2. LITERATURE REVIEW

Water is an essential substance in the daily life of each and every one of us and life on this planet would not be possible without this precious liquid. Water is also constantly being cycled between atmosphere, oceans, continental water bodies and land due to its ability to change very easily from solid to liquid to gas at the earth's temperature conditions. Most of the water on earth is saline (97.5 %) and is found in the oceans, whilst the remaining fraction corresponds to fresh water (2.5%) which is not easily accessible. In fact, 68.9 percent of all fresh water is found in the solid form in ice caps and glaciers, 29.9 percent is fresh groundwater and only 0.26 percent is available in lakes, reservoirs and river systems (Shiklomanov, 1998).

The development of human societies has been historically supported by fresh water which explains why most of the largest human settlements are located nearby surface water bodies. However, the current rapid world population growth is driving water resources to the limit, in order to cope with human water needs and uses (domestic, industrial and agriculture). The world's population has increased from 2.5 billion to over 6 billion in the past 50 years and furthermore since mid-2007 more than 50 percent of global population has been located in urban areas (≈3% of the earth's land surface) (UN-Water, 2006) − this is making water supply and wastewater management a real challenge. Moreover, the impact of extreme climates − floods and droughts − is also contributing to fresh water being an increasingly scarce resource.

In fact, although there are regions where water resources are sufficient to meet current needs, surface water bodies are becoming increasingly polluted and unfortunately there are many examples where the self-purification capacity of rivers and lakes has become overwhelmed. The most frequent sources of water pollution are wastewater discharges from domestic and industrial activities (point sources) and agricultural and urban runoff (non-point sources). Originally the main concerns about water quality were related to the presence of disease-producing organisms and oxygen depletion in water bodies, which were linked directly to untreated domestic wastewater discharges. However, even though actions have been taken to minimise the impact of biodegradable organic matter and faecal pathogens from domestic wastewaters on surface water bodies, it is still a problem in less developed countries where more than 65 percent of cities discharge untreated wastewater (UNHSP, 2001).

On the other hand, a new pool of pollutants from intensive agriculture, industrial activities and modernised life-style habits has emerged in both industrialised and developing countries. Nutrients (nitrogen and phosphorus), pesticides, pharmaceuticals and toxic compounds are now the target of pollution-control policies, regulation laws and control technologies. In fact, nutrient control has become one of the biggest challenges facing water resources in many parts of the world (Shiklomanov, 1997) and undoubtedly it is on top of the environmental agenda of industrialised countries.

This chapter presents the environmental significance of nitrogen compounds and principles regarding feasible pathways involved on nitrogen transformation in natural environments and removal mechanisms in domestic wastewater treatment systems. Moreover, there is an introduction to tracer experiments with stable nitrogen isotopes which were carried out as part of the research approach undertaken.

2.1 NITROGEN

Nitrogen is an extremely versatile element due to its electronic formula ($1s^2\ 2s^2\ 2p^3$), which allows it to have six different valence numbers (0, –3, +3, +5, +4 and +2) and hence nitrogen can form both inorganic and organic compounds (Faure and Mensing, 2005). Although nitrogen appears as the largest single component of the Earth's atmosphere (78.1% by volume, 75.5% by weight), it is not a common element in the Earth's crust (Table 2.1). In the air, nitrogen forms a diatomic molecule (N_2) with a strong triple bond between the nitrogen atoms which makes it relatively inert.

Table 2.1 Nitrogen concentrations in the environment

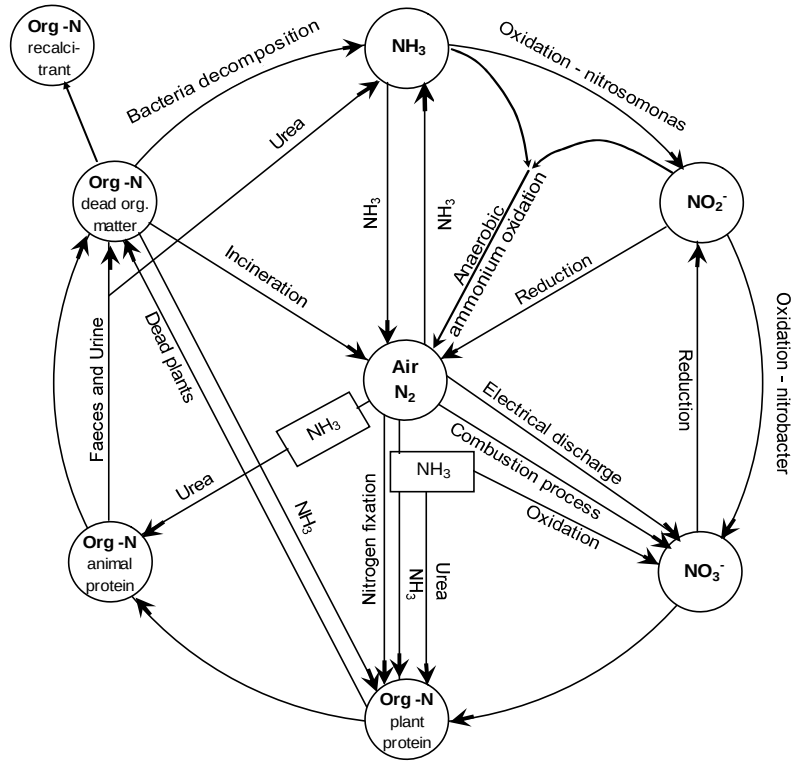
Location	Concentration
Earth's crust	25 ppm
Sea Water:	
surface	0.1 ppb
deep ocean	0.5 ppm
Fresh waters:	
unpolluted	< 0.5 ppm
agricultural land	1 – 100 ppm
Atmosphere	78 %
Human body	2.5 %

Source: Cox, 1995.

Even though nitrogen is the fourth most abundant element in living organisms, after carbon, oxygen and hydrogen, the inert nature of molecular nitrogen means that biologically available nitrogen is often in short supply in natural ecosystems, thus limiting plant growth and biomass accumulation. However, the combination of inertness and a low boiling point (−198ºC) makes N_2 an ideal coolant for some industrial applications such as steel making and food freezing; liquid N_2 is obtained for industrial applications from air by liquefaction and fractional distillation. Liquid nitrogen production is an intermediate step for synthetic ammonia and nitric acid production which are certainly the most important nitrogen compounds in industry. Their uses include the production of explosives, rocket fuels, plastics and others petrochemicals, but some 80 percent of all industrial production of nitrogen compounds are fertilizers (Cox, 1995).

2.1.1 The Nitrogen Cycle

The movement of nitrogen between the atmosphere, biosphere and geosphere in different forms is described by the nitrogen cycle (Figure 2.1) which represents one of the most important nutrient cycles found in terrestrial and aquatic ecosystems.



Adapted from: Sawyer *et al* (1994).

Figure 2.1 Redox cycle for nitrogen

The atmosphere serves as a reservoir of nitrogen and almost all of the nitrogen found in any terrestrial ecosystem originally comes from it. Naturally nitrogen is constantly fixed from the atmosphere either by the action of electrical discharge (lightning) or by biological processes. Nitrogen fixation occurs chemically, to a small extent, when molecular nitrogen (N_2) is oxidized to dinitrogen pentoxide (N_2O_5) during electrical storms in the atmosphere. This nitrogen oxide reacts with water and produces nitric acid (HNO_3) which is carried to soil and surface water bodies in the rain where it is finally fixed as nitrate (NO_3^-).

On the other hand, nitrogen is also fixed biochemically as ammonia (NH_3) by specialized prokaryotic bacteria called diazotrophs; although ammonia is the first product of biological nitrogen fixation, it is nearly always assimilated as rapidly as it is formed (Postgate, 1998). A variety of free-living prokaryotes fixes nitrogen either under aerobic conditions (e.g., *Cyanobacteria, Azotobacter, Azomonas, Azopirillum, Derxia, Klebsiella* and *Beijerinckia*) or in anaerobic environments (e.g., *Desulfovibrio, Clostridium,* purple sulphur bacteria, purple non-sulphur bacteria and green sulphur bacteria) (Brock *et al.*, 1994). There is also a group of bacteria working in symbiosis with plants (e.g., *Rhizobium* and *Bradyrhizobium*) that fixes nitrogen only when present in nodules or on roots of specific leguminous plants. It is estimated that biological fixation globally adds approximately 240 Mt of nitrogen to ecosystems every year (Mulder, 2003).

Once nitrogen has been fixed within the soil, it could be transformed into organic nitrogen as plant protein. However, taking into consideration that most of the plants can assimilate nitrogen only in the form of ammonia or nitrate (NO_3^-) which occur in soil in only limited concentrations, it is not surprising that nitrogen is often the most limiting nutrient for plant growth. Animals and humans receive the nitrogen they need for metabolism, growth and reproduction by the consumption of living or dead organic matter containing molecules composed partially of nitrogen (plant and animal protein); nitrogen is required in large amounts as an essential component of proteins, nucleic acids and other cellular constituents. In contrast, animals and humans release waste products which contain nitrogen compounds resulting from the metabolic breakdown of proteins (e.g., urea in urine) and appreciable amounts of unassimilated protein (e.g., organic nitrogen in faeces).

In most ecosystems nitrogen is primarily stored in living organic matter, but at the end of the life cycle it becomes dead organic matter, which is the starting point for the next step within the nitrogen cycle known as ammonification. Nitrogen from dead organic matter is converted in large measure to ammonium nitrogen by the action of heterotrophic

bacteria, under either anaerobic or aerobic conditions, although some dead organic nitrogen can remain as non-biodegradable organic matter (recalcitrant organic matter) and it becomes part of the detritus in water or sediments, or the humus in soils (Sawyer *et al.*, 1994). Ammonium nitrogen can be partially absorbed onto the surfaces of clay particles in the soil, whilst it remains soluble in water.

Ammonium nitrogen can be biochemically altered by a specific type of autotrophic bacteria (*Nitrosomonas* spp and *Nitrospira* spp) into nitrite (NO_2^-). Further modification by another type of bacteria (*Nitrobacter* spp) converts the nitrite to nitrate. Both of these processes involve chemical oxidation under aerobic conditions and they are known as nitrogen nitrification. Nitrate is very soluble and it is easily lost from the soil system by leaching to groundwater and surface water bodies. Under anaerobic (or least anoxic) conditions nitrate can be returned to the atmosphere as molecular nitrogen. This process is called denitrification and it is carried out in soils and waters by many species of anaerobic and facultative heterotrophic proteobacteria, including those in the genera *Achromobacter, Alcaligenes, Micrococcus, Pseudomonas* and *Thiobacillus*. The process of denitrification involves the reduction of nitrate to nitrite and then to molecular nitrogen and nitrous oxide (N_2O) gas (Brock *et al*., 1994). Recently, a short cut in the denitrification process was discovered (Mulder *et al*., 1995): N_2 can be also produced by combining ammonium nitrogen and nitrite directly into molecular nitrogen under anaerobic conditions (van de Graaf *et al.*, 1997).

2.1.2 Current Environmental Significance of Nitrogen

The activities of humans have severely altered the nitrogen cycle and some of the major processes involved in this alteration include: forest burning, fossil fuel combustion, industrial nitrogen fixation and world population growth. The natural loss of nutrients from the soil-water system is affected by forest burning and rainforest reduction; deforestation has decreased the plant nitrogen uptake rate on a global scale and forest burning releases a variety of solid forms of nitrogen through combustion.

Fossil fuels (oil, coal and natural gas) are the most important source of energy all over the world and its combustion is responsible for most of the nitrogen oxides released into the atmosphere, although nearly 25 percent of N_2O and NO emissions come from sub-optimally managed waste treatment plants (Robertson and Kuenen, 1992). Nitrogen oxides contribute to ground-level ozone pollution (photochemical smog) which may cause serious respiratory problems, especially for young children, the elderly and even

healthy adults that are active outdoors. Ozone pollution may also decrease the ability of plants to convert sunlight to energy and hence it may reduce plant growth.

Nitrogen oxides are transformed in the atmosphere into unhealthy nitrate aerosol particulates which can affect respiratory function, damage lung tissue and lead to premature death; moreover, nitrogen oxides are now held responsible by some for contributing significantly towards the greenhouse effect, the depletion of the stratosphere ozone layer and the formation of acid rain (Robertson and Kuenen, 1992). Acid rain contributes to acidifying nitrate deposition which changes water and soil chemistry as it can lead to fish kills, alter sensitive plant communities (e.g., alpine plants) and reduce tree growth. Nitrate deposition results in excess nitrogen in ecosystems which can also cause changes in vegetation and loss of biodiversity (Seinfeld and Pandis, 1998). Although the emissions of nitrogen oxides (NOx) are concentrated in specific areas around the world (in industrialized countries mainly), they produce environmental problems globally: according to UNECE and EMEP (2005), the USA and the EU delivered 78 percent of world NOx emissions in 2003 (6.0 and 3.0 Mt NOx as N, respectively).

The urgent need for food production has generated an intensive agricultural activity which requires huge amounts of nitrogen fertilizers obtained via industrial fixation. Industrial nitrogen fixation is carried out by the Haber–Bosch process and represents 37 percent of the natural nitrogen fixation (Gijzen and Mulder, 2001). The world consumption of nitrogen fertilizers increased ninefold from 10 Mt N in 1960 to 91 Mt N in 2006, but even more alarming is the fact that in the same period of time it increased 22 times (from 2.3 to 63.7 Mt N/year) in developing countries (IFA, 2007) (Figure 2.2).

Nevertheless, once that enormous amount of reactive forms of nitrogen (ammonium and nitrate) is released into soils, only a very low fraction (10–15%) is finally incorporated into plant tissue; nitrogen is lost from soils through erosion, denitrification and leaching (Gijzen, 2001). Nitrate leaching is an agricultural concern because excess leaching leads to potential water and groundwater pollution which may cause a dramatic regional imbalance in the nitrogen cycle affecting water bodies' uses and ecosystems, mainly in countries with intensive agricultural and factory farming. In the near future, it is expected that nitrogen fertilizer consumption will rise even faster not only to cover the world's growing food needs but also the emergent bio-fuel production based on crops such as corn, wheat, rice, soybeans, sugar cane and oil palm trees. In the USA for instance, 70 percent of the whole corn production in 2006 (14 million tons) was used to produce 4,855 million gallons of ethanol (Brown, 2006; RFA, 2008).

On the other hand, nitrogen excretion by human beings in urine and faeces contributes increasing nitrogen discharge in domestic wastewater, to an estimated human discharge of 4.75 kg N per capita per year (Mulder, 2003). If that figure is used to estimate the total human nitrogen excretion based on world population [3] (see Figure 2.2), the current nitrogen fertilizer consumption by developed countries would correspond to the total nitrogen excreted by human beings. Therefore, domestic wastewater is one of the primary sources of nitrogen in watersheds with low agricultural activity and it may become a major environmental problem due to many of the existing domestic wastewater treatment plants not being equipped to control nutrients − only 5% of the total volume of wastewater receives tertiary treatment on global scale (Gijzen and Mulder, 2001).

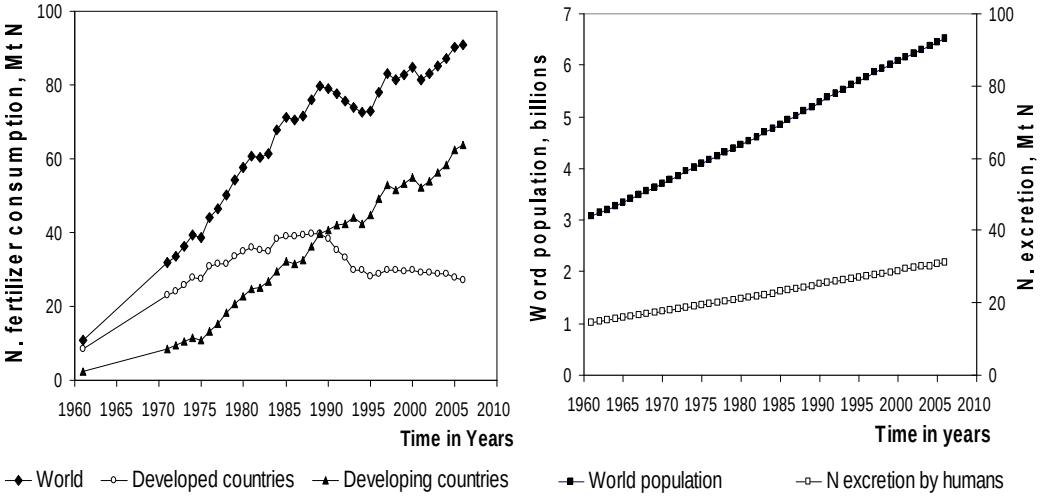


Figure 2.2 Nitrogen fertilizer consumption and nitrogen excretion by humans

Considering that the population growth is not homogeneous and has a tendency to rise faster in urban centres than in rural areas, the discharge of domestic wastewater (untreated and secondary effluents) is responsible for a substantial contribution of ammonium nitrogen. The discharge of nitrogen compounds like ammonium is a well known cause of kill fish and its subsequent oxidation to nitrites and nitrates may contribute to reduce dissolved oxygen levels and increase levels of eutrophication in receiving water bodies. The presence of nitrites and nitrates in drinking water supplies is undesirable (>1.0 mg NO_2^--N/L and >10 mg NO_3^--N/L; WHO, 2006) as it may become a public health risk as nitrate is the causative agent of methaemoglobinemia, a rare but serious illness that has been proven fatal in infants.

[3] *Source:* U.S. Census Bureau (2008)

Eutrophication is a condition in aquatic ecosystems where high nutrient concentrations stimulate excessive plant growth (e.g., phytoplankton, periphyton and macrophytes), creating conditions that interfere with surface water uses (e.g., transportation, fishing, recreational, water supply, etc.) and the health and diversity of indigenous fish, plant and animal populations. Although eutrophication is a natural process in lakes, some estuaries and slow-moving streams, human activities described above can greatly accelerate eutrophication by increasing the rate at which nutrients and organic substances enter into aquatic ecosystems from their surrounding watersheds.

When the primary productivity of a water body is over-stimulated, aquatic plants (floating and rooted) and algal blooms affect aquatic ecosystems due to blocking sunlight and causing underwater grasses to die; these grasses are important in aquatic ecosystems as they are a habitat for aquatic creatures providing food and shelter. Algae turn the water column cloudy and may impart taste and odour to water supplies; on the other hand, large floating algal masses are concentrated by wind action and interfere with water-based recreational activities. Also, when underwater grasses and algal masses die, decomposition releases foul odours and may deplete dissolved oxygen to such a low level as to cause fish kills.

Generally speaking, it is believed that eutrophication is more influenced by phosphorus than nitrogen enrichment. This is based on the fact that even when nitrogen has been removed, cyanobacteria are able to fix atmospheric nitrogen. Also experiments with large water reservoirs have shown no eutrophication when the phosphorus concentration is reduced to 8−10 μg P/L even when the nitrogen concentration amounts to 4−5 mg N/L (Kortstee *et al.*, 1994). However, sustainable wastewater management should include both nitrogen and phosphorus control strategies.

Nitrogen removal from industrial and domestic wastewaters contributes to mitigate regional environmental and public health effects related to reactive forms of nitrogen which enter into the nitrogen cycle via industrial nitrogen fixation. Nevertheless, although advanced treatment for wastewaters by nitrification-denitrification processes has been achieved in most of the industrialised countries, effects such as eutrophication of surface waters could not be controlled because of the imbalance between natural and anthropogenic nitrogen fluxes. The average retention time of fixed nitrogen in the biosphere is over 7,000 years before it is eventually recycled to atmospheric molecular nitrogen (Brock *et al.*, 1994). Therefore, a strategy to achieve a sustainable nitrogen management should include not only a reduction of anthropogenic nitrogen fixation, but

also processing and reuse in food production of reactive forms of nitrogen available in the biosphere (e.g., safe wastewater reuse in agriculture and aquaculture).

2.1.3 Nitrogen Transformations in Water and Wastewater

The biochemical cycle of nitrogen is one of the most complexes in nature because of the different oxidation states of nitrogen and its associated compounds as mentioned previously. Nitrogen transformations in natural aquatic environments have been well studied and their principles are used to design wastewater treatment facilities; such designs take advantage of the natural, physical, chemical and biological processes which are addressed to achieve specific aims by wastewater treatment engineers. Nitrogen compounds found in surface waters and wastewaters are transformed by a wide and diverse population of aerobic, anoxic and anaerobic micro-organisms, phytoplankton, zooplankton, periphyton and macrophytes. The most important transformations of nitrogen occurring in natural waters and wastewaters are briefly shortly described below.

Biological nitrogen fixation

Molecular nitrogen can be biologically reduced to ammonia (NH_3) via the enzyme nitrogenase, which consists of two separated proteins called dinitrogenase and dinitrogenase reductase, and finally fixed as ammonium (NH_4^+) in aquatic ecosystems. Biological nitrogen fixation is a strictly anaerobic process and it is inhibited by oxygen since dinitrogenase reductase is rapidly and irreversibly inactivated by oxygen (Postgate, 1998). Aerobic bacteria protect nitrogenase from oxygen either by removal of oxygen by respiration, the production of oxygen-retarding slime layers or by compartmentalising nitrogenase in a special type of compartment (Brock *et al.*, 1994). The overall reaction for biological nitrogen fixation is presented as follows:

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2 \qquad \textbf{(2.1)}$$

Cyanobacteria appear responsible for most planktonic nitrogen fixation in aquatic ecosystems; however, nitrogen fixation rates are high only when these micro-organisms make up a major percentage of the planktonic biomass. In fact, planktonic nitrogen fixation is higher in eutrophic lakes (0.2−9.2 g N/m^2 year) than in oligotrophic and mesotrophic lakes (<<0.1g N/m^2 year) (Howarth *et al.*, 1988). It is due to ammonia not being in excess under eutrophic conditions and, as soon as ammonia is produced by catalysed nitrogenase reaction, it is rapidly incorporated in organic form and used in biosynthesis. Additionally, nitrogenase synthesis is quickly repressed when ammonia is

in excess and nitrogen fixation may not represent an important nitrogen input in aquatic environments (Brock *et al.*, 1994).

Ammonification

Ammonia is the final product of this process called *ammonification* which comprises autolysis, decay and putrefaction of biological material by the action of heterotrophic bacteria either under aerobic or anaerobic conditions. The enzyme glutamate dehydrogenase is typical of the enzymes responsible for this process and, as shown in the following reaction, it forms a keto acid from an amino acid:

$$NAD + HOOC.(CH_2)_2.CHNH_2.COOH + H_2O \rightarrow HOOC.(CH_2)_2.CO.COOH + NH_3 + NADH \quad \textbf{(2.2)}$$

glutamate **2-oxoglutarate**

Nicotinamide adenine dinucleotide (NAD) is a coenzyme found in all living cells and it is involved in redox reactions. NAD is therefore found in two forms in cells: NAD is the reduced form (oxidizing agent) and NADH is the oxidized form (reducing agent) (Postgate, 1998).

In addition, ammonia can be released from amino acids by a hydrolytic reaction catalysed by deaminases and from amides by deamidases; a well known deamidase is urease which hydrolyses the urea in urine into ammonia. Ammonia exists at pH neutral as the ammonium ion which is highly soluble in water, but under alkaline conditions ammonia is volatile and it may be released to the atmosphere. On a global basis, ammonia losses constitute only 15 percent of the nitrogen released to the atmosphere, mainly from highly alkaline soils and areas of dense animal population (e.g., cattle farms and intensive piggeries); the majority of the nitrogen is released to the atmosphere in the form of N_2 and N_2O by denitrification processes (Brock *et al.*, 1994).

Ammonia volatilisation

Ammonia in an aqueous solution acts as a weak base in a dynamic equilibrium between two species: ammonium (ionic form) and ammonia (un-ionized gaseous form); this equilibrium (equation 2.3 in Table 2.2) depends mainly on pH but also on the dissociation constant (K_b) which is temperature-dependent (equations 2.4 and 2.5; Emerson *et al.*, 1975). For many practical purposes the percentage of un-ionized ammonia can be expressed as a function of pH and K_b values at a specific water temperature (equation 2.6). At appropriate temperature and pH values, ammonia can be released from water by volatilisation to the atmosphere; the ammonia volatilisation rate depends on the mass transfer coefficient (K_l) which is enhanced by the mixing effect of wind action and high

temperatures. K_l values in ponds can be calculated from an equation reported by Ferrara and Avci (1982) (equation 2.7), which was based on Stratton's work (1968, 1969). Equations related to ammonia volatilisation in aquatic environments are summarised in Table 2.2.

Table 2.2 Fundamental equations to calculate ammonia volatilisation rates in ponds

Meaning	**Equation***	
Ammonia–ammonium equilibrium reaction	$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^-$	**(2.3)**
Ammonia dissociation – ionization equilibrium constant (K_b)	$K_b = \frac{[NH_4^+] \times [OH^-]}{[NH_3]}$	**(2.4)**
K_b as a function of water temperature	$pK_b = 0.09018 + \frac{2729.92}{273.2 + T}$	**(2.5)**
Percentage of free ammonia in an aqueous solution	$\%NH_3 = \frac{100}{1 + 10^{(pK_b - pH)}}$	**(2.6)**
Ammonia mass transfer coefficient in ponds	$K_l = \frac{0.0566}{d} \exp[0.13(T - 20)]$	**(2.7)**

* $[NH_4^+]$, $[OH^-]$ and $[NH_3]$ are molar concentrations; $pK_b = -\text{Log}_{10}K_b$; T is water temperature (°C); d is the depth of the water column in the pond (m).

Nitrification

Nitrification is an aerobic biochemical process by which ammonia is oxidized to nitrate. There is a range of autotrophic and heterotrophic bacteria widely distributed in soil and water capable of nitrification (Castignetti and Hollocher, 1984; Halling-Sørensen and Jørgensen, 1993). Autotrophic nitrifiers require an inorganic source of carbon (e.g., CO_2 or HCO_3^-), whilst heterotrophic nitrifiers nitrify using either an organic or inorganic pathway. In wastewater treatment systems, autotrophs constitute only a small percentage of the microbial community, but they are responsible for the bulk of nitrification (Blackall and Burell, 1999). Nitrification process comprises the conversion of ammonia to nitrite (ammonia oxidation) and nitrite to nitrate (nitrite oxidation).

Autotrophic ammonia oxidation takes place in two steps: (a) ammonia oxidation to hydroxylamine (NH_2OH) and (b) hydroxylamine oxidation to nitrite. The former process is catalyzed by the membrane-bound enzyme ammonia mono-oxygenase and requires molecular oxygen (O_2) and the input of electrons (equations 2.8). The required electrons are provided by the second step, the conversion of NH_2OH into NO_2^-, which is catalyzed by the enzyme hydroxylamine oxidoreductase (HAO) located in the periplasm (equation

2.9) (Kool *et al*, 2007). The two-step ammonia oxidation is attributed to ammonia-oxidizing bacteria which belong to the gamma (*Nitrosococcus oceanus and N. halophilus*) and beta (*Nitrosomonas, Nitrosospira, Nitrosovibrio, Nitrosolobus* and *Nitrosococcus mobilis*) subclasses of the class Proteobacteria (Juretschko *et al*., 1998). However, it has been recently found that ammonia can be oxidized to nitrite in one single step by ammonia-oxidizing archaea (Treusch *et al*., 2005; Könneke *et al*, 2005).

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad \textbf{(2.8)}$$

$$NH_2OH + H_2O \leftrightarrow NO_2^- + 5H^+ + 4e^- \quad \textbf{(2.9)}$$

$$NO_2^- + H_2O \leftrightarrow NO_3^- + 2H^+ + 2e^- \quad \textbf{(2.10)}$$

On the other hand, nitrite is oxidized to nitrate and the oxygen in the nitrate is derived from water and not from molecular oxygen − see equation 2.10 (Aleem *et al*., 1965; Kumar *et al*., 1983). Autotrophic nitrite oxidation is catalysed by the membrane-bound nitrite oxidoreductase located in the periplasm of organisms of the genus *Nitrobacter, Nitrospina, Nitrococcus, and Nitrospira* (Juretschko *et al*., 1998). Considering that molecular oxygen is the preferred electron acceptor under aerobic conditions, the overall expressions for ammonia oxidation (equation 2.11) and nitrite oxidation (equation 2.12) show that the theoretical oxygen demand for each step is 3.43 g O_2/ g NH_4^+-N and 1.14 g O_2/ g NO_2^-, respectively (4.57 g O_2/ g NH_4^+-N for full nitrification).

However, a more comprehensive expression (equation 2.13) including the nitrification synthesis-respiration reaction was reported by the United States Environmental Protection Agency – USEPA (Hall and Murphy, 1980; Ahn, 2006); it includes the formation of nitrifying bacterial cells ($C_5H_7O_2N$) and considers that yields for ammonia oxidation by *Nitrosomonas* and nitrite oxidation by *Nitrobacter* are 0.15 mg cells/mg NH_4^+-N and 0.02 mg cells/mg NO_2^--N, respectively. Based onto that, the nitrification process demands 4.18 g of O_2 and 7.07 g of alkalinity as $CaCO_3$ per 1 g of ammonium-N oxidized.

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad \textbf{(2.11)}$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad \textbf{(2.12)}$$

$$NH_4^+ + 1.83O_2 + 1.98HCO_3^- \rightarrow 0.02C_5H_7O_2N + 0.98NO_3^- + 1.04H_2O + 1.88H_2CO_3 \quad \textbf{(2.13)}$$

The nitrification process has been shown to be strongly dependent on temperature, pH and dissolved oxygen (DO) levels. Although nitrifiers can be found in extreme habitats at high temperatures and in Antarctic soils, nitrification occurs over a range of approximately 4–45ºC , with about 35ºC optimum for *Nitrosomonas* and 35−42ºC optimum to *Nitrobacter*. Also nitrification has an optimum pH of between 7.0 and 8.0

and it can be limited at DO levels less than 0.5 mg/L (USEPA, 1993; Blackall and Burell, 1999; Schmidt *et al.,* 2002).

Heterotrophic nitrification comprises the oxidation of ammonium, hydroxylamine or nitrogen organic compounds (e.g., oximes) mainly into nitrite as their final product, with smaller amounts of nitrate and nitrogenous organic compounds and these compounds may in turn then support autotrophic nitrite oxidizers and / or heterotrophic denitrifiers. It is carried out by a wide range of micro-organisms including bacteria, fungi and algae. Heterotrophic nitrification rates are lower in comparison with autotrophic nitrification; therefore, heterotrophic nitrification was thought to occur preferentially under conditions which are not favourable for autotrophic nitrification (e.g., acidic environments) (Blackall and Burell, 1999; Schmidt *et al.*, 2003).

Conventional denitrification

Nitrite and nitrate are alternative electron acceptors in energy generation under anoxic conditions and they are converted into reduced gaseous forms of nitrogen (e.g., N_2O, NO and N_2) which can easily be lost to the atmosphere (Brock *et al.*, 1994). Molecular nitrogen is the main end product of denitrification while nitric oxide (NO) and nitrous oxide (N_2O) are occurring as intermediates in low concentrations; however, when DO levels are too high these gases are also released as end products (Schmidt *et al.,* 2003). Although denitrification is restricted to prokaryotes, a wide diversity of such organisms can carry out that process. Only few obligate denitrifiers have been identified (e.g., *Propionibacterium*) whilst all other denitrifiers are facultative, meaning that they will preferentially use oxygen for respiration but they will switch to anaerobic respiration once the available oxygen has been depleted (Ritter and Eastburn, 1988).

During the denitrification process, nitrate is reduced to nitrite by the enzyme nitrate reductase (NaR), which is located in the cytoplasmic membrane with its active site inside the cytoplasm. Nitrite is reduced to nitric oxide by both the cytochrome cd_1 and the Cu nitrite reductase (cd_1NiR and CuNiR); these are soluble enzymes located in the periplasmic space. Nitric oxide is transformed into nitrous oxide by nitric oxide reductase (NOR) which is bound to the cytoplasmic membrane, but has its active site in the periplasm. Nitrous oxide reductase (NOS) catalyses the last step in denitrification, the reduction of nitrous oxide to molecular nitrogen (Butler and Richardson, 2005; Kool *et al.*, 2007).

The heterotrophic denitrification process requires an organic source of carbon and generates alkalinity; combined dissimilation-synthesis equations for denitrification of

nitrite and nitrate using methanol as an electron donor are given in equations 2.14 and 2.15, respectively (Ahn, 2006).

$$NO_2^- + 0.53CH_3OH + 0.67H_2CO_3 \rightarrow 0.04C_5H_7O_2N + 0.48N_2 + 1.23H_2O + HCO_3^- \quad \textbf{(2.14)}$$

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow 0.06C_5H_7O_2N + 0.47N_2 + 1.68H_2O + HCO_3^- \quad \textbf{(2.15)}$$

Hence, for every gram of nitrite-N denitrified, 1.21 g of methanol (1.8 g BOD) is consumed, 0.32 g of biomass ($C_5H_7O_2N$) is produced as well as 3.57 g of alkalinity ($CaCO_3$); corresponding figures for the reduction of 1 g of nitrate-N are 2.47 g of methanol (3.67 g BOD) consumed and 0.45 g of biomass and 3.57 g of alkalinity produced.

Denitrification by nitrifiers

Considering that *Nitrosomonas* and *Nitrobacter* are capable of growth even in environments where no oxygen can be detected (Laanbroek *et al*., 1994), *Nitrosomonas europaea* has shown be able to use nitrite as electron acceptor and produce N_2 under oxygen stressed or microaerophilic conditions (Poth, 1986) or NO and N_2O under strict anaerobic conditions (Ritchie and Nicholson, 1972; Aveliovich and Vonshak, 1992). On the other hand, *Nitrobacter* performs the anaerobic reduction of nitrate and produces ammonia, nitrite, N_2O and NO_2 as final products (Freitag *et al*, 1987; Bock *et al*., 1988). In fact, denitrification activity from *Nitrosomomas*-like bacteria can be controlled and stimulated by adding trace amounts of nitrogen oxides into wastewaters (NOx process), in order to achieve nitrogen removal targets by simultaneous nitrification-denitrification under fully oxic conditions; the NOx process is carried out largely by *Nitrosomonas eutropha* which has shown to be one of the most capable denitrifiers among nitrifiers (Ahn, 2006; Schmidt *et al.*, 2003).

Nitrification–denitrification by methanotrophs

Methanotrophs may also contribute to ammonium oxidation in nature which can lead to production of nitrous oxide (N_2O) and molecular nitrogen (N_2). Amaral *et al.* (1995) studied the impact of methanotrophs on denitrification and isolated at least three denitrifiers associated with methanotrophic growth and activity. It was found that methanotrophic activity supported denitrification by reducing the oxygen-tension and by supplying organic compounds to the denitrifiers. In addition, the co-metabolism of ammonium by methane-oxidising bacteria as methane mono-oxygenase is very similar to ammonium mono-oxygenase; therefore, methanotrophs can also catalyze the oxidation of ammonium (nitrification) and produce nitric and nitrous oxides (Murrell and Radajewski, 2000). The nitrate formed is rapidly denitrified as ammonia oxidation is the limiting step

in this nitrogen removal mechanism (Pel *et al*., 1997). Although methanotrophs are not themselves known to carry out denitrification, there is good evidence that denitrifying bacteria can be associated with methanotrophs and can use simple carbon compounds released by the methanotrophs as substrates for the denitrification reactions and for growth in natural environments (Knowles, 2005). Such denitrification associated with methanotrophs can release nitrogen gases (e.g., NO, N_2O, N_2) and so contribute to permanent nitrogen removal from WSP.

Anaerobic ammonium oxidation

Oxidation of ammonium had only been known to proceed under aerobic conditions, before Mulder *et al*. (1995) showed evidence for a new anaerobic ammonium oxidation process, which was named and patented as "anammox", based on the metabolism that was thought to be involved (Mulder, 1992). The anammox process combines ammonium and nitrite directly into molecular nitrogen gas and does not require biodegradable organic matter as electron donor (van de Graff *et al*., 1997). The anaerobic ammonium oxidation process is carried out by a group of planctomycete bacteria (Strous *et al.*, 1999), two of which have been named provisionally as *Candidatus* Brocadia anammoxidans (Schmid *et al.*, 2000) and *Candidatus* Kuenenia stuttgartiensis (Jetten *et al.*, 2001). Particularly, *Candidatus* B. anammoxidans has an extremely low growth rate, with a doubling time of approximately 2 weeks, and also its growth is reversibly inhibited even by oxygen concentrations below 0.5 percent air saturation (Strous *et al.*, 1997).

An analysis of mass balances showed that *Candidatus* B. anammoxidans uses carbon dioxide as its carbon source to produce biomass ($CH_2O_{0.5}N_{0.15}$) and that nitrite not only functions as an electron acceptor for ammonium oxidation, but also as an electron donor for the reduction of carbon dioxide according to equation 2.16 (Kuenen, 2008).

$$NH_4^+ + 1.32NO_2^- + 0.07HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.07CH_2O_{0.5}N_{0.15} \quad \textbf{(2.16)}$$

Recently, experiments that used an oxygen-limited reactor, which was fed with ammonium only, revealed that at low oxygen concentrations (up to 2 mg per litre in the bulk phase) coexisting small clumps of *Candidatus* B. anammoxidans and separate clumps of a *Nitrosomonas eutropha* strain dominated the reactor biomass; a few nitrite oxidizers were also found. In this particular case, the removed ammonia was converted to molecular nitrogen gas by a cooperative work between nitrite-formation and anaerobic ammonium oxidation bacterial groups. This oxygen-limited combined process for the removal of ammonium has been named and patented as the CANON process (completely autotrophic nitrogen removal over nitrite) (Sliekers *et al.*, 2002; Third *et al*., 2001).

Biological nitrogen uptake

Microorganisms utilize nitrogen to synthesise those amino acids that they cannot obtain from the environment. Basically, there are two aspects of amino acids biosynthesis: the synthesis of the carbon skeleton of each amino acid and the manner by which the amino group is incorporated. There are several enzymes which are able to catalyze the addition of ammonia to a carbon skeleton and one of them is glutamate dehydrogenase. In amino acid biosynthesis, glutamate dehydrogenase plays an inverse role to that in the ammonification process described previously; therefore, once ammonia has been incorporated into the amino group of glutamate, the amino group can be transferred to other carbon skeletons by enzymes called transaminases, leading to the formation of a new amino acid and regeneration of α–ketoglutaric acid from glutamate (Brock *et al.*, 1994).

Next to carbon, nitrogen is quantitatively the most important element in microbial nutrition; for instance, the average nitrogen requirement for many green algae is approximately 5−10 percent of dry weight (Becker, 1994). Algae and bacteria are able to utilize ammonium, nitrate or other organic source of nitrogen such as urea, although ammonia (NH_3) and ammonium (NH_4^+) are invariably the preferred form of nitrogen supply when it is available. Nitrite can also be taken up but its toxicity at higher concentrations makes it less convenient (Becker, 1994). Algae and bacteria will preferentially take up ammonium nitrogen until the concentration of that nitrogen source becomes low (<1mM for bacteria and <1μM for algae – Magasanik, 1988; Flynn, 1991) when they commence the uptake of nitrate, which should be firstly reduced to ammonium. In fact, nitrate will be reduced to ammonium only when ammonium is limiting as the presence of ammonium represses the action of the nitrate reductase system (Bagchi *et al.*, 1985).

Recent studies have demonstrated that ammonia can diffuse freely through cell membranes due to specific ammonia transporters or Amt proteins, which act as channels enabling the controlled uptake of ammonia rather than the active transport of ammonium ions (Khademi *et al*, 2004). Each Amt protein monomer has an ammonium-binding site at the extracellular pore entrance that might transform ammonium ions into ammonia for passage through the channel; therefore, the solution equilibrium between ammonia and ammonium (or pH-dependant effect) has little to do with the ammonia uptake mechanism because both ammonia and ammonium can enter the extracellular pore to become NH_3 (Khademi *et al.*, 2004; Yildiz *et al.*, 2007).

2.1.4 Nitrogen Removal Processes in Domestic Wastewater Treatment

Important economic resources and technical efforts are combined everyday to reduce the environmental impact that domestic wastewater can cause on receiving water bodies. Wastewater pollution control is most commonly achieved in industrialised countries through centralized treatment systems, which have been mainly based on technologies with high construction costs and high energy consumption and which require qualified personnel for good operation and maintenance. Generally speaking, centralized wastewater treatment works in industrialised countries aim to achieve secondary treatment goals mostly by aerobic technologies, such as trickling filters, rotating biological contactors, aerated lagoons and especially activated sludge. However, as a result of increasingly stringent discharge consents for large wastewater treatment plants (>10,000 p.e.), especially for nutrients (tertiary treatment goals), the water industry is facing new challenges.

Consequently, and considering that domestic and industrial wastewaters contain significant quantities of reactive forms of phosphorus and nitrogen, large wastewater treatment plants (WWTP) have opted for including either biological or chemical nutrient removal processes such as modified activated sludge or chemical precipitation processes for nutrient removal. The removal of phosphorus can be achieved both chemically and biologically, whilst nitrogen is almost exclusively removed by biological processes. Biological removal of nitrogen is most commonly carried out by nitrification-denitrification in two-stage activated sludge systems. In these systems, ammonium is firstly oxidized to nitrate (nitrification) under aerobic conditions by an autotrophic process and in the second stage (denitrification) nitrate removal by conversion to molecular nitrogen gas is accomplished biologically under anoxic conditions (Blackall and Burrell, 1999).

Modified forms of the conventional nitrification-denitrification process have emerged in order to improve the sustainability of nitrogen removal systems. They have demonstrated that it is possible to increase nitrogen loads, keeping the same nitrogen removal performance, but reducing energy consumption and sludge production. Nevertheless, this certainly does not mean inexpensive; indeed, even improved and innovative nitrogen removal processes are still highly energy demanding and therefore costly. Modified processes include among others: (a) partial nitrification and denitrification, best known as the SHARON process (a single reactor system for high-rate ammonium removal over nitrite; Logemann *et al.*, 1998); (b) partial nitrification followed by anaerobic ammonium oxidation in separated reactors (a combination of SHARON and ANAMMOX); (c)

anaerobic ammonium oxidation (ANAMMOX); (d) partial nitrification and anaerobic ammonium oxidation in a single aerated reactor (the CANON process); (e) simultaneous nitrification-denitrification under fully oxic conditions (the NOx process). Unfortunately for small communities (<2,000 p.e.) and low-income countries, those processes are even more expensive and demand highly skilled personnel for their operation and maintenance.

Nitrogen removal can occur in constructed natural systems used either to provide secondary and advanced treatment or to improve secondary effluent quality as a separate unit (secondary effluent polishing). Constructed natural systems for wastewater treatment are designed to take advantage of natural physical, chemical and biological processes which occur when water, soil, atmosphere and biomass interact. That interaction is complex and very versatile at the same time; in fact, it can include simultaneously processes such as sedimentation, filtration, gas transfer, adsorption, ion exchange, chemical precipitation, chemical oxidation and reduction, biological degradation and conversion, photosynthesis, photochemical oxidation and biological uptake among others (Metcalf and Eddy, 1991).

Table 2.3 Typical values of operational parameters from nitrogen removal systems

System	**N load, kg N/ha d**	**Energy consumption, kWh/kg N**	**Sludge/biomass production, kg dw/kg N***	**N_{total} removal efficiency, %**
Conventional nitrification-denitrification	200-700	2.3	1.0-1.2	>75
SHARON process	200-700	1.7	0.8-0.9	>75
ANAMMOX process	>200-700	0.9	<0.1	>75
WSP	15-30	0.1-1.0	10-15	23-78
Duckweed pond	3-4	<0.1	20-26	74-77
Constructed wetland	3-36	<0.1	- -	30-70

*dw = dry weight. Adapted from Mulder (2003).

In a constructed natural system, nitrogen removal does not occur as result of sequential processes in separate reactors at accelerated rates, as does in electromechanical systems, but it occurs at innate rates and involves different processes which occur concurrently in a single reactor. For instance, waste stabilisation ponds (WSP), duckweed ponds and constructed wetlands, all of them classified as constructed natural systems, have demonstrated be able to remove nitrogen compounds. Also, they are a very attractive option for small and decentralised wastewater treatment systems, because of their advantages related to low capital and operation and maintenance costs (Crites *et al.*,

2006). Specific operational parameters for nitrogen removal systems in domestic wastewater treatment are summarized in Table 2.3.

Natural wastewater treatment plants, such WSP and duckweed ponds, can also make an important contribution in the reduction of industrial nitrogen fixation, by recycling nutrients for agriculture and aquaculture from their final effluents; in activated sludge for instance, ammonium nitrogen is returned as nitrogen gas and nitrogen oxides to the atmosphere. In fact, wastewater treatment plants contribute up to 21 percent of N_2O emissions per year, excluding emissions due to fossil fuel combustion (Mulder, 2003). Evidently, the large land area requirement could be seen as the main disadvantage for natural wastewater treatment systems, but considering energy consumption and nitrogen removal efficiency from Table 2.3, this is probably more a myth rather than a solid argument.

2.1.5 Tracer Experiments with Nitrogen Stable Isotopes

In nature nitrogen exists in both stable and unstable (radioactive) forms. There are two stable nitrogen isotopes (^{14}N and ^{15}N), with the lightest of these present in much greater abundance in atmospheric N_2 gas (^{14}N abundance: 99.6337% vs. ^{15}N abundance: 0.3663%) (Rundel *et al.*, 1989). Among stable isotopes the most useful as biological tracers are the heavy isotopes of carbon (^{13}C) and nitrogen (^{15}N). The isotopic composition of nitrogen in any sample is expressed as delta values in parts per thousand ($\delta^{15}N$, ‰), defined as follows:

$$\delta^{15}N, {}^{0}/_{00} = \left[\frac{\left(^{15}N/^{14}N\right)_{sample} - \left(^{15}N/^{14}N\right)_{std}}{\left(^{15}N/^{14}N\right)_{std}} \right] \times 1000 \qquad \textbf{(2.17)}$$

$\delta^{15}N$ values are not concentrations of the ^{15}N isotope but differences between ^{15}N:^{14}N ratios in the sample ($^{15}N/^{14}N_{sample}$) and atmospheric N_2 gas ($^{15}N/^{14}N_{std}$), which has a known ^{15}N:^{14}N ratio (0.0036765) and acts as a standard.

In comparison with atmospheric N_2 gas ($\delta^{15}N$ = 0‰), the ratio of stable nitrogen isotopes in environmental samples varies due to physical, chemical and biological processes; these changes are called isotopic fractionations and are indicative of the progression of reactions. The isotopes of nitrogen are lightly fractionated during nitrogen fixation but it becomes larger during biochemical oxidation of organic nitrogen to nitrate ($+8 < \delta^{15}N < +22$ ‰); on the other hand, denitrification and ammonia volatilisation cause extensive depletion in ^{15}N content of N_2 and NH_3, respectively. Each stable nitrogen isotope

behaves slightly different under the same environmental conditions and hence, isotopic fractionation is expected due to variations on mass transfer and kinetic coefficients (Gunter and Mensing, 2005).

It is feasible to track nitrogen transformations based on isotopic fractionation since the $\delta^{15}N$ of both nitrogen species and microorganisms involved varies as the reactions proceed. Nevertheless, the $\delta^{15}N$ in the microorganisms involved in nitrogen transformation processes may be affected not only by the $\delta^{15}N$ in the nitrogen source but also by the isotope fractionation of assimilation and advection of microorganisms among other factors. This may make the quantitative estimation of nitrogen transformation rates difficult, particularly in natural environments where influent loads are difficult to measure or control; therefore, the use of the isotopic fractionation method in a well controlled system, such as a wastewater treatment plant, may help to improve the understanding of nitrogen removal mechanisms. Indeed, Kanazawa and Urushigawa (2007) made a study in an oxidation ditch and a conventional activated sludge plants based on the isotopic fractionation method, in order to determine the relationship between the ammonia removal rates and the $\delta^{15}N$ values from biomass.

Another method to track nitrogen transformations is a tracer method, which has been used by ecologists for the simultaneous study of the dynamics of inorganic forms of dissolved nitrogen and their uptake and transformations by stream biota (Ashkenas *et al.*, 2004). Tracer experiments with ^{15}N include the addition of small amounts of ammonium or nitrate salts with high ^{15}N enrichment levels, resulting in a negligible increment of ammonium or nitrate concentrations and avoiding the stimulatory effect of added nutrients (Mulholland *et al.*, 2000). The ^{15}N labelling experiments have been very important in the discovery of novel nitrogen removal pathways in wastewater treatment systems − for instance the anaerobic ammonium oxidation process (van de Graaf *et al.*, 1997).

In the literature there are no reported research works based on ^{15}N stable isotopes in WSP systems, apart from a bench-scale experiment in a greenhouse with agricultural drainage effluent as a wastewater, on a reactor seeded with algae as a control ($1.2 \times 0.6 \times 0.6$ m), in order to determine the fate of ^{15}N labelled ammonium and nitrate under batch conditions for 27 days (Reddy, 1983). Therefore an outdoor ^{15}N tracer study of nitrogen transformation pathways and removal mechanisms in WSP systems under pilot-scale and continuous flow conditions, which is reported herein, is likely to be very beneficial in improving our understanding of nitrogen removal in WSP systems.

2.2 WASTE STABILISATION PONDS

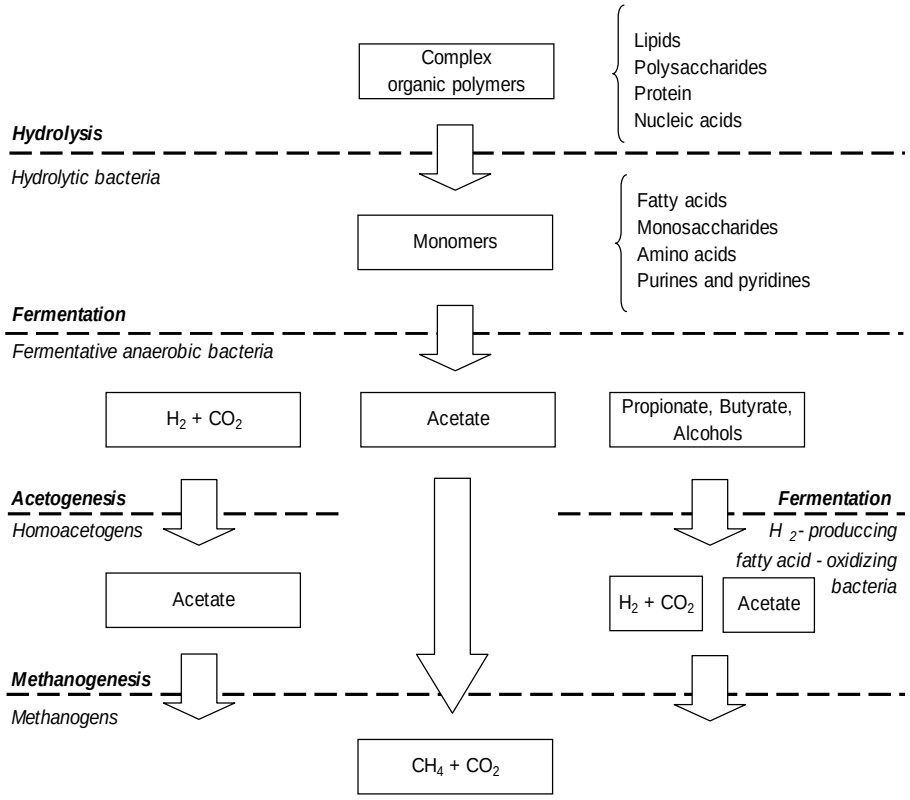
Waste stabilization ponds (WSP) have been used as a natural wastewater treatment process for over 3,000 years. In the USA, the first 'modern' WSP system was constructed at San Antonio, Texas, in 1901. Later in the 1920's, more WSP were designed and constructed to receive and stabilise domestic and industrial wastewaters based on prior results which were very promising. By 1950 the use of ponds had become recognized as an economical wastewater treatment method for small municipalities and industries. In the 1980's approximately 7,000 WSP systems were in use in the USA and today one third of all secondary wastewater treatment facilities (more than 7,300 systems) include a pond system of one type or another (US EPA, 1983, 2002).

Throughout the world large numbers of pond systems are successfully used for domestic and industrial wastewater treatment and more of these systems are being designed and constructed every year as the understanding of pond operation mechanisms has increased. The major reasons for their popularity are simplicity of the process, construction, operation and maintenance; high efficiency in removing BOD, suspended solids and faecal coliforms under a wide range of weather conditions ranging from tropical to arctic; low capital, operation and maintenance costs and energy requirements; and robustness in terms of their resilience to both organic and hydraulic shock loads (US EPA, 1983; Mara, 2004).

WSP are large shallow earthen basins which can be used alone, comprising at any one location one or more series of ponds, or in combination with other wastewater treatment processes as a storage unit, primary and secondary treatment or polishing component. WSP technology is entirely a natural processes in which wastewater is treated by biochemical reactions involving both algae and bacteria. For that reason, the rate of oxidation is slower and as a consequence hydraulic retention times are longer than in conventional electromechanical wastewater processes. WSP can be classified based on their depth and the biochemical reactions that occur inside the pond; according to that, the main types of ponds are: anaerobic, facultative and maturation ponds. In a WSP system, the wastewater is first subjected to preliminary treatment (screening and grit removal), followed by the different pond types which are arranged in a series or several series in parallel. Basically, primary treatment is carried out in anaerobic ponds or in primary facultative ponds, secondary treatment in facultative ponds and tertiary treatment in maturation ponds (Mara, 2004).

2.2.1 Anaerobic Ponds

Anaerobic ponds are heavily loaded with organic matter and operated in series with aerated or facultative ponds. They do not contain dissolved oxygen and their biological activity is typically lower than that in an anaerobic digester. Anaerobic ponds are mainly designed to remove organic matter and that is achieved by the sedimentation of the settleable fraction and its subsequent anaerobic digestion in the resulting sludge layer. Moreover, soluble organic matter is also transformed by anaerobic processes to carbon dioxide (CO_2) and methane (CH_4); about 30 percent of influent BOD is transformed into this *biogas*. Other end products such as hydrogen sulphide (H_2S), volatile fatty acids (VFAs), NH_4^+, simple organic compounds and cell tissues are produced following a complex and syntrophic biochemical process carried out by a consortium of microorganisms (Metcalf and Eddy, 1991; Mara, 2004; Crites *et al.*, 2006).



Adapted from: Brock *et al.* (1994).

Figure 2.3 Anaerobic digestion process

The anaerobic process involves a pool of anaerobic microorganisms working together in four consecutive steps: hydrolysis, acidogenesis (fermentation), acetogenesis and methanogenesis (Figure 2.3). One group of microorganisms is responsible for hydrolyzing organic polymers (proteins, polysaccharides, nucleic acids, etc) and lipids to

basic monomers such as monosaccharides, aminoacids and related compounds. A second group of anaerobic bacteria ferments the breakdown products to simple organic acids such as propionic, lactic, butyric and acetic. Acetic acid can also be produced by a third group of microorganisms (homoacetogen bacteria) from molecular hydrogen (H_2) and CO_2. Finally, a fourth group of microorganisms converts H_2, CO_2 and acetic acid formed by fermentative bacteria to CH_4 and CO_2 (Brock *et al.*, 1994).

The bacterial groups involved are equally sensitive to toxic compounds as those in any anaerobic reactor. Therefore, anaerobic ponds should be devoid of dissolved oxygen throughout their depth and free from inhibitory concentrations of heavy metals and sulphides. pH is an important environmental parameter and it should not drop below 6.2 because methanogenesis is inhibited (Metcalf and Eddy, 1991). Furthermore, at the pH values normally found in well designed anaerobic ponds (around 7.5), the offensive odour caused by escaping H_2S molecules is negligible because most of the sulphide is present as the odourless bisulphide ion (HS^-) (Mara, 2004). Water temperature is also a key variable and it should be above 15 °C which explains why anaerobic ponds are especially suitable for hot climates. Typical design values and operation information for anaerobic ponds are showed in Table 2.4.

Table 2.4 Design and operation values for anaerobic ponds

Item	**Value**		
Temperature, °C	Mean air temperature of the coldest month		
Depth, m	2 – 4		
Hydraulic retention time, d	1		
Desludging	Once every 1-3 years		
pH, units	7.5		
Sulphate concentration in influent, mg/l	<500		
Design values of volumetric organic load (λ_v)	λ_v, g BOD/m^3 d	BOD removal, %	Temperature (T), °C
	100	40	<10
	20T - 100	2T + 20	10 – 20
	10T + 100	2T + 20	20 – 25
	350	70	>25

Source: Mara (2004) and Peña Varón (2002).

Organic matter removal is more efficient in anaerobic ponds and sludge production is less than in primary facultative ponds. However, one disadvantage of anaerobic ponds is that they are only good at removing organic matter and suspended solids, and not other sorts

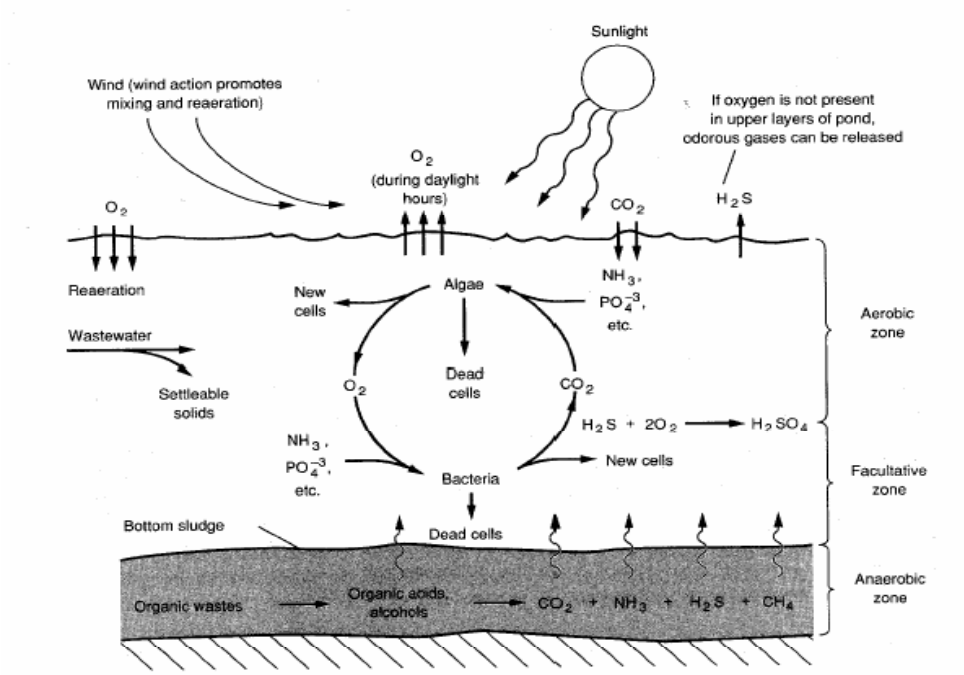
of pollution such as nutrients or pathogens. Nitrogen and phosphorus compounds from sewage are not effectively removed in anaerobic ponds, although they are mainly transformed into ammonium and orthophosphate by hydrolytic bacteria. For this reason, the use of anaerobic ponds in isolation is not sufficient enough and their effluent demands additional treatment to achieve required quality standards (Metcalf and Eddy, 1991; Mara, 2004).

2.2.2 Facultative Ponds

Facultative ponds are designed to achieve specific organic matter removal targets (BOD removal). They can be classified as primary and secondary facultative ponds. Primary facultative ponds (PFP) are fed with wastewater after preliminary treatment and secondary facultative ponds (SFP) receive pre-treated wastewater from primary treatment units; usually in a WSP system, SFP are fed with anaerobic pond effluents. Three zones exist in a facultative pond: aerobic, facultative and anaerobic (Figure 2.4). The aerobic layer is near to the surface and contains dissolved oxygen due to atmospheric re-aeration and algal photosynthetic oxygen production. In this layer, the oxygen is used by bacteria in the aerobic degradation of organic matter and nutrient assimilation; CO_2 is released in this degradation and it is used by the algae to produce oxygen in a harmonious symbiotic relationship.

The facultative zone is an intermediate layer which is partly aerobic near the top and partly anaerobic at the bottom; under these conditions, aerobic and facultative organisms and their biochemical processes are supported. The anaerobic zone is located below the oxypause (the depth at which the dissolved oxygen concentrations reaches zero); it includes sludge deposits and supports anaerobic and anoxic organisms. Anaerobic degradation is carried out as previously discussed. Therefore the sludge layer contributes to organic matter removal through sedimentation of both settleable fraction and dead algae and heterotrophic bacteria and their subsequent anaerobic digestion.

Facultative ponds are mainly designed for organic matter removal on the basis of a low organic surface load to permit the development of an active algal population. Healthy facultative ponds have a dark green colour given by algae populations but occasionally they can turn red or pink due to the presence of purple sulphide-oxidising photosynthetic activity. The concentration of algae in an optimally performing facultative pond depends on organic load and temperature, but is usually in the range 500 to 2,000 μg/l of chlorophyll-*a*; a minimum of 300 μg/l has been suggested for a correctly operating facultative pond (Mara and Pearson, 1986).



Source: Metcalf and Eddy (1991)

Figure 2.4 Schematic representation of a facultative pond

Facultative ponds have shown diurnal variations in effluent quality related to parameters such as dissolved oxygen, pH, total and soluble phosphorous, filtered and unfiltered BOD_5, suspended solids (SS), chlorophyll-*a* and faecal coliforms (Mara, 2004). Thermal inversion can occur in the spring and fall when the surface water layer may have a higher density than lower layers due to temperature fluctuations. This higher density water sinks during these unstable periods, creates turbidity due to sediments from the bottom being re-suspended (sludge feedback), and it may produce objectionable odours which are associated with released gases from the anaerobic layer. Generally speaking, algae present in facultative pond effluent may compromise the pond's reliability in terms of total SS and total BOD removals (US EPA, 1983; 2002).

A number of empirical and rational models exists for the design of facultative ponds. These include first-order plug flow reactor, first-order completely mixed reactor, first-order dispersed flow and surface organic loading (see Table 2.5). All of these provide a reasonable design as long as the basis for the formula is understood, proper parameters are selected and sludge retention characteristics of the system are known. Facultative ponds in operation have reported that an effluent with less than 30 mg/l BOD can usually be achieved (BOD removal between 50-90%), while SS in the effluent may range from less than 30 mg/l to more than 100 mg/l, depending on the algal concentrations and

design of discharge structures (USEPA, 2002); this wide variation reflects the variety in design and a heavy reliance on local climatic conditions.

Table 2.5 Design models for facultative ponds

Model	Characteristics
Marais and Shaw (1961) $C_n = C_o / (1 + k_{(T)}\,\theta)^n$ (2.18) where: C_n = effluent BOD_5 from pond *n*, mg/l C_o = influent BOD_5, mg/l $k_{(T)}$ = reaction rate constant at temperature *T*, d^{-1} θ = hydraulic retention time, d n = number of ponds in series	Model based on first-order kinetics in *n* ideal completely mixed reactors in series with equal retention time. This model assumes light penetration to the pond bottom. The value of $k_{(T)}$ is temperature dependent as follows: $k_{(T)} = k_{(35)}\,(1.085)^{(35\,-\,T)}$ (2.19) where: $k_{(35)}$ = reaction rate constant at 35°C = 1.2 d^{-1} *T* = minimum operating water temperature, °C
Reed *et al.* (1988) $C_e = C_o e^{-k_{(T)}\theta}$ (2.20) where *Ce* = effluent BOD_5, mg/l *Co* = influent BOD_5, mg/l $k_{(T)}$ = reaction rate constant at temperature *T*, d^{-1} θ = hydraulic retention time, d	Model based on first-order kinetics in ideal plug flow reactors. The value of $k_{(T)}$ is temperature dependent as follows: $k_{(T)} = k_{(20)}\,(1.09)^{(T\,-\,20)}$ (2.21) where $k_{(20)}$ = reaction rate constant at 20°C, d^{-1} *T* = minimum operating water temperature, °C $k_{(20)}$ depends on the BOD_5 surface loading rate, but if this is not known, a value of 0.1 d^{-1} may be used.
Thirumurthi (1969), based on Wehner and Wilhelm's equation (1956) $\frac{C_e}{C_o} = \frac{4a\exp(1/2\delta)}{(1+a)^2\exp(a/2\delta) - (1-a)^2\exp(-a/2\delta)}$ (2.22) where *Ce* = effluent BOD_5, mg/l *Co* = influent BOD_5, mg/l $a = (1 + k_{(T)}\theta\delta)^{0.5}$ $k_{(T)}$ = reaction rate constant at temperature *T*, d^{-1} θ = hydraulic retention time, d δ = dispersion number	This is a dispersed flow model based on first-order kinetics. The value of δ is unknown at design stage; it can be determined directly by tracer studies when pond is in operating. The value of *k* is temperature dependent as follows: $k_{(T)} = k_{(20)}\,(1.09)^{(T\,-\,20)}$ (2.23) where $k_{(20)}$ = reaction rate constant at 20°C, d^{-1} *T* = minimum operating water temperature, °C
McGarry and Pescod (1970) $\lambda_s = 60\,(1.099)^{\,T}$ (2.24) where λ_s = max. BOD_5 loading before failure, kg/ha d *T*= temperature, °C	Model based on the maximum surface BOD loading rate that ca be applied to a facultative pond before it fails.
Mara (1987) $\lambda_s = 350\,(1.107 - 0.002T)^{\,T-25}$ (2.25) where λ_s = BOD_5 loading rate, kg/ha d *T* = temperature, °C	This surface BOD loading rate model is based on McGarry – Pescod's model and incorporates a safety factor to give a global design equation for facultative ponds loading.

The quality of the facultative pond effluent is deeply affected by algal productivity inside the ponds which is climate dependent. In cold climates, primary productivity and fermentation reaction rates are significantly reduced during the winter and early spring and effluent quality may be reduced to the equivalent of primary effluent when an ice cover persists on the water surface. As a result, many states in the northern of the USA and Canada have prohibited discharge from facultative ponds during the winter (US EPA, 2002). In Europe, discharges consents required by the EU Urban Waste Water Treatment Directive (UWWTD) (CEC, 1991; 1998) have been relaxed for effluents from pond systems (≤25 mg filtered BOD_5/l and ≤150 mg SS/l), because algal SS and BOD should not be subject to conventional effluent requirements.

Facultative ponds play an important role in achieving quality standards in the final effluent of a WSP system, as they have additionally demonstrated an effective contribution to pathogen removal (faecal viruses, faecal bacteria and helminth eggs). Implicated mechanisms in the die-off of pathogens and the design methods for pathogen removal in facultative ponds are the same as those for maturation ponds and they will be discussed later (section 2.2.3). Nitrogen and phosphorus are also removed by facultative ponds but their removal capability has been given little consideration in system designs. Mechanisms by which nitrogen may be removed from facultative ponds have been studied over the last 20 years or so; unfortunately, there is disagreement in the literature about which factor is, or which factors are, the most important.

2.2.3 Maturation Ponds

Maturation ponds are wastewater treatment units which are mainly designed to reduce the number of pathogenic organisms and provide secondary effluent polishing. The size and number of these ponds working in series are normally determined by the required microbiological quality of the final effluent. In a WSP system, maturation ponds are fed with the effluent from a facultative pond in order to improve its quality not only in terms of excreted pathogen indicators (faecal coliform bacteria and helminth eggs), but also BOD, SS and nutrients (nitrogen and phosphorus) prior to being reused or discharged to a surface water (Mara, 2004).

Maturation ponds are mainly aerobic and show low vertical stratification as they are shallow (1.0−1.5 m depth) and operate with a very low surface BOD loading. Biological processes are similar to, but distinguishable from, those in facultative ponds; oxygen supply (by atmospheric re-aeration and algal photosynthetic oxygen production) and aerobic degradation of organic matter (by heterotrophic bacteria) take place in maturation

ponds, but sludge accumulation, anaerobic digestion and sulphide reduction are minimal. Algal populations in maturation ponds are much more diverse than in facultative ponds and this algal diversity increases from pond to pond along the series; however, algal biomass is lower in maturation ponds. This typical condition of maturation ponds could be partially explained due low concentrations of fatty acids which may reduce the level of heterotrophic nutrition by algae (Silva, 1982; Trousellier *et al.*, 1986).

Maturation ponds in operation have reported effective pathogen indicators removal, a small removal of BOD and SS, a significant contribution to cumulative nitrogen and phosphorus removal in WSP systems, and low maintenance and operation costs (Mara and Pearson, 1986, 1998; Pearson *et al.*, 1987c; Mara *et al.*, 1992). Several factors have been proposed to explain the precise mechanisms controlling the die-off of excreted pathogens but they are in many cases only tentative. For faecal bacteria removal in WSP, these factors have been conveniently grouped into light-independent processes and light-mediated processes (Mara, 2004). Light-independent processes include sedimentation of the faecal bacteria adsorbed on and/or contained within settleable solids and flocs, predation by protozoa and micro-invertebrates, and death due starvation and senescence. Factors related to light-mediated processes which increase faecal bacterial die-off rate are: long retention time, high temperature and pH and high light intensity and dissolved oxygen.

The removal of excreted pathogens has had much greater importance in the design of maturation ponds than the removal of BOD, SS or nutrients. Marais (1974) proposed a model for *E. coli* die-off based on first-order kinetics in an ideal completely mixed reactor. The faecal coliform (FC) removal is express as follows:

$$Ne = \frac{No}{1 + k_{b(T)}\theta} \qquad \textbf{(2.26)}$$

$$k_{b(T)} = 2.61(1.19)^{T-20} \qquad \textbf{(2.27)}$$

where *Ne* and *No* refer to the number of FC in effluent and influent respectively (FC/100ml), $k_{b(T)}$ is the reaction rate constant at temperature *T* (day^{-1}), θ is the hydraulic retention time (days) and *T* is the average water temperature (°C).

At the design stage of a WSP system with ponds in series, the number of maturation ponds and corresponding theoretical hydraulic retention time can be estimated using Marais' method; partial and cumulative FC removal a long the series can be also

calculated. Marais' model has been criticised because time and temperature are the only two factors included in his equations. For instance, Silva (1982) found that in primary facultative ponds the value of k_b depended on surface BOD loading (λ_s) and decreased by increasing loading; and Polprasert *et al.* (1983) found that the value of k_b depended on temperature, surface chemical oxygen demand (COD) loading (λ_{sCOD}) and the concentration of algae in the pond according to the equation:

$$e^{k_b} = 0.716(1.0281)^T (1.0016)^C (0.9994)^{\lambda_{sCOD}} \quad \textbf{(2.28)}$$

where k_b is the reaction rate constant (day^{-1}), T is water temperature (°C), C is the algal concentration (mg/l) and λ_{sCOD} is the surface COD loading (kg COD/ha d). That model was tested in conjunction with a sub-model for the prediction of C and an equation for first-order kinetics removal rate in a dispersed flow reactor based on Wehner and Wilhelm's equation (1956) (Dissayanake, 1980). Results were in good agreement with data of Silva (1982) but the difficulty of predicting the dispersion number restricts its use (Curtis and Mara, 1994). Thirumurthi (1969) found that if the value of the dispersion number is less than 2.0, the second term in the denominator of his model (equation 2.22) shall be negligibly small and as a result, it can be rewritten for FC removal as follows:

$$\frac{Ne}{No} = \left[\frac{4a}{(1+a)^2}\right] \exp\left[\frac{1-a}{2\delta}\right] \quad \textbf{(2.29)}$$

where *Ne* and *No* are the number of FC in the pond effluent and influent, respectively (FC/100ml), $a = (1 + k_{b(T)}\theta\delta)^{0.5}$, θ is the hydraulic retention time (days), δ is the dispersion number and $k_{b(T)}$ is the reaction rate constant (day^{-1}) at temperature T (ºC). von Sperling (1999) has proposed an alternative approach to estimate dispersion number (equation 2.30) and $k_{b(T)}$ (equations 2.31 and 2.32) values for facultative and maturation ponds which are based on pond geometry and FC removal data from 33 ponds in tropical and subtropical Brazil. According to Mara (2004), the von Sperling's method can be recommended in preference to Marais's method.

$$\delta = \left(L/B\right)^{-1} \quad \textbf{(2.30)}$$

$$k_{b(T)} = k_{b(20)}(1.07)^{T-20} \quad \textbf{(2.31)}$$

$$k_{b(20)} = 0.92H^{-0.88}\theta^{-0.33} \quad \textbf{(2.32)}$$

where *L*, *B*, and *H* are pond length, width and depth in m, respectively.

Organic matter removal in maturation ponds is much slower than in facultative ponds as easily biodegradable organic compounds have been previously oxidised by the heterotrophic bacteria in the preceding facultative pond. For that reason, maturation ponds only achieve a small removal of filtered BOD_5 and secondary effluents should be at, or below, discharge consent level for BOD because an increase in the BOD and SS in the final effluent from maturation ponds can occur if there is a high algal content (Mara, 2004). Bradley (1983) and Mara (2004) stated that between 50 and 90 percent of BOD in a maturation pond effluent is due to its algal content. Also, Mara (2004) suggests using a first-order kinetic model in an ideal completely mixed reactor to estimate unfiltered BOD removal with a value of the reaction rate constant (k_1) of ~0.05 d^{-1} for temperatures of 15−25°C; while for primary and secondary facultative ponds, values suggested of k_1 for the same temperature range are 0.24–0.38 d^{-1} and 0.08–0.13 d^{-1}, respectively.

2.2.4 Nitrogen Removal in Facultative and Maturation Ponds

Nitrogen removal occurs in facultative and maturation ponds; unfortunately, all available models for ammonium and/or total nitrogen removal have failed to determine the relative importance of the various pathways through which, and the mechanisms by which, nitrogen is transformed and removed in WSP. The most accepted models for ammonium and nitrogen removal were proposed by Pano and Middlebrooks (1982), Ferrara and Avci (1982) and Reed (1985). Data utilized to develop and validate these models were taken from four WSP systems located at Peterborough, New Hampshire (US EPA, 1977a); Eudora, Kansas (US EPA, 1977b); Corinne, Utah (US EPA, 1977c); and Kilmichael, Mississippi (US EPA, 1977d). These studies included a full year of data collection, including four separated 30-consecutive-day and 24-hour composite sampling periods, one each season (US EPA, 1983).

Pano and Middlebrooks (1982) studied data from WSP systems located at Peterborough (New Hampshire), Eudora (Kansas) and Corinne (Utah). They proposed that ammonia volatilisation is the main removal mechanism in WSP systems for ammonia removal and based on that assumption, a mass balance equation (equation 2.33) for ammonia stripping assuming steady-state in a completely mixed flow reactor was developed. The corresponding expression for temperatures below 20ºC is reported in equation 2.34.

In equations 2.33 and 2.34, *Ce* and *Co* are the total ammonium concentration (NH_4^+ + NH_3) in mg N/l from pond effluent and influent, respectively; *A* is the surface area of the pond (m^2), *Q* is the inlet flow rate (m^3/d), *K* is the ammonia mass transfer coefficient

(m/d), K_w is the ion product for water ($pK_w = -\text{Log } K_w$), K_b is the ammonia dissociation constant ($pK_b = -\text{Log } K_b$) and T is water temperature (°C).

$$\frac{Ce}{Co} = \frac{1}{1 + \frac{A}{Q} K \left[\frac{1}{1 + 10^{pK_w - pK_b - pH}} \right]} \quad \textbf{(2.33)}$$

$$\frac{Ce}{Co} = \frac{1}{\left\{ 1 + \frac{A}{Q} (0.0038 + 0.000134T) \exp[(1.041 + 0.044T)(pH - 6.6)] \right\}} \quad \textbf{(2.34)}$$

Another model to estimate total nitrogen removal from WSP was proposed by Reed (1985) based on data from WSP systems located at Peterborough (New Hampshire), Eudora (Kansas), Corinne (Utah) and Kilmichael (Mississippi). Total nitrogen values from influent and effluent were obtained by adding the figures of total Kjeldhal nitrogen (TKN), NO_2^- and NO_3^- concentrations. Reed's model (equation 2.35) is based on a first-order kinetics removal rate which is a function of temperature, hydraulic retention time and pH and also it assumes that an ideal plug flow is the predominant flow regimen.

$$Ce = Co\ exp\ \{-[0.0064 \times (1.039)^{T-20}]\ [\theta + 60.6 \times (pH - 6.6)]\} \quad \textbf{(2.35)}$$

where Ce and Co are total nitrogen concentrations (mg N/l) in the pond effluent and influent, respectively; T is the water temperature (°C) and θ is the hydraulic retention time (days).

Reed's model is the best equation (significant at the 0.05 level) to describe how total nitrogen concentrations from influent and effluent in WSPs used in his study are related to water temperature, hydraulic retention time and pH. Therefore, it is not possible to determine what mechanism dominates total nitrogen removal, because most of the feasible biochemical mechanisms in WSPs depend on these same variables. However, Reed (1985) concluded that nitrification and denitrification were minimal because pond effluents contained low concentrations of nitrite and nitrate (in agreement with Pano and Middlebrooks, 1982), and that any nitrogen in the pond effluent will tend to be in either the ammonia or the organic form, or both. Moreover, Reed (1985) suggested that volatilisation and benthic deposition, or both, could be pathways for nitrogen removal depending on the environmental conditions.

On the other hand, Ferrara and Avci (1982) applied a completely mixed flow and time-variable model developed by Ferrara and Harleman (1978, 1981) to data from a WSP system located in Corine, Utah. That study paid particular attention to the relative importance of ammonia volatilisation, biological nitrogen uptake and sedimentation of dead biomass as the most important nitrogen removal mechanisms in WSP systems. Ferrara and Avci (1982) concluded that the primary mechanism for total nitrogen removal was sedimentation of organic nitrogen via biological uptake and emphasised that ammonia volatilisation cannot account for very much ammonia removal.

The final form of the ammonia removal model proposed by Pano and Middlebrooks (1982) does not prove that the most important mechanism in ammonia nitrogen removal in WSP is ammonia volatilisation. In fact, their work only shows that ammonia removal has a statistically significant relationship with pH, water temperature and hydraulic loading rate, and that ammonia nitrogen removal increases as the pH, detention time and temperature increase (e.g., summer conditions). Indeed, as an increasing pond water temperature increases biological activity, it can be also expected that a higher ammonia uptake rate by the pond algae can explain a higher ammonia removal rate (Pearson *et al.,* 1988).

Moreover, Pano and Middlebrooks' model does not explain why if ammonia volatilisation is the main mechanism for permanent ammonium removal in WSP, the annual average ammonium removal rates in the WSP under study (52.8% in Peterborough, NH; 96.1% in Eudora, KS; and 99% in Corin, UT; Pano and Middlebrooks, 1982; Ferrara and Avci, 1982) are higher that the corresponding total nitrogen removal figures (43, 82 and 91%, respectively; Reed, 1985; Ferrara and Avci, 1982). This could infer that, although ammonium nitrogen is removed, it may be transformed into other nitrogen specie (e.g., organic nitrogen), which is leaving in the pond effluent and therefore, total nitrogen removal is lower than ammonium removal. Nevertheless, many authors have reported ammonia volatilisation as the dominant nitrogen removal mechanism in WSP (Soares *et al.,* 1996; Rockne and Brezonik, 2006), despite opposite evidence based on direct-on site measurements which have reported insignificant nitrogen removal via ammonia volatilisation (Zimmo *et al.* 2003; Caicedo Bejarano, 2005).

Most of the studies reported previously have not quantified the amount of nitrogen that follow each feasible nitrogen removal route and their conclusions are based on the comparison of inlet and outlet nitrogen concentrations. In other words, WSP systems have been studied as big black boxes. The ^{15}N tracer experiments carried out by Reddy (1983) reported nitrogen losses in a control reactor with algal biomass for agricultural

drainage effluent treatment. After the system was spiked with labelled $^{15}NO_3^-$, 28.9 percent of ^{15}N was unaccounted after a net mass balance, suggesting that denitrification can be also a feasible nitrogen removal mechanism in WSP.

Nitrification and denitrification rates in WSP were estimated by Zimmo *et al.* (2004); the reported net in situ nitrification rate (nitrification rate minus denitrification rate) for algal-based ponds was between –130 and 570 g N/ha d, while the denitrification rate ranged between 1,800 and 5,890 g N/ha d. These figures could explain why the denitrification process has not been included in many research studies which argue for the absence of nitrification in WSP because low nitrite and nitrate concentrations have been reported in pond effluents. In fact, simultaneous processes such as nitrate uptake or denitrification are consistent with these low nitrite and nitrate concentrations.

Therefore, and based on the literature reviewed, feasible transformation pathways and removal mechanisms for nitrogen removal in WSP could include: (a) ammonia volatilisation, (b) biological ammonium uptake, (c) conventional nitrification-denitrification, (d) nitrification and simultaneous biological uptake, (e) sedimentation of dead biomass and accumulation on sludge layer after partial hydrolysis, (f) denitrification by denitrifiers, (g) anaerobic ammonia oxidation. However, current evidence is far from being able to determine which mechanism(s) dominate(s) nitrogen removal in WSP and most importantly, under which operational and environmental conditions. Once we have a clear knowledge about how nitrogen is transformed and removed, it will be an important contribution to wastewater engineers designing new, and improving existing, WSP systems.