological filters entrap larvae and feed particles and make the whole process non-viable. Therefore the need for a viable user friendly and commercially viable technology for the rapid and continuous removal of ammonia and nitrite from penaeid and non-penaeid hatchery system has been felt for very long time and the present research programme was conceptualized and executed that end. The work was oriented towards developing bioreactors for nitrifying the larval rearing water continuous by, during larval rearing itself and also for nitrifying freshly collected sea water and spent water as that the water can be reused and there by system can be made a closed and recirculating one.

As part of maintenance of biological filter detritus has to be removed from the filter bed every two weeks after stirring the media, usually gravel. Some of the filter bacteria may be removed along with excess detritus; then the population density decreases and sometimes a detectable lag in nutrient conversion may occur.

Hence, this work is an attempt in this direction to cater to the needs of aquaculture industry for treatment and remediation of ammonia and nitrite in penaeid and non-penaeid hatcheries, by developing nitrifying bacteria allochthonous to the particular environment under consideration, and immobilizing them on an appropriately designed support materials configured as reactors.