The removal of ammonia-nitrogen and degradation of 17alpha-ethynylestradiol and mestranol using partial fixed bed continuous reactor (PFBR) and moving bed continuous reactor (MBBR).

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The Removal of Ammonia - Nitrogen and Degradation of 17α – Ethynylestradiol and Mestranol Using Partial Fixed Bed Continuous Reactor (PFBR) and Moving Bed Continuous Reactor (MBBR)

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ABSTRACT

Effective treatment of wastewater is an important process in reducing the environmental impact of industry and human activity. Although conventional water treatment systems can adequately remove the principle components of waste (i.e. substances that can be represented the majority of biological and chemical oxygen demand) several materials are poorly or slowly removed. Tertiary treatment polishing processes are therefore required to remove these contaminants to ensure complete wastewater treatment. This thesis reports investigations made using film reactors that are used to remove recalcitrant materials such as ammonia- nitrogen and endocrine disrupters that although present in low concentrations, if left untreated can have a strong impact on the environment. Film reactors potentially offer several process advantages over conventional activated sludge treatments systems as they allow very long residence time and contact with high concentrations of fixed microbes with the low concentrations of pollutants so enhancing kinetic performance and efficiency of the process. Two reactor configurations, a partial fixed bed (PFBR) and moving bed biofilm reactors (MBBR) were investigated. A thirty liter reactor with a working volume of 16 liters was constructed and contained fixed microbial films on either free suspended or fixed beds plastic packing (K2 AnoxKaldnes). The investigation of ammonia - nitrogen oxidation showed that after a suitable acclimation period (2 weeks) that ammonia was oxidise rapidly reducing the feed concentrations of 35 mg/L to < 2 mg/L in the effluent. To assess the performance for ammonia – nitrogen removal the reactors operated for long periods (up to 3 months) with continuous feed using the reactor in either PFBR or MBBR modes in addition of 17α – ethynylestradiol (EE2) and mestranol (MeEE2), the endocrine disrupting compounds commonly found in municipal wastewater. These substances is derived from a synthetic hormones if found in the natural environment can reduced the productivity of the fish as this can cause feminization in aquatic organisms with disastrous consequences on fish populations. The MBBR and PFBR systems were used to investigate the co-metabolism of ammonia – nitrogen, 17α – ethynylestradiol (EE2) and mestranol from model waste water feed containing 35 mg/L of ammonia - nitrogen and 100 μg/L of 17α – ethynylestradiol (EE2) and mestranol (MeEE2). A kinetic analysis of the systems were made and for the PFBR reactor, the specific growth rate, μ_{max} of 7.092 d^{-1} with saturation constants, Ks of 1.574 mg/L. The kinetic analysis for the MBBR system was 6.329 d^{-1} for the μ_{max} with the Ks of 0.652 mg/L. When the PFBR was used removal of EE2 represents ≤ 90% removal and MeEE2 represents ≤ 70% removal. With the MBBR ≥ 90% EE2 was removed and ≥ 70% MeEE2 was removed. MBBR were shown to be more effective and efficient in removing ammonia – nitrogen reducing the levels under good conditions to < 2 mg/L while the PFBR could also achieve 2 mg/L. The MBBR system was also more competent in the removal of 17α – ethynylestradiol (EE2) and mestranol compared to PFBR. This work demonstrates that there are considerable advantages to using thin film reactors as polishing step for the tertiary treatment of waste waters when to compared to other processes in reducing the inorganic pollutants as endocrine disrupting compounds. The significance of these results is discussed in this context.

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Abbreviations

PFBR	partial fixed bed continuous reactor
MBBR	moving bed continuous reactor
EE2	17α – ethynylestradiol
MeEE2	mestranol
H_2O	water
DDT	dichlorodiphenyltrichloroethane
NDMA	n – nitrosodimethylamine
MTBE	methyl tertiary butyl ether
N_2	nitrogen gas
NH_3	ammonia
NH_4^+	ammonium ion
NO_2^-	nitrite
NO_3^-	nitrate
NH_3-N	ammonia – nitrogen
NH_4^+ - N	ammonium – nitrogen
TAN	total ammonia nitrogen
NO_2^- - N	nitrite - nitrogen
NO_3^- - N	nitrate - nitrogen
TIN	total inorganic nitrogen
TKN	total Kjeldahl nitrogen
Organic N	organic nitrogen
TN	total nitrogen
COD	chemical oxygen demand
BOD	biochemical oxygen demand
TSS	total suspended solids
VSS	total volatile solids
$CaCO_3$	calcium carbonate
C/N	carbon and nitrogen ratio
NH_2	amines
OH	hydroxyl functional group

$CHNH_2$	amino acid oxidoreductases
COOH	carboxylic acids
EDCs	endocrine disrupting chemicals
AMO	ammonium monooxygenase
O_2	oxygen
E2	17β – estradiol
E1	estrone
WWTP	wastewater treatment plant
TAN	total ammonium nitrogen
TNO_2	total nitrite nitrogen
BOD_5	Five – day biochemical oxygen demand
RBC	Rotating Disc Scheme
N	Nitrogen
MLSS	Mixed Liquor Suspended Solids
DO	Dissolved Oxygen
NOD	Nitrogenous oxygen demand
FA	Free ammonia
FNA	Free nitrous acid
MLVSS	Mixed Liquor Volatile Suspended Solids
USEPA	United State Environmental Protection Agency
PPCP	Pharmaceutical and Personal Care Product
aE2	17α – estradiol
bE2	17β – estradiol
AC	Activated Carbon
PAC	Powdered Activated Carbon
GAC	Granular Activated Carbon
BAF	Biological Aerated Filter
IFAS	Integrated Filter Activated Sludge
SCOD	Soluble Chemical Oxygen Demand
TCOD	Total Chemical Oxygen Demand
MBR	Membrane Bioreactor

AS	Activated Sludge
p.e	person equivalent
OLR	Organic Loading Rate
RBC	Rotating Biological Contactor
HDPE	High Density Polyethylene
DO	Dissolved Oxygen
ANAMMOX	Anaerobic ammonia oxidation
TAN	total ammonium
TNO_2	total nitrite
WHO	World Health Organization
MCL	maximum contaminant level
CANON	Completely autotrophic nitrogen removal over nitrite
BOD	Biochemical Oxygen Demand
TKN	Total Kjeldahl Nitrogen
SHARON	Single Reactor High Activity Ammonium Removal Over Nitrite
RBC	Rotating Biological Contactor

CHAPTER 1

INTRODUCTION

1.1 Background

Water is the common name applied to the liquid form (state) of the compound consisting of two atoms of hydrogen and one atom of oxygen, H_2O. Pure water is an odourless, tasteless, clear liquid. Water is one of nature's most important gifts to mankind; a person's survival depends on drinking water. Water is one of the most essential elements to good health and water is necessary for the digestion and absorption of food. Water helps to maintain proper muscle tone and supplies oxygen and nutrients to the cells. Water rids the body of wastes and serves as a natural air conditioning system. Health officials emphasise the importance of drinking at least eight glasses of clean water each and every day to maintain good health (WHO, 2002).

Water is an important component in determining the quality of our lives. Today, people are concerned about the quality of the water that they drink. Although water covers more than 70% of the Earth, only 1% of the Earth's water is available for drinking (WHO, 2004). However, today, humans continue to contaminate this precious resource. Water is known as a natural solvent. Before this liquid reaches the consumer's tap, water comes into contact with many different substances, including organic and inorganic matter, chemicals, and other contaminants. All of these organic pollutants, although in a

low concentration, will deteriorate human health and destabilise the metabolism of any organisms (Novak, 2012).

Some of these toxic inorganic chemicals, including metals, non-metallic salts, acids and bases, are released from rocks by weathering, carried by runoff into lakes or rivers, or percolate into groundwater aquifers. Humans can accelerate the rate of release of these inorganic chemicals through mining, processing, using, and discarding of minerals. However, without proper care when utilising and composting these toxic compounds, these heavy pollutants, even at low levels, will dissipate through runoff and endanger any living organisms. For example, many metals such as mercury, lead, cadmium, and nickel are highly toxic. A famous case of mercury poisoning occurred in Japan in the 1950s (McCurry, 2006). Heavy metals released as a result of human activities also are concentrated by hydrological and biological processes, meaning that they become hazardous to both natural ecosystems and human health (Hu *et al.*, 2004 and Honda *et al.*, 2006). Mine drainage and leaching of mining wastes are serious sources of metal pollution in water (EPA, 2009).

Desert soils often contain soluble salts, including toxic selenium and arsenic (Golka *et al.*, 2010). Salts such as sodium chloride, which are non-toxic at low concentrations, can also be mobilised by irrigation and concentrated by evaporation, reaching levels that are toxic for plants and animals (Zhao *et al.*, 2009). Acids are released as by-products of industrial processes (e.g. leather tanning, metal smelting and planting) Coal and oil combustion also leads to the formation of atmospheric sulphuric and nitric acids, which are disseminated by long-range transport processes (EPA, 2009).

There are many type of chemicals used in the chemical industry that are highly toxic; for example DDT, atrazine and diuron for making pesticides (Gilden *et al.*, 2010), ethylene, propylene and xylenes for the production of plastics (Matar and Hatch, 2001), 17α-ethynylestradiol and mestranol in the production of pharmaceuticals drugs (Yu *et al.*, 2007), and titanium dioxide in pigment processing (Berglund and Carlmark, 2011). The two most important sources of toxic organic chemicals in water are the improper disposal of industrial and household wastes and runoff from pesticides. Many of the toxic organic chemicals (e.g. DDT, dioxins, and other chlorinated hydrocarbons such as endocrine-disrupting chemicals) in water are passed through ecosystems and accumulate, even

though low levels will change the metabolic function and reproduction of any target organisms (Roefer *et al.*, 2000).

Wastewater has been defined as a combination of liquid- or water-carried wastes removed from residences, institutions, and commercial and industrials establishments, together with whatever groundwater, surface water, and storm water may be present. When untreated wastewater accumulates and is allowed to go septic, wastewater can lead to nuisance conditions including the production of malodorous gaseous (Grady *et al.*, 2011).

The untreated wastewater contains pathogenic microorganisms that came from the human intestinal tract, nutrients and may also contains toxic compounds that are potentially mutagenic or carcinogenic (Khin and Annachhatre, 2004). From the above reasons and definitions, the treatment of wastewater is one of the key ways to protect public health and the environment. The wastewater treatment principles are applied to treat and reuse wastewater.

Nowadays, research into the characteristics of wastewater has become more extensive and the techniques for analysing specific constituents and the potential hazards to health and the environment have become more comprehensive. New treatment methods have been designed and developed to deal with the health and environmental issues that have been raised with the findings of recent research (Schmidt *et al.*, 2003; Mulder, 2003; Ahn, 2006; Chang *et al.*, 2009).

The developments of new and more sensitive methods for detecting chemicals are available and methods have been developed to better determine biological effects; thus, constituents that were previously undetected are now of concern (Kuch and Ballschmiter, 2001; Metcalf and Eddy, 2004). All of the chemicals that have a high impact on the environment, even in low concentrations, can now be detected with highly efficient detection equipment that has significantly expanded the scientific research for new treatment methods of wastewater (Kuch and Ballschmiter, 2001; Chang *et al.*, 2009).

As the population increases over time and the high standard of living changes along with technological advances, with many industrial wastes, changes also occur in the compounds of effluents and effects on wastewater characteristics. Such examples of

chemical constituents found in both surface and groundwater include: n-nitrosodimethylamine (NDMA), a principal ingredient in rocket fuel, methyl tertiary butyl ether (MTBE), a highly soluble gasoline additive, medically active substances including endocrine disruptors, pesticides, industrial chemicals, and phenolic compounds that are commonly found in non-ionic surfactants (Richardson and Ternes, 2005)

The hazardous chemicals, especially endocrine-disruptive compounds, are of major concern as they can mimic hormones produced in vertebrates by causing an exaggerated response, or can block the effects of a hormone on the body (Trussell, 2001). These chemicals can cause problems with development, behaviour, and reproduction in a variety of species. Increased rates of testicular, prostate and breast cancers have been blamed on endocrine-disruptive chemicals (Roefer *et al.*, 2000).

Effluents containing total ammonia-nitrogen in the water, even in low concentrations, are toxic to fresh water organisms, especially to fish (Randall and Tsui, 2002). The concentration of ammonia-nitrogen in the water, reported to be in the range of 3 mg/l to 22.8 mg/L, is toxic to fish (Durborow *et al.*, 1992; Randall and Tsui, 2002). Toxic levels are both pH- and temperature-dependent and toxicity increases as pH decreases and as temperature decreases; therefore, hatching and growth rates of fishes may be affected (Durborow *et. al.*, 1992; Randall and Tsui, 2002; Timmons *et al.*, 2002).

In the structural development of fish, changes in the tissues of gills, liver, and kidneys may also occur (Bartoli, 2007). High levels of toxic concentrations of ammonia-nitrogen in the form of ammonia in humans may also cause a loss of equilibrium, convulsions, coma, and death (Randall and Tsui, 2002).

1.2 Nitrogen cycle

Nitrogen is a naturally occurring element that is essential for growth and reproduction in living organisms. Nitrogen is also a key component of proteins and nucleic acids, and without them, no life can exist. Nitrogen is the most abundant compound in the atmosphere; gaseous nitrogen (N_2) consists of two nitrogen atoms and comprises 79% of the air volume (WEF, 2005).

This large amount of nitrogen in the atmosphere, however, is not readily available to most organisms. Certain groups of organisms assimilate nitrogen gas to form organic nitrogen that is then available to other organisms as their source of food. This process is termed nitrogen fixation. Lightning contributes to nitrogen fixation; however, most of the nitrogen fixation is either of biological or industrial origin. In biological nitrogen fixation, atmospheric nitrogen is converted to ammonia by enzymes. The major group of nitrogen-fixing organisms (diazotrophs) live in close proximity to plant roots and obtain energy from the plants. Industrial fixation processes ammonium and nitrate from the air through various chemical processes (WEF, 2005).

The major sources of nitrogen are of plant, animal and human origin (decaying plant material, and animal and human wastes), industrial and agricultural origin, and atmospheric origin. Nitrogen compounds in human and animal waste are associated with proteins and nucleic acids. Ammonia is formed as a result of protein and nucleic acid decomposition. Volatile organic nitrogen is released into the atmosphere during plant decay. Industrial emissions and fuel combustion contributes gaseous nitrous oxide and nitric acid. Many forms of nitrogen are used for agricultural purposes as fertiliser. The common nitrogen compounds used in fertilisers are urea, ammonium phosphate, ammonium sulphate and ammonium nitrate. Atmospheric deposition can also contribute to the nitrogen balance (Sedlak, 1991 and Zhang *et al.*, 2012).

All higher plants and microorganisms depend on combined nitrogen for their nutrition. Combined nitrogen in the form of ammonia, nitrate and organic compounds often becomes the limiting factor for biological processes in soil (Randall *et al.*, 1992).For this reason, the cyclic transformations of nitrogenous compounds, including the mineralisation of nitrogenous organic matter, are of great importance in the total turnover of this element in soil.

Nitrogen mineralisation consists of two different processes: the ammonification of organic compounds by a large number of heterotrophic microorganisms, and the oxidation of released ammonia to nitrite and nitrate (nitrification), mainly by autotrophic bacteria. The mineralisation of organic nitrogen depends mainly on temperature, moisture, aeration, type of organic nitrogen and pH value. The inorganic nitrogen produced by mineralisation is subject to nitrogen immobilisation and fixation by clays.

However, inorganic nitrogen can be lost through denitrification and leaching (WEF, 2005). Figure 1.1 shows the processes of the nitrogen cycle.

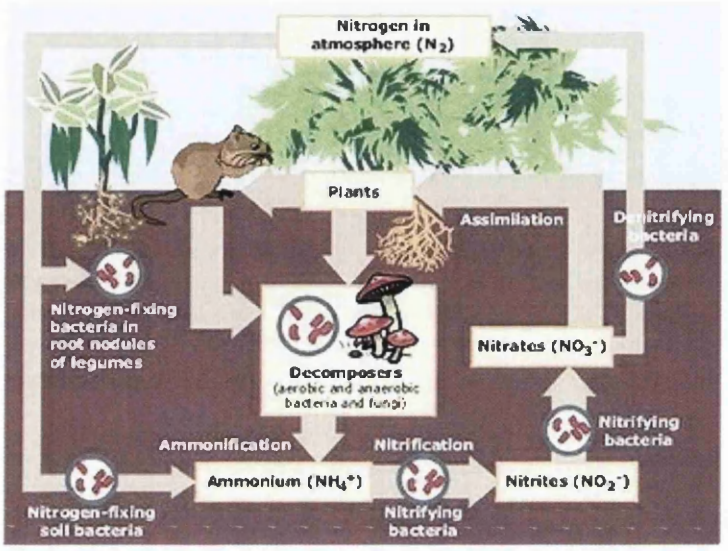


Figure 1.1: Nitrogen cycle (WEF, 2005)

1.2.1 Wastewater nitrogen removal

Nutrients or biostimulants are the most important elements for the growth of microorganisms, plants and animals. Both nitrogen and phosphorus play a major role in nutrient requirements. Nitrogen is an essential building block in the synthesis of proteins; nitrogen data will be required to evaluate the treat ability of wastewater by biological processes. Insufficient nitrogen can necessitate the addition of nitrogen to make waste treatable (Mulder, 2003).

The most common forms of nitrogen in wastewater are ammonia (NH_3), ammonium ion (NH_4^+), nitrogen gas (N_2), nitrite (NO_2^-), nitrate (NO_3^-) and organic nitrogen. Municipal wastewater primarily contains ammonium and organic nitrogen, whereas some industrial wastewater contains appreciable amount of nitrate-nitrogen (Khin and Annachhatre, 2004). Table 1.1 shows the forms of nitrogen and their definitions (Metcalf and Eddy, 2004).

Table 1.1: Forms of nitrogen and their definitions (Metcalf and Eddy, 2004).

Compound	Abbreviation	Form	Definition
Ammonia-nitrogen	NH_3-N	Soluble*	NH_3-N
Ammonium-nitrogen	NH_4^+ - N	Soluble	NH_4^+-N
Total ammonia nitrogen	TAN	Soluble*	NH_3-N + NH_4^+-N
Nitrite	NO_2^- - N	Soluble	NO_2^--N
Nitrate	NO_3^- - N	Soluble	NO_3^--N
Total inorganic nitrogen	TIN	Soluble*	NH_3-N + NH_4^+-N + NO_2^--N + NO_3^--N
Total Kjeldahl nitrogen	TKN	Particulate, Soluble*	Organic N + NH_3-N + NH_4^+-N
Organic nitrogen	Organic N	Particulate, Soluble*	TKN-NH_3-N + NH_4^+-N
Total nitrogen	TN	Particulate, Soluble*	Organic N + NH_3-N + NH_4^+-N + NO_2^--N + NO_3^-N

*In neutral pH range, the gas form of ammonia (NH_3-N) is negligible.

Key wastewater constituents for process design have been grouped into the following categories: carbonaceous substrates, nitrogenous compounds, phosphorous compounds, total and volatile suspended solids (TSS and VSS) and alkalinity, as shown in Table 1.2 (Metcalf and Eddy, 2004).

Table 1.2: The example of typical domestic wastewater characterisation parameters and typical values (Metcalf and Eddy, 2004).

Component	Concentration, mg/L[a]
COD	430
BOD	190
TSS	210
VSS	160
TKN	40
NH_4-N	25
NO_3-N	0
Total phosphorus	7
Alkalinity	200 (as $CaCO_3$)

[a] Typical medium-strength wastewater in the United States

The composition of nitrogen in wastewater is illustrated in Figure 1.2. The total Kjeldahl nitrogen (TKN) is a measure of the sum of the ammonia and organic nitrogen. About 60 to 70 percent of the influent TKN concentration will be as NH_4-N, which is readily available for bacterial synthesis and nitrification. Organic nitrogen is present in both soluble and particulate forms and some portions of each of these is non-

biodegradable. The particulate degradable organic nitrogen will be removed more slowly than the soluble degradable organic nitrogen because a hydrolysis reaction is necessary first (Brion and Billen, 2000; Metcalf and Eddy, 2004).

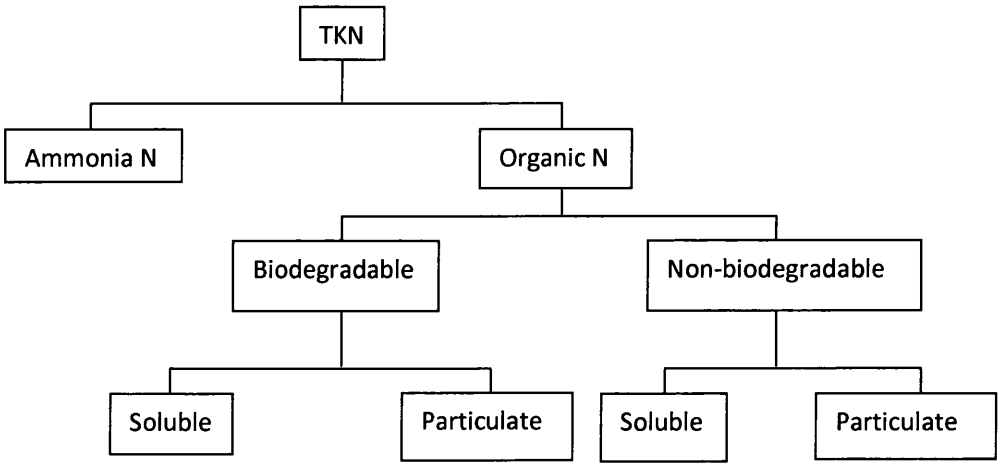


Figure 1.2: Fraction of nitrogen in wastewater. Information on the nitrogen fractions is used in the detailed design of nitrification and denitrification processes (Metcalf and Eddy, 2004).

1.2.2 Description of the biological nitrification process

Organic nitrogen (e.g. protein and nucleic acids) is converted by microbial decomposition to ammonia. The first step of protein hydrolysis e.g. is the release of amino acids, which are then hydrolysed under aerobic or anaerobic conditions to ammonia (Shukla and Varma, 2010). The ammonification rates depend on the C/N ratio of the organic compound; high rates generally occur at low C/N ratios. Except for the hydrolysis of urea by extracellular urease, the ammonification is bound to the metabolism of active cells (Nannipieri *et al.*, 1990; Hanson *et al.*, 2013).

Ammonia can be released from organic N by different mechanisms:

1. Hydrolytic deamination:

$$R - NH_2 + H_2O \rightarrow R - OH + NH_3$$

2. Oxidative deamination:

$$R - CHNH_2 - COOH + H_2O \rightarrow R - CO - COOH + 2H^+ + NH_3$$

3. Reductive deamination:

$$R - CHNH_3 - COOH + 2H^+ \rightarrow R - CH_2 - COOH + NH_3$$

4. Desaturative deamination:

$$R - CH_2 - CHNH_2 - COOH \rightarrow R - CH = CH - COOH$$

The ammonification of amino acids has been proposed as simple and rapid parameter to estimate microbial activity in soils (Zhang *et al.*, 2011; Hanson *et al.*, 2013). Burger and Jackson (2003) have investigated the ammonification and nitrification rates in organic and conventional cropping systems.

Nitrification is the two-step biological conversion of ammonia to nitrite and then to nitrate under aerobic conditions. The conversions to nitrite and nitrate involve two specific groups of autotrophic bacteria: *Nitrosomonas* bacteria and *Nitrobacter*. Autotrophic bacteria, specifically chemoautotrophic bacteria, differ from the heterotrophic bacteria that consume organic material, biological oxygen demand (BOD), in that chemoautotrophic bacteria use carbon dioxide as their carbon source and specific inorganic chemicals as a source of energy for growth. In the case of *Nitrosomonas* and *Nitrobacter*, the inorganic chemicals used are ammonia and nitrite, respectively (Grunditz and Dalhammar, 2011).

The microorganisms that perform nitrification and denitrification are only able to act on the inorganic forms of nitrogen (ammonia, nitrite and nitrate). Therefore, any portion of the influent nitrogen remaining in the organic from – either particulate or soluble – has the potential of passing through the process untreated (Grady et al., 2011). Generally, particulate organic nitrogen is incorporated to the solids that are removed by clarification or filtration.

Endocrine disrupting chemicals (EDCs) have been shown to produce changes in the endocrine system of organisms, leading to an increase in cancers and abnormalities in

reproductive structure and function. In wastewater treatment, the nitrification process that occur in the secondary treatment can have benefits to the removal of organic contaminants, such as endocrine disrupting chemicals (EDCs) (Langford and Lester, 2003).

Co-metabolism is defined as the simultaneous degradation of two compounds, in which the degradation of the second compound (the secondary substrate) depends on the presence of the first compound (the primary substrate). This biotransformation process, where the second compound is modified but not utilised for growth, is another important process of biodegradation (Alexander, 1994). The knowledge of co-metabolism transformation processes for endocrine disrupting chemicals (EDCs), especially for 17α-ethynylestradiol (EE2) and mestranol (MeEE2), is essential to assess the fate and potential effects of these compounds (Vader *et al.*, 2000; Pauwels *et al.*, 2008).

Several bacterial strains in the wastewater that produce monooxygenase enzymes are known to aerobically co-metabolise organic compounds (Yi and Harper, 2007). *Nitrosomonas europea* is a ubiquitous monooxygenase-producing bacterium which catalyses the oxidation of ammonium in soils, natural waters and nitrifying activated sludge. The enzyme of ammonium monooxygenase (AMO) in the cells of *Nitrosomonas europea* is capable of co-oxidising many organic compounds, particularly endocrine disrupting chemicals (EDCs) (Vader *et al.*, 2000; Shi *et al.*, 2004; De Gusseme *et al.*, 2009; Forrez *et al.*, 2009).

1.2.2.1 Stoichiometry of nitrification process

The stoichiometry of the biochemical reactions associated with nitrification defines the proportion of reactants and products involved with this process. The understanding of stoichiometry is important as the basic inputs and outputs for each of the steps in the process can be defined and determined to see which of the inputs will limit the reaction process.

The stoichiometric equation that defines the molar ratios for the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) by *Nitrosomonas* is as follows:

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O$$

Similarly, the stoichiometric equation that describes the oxidation of nitrite to nitrate (NO_3^-) by *Nitrobacter* is the following:

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$$

These reactions also generate biomass associated with the growth of *Nitrosomonas* and *Nitrobacter* (i.e., nitrifiers). Unlike heterotrophs, *Nitrosomonas* and *Nitrobacter* obtain the carbon for cell growth from an inorganic source – carbon dioxide (WEF, 2005).

1.2.2.2 Nitrification kinetics

Growth of *Nitrosomonas* and *Nitrobacter* is the result of the oxidation of ammonia and nitrite, respectively. The growth of either of these species of bacteria is limited by the concentration of the respective substrate (food source), as defined by the Monod equation (USEPA, 1993a), which is illustrated as follows:

$$\mu = \frac{\mu max\ S}{Ks + S}$$

Where:

μ = specific growth rate of microorganisms (g nitrifiers/g nitrifiers in system.d);

μmax = maximum specific growth rate of microorganisms (g nitrifiers/g nitrifiers in system.d);

Ks = half-saturation coefficient for ammonium-nitrogen (mg/L); and

S = growth-limiting substrate (NH_4^+-N) concentration (mg/L).

1.2.2.3 Wastewater temperature for nitrification

The growth rate of *Nitrosomonas* and *Nitrobacter* are particularly sensitive to the liquid temperature in which they live. Nitrification has been shown to occur in wastewater temperatures from 4°C to 45°C, with an optimum growth rate occurring in the temperature range 35°C to 42°C (USEPA, 1993a; Zhu and Chen, 2002).

1.3 Endocrine Disruptive Compounds

Steroid hormones are naturally occurring organic substances which are mainly produced by humans and animals; these are human sexual hormones. Oestrogens are the most important female sexual hormones which are produced in the female ovaries. The most abundant oestrogens are 17β–oestradiol (E2) and oestrone (E1) (Yu *et al.*, 2007; Moschet and Hollender, 2009). Male sexual hormones are called androgens and are produced in the testes; the most important androgen is testosterone (Fahrbach, 2006; Young and Borch, 2009). These hormones are excreted in the urine of humans or animals, normally in a conjugated form as soluble and inactive glucorinides (Pauwels *et al.*, 2008).This conjugated form is then microbially-cleaved in the environment or in wastewater treatment plants (WWTP) (Jurgens *et al.*, 2002).

The hormones that are naturally produced by humans and animals act as endocrine disruptors, especially to water organisms. The impact of endocrine disruptors on the water environment has become a critical concern (Hashimoto and Murakami, 2009). Since the population has grown and more people now live in large cities, all excreted hormones are entering the environment at one point: the effluent of WWTPs (Ternes *et al.*, 1999b). In addition, 17α–ethinylestradiol (EE2) and mestranol (MeEE2) have become the major compounds of contraceptive pills (which are also excreted by the urine), thus enhancing the concentration of hormone-like substances in the WWTP effluent (Yu *et al.*, 2007; Moschet and Hollender, 2009). However, only 1% of all excreted oestrogens are EE2, with 80% being oestrogens produced by women (De Mes *et al.*, 2005).

The concentration of oestrogens in the water environment is mostly lower than 5 ng/L, whereas the concentration of oestrogens in the WWTP effluent can exceed 50 ng/L (Yu *et al.*, 2007). Due to the higher persistence of EE2 in the WWTP, the concentrations of synthetic oestrogens in the environment is analogous to concentrations of natural oestrogens, despite the fact that this synthetic hormones excreted in a much smaller amount (Yu *et al.*, 2007; Moschet and Hollender, 2009).

1.3.1 Structure, stability and degradation process

The steroid hormones are all built from cholesterol; the A ring shows phenolic properties, whereas the other rings do not contain any double bonds. Due to the four linked rings, the steroid skeleton is very stable (Fahrbach, 2006). Oestrogens and androgens contain three quaternary C-atoms: C-5, C-10 and C-13 (Figure 1.3). Not many sites are available where the simple oxidation of a C-atom can take place, as the rings are hardly substituted. Thus, the energy gained by the degradation of steroids is very low. In the case of EE2, degradation is even more difficult, since the introduction of the ethinyl group makes the ring very stable against oxidation at position 17. (De Mes *et al.*, 2005; Fahrbach, 2006).

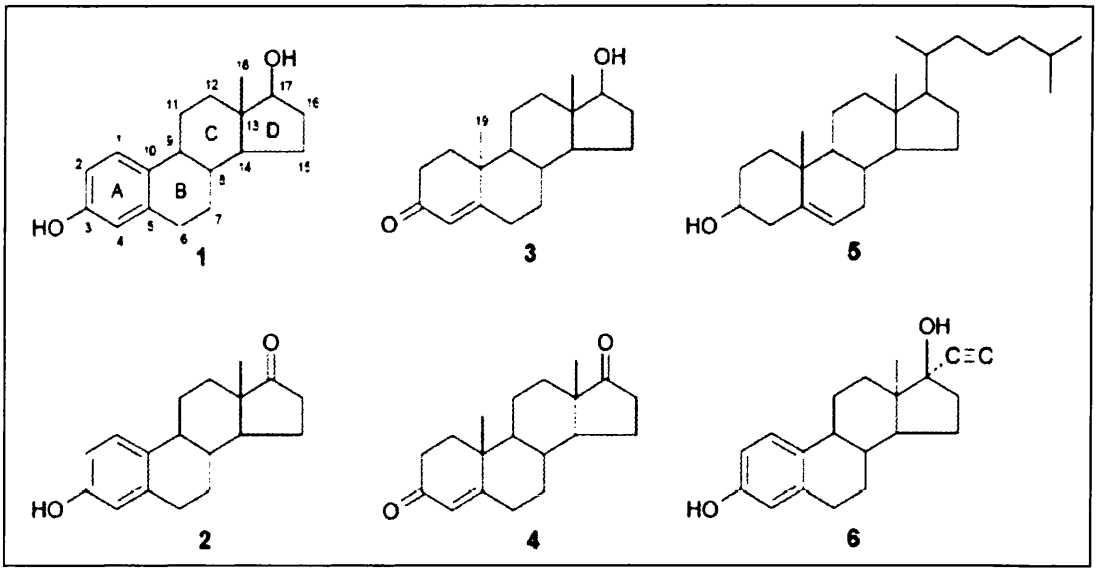


Figure 1.3: Molecular structures of the steroid hormones 1) Oestradiol (E2); 2) Oestrone (E1); 3) Testosterone (T); 4) 4-androstene-3, 17-dione; 5) Their precursor cholesterol; and 6) The main substance of the contraceptive pill, 17α–ethinylestradiol (EE2) (Fahrbach, 2006).

In the environment, steroid hormones can be removed differently. There are several other processes that include sorption, photolytic degradation as well as microbial degradation (Chang *et al.*, 2009; Liu *et al.*, 2009; Clouzot *et al.*, 2010). In WWTPs, the natural processes sorption and degradation are the two main processes; in addition, there

are different technical elimination processes that have been tested as tertiary treatments to date (Yu *et al.*, 2007; Moschet and Hollender, 2009; Chang *et al.*, 2009; Liu *et al.*, 2009).

1.4 Suspended and Attached Growth System

Suspended-growth biological treatment systems operate in a fashion that allows control of the amount of biomass in the process and, therefore, the net growth rate of the biomass (WEF, 2005). Suspended-growth nitrification can be achieved in various reactor configurations; however, these configurations must be designed and operated to meet two overriding criteria (Wang *et al.*, 2012). High hydraulic retention time (HRT) must be adopted to achieve the simultaneous biological oxidation of organic and nitrogenous compounds in a single sludge system because of inhibitory factors in activated sludge systems (Gheewala *et al.*, 2004; Jubany *et al.*, 2005; Kim *et al.*, 2006). The high sludge recycle ratio should also be adopted in activated sludge in suspended-growth systems to keep a high biomass concentration in the reactor (Philips and Verstraete, 2000; Jubany *et al.*, 2008; Liwarska-Bizukojc and Biernacki, 2010).

The biomass inventory must be retained in the system for a sufficient period of time to allow a stable population of nitrifiers to develop and to be maintained in the process of nitrification. In suspended-growth systems for activated sludge reactors, the hydraulic retention time of the system must be high so that the biomass provided is capable of reacting to the quantity of pollutants entering the system to result in the necessary process to achieve a high quality of effluents (Wang *et al.*, 2012).

Biological wastewater treatment processes with the biomass attached to some types of inert media are termed fixed-film, attached-growth reactors (Akhbari *et al.*, 2011). True fixed-growth systems are differentiated from coupled treatment systems that incorporate a separate fixed-growth reactor followed by an activated sludge process without intermediate clarification and hybrid systems that include suspended and fixed-growth processes within the same reactor (Jahren *et al.*, 2002; Wang *et al.*, 2012).

The fixed-growth biological treatment processes are generally very easy to operate and are more flexible. In the suspended-growth nitrification systems, the onset and accumulation of autotrophic bacteria that accomplish nitrification are limited by the

growth of heterotrophic bacteria that predominate until the substrate (food source) for those microorganisms are mostly exhausted (Leiknes and Ødegaard, 2007). The biofilm treatment system, the advantage of a hybrid system of suspended and attached growth reactors, is more compact than the activated sludge system. This is because the treatment efficiencies of the biofilm reactor are less dependent on the sludge separation characteristics to prevent sludge bulking and the low hydraulic retention time needed to operate the system (Johnson *et al.*, 2000; Jahren *et al.*, 2002; Li *et al.*, 2011).

1.5 Problem Statement

There are recent environmental issues being raised in Malaysia regarding the water quality in rivers and lakes due to increasing pollutants such as ammonia from the leachate of dumping grounds and fish farms. There is also critical concern about the existence of synthetic drugs from contraception pills, such as 17α-ethynylestradiol and mestranol, in wastewater effluents. Due to these problems, the removal of ammonia, 17α-ethynylestradiol and mestranol is very important as these pollutants can cause hazards in aquatic environments. The ammonia concentration in the water bodies also needs to be maintained at a low level, especially in fish farms, to prevent toxicity to aquatic organisms. The endocrine disruptive compounds from 17α-ethynylestradiol and mestranol need to be removed from the wastewater effluent as prolonged exposure to both substances will decrease the quality and productivity of aquatic organisms in water bodies.

The presence of ammonia-nitrogen may deteriorate the quality of receiving water; some technologies such as nitrification/denitrification and breakpoint chlorination convert ammonia-nitrogen to nitrogen gas. However, an approach based on resource recovery is preferable and may contribute positively to an overall nitrogen balance as well as to the economics of wastewater treatment, leading to sustainable technologies. Tertiary treatment polishing processes using film reactors potentially offer several process advantages in removing the hazardous contaminants, especially ammonia-nitrogen and endocrine disruptive compounds of 17α-ethynylestradiol (EE2) and mestranol (MeEE2). In this study, packing materials were used in batch cultures and continuous cultures for the partial fixed-bed system (PFBR) and moving bed system (MBBR). The packing material was used to retain the nitrifying bacteria in the batch culture and continuous

culture systems so that the process of nitrification could be enhanced to remove the ammonia-nitrogen and synthetic drugs.

The nitrifying bacteria were grown in enrichment culture. The process of nitrification was observed in batch cultures and continuous cultures of a partial fixed-bed system (PFBR) and moving bed system (MBBR). The removal process of 17α-ethynylestradiol and mestranol were also investigated in batch culture and continuous culture systems.

1.6 Organisation of Thesis

Chapter 1 introduces and outlines the principle aims and objectives of the principal project. The scientific background and relevant literature have been summarised to understand the characteristics and growth of nitrifying bacteria and the nitrification process. This chapter also explains the role of nitrification and possible solutions to the problems associated with nitrification.

Chapter 2 represents the literature review of the case study, especially the technology of hybrid biofilm bed bioreactors using packing materials.

The experimental results of the project are documented in Chapter 3, Chapter 4 and Chapter 5. Chapter 3 provides a detailed account of the methods and materials for the measurement of the growth of organisms, the analysis of chemicals and the use of equipment. Chapter 3 also reports the growth and characterisation of nitrifying bacteria. This focuses on optimisation of the growth medium and optimal growth conditions as well as the growth and metabolism of nitrifying bacteria with respect to synthetic medium use and the performance of the nitrifying bacteria grown in the presence and absence of packing materials.

Chapter 4 investigates the growth of nitrifying bacteria in batch. The emphasis was on monitoring cell growth, substrate consumption and product formation, as well as determining the growth kinetics of nitrifying bacteria in a particular packing material. This was to further understand the physiological and kinetic characteristics of the growth of nitrifying bacteria and the performance of 17α-ethynylestradiol and mestranol, two

contraceptive hormones of the endocrine-disruptive family of compounds, with regard to degradation of the batch culture of nitrifying bacteria.

Chapter 5 concerns studies involving the growth of nitrifying bacteria in chemostat continuous culture for two systems. Partial fixed-bed and moving bed biofilm reactors were runs as continuous reactors; both reactors were studied for the growth kinetics of the ammonia-oxidising bacteria (AOB) for substrate removal in addition to 17α-ethynylestradiol and mestranol as two contraceptive hormones of the endocrine-disruptive family of compounds. The study focused on the performance of the following two systems: partial fixed-bed reactor of K2 AnoxKaldnes packing materials and the moving bed reactor of K2 AnoxKaldnes packing materials. These were assessed for the low substrate utilisation of ammonia-nitrogen and the end products for the ammonia-oxidising bacteria in synthetic medium until a steady state was reached along with the degradation of 17α-ethynylestradiol and mestranol.

The discussion and conclusion for the overall study together with suggestions for future work are included in Chapter 6.

CHAPTER 2

LITERATURE REVIEW

2.1 Nitrogen Origins and Effects

All organisms need nitrogen for the synthesis of proteins, nucleic acids and other essential nitrogen-containing compounds. Nitrogen exists in the form of nitrogen gas (N_2) and makes up 78% of the earth's atmosphere; even though the level of N_2 is high, nitrogen cannot be used directly, except by a few specialised organisms (Pynaert *et al.*, 2003 and 2004). Nitrogen fixation by these organisms is limiting by the nutrient itself because the availability is usually smaller than the potential uptake.

The supply of protein for the global population by agriculture is largely dependent on the use of synthetic nitrogen fertilisers produced from atmospheric N_2 by the Haber-Bosch process. In the last century, the world's annual industrial output of nitrogenous fertiliser increased from 10 Mt N in 1960 to about 90 Mt N in 1998 (Mulder, 2003). The global estimate for biological nitrogen fixation is in the range of 200-240 Mt N, which shows that the anthropogenic mass flows for nitrogen have a major impact on the global nitrogen cycle (Gijzen and Mulder, 2001).

The consumption of protein will ultimately result in the discharge of the protein's nitrogen in wastewater. In European countries, approximately 18% of fertiliser nitrogen ends up in wastewater in the form of total ammonia nitrogen (TAN) or inorganic nitrogen (Mulder, 2003). Other polluting nitrogen compounds are nitrite and nitrate. Nitrate is primarily used to make fertiliser, although nitrate is also used to make glass and explosives as well as in other chemical production and separation processes. Nitrite is manufactured mainly for use as a food preservative, and both nitrate and nitrite are used

extensively to enhance the colour and extend the shelf-life of processed meats (WHO, 2004).

The discharge of nitrogen compounds into the receiving waters could lead to several environmental and health risks. Ammonia is an essential plant nutrient and, after nitrification to nitrate, is responsible for eutrophication, i.e. undesirable growth of aquatic plants and algae (Weiss *et al.*, 2008). The enrichment of nutrients causes the existence of blue-green algae which can produce algal toxins, killing animals and poisoning freshwater reservoir supplies (Thieu *et al.*, 2010). The breakdown of these cells when they die can cause a depletion of oxygen in the water, which takes a heavy toll on fish. Ammonia toxicity, expressed as total ammonia (the sum of NH_3 and NH_4^+) in the environment, increases with water pH, because ammonia/ammonium reaction is around 9.5 and varies with ionic strength, pressure and temperature. This can upset metabolic processes in an organism, which can result in disease or death (Randall and Tsui, 2002). Ammonia itself is also toxic to aquatic organisms at concentrations below 1 mgNH_3-N/L (Sinha *et al.*, 2012).

Nitrate pollution impedes the production of drinking water. Nitrites in drinking water can lead to oxygen shortage in newborns ('blue baby syndrome') and during the chlorination of drinking water, carcinogenic nitrosamines may be formed by the interaction of nitrite with compounds containing organic nitrogen. The World Health Organisation (WHO) guideline of 50 mgNO_3^- N/L and the US maximum contaminant level (MCL) of 45 mgNO_3^- N/L for nitrate in drinking water have been established for the protection of infants from methaemoglobinaemia. The WHO guideline for nitrite is 3 mgNO_2^- N /L and for MCL is 1 mgNO_2^- N /L. The water must also be microbiologically safe. The toxicity results from the conversion of nitrate to nitrite *in vivo*, with reduced oxygen transport by haemoglobin to tissues in the body producing the symptoms of oxygen deficiency (Fan, 2011).

Nitrogen compounds therefore need to be removed from wastewater, as the effect of wastewater discharges containing nitrogen can be toxic to aquatic life. These compounds can be eliminated from wastewater by a variety of physicochemical and biological processes (Ahn, 2006).

2.2 Nitrogen in Wastewater

For the removal of total ammonia nitrogen from wastewater, a wide variety of biological and physicochemical removal systems are available (Henze *et al.*, 2002 and Cole *et al.*, 2002). For a specific application, the available alternatives will be evaluated on cost aspects, chemical and energy requirements, operational experiences and process reliability. However, in practice, the most optimal alternative is generally based on cost-effectiveness. The selection of either a biological or physicochemical method is determined by the nitrogen concentration in wastewater. The range of concentrations can be distinguished (Mulder, 2003):

- Diluted wastewater with a total ammonia nitrogen concentration up to 100 mgTAN/L. In this range, biological N-removal is the preferred process based on cost-effectiveness. Domestic wastewater is within this range.
- Concentrated wastewater with TAN concentrations in the range of 100-5000 mgTAN/L. A typical example is sludge reject water for which, after extensive investigations, biological treatment is preferred (Janus *et al.*, 1997). Although ammonia stripping and the production of $MgNH_4PO_4$ were identified as interesting alternatives for resource recovery, these options were not cost-effective (Priestley *et al.*, 1995; Janus *et al.*, 1997). Recently, several novel and cost-effective biological nitrogen elimination processes have been developed, including partial nitritation, nitrifier-denitrification, anaerobic ammonium oxidation (ANAMMOX), and a combined system for complete autotrophic nitrogen removal of nitrite (CANON) (Ahn, 2006).
- Concentrated wastewater with total ammonia nitrogen (TAN) higher than 5000 mgTAN/L. In this range, physicochemical methods are technically and commercially feasible. A successful example is the stream stripping of a wastewater with total ammonia nitrogen concentration of 1.5% followed by ammonia recovery, which has been in operation on an industrial scale since 1985 (Mulder, 2003).

2.3 Biological Nitrogen Removal Systems

The nitrogen concentrations studied in this PhD are below 500 mgTAN/L for the tertiary treatment as the polishing step of wastewater effluent. The focus will be on the biological treatment systems as the cheapest and most economical in practice. A review of the N-removal systems is considered (Mulder, 2003).

- Activated sludge with conventional nitrification and denitrification. Around the world, activated sludge with nitrification and denitrification is the most widely used system for N-removal with many design variations (Henze *et al.*, 1996). In the nitrification process, ammonium is converted to nitrate via nitrite: $NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$. According to this reaction, the oxidation of 1 kg N requires 4.57 kg O_2. For denitrification, the required COD/N ratio varies from 3-6.
- Activated sludge with nitrification denitrification via nitrite. In this process, ammonium is oxidised into nitrite: $2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O$ Oxidation of 1 kg N requires 3.42 kg O_2. Under anaerobic conditions, nitrite is reduced into nitrogen gas and the required COD/N ratio is 2-4.
- Activated sludge with autotrophic N-removal. Recently, the ANAMMOX process has been developed in which ammonium is oxidised under anaerobic conditions (Mulder *et al.*, 1995; Ahn 2006). First, ammonium must be oxidised to nitrite and then with ANAMMOX the overall reaction of the autotrophic nitrogen removal process is: $4NH_4^+ + 3O_2 \rightarrow 2N_2$. According to this reaction, the removal of 1 kg N requires 1.71 kg O_2.
- Algal ponds. In algal ponds, ammonia is assimilated into algal biomass according to the equation: $NH_3 + 5CO_2 + 2H_2O \rightarrow C_5H_7O_2N + 5O_2$. The energy use in the algal pond is required for mixing and pumping. The highest value of the N load corresponds with the lowest efficiency.
- Duckweed ponds. From the applied N-load 41-68% is assimilated in duckweed (Alaerts *et al.*, 1996).
- Constructed wetlands. In constructed wetlands, biomass is not recovered. The highest value of the N-load corresponds to the lowest removal efficiency (Brouwer, 1995).

2.3.1 Removal of nitrogen and organic matter

There are two ways of combining the removal of ammonium and organic matter with activated sludge and biofilms (Hu *et al.*, 2000). All wastewater containing ammonium also contains organic compounds, as nitrification normally takes place concurrently with oxidation of organic matter. As for the operation of activated sludge treatment plants for nitrification of wastewater, the only oxygen-consuming substance is ammonium, which is very difficult to use in practice. In a normal situation, the settling behaviour of the sludge is not good and due to the very small sludge production in the process, a reasonable sludge mass is difficult to maintain in the plant. In practice, plants for this type of wastewater are always designed as biofilters (Henze *et al.*, 2002).

2.3.1.1 One-sludge nitrification

One-sludge nitrification is a process configuration in which heterotrophic and nitrifying bacteria co-exist in a single mixed liquor that simultaneously oxidises ammonium and organic BOD. Figure 2.1 schematically illustrates the one-sludge approach, which has one reactor and one settler for all types of biomass. One-sludge nitrification can be carried out in sequencing batch reactors, which involve sequential periods of filling, aerobic reaction, settling, and effluent draw-off in one tank (Ritmann and McCarty, 2001).

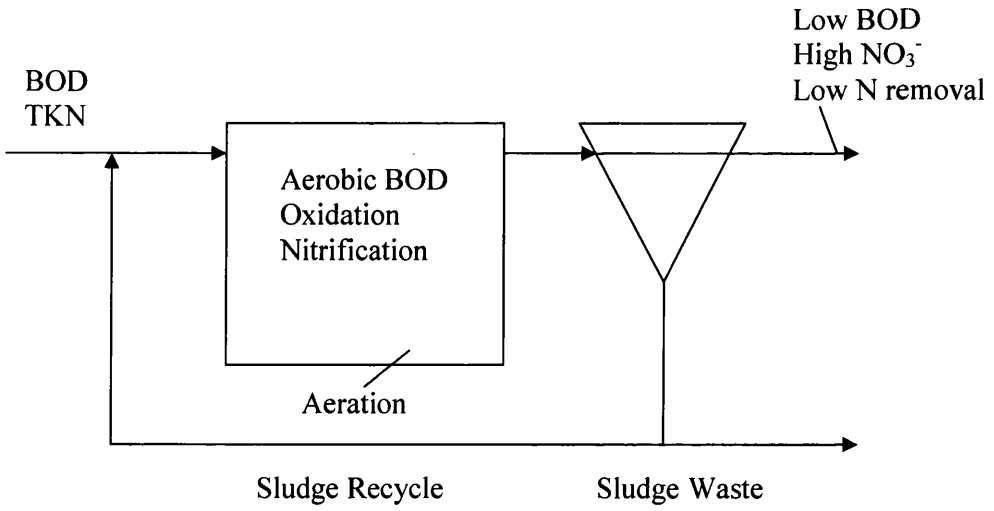


Figure 2.1: Schematic of the one-sludge system (Rittmann and McCarty, 2001)

2.3.1.2 Two-sludge nitrification

The two-sludge nitrification reactor configuration is an attempt to reduce the competition between heterotrophs and nitrifiers by oxidising most of the organic BOD in the first stage, while the ammonium is oxidised in the second stage. Figure 2.2 shows how the two-sludge process is comprised of two complete activated-sludge systems in series. The biomass in each stage is captured and recycled solely within the stage, with each stage developing its own biomass. There are two different communities, one for each stage. The first stage is essentially free from nitrifiers, while the second stage has a major fraction of nitrifiers; the process of nitrification occurs mostly in this stage (Ritmann and McCarty, 2001).

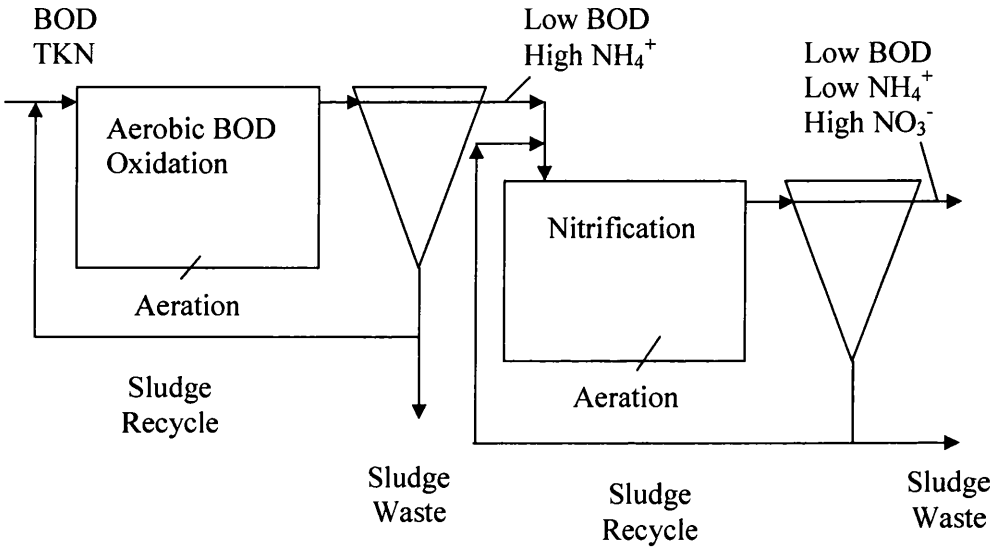


Figure 2.2: Schematic of the two-sludge system (Rittmann and McCarty, 2001)

2.3.1.3 Biofilm Nitrification

Biofilm processes are useful for aerobic BOD oxidation and can be employed to perform nitrification, provided that the process design appropriately considers the slow specific growth rate that is inherent to nitrifiers and the competition between nitrifiers and heterotrophs for oxygen and space. Table 2.1 summarises successfully used N and BOD_L surface loads for various biofilm processes (Ritmann and McCarty, 2001).

Table 2.1: Nitrogen and BOD loadings used successfully in various nitrifying biofilm processes (Ritmann and McCarty, 2001).

Process Type	N Surface Loading* kg N/1000 m^2-d	BOD Surface Loading kg BOD_L/1000 m^2 - d
Trickling filters	0.5 – 0.8	< 4.4
Rotating biological contactors	0.2 – 0.6	< 6
Biolite granular filters	< 0.7	< 6
Fluidised beds	0.5	Not given
Circulating beds	< 1	Not given

*The N surface loading should be computed from TKN concentration, not only the NH_4^+-N concentration, since organic N can be hydrolysed to NH_4^+-N.

2.3.1.4 Hybrid biofilm/suspended-growth processes

Nitrification is one of the applications for which hybrid suspended-growth/biofilm processes are used to increase the volumetric loading rate. For the existing activated sludge processes, the performance can be upgraded by adding a biofilm surface area inside the aeration basin. Immobile biofilms include screens, ribbons, or lace strings that are held inside the aeration basin by fixed frames immersed in the mixed liquor. Mobile biofilm carriers are mixed into and travel with the mixed liquor. They include sponges, plastic mesh-cubes or cylinders, porous cellulosic pellets, and polyethylene glycol pellets. The latter can be imbedded with bacteria, whilst the mobile biofilm carriers must be easily separated from the mixed liquor and held in the aeration basin. The screens or wire wedges at the aeration basin outlet are effective. The system for the mobile carriers is a moving-bed biofilm reactor (Ritmann and McCarty, 2001). These removal systems are discussed further (Ødegaard *et al.*, 1994; Hem *et al.*, 1994; Melin and Ødegaard, 2000).

2.3.1.5 The anaerobic ammonium oxidation process (ANNAMOX)

Recently, a novel bacterium in the planctomycetes group has been discovered for the ability to anaerobically oxidise NH_4^+-N to N_2 rather than to NO_2^- (Strous *et al.*, 1999). This new removal system is an emerging tool for the biological nitrogen removal process (Mulder *et al.*, 2008). In addition, the ANAMMOX reaction is an autotrophic process

without external organic matters. The power consumption can be reduced by 60% in industrial applications (Chamchoi *et al.*, 2008). The ANAMMOX process was considered an environmental friendly sustainable process (Ni *et al.*, 2010). However, the ANAMMOX process can be inhibited by some environmental factors, e.g. temperature, organic matter, dissolved oxygen (DO), light and etc., which must be overcome by artificial measures in industrial applications. The synergistic action of bacteria can eliminate the inhibited effects of organic matters, DO and temperature (Van Hulle, 2004 and Gao *et al.*, 2012).

2.3.1.5.1 Single reactor high activity ammonium removal over nitrite (SHARON)

Nitrogen compounds can be eliminated from ammonium-rich wastewater by anaerobic ammonium oxidation (ANAMMOX). However, ammonium in substrates must partly be pre-oxidised to nitrite (50-60% ammonium), but not to nitrate, before feeding into the ANAMMOX process. Thus, the ANAMMOX process needs to be applied by a series of operations with partial nitritation processes such as a partial SHARON (single reactor high activity ammonium removal over nitrite) process (Ahn, 2006).

The SHARON process was originally developed for the removal of ammonia via the so-called 'nitrite route'. In this process, both autotrophic nitrification and heterotrophic de-nitrification take place in a single SHARON reactor system using intermittent aeration (Jetten *et al.*, 2002; Hellinga *et al.*, 1998). The drawbacks of this system are the de-nitrification (with added methanol) in the SHARON process, which is primarily required for pH control and alkalinity production, allowing the complete compensation of the acidifying effect in the nitrification phase. In many cases, this process is not suitable for all wastewater due to high temperature dependency (Ahn, 2006). A batch system in SHARON showed that ammonia rather than ammonium is the actual substrate and nitrous acid rather than nitrite is the actual inhibitor (Van Hulle *et al.*, 2007).

2.3.1.5.2 Completely autotrophic nitrogen removal over nitrite (CANON)

The concept of the CANON process (completely autotrophic nitrogen removal over nitrite) is the combination of partial nitritation and ANAMMOX. However, this process performs two sequential reactions in a single and aerated reactor, implying the two groups of bacteria (*Nitrosomonas* –aerobic microorganisms – and *Planctomycete* – anaerobic bacteria) cooperate in the whole process (Figueroa *et al.*, 2012).The nitrifiers oxidise ammonia to nitrite, consume oxygen and create anoxic conditions that the ANAMMOX process needs (Ahn, 2006). The CANON process has quite sensitive operational characteristics in dissolved oxygen, nitrogen-surface load, biofilm thickness and temperature (De Kreuk and Loosdrecht, 2004).

2.3.1.5.3 Oxygen-Limited autotrophic nitrification-denitrification (OLAND)

The process in which NH_4^+ is autotrophically oxidised to N_2 with NO_2^- as the electron acceptor under oxygen-limited conditions is referred to as oxygen-limited autotrophic nitrification denitrification (OLAND) (Kuai and Verstraete, 1998). This autotrophic process consumes 63% less oxygen and 100% less biodegradable organic carbon compared to the conventional nitrification-denitrification process (Verstraete and Philips, 1998). The OLAND process was first described for a mixed culture of nitrifying bacteria (Kuai and Verstraete, 1998), but was subsequently examined in more detail in a mixed community of a lab-scale rotating biological contactor (RBC) (Pynaert *et al.*, 2002a, 2000b, 2003, and 2004; Windey *et al.*, 2005).

2.4 Nitrification Principles

Nitrification is a microbiological process that converts ammonium to nitrite and eventually nitrite to nitrate. The process occurs anywhere in the biosphere, provided that the environments are such that the nitrifying bacteria can exist. The nitrification process is very important for the oxygen conditions in soil, streams, lakes and biological wastewater treatment plants (USEPA, 1993a, 1993b and Henze *et al.*, 2002).

The overall stoichiometric reactions in the oxidation of ammonia to nitrate can be written as follows (EPA, 2002):

$$NH_4^+ + 1.5O_2 \rightarrow 2H^+ + H_2O + NO_2^- + 58 - 84 \text{ kcal} \quad (2.1)$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- + 15.4 - 20.9 \text{ kcal} \quad (2.2)$$

Reactions (2.1) and (2.2) are believed to serve as energy-yielding reactions for two autotrophic bacteria, represented by the genera *Nitrosomonas* and *Nitrobacter*, respectively. In soils, streams and treatment plants, with conditions permitting, ammonia and nitrite oxidation can be carried out by a variety of microorganisms, both heterotrophic and autotrophic. The formation of oxidised nitrogen by some *Aspergillus* and *Arthrobacter* species has been demonstrated and this has been shown that heterotrophs contribute significantly to nitrification nature (Brierley and Wood, 2001; Effmert *et al.*, 2012).

On a relative basis, ammonia and nitrite oxidation in nature is carried out mainly by autotrophs of the types *Nitrosomonas sp.* and *Nitrobacter sp.*(De Boer and Kowalchuk, 2001; Effmert *et al.*, 2012); thus, in the literature, the words *Nitrosomonas* and *Nitrobacter* have been used to imply organisms that are capable of oxidising ammonia and nitrite.

If the empirical formulation $C_5H_7NO_2$ for the gross composition of biomass is considered acceptable for ammonia and nitrite oxidisers, the following reactions can be written to represent growth (EPA, 2002):

$$15CO_2 + 13NH_4^+ \rightarrow 10NO_2^- + 3C_5H_7NO_2 + 23H^+ + 4H_2O \quad (2.3)$$

$$5CO_2 + NH_4^+ + 10NO_2^- + 2H_2O \rightarrow 10NO_3^- + C_5H_7NO_2 + H^+ \quad (2.4)$$

On the basis of the representative measurement of the yields and oxygen consumption, the following overall equation for nitrifier synthesis and nitrification has been suggested (EPA, 2002):

$$NH_4^- + 1.83O_2 + 1.98HCO_3^- \rightarrow 0.021C_5H_7NO_2 + 0.98NO_3^- + 1.041H_2O + 1.88H_2CO_3 \quad (2.5)$$

The alkalinity-pH relationship, expressed in Equation (2.5), is particularly important from the viewpoint of wastewater treatment plant operation. As a result of a number of experimental studies, on the suspended and as well as attached-growth systems, the results has found that 6.0 to 7.4 mg alkalinity is destroyed per milligram NH_4^+-N oxidised to nitrate (Henze *et al.*, 2002; EPA, 2002).

2.4.1 Limiting factors of biological nitrification

Parameters affecting nitrifying bacteria or their activity (nitrification) as observed by various researchers are summarised and the circumstance of observation must be considered carefully to achieve the optimal values (Henze *et al.*, 2002; Zhu and Chen, 2002; Zhang *et al.*, 2014).

The most environmental parameters influencing nitrification are the dissolved oxygen (DO) concentration, temperature, pH and free ammonia (FA) and free nitrous acid (FNA) concentration (Zhu and Chen, 2002; Yusof *et al.*, 2010; Zhang *et al.*, 2014). Other nitrogen components such as hydroxylamine also influence nitrification, but to a lower extent. Further, nitrification is partially inhibited by volatile fatty acids (Eilersen *et al.*, 1994) and other organic matter, phosphate and light (Philips *et al.*, 2002). The difference in the sensitivity of ammonium and nitrite oxidisers towards these influences determines whether there will be TNO_2 accumulation in a nitrifying system. Indeed, generally, nitrite oxidisers are more sensitive to detrimental environmental conditions than ammonium oxidisers (Jubany *et al.*, 2008).

2.4.1.1 Dissolved oxygen

Oxygen is utilised in the oxidation reactions carried out by nitrifying bacteria. The stoichiometric quantities of oxygen required according to Equation 2.1 and 2.2 are: 3.43 mg for nitrification of 1 mg NH_3-N and 1.14 mg for nitrification of 1 mg NO_2-N.The theoretical nitrogenous oxygen demand (NOD) is 4.57 mg per milligram of NH_3-N. A factor found interesting by a number of workers is the actual ratio of oxygen consumed to nitrogen oxidised, which is often different than predicted stoichiometrically (Jianlong and Ning, 2004; Guo *et al.*, 2009; Murat Hocaoglu *et al.*, 2011).

The dissolved oxygen concentration is of utmost importance for ammonium oxidising and nitrite oxidising bacteria (Philips *et al.*, 2002; Pynaert *et al.*, 2002a and

2000b). The ammonia oxidisers seem to be more robust towards low DO than nitrite oxidisers. Accumulation of nitrite at low DO is usually explained by the difference in oxygen half saturation constant DO for ammonium oxidisers and nitrite oxidisers (Chandran and Smets, 2001).In other words, oxygen deficiency due to low DO influences the activity of nitrite oxidisers more significantly than that of ammonium oxidisers (Philips *et al.*, 2002). The ammonia oxidiser community in activated sludge was dominated by members of the *Nitrosomonas europea* lineage at a low DO (0.12–0.24 mg/L), while at high DO (up to 8.5 mg/L), members of the *Nitrosomonas oligotropha* lineage were prevalent (Park and Noguera, 2004).

In conventional wastewater treatment, to remove organic and nitrogen compounds, alternating anoxic-aerobic conditions are commonly used, e.g. in flow reactors, with above 2 mg O_2/L in the aerobic tank. Nitrogen removal (nitrification, de-nitrification and biomass synthesis) in a single reactor under the same operating conditions allows for savings in aeration costs for nitrification and limits organic requirements for de-nitrification (Zielińska *et al.*, 2011).

The OLAND process (Oxygen Limited Autotrophic Nitrification – De-nitrification) (Kuai and Verstraete, 1998; Windey *et al.*, 2005; Monballiu *et al.*, 2013) and CANON (Completely Autotrophic Nitrogen removal Over Nitrite) (Sliekers *et al.*, 2002) processes are based on this oxygen limitation. In these systems, the amount of oxidised TAN can be controlled by varying the amount of oxygen supplied to the system.

2.4.1.2 Temperature

Temperature is a key parameter in the nitrification process, but the exact influence is hard to determine because of the interaction between mass transfer, chemical equilibrium and growth rate dependency. A temperature rise creates two opposite effects: increased NH_3 inhibition, as explained above, and activation of the organism according to the Arrhenius principal. This increased activity only holds up to a certain critical temperature, above which biological activity decreases again. Thus, nitrification reactions follow the Van't Hoff – Arrhenius law up to 30°C (Zhu and Chen, 2002; Qiao *et al.*, 2010; Gu *et al.*, 2012; Zhang 2014).

The test of pure culture for *Nitrosomonas* and *Nitrobacter* were reported to be in the highest activity at a temperature of 35°C for *Nitrosomonas* and at 38°C for *Nitrobacter* (Grunditz and Dalhammar, 2001).These optimum temperatures for

experiments of pure culture can be seen in Figure 2.3. This is in agreement with the results of Groeneweg *et al.* (1994), Grunditz *et al.* (1998), Kim *et al.* (2006) and Yang *et al.*, (2007).

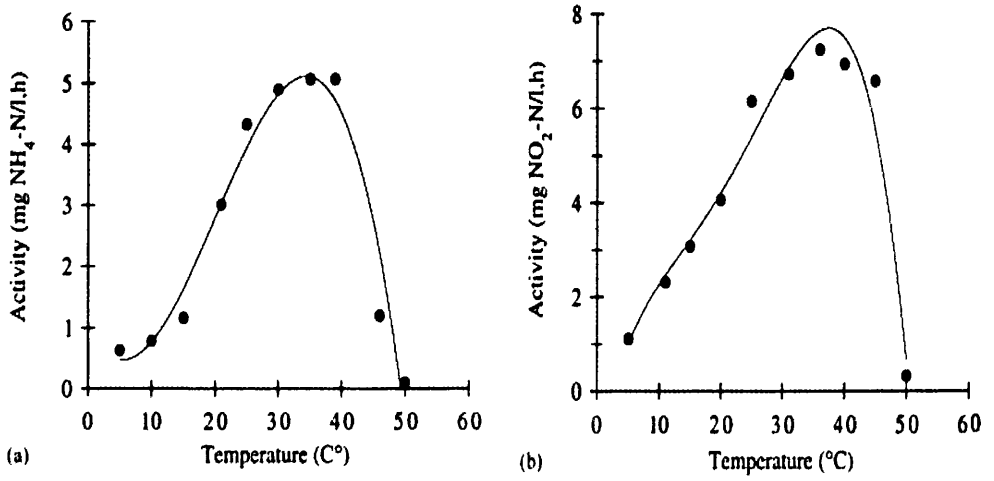


Figure 2.3: a) Effect of temperature on the activity of ammonium oxidisers. b) Effect of temperature on the activity of nitrite oxidisers (Grunditz and Dalhammar, 2001)

The optimal temperature for the activity of ammonium oxidisers was in the range from 35-42°C for sludge cultivated at 30°C; however, the investigation was in the short-term. Long-term exposure to temperatures above 40°C is expected to lead to deactivation (Kim *et al.*, 2006).

2.4.1.3 Effect of pH

Tanwar *et al.* (2008) showed the previous work of numerous researchers on the effect of pH on the short-term activity of pre-grown cells. The pH optima for the overall nitrification reaction appears to be slightly on the alkaline side. As has been summarised by Green et al.(2001 and 2002) the following sources of error must be recognised: i) Local hydrogen ion concentrations change while adjustments are made to the pH level of a culture; and ii) The pH of the system is lowered during nitrification reactions. However, if the system is not buffered, the activity in the system will change, whereas if a buffer is used, the anion may have an influence on culture activity.

Despite a wide divergence of the reported effects of pH on nitrification, there seems to be a consensus that the optimum pH for both ammonium and nitrite oxidisers

lies between 7 and 8. The results from experimental pure cultures from Grunditz and Dalhammar (2001) are depicted in Figure 2.4 for effect of pH on the activity of ammonium oxidisers and nitrite oxidisers.

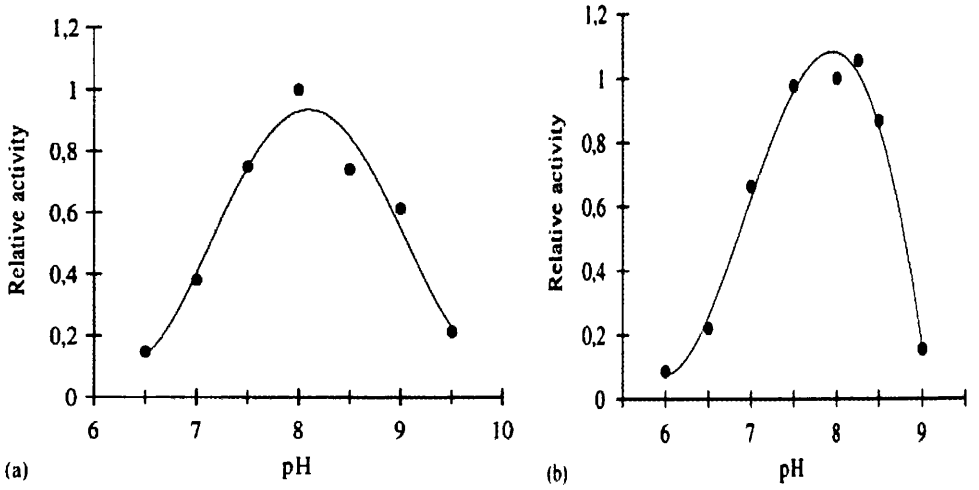


Figure 2.4: Effect of pH on the activity of ammonium oxidisers a) and nitrite oxidisers. b) the activity at pH 8.0 was used as the reference activity (Grunditz and Dalhammar, 2001).

The explanation for the preference of ammonium oxidisers for slightly alkaline environments could be the fact that these organisms use NH_3 as a substrate (Ruiz *et al.*, 2003). This optimum pH range is also linked with the pH-dependent NH_4^+/NH_3 and HNO_2/NO_2^- equilibria, where NH_3 and HNO_2 can exhibit inhibitory effects starting from a certain pH, as explained above (Vadivelu *et al.*, 2006; Gu *et al.*, 2012). Apart from the influence of pH on chemical equilibrium, pure pH effects also exist. Below pH 7, the nitrification rate will decrease, although high nitrification rates at low pH were detected in a fluidised bed reactor with chalk as the biofilm carrier (Tarre *et al.*, 2004). In this system, the chalk probably acted as local buffer system (Green *et al.*, 2001 and 2002; Tarre *et al.*, 2004).

By working at a pH between 7.5 and 8, the growth of ammonium oxidisers is favoured over the growth of nitrite oxidisers because of the substrate availability and NH_3 inhibition (Van Hulle, 2010).

2.4.1.4 Effect of free ammonia (FA) and free nitrous acid (FNA) concentrations

Both *Nitrosomonas sp.* and *Nitrobacter sp.* are sensitive to their own substrate and more so to the substrate of the other (Vadivelu *et al.*, 2007). According to Athonisen *et al.* (1976), as above, the degree of inhibition depends upon the ammonia-ammonium and the nitrite-nitrous acid equilibrium. Other researchers (Ruiz *et al.*, 2003; Vadivelu *et al.*, 2006 and 2007; Jubany *et al.*, 2005) support the suggestion that inhibition is due to free ammonia and un-dissociated nitrous acid; concentrations of these species have significance in the inhibition of nitrification. Jubany *et al.* (2008) also reported that the start-up of biological nitrifying systems to treat high-strength ammonium wastewater must be done carefully to avoid ammonium or nitrite build-up and subsequently system destabilisation due to the inhibition of both substances.

Wide ranges of ammonium and nitrite ion concentrations can be oxidised by the nitrifiers (Qiao *et al.*, 2010; Posmanik *et al.*, 2014). Differences in conditions can account for the apparent discrepancies. Normal ammonia and nitrite ion concentrations in domestic wastewaters are not in the inhibiting ranges (Philips and Verstraete, 2000; Vadivelu *et al.*, 2006). However, substrate and product inhibition are of significance in treatment of industrial, poultry and agricultural wastes (Scaglione *et al.*, 2013; Posmanik *et al.*, 2014).

One mechanism by which the 'free ammonia' (NH_3) and 'free nitrous acid' (HNO_2) affects the pH for the rate of nitrification has been proposed by Anthonisen *et al.* (1976). The hypothesis is based on the fact that ammonia-ammonium and nitrite-nitrous acid equilibrium depends on pH (Vadivelu *et al.*, 2007). To borrow Anthonisen's terminology, both "free ammonia" (NH_3) and "free nitrous acid" (HNO_2) inhibit nitrifying organisms. When the intracellular pH of nitrifying organisms is lower than the pH of the extracellular environment, free ammonia (FA) will penetrate the cell membrane. Ionised ammonia (NH_4^+) is postulated to remain in the extracellular environment. Similarly, when intracellular pH is higher than that of the extracellular environment, free nitrous acid (FNA) permeates the cell, rather than nitrite ions. Anthonisen *et al.* (1976) proposed that the ability of FA and FNA to penetrate the nitrifying organisms makes them inhibitory to ammonium and nitrite ions.

On the basis of batch and continuous studies with several types of wastes, Anthonisen *et al.* (1976) prepared an operational chart for evaluating nitrifying systems.

According to operational charts proposed by Anthonisen *et al.* (1976),which are shown in Figure 2.5, Zone 1: complete inhibition by free nitrous acid, no nitrification; Zone 2: complete inhibition by free ammonia, no nitrification; Zone 3: relatively greater inhibition of nitrite oxidisers and nitrite accumulation; Zone 4: no inhibition; complete nitrification.

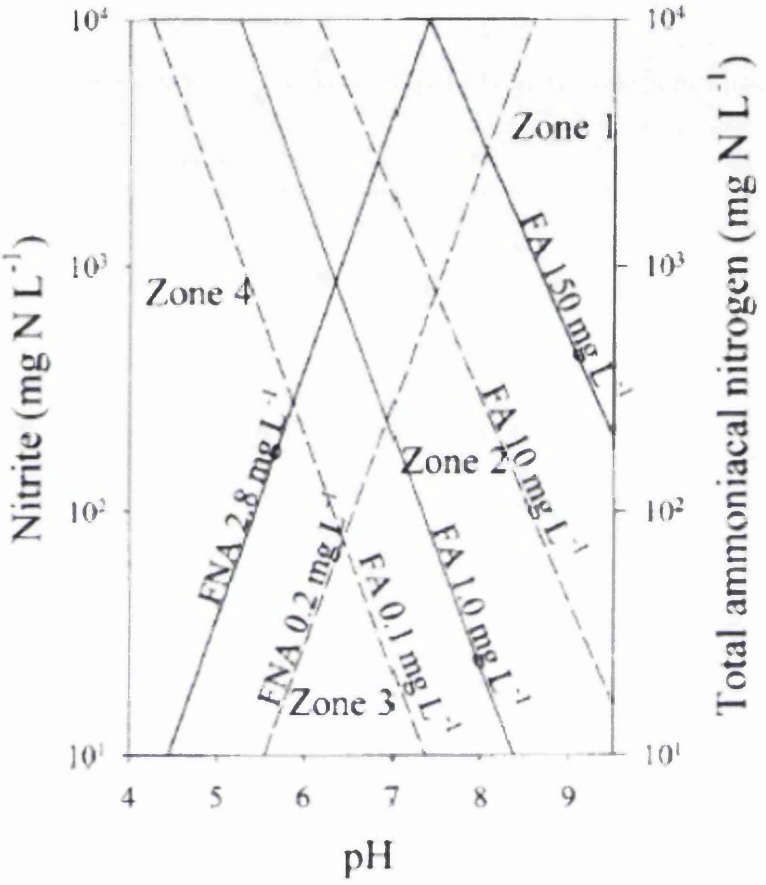


Figure 2.5: Relationship between concentration of ammonia (FA) and nitrous acid (FNA) and the inhibition of nitrifiers at ambient conditions. The dashed lines mark the lower limit and the solid lines mark the upper limit of the range of boundary conditions of zones of nitrification inhibition (Anthonisen *et al.*, 1976). *See text for further explanation.*

2.4.1.5 Concentration of nitrifiers

The number (or mass) of nitrifying organisms influences the rate of nitrification observed (Hanaki *et al.*, 1990 and Fang *et al.*, 2009). The concentration of nitrifying bacteria is estimated by the most probable number technique, where the highest detected MPN counts of ammonia-oxidising bacteria (AOB) were 500 MPN/cm^2 and 1.0 x 10^6

MPN/cm^2, and those of nitrite-oxidising bacteria (NOB) were 96 MPN/cm^2 and 2.2 x 10^3 MPN/cm^2, respectively, in the biofilms of drinking water (Lipponen *et al.*, 2004). There is a paucity of quantitative information on this point, perhaps due to difficulties in enumerating nitrifiers. Much of the available data, with some exceptions, is on the basis of volatile suspended solids (VSS) and the spatial distribution of nitrifying populations by quantification using oxygen uptake rates (OUR) (Fdz-Polanco *et al.*, 2000). However, with new incoming technology for image analysis, the quantification of nitrifying bacteria can be done by using Fluorescence in Situ Hybridisation (FISH) and confocal laser scanning microscopy (CLSM). The disadvantages of these new technologies are that this image analysis is very time-consuming and expensive, but these have been overcome by a rapid method that has been recently developed (Manser *et al.*, 2005).

Lee and Oleszkiewicz (2003) reported that the initial rate of ammonia oxidation increased with increasing initial seed concentration in VSS. The time required for a given degree of ammonia removal was inversely proportional to the concentration of nitrifiers present. Similarly, Lee *et al.* (2002) found that the time to completely nitrify a given amount of ammonia-nitrogen per gram of mixed-liquor VSS (MLVSS) was constant, given the same environmental conditions with the maximum value of 7100 mgMLVSS/L for the biofilm that was formed in the hybrid biological reactor using powdered minerals.

The rate of nitrification probably does not remain first-order with respect to nitrifier concentration and also depends on the ratio of ammonia-to-nitrite oxidisers. Typical values for the rates of nitritification-nitratification, expressed as mg N oxidised/g cells/h at 20°-30°C are in the range of 110-250 for pure cultures and 0.5-6 for activated sludge-type systems (EPA, 2002), although rates up to 20-25 were observed in one case (EPA, 2002).

The influence of salt has been demonstrated in partial nitrification in a sequencing batch reactor treating saline sewage with the microbial population tests, supporting that NOB is strongly inhibited in salt contents below 1% (w/v) (Ye *et al.*, 2009).A similar kinetics behaviour pattern of nitrifiers was observed in the enriched cultures of nitrifiers tested in sequencing batch reactors with high concentrations of salt (NaCl); these ascertained that the ammonia oxidation rate has a Monod-type relationship (Moussa *et al.*, 2006). *Nitrosomonas europaea* and *Nitrobacter sp.* (fluorescent in situ hybridisation) were the only nitrifiers present at high salt levels, whereas the increased salt concentrations resulted in better settling characteristics of the nitrifying sludge (Moussa *et*

al., 2006). This situation showed that the nitrifiers are more resilient and capable of growing under extreme conditions.

The influence of nitrifier concentrations of nitrification has added significance in the case of biochemical oxygen demand (BOD) tests. The nitrification can be done by autotrophs under aerobic conditions and de-nitrification by heterotrophs under anaerobic conditions. However, recent investigations have shown that some bacteria are capable of combining heterotrophic nitrification and aerobic de-nitrification. These microorganisms could be a potential breakthrough in biological nitrogen removal systems (Ahmad *et al.*, 2008; De Gusseme *et al.*, 2009; Chen and Ni, 2012; Yongkang *et al.*, 2012; Yao *et al.*, 2013).Several bacteria strains have been identified; for example, the heterotrophic nitrifying strain W1, identified as *Alcaligenes sp.* (Yonkang *et al.*, 2012), the ammonium removal by *Agrobacterium sp.* LAD9, which is capable of heterotrophic nitrification and aerobic de-nitrification (Chen and Ni 2012), heterotrophic nitrifying and aerobic denitrifying bacteria strain CF-S9 of *Klebsiella pneumoniae* for the bioremediation of nitrogenous compounds from domestic wastewater (Padhi *et al.*, 2013), and heterotrophic nitrification and aerobic de-nitrification by an isolated bacterium of *Acinetobacter sp.* HA2 (Yao *et al.*, 2013).

The disappearance of ammonia-nitrogen and co-metabolic activities of autotrophic and heterotrophic nitrifying bacteria in the biodegradation of emerging trace organic contaminants (EOCs) has raised numerous concerns among researchers. The metabolism of EOCs can only be observed by heterotrophic nitrifying bacteria and for autotrophic ammonia oxidising bacteria (AOB); a variety of EOCs are co-metabolised via non-specific enzymes, such as ammonia monooxygenase (AMO). Higher biodegradation of EOCs is often noted under nitrification at a high ammonia loading rate. The presence of a growth substrate, ammonia-nitrogen, promotes co-metabolic biodegradation of EOCs (Tran *et al.*, 2013).

High density of nitrifying bacteria populations increased the nitrification process in optimised conditions with less inhibitory effects (Chandran and Smets, 2005). The nitrifying bacteria appear to be influenced by the start-up period conditions, whereas the inhibition factor was caused by the free ammonia; however, the colonisation of the nitrifying bacteria in submerged nitrifying bio-filters showed the increasing activity after the steady state, even when in high concentrations of free ammonia (Villaverde *et al.*, 2000).

2.4.1.6 Sludge age, organic loading and detention time

In the operation of suspended-growth treatment plants, several operational parameters indirectly reflect the effect of nitrifier concentrations on nitrification. Among these are the sludge age, retention time and organic loading required to achieve a given degree of nitrification. With other parameters (temperature, pH, dissolved oxygen, etc.) held constant, the degree of nitrification decreases significantly with an increase in organic loading (Jubany *et al.*, 2008). Similarly, a sludge age of approximately three to four days appears to be required in most suspended-growth systems to achieve a high degree of nitrification (Fang *et al.*, 2009).

In itself, retention time is not an inclusive parameter; the value depends upon a number of system parameters. High detention times may be needed if: i) the concentration of nitrifying bacteria is low, ii) sludge age is low, or iii) system temperature is low (Henze *et al.*, 2008). Operationally, the retention times employed for nitrification in suspended-growth systems are usually greater than those in attached growth systems. This is easily understood because the basic issue is whether there are enough nitrifiers in the system at a given time, on an absolute basis or relative to heterotrophs, notwithstanding the presence of inhibitors. This is reflected in the solid retention time or sludge age, but not by the hydraulic retention time. The latter and organic loading are the only tools to achieve the desired sludge age and, hence, the concentration of nitrifiers (Grady *et al.*, 2011). In attached growth systems, the same point is seen in the form of filter start-up time in the range of 25 to 30 days and above (Ritmann and McCarty, 2001).

High hydraulic retention time must be adopted in treating organic and nitrogenous compounds in a single activated sludge system compared to the biofilm process due to the presence of toxic and inhibitory compounds (Kumar *et al.*, 2000; Li *et al.*, 2011). A high sludge recycle ratio has to be adopted in activated sludge nitrification systems to keep a high biomass concentration in the reactor. However, high sludge recycling often leads to the growth of filamentous bacteria and sludge bulking in the activated sludge nitrification system and the fast growth of competitive bacteria, which the nitrifying bacteria easily wash out from the activated sludge system (Kim *et al.*, 2007; Li *et al.*, 2011).

A recent revolution of granular sludge technology as a novel promising wastewater treatment process has shown better performance compared to the conventional activated sludge process. However, this technology has a drawback that needs to be overcome by the start-up period that needs to be enhanced in adjusting the

settling time and food/microorganism (F/M) ratio (Fang *et al.*, 2009; Liu *et al.*, 2011). Alternatively, more robust technology of the biofilm process has been proven to be more reliable for organic carbon and nitrogen removal without some problems faced by the activated sludge system (Yang *et al.*, 2000; Wang *et al.*, 2012).

2.4.1.7 Effect of light

In drinking water distribution systems, ammonia-nitrogen is deliberately added in a chloraminated water supply as a result of natural processes. Monochloramine is an inorganic compound with the formula NH_2Cl (EPA, 2002). Although monochloramine will degrade when exposed to the atmosphere at varying rates, depending on the amount of sunlight, wind and temperature (Wilczak, 2001), nitrifiers are very sensitive to near UV, visual, and fluorescent light. Consequently, nitrification episodes in distribution systems occur in the dark, for example in covered reservoirs, pipelines and taps (Wolfe *et al.*, 2001). However, the nitrifiers have an excision repair mechanism for DNA repair; therefore, low levels of nitrifiers may be recovered from partially shaded reservoirs or channels (Wolfe *et al.*, 2001)

High light intensities have been identified to be a major factor inhibiting nitrification in a wastewater reservoir during summer weather compared to the cold winter weather, along with other parameters of high temperatures and high concentrations of free ammonia (FA),causing the pH value to become acidic. All of these factors contributed to the disruption of the balance between the two steps of the nitrification process, resulting in high nitrite accumulation (Kaplan *et al.*, 2000).

The investigation performed by Guerrero and Jones (1996) showed that the nitrite oxidising bacteria (NOB) were less sensitive to blue light spectrum (400 to 475 nm) inhibition than ammonium oxidising bacteria (AOB). Furthermore, cool-white fluorescent light inhibited the activity of ammonium oxidising bacteria. In characteristics, the extent of photo-inhibition is dependent on the wavelength and is species-specific. However, from the data gathered by Guerrero and Jones (1996), exposure to sunlight resulted in the inhibition of activity in both types of nitrifying bacteria. These data demonstrate that the effect of light on autotrophic nitrification depends not only on the type of nitrifying bacteria (AOB and NOB), but also on the conditions of their environment.

2.4.1.8 Micronutrients

The apparent inconsistencies in results obtained by workers in the field of nitrification may be related to the presence or absence of micronutrients (or micro-inhibitors). There have been a number of attempts to identify substances that stimulate or inhibit the growth or activity of nitrifiers. Whether slow growth and low yield of cells per unit of energy source oxidised, i.e. free-energy efficiency, are inevitable results of the autotrophic mode of life is not certain (Grady *et al.*, 2011).

The degree of effectiveness of an inhibitor is affected by: i) presence of microorganisms other than nitrifiers, ii) concentration of the inhibitor, iii) duration of exposure, i.e. whether the nitrifiers are in a batch or continuous system, and v) presence of other inhibitors; e.g. copper may void the inhibition due to thiourea, and mercury due to mercaptobenzthiazole (McCurry, 2006; Henze *et al.*, 2008).Metals can inhibit the nitrification, but this is due to the difference between pure culture and activated sludge cultures (Henze *et al.*, 2002). The nitrifying bacteria cannot be expected to be less susceptible to the influence of metals, as the metal ion activities in the liquid phase and in the sludge phase are very different; the bacteria in sludge cultures can resist higher metal ion concentrations, as shown in Table 2.2 (Henze *et al.*, 2002).

Table 2.2: Inhibition of nitrification with metals (Henze *et al.*, 2002)

Metal	Concentration (g/L)	Effect
Copper (Cu)	0.05-0.56	*Nitrosomonas* activity inhibited (pure culture)
Copper (Cu)	4	No essential inhibition in activated sludge
Copper (Cu)	150	75% inhibition of activated sludge
Nickel (Ni)	> 0.25	*Nitrosomonas* growth inhibited (pure culture)
Chromium (Cr)	> 0.25	*Nitrosomonas* growth inhibited (pure culture)
Chromium (Cr)	118	75% inhibition of activated sludge
Zinc (Zn)	0.08-0.5	*Nitrosomonas* growth inhibited (pure culture)
Cobalt (Co)	0.08-0.5	*Nitrosomonas* growth inhibited (pure culture)

A list of some organic compounds that may have inhibitory action to the nitrifying bacteria has been identified (WEF, 2005). In the case of cystine and cysteine, and to a lesser degree methionine, the result has been shown that allylthiourea, diethyldithiocarbamate, ethyl xanthane and a host of other compounds are potent

inhibitors of ammonia oxidation by *Nitrosomonas* but do not inhibit the oxidation of hydroxylamine. Hydroxylamine exhibited acute toxicity to *Nitrobacter* and this may also cause TNO_2 build-up in a nitrifying system (Hu *et al.*, 2000).

The nitrification process can be inhibited by the influence of liberated silver from silver nanoparticles. The addition order of Ag-NP and the media constituents had a profound influence on the stability of the Ag-NP suspension and the corresponding repeatability of results and sensitivity of *Nitrosomonas europaea.* This ammonia oxidising bacteria (AOB) was found to be extremely sensitive to ionic silver (Ag^+) and two sizes of Ag-NPs (20 and 80 nm). The metal, Ag^+ exposures resulted in the highest level of toxicity with smaller Ag-NPs (20 nm) being more toxic than larger Ag-NPs (80 nm) (Radniecki *et al.*, 2011).

The influence of cadmium (Cd) in nitrification inhibition was investigated in batch-suspended growth activated sludge systems, which contain biomass enriched with nitrifying bacteria (Semerci and Cecen, 2007).The cadmium metal was adjusted with a strong complexing agent, ethylenediaminetetraacetic acid (EDTA). The presence of EDTA decreased nitrification inhibition by lowering the available cadmium species and preventing the biosorption of cadmium. The observation of almost complete recovery from inhibition was attained by EDTA addition to nitrifying bacteria which were inhibited by cadmium for a certain time (Semerci and Cecen, 2007).

The toxicity of copper (II) ions to microorganisms in biological wastewater treatment systems has been studied by Ochoa-Herrera *et al.* (2011), who found that the denitrifying and nitrifying bacteria showed the considerable recovery of their metabolic activity after only several days of exposure to high copper levels (up to 25 and 100 mg Cu(II)/L for de-nitrification and nitrification processes, respectively). The recovery of both denitrifying bacteria and nitrifying bacteria could be due to the attenuation of soluble copper or to microbial adaptation (Ochoa-Herrera *et al.*, 2011).

2.4.1.9 Organic matter as an inhibitor

The batch test experiment was performed by Leu *et al.* (1998) to examine the effects of organic matter, with and without the addition of glucose on variations of nitrogenous compounds. The experimental results indicated that increasing the organic matter concentration decreased the concentrations of nitrate and total nitrogen with an

enormous build-up of nitrite occurring in the water. This showed that high organic matter inhibited the nitrification process.

In a column experiment of a tidal flow constructed wetland system for the removal of ammonia-nitrogen, result investigation has demonstrated a high content of organic matter in the wastewater may have inhibited nitrification. The oxygen available in the wetland is mostly used by heterotrophic microorganisms to remove organic matter and significant nitrification cannot take place until the BOD_5 drops to 200 mg/l (Sun *et al.*, 2005). This indicated that the inhibitory effect of organic matter on nitrification may be due to dissolved oxygen concentration limitations and the situation seems possible due to localised competition between nitrifiers and common heterotrophs (Stuven *et al.*, 1992).

In the shortcut nitrification of the sequence batch reactor (SBR), the result was shown that the competition of aerobic bacteria was increased and the degradation rate of ammonia-nitrogen was reduced in the system with an increasing chemical oxygen demand (COD) associated with the high content of organic matter concentration and the loading of sludge (Tang *et al.*, 2010).

2.5 Endocrine Disruptive Compounds

Endocrine disrupting compounds (EDCs) are chemicals with the potential to elicit negative effects on the endocrine systems of humans and wildlife. In the past few decades, research efforts to combat this problem have grown immensely. Key to the solution for this problem is the identification of EDCs, the accurate measurement of their presence in aquatic systems, and the development of methods for their elimination from the environment (Liu *et al.*, 2009).

Various natural and synthetic chemical compounds have been identified that induce oestrogen-like responses, including pharmaceuticals, pesticides, industrial chemicals, and heavy metals (Giesy *et al.*, 2002). EDCs have been attributed as a cause of reproductive disturbance in humans and wildlife (Hayes *et al.*, 2002; Oak *et al.*, 2004; Samir *et al.*, 2006; Campbell *et al.*, 2006). Human exposure to these chemicals in the environment is a critical concern with unknown long-term impacts. Natural and synthetic EDCs are released into the environment by humans, animals and industry; mainly through sewage treatment systems, before reaching the receiving bodies (soil, surface water,

sediment and ground water). The main distribution of EDCs in the environment is illustrated in Figure 2.6 (Fleming and Bent, 2003).

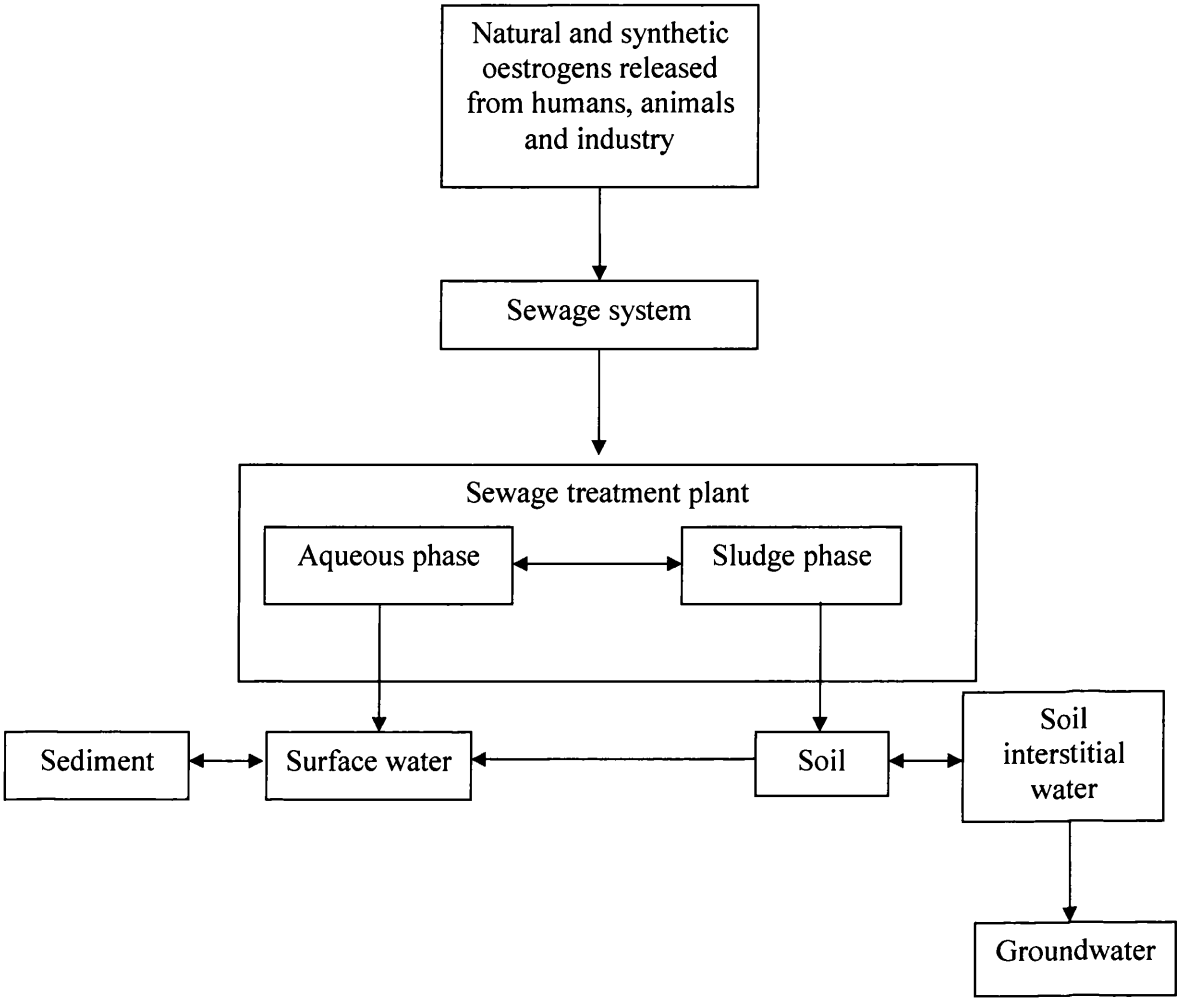


Figure 2.6: EDCs distribution in the environment (Fleming and Bent, 2003).

2.5.1 Definition and characteristic of EDCs

The US Environmental Protection Agency (USEPA) defines an EDC as an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour (USEPA, 1997).

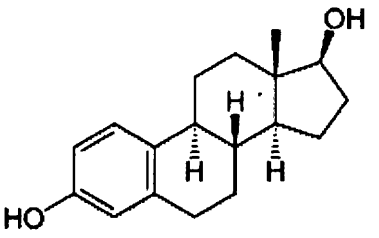
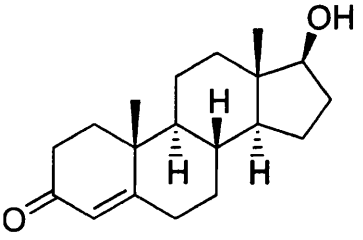
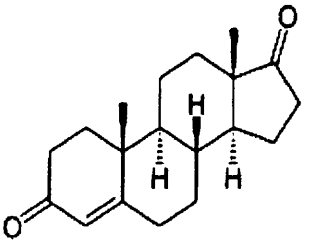
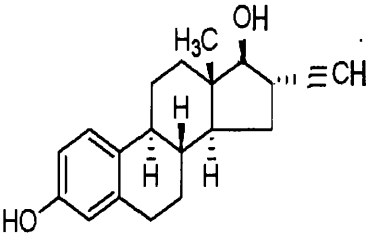
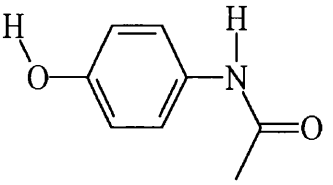
Oestrogens are a group of steroid hormones defined by their chemical structure and by their effect on the oestrous cycle. They act as endocrine disruptors (i.e., substances that interfere with the endocrine system) and disrupt the physiological functions of

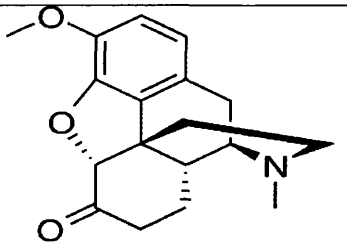
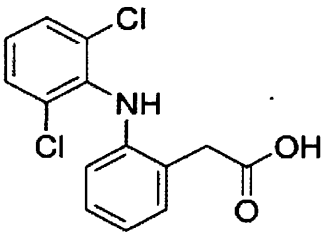
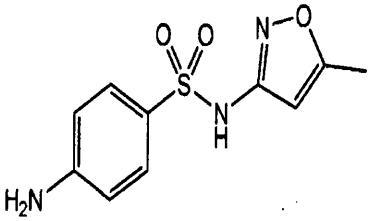
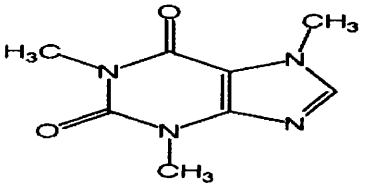
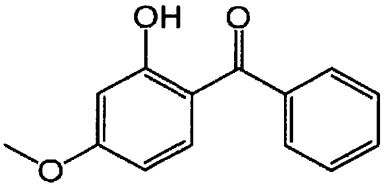
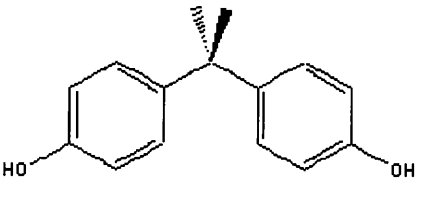
hormones. Oestrogens have moderate to high hydrophobicity, with an octanol-water partition coefficient generally in the range 3-6 (Gabet *et al.*, 2007).

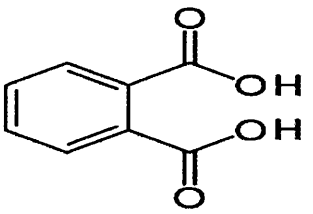
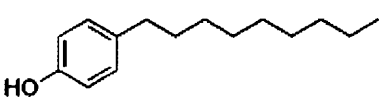
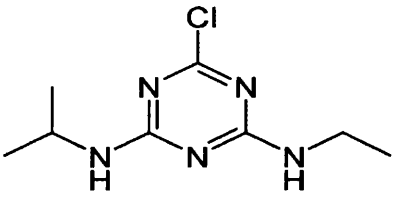
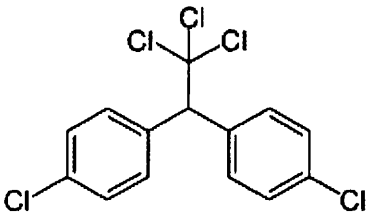
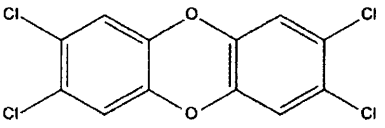
The primary effects of EDCs, as described earlier, are either mimicking the inhibition of the behaviour of natural hormones, such as oestrogens, testosterone and/or thyroid hormones. Depending on the endocrine endpoints, they can be oestrogenic, androgenic, or thyroidal compounds (Chang et. al 2009). Although the disruption of the androgen and thyroid functions might exert greater or equal impacts on the environment, most research studies so far have focused on oestrogenic EDCs (Chang *et al.*, 2009).

In Chang *et al.* (2009), the molecular structures of several EDCs, with accompanying varying functionalities, were characterised, and are summarised in Table 2.3. They are broadly classified into several categories, such as hormones (natural and synthetic oestrogens or steroids), pharmaceuticals and personal care products (PPCPs), industrial chemicals, pesticides, combustion by-products, and surfactants (Campbell *et al.*, 2006; Giesy *et al.*, 2002).The EDCs shown in Table 2.3 have at least one aromatic structure in their molecular structures. There is a possibility that the hydrophobic properties might comprise an important characteristic in studying and controlling EDCs in both natural and engineered environments (Chang *et al.*, 2009).

Table 2.3: Examples of various types of EDCs classified (Chang *et al.*, 2009)

Class (use)	Compound	Structure
Steroid (Oestrogen)	Oestradiol	
(Androgen)	Testosterone	
	Androstenedione	
(Synthetic oestrogen)	Ethynylestradiol	
Pharmaceutical (Analgesic)	Paracetamol	

	Hydrocodone	
(Anti-arthritic)	Diclofenac	
(Antibiotic)	Sulphamethoxazole	
Personal care product (Stimulant)	Caffeine	
(Sun screen)	Oxybenzone	
Industrial chemicals (Plastics)	Bisphenol A	

	Phthalate	
(Surfactant)	Nonylphenol	
Pesticides (Pesticide)	Atrazine	
	DDT	
Combustion by-product	Dioxin	

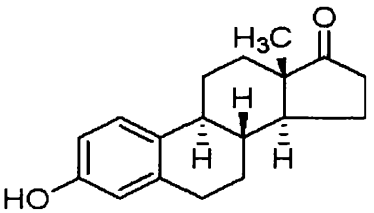
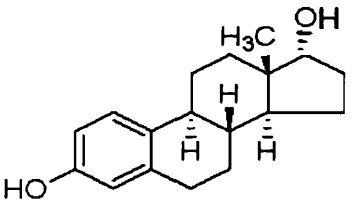
2.5.2 Toxicological effects and determination of EDC

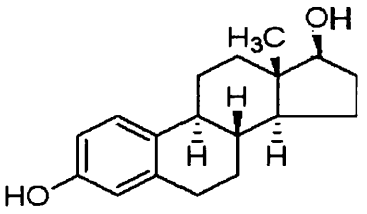
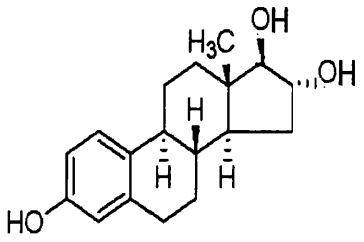
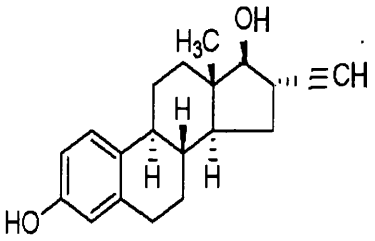
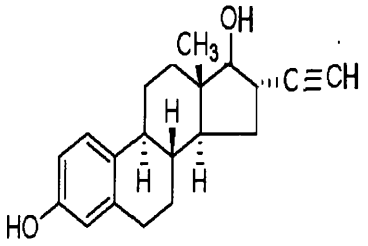
The adverse effects of EDC regarding reproductive health in humans and wildlife have become a major concern among the public (Chang *et al.*, 2009).The correlation between exposure to EDC and the health of humans and wildlife, including any unknown long-term impacts, is a very complicated and controversial issue which is difficult to confirm. The actual effects of exposure to EDC, based on toxicological tests, have been reported (Chang *et al.*, 2009).

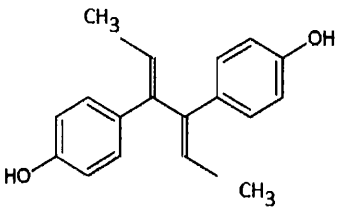
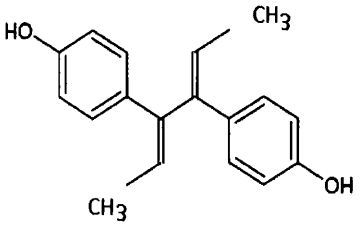
Diethylstilbestrol is one of several EDCs which have been prescribed as orally active synthetics of non-steroidal oestrogens. The compound of diethylstilbestrol may cause decreased sperm counts in human males (Sharpe *et al.*, 1993). However, these results were slightly suspicious because they have not been confirmed by further studies (Itoh *et al.*, 2001; Mantovani *et al.*, 2005). On the other hand, similar effects caused by other EDCs have been encountered in different regions, and the trend of reanalysed data for male sperm counts showed a decline in sperm density in the United States and Europe (Swan *et al.*, 1997). A correlation between lower sperm counts and high concentrations of polychlorinated biphenyl (PCB) was found in blood serum studies conducted in the Netherlands (Dallinga, 2002).

Many human tissues show oestrogen receptor sites, including the brain, immune system, cardiovascular system, lungs, mammary glands, liver, kidneys, reproductive tract (ovaries, testes, uterus, prostrate), adipose tissue, and bones (Müller, 2004).The transport of EDCs to offspring has also been reported through a study of rat tissues (Kuriyama *et al.*, 2007).Other detailed discussions on a variety of aspects of human health, affected by exposure to EDCs, have been reported by other studies (Chang *et al.*, 2009). Table 2.4 showed the chemical structures of oestrogens of interest (Gabet *et al.*, 2007).

Table 2.4: Chemical structures of oestrogens of interest (Gabet *et al.*, 2007).

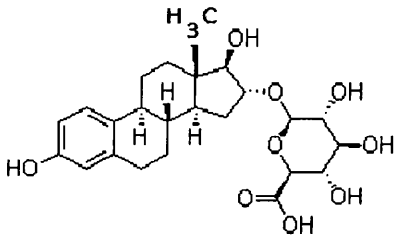
Oestrogen	Abbreviation	Chemical Structure	Examples of conjugated forms
Oestrone	E1	Natural oestrogenic compounds 	Oestrone-3-sulphate Oestrone-3-glucuronide
17α-Oestradiol	αE2		Oestradiol-3-sulphate Oestrone-3-glucuronide Oestradiol-17-sulphate Oestradiol-17-glucuronide

17β-Oestradiol	βE2		Oestradiol-3-sulphate-17-glucuronide Oestradiol-3-glucuronide-17-sulphate Oestradiol-3,17-disulphate Oestradiol-3,17-diglucuronide
Oestriol	E3		Oestriol-3-glucuronide Oestriol-16-glucuronide Oestriol-17-glucuronide Oestriol-3,17-disulphate Oestriol-3-sulphate-17-glucuronide
17α-Ethylnylestradiol	EE2	Synthetic oestrogenic compounds 	Ethynylestradiol-3-glucuronide Ethynylestradiol-3-sulphate
Mestranol	MeEE2		

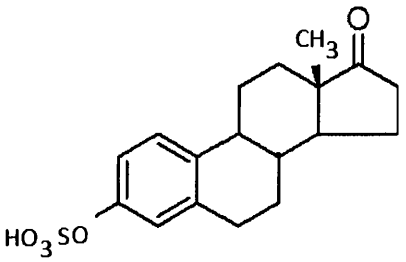
Dienestrol	DIE		
Diethylstilbestrol	DES		

Structure of two conjugated forms:

Oestriol-16-glucuronide



Oestriol-3-sulphate



2.5.3 17α-Ethinylestradiol

17α-ethinylestradiol (EE2), is a derivative of oestradiol.17α-ethinylestradiol is an orally bio-active oestrogen used in almost all modern formulations of combined oral contraceptive pills and it is one of the most commonly used medications (Gabet *et al.*, 2007). The occurrence of this type of endocrine-disrupting chemical in the environment has led to a growing awareness that both animals and humans may be adversely affected, leading to cancer, reproductive tract disorders, reduced sperm counts, and a reduction of reproductive fitness (Noppe *et al.*, 2005). Mestranol is the 3-methylether of ethinylestradiol. Mestranol is often used in many oral contraceptives.

From the large group of substances that are suspected or known to be endocrine disruptors, natural and synthetic oestrogens have been reported as compounds with high potent oestrogenic properties. The latter are used in birth-control pills and for the management of menopausal syndromes and cancers (Noppe *et al.*, 2005).

The compounds 17α-oestradiol (aE2), 17β-oestradiol (bE2) and oestrone (E1) are natural female sex hormones produced by humans, mammals, and other vertebrates (Vethaak *et al.*, 2005; Belfroid *et al.*, 1999; Noppe *et al.,* 2005).These oestrogens are lipophilic, fat soluble molecules. They are excreted unchanged or, mainly, as water-soluble inactive polar glucuronates or sulphate conjugates (Ternes *et al.*, 1999a; Noppe *et al.,* 2005). Under experimental conditions, these conjugates are quickly hydrolysed, leading to the free hormones or their metabolites (Vethaak *et al.*, 2002; Lintelmann *et al.*, 2003; Noppe *et al.,* 2005). On the basis of current evidence, degradation in the environment is expected to take several days when circumstances are optimum, or to be far slower in less ideal circumstances (Vethaak *et al.*, 2002; Fine *et al.*, 2003; Noppe *et al.,* 2005).

Oestrogens enter environmental compartments directly or after they have passed through wastewater treatment plants (WWTP) (Lòpez *et al.*, 2001; Noppe *et al.,* 2005). Once in the environment, they can undergo degradation or transfer processes or can be distributed between the environmental compartments water, sediment, suspended matter and animals (Vethaak *et al.*, 2002; Lintelmann *et al.*, 2003; Noppe *et al.,* 2005).

A quantitatively important source of natural oestrogens is livestock husbandry. These animals are often kept at one site, which results in sewage and manure that contains high concentrations of sex steroids which, depending on the source, enter the environment by different pathways (Lintelmann *et al.*, 2003; Fine *et al.*, 2003; Noppe *et al.,* 2005).

Pharmaceuticals, personal care products, disinfection by-products, and many household and industrial chemicals are often recalcitrant and not sufficiently removed in conventional WWTPs. This potential hazard has been studied extensively in recent years (Garric *et al.*, 1996; Forrez *et al.*, 2009).

EDCs are of increasing concern for the environment, since these have been known for some decades to cause feminisation of aquatic organisms (Rodgers *et al.*, 2000). The major contributor is 17α-ethinylestradiol, the synthetic contraceptive pill hormone which is one of the most important oestrogenic compounds presents in WWTP effluents (Forrez *et al.*, 2009). As for this growing problem, this research will focus on ways to minimise this type of synthetic drug in the wastewater system. The idea is to study the maximum capacity of the nitrifier to co-metabolism of both ammonia-nitrogen and 17α-ethylestradiol in wastewater synthetic medium in the single reactor.

2.5.4 Removal of Endocrine Disrupting Compounds

There are various treatments that can be applied to remove EDCs; separation and oxidation techniques have been considered potential treatment options to effectively remove EDCs. The processes of separation, adsorptive removal and biological conversion have been discussed in Chang *et al.* (2009).

2.5.4.1 Removal of EDCs by physical methods

The use of activated carbon (AC) is a well-known process for removing various organic contaminants. AC is most commonly applied as a powdered feed (powder activated carbon, PAC) or in a granular form (granular activated carbon, GAC) in packed bed filters (Liu *et al.*, 2009).Several authors have demonstrated the efficiency of AC, both as PAC and GAC, for the removal of trace organic pollutants from water (Matsui *et al.*, 2000; Asada *et al.*, 2004; Westerhoff *et al.*, 2005; Zhou *et al.*, 2007).

In the past few years, many researchers have demonstrated that AC also has a strong capability of removing a broad range of representative EDCs for artificial and real wastewater in the laboratory as well as in pilot and full–scale plants (Nakanishi *et al.*, 2002; Iwasaki *et al.*, 2001; Zha and Wang, 2005; Choi *et al.*, 2005; Fukuhara *et al.*, 2006; Tsai *et al.*, 2006; Snyder *et al.*, 2003 and 2007).

2.5.4.2 Rejection by membranes

Severe deterioration of the adsorption capacity of activated carbon (AC) was found in the effluent of WWTP by Snyder *et al.* (2007), when the EDC removal efficiencies of effluent of WWTP were compared to those of surface water. To overcome the problem of deterioration of AC adsorption on complex wastewater systems, one of the effective alternatives is the combination of AC with membrane filtration.

The membrane process is gaining wide use for contaminant removal in advanced water and wastewater treatments. Compared to conventional processes, the membrane process has a high advantage in the quality of effluent. There is a lot of research data on membrane processes for the effective removal of organic micro-pollutants from pesticides to pharmaceuticals and personal care chemicals (Van der *et al.*, 1998; Snyder *et al.*, 2007).

2.5.4.3 Removal of EDC by biodegradation

The objective of wastewater treatment systems is to remove organic substances, such as phosphorus and nitrogen, but recent research has discovered that EDCs can also be reduced by wastewater treatment systems (Liu *et al.*, 2009). Among wastewater treatment systems, the activated sludge process is the most widely used, as the proportion of removal by primary settling, chemical precipitation, aerating volatilisation and sludge absorption was small; the majority of EDCs in wastewater are regarded as removed by biodegradation (Svenson *et al.*, 2003; Andersen *et al.*, 2003; Braga *et al.*, 2005).

With regard to the progress of the chemical detection of EDC in wastewater effluents, numerous researches have turned to the biodegradation of EDC in wastewater treatment systems (Liu *et al.*, 2009).In early 1999, EDC removal via the activated sludge process was studied in Germany, Canada and Brazil, and was evaluated by GC-MS/MS. The removal efficiency for E1, E2 and EE2 was 83%, 99.9% and 78%, respectively (Ternes *et al.*, 1999a).

In 2003, the investigation of chemical precipitation in 20 wastewater treatment plants in Sweden was done by Svenson *et al.* (2003). From these observations, the activated sludge process gave the highest oestrogenic removal and trickling filters were better than chemical precipitation; the corresponding mean removal rates were 81% for E1, 28% for E2 and 18% for EE2 (Svenson *et al.*, 2003).

Andersen *et al.* (2003) investigated the fate of E1, E2 and EE2 at one German sewage treatment plant. They observed that an overall elimination efficiency of E1 and E2 was above 98%, while EE2 elimination was slightly lower. About 90% of E1 and E2 were found to be degraded in the activated sludge system, while EE2 was primarily degraded only in the nitrifying tank. This was proven by Yi and Harper (2007), who reported that the biotransformation rate of EE2 had a linear relationship with the NH_3-N biotransformation rate in a series of batch experiments.

2.6 Attached Growth Processes

There are three general classes for the attached growth processes which were discussed thoroughly in Metcalf and Eddy (2004). The three classes are non-submerged attached growth processes, suspended growth processes with fixed-film packing and submerged attached growth aerobic processes.

2.6.1 Submerged attached growth processes

In the early 1970s and the 1980s, aerobic attached growth processes became established alternatives for biological wastewater treatment. These are up flow and down flow packed-bed reactors and fluidised reactors that omitted secondary clarification. Their advantage is a small footprint with an area requirement that is a fraction (one-fifth to one-third) of that needed in activated-sludge treatment. This process is suitable for use as a tertiary treatment, especially for nitrification and de-nitrification following suspended or attached growth nitrification (Grady *et al.*, 2011).

2.6.2 Moving-bed biofilm reactor (MBBR)

A moving-bed biofilm reactor has been developed by a Norwegian company, Kaldnes Miljøteknologi. The process consists of adding small cylindrical-shaped polyethylene carrier elements with a specific density of 0.96 g/cm^3 in aerated or non-aerated basins to support biofilm growth. The small cylinders are about 10 mm in diameter and 7 mm in height with a cross inside the cylinder and longitudinal fins on the outside (Ritmann and McCarty, 2001).

The biofilm carriers are maintained in the reactor by a perforated plate in the outlet tank. Air is introduced to the bottom of the reactor, mixing the reactor contents and keeping the carriers, which have a density close to water, in suspension and continuous movement. The packing may fill 25% to 50% of the tank volume and the specific surface area is about 500 m^2/m^3 of bulk packing volume. The MBBR does not require any return activated-sludge flow or backwashing. Figure 2.7 shows the moving-bed-biofilm reactor (MBBR) concept and an illustration of carrier types (Ødegaard *et al.*, 2000). A final clarifier is used to settle sloughed solids (Ritmann and McCarty, 2001).

The MBBR process provides an advantage for plant upgrading by reducing the solids loading on existing clarifiers (Rusten *et al.*, 1995; 1998; 1999 and 2006). This presence of packing materials discourages the use of more efficient fine bubble aeration equipment, which requires periodic drainage of the aeration and removal of packing for diffuser cleaning (Grady *et al.*, 2011).

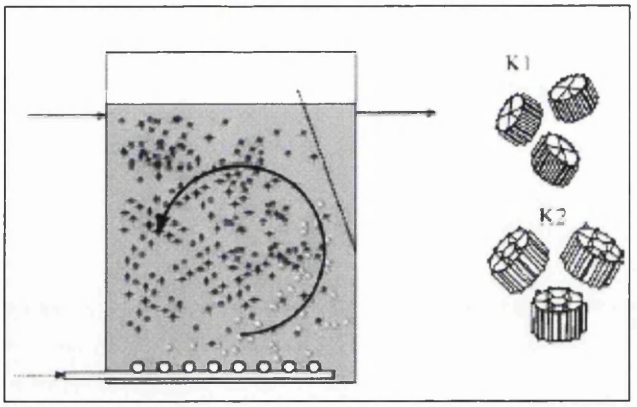


Figure 2.7: The moving-bed-biofilm reactor (MBBR) concept and illustration of carrier types (Ødegaard *et al.*, 2000).

2.6.2.1 Principles of moving-bed biofilm reactor (MBBR)

The principle with moving-bed biofilm reactor (MBBR) brings the following advantages compared to conventional biological methods: i) the amount of active biomass brought into contact with the wastewater is very high; ii) the biological process is very fast, thus a very short retention time is needed, which gives small plants with low investment costs; iii) the process is in contrast to activated sludge which needs pre-sedimentation that is not disturbed by solid particles in the wastewater; iv) the process is insensitive to disturbances and variations in the incoming wastewater - in the MBBR, the biological reduction normally reaches full capacity in less than 24 hours by the accidental elimination of bacteria compared to the activated sludge process which takes around 1-2 weeks; v) there is no risk of clogging, as in the fixed-bed processes; vi) there is no circulating sludge, as in the activated sludge process, which could improve the availability and simplify the process control - there is less risk due to the lack of oxygen and the following problems with odorous gases, since the amount of organic material is relatively small, i.e. the sludge age is low, and the capacity of the reactor can easily be enlarged in case the flow and/or organic load increase in the future; and vii) the carriers can, if necessary, be easily pumped in and out for the continuous cleaning of carriers (Ødegaard *et al.*, 2000).

2.6.2.2 Method of MBBR using Kaldnes carriers

The system for only pure Kaldnes carriers of moving-bed biofilm reactors is a system for nitrification that is started with virgin biofilm carriers; the important aspect is

to have a start-up period with a very low and gradual increase in total ammonia-nitrogen (TAN) load. Otherwise, the nitrite peak during start-up may be so high that this substance is toxic to the aquatic organisms (Rusten *et al.*, 2006). For a compact plant, only the moving-bed biofilm reactor system is typically used for new nitrogen removal plants, for example the new BOD/COD removal plants, and to upgrade the old wastewater plants (Rusten *et al.*, 2006).

The second method for using the moving-bed biofilm is the combination of MBBR and activated sludge in the same reactor. This solution combines the benefits of the conventional activated sludge process with a biofilm process in the same reactor. This process set-up could be suitable for upgrading existing activated sludge to achieve nitrification of higher BOD/COD capacity and phosphorus removal (Gieseke *et al.*, 2003; Ødegaard *et al.*, 2004; Rusten *et al.*, 2006). The third process scheme is the MBBR process followed by the activated sludge process; this combination of biofilm processes will work as a pre-treatment to relieve the load on the activated sludge reactor and this is typically used for new plants where the biofilm process works as a pre-treatment and to upgrade the existing activated sludge to achieve nitrification or higher capacity (Rusten *et al.*, 2000).

The fourth scheme is nutrient removal for pre-denitrification, post-denitrification and combined both processes in the same reactor. As for pre-denitrification, the carbon source comes from the raw wastewater. For the post-denitrification process, the carbon source comes from external COD such as methanol or ethanol; this process stage is more efficient for nitrate removal (Ødegaard *et al.*, 2004; Rusten *et al.*, 2006).

2.6.2.3 Biofilm technology

When communities of microorganism grow on a surface, they are called biofilms. Microorganisms in a biofilm wastewater treatment process are more resilient to process disturbances compared to other types of biological treatment processes. Thus, biofilm wastewater treatment technologies can be considerably more robust, especially when compared to conventional technologies like activated sludge (Ødegaard *et al.*, 1994; 1999; 2000; Ødegaard, 2006).

There are different types of reactors which have been developed for the beneficial use of biofilms in wastewater treatment. The very first biofilm process, the trickling filter,

was invented towards the end of the 19th century. The trickling filter is reliable and stable but suffers from one serious drawback: this system can easily become clogged and septic, even under moderate loading conditions (Henze *et al.*, 2008; Grady *et al.*, 2011).

The MBBR biofilm technology is efficient, compact and easy to operate. The MBBR system can be an excellent solution as a stand-alone process, and can be used to specifically enhance or upgrade the treatment potential of activated sludge processes. The MBBR biofilm technology can be used as a preliminary treatment stage biological aerated filter (BAF), combined with hybrid biofilm activated sludge (HYBAS) (Ødegaard *et al.*, 1994; 1999; 2000; 2006; Colt *et al.*, 2006; Drennan *et al.*, 2006).

In the moving-bed biofilm reactor, the Kaldnes carriers need to be mature as the delicate ecosystem naturally develops for the bacteria involved in the nitrification process. Kaldnes media provides the maximum active surface area for the bacteria to colonise, and provides more of an area than other types of static media (Ødegaard *et al.*, 2004; Rusten *et al.*, 2006; Drennan *et al.*, 2006).

As the Kaldnes media moves within the filter, this causes the old dead bacteria on the outside to be displaced by attrition. This makes space for new younger more vital and heavier feeding bacteria to rapidly colonise. Within the wheel is a protected surface which enables colonies of bacteria to naturally follow their lifecycle of maturing, dying and then helping to fuel the latter stages of the nitrification cycle. This carrier has been designed to provide the best possible habitat for both young and mature beneficial bacterial colonies (Ødegaard *et al.*, 1994; 1999; 2000).

This media is designed to move freely within the filter and with constant chaotic movement of the air from the pump, causing the media to self-clean and thus requiring no maintenance. This allows the filter to reach optimum effectiveness without the disturbance of periodic cleaning, avoiding the unnecessary loss of bacteria within the filter (Ødegaard, 2006; Dupla *et al.*, 2006).

2.6.2.4 MBBR reactor

According to Jahren *et al.* (2002), the continuously operated laboratory scale of Kaldnes moving-bed biofilm reactor (MBBR) was used for thermophilic (55°C) aerobic treatment of TMP whitewater. In the MBBR, the biomass is grown on carrier elements that move along with the water in the reactor in Figure 2.8.

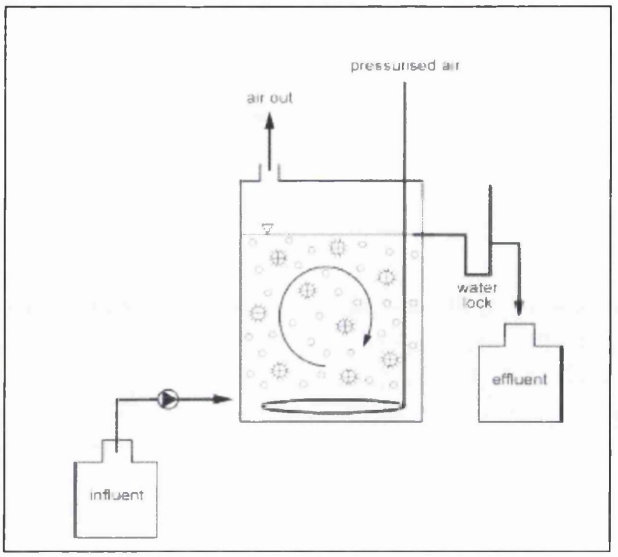


Figure 2.8: Experimental set-up (Jahren *et al.*, 2002)

The biofilm treatment plants are more compact than activated sludge plants and the treatment efficiencies are less dependent on the sludge separation characteristics. The Kaldnes moving-bed biofilm reactor (MBBR) is a completely mixed continuously operated biofilm reactor where the biomass is grown on small carrier elements that move along with the water in the reactor (Ødegaard *et al.*, 1994; 1999; 2000). No sludge recycling is necessary for keeping the biomass per unit volume at a high level.

The Kaldnes polyethylene carrier elements are shaped like small cylinders with a cross inside and longitudinal fins on the outside. The bulk carrier volume relative to reactor volume was 40-70%. The carriers have a potential growth area for a biofilm of about 490 m^2/m^3 at 70% filling, while the efficient growth area is calculated to be about 350 m^2/m^3 (Jahren *et al.*, 2002).The movement in the reactor is caused by aeration in an aerobic reactor and by a mechanical mixer in an anaerobic or anoxic reactor. The MBBR has been successfully applied under aerobic and anoxic conditions for the full-scale treatment of municipal and industrial wastewaters (Ødegaard *et al.*, 1994;1999; 2000).

Jahren *et al.* (2002) were the first to demonstrate the operation of an aerobic biofilm process for the treatment of pulp and paper mill wastewater or process water under thermophilic conditions. In the experiment of Jahren *et al.* (2002),the reason for the low biomass concentration in the thermophilic aerobic MBBR (1400-1700 mg VSS/L) could be the low loading rates applied or nutrient limitation, resulting in low biomass formation. When the biofilm on the carrier is sloughed off, the biomass leaves the reactor with the effluent. This is recognised as temporarily increased effluent solids and total

chemical oxygen demand (TCOD), as shown in Figure 2.9. This, however, did not cause any decrease in the treatment efficiency based on soluble chemical oxygen demand (SCOD) values. A separation system for excess sludge would be needed when constructing a full-scale plant (Ferrai *et al.*, 2010). Chemical addition has been found to improve the precipitation of biomass from mesophilic MBBR treating integrated newsprint mill wastewater (Broch-Due *et al.*, 1997).

The conclusion of the study of Jahren *et al.* (2002), as shown in Figure 2.9, is that Kaldnes MBBR is feasible for the treatment of pulp and paper mill (TMP) whitewater under thermophilic aerobic conditions. Rapid start-up with initial SCOD removals of 60-65% was achieved with mesophilic inoculum. Removal rates of 1.5-2.4 kg $SCODm^{-3}$ d^{-1} were obtained at loading rates of 2.3-3.8 kg $SCODm^{-3}d^{-1}$. Around 25% of the whitewater SCOD were not biodegradable. The removal rates increased linearly with increasing loading rates and there were no signs that the maximum loading rates were similar. The thermophilic aerobic Kaldnes moving-bed biofilm process gave sludge yields and sludge activities comparable to those from mesophilic activated sludge treatments of similar waters.

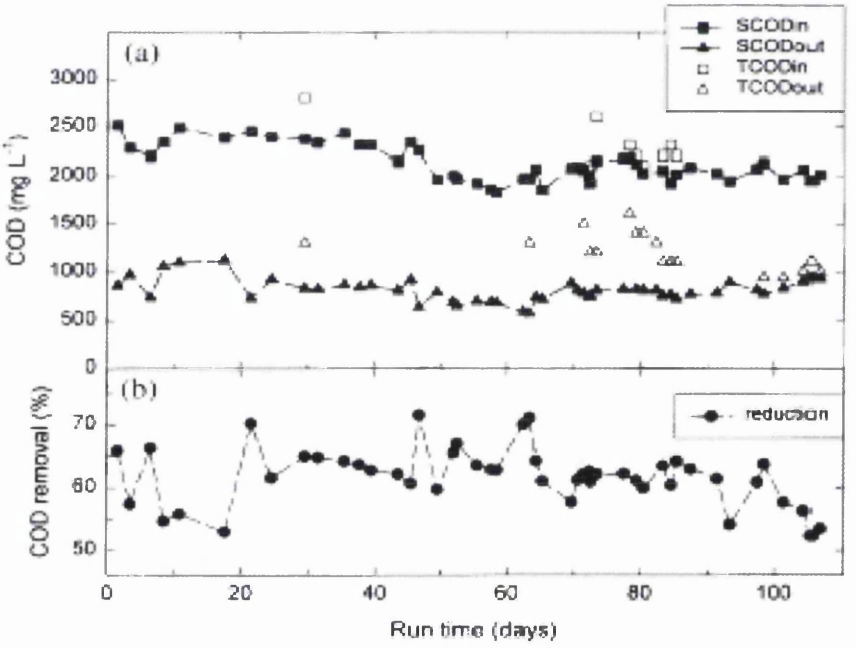


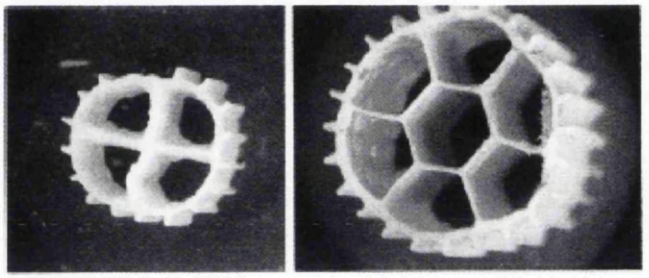
Figure 2.9: Influent and effluent soluble chemical oxygen demand (SCOD) and total chemical oxygen demand (TCOD) (a) and% SCOD removals (b) in the thermophilic aerobic MBBR (Jahren *et al.*, 2002).

The upgrading of activated sludge wastewater treatment plants with hybrid moving-bed biofilm reactors was studied by Falletti and Conte (2007). For the research, a pilot-scale activated sludge plant with an anoxic tank (1 m^3), an aerated tank (1 m^3), and settler (1 m^3) was arranged and fed with municipal wastewater (at a rate of 300 L/h). This plant removed only 11% of the NH_4-N, so the aerated tank was divided into two reactors and polyethylene carriers were placed into each reactor. The upgraded plant removed 86% of the ammonium and 73% of the total nitrogen. The following step was a full-scale plant. A municipal activated sludge wastewater treatment plant, originally built for 3000 p.e., received a nitrogen load corresponding to 5800 p.e. (The term "p.e." represents the persons equivalent). A sector of each aerated tank was transformed to a hybrid MBBR: the aeration equipment was changed and polyethylene carriers were placed in these sectors. With an incoming total Kjeldahl nitrogen (TKN) concentration of 79 mg/L, the outgoing NH_4-N concentration was <1 mg/L (Falletti and Conte, 2007).

In almost all plants, nitrification was achieved in the same aerated tanks where the biological oxygen demand (BOD) oxidation occurred. Nitrifying bacteria are slow-growth microorganisms, meaning that they are only a small fraction of the activated sludge (4%-5% of total suspended solids (TSS)); a low organic loading rate is needed to allow nitrification (no more than 0.15-0.20 $kg_{BOD}/(kg_{TSS}$ d)) and to avoid the washing-out of nitrifying bacteria (Wang *et al.*, 2012). Therefore, an activated sludge aerated tank for both BOD oxidation and nitrification can require double the volume of an aerated tank that only removes BOD because the tank volume is inversely proportional to organic loading rate. In highly populated zones, a large area is needed to build a larger activated sludge reactor; however, the situation is very difficult due to the environmental and town-planning restrictions. The problem can be overcome by implementing the moving-bed biofilm reactor (MBBR) system as the possible solution (Falletti and Conte, 2007; Leiknes and Ødegaard, 2007).

In pure biofilm reactors, the biomass grows only on carriers, whereas in hybrid reactors, there are both biofilms and suspended biomass in the same tank (Leiknes and Ødegaard, 2007). Several processes with different types of floating carriers have been developed, both with porous materials such as expanded polyurethane and nonporous materials such as polyethylene and polypropylene (Ødegaard *et al.*, 1994; Andreottola *et al.*, 2000a, 2000b, 2002 and 2003).The process with the widest number of applications is the AnoxKaldnes moving-bed (MBBR) process, with more than 400 plants worldwide.

The AnoxKaldnes moving-bed (MBBR) was developed in Norway as a pure biofilm process and was patented in 1991-1994 (Ødegaard *et al.*, 1999; Lekang and Kleppe, 2000). The carriers are called K1 and K2; their shape is represented in Figure 2.10, and their characteristics are listed in Table2.5.



K1 K2

Figure 2.10: AnoxKaldnes carriers with biomass (Ødegaard *et al.*, 1999).

Table 2.5: Characteristics of the AnoxKaldnes Polyethylene Carriers (Ødegaard *et al.*, 1999).

parameter	Value	
	carrier K1	carrier K2
length	7 mm	15 mm
diameter	10 mm	15 mm
density	0.95 g/cm^3	0.95 g/cm^3
number of carriers per m^3	1030000	159000
maximum filling degree	66%	66%
intrinsic specific surface		
total	690 m^2/(m^3 of carrier)	550 m^2/(m^3 of carrier)
effective	500 m^2/(m^3 of carrier)	315 m^2/(m^3 of carrier)

2.6.2.5 Nitrification in MBBR reactors

Several studies of nitrification with a pure biofilm MBBR have shown that this process is strongly influenced by oxygen concentration (which is the limiting substrate when the O_2/N ratio is <2), and the global kinetic is often controlled by oxygen diffusion instead of the biological metabolism itself (Hwang *et al.*, 2009; Mašic´*et al.*, 2010; Ferrai *et al.*, 2010). Biofilm internal and external mass transfer resistance was investigated in laboratory-scale nitrifying suspended carrier reactors (SCR). Controlled respirometric experiments revealed that oxygen mass transfer resistance regulated the process performance up to a DO concentration of 20 mg/L. External mass transfer exerts significant control over the overall reaction rate; thus, biofilm models must adequately

account for this resistance (Gapes and Keller, 2009). Whilst carrier type and characteristics have some influence, biofilm structure seems primarily responsible for differences in mass transfer and nitrification performance. Heterogeneous biofilms grown under high ammonium loadings had much greater area-specific rates than the gel-like biofilms sourced from low loaded systems (Gapes and Keller, 2009; Colt *et al.*, 2006; Aygun *et al.*, 2008).

The temperature negatively affects the biological process, but positively affects oxygen solubility in water. The specific superficial nitrification rate at 15°C and 5 mg/L O_2 is 0.7-1.2 $g_N/(m^2.d)$; however, the nitrification rate decreases with high organic loading and almost inhibited at 5 g $BOD/(m^2.d)$ (Hem *et al.*, 1994; Rusten *et al.*, 1995; Hwang *et al.*, 2009). On-site post-treatment of anaerobic pre-treated dairy parlour wastewater at a temperature of 10°C and using mixture of kitchen waste and black water at the temperature of 20°C was studied in moving-bed biofilm reactors (MBBR) to remove nitrogen and residual chemical oxygen demand (COD) (Luostarinen *et al.*, 2006). The results reported by Luostarinen *et al.* (2006) showed that the MBBR reactor removed 50-60% of nitrogen and 40-70% of total COD. Complete nitrification was achieved, but denitrification was restricted by a lack of carbon.

The nitrification performance of a fixed-bed biofilm reactor (FB) and moving-bed biofilm reactor (MBBR) was observed with incoming influent water from a commercial outdoor return activated sludge (RAS) facility producing rainbow trout (average density 32 kg/m^3) at a temperature of 8°C (Suhr and Pedersen, 2010). The results indicated that the more heterogeneous and stratified biofilm to be expected in FB can react more flexibly when challenged with changes such as differences in TAN loading. The effect of dissolved oxygen level on FB filter nitrification rates was additionally tested at TAN 5.35 mg O_2/L. Below approximately 60% saturation (7.1 mg O_2/L), measured at the filter outlet, nitrification rates started decreasing rapidly compared to the MBBR system (Suhr and Pedersen, 2010).

Studies on denitrification with a pure biofilm MBBR have shown that this process is strongly affected by the type and concentration of the organic substrate. With the pre-denitrification scheme (with raw wastewater as the carbon source), the specific superficial nitrification rate at 15°C is 0.40-0.70 $g_N/(m^2.d)$, with COD/N >3.5. With the post-denitrification scheme, where, in addition to sodium acetate, that provides the carbon

source, the specific denitrification rate at 15°C is 1.20 $g_N/(m^2.d)$; however, this solution is expensive (Ødegaard *et al.*, 1994; Rusten *et al.*, 1995; Ødegaard, 2006).

In 2004, a new type of carrier was developed by AnoxKaldnes and applied in a hybrid MBBR process to improve nitrification (Wessman *et al.*, 2004). In the hybrid aerated reactor, suspended sludge concentration was in the range of 2.1-3.1 kg_{TSS}/m^3, and 43% of the volume was filled with carriers with effective specific surface 900 m^2/m^3. The nitrification rate was in the range of 19-23 $g_N/(m^3.h)$ at 12°C-20°C with 5-6 mg/L O_2. In the batch test, the result investigation was estimated that 80% of the ammonium-nitrogen was nitrified by the biofilm (Wessman *et al.*, 2004).

A pilot hybrid MBBR was successfully tested by Falletti and Conte (2007), as shown in Figure 2.11, with very positive results which encouraged the application of this technology to upgrade a full-scale overloaded municipal wastewater treatment plant in Masera (PD, Italy). The pilot plant was originally made from an anoxic tank (with a volume of 1 m^3) with a mechanical stirrer, an aerated tank (with a volume of 1 m^3), and a sludge settler (1 m^3). The pilot plant was fed with municipal wastewater at a rate of 300 L/h, where the COD concentration was 270-980 mg/L (the average value was 615 mg/L, so the organic loading rate (OLR) was 0.88 $kg_{COD}/(kg_{TSS}d)$ and the NH_4-N concentration was 35-71 mg/L (with an average value of 46 mg/L). The suspended activated sludge concentration was maintained at ~5 kg_{TSS}/m^3, and the sludge recirculation feed rate was 600 L/h (so the recirculation ratio was 2:1); the sludge age was 5-6 days (Falletti and Conte, 2007).

Under these conditions, the activated sludge pilot plant removed only 11% of the ammonium-nitrogen (the average influent concentration was 46.05 mg/L, and the effluent concentration was 40.91 mg/L); the oxidation tank did not produce nitrates. Batch ammonium uptake rate tests on activated sludge showed no nitrifying activity, so that low nitrogen removal was explained by assimilation for heterotrophic cell growth of nitrification (Falletti and Conte, 2007).

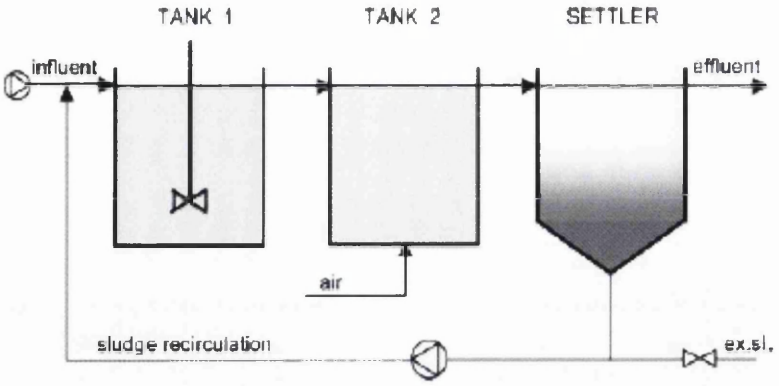


Figure 2.11: Scheme of the pilot plant without carriers (Falletti and Conte, 2007).

Therefore, the plant was modified to increase the nitrogen removal. First, Kaldnes K2 polyethylene carriers were placed into the second tank with a filling degree of 60%. After five weeks, the biofilm had visibly grown. The ammonium removal efficiency was 30% (the average influent concentration was 58.49 mg/L, and the effluent concentration was 40.74 mg/L), but NO_3-N was produced in the aerated tank and the value was still <1 mg/L. The biofilm concentration was 39.3 $g_{TSS}/(m^2$ of carrier) (8.24 kg_{TSS}/m^3 of reactor); a batch ammonium uptake rate test on the biofilm showed a nitrification rate of 0.64 $mg_N/(g_{TSS}$ h) at 20°C. That low nitrate production, despite the nitrifying activity of the biofilm under batch conditions, was explained considering that the wastewater treatment plant (Falleti and Conte, 2007) often received high-load wine industry wastewater and septic tank liquid wastes; these wastes caused high and frequent COD peaks, which created unfavourable conditions for nitrification (Falletti and Conte, 2007).

The first tank was then also aerated, and the second tank was divided into two reactors (with volumes of 600 and 400L, respectively, both with a filling degree of 60%), to facilitate the growth of a more-specialised nitrifying biomass (Figure 2.12). After two months, the ammonium removal efficiency reached 56%, and the average NO_3-N production in the aerated tanks was ~5 mg/L; the average results are listed in Table 2.6. The biofilm concentration was 8.39 $g_{TSS}/(m^2$ of carrier) in tank 2 (1.76 $kg_{TSS}/(m^3$ of reactor)), and 7.64 $g_{TSS}/(m^2$ of carrier) in tank 3 (1.60 $kg_{TSS}/(m^3$ of reactor)). Batch ammonium-nitrogen uptake rate tests on the biofilm from tanks 2 and 3 showed nitrification rates of 1.40 and 2.41 $mg_N/(g_{TSS}$ h), respectively, at 20°C; these values were much higher than those obtained before this modification of the plant, whereas the biofilm concentrations were much lower than those previously observed. The suspended solids concentration was 4.6-5.4 g_{TSS}/L (with an average value of 5 g_{TSS}/L). These results

proved that (i) nitrification effectively started in the two hybrid MBBR tanks and (ii) a specialised nitrifying biofilm was developed (Falletti and Conte, 2007).

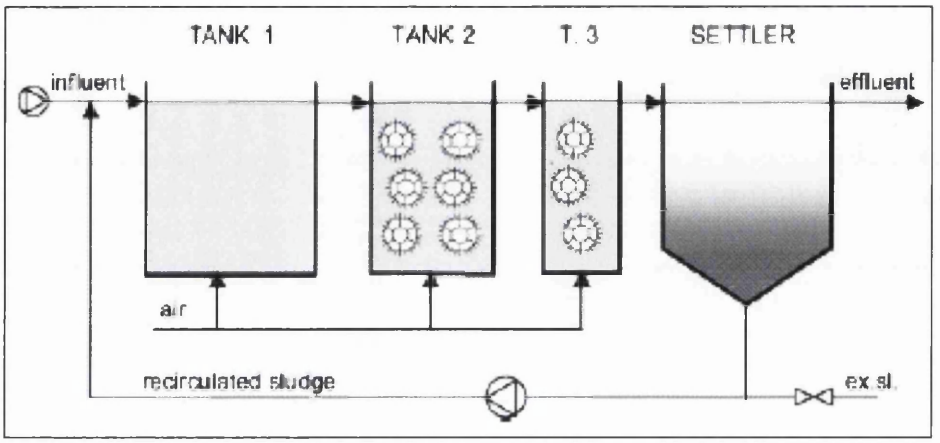


Figure 2.12: Pilot plant after the division of the aerated tank (Falleti, 2007).

Table 2.6: Results after the division of the aerated tank into two tanks(Falletti 2007).

	COD (mg/L)	NH_4-N (mg/L)	NO_2-N (mg/L)	NO_3-N (mg/L)	Tot-N (mg/L)	O_2 (mg/L)	temperature, T(°C)
influent	546	40.08	0.02	0.32	40.42		20.5
tank 1	193	28.30	0.62	1.88	30.80	3.6	21.8
tank 2	169	24.34	1.34	2.80	28.88	5.9	21.5
tank 3	130	18.49	1.34	4.27	24.10	6.1	21.2
effluent	120	17.65	1.33	4.16	23.14		
removal percentage	78.0%	56.0%			42.8%		

In the following phase of this research by Falletti and Conte (2007), K2 carriers were also placed in the first tank, with a filling degree of 60%; after three weeks, the biofilm had visibly grown, so, in the first tank, aeration was stopped and the mechanical stirrer was switched on, to improve denitrification (Figure 2.13). The results are listed in Table 2.7.

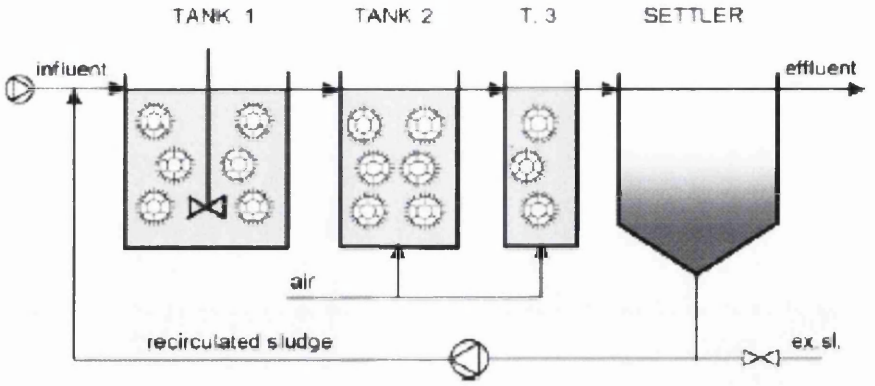


Figure 2.13: Pilot plant in its configuration (Falletti and Conte, 2007).

Table 2.7: Results of the final phase of the pilot-scale research (Falletti and Conte, 2007).

	COD (mg/L)	NH_4-N (mg/L)	NO_2-N (mg/L)	NO_3-N (mg/L)	Tot-N (mg/L)	O_2 (mg/L)	temperature, T (°C)
influent	548	44.28	0.02	0.34	44.64		23.1
tank 1	235	21.90	0.54	0.71	23.15	0.5	23.0
tank 2	156	12.77	0.49	3.25	16.51	4.5	23.0
tank 3	133	6.80	0.51	5.07	12.38	5.2	22.9
effluent	132	6.22	0.51	5.18	11.91		
removal percentage	75.7%	86.0%			73.3%		

Under these conditions, ~86% of the ammonium was removed, and 73% of the total nitrogen was also removed. The biofilm concentration was 1.59 $g_{TSS}/(m^2$ of carrier) (0.33 $kg_{TSS}/(m^3$ of reactor)) in the anoxic tank (tank 1), 16.16 $g_{TSS}/(m^2$ of carrier) (3.39 $kg_{TSS}/(m^3$ of reactor) in the aerated tank (tank 2), and 12.99 $g_{TSS}/(m^2$ of carrier) (2.73 $kg_{TSS}/(m^3$ of reactor) in the aerated tank (tank 3). Batch ammonium uptake rate tests on biofilm from tanks 2 and 3 showed nitrification rates of 0.54 and 0.67 $mg_N/(g_{TSS}$ h), respectively, at 20°C. These values were lower than those determined when all three tanks were aerated, so the anoxic conditions of tank 1 caused a depletion in the biofilm-specific nitrifying activity of tank 2 and 3; however, the biofilm was more developed and this higher concentration compensated for this effect. The suspended solids concentration was 4.8-5.4 g_{TSS}/L (with an average value of 5 g_{TSS}/L) also in this final pilot-plant configuration (Falletti and Conte, 2007).

2.6.2.6 MBBR reactor configuration

The air requirement is usually higher in a hybrid MBBR than in an activated sludge tank of the same volume; in terms of m^3/h, the air requirement of a hybrid MBBR is approximately 25%-30% higher and this contributed to the main cost of the system. However, higher oxygen concentrations, which are needed for nitrification (4-6 mg/L instead of the 2 mg/L typical of activated sludge plants), shall be maintained in a reactor whose volume is ~50% of the volume of an equivalent activated sludge tank. Moreover, with simple regulation instruments (such an oxygen electrode and an inverter), this is possible to control the air supply based on the pollutants concentration (e.g., with a low ammonium concentration, nitrification rate is limited by ammonium; therefore, this is not necessary to maintain high oxygen concentrations) and temperature (e.g., in the winter, a higher oxygen concentration can compensate for the negative effect of low temperature) (Hem *et al.*, 1994; Ødegaard, 2006; Watten and Sibrell, 2006; Borkar *et al.*, 2013).

The research performed by Andreottola *et al.* (2000a; 2000b; 2002; 2003) was to evaluate the performance of a full-scale upgrading of an existing RBC wastewater treatment plant with an MBBR (Moving-bed Biofilm Reactor) system, installed in a tank that was previously used for sludge aerobic digestion. Due to the fact that the people varied during the year's seasons (2000 resident people and 2000 tourists) the RBC system was insufficient to meet the effluent standards. The MBBR applied system consists of the FLOCOR-RMP® plastic media with a specific surface area of 160 m^2/m^3 (internal surface only).

Nitrogen and carbon removal from wastewater was investigated over a 1-year period, with two different plant lay-outs: one-stage (only MBBR) and two-stage systems (MBBR and rotating biological contactors in series). The systems were operated at low temperature (5 to 15°C). Approximately 50% of the MBBR volume (V=79 m^3) was filled. The organic and ammonium loads were in the average of 7.9 gCOD m^{-2} d^{-1} and 0.9 g NH_4-N m^{-2} d^{-1}. Typical carbon and nitrogen removals in the MBBR at temperatures lower than 8°C were 73% and 72%, respectively (Andreottola *et al.*, 2000a, 2000b, 2002 and 2003).

The upgrading of the plant consisted of two modifications: an aerobic digestion tank into an MBBR and the thickening tank into a settler for separating the detached biomass. The separated sludge was sent into the aerobic digester (Figure 2.14). The intervention of the tank to be prepared for MBBR consisted of the use of a sieve (mesh:

10 mm^3 10 mm) in order to avoid the exit of the plastic elements in the effluent. The available volume of the reactor was 79 m^3. Moreover, the air diffusing system was changed to the Messner®, where diffusing plates were placed on the bottom (each plate measure 2 m^2; the diffusing surface resulted 75% of the bottom surface). According to the results, complete mixing was guaranteed. Only 53% of the tank volume was occupied by elements. As a consequence, the surface available for the biofilm growth was 85 m^2/m^3(Andreottola *et al.*, 2000a; 2000b; 2002; 2003).

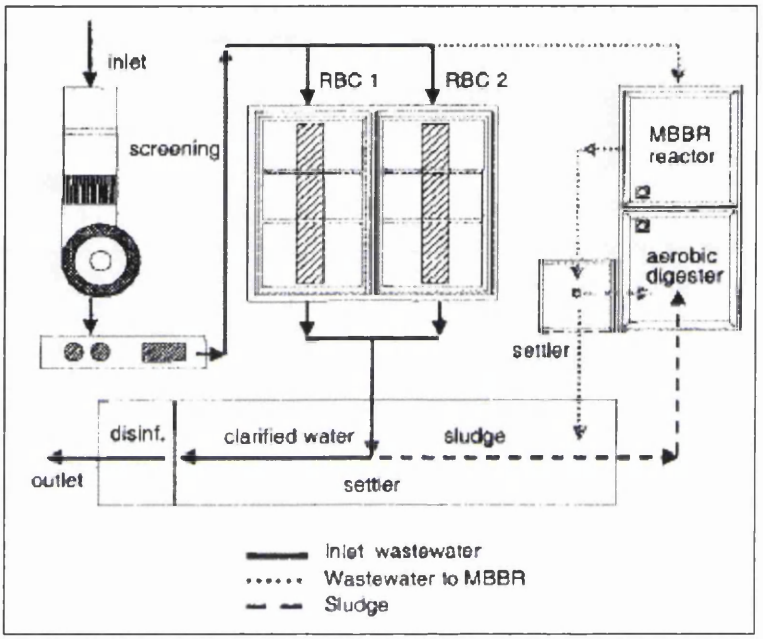


Figure 2.14: WWTP scheme after upgrading and overall building view (Andreottola *et al.*, 2000a).

Andreottola *et al.* (2000a) reported that for both the sequencing and parallel solution, the MBBR system showed a steady efficiency in spite of significant fluctuations of the hydraulic and pollution load. Good efficiency was also shown with respect to the seasonal fluctuations in temperature. When HRT is <5 h the efficiency at low temperatures showed a significant decrease. When MBBR is designed, sludge recycling can be avoided and final settlers can be reduced in volume. Another advantage of the used MBBR is the easy management (no clogging, no rotating shafts). For this reason, the MBBR can be considered an interesting solution when biological plants must be upgraded.

2.7 Aims and Objectives

1) The aim of this research is to develop and investigate the ability of continuous reactors of partial fixed-bed (PFBR) and moving-bed biofilm (MBBR) reactors in the removal of ammonia-nitrogen and synthetic oestrogens of 17α-ethynylestradiol and mestranol.
2) To ensure the high performance of nitrification, the optimisation of the growth medium and optimal growth conditions of nitrifying bacteria need to be evaluated.
3) The investigation of the growth of nitrifying bacteria in batches needs to be done to monitor cell growth, substrate consumption, product formation and to determine the growth kinetics of nitrifying bacteria in a particular packing material with the degradation of 17α-ethynylestradiol and mestranol.

Having completed this work, the effectiveness of these processes for ammonia-nitrogen oxidation and hormone destruction will be better understood. The potential and design of these processes will be clarified.

CHAPTER 3

PRELIMINARY STUDY OF ENRICHMENT CULTURE

All experimental work was carried out within the Centre for Complex Fluids Processing at the College of Engineering, the University of Swansea and the Environmental Laboratory II at the Department of Civil & Environmental Engineering, the National University of Malaysia. The nitrification inoculum was provided from the effluent waste from the Centre for Sustainable Aquatic Research, College of Science, Swansea University.

3.1 Preparation of Microbial Growth Media

3.1.1 Medium composition and formulation

Nitrifying bacteria need a complete inorganic medium as these organisms grow very slowly and are therefore often overgrown by contaminants. Nitrification Medium A (Reddy *et al.*, 2007) was used for the growth of nitrifying bacteria. This medium was compared with Nitrification Medium B for nitrifying bacteria (Alef and Nannipieri, 1995).

3.1.1.1 Nitrification medium A

The formulation of nitrification medium A was as follows; Na_2HPO_4, 13.5 g; KH_2PO_4, 0.7 g; $NaHCO_3$, 0.5 g; $MgSO_4.7H_2O$, 0.1g; $FeCl_3.6H_2O$, 0.014 g; $CaCl_2.2H_2O$, 0.18 g and 1000 mL of distilled water (Reddy *et al.*, 2007).

Aliquots of 100 mL of medium were put into 250 mL Erlenmeyer flasks, and sterile at 121°C for 15 min. For ammonium oxidizers, a sterile stock solution of $(NH_4)_2SO_4$ was prepared separately from the basal medium. The stock solution of $(NH_4)_2SO_4$ was added aseptically to give the final concentration of 0.5 g/L. The pH of the final media was 8.0.

3.1.1.2 Nitrification medium B

A stock solution for nitrification medium B was prepared as follows; Na_2HPO_4, 3.5 g; KH_2PO_4, 0.7 g; $NaHCO_3$ 0.5 g; $MgSO_4.7H_2O$, 0.1 g; $FeCl_3.6H_2O$, 0.014 g; $CaCl_2.2H_2O$, 0.18 g and 1000 mL of distilled water. For ammonium oxidizers, a sterile stock solution of $(NH_4)_2SO_4$ was prepared separately from the basal medium and added aseptically to give the final concentration of 0.5 g/L. The final pH was 7.8 (Alef and Nannipieri, 1995).

3.2 Culture Techniques for Enrichment Culture

The experiment was carried out to test the enrichment culture of nitrifying bacteria which capable of oxidizing ammonia-nitrogen in the form of a substrate. The inocula for enrichment were from soil collected locally and from fish effluent provided by the Centre for Sustainable Aquatic Research, College of Science, Swansea University.

3.2.1 Enrichment Culture in Serial Batches

The isolation of bacteria from soils was carried out by adding 0.1 to 0.3 g of soil into serial batches of culture medium with and without packing materials. The flasks were then incubated for 2 weeks in a continuous mechanical shaker at a temperature of 30°C. To determine the free suspended solids, 100 mL of the liquid suspension was then filtered through Whatman filter paper (0.2 μm) and washed before being dried to a constant

weight in an oven at 90°C to 105°C. The sample then was put in a desiccator and the weight of the sample is taken.

The flasks of serial batch cultures with and without packing materials were then filled again with fresh media with the addition of 10 mL of the liquid suspension retained from previous batch cultures. The enrichment procedure for fish effluent was exactly the same as the enrichment culture for soils.

3.2.1.1 Preparation of aerobic liquid medium culture

Prepared media, including packing materials, were sterilised by autoclaving at 121°C for 15 min. For enrichment cultures from waste, the effluent of a fish tank was used to inoculate sterile medium in 250 mL Erlenmeyer flasks. There were five sets of flasks which contained either K1 packing materials, K2 packing materials or Flocor packing materials; one set contained no packing materials and the last set was used as a blank un-inoculated control. The specifications of the solid media are given in Table 3.1 below and are illustrated in Figure 3.1.

Table 3.1: Specifications of the solid media used in this work

Materials	Characteristics
FLOCOR-RMP® plastic media	Polypropylene : density = 0.94 g/cm^3 Shape : Corrugated cylinder Dimensions : Length = 20-30 mm Diameter : 15-20 mm Specific surface : 160 m^2/m^3 Filling rate : 70%
AnoxKaldnesBiocarrier K1	Polyethylene : density = 0.95 g/cm^3 Shape : small cylinders with a cross on the inside of the cylinders and 'fins' on the outside Dimensions : Length = 7 mm

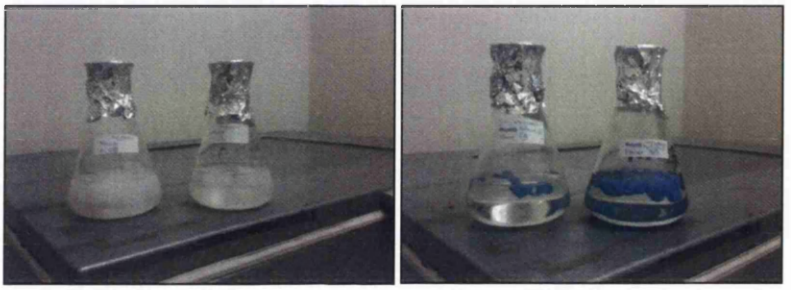
	Diameter : 10 mm Specific surface : Total : 690 m^2/m^3 Effective : 500 m^2/m^3 Filling rate : 70%
AnoxKaldnesBiocarrier K2	Polyethylene : density = 0.95 g/cm^3 Shape : Cylinders with a cross on the inside of the cylinders and 'fins' on the outside Dimensions : Length = 15 mm Diameter : 15 mm Specific surface : Total : 550 m^2/m^3 Effective : 315 m^2/m^3 Filling rate: 66%

A B C

Figure 3.1: Image A) K1 AnoxKaldnes packing material, Image B) K2 AnoxKaldnes packing material, Image C) Flocor packing material. These pictures are not to scale.

All sets of flasks contained 100 mL of medium, and 10 mL of inoculum were injected into each flask except for the blank control. The stoppers for the flasks were made of non–absorbent cotton and aluminium foil.

The flasks were then placed in a mechanical shaker incubator set at 180 rpm and 28°C. Samples were taken daily to determine the concentrations of ammonium nitrogen, nitrite-nitrogen and nitrate-nitrogen. To encourage surface growth on the packing material, the liquid medium was drained on a daily basis and replaced with new sterile medium. In the flasks containing no packing material, 10 mL of the solution was retained and new sterile medium was added to the flasks.



A B

Figure 3.2: Picture of flasks containing sterile medium and packing materials; A) K1 and K2 AnoxKaldnes packing materials, B) Flocor packing materials

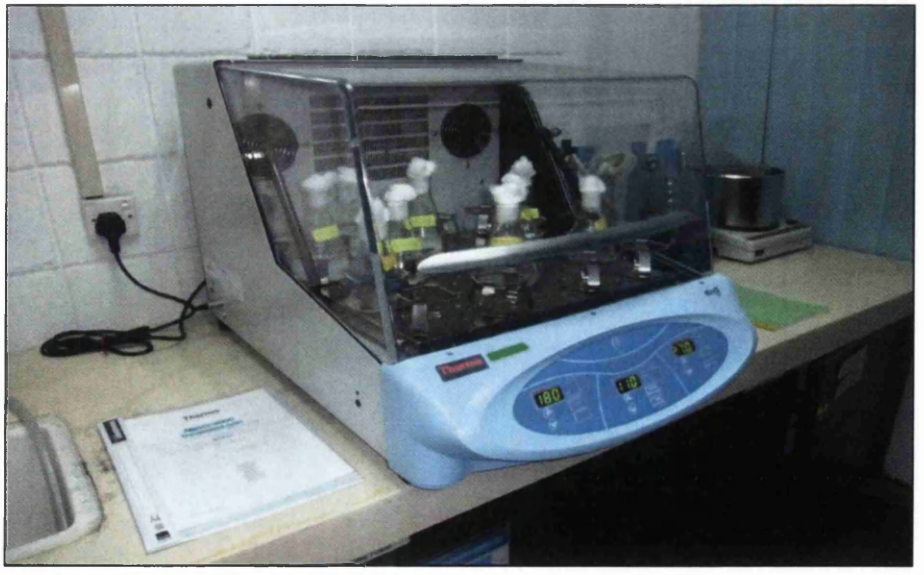


Figure 3.3: Mechanical stirrer/incubator with culture flasks

After enrichment, the cultures were grown routinely by taking 10 mL as the inoculum and growing each flask for 1 week. The medium after sterilization and cooling could then be inoculated with nitrifying bacteria from the medium culture. The inoculum was taken from active culture flasks using a syringe and injected into fresh new sets of flasks.

After experimentation, the flasks and packing materials were autoclaved to kill any microbes that may have been present in the flasks before the medium was added with the nitrifying bacteria. This was done to limit any possible source of contamination.

3.2.1.2 Preparation of aerobic agar plate culture

Further procedure was necessary to confirm the composition of the culture and to ensure that only nitrifying bacteria were grown in the enrichment culture media. To do this, samples were plated out from the liquid medium culture from the flask onto agar medium. The agar plates were incubated at 28°C in an incubator with an aerator. The agar plate was incubated for 4-7 days in order to isolate and prevent any possible contaminants and to confirm the purity of the desired nitrifying bacteria.

The plates were prepared by dissolving 15 g of pure agar (Sigma Aldrich) in 1 L of modified nitrification medium (section 3.1.1); after autoclaving, this was poured into sterile petri dishes in a sterile air cabinet ensuring aseptic conditions at all times. After cooling and solidification, the agar was left to dry with the lids resting on top on a slant for 10 to 15 minutes. The culture medium from the flask was streaked onto the surface of the agar. The plates were then placed in the incubator at 28°C and were tested for the presence of nitrite - nitrogen after 4-7 days.

3.2.2 Test of Purity

To ensure that the organisms present during the experimental studies were the desired organisms, the following tests were carried out at the start, end and during each investigation. Two tests were applied. The first was colony examination on agar plates while the second involved staining of the bacteria to observe the organisms under the microscope.

Figure 3.4 shows the typical colony morphology observed on an agar plates from the experimental study. Gram staining was used to visualise the microbes under the microscope.

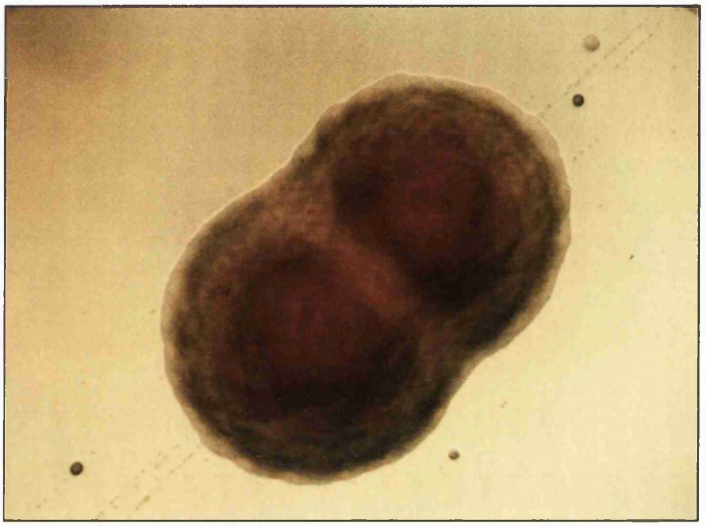


Figure 3.4: Image of an agar colony of nitrifying bacteria from the microscope

3.2.2.1 Microscopic examination

At the end of each experiment, a sample of the culture was taken and placed under the microscope for analysis. This was to check if there was any contamination in the agar plate, and most importantly to identifying the presence and the morphology of nitrifying bacteria. When observing the cells taken from the culture medium, the cells were placed onto a slide with a flamed loop and the staining procedure was performed. The Gram staining using the Hucker staining method was used in the study (Reddy *et al.*, 2007). ***The procedure is explained in Appendix A.***

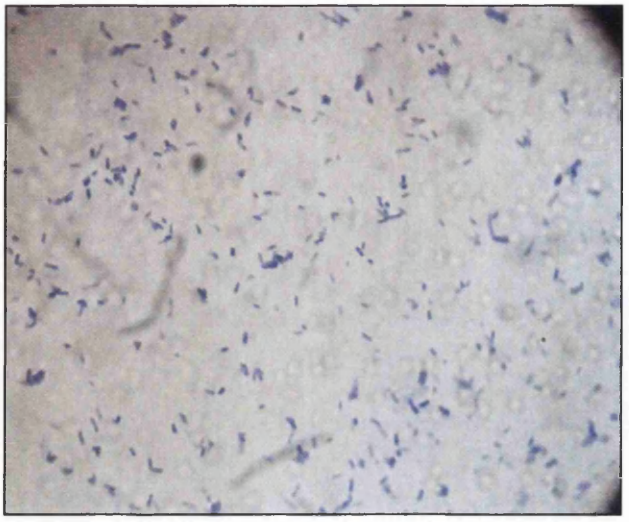


Figure 3.5: Nitrifying bacteria after Gram staining

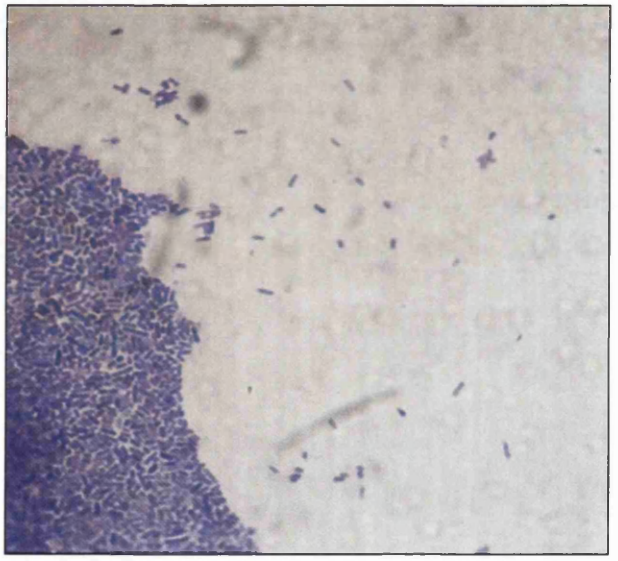


Figure 3.6: Nitrifying bacteria, rod-shaped

3.2.2.2 Agar plates

When the organisms were cultured on the nitrification medium agar, the colonies that grew on the agar plate were evaluated visually. If there were two different types of colonies located on a plate, sub-cultures were taken off the plates for the isolation of

contaminants. This involved using a sterile loop to pick a single colony, which was streaked onto a fresh plate.

The colony morphology was obtained by placing cells taken from the colonies and studying them under a microscope. The detection of nitrite - nitrogen and nitrate – nitrogen using API strips was also used to check the existence of nitrifying bacteria in the colonies on the agar plates.

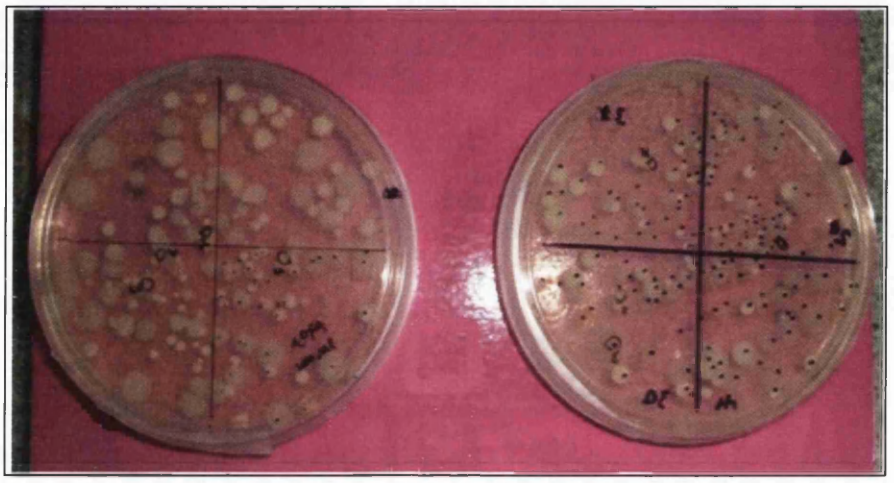


Figure 3.7: Colony count in agar medium

Figure 3.8: Streak plate of nitrifying bacteria

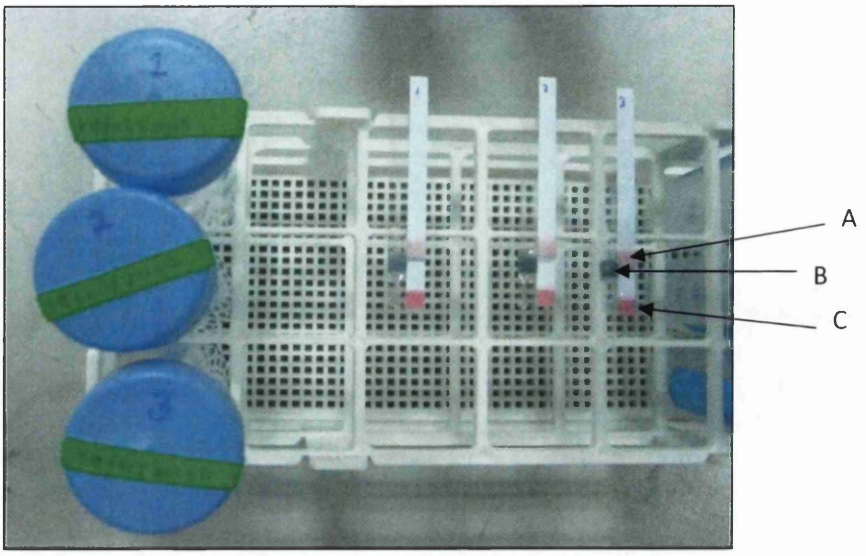
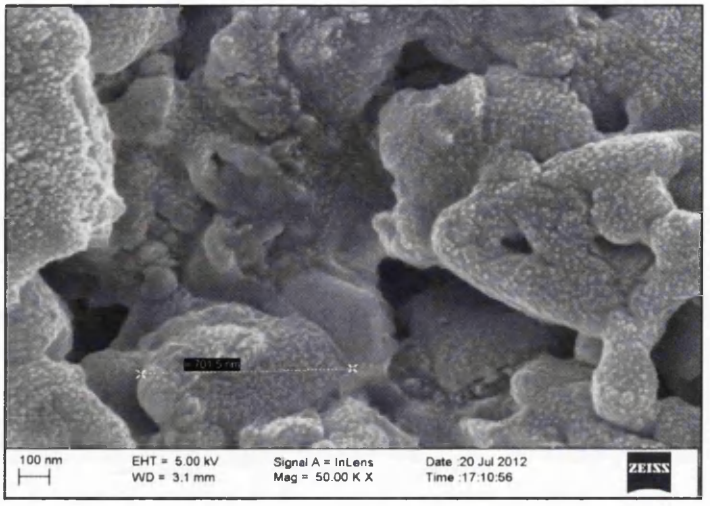


Figure 3.9: Qualitative detection of A) nitrite–nitrogen (NO_2-N), B) ammonia-nitrogen (NH_3-N) and C) nitrate-nitrogen (NO_3-N). Test performed using API strips. The test strip gave three colour responses, as indicated by A, B and C.

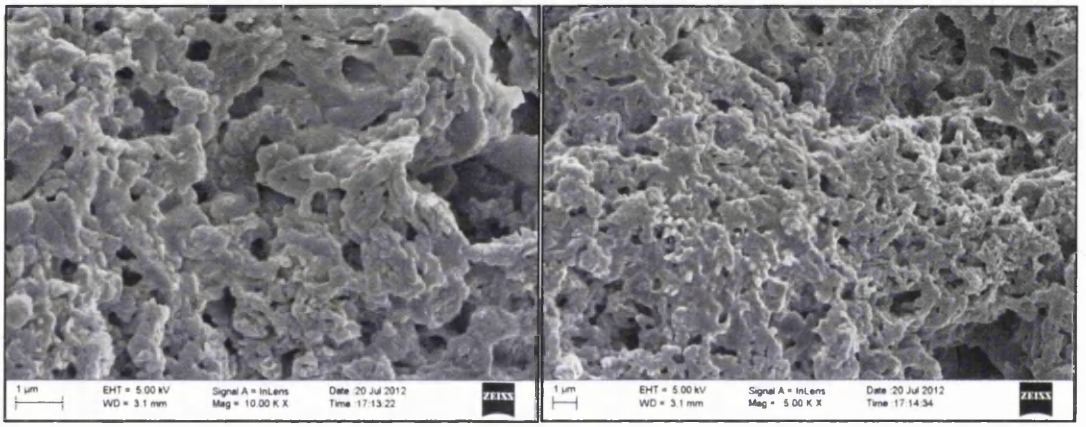
3.2.2.3 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to capture the image of biofilm on the K1 AnoxKaldes packing material. This was done to determine the morphology and the thickness of the biofilm attached on the biofilter of the K1 AnoxKaldes packing material.

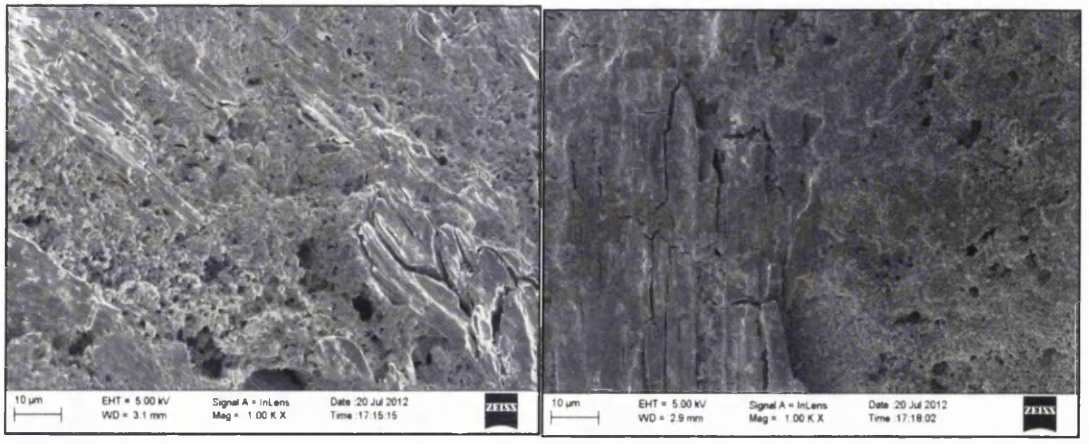
First, the K1AnoxKaldnes packing material was dried in an oven for 24 hrs at 105°C. The K1AnoxKaldnes biofilter was then fixed on a conductive surface such as graphite that can be affixed to the specimen holder. The specimen then was given a thin metal coating (sputtered in a vacuum with gold). This procedure is required to avoid specimen charging and to give a strong surface signal for imaging. Then, the sample image was captured by the scanning electron microscope. Figure 3.10 shows the SEM image of the biofilm attached to the K1 AnoxKaldnes packing material.



A



B C



D E

Figure 3.10: Images of the biofilm attached to the K1 biofilter. A) Image of the biofilm (701.5 nm) captured at 50 000 X, B) 10 000 X, C) 5 000 X, and D and E) 1 000 X

3.3 Biomass Determination of Nitrifying Bacteria

Growth (cell density) was measured by dry weight. The nominal dry weight (solids content) of bacterial cells originally in 100 mL of a liquid suspension was obtained by drying a measured wet weight or volume in an oven at 90-105°C to a constant weight. A filtration step using Whatmann filter paper (45μm) was followed by washing then drying the filter paper in the oven. The sample was then placed in the desiccator and the weight of the sample was taken.

3.3.1 Protein assay

The Bradford protein assay was used to determine the amount of solubilised protein in the enrichment culture. The Bio-Rad Protein Assay Method was used, which involves the addition of an acidic dye to a protein solution and subsequently measuring the sample at 595 nm with a spectrophotometer. Beer's law is applied for accurate quantification of protein by selecting an appropriate ratio of the dye volume to sample concentration. The comparison to a standard curve using bovine serum albumin (BSA) provides a relative measurement of the protein concentration (Bio-Rad Protein Assay Manual, 2000). ***Appendix B shows the calibration curve for BSA.***

However, the results of the Bradford protein assay showed negative values for the nitrifying enrichment culture. This may have occurred due to interference caused by chemical-protein and/or chemical-dye interactions. There are some chemical reagents that directly affect the development of the dye colour, and basic buffer conditions and detergents can interfere with this protein assay. A list of compatible chemical reagents is provided in the Bio-Rad Protein Assay manual. There is possibility that a high concentration of ammonium sulphate in the medium may interfere with the results of this protein assay.

3.3.2 Chemical oxygen demand

Chemical oxygen demand (COD) is defined as the amount of a specific oxidant that reacts with the sample under controlled conditions. The quantity of the oxidant consumed is expressed in terms of oxygen equivalence. The dichromate ion ($Cr_2O_7^{2-}$) is the specific oxidant and the ion is reduced to the chromic ion (Cr^{3+}) in these tests. (Standard Methods, 2005)

The determination of COD for the sample in this PhD study was performed using the COD Digestion Reagent Vials Kit (low range 3 to 150 mg/L) according to the HACH Manufacturing for United States Environmental Protection Agency (USEPA) Digestion Reactor method. The method used the DRB200 Reactor, which was preheated to 150°C. The caps from the COD Digestion Reagent Vials were removed and 2 mL of prepared samples were added to each vial. For preparation of the blank, 2 mL of deionized water was inserted into a vial. The vials were capped tightly and were inverted gently several times to mix. Through mixing, the sample became hot and all the vials including the blank sample were inserted in the DRB200 Reactor. The samples were then heated for 2 hours. After the heating step was finished, the vials were placed into a rack to cool to room temperature.

The colorimetric determination of the COD Digestion Reagent Vials Kit (low range 3 to 150 mg/L) was performed using a spectrophotometer. For the COD low range, the programme was chosen from the display menu of the spectrophotometer and the blank sample was inserted first for zeroing, then the samples were read. Figure 3.11 shows the apparatus used for COD determination.

A B

Figure 3.11: A) DRB 200 Reactor Hach. B) Spectrophotometer for COD LR Hach

3.3.3 Dissolved oxygen

The dissolved oxygen (DO) level was measured using a digital portable DO meter (NeotekPonsel). The measurements were taken daily for the batch reactor and continuous reactor. The results are expressed in mg/L and percent (%) saturation.

3.3.4 Other measurements

3.3.4.1 pH and temperature

The pH and temperature was determined using a digital and portable pH meter, Model CyberScan pH 110 (Eutech Instrument). The pH meter was calibrated daily with standard buffer solutions at pH 4, 7 and 10 to ensure an accuracy of $\pm$0.1 pH units.

3.3.4.2 Total dissolved solids (TDS), conductivity and salinity

The total dissolved solids (TDS), conductivity and salinity were determined using a multi-parameter pocket tester, Model Multi-Parameter Testr™35 Series (Eutech Instrument) (Yusof *et al.*, 2010).

3.4 Colorimetric Analysis

There are two major factors that influence the selection of the method to determine the ammonia concentration and the presence of interfering substances. In general, direct manual determination of low concentrations of ammonia is confined to drinking water, clean surface or groundwater, and good-quality nitrified wastewater effluent. When interfering substances are present, greater precision is necessary, and a preliminary distillation step is required (Standard Methods, 2005). In this experimental study, the distillation step was omitted as all samples were prepared with deionized water (18.2 milliΩ).

Colorimetric analysis was used for the determination of ammonium-nitrogen (NH_4-N) and nitrite-nitrogen (NO_2-N) in the concentration range of 5 to 1000 μg NO_2^--N/L and nitrate-nitrogen (NO_3-N) in the concentration range of 0.01 to 1.0 mg NO_3^--N/L (Standard Methods, 2005). The absorbance data for all the samples were read using a spectrophotometer (Biorad SmartSpecPlus UV/VIS Spectrophotometer 200-800 nm). ***Further details on the colorimetric method and the calibration graph are provided in Appendix C, D and E.***

For high concentrations of nitrite-nitrogen and nitrate-nitrogen above the stated level, an ion chromatograph procedure was used. The ion chromatograph procedure for the determination of nitrite-nitrogen and nitrate-nitrogen is described in the next section.

3.5 Ion Chromatograph Analysis of Nitrite-nitrogen and Nitrate-nitrogen

The Dionex ICS-90 Ion Chromatography System (ICS-90) performs isocratic ion analyses using suppressed conductivity detection. An ion chromatography system typically consists of a liquid eluent, a high-pressure pump, a sample injector, a separator column, a chemical suppressor and a conductivity cell. Before running a sample, the ICS-90 is calibrated using a standard solution. By comparing the data obtained from a sample to that obtained from the standard, sample ions can be identified and quantified. A computer running chromatography software automatically integrates each peak in the chromatogram to determine the sample concentration and produces a tabulated printout of the results (Operating Manual Dionex, 2006).

3.5.1 Principle of the method

IC analysis consists of four stages. 1) Eluent delivery, where a carrier liquid helps to separate the sample ions and carries the sample through the ion chromatograph system. The ICS-90 is an isocratic delivery system. The liquid sample is injected into the eluent stream either manually or automatically (when an automated sampler is installed). 2) Separation occurs as the eluent and sample are pumped through the separator column, and the sample ions are separated. In the ICS-90, the mode of separation occurs by ion exchange and is based on the premise that different sample ions migrate through the IC column at different rates, depending upon their interactions with the ion exchange sites. 3) The detection stage occurs after the eluent and sample ions leave the column; they flow through a suppressor that selectively enhances the detection of sample ions while suppressing the conductivity of the eluent. A conductivity cell monitors and measures the electrical conductance of the sample ions as they emerge from the suppressor and produces a signal based on a chemical or physical property of the analyte. 4) Data are collected when the conductivity cell transmits the signal to a computer running chromatography software. The chromatography software (for the ICS-90, this is Chromeleon®) analyses the data by comparing the sample peaks in the chromatogram to those produced from a standard solution. The software identifies the ions based on retention time, and quantifies each analyte by integrating the peak area or peak height. The results are displayed as a chromatogram, with the concentrations of ionic analytes automatically determined and tabulated. Figure 3.12 shows the apparatus and the principle of operation of ion chromatography using a Dionex ICS 90. ***Further details are provided in Appendix F.***

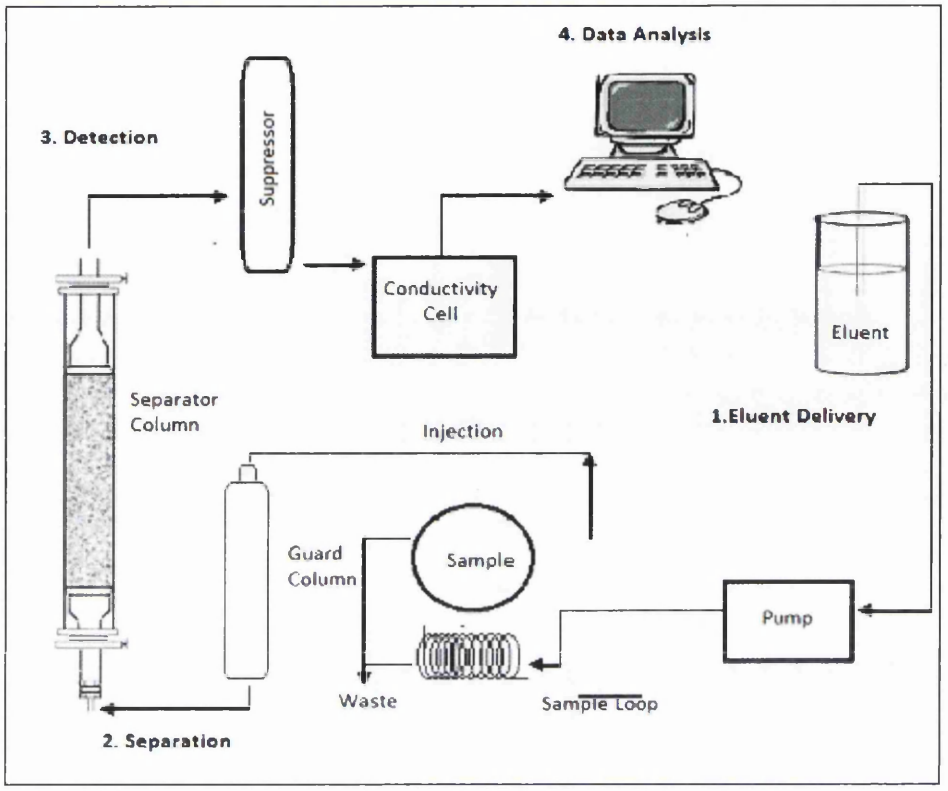


Figure 3.12: Arrangement and principle of the ion chromatograph system, Dionex (ICS-90)

3.6 Enrichment Culture in Serial Flasks

The aim of batch enrichment studies is to establish growth and determine the activity of a culture capable of ammonia oxidation. The basic strategy is to enrich for a culture that would grow on ammonia-nitrogen media using the packing materials in flask culture.

Once the culture of bacteria is active, the activity is determined by a number of methods including ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen measurements to establish the growth conditions. Serial batch cultures were carried out throughout the experimental work to acquire suitable active bacteria for ammonia oxidation.

Ammonia is a major industrial chemical that is mainly used as a fertilizer, directly applied as anhydrous ammonia, or further processed into urea, ammonium nitrates, ammonium phosphates, and other nitrogen compounds. Ammonia also is used to produce plastics, synthetic fibres and resins, and explosives (EPA, 2009). Even though ammonia-

nitrogen is important in the chemical industry, when released into the environment, ammonia-nitrogen is very toxic to aquatic organisms, especially fish, even at low concentrations (Randall and Tsui, 2002).

Ammonia-nitrogen at a concentration greater than 3 mg/L is toxic to fish (Durborow *et al.,* 1992; Randall and Tsui, 2002). The hatching and growth rates of fish are affected by toxic levels, and these toxic levels are pH- and temperature-dependent. When the pH and temperature are low, the toxicity will be high (Durborow *et. al.,* 1992; Randall and Tsui, 2002; Timmons *et al.,* 2002). Ammonia-nitrogen is abundant in many industrial and agricultural wastewaters, and must be removed from the environment to prevent oxygen depletion and the eutrophication of surface waters (Hosseini *et al.,* 2013). In the present study, ammonia-nitrogen oxidation was investigated in order to eliminate or reduce this pollutant in water so as to reduce toxicity in the fish population and also to reduce the impact of oxygen depletion and eutrophication in aqueous environments.

3.6.1 Preliminary Studies on Nitrifying Enrichment Cultures

The growth of enrichment cultures from the different inocula of soils and fish farm effluent were carried out. The enrichment procedures were explained earlier in this chapter, and nitrification medium A and nitrification medium B used in this chapter are described in section 3.1. All media were adjusted to pH 8.0 after being autoclaved at 121°C for 15 minutes. Next, 10 mL of nitrifying bacteria culture were inoculated into 100 mL of growth medium in 250 mL Erlenmeyer flasks, each containing three different packing materials and one flask containing no packing materials. A sterile blank was also prepared. Flasks were cultivated at 30°C on a rotary shaker at 180 rpm. All batch experiments were performed in triplicate.

3.6.1.1 Influence of packing materials on nitrification enrichment cultures

3.6.1.1.1 Enrichment culture from soil in serial batches

Two set of flasks containing nitrification medium A and nitrification medium B that had been autoclaved were inoculated with soil (Section 3.2.1). The serial batch contained 0.5 g of $(NH_4)_2SO_4$, equivalent to 100 mg/L of NH_4-N. Fresh medium from nitrification medium A and nitrification medium B with 100 mg/L of NH_4-N was added each time after the free biomass determination. The dry weight of free biomass in the serial batch culture for three different packing materials and without any packing material had been determined for the two sets of flasks containing nitrification medium A and nitrification medium B according to the procedure explained in section 3.3. Ammonia-nitrogen and nitrite-nitrogen were measured using the colorimetric methods described in section 3.4 and ion chromatography as explained in section 3.5. There were 12 serial batched performed for nitrification medium A and nitrification medium B. The experiment was run for approximately 22 days, with measurements taken in two-day intervals.

Figure 3.13 shows the results of soil enrichment culture for nitrification medium A and nitrification medium B for 12 serial batches over a period of 22 days. The biomass for the serial batches in nitrification medium A was slightly higher than the serial batches in nitrification medium B, with a maximum value of 80 mg/L for the K1 AnoxKaldnes and K2 AnoxKaldnes packing materials. The serial batch without packing material for nitrification medium B showed the lowest value of 25 mg/L after 12 serial batches. In the experiment with nitrification medium B, the K2 AnoxKaldnes packing material had the same value of biomass as the Flocor packing material in the serial batch at the end of 22 days after 12 serial batches, with a value of 55 mg/L. Both nitrification medium A and nitrification medium B for the serial batch with packing materials showed a good value for biomass, which was better than the serial batch culture without packing material. Based on the results shown in Figure 3.13, this was concluded that the bacteria easily attached to the packing materials which stimulated the growth and activity of the nitrifying bacteria.

The experimental results show that the biomass content was high on the packing materials compared to the results without packing material. The packing material in the

flasks eliminated the wash-out of biomass from the system. Hence, the high concentration of biomass in the flasks containing packing materials was maintained by the attachment of the microorganisms on the surface and the subsequent growth of biofilm on the material itself. However, in the flask without packing material, possible wash-out of biomass took place. This reduced the content of biomass in the flasks without packing materials. Here, we can clearly see the disadvantages of the suspended-growth system compared to the attached growth system, in that the suspended-growth system required recirculation of the concentrated microbial solids to maintain a desirable content of biomass in the reactor (Ritmann and McCarty, 2001; Mara and Horan, 2003; Grady *et al.*, 2011).

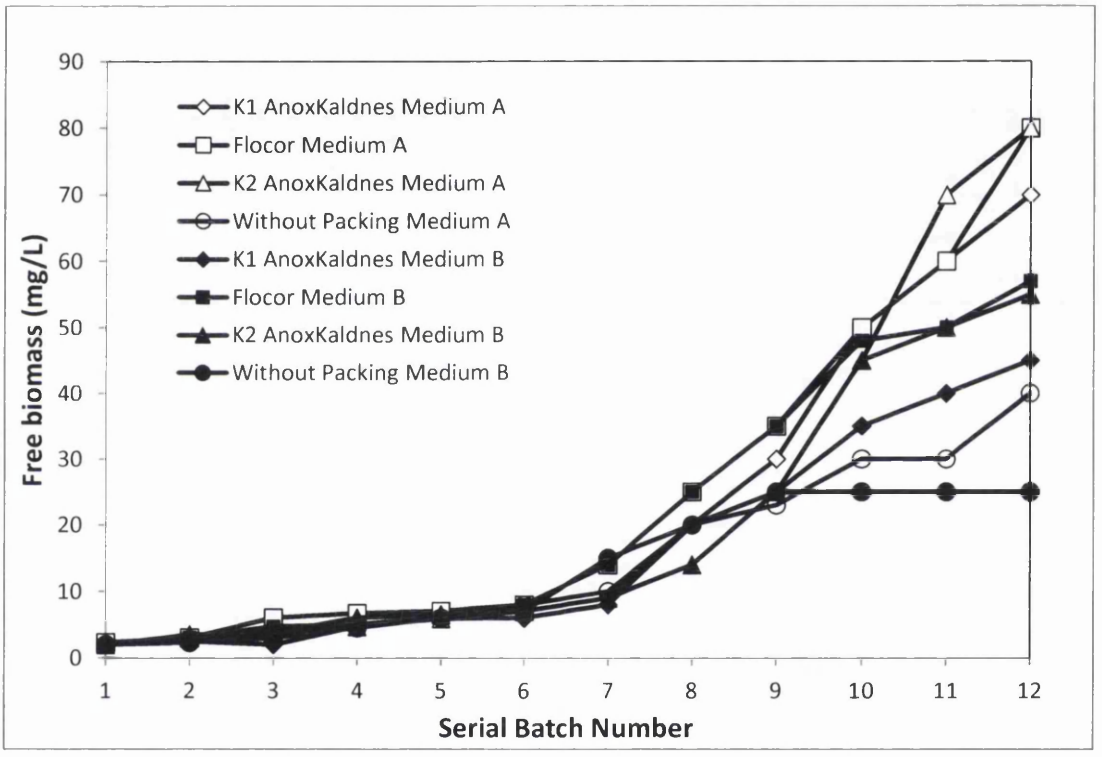


Figure 3.13: Measurement of free biomass over time in serial batch culture following soil enrichment in nitrification medium A and medium B after 22 days of incubation.

Figure 3.14 shows the ammonia-nitrogen uptake while Figure 3.15 shows the production of nitrite-nitrogen in the serial batch culture with and without packing material

in nitrification medium A and nitrification medium B for the soil enrichment culture. As shown in Figure 3.14 and Figure 3.15, the ammonia-nitrogen uptake and the production of nitrite-nitrogen in nitrification medium B for the soil enrichment culture were similar to the values with nitrification medium A. As the substrate of ammonia-nitrogen decreased, the production of nitrite-nitrogen increased, showing that the bacterial suspensions in the experiment for soil enrichment culture were capable of oxidizing ammonia-nitrogen.

The experimental results show that the production of nitrite-nitrogen was due to the nitrification process, in which ammonia-nitrogen was converted to nitrite-nitrogen. Clearly, we can see from Figure 3.14 that ammonia-nitrogen decreased by nearly 40% with the highest decrease in the serial flask containing Flocor in nitrification medium B throughout the experimental process in 12 serial flasks with packing materials and in the serial flasks without packing materials for both media. The amount of ammonia-nitrogen introduced in every serial batch culture was 100 mg/L; this concentration was quite high for the flasks containing 100 mL of nitrification medium A and nitrification medium B with 2 mL of the bacterial suspension. A longer incubation period was needed to oxidize all ammonia-nitrogen in the serial batch experiment. A high amount of nitrifying bacteria was also needed to complete the nitrification process.

Smet *et al.* (2000) used fresh bio-waste compost as the inoculum for the enrichment of nitrifying micro-organisms in biofilters for the purification of waste gases containing high (>70 mg/m^3) ammonia concentrations. The results from Smet *et al.* (2000) regarding the effect of these high ammonia concentrations on the odour removal potential using dimethyl sulphide as the model odorant compound showed that the removal of the odorant dimethyl sulphide (Me_2S) in a *Hyphomicrobium* MS3-inoculated compost biofilter was completely inhibited due to ammonia toxicity at a waste gas concentration of 100 mg NH_3/m^3. Furthermore, the ammonium and nitrate concentrations were shown to inhibit nitrification, which also strongly affected the Me_2S-degrading activity of *Hyphomicrobium* MS3.

The changes to the total bacterial community and ammonia-oxidizing bacterial community as biofilters are exposed to increasing ammonia concentrations was studied by Gregory *et al.*, (2010). At higher concentrations of ammonia, a lower percentage of ammonia was removed, and on occasion nitrite accumulation was observed with a drop in

the number of operational taxonomic units (OTUs) detected in the bacterial community; this was observed concurrently with a decrease in the percentage ammonia removal (Gregory *et al.*, 2010).

Ammonia-oxidizing bacteria (AOB) can experience fluctuating substrate concentrations with differences in adaptation to and competitiveness under conditions of high or low ammonia or oxygen concentrations within ammonia – oxidizing bacteria (AOB) groups (Geets *et al.*, 2006). In addition, ammonia – oxidizing bacteria (AOB) seem to be able to communicate through cell-to-cell signalling and move towards a more favourable environment; ammonia – oxidizing bacteria (AOB) have the tendency to grow in cell clusters. Here, we observed that ammonia oxidizers play an important role in the treatment of nitrogenous wastewater (Geets *et al.*, 2006).

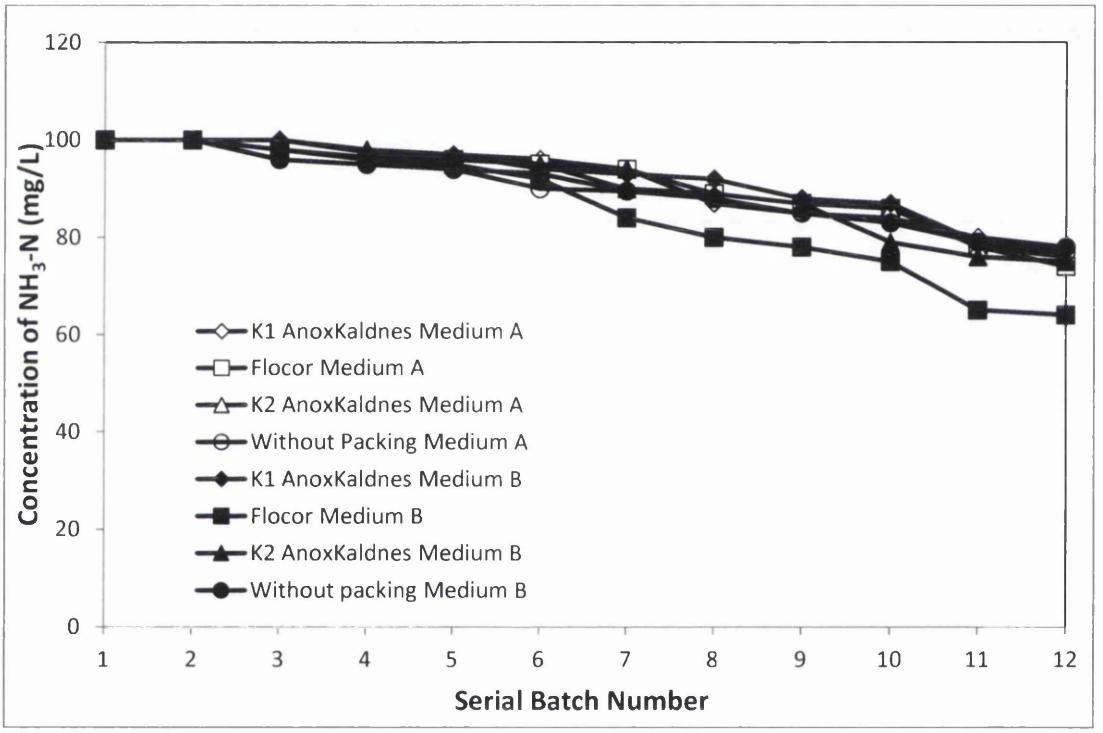


Figure 3.14: Ammonia-nitrogen uptake over time in a serial batch culture for soil enrichment culture in nitrification medium A and medium B over an incubation period of 22 days.

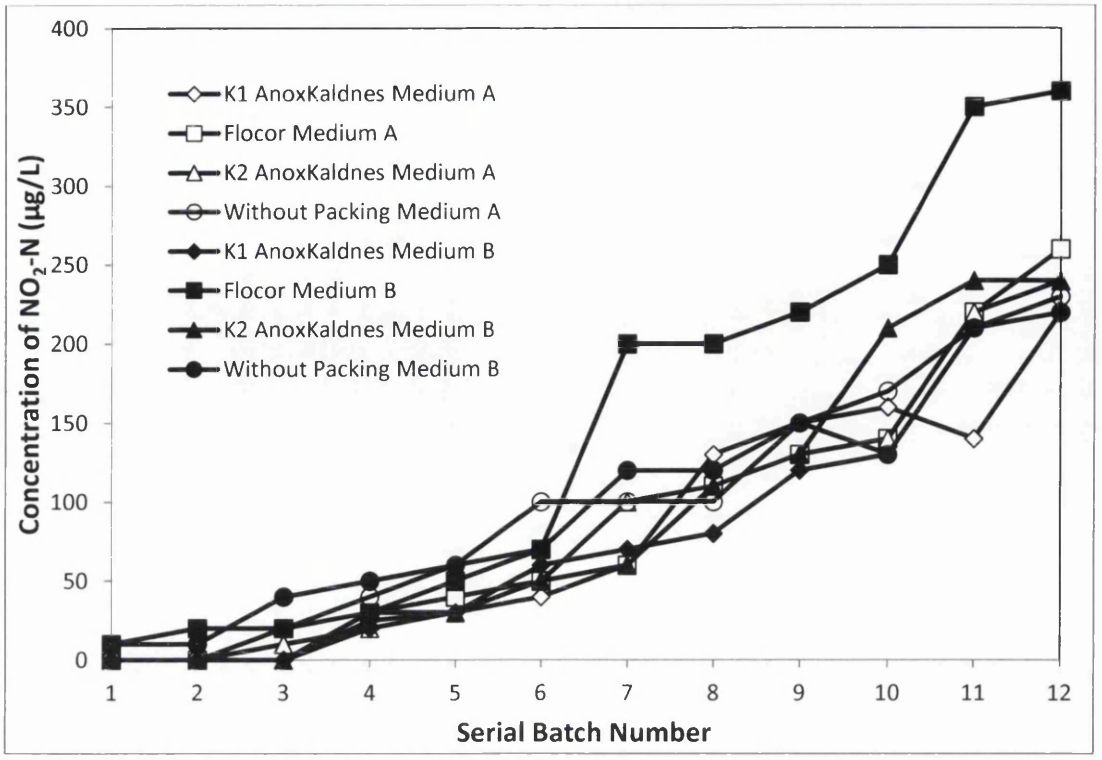


Figure 3.15: The production of nitrite-nitrogen over time in a serial batch culture for soil enrichment culture in nitrification medium A and medium B over an incubation period of 22 days.

Based on these experimental results, enrichment culture for nitrifying bacteria from soil can be performed as soil contains considerable amounts of ammonia – oxidizing bacteria (AOB), which drive the nitrification process. However, three potentially distinguishing characteristics have been suggested regarding the cultivation of ammonia – oxidizing bacteria (AOB) from soil: ammonia affinity, mixotrophy, and the optimum pH for growth. Furthermore, analysis of soil heterogeneity and microenvironments is necessary to understand the mechanisms controlling the ammonia-oxidiser community composition and activity to increase the growth of ammonia – oxidizing bacteria (AOB) (Prosser and Nicol, 2012).

3.6.1.1.2 Enrichment culture from fish farm effluent in serial batches

The same experiment was done for the enrichment culture from a fish farm in serial batches containing two sets of flasks with nitrification medium A and nitrification medium B. The experiment procedures are explained in section 3.6.1.1.1; the fish effluent inocula were provided from the effluent waste of the Centre for Sustainable Aquatic Research, College of Science, Swansea University.

The enrichment cultures for the fish effluent are shown in Figure 3.16 for the growth of bacteria in nitrification medium A and nitrification medium B, while ammonia-nitrogen removal and nitrite-nitrogen production are shown in Figure 3.17 and Figure 3.18, respectively. As shown in Figure 3.16, the biomass values for the fish enrichment culture were higher for both serial batches, both with and without packing material, compared to the enrichment culture from soil.

A same pattern was shown for the amount of biomass in nitrification medium A for the enrichment culture of fish effluent compared to the enrichment culture from soil in serial batches, both with and without packing material. The results show that a greater amount of biomass was found in nitrification medium A compared to nitrification medium B. The biomass on the K2 AnoxKaldnes packing material for the serial batch culture was highest in both nitrification medium A and nitrification medium B. However, the biomass was low in the serial batch culture without packing material in both nitrification medium A and nitrification medium B for the fish effluent enrichment culture, with the value of 40 mg/L of biomass in nitrification medium A and 25 mg/L of biomass in nitrification medium B.

However, the results show that the biomass reached a maximum value above 120 mg/L for the K1 and K2 AnoxKaldnes packing materials and Flocor packing material for the serial culture in nitrification medium A in the fish enrichment culture. In contrast, the maximum value that was achieved in nitrification medium A in the soil enrichment culture was 80 mg/L for both the K1 and K2 AnoxKaldnes packing materials, according to Figure 3.13.

The ammonia-nitrogen concentration was reduced by nearly 50% from the initial value of 100 mg/L ammonia-nitrogen, as shown in Figure 3.17. The greatest reduction in ammonia-nitrogen was shown in three serial flasks containing the K2 AnoxKaldnes and Flocor packing materials in nitrification medium A and the Flocor packing material in nitrification medium B. In Figure 3.17, the lowest decrease in ammonia-nitrogen was found in the serial flask without packing material in nitrification medium B.

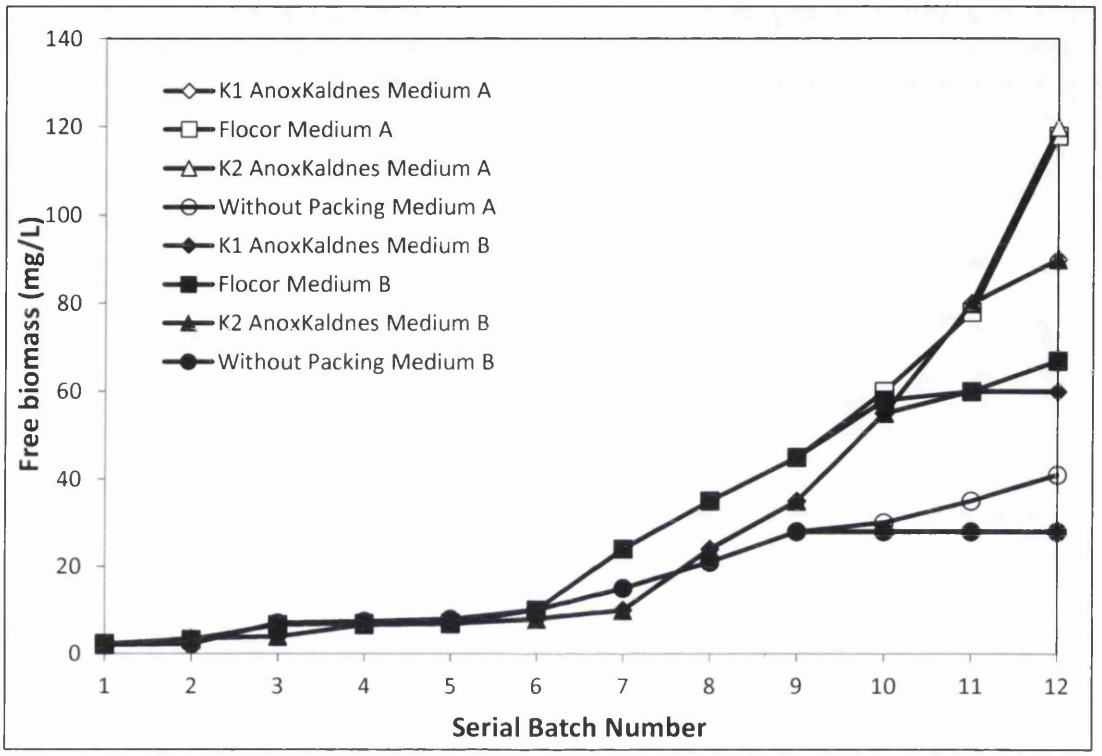


Figure 3.16: Measurement of free biomass over time in a serial batch culture with fish effluent enrichment in nitrification medium A and medium B over an incubation period of 22 days.

The production of nitrite-nitrogen in every serial flask both with and without packing material in both nitrification medium A and medium B is shown in Figure 3.18. The accumulation of nitrite-nitrogen was very high with the K2 AnoxKaldnes and Flocor packing materials in nitrification medium A and with the Flocor packing material in nitrification medium B for the serial batch enrichment culture with fish effluent. However, a slow build-up of nitrite-nitrogen was obtained in serial flasks without packing material in nitrification medium B, as shown in Figure 3.18.

Repeatedly, the serial flasks without packing materials showed poorer performance compared to the serial flasks with packing materials in the enrichment culture of fish effluent and as well as enrichment culture from soil. The suspended system in serial flasks without packing material was influenced by the vigorous mixing and/or aeration that reduced the thickness of the liquid film surrounding the flocs and the size of the flocs which, in turn, reduced the effects of mass transfer. Here, the inter- and intra-phase mass transfer was insignificant and the process was treated as a homogenous system. However, in the attached growth system in the serial flasks with packing materials, the inter- and intra-phase mass transfer was significant because the biofilm structure tends to retard the rate of transport of the substrate through the carriers. The liquid-biofilm interface is another source of resistance to the transport of substances. Therefore, biofilm systems are heterogenous systems (Mara and Horan, 2003).

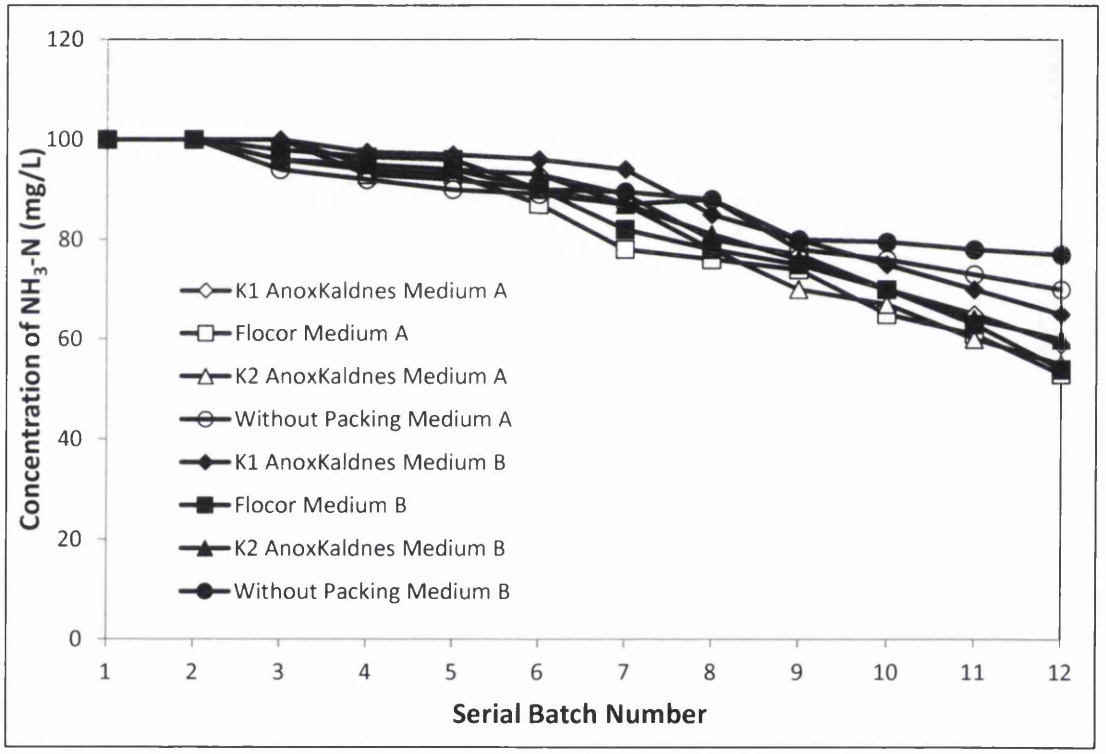


Figure 3.17: Ammonia-nitrogen uptake over time in a serial batch culture with fish effluent enrichment in nitrification medium A and medium B over an incubation period of 22 days.

Here, the fish effluent was chosen as the source of the inoculum to observe the growth of nitrifying bacteria in an enrichment culture. Based on the results shown in Figure 3.17 and Figure 3.18, the bacteria present in the serial flasks with and without packing materials were able to convert ammonia-nitrogen to nitrite-nitrogen through a nitrification process.

Enrichment culture is used as a method to revive or start a bio-filter; it is also called a 'starter' for a bacterial population derived; here in this research study, the 'starter' were from soils and fish effluent, and is used to feed new ammonia-nitrogen bio-filters to create optimal conditions for the development of a nitrifying bacterial population (Wheaton *et al.,* 1994; Van Rijn, 1996; Brion and Billen, 2000; Van Rijn *et al.,* 2006). The development of pre-coating nitrifying bacteria on a solid support has also been tried for use in aquaculture bio-filters to enhance nitrification, using mixed enrichment cultures from natural saltwater and aquaculture facilities (Horowitz *et al.,* 2001). Soil is also a potential starter for bacterial populations for nitrifying bacteria enrichment in aquaculture bio-filters (Gross *et al.,* 2003).

The most important aspects for a successful starter in bacterial amendments for aquaculture must be a lack of pathogenicity, a short lag period and tolerance to a variety of environmental conditions in ponds such as diurnal changes in temperature, radiation and salinity (Gross *et al.,* 2003). Hence, the nitrifying bacteria that developed under these extreme environmental conditions have the potential to be tolerant to stresses and can be used as starters for bio-filters that operate in ponds under extreme environmental conditions.

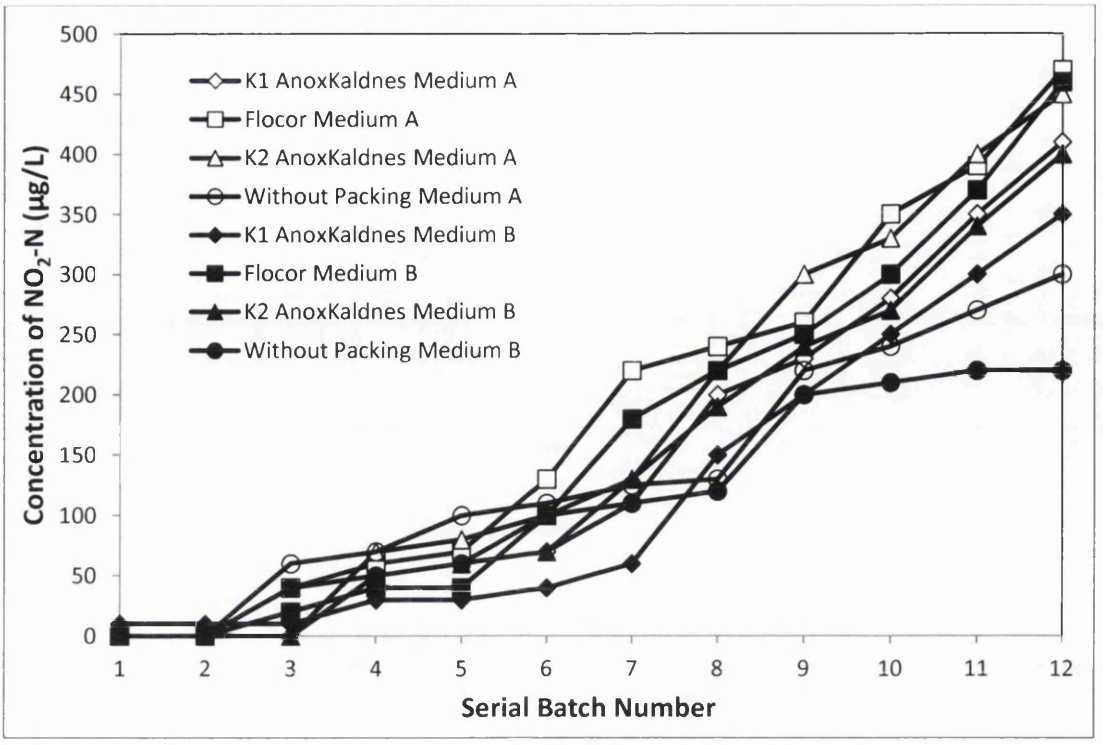


Figure 3.18: Production of nitrite-nitrogen in a serial batch culture with fish effluent enrichment culture in nitrification medium A and medium B

The studies on the enrichment culture with fish effluent and soil were done to examine if there is any impact of the source of the inoculum and to prepare for the next step, i.e. the design of batch and continuous experiments.

From all the results displayed, for the enrichment cultures from soil and fish effluent, the fish effluent culture showed greater production of biomass. A higher value of ammonia-nitrogen uptake and nitrite-nitrogen production were achieved in the enrichment culture from fish effluent compared to the enrichment culture from soil in the serial batch culture with and without packing material. Therefore, from the experimental results, the bacteria capable of oxidizing ammonia in fish effluent enrichment culture were much more numerous compared to the amount of bacteria in the soil enrichment culture. For this reason, enrichment culture from fish effluent was used in subsequent studies.

As for the results of the enrichment culture from soil and fish effluent, the ammonia oxidisers were relatively slow-growing bacteria, and the concentration of 100 mg/L ammonia-nitrogen in 100 mL of bacteria suspension was too high for complete

ammonia oxidation. To optimize the growth of ammonia oxidizers in the serial batch culture with and without packing materials, lower concentrations of ammonia-nitrogen were investigated. The nitrifying bacterium also had a low maximum growth rate and biomass yield compared to other bacteria, so retaining sufficient nitrifying bacteria in a bioreactor is difficult to achieve (Hosseini *et al.,* 2013). However, from the experimental observations, the use of packing materials advantageous compared to the system without packing materials in retaining these slow-growing bacteria.

3.7 Further Optimization of the Growth Media for Ammonia-Oxidizing Bacteria

3.7.1 Alternative nitrogen sources

Autotrophic nitrifying bacteria (ammonia oxidizers) need inorganic salts for growth as nitrifiers are unable to utilize organic substrates. Moreover, these organisms are actually inhibited by low concentrations of glucose, asparagines or sodium butyrate and by higher concentrations of urine, broth or sodium acetate. A nitrogen source from peptone or meat extract cannot be used as peptone is toxic to nitrifying bacteria (Moussa, 2004). Therefore, inorganic salts in the form of ammonium salts such as ammonium sulphate, $(NH_4)_2SO_4$, are used as the nitrogen source.

3.7.2 Influence of $(NH_4)_2SO_4$ concentration on growth

The yield of biomass in all nitrifying bacteria (ammonia oxidizer bacteria) is strongly dependent on the nitrogen source (Grady *et al.,* 2011). The effect of a low concentration of ammonia-nitrogen (NH_3-N) from the ammonium salts of $(NH_3)_2SO_4$ was studied in the fish effluent enrichment culture. Concentrations of 30 mg/L, 70 mg/L and 100 mg/L of ammonia-nitrogen were investigated.

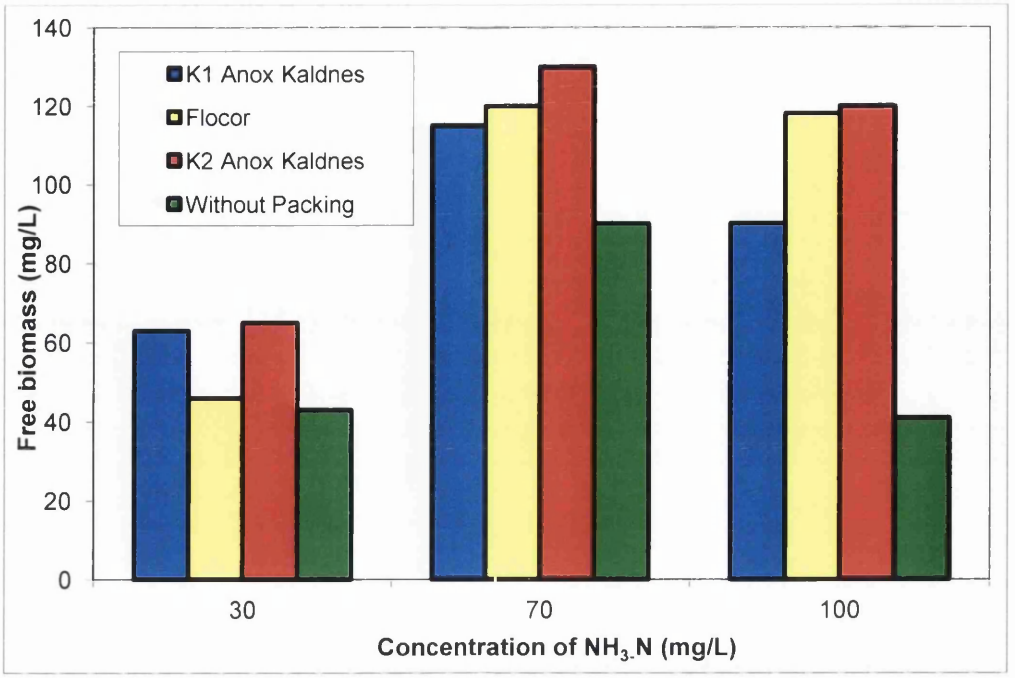


Figure 3.19: The effect of the ammonia-nitrogen concentration on the free biomass of fish effluent enrichment serial batch culture with and without packing materials.

Figure 3.19 shows that the concentration of 70 mg/L of ammonia-nitrogen provided the highest yield of biomass compared to 30 mg/L and 100 mg/L of ammonia-nitrogen. As for the packing materials, the K2 AnoxKaldnes packing material showed the best results regarding biomass of the three concentrations of ammonia-nitrogen. The free biomass results were taken from three different experiments of serial batch culture with and without packing materials for different concentrations of ammonia-nitrogen consists; 30 mg/L, 70 mg/L and 100 mg/L ammonia-nitrogen using nitrification medium A. The free biomass was assessed as dry weight at the end of each experiment according to the procedure in section 3.3.

This work focused on the biological treatment of different concentrations of ammonia-nitrogen. The results show the nitrification process in synthetic nitrification medium A using both freely suspended and immobilised bacteria in flasks with and without packing material. Hence, the aim of the work was to determine if immobilisation conferred an advantage on the degradation of different concentrations of ammonia-nitrogen and to test which of the systems, with or without immobilisation media, performed best under different ammonia-nitrogen concentration. Figure 3.19 shows that

the K2 AnoxKaldnes packing material was the best choice of biomass support system as this carrier favoured the growth of nitrifying bacteria.

A study on immobilisation media under a variety of conditions in continuously stirred tank reactors (CSTR) was performed by Rostron *et al.* (2001). The immobilisation media were Linpor (poly-urethane foam cubes), Kaldnes (polyethylene 'pasta' shapes 1 cm in diameter) and both commercial adsorption particles encapsulated in PVA cubes. The findings of Rostron *et al.* (2001) showed that Linpor and Kaldnes in PVA cubes provided the best nitrification performance. However, the results from Rostron *et al.* (2001) did not compare the K1 AnoxKaldnes packing material, K2 packing material and Flocor packing material. The K1 AnoxKaldnes packing material and K2 AnoxKaldnes packing material were patented in 2004 (Wessman *et al.*, 2004).

The use of a biomass support is to meet a certain objective, and thus an increase the stability and performance of the treatment system. The main advantage of immobilized systems is to improve nitrification and increase sludge settleability. Additional advantages are the ability to resist failure from large hydraulic surges, stable nitrification under transient inhibitory conditions resulting from temperature changes, hydraulic surges and/or toxic chemicals, and the ability to establish stable operating conditions with respect to both carbon oxidation and nitrification with short hydraulic retention times and young sludge ages (Mara and Horan, 2003).

Hence, based on the results shown in Figure 3.19, the immobilization system using suspended carriers gave a high content of free biomass in contrast to the flask without packing materials at all concentrations of ammonia-nitrogen due to the attachment of biomass in the carrier systems.

Figures 3.20 and 3.21 show the results of serial batch culture experiments for fish effluent enrichment culture with and without packing material at the concentrations of 30 mg/L ammonia-nitrogen and 70 mg/L ammonia-nitrogen, respectively. For 30 mg/L ammonia-nitrogen, the amount of nitrite-nitrogen was the highest with the K1 AnoxKaldnes packing material, followed by the K2 AnoxKaldnes packing material and Flocor packing material. The nitrate-nitrogen production with 30 mg/L ammonia-nitrogen was the highest with the K2 AnoxKaldnes packing material. At a concentration of 70 mg/L ammonia-nitrogen, the nitrite-nitrogen value was the highest in the enrichment

culture serial batch experiment for the Flocor packing material. The production of nitrate-nitrogen at a concentration of 70 mg/L was the highest in the serial batch experiment with the K2 AnoxKaldnes packing material.

All the serial batch experiments at both 30 mg/L and 70 mg/L of ammonia-nitrogen produced nitrite-nitrogen and nitrate-nitrogen. Here, the results show that the levels of ammonia-nitrogen decreased, leading to the conclusion that the serial batch experiment with and without packing materials in these enrichment cultures contained nitrifying bacteria that were capable of the nitrification process. The packing material systems were more efficient compared to the enrichment cultures of the serial batch experiments without packing material, even at different concentrations of ammonia-nitrogen.

The activated sludge process for the biological treatment of ammonia-rich wastewater (up to 1000 mg NH_3-N/L) requires long solid retention times for the optimization of a two-stage treatment scheme of an activated sludge unit consisting of a mixing basin (Campos *et al.,* 2002). With a high concentration of ammonia-nitrogen, would added to the high ammonia (HA) accumulation in a nitrifying pilot-scale sequencing batch reactor and led to a total loss of nitrogen (N). In contrast to the situation, which was not observed with a low concentration of ammonia-nitrogen. Additionally, the results indicated the inhibition of nitrite-oxidizing bacteria (NOB), but not of ammonia-oxidizing bacteria (AOB), by free ammonia under HA conditions (Gieseke *et al.,* 2003).

The results shown in Figure 3.20 and 3.21 indicate that nitrite-nitrogen simultaneously decreased when the nitrate-nitrogen concentration became higher. These findings support the findings of Gieseke *et al.* (2003) regarding the inhibition of nitrite-oxidizing bacteria (NOB), which are influenced by high concentrations of ammonia-nitrogen; i.e., the nitrate-nitrogen build-up is dependent on the NOB population. Here, the ammonia-nitrogen concentrations of 30 mg/L and 70 mg/L are considered low compared to 100 mg/L ammonia-nitrogen. In these low ammonia-nitrogen conditions, the growth of NOB populations was not affected.

Ammonia-nitrogen removal from prawn aquaculture water has been demonstrated using immobilized nitrifying bacteria capable of removing ammonia-nitrogen up to 200 mg/L. The cultures were immobilized onto porous clay pellets to enhance cell density and applied to culture medium under aerobic conditions to determine the total ammonia-nitrogen removal proficiency (Shan and Obbard, 2001). High concentrations of ammonia-nitrogen are associated with intensive of prawn aquaculture in tropical regions as a result of high rates of prawn excretion and feed loading (Shan and Obbard, 2001).

A study performed by Bollmann and Laanbroek (2001) using an enrichment culture of ammonia oxidizing bacteria (AOB) at a low ammonia-nitrogen concentration showed that AOB have competitive abilities depending on the different bacteria present at different ammonia-nitrogen concentrations in continuous and batch cultures. The results from this study also indicated that the growth of the *Nitrosomonas cluster 6a-A* is inhibited at ammonium concentrations of 10 millimolar (mM).

The attachment of ammonia-oxidizing bacteria to immobilized packing materials can have several positive effects on ammonia-oxidizing bacteria, such as an increase in ammonia-oxidizing activity, a reduction in the lag phase and an increase in activity at low pH. Furthermore, the attachment of ammonia-oxidizing bacteria could decrease the growth-limiting substrate concentration (Bollmann and Laanbroek, 2001). This selectivity of the ammonium concentration on the enrichment of ammonia-oxidizing bacteria could be caused by the better competitive abilities of certain nitrifying bacteria under the prevailing conditions, but this could also be due to different sensitivities against the toxic effects of ammonia-nitrogen.

In the present study, the result shown that using immobilized packing materials can improve the degradation of ammonia-nitrogen; however, the degree of degradation depends on the concentration of the substrate (the ammonia-nitrogen level), the incubation period and the population density of nitrifying bacteria. In the next chapter, higher concentrations of ammonia-nitrogen were studied (up to 500 mg/L) in the batch culture process.

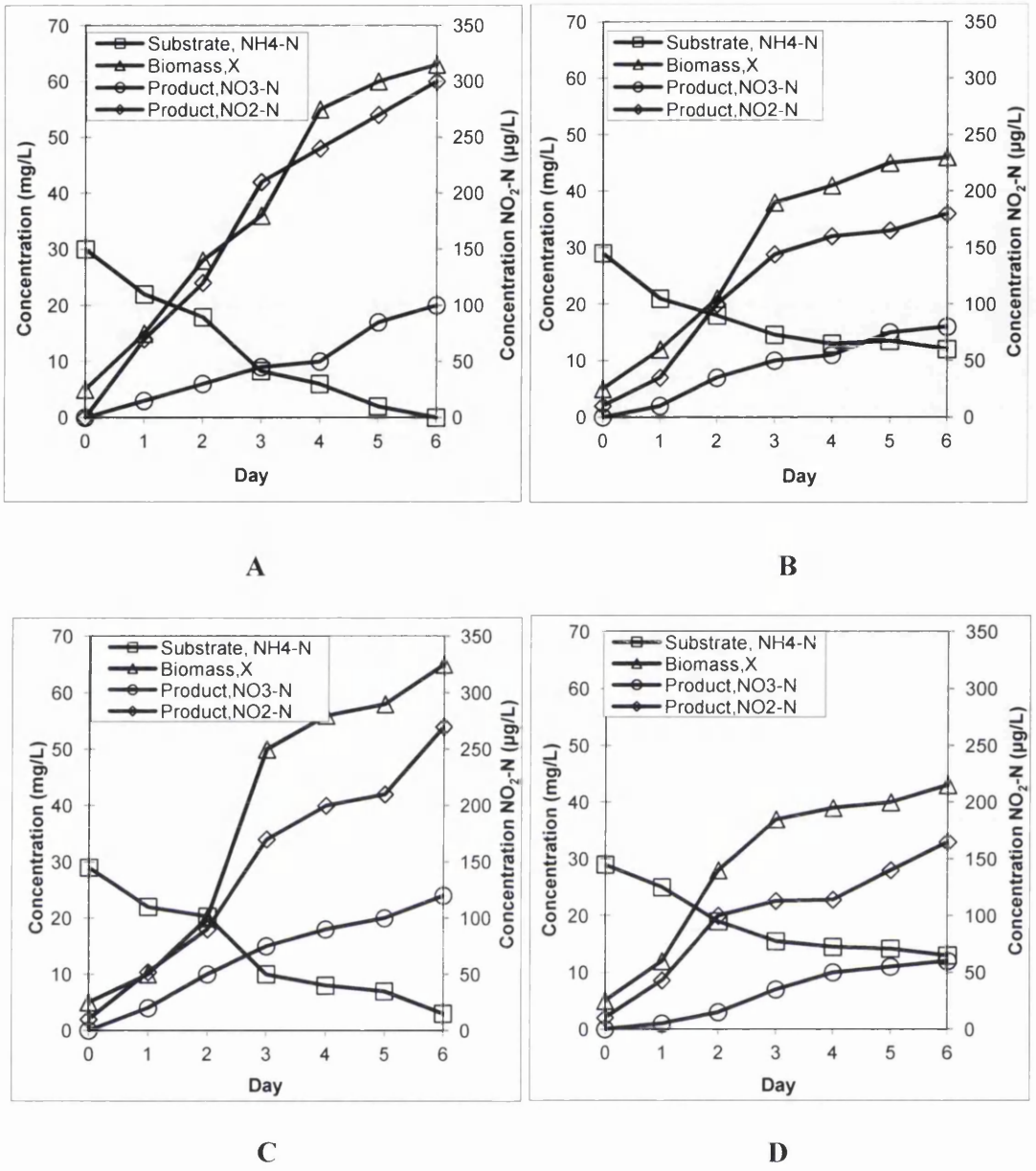


Figure 3.20: The growth and activity of fish effluent enrichment culture in a serial batch culture experiment using 30 mg/L ammonia-nitrogen A) K1 AnoxKaldnes packing material B) Flocor C) K2 AnoxKaldnes D) Without packing material.

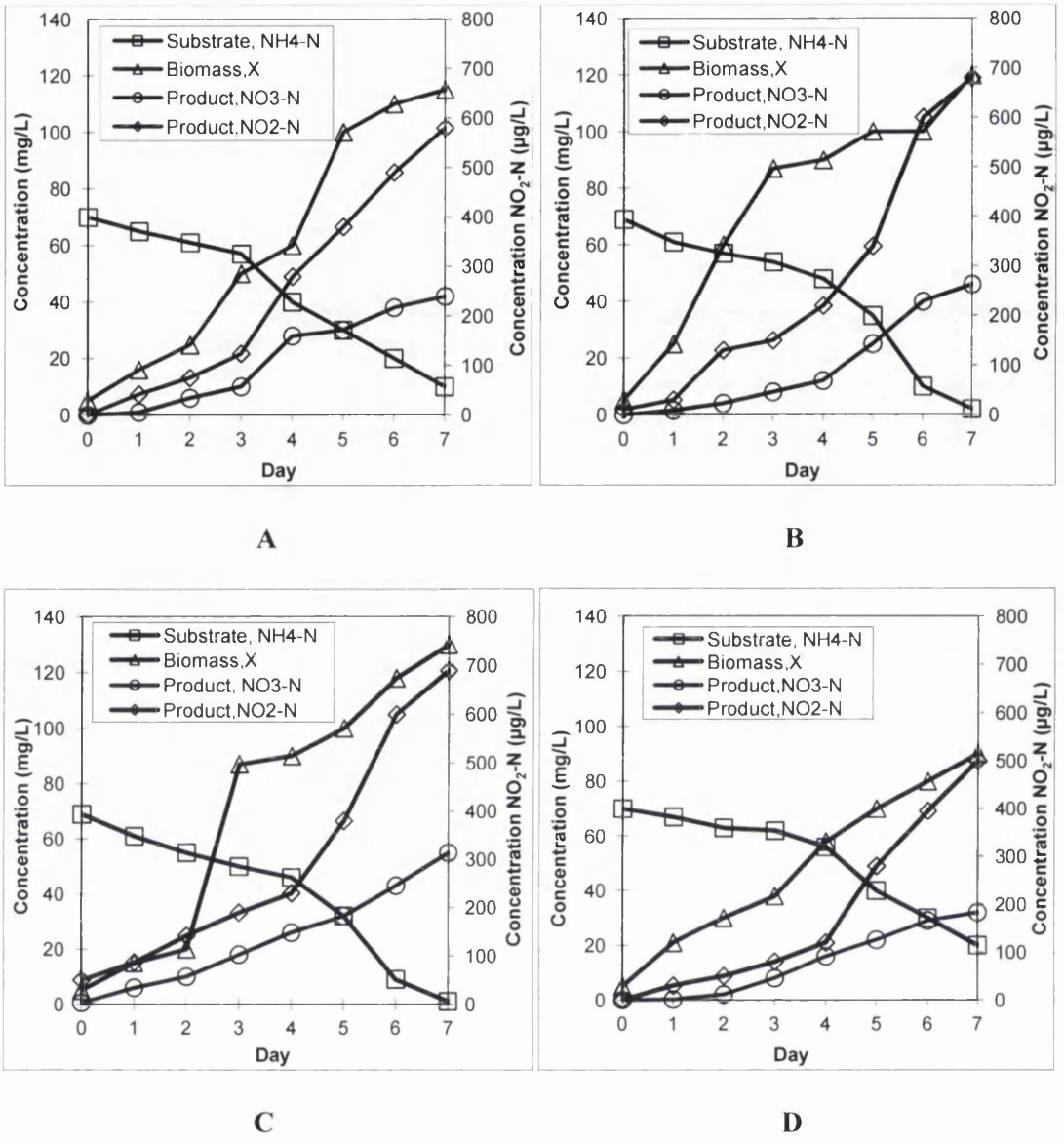


Figure 3.21: The growth and activity of fish effluent enrichment culture in a serial batch culture experiment using 70 mg/L ammonia-nitrogen A) K1 AnoxKaldnes packing material B) Flocor C) K2 AnoxKaldnes D) Without packing material.

3.8 Influence of temperature on the growth of nitrifying bacteria in batch culture

The temperature of wastewater is commonly higher than the local water supply due to the addition of warm water from households and industrial activities. The temperature of wastewater is also greater than the local air temperature due to the fact that the specific heat of water is much greater than that of air during most of the year, although this decreases during the summer (Grady *et al.*, 2011). Wastewater temperature depends on the geographic location as well; the annual temperature of wastewater in the United States varies from 3°C to 27°C, and the temperature of wastewater is often above 30°C in countries in Africa and the Middle East (WEF, 2005). In Malaysia, most wastewater treatment plants operate in the range of 25°C to 30°C.

Nitrifying bacteria are sensitive to temperature, which makes temperature an important parameter that influences ammonia-nitrogen removal rates in wastewater treatment systems, particularly in attached growth systems (Zhu and Chen, 2002; Salvetti *et al.*, 2006; Zhang *et al.*, 2014). Nitrification has been shown to occur in the temperature range from 4°C to 45°C with the optimum growth rate occurring in the temperature range from 35°C to 42°C (WEF, 2005). With an increase in temperature to 50°C, the processes of aerobic digestion and nitrification will be retarded; lowering the temperature to about 5°C will practically cease the function of autotrophic nitrifying bacteria (Henze *et al.*, 2008). The nitrifying bacteria growth rate (μ) is related exponentially to temperature, T, by a form of the Van't Hoff-Arrhenius equation (Metcalf and Eddy, 2004):

$$\mu = \mu_{15} e^{kt\,(T-15)} \qquad (3.1)$$

where kt is the temperature coefficient (units $°C^{-1}$). The equation applies for temperatures between 8°C and 30°C.

For the experiments on the effects of temperature on the growth of nitrifying bacteria in fish effluent enrichment culture, two different concentrations of ammonia-nitrogen were prepared, i.e. 30 mg/L and 70 mg/L. This was added to 100 mL of Medium A in a 250 mL volumetric flask. Each set included flasks containing the K1 AnoxKaldnes packing material, K2 AnoxKaldnes packing material, Flocor packing material and one

without packing material. Two set of flasks with ammonia-nitrogen concentrations of 30 mg/L and 70 mg/L were inserted into a refrigerator at a temperature between 0°C and 4°C. The other sets of flasks for both concentrations were incubated in a mechanical stirrer for experiments at various temperatures ranging from 15°C to 35°C. At the temperatures of 40°C and 50°C, two sets of flasks for both concentrations were placed in water baths, as shown in Figure 3.22.

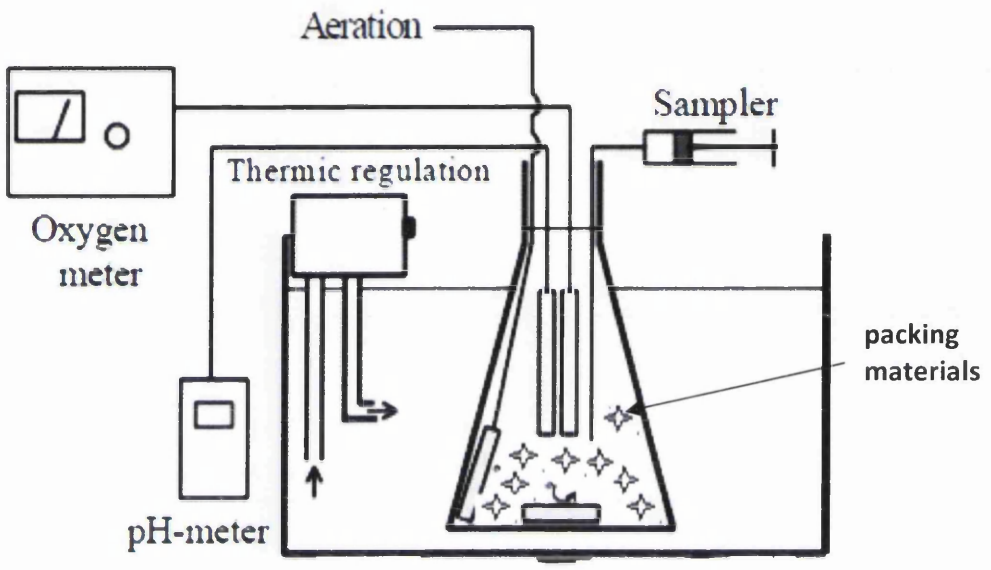


Figure 3.22: Schematic diagram of the experiment with 30 mg/L and 70 mg/L ammonia-nitrogen at 40°C and 50°C in a water bath.

The fish effluent inocula for the serial batches were inserted into the sets of flasks in the amount of 10 mL, when the flasks had reached their respective temperatures. The determinations of ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were taken immediately and at 1-day intervals, as explained in section 3.4 and section 3.5.

The detailed results of nitrate-nitrogen accumulation at various temperatures for the different packing materials and without packing material for the ammonia-nitrogen concentrations of 30 mg/L and 70 mg/L are shown in *Appendix H* and *Appendix I*, respectively.

The tables in *Appendix H* and *Appendix I* for the maximum percentage nitrate-nitrogen production are illustrated in the graphs shown in Figure 3.23 and Figure 3.24. These graphs show the maximum percentage of nitrate-nitrogen production in the flasks

with and without packing materials at different temperatures with 30 mg/L and 70 mg/L ammonia-nitrogen.

Figure 3.23 and Figure 3.24 showed the maximum percentage production rate of nitrate-nitrogen at the various temperatures studied. The calculation to obtain the maximum percentage nitrate-nitrogen production rate was done according to Equation 3.2 and Equation 3.3. The nitrate-nitrogen production rate (mg/L.day) was calculated using the geometric average calculation with respect to temperature.

$$NO3 - N\ production\ rate\ (mg/L.day) = \frac{Sum\ of\ NO3{-}N\ (mg/L)}{Sum\ of\ days} \quad (3.2)$$

The percentage of maximum value for the nitrate-nitrogen rate (%) at a particular temperature was calculated from the following equation;

$$\%\ of\ NO3 - N = \frac{NO3{-}N\ production\ rate\ (mg/L.day)}{The\ highest\ value\ of\ NO3{-}N\ production\ rate\ (mg/L.day)} \times 100 \quad (3.3)$$

From the results shown in Figure 3.23 and Figure 3.24 for the concentrations of 30 mg/L and 70 mg/L ammonia-nitrogen, no sign of nitrate-nitrogen was found in the experimental flasks with and without packing materials at 0°C. However, the production rate of nitrate-nitrogen slowly built up in the flasks at both concentrations (30 mg/L and 70 mg/L of ammonia-nitrogen) at 4°C and 15°C. Furthermore, the production rate of nitrate-nitrogen increased tremendously for the flasks at 20°C and 30°C for both concentrations (30 mg/L and 70 mg/L of ammonia-nitrogen). The results in Figure 3.23 and Figure 3.24 show that the highest production rate of nitrate-nitrogen was in the flasks at a temperature of 35°C for both concentrations (30 mg/L and 70 mg/L of ammonia-nitrogen). However, based on the results in Figure 3.23 and Figure 3.24, the production rate of nitrate-nitrogen in all flasks declined at a temperature of 40°C and no nitrate-nitrogen was produced in the flasks at 50°C for both concentrations (30 mg/L and 70 of mg/L ammonia-nitrogen).

The results demonstrate the effect of temperature on nitrate production rate by nitrifying bacteria in fish effluent enrichment culture with and without packing material at the concentrations of 30 mg/L and 70 mg/L ammonia nitrogen. There was an approximately logarithmic increase in the nitrate-nitrogen production rate, up to a maximum level at 35°C for both concentrations. However, the production rate of nitrate-

nitrogen at the concentrations of 30 mg/L and 70 mg/L ammonia-nitrogen decreased when the flasks were incubated at 40°C and rapidly declined at a temperature of 50°C.

Here, we can see from Figure 3.23 and Figure 3.24 that the nitrifying bacteria achieved the highest growth at 35°C. These results agree with the experimental results reported by Grunditz and Dalhammar (2001) in a pure culture of *Nitrosomonas* and *Nitrobacter*. The observations in Figure 3.23 and Figure 3.24 indicate that the ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) have different sensitivities to various temperatures. Shifting the temperature from low to high changed the dominant growth of the nitrifier population.

The effects of temperature and free ammonia of landfill leachate on nitrification and nitrite accumulation using a semi-pilot scale biofilm airlift reactor has been studied by Kim *et al.* (2003). The nitrification rate of landfill leachate increased with temperature when free ammonia in the reactor was below the inhibition level for nitrifiers. The leachate was completely nitrified at a temperature of 28°C. However, the nitrification of the leachate treatment plant was severely affected by seasonal temperature variations. The average winter temperature was 5°C and dropped to 1-2°C, which led to variations in free ammonia (NH_3-N) which inhibited not only NOB but also AOB. The activity of NOB was inhibited by NH_3-N, resulting in the accumulation of nitrite with a decrease of NOB activity in the value of more than 50% (Kim *et al.,* 2006).

The temperature and free ammonia concentration control the nitrification rate, nitrite-nitrogen accumulation and nitrate-nitrogen build-up. AOB have superior growth rates at higher temperatures than nitrite oxidizers (Kim *et al.,* 2008). The phenomenon of nitrite-nitrogen accumulation is contributed to by some conditions involving low DO concentrations, high temperatures and an inhibitory nitrite-oxidization environment (Kim *et al.,* 2008). However, by providing the optimum growth conditions, the nitrification process can be completely carried out with the outcome of excessive nitrate-nitrogen production. To achieve a high nitrification rate, proper control of operating variables such as hydraulic residence time, solid retention time, dissolved oxygen, temperature, and free ammonia is used to suppress nitrite oxidation without excessively retarding ammonia oxidation.

The outcome shown in Figure 3.23 and Figure 3.24 can be interpreted that temperature has a great influence on the growth of nitrifying bacteria (AOB and NOB). The nitrifying bacteria could not survive even a short incubation period at low temperatures below 4°C. The nitrification process also dropped at temperatures above 40°C, and the nitrifying processes could not take place at thermophilic temperatures in the range of 50°C to 60°C (Henze *et al.*, 2002). However, research study done by Jahren *et al.* (2002) using an aerobic moving bed biofilm reactor to treat thermomechanical pulping whitewater under thermophilic conditions at a temperature of 55°C provided 60 to 65% removal of soluble chemical oxygen demand (SCOD). This confirms that the biomass grown on Kaldnes carrier elements in the moving bed reactor (MBBR) could be successfully operated under thermophilic conditions. However, the nitrification process needs an optimum temperature, i.e. a temperature below 40°C, or 28°C to 35°C on average, to maintain a high rate of activity of nitrifying bacteria (WEF, 2005).

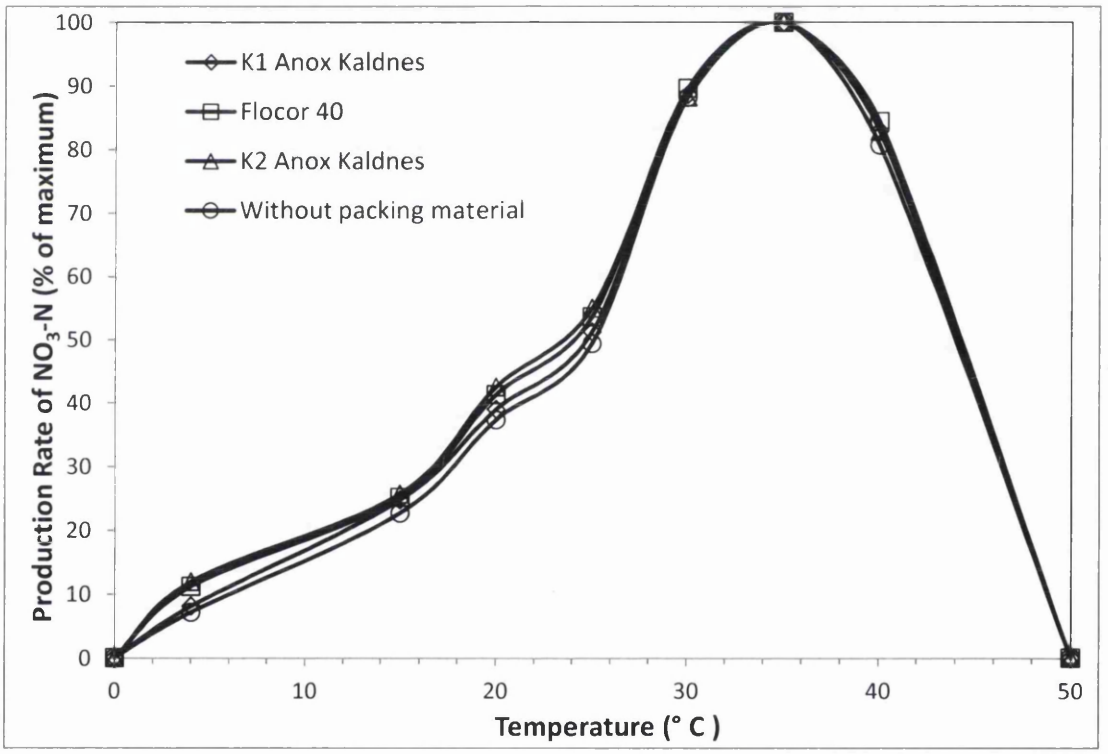


Figure 3.23: A comparison of the effect of the production rate of nitrate-nitrogen in a batch cultures with and without packing material at the concentration of 30 mg/L ammonia-nitrogen in fish effluent enrichment culture. ***See Appendix H.***

Using the batch culture method, these investigations into the effect of various temperatures on the nitrification process in the enrichment culture of nitrifying bacteria are important to have a clearer understanding and to provide further insight into identifying the key parameters controlling nitrification. To achieve lower energy consumption and reduce the requirements for the organic substrate, the enrichment culture of nitrifying bacteria in the present study was grown on a suspended carrier of packing materials and at a 30°C of temperature. Through this strategy, an economical start-up method of bacterial culture could be obtained.

By considering the cost of operation and maintenance in a wastewater treatment plant, an adequate operation system needs to be developed to reduce the investment required. The application of a biological process system using carriers in an attached growth system may be the best solution to tackle the problems of a growing population and rapid urbanization, which requires greater land availability to set up centralized sewage treatment plants (Azizi *et al.,* 2013). In applying the attached growth system, an effective cost reduction could be achieved.

The process efficiency in treating wastewater primarily depends on the biomass concentration and specific conversion rate of the microorganisms. The attached growth system is an advanced approach, preferred over the suspended biomass processes, since an attached growth system creates a biofilm on the support media which provides better treatment efficiency due to the accumulation of a large microbial population on a large surface area (Azizi *et al.,* 2013). The shape and size of the biomass-supporting media plays a significant role in the design of biofilm processes in order to meet an obligatory surface area for microbial growth. The microorganisms secrete a sort of natural polymer to facilitate firm adhesion on the inert support matrix, which enhances biofilm development and the bio-oxidation mechanism.

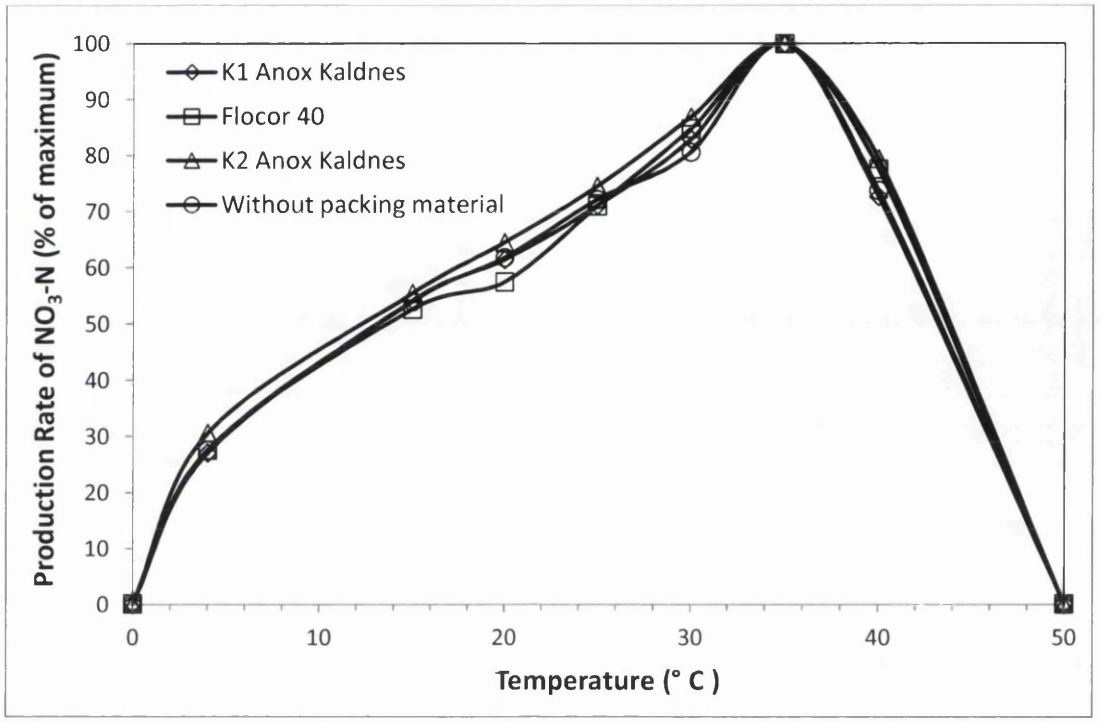


Figure 3.24: The effect of temperature on the production rate of nitrate-nitrogen in a batch culture with and without packing material at the concentration of 70 mg/L ammonia-nitrogen in the fish effluent enrichment culture. ***See Appendix I***

The measured nitrate-nitrogen production rates can be used to determine a value for the constant, kt, in the Van't Hoff-Arrhenius equation (equation 3.1), by plotting the natural logarithm of the reaction rate against temperature for the logarithmic section of the temperature relationship, as shown in Figure 3.25. The value of kt for the production rate of nitrate-nitrogen with the K1 AnoxKaldnes packing material at an ammonia-nitrogen concentration 30 mg/L was thus derived from the equation 3.1; according to the linear value of the graph, the value of kt is $0.126°C^{-1}$. This value is accordance with the reported values for the growth of nitrifying bacteria in the range of $0.095\text{-}0.12°C^{-1}$ (Henze *et al.*, 2002 and Henze *et al.*, 2008).

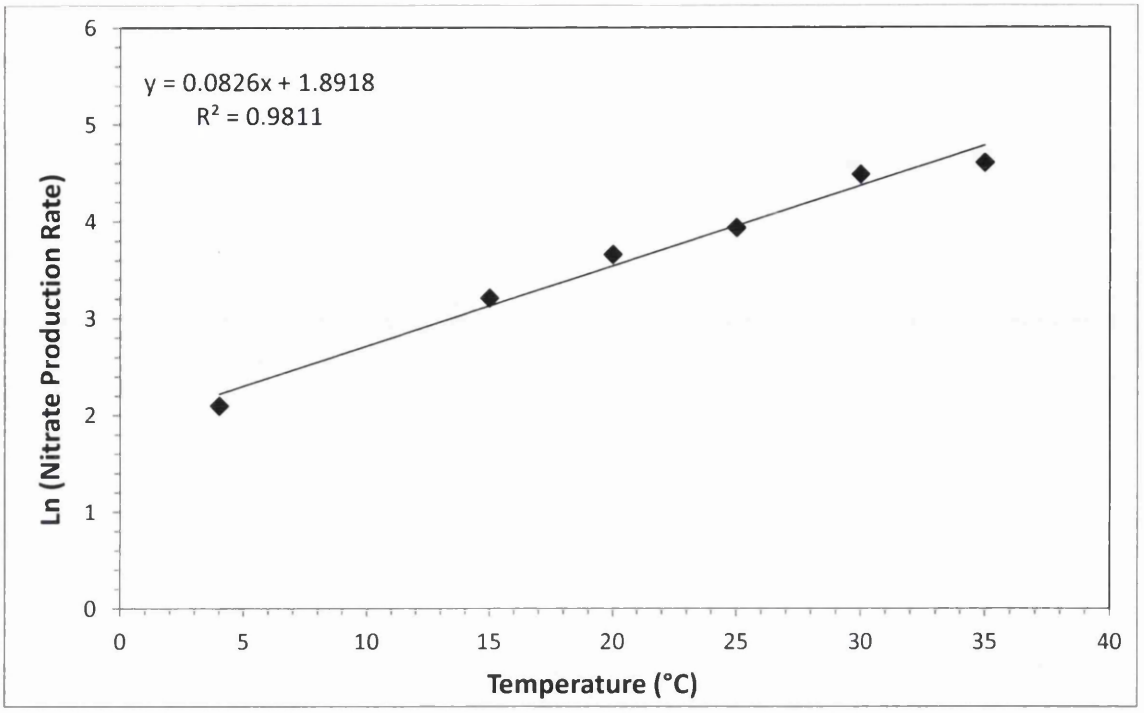


Figure 3.25: Arrhenius plot of Ln (reaction rate) for the production of nitrate-nitrogen in a batch culture with the K1 AnoxKaldnes packing material at an ammonia-nitrogen concentration of 30 mg/L in the fish effluent enrichment culture plotted against temperature to determine kt, the constant in the Van't Hoff-Arrhenius equation.

3.9 Influence of pH on the growth of nitrifying bacteria in batch culture

The parameter of pH plays a major role in the nitrification process, since nitrifying bacteria generally increase in a pH range of 6.5 to 8.0 standard pH units. *Nitrosomonas* has an optimal pH between approximately 7.0 and 8.0, and the optimum pH range for *Nitrobacter* is approximately 7.5 to 8.0. An increase in pH above 9 pH units would cause a reduction in the nitrification rate. However, many other factors contribute to the viability of nitrifying bacteria and, as a result, nitrification has been observed at pH levels ranging from 6.6 to 9.7 (EPA, 2002).

Wilczak (2001) have stated that pH appears to be the most important factor controlling the rate of chloramine auto-decomposition. The effect of low alkalinity

associated with a reduction in buffering capacity, which can impact pH stability, contributed to a decrease in the nitrification process. Therefore, sufficient alkalinity is needed to maintain nitrifying bacteria in the wastewater system (USEPA 1993a, 1993b and EPA, 2002).

For the determination of pH on the growth of enrichment bacteria culture for nitrifying bacteria from fish effluent, two sets of experimental batch cultures were done at two different concentrations of ammonia-nitrogen, i.e. 30 mg/L and 70 mg/L ammonia-nitrogen. These two sets of batch culture were placed in 250 mL volumetric flasks containing the K1 AnoxKaldnes packing material, Flocor packing material, K2 AnoxKaldnes packing material and one flask without packing material; the volume of each flasks were set at 100 mL. A 10 mL aliquot of fish effluent enrichment culture from serial batch culture was inserted in each flask. A set of blank flasks was also prepared containing only medium without packing material and culture bacteria.

These experimental batch cultures were prepared in the pH range from 6 to 8.5. During the experiments, the temperature of the batch culture flasks was maintained at 30°C and the aeration was maintained above 2.0 mg/L. All sets of flasks were stirred by a mechanical stirrer to provide aeration and temperature control. The pH value was controlled by adding 0.01 M hydrochloric acid (HCl) and/or 0.01 sodium hydroxide solution (NaOH), as required. The levels of ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were evaluated daily over a certain incubation period.

The temperature 30°C was chosen, as explained in Section 3.8 to allow for lower energy consumption, for further investigation of the nitrifying bacteria grown on the suspended carrier packing materials. The aeration rate provided was greater than 2.0 mg/L dissolved oxygen to prevent any problems with oxygen transfer limitations into the biofilms of the suspended carrier packing materials. By providing these conditions, this possibility in using these parameters would lead to greater performance and overcome some limitations. Moreover, the comparative investigation could also provide information in the appropriate selection and operation parameters for further experimentation.

The nitrite-nitrogen rate (mg/L.day) was calculated according to the geometric average calculation with respect to the pH:

$$NO2 - N\ production\ rate\ (mg/L.day) = \frac{Sum\ of\ NO2{-}N\ (mg/L)}{Sum\ of\ days} \quad (3.4)$$

The maximum percentage of the nitrite-nitrogen rate (%) with respect to pH was calculated from the following equation:

$$\%\ of\ NO2 - N = \frac{NO2{-}N\ production\ rate\ (mg/L.day)}{The\ highest\ value\ of\ NO2{-}N\ production\ rate\ (mg/L.day)} \text{X } 100 \quad (3.5)$$

For the production rate of nitrate-nitrogen with respect to pH at the concentrations of 30 mg/L and 70 mg/L ammonia-nitrogen, the formulas are given in equation 3.2 and equation 3.3.

Figure 3.26 and Figure 3.27 show the outcome of the results from the tables illustrated in *Appendix J* and *Appendix K*, respectively, for the flasks with and without packing materials using 30 mg/L of ammonia-nitrogen over a range of pH from 6 to 8.5 pH units. This plot of the graph in Figures 3.26 and Figure 3.27 was calculated using Equation 3.2 to Equation 3.5. The results shown in Figure 3.26 show the maximum percentage nitrite-nitrogen production rate at various pH levels. The accumulation of nitrite-nitrogen was slow in the beginning at pH levels between 6 to 7 pH units. However, an increase in nitrite-nitrogen build-up was found at pH 7.5 and the maximum value of nitrite-nitrogen was obtained at pH 8. On the other hand, at pH 8.5, the nitrite-nitrogen level was reduced.

The production rate of nitrate-nitrogen in flasks with and without the support carrier at the ammonia-nitrogen concentration of 30 mg/L is shown in Figure 3.27. The same patterns of nitrate-nitrogen build-up can be seen in Figure 3.27 following the results shown in Figure 3.26 for nitrite-nitrogen production in the immobilized system using the K1 AnoxKaldnes packing material, Flocor packing material, K2 AnoxKaldnes packing material and flasks without the support carrier. The highest yield of nitrate-nitrogen was discovered at pH 8 for all flasks.

The detailed results for the ammonia-nitrogen concentration of 70 mg/L on the production of nitrite-nitrogen and nitrate-nitrogen with and without packing material are

shown in *Appendix L* and *Appendix M.* Figure 3.28 and Figure 3.29 illustrate the results gathered from the tables shown in *Appendix L* and *Appendix M,* respectively.

In Figure 3.28, nitrite-nitrogen increased tremendously at pH 8 after gradually increasing from pH 6 to 7.5. The maximum yield of production for nitrite-nitrogen was at the optimum pH 8 units in the flasks with immobilized packing materials and also in the flasks without the packing material. Following this and regarding the results in Figure 3.28, the nitrifying bacteria achieved the greatest growth at pH 8. Nevertheless, from the experimental observations, the nitrifiers demonstrated good growth at pH 7.5 to 8.5.

Moreover, Figure 3.29 shows the effects of pH on the nitrate-nitrogen production rate in the process of the ammonia oxidation by nitrifying bacteria. This is the outcome of the batch culture with and without packing material at an ammonia-nitrogen concentration of 70 mg/L, according to *Appendix M.* In the immobilized system with packing materials and without packing materials, there was a slightly different level of nitrate-nitrogen production at pH 6.5 in the flasks without the carrier packing, which produced a lower level of nitrate-nitrogen compared to the experimental flasks with the suspended carriers. However, at pH 8, the whole system, i.e. with and without packing materials, displayed high yield of nitrate-nitrogen.

Taken together, all the results in Figure 3.26 to Figure 3.29 show that the pH value is a very important parameter influencing the level of ammonia oxidation by nitrifying bacteria in batch culture with and without packing material at the ammonia-nitrogen concentrations of 30 mg/L and 70 mg/L. The inquest to determine the optimum pH conditions for nitrifiers is extremely critical to achieve a high rate of nitrification. Furthermore, the experimental results demonstrate that the activity of the nitrifying bacteria increased gradually in the pH range of 7 to 8.5. The support media in the experimental flasks and in the flasks without a carrier containing 30 mg/L and 70 mg/L ammonia-nitrogen produced a comparable amount of nitrite-nitrogen and nitrate-nitrogen at pH levels between 7.5 and 8.

From the results, the parameter of pH did not significantly contribute to the production of nitrite-nitrogen and nitrate-nitrogen using the immobilized system and/or without a support carrier. However, the growth of nitrifying bacteria is controlled by the optimum pH. At a pH lower than 6.5 and greater than 8.5, complete inhibition of

nitrification takes place (Ruiz *et al.*, 2003). In spite of this, nitrifying bacteria are slow-growing bacteria, so by providing a support media in the system could retain the bacteria after wash-out and promote higher growth, assuming that a long incubation period of time is given (Rittmann and McCarty, 2001).

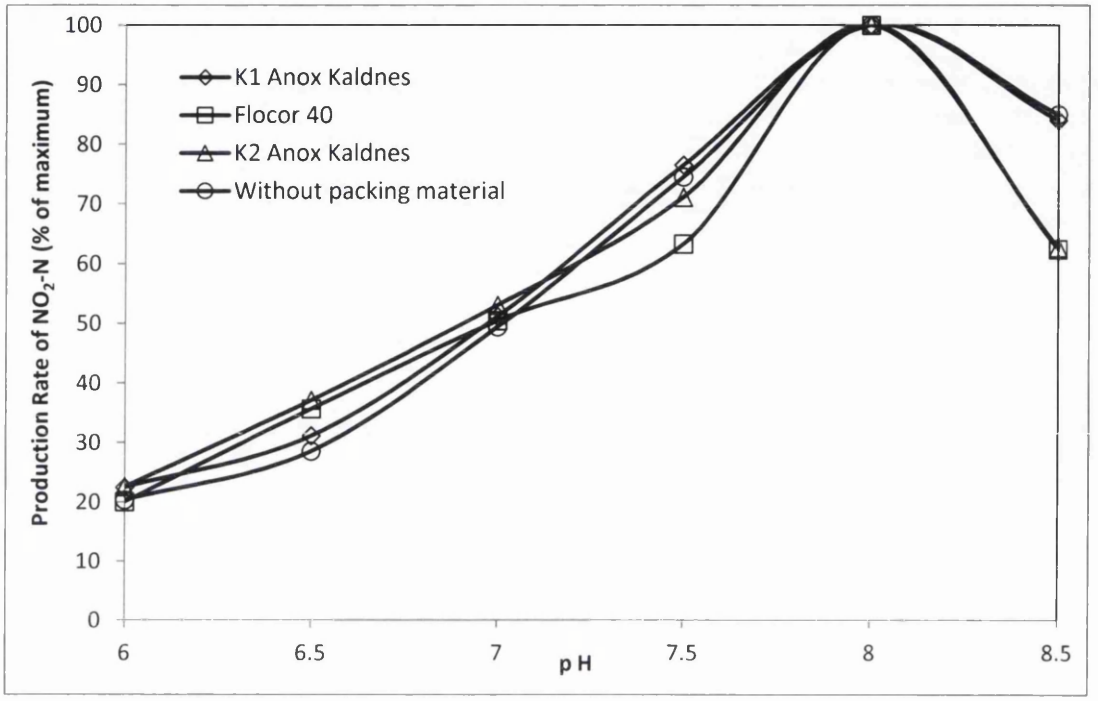


Figure 3.26: The effect of pH on the production rate of nitrite-nitrogen in a batch culture with and without packing material at a concentration of 30 mg/L ammonia-nitrogen in fish effluent enrichment culture.

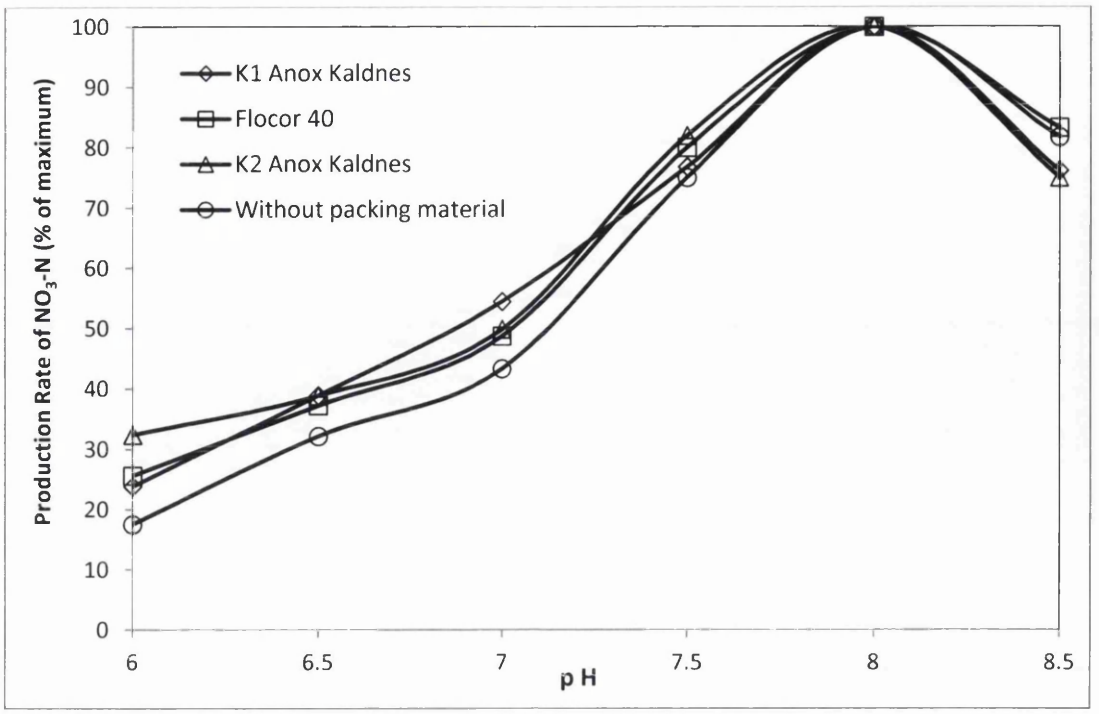


Figure 3.27: The effect of pH on the production rate of nitrate-nitrogen in a batch culture with and without packing material at a concentration of 30 mg/L ammonia-nitrogen in fish effluent enrichment culture.

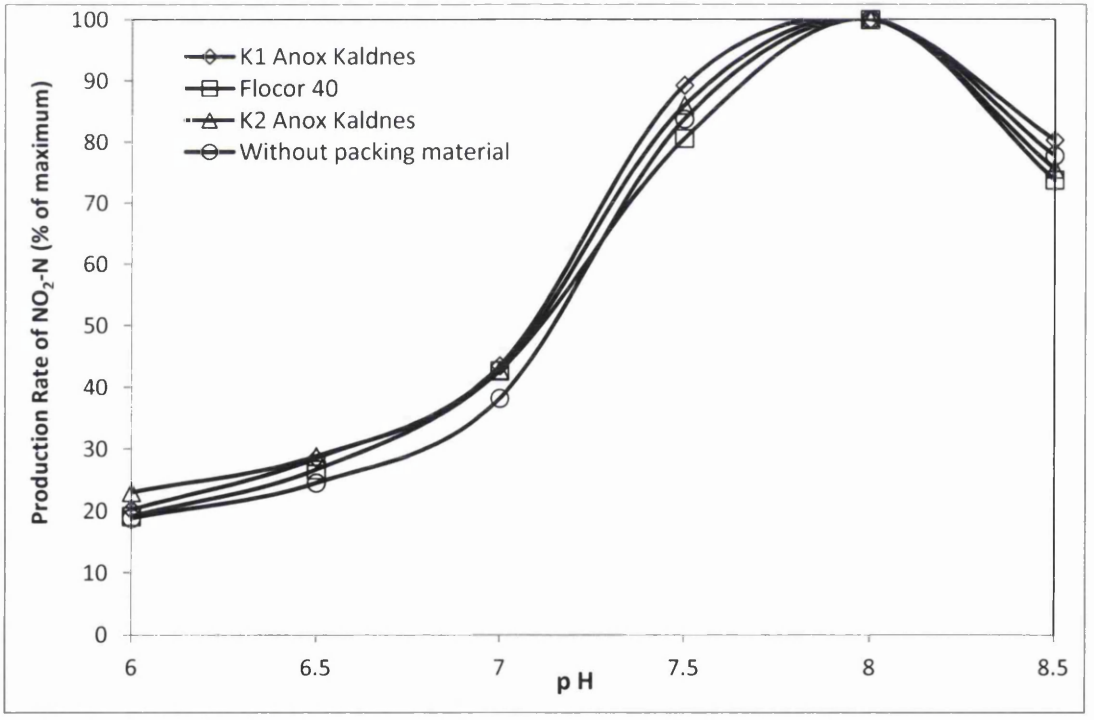


Figure 3.28: The effect of pH on the production rate of nitrite-nitrogen in a batch culture with and without packing material at a concentration of 70 mg/L ammonia-nitrogen in fish effluent enrichment culture.

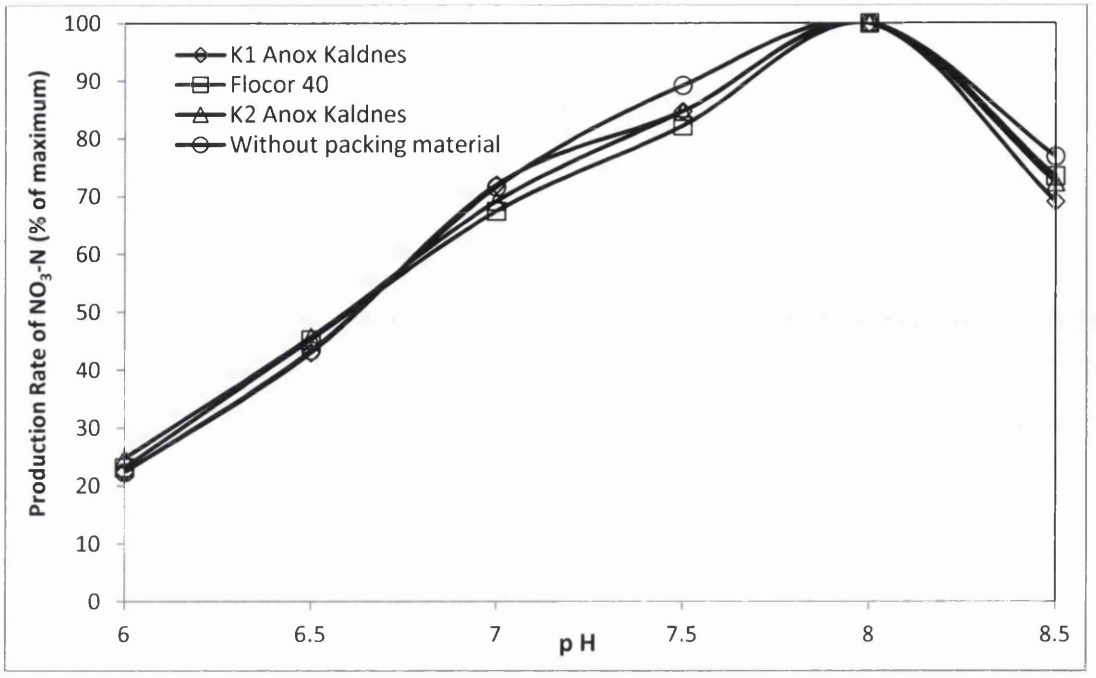


Figure 3.29: The effect of pH on the production rate of nitrate-nitrogen in a batch culture with and without packing material at a concentration of 70 mg/L ammonia-nitrogen in fish effluent enrichment culture.

The results indicate that the optimal pH for nitrification at 30°C for both concentrations of 30 mg/L and 70 mg/L ammonia-nitrogen lies in the range of 7.5 to 8.5 pH units. These data agree well with the results presented by Grunditz and Dalhammar (2001) for experimental pure cultures of *Nitrosomonas* and *Nitrobacter*. As for the response, the batch enrichment culture with fish effluent for all packing materials and without packing material showed significant ammonia oxidation at the optimum pH 8.

Ciudad *et al.* (2007) investigated the modes of operation and pH control for partial nitrification under oxygen transport limitation in a sequencing batch reactor (SBR) and in continuous operation mode. In the SBR system studied by Ciudad *et al.* (2007), different pH control strategies were applied to enhance partial nitrification in a biofilm rotating disk reactor. The pH was controlled in the range of 7.5 to 8.6 and fixed pH at 7.5 and 8.5, with the dissolved oxygen (DO) concentration in the range of 0.6–5.0 mgO_2/L. Based on the observations of Ciudad *et al.* (2007), both operation modes were simultaneously controlled by oxygen transport and micro-kinetics (influenced by pH and NH_3). However, a suitable pH control strategy can act as an enhancement factor for partial nitrification even under oxygen transport-limiting conditions.

The effect of pH and seasonal temperature variation on simultaneous partial nitrification and anammox in free-water surface wetlands has been studied by He *et al.* (2012). In this investigation, a pH between 7.5 and 7.8 favoured partial nitrification over nitrite oxidation; however, the pH should be controlled along with the ammonium concentration and temperature to avoid toxicity of free ammonia to nitrifying bacteria and also to anammox bacteria (He *et al.*, 2012).

A key advantage in the experimental observations regarding the influence of pH in the enrichment culture of nitrifying bacteria in flasks with an immobilized system and without a suspended carrier was to establish the optimum pH value. In obtaining the optimum pH value for nitrifying bacteria, an evaluation of the apparent limitations can be performed.

In considering the limiting factors for the growth of nitrifying bacteria, further steps to achieve high performance in biological treatment systems should be pursued. Hence, based on the results gathered from the present experimental study, the most suitable conditions for the enrichment culture for nitrifying bacteria were established. Furthermore, a high population of nitrifiers in the enrichment culture was established to fulfil the aim of the study.

3.10 Conclusions

The results of this study on enrichment cultures show the possibility of producing ammonia oxidising enrichments that are stimulated by the use of packing materials in the cultures. Clearly, the packing materials enhanced the enrichment process, allowing for greater nitrifying activity to develop. This occurred through an increase in the amount of biomass production by allowing surface-growing bacteria to be retained in the culture, as indicated by the release of cells into the free liquid phase. In the initial enrichment studies, the cultures grew slowly and took many days to develop healthy populations with good activity. These studies also established the growing methods and the analytical techniques for monitoring these cultures.

The results on the production rate of nitrate-nitrogen in batch cultures with and without packing materials for ammonia-nitrogen concentrations of 30 mg/L and 70 mg/L

in the fish effluent enrichment culture clearly show that the highest production rate of nitrate-nitrogen for both concentrations was found at an optimum temperature of 35°C. This result confirms the work of other studies on nitrifying bacteria with respect to the optimum temperature of 35°C to 38°C (Grunditz and Dalhammar, 2001). A high temperature in the range of 30°C to 35°C represents the optimal conditions for achieving nitrification (Gu *et al.,* 2012). However, several investigations on the growth of nitrifying bacteria at milder temperatures between 20°C to 25°C were done by Guo *et al.* (2009), along with some limited studies on attaining nitrification at low temperatures (Qiao *et al.,* 2010).

On the other hand, the important aspect is to reduce the operation costs of biological treatment systems for wastewater. An economical solution in selecting a temperature of 30°C as the working conditions of the enrichment culture and providing support materials in the system would lead to a better strategy in the start-up of bacterial culture.

The use of support materials is the best solution to obtain a high concentration of biomass. As the biofilm was attached to the surface of the carrier, an effective biological process of ammonia-nitrogen degradation could be established. The immobilized system maintained the process of nutrient transfer, and the ideal relatively thin biofilm was evenly distributed over the surface carrier. Providing high aeration may have created turbulence in the flasks, which influenced substrate and oxygen transfer on the support carriers. Moreover, nitrifiers are chemolithoautotrophic and they grow slowly. In implementing these immobilization techniques, biofilm adaptation may help to overcome this drawback by increasing the nitrifier concentrations in the enrichment culture and thus improving the efficiency of the treatment.

Based on the experimental results, the K2 AnoxKaldnes packing material showed the best performance in producing a large amount of free biomass at the different ammonia-nitrogen concentrations of 30 mg/L, 70 mg/L and 100 mg/L, as discussed in Section 3.7.2. The K2 AnoxKaldnes packing material showed a significant benefit in terms of ammonia oxidation compared to the other packing materials and the system without a support carrier. In the experiment investigating temperature and pH, K2 AnoxKaldnes showed slightly different and better performance in comparison to the other packing materials and without the immobilization system. Therefore, the K2

AnoxKaldnes packing material was used in the further study of batch culture and continuous culture in subsequent experiments.

Regarding the effect of pH on the production rate of nitrate-nitrogen in batch culture with and without packing materials at the ammonia-nitrogen concentrations of 30 mg/L and 70 mg/L in the fish effluent enrichment culture, a pH value of 8 was found to be optimum. This is similar to those found in the literature (Vadivelu *et al.*, 2006; Ciudad *et al.*, 2007; Gu *et al.*, 2012). The optimum values correspond to the conditions in the bioassays presented here with regards to the low concentration of ammonia-nitrogen (30 mg/L and 70 mg/L) in the batch culture.

The nitrifying bacteria often referred to as *Nitrosomonas* and *Nitrobacter* are slow-growing bacteria, which make the nitrification process very susceptible to inhibition (Grunditz and Dalhammar, 2001). The most important aspect is that the medium does not contain toxic compounds (Henze *et al.*, 2002). Hence, the optimum conditions for the growth of nitrifying bacteria need to be sustained. Therefore, the next goal is to obtain ammonia oxidation. As a consequence, the nitrifying bacteria will be able to grow successfully and a high growth rate of nitrifying bacteria can be achieved. Furthermore, a high nitrification rate can be achieved during stable long-term operation.

CHAPTER 4

BATCH CULTURE

In this chapter, further investigations into batch culture for nitrifying bacteria were conducted. Moreover, an analysis of the growth kinetics of nitrifying bacteria was performed and the performance of batch culture in the degradation of oestrogens was assessed.

4.1 Background Theory

Culturing cells in a vessel with an initial charge of medium that is not altered by further nutrient addition or removal is a basic principle of batch culture (Shuler and Kargi, 1992). A batch culture is a closed system, such that the growth rate of the biomass must tend towards zero, either because of a lack of nutrients or later by the accumulation of products that cannot be tolerated. Hence, this system is always in a transient state (Pirt, 1975).

4.2 Batch Culture of Nitrifying Bacteria in a One Litre Reactor

In the present study, literally the oxidation of ammonia-nitrogen and nitrite-nitrogen were performed only by *Nitrosomonas* species and *Nitrobacter* species, respectively. Nevertheless, the existence of at least several other types of nitrifying bacteria, i.e. ammonia oxidizing organisms, is reasonably well established (see Chapter 2-

Literature Review). Since there are possibility that other types of bacteria were active in these samples, the observed growth constants may in fact represent an average for a mixed population.

The batch experiments were performed to assess ammonia-nitrogen concentrations and the effects of nitrifying bacteria using fish effluent culture as the substrate for the ammonia oxidation process. There is consensus that ammonia oxidation by the group of nitrifying bacteria follows Michaelis–Menten kinetics (Henze et al., 2002). Ammonia-nitrogen concentrations up to 500 mg/L ammonia-nitrogen were used, and assays were carried out at 30°C in an incubator and at the optimum pH 8 with dosing of sodium hydroxide (NaOH) and/or hydrochloric acid (HCl). The choice of temperature and pH were made according to the results presented in Chapter 3 - Preliminary Study of Enrichment Culture. The K2 AnoxKaldnes packing material was chosen to be the best suitable packing material in batch culture, as shown in the results in Chapter 3- Preliminary Study of Enrichment Culture. Cultures without packing material were used for comparison in the batch culture experiments.

A one litre culture bottle was used as the reactor for the batch culture experiments, with one litre of Medium A which had been autoclaved for 15 minutes. Nitrification Medium A was added to the reactor together with 100 mL of an enrichment culture of fish effluent from the serial batch reaction. The reactor was aerated through a pumice stone using an aeration pump connected by silicone tubing through a hole made on the top of the cap of the reactor. The dissolved oxygen was closely monitored with a dissolved oxygen probe on a daily basis so that the dissolved oxygen in the reactor was maintained above 6 mg/L.

Dissolved oxygen is an extremely important parameter as the dissolved oxygen (DO) concentration has to be relatively high; this is the limiting factor in biofilm processes and in an immobilized reactor system (Rusten *et al.,* 1994). A high driving force in terms of DO concentration across the biofilm is therefore required. Typically for an immobilized system of moving particles with a suspended carrier, the operation of the system demanded a high DO concentration between 5-7 mg/L (Ødegaard *et al.,* 2000; Zafarzadeh *et al.,* 2010).

The conditions regarding the oxygen transfer rate for an immobilized system with moving suspended particles is similar to those achieved in conventional aeration tanks by fine bubble aeration, although coarse bubble aeration can be used in an immobilized system with moving suspended particles. On the other hand, the biofilm on the Kaldnes particles becomes thinner at higher loading rates because of the mixing intensity in aerated or stirred tanks compared to attached growth systems using trickling filters or rotating biological contactors (Ødegaard *et al.*, 2000; Ritmann and McCarty, 2001).

The bottles were capped to prevent spillage of the medium and to ensure that the aeration in the reactor containing the K2 AnoxKaldnes packing material provided constant motion of the packing material in the submerged stage. The experiments in one litre reactors were operated for more than two weeks. Figure 4.1 shows a diagram of the batch culture using K2 AnoxKaldnes packing material in the reactor.

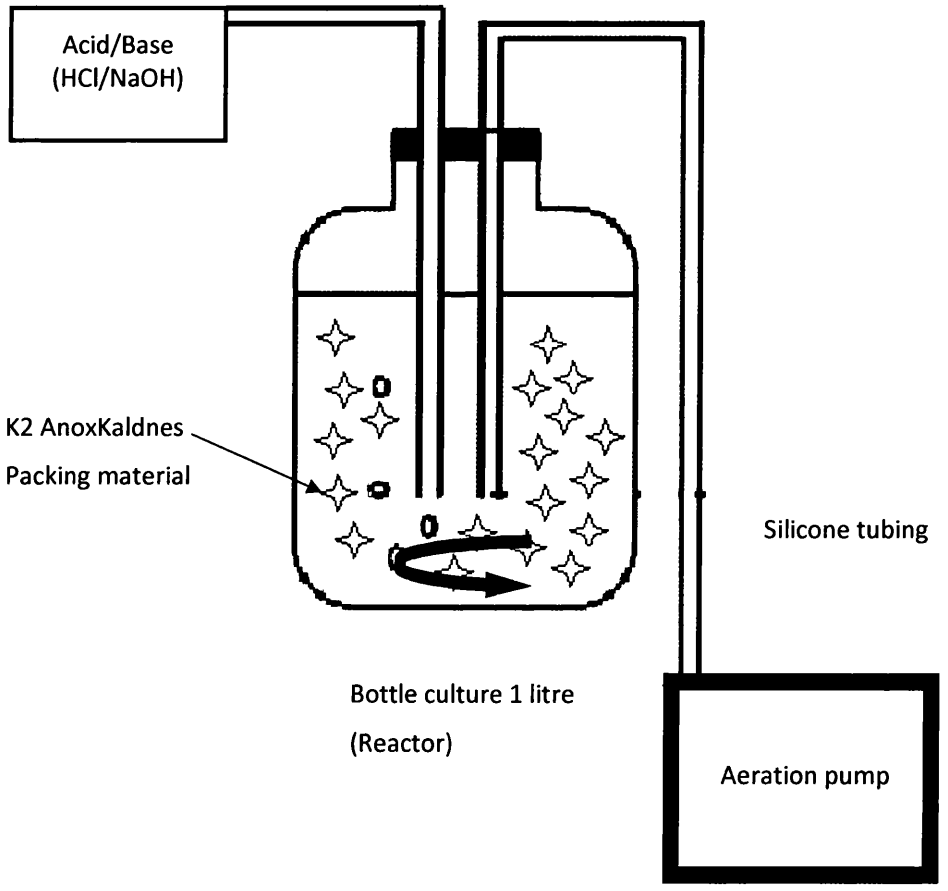


Figure 4.1: Diagram of the batch culture using K2 AnoxKaldnes packing material in a moving bed batch reactor.

The levels of ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were determined on a daily basis. The pH was monitored using a pH probe and the chemical oxygen demand was determined according to the methods outlined in Chapter 3-Preliminary Study of Enrichment Culture.

4.2.1 Low substrate concentration of ammonia-nitrogen (NH_3-N)

Two stages of the experiments were carried out to assess the effect of different substrate concentrations in ammonia oxidation using a moving bed batch reactor with nitrifying bacteria on the K2 AnoxKaldnes packing material. Stage 1 involved low concentrations of ammonia-nitrogen from 5 mg/L ammonia-nitrogen to 100 mg/L ammonia-nitrogen. Stage 2 involved high concentrations from 200 mg/L ammonia-nitrogen up to 500 mg/L ammonia-nitrogen.

The results from the attached growth for nitrifying bacteria in the batch reactor are shown in Figure 4.2 for the K2 AnoxKaldnes packing material with low concentrations of ammonia-nitrogen. A) 5 mg/L ammonia-nitrogen; B) 15 mg/L ammonia-nitrogen; C) 25 mg/L ammonia-nitrogen. Figure 4.3 shows the results for concentrations of A) concentration of 35 mg/L ammonia-nitrogen; B) 65 mg/L ammonia-nitrogen; C) 85 mg/L ammonia-nitrogen and D) 100 mg/L ammonia-nitrogen.

From the results with low concentrations of ammonia-nitrogen, the nitrification process were detected in all the concentrations for ammonia-nitrogen in the attached growth of nitrifying bacteria in batch reactor for K2 AnoxKaldnes packing material. As shown in Figure 4.2 for the concentrations of A) 5 mg/L and B) 15 mg/L ammonia-nitrogen, respectively, ammonia-nitrogen was found to decrease rapidly within a short incubation period with a simultaneous increase in the amounts of nitrate-nitrogen. For the concentration of C) 25 mg/L ammonia-nitrogen, ammonia oxidation required a longer incubation period of nearly 12 days to complete the nitrification process.

In Figure 4.3, the results showed that the concentration of nitrate-nitrogen increased steadily for the concentrations of A) 35 mg/L ammonia-nitrogen and B) 65 mg/L ammonia-nitrogen as the ammonia oxidiser bacteria consumed the ammonia-nitrogen. The amounts of nitrite-nitrogen at all concentrations declined sharply as the nitrate-nitrogen level increased. This condition is presumed to be caused by nitrite

oxidisers converting nitrite-nitrogen to nitrate-nitrogen. The amount of nitrite-nitrogen in Figure 4.3, D) 100 mg/L of ammonia-nitrogen concentration, was at a very high level at approximately nearly 950 µg/L nitrite-nitrogen on day 3 of the incubation period. However, this decreased rapidly by day 5, such that this value was almost zero by the end of the incubation period.

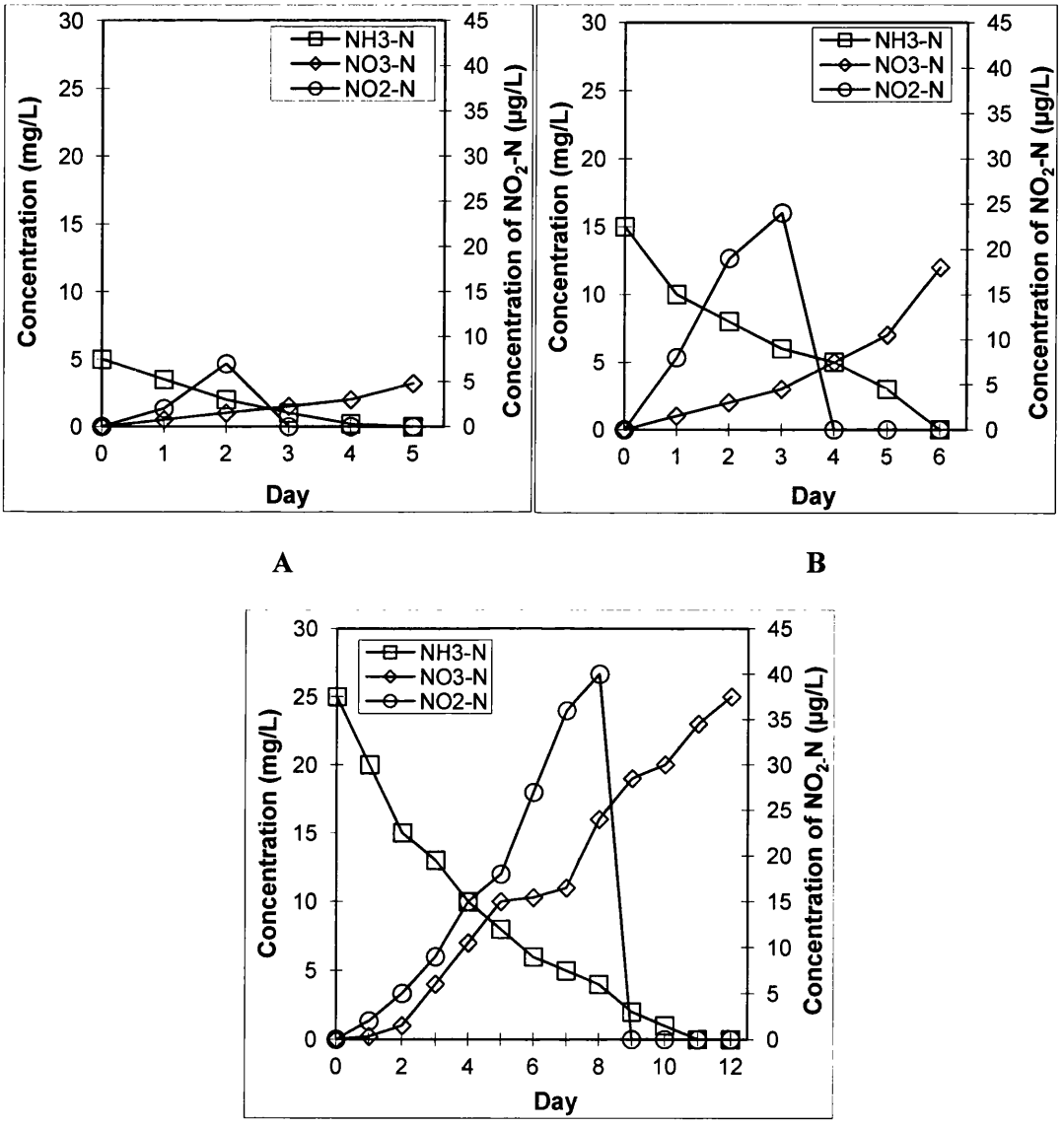


Figure 4.2: The effect of low concentrations of ammonia-nitrogen on nitrification in batch reactor experiments using the K2 AnoxKaldnes packing material; A) 5 mg/L ammonia-nitrogen, B) 15 mg/L ammonia-nitrogen, C) 25 mg/L ammonia-nitrogen.

Ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) are thought to exist in the attached growth batch culture on the K2 AnoxKaldnes packing material. Nitrite-nitrogen does not accumulate in large concentrations under stable conditions because the maximum growth rate for NOB is significantly higher than that of AOB. As the result, the growth rate of AOB generally controls the overall rate of nitrification (WEF, 2005). This was observed in Figure 4.2 and Figure 4.3, as all the nitrite-nitrogen concentrations achieved maximum values at intermediate incubation periods before levels declined sharply as nitrate-nitrogen built up. In the nitrification process, nitrite-nitrogen oxidation occurs faster than ammonia-nitrogen oxidation; therefore, nitrite-nitrogen rarely increases in the environment. This condition is very likely due to a minimum substrate concentration and the relatively high substrate uptake rate of nitrite oxidizers (Rittmann and McCarty, 2001).

A low dissolved oxygen concentration could affect the specific growth of AOB and NOB, depending on the saturation constant (Zafarzadeh *et al.,* 2010). The population structure of nitrifiers changes when the concentration of dissolved oxygen is low. This condition could affect the nitrite – nitrogen accumulation rate (Park and Noguera, 2004). By providing a high level of dissolved oxygen in the batch culture system, i.e. above 6 mg/L, this should increase the population of nitrifiers such that the complete process of nitrification can be achieved.

The immobilized system adapted in the batch culture also contributed to a high density of nitrifiers. Nitrifiers can easily attach to the support media, and the large surface area provided by the media increases the growth of nitrifying bacteria. Furthermore, the reactor volume in the immobilized reactor with a moving suspended carrier is totally mixed with dissolved oxygen, so new bacteria can increasingly occupy the surface area. Consequently, there is no dead or unused space in the reactor system, so the reactor can function at full capacity. In addition to that, the immobilized system has small head loss and there is no need to recycle the biomass or sludge back into the system due to the high density of nitrifying bacteria that can be grown on the support media (Xiao *et al.*, 2007).

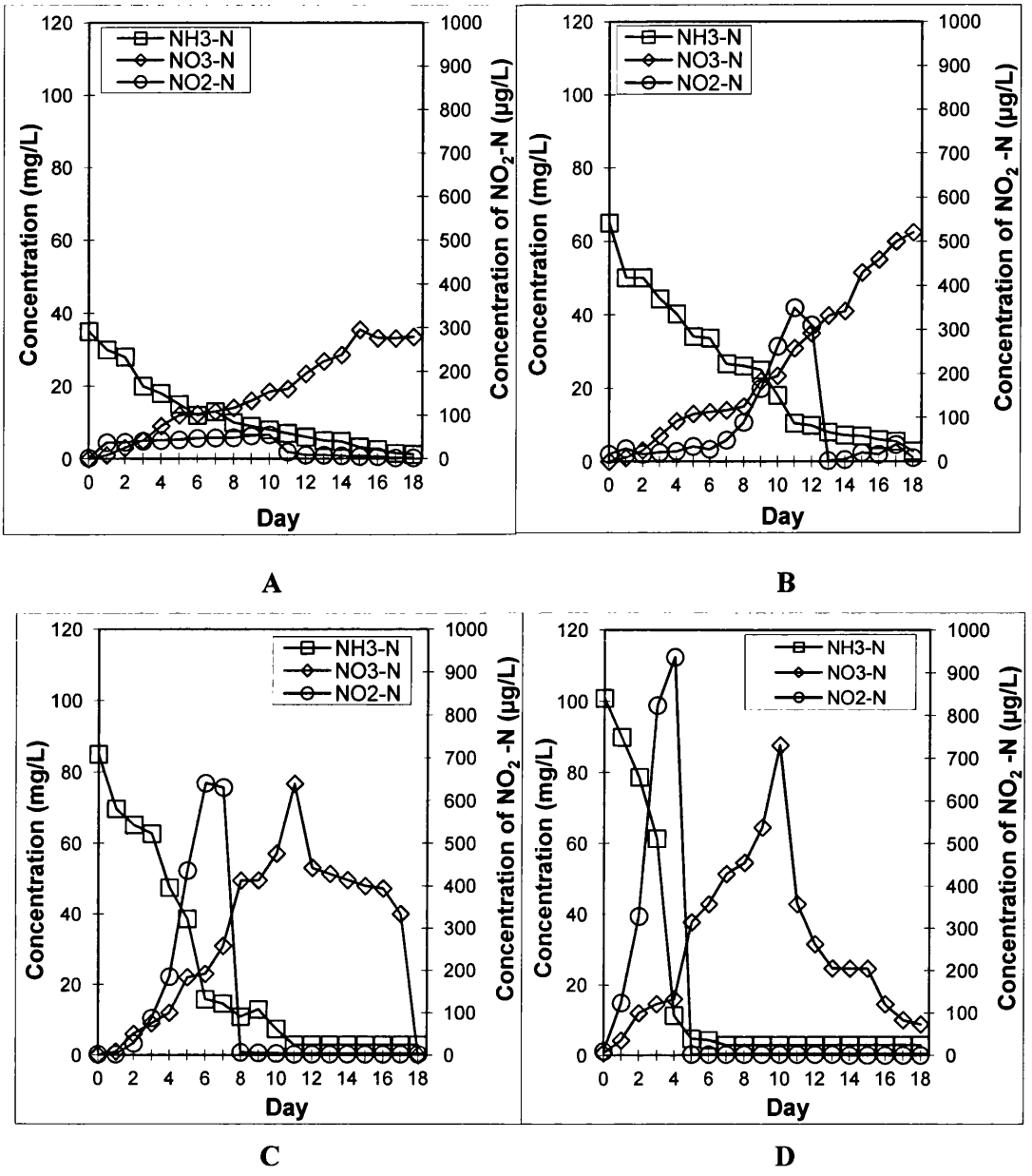


Figure 4.3: The effect of high concentrations of ammonia-nitrogen on nitrification in batch reactor experiments using the K2 AnoxKaldnes packing material; A) 35 mg/L ammonia-nitrogen, B) 65 mg/L ammonia-nitrogen, C) 85 mg/L ammonia-nitrogen, D) 100 mg/L ammonia-nitrogen.

Figure 4.4 A) shows a comparison of ammonia-nitrogen at different concentrations from 5 mg/L to 100 mg/L for a specific incubation period. The rate of ammonia-nitrogen removal was directly related to the initial concentration of ammonia-nitrogen. In all cases, the ammonia-nitrogen level reduced from < 2 mg/L to undetectable levels. At low concentrations, ammonia-nitrogen was generally reduced in less than a week, while at high concentrations, this took up to 2 weeks of incubation. The results indicate that an enhanced rate of ammonia oxidation is proportionate to the length of time needed for the nitrifying bacteria to consume ammonia-nitrogen.

However, in response to the changes of the ammonia-nitrogen concentration introduced in the batch culture system, a long incubation time was needed for the nitrifying bacteria to consume high concentrations of ammonia-nitrogen. On the other hand, from the experimental observations with an initial concentration of 100 mg/L ammonia-nitrogen, the nitrifying bacteria could utilize ammonia-nitrogen in one week's time. The incubation time for nitrifying bacteria with 100 mg/L ammonia-nitrogen was shorter compared to the other initial concentrations of ammonia-nitrogen. This situation could be explained since the enrichment culture of the nitrifying bacteria was previously grown with an ammonia-nitrogen concentration of 100 mg/L. Under these conditions, the nitrifying bacteria were already adapted to a high concentration of ammonia-nitrogen (100 mg/L), so these bacteria could assimilate ammonia-nitrogen faster compared to conditions with different ammonia-nitrogen concentrations. Furthermore, in the next section, ammonia-nitrogen concentrations above 100 mg/L were investigated, in which a concentration up to 500 mg/L ammonia-nitrogen was used for the experimental study of ammonia oxidation by nitrifying bacteria.

The ammonia-nitrogen (NH_3-N) removal rate (mg/L.day) was calculated according to the geometric average calculation with respect to time:

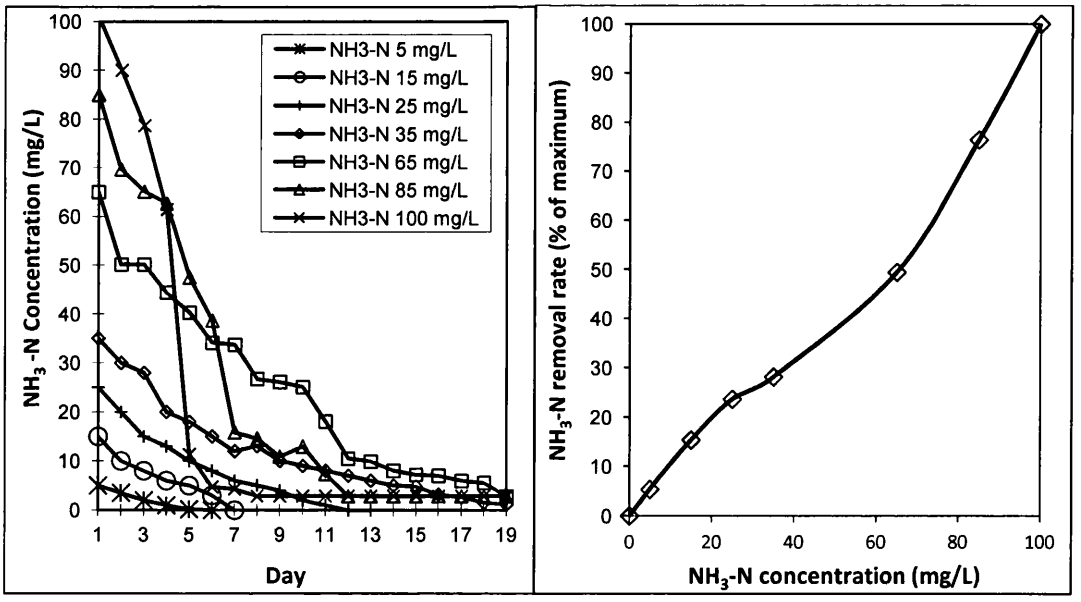
$$NH3-N\ removal\ rate\ \left(\frac{mg}{L.day}\right) = \frac{\left((Initial\ of\ NH3-N)-(NH3-N\ remain)\right)\left(\frac{mg}{L}\right)}{Sum\ of\ days} \quad (4.1)$$

The percentage value for the ammonia-nitrogen removal rate (%) with respect to time was calculated using the following equation:

$$\%\ of\ NH3-N\ removal\ rate = \frac{NH3-N\ removal\ rate\left(\frac{mg}{L.day}\right)}{The\ highest\ value\ of\ NH3-N\ removal\ rate\left(\frac{mg}{L.day}\right)} \times 100 \quad (4.2)$$

Figure 4.4 B) shows the removal rate of ammonia-nitrogen that was calculated according to the percentage maximum removal of ammonia-nitrogen according to the Equation 4.1 and Equation 4.2. The results for ammonia-nitrogen concentrations can be found in *Appendix N* for ammonia-nitrogen concentrations from 5 mg/L to 100 mg/L. For all the low concentrations that were investigated, the removal rates of ammonia-nitrogen increased steadily with the highest removal rate at 100 mg/L. This showed that the nitrifying bacteria were capable of nitrifying ammonia-nitrogen up to a concentration of 100 mg/L in the batch reactor with an attached growth system on the K2 AnoxKaldnes packing material.

The graphs shown in Figure 4.4 A) and Figure 4.4 B) were used to obtain data for determining the results shown in Figure 4.5. A further explanation of the ammonia-nitrogen removal rate versus substrate concentrations from the experiment investigations is illustrated in Figure 4.5 for the ammonia-nitrogen concentrations of 5 mg/L to 100 mg/L.



A B

Figure 4.4: The effect of the ammonia-nitrogen concentration in the K2 AnoxKaldnes packing material in batch reactor. A) Different concentrations of ammonia-nitrogen in a certain incubation period. B) The removal rate of ammonia-nitrogen (nitrification process) vs. the ammonia-nitrogen concentration.

The rate of ammonia-nitrogen removal in the nitrification process with attached growth in the batch reactor using the K2 AnoxKaldnes packing material was shown to follow Michaelis-Menten kinetics by replotting the results on a double-reciprocal plot (Lineweaver & Burk, 1934), as shown in Figure 4.5. The derivation for a double-reciprocal plot (Lineweaver & Burk, 1934) is shown in the following equation:

$$V = \frac{VmaxS}{Ks+S} \tag{4.3}$$

$$\frac{1}{V} = \frac{Ks+S}{V\,max+S} = \frac{Ks}{Vmax}\frac{1}{S} + \frac{1}{Vmax} \tag{4.4}$$

where

V = reaction rate

V_{max} = maximum rate

Ks = Michaelis-Menten half saturation constant

S = substrate concentration

The value used to plot the x-axis is *1/S* and that for the y-axis is *1/v* in a double-reciprocal plot (Lineweaver & Burk, 1934). The experimental results from the double-reciprocal plot show the effect of the substrate concentration on the ammonia-nitrogen removal rate (nitrification process). According to Figure 4.5, the values obtained for the maximum rate, V_{max}, and half saturation constant, *Ks*, were 76.92 mg NH_3-N/L.day and 49 mg NH_3-N/L, respectively.

The results from the double-reciprocal plot (Lineweaver & Burk, 1934) in Figure 4.5 were fit to the best linear equation to determine the maximum rate, V_{max}, and the half saturation constant, *Ks*. However, the graph of the double-reciprocal plot in Figure 4.5 tended to curve in the beginning of the plot. Yet, the results plotted is important to obtain the optimum value for the calculation of the intercept for the maximum rate, V_{max}, and slope to verify the half saturation constant, *Ks*.

Data points restricted to the lower and upper portions of the double-reciprocal plot (Lineweaver & Burk, 1934) gave the half saturation constant, *Ks*. In spite of that, to obtain a high substrate removal rate, the *Ks* value has to be low. The experimental results show that the half saturation constant, *Ks*, was very high with a value of 49 mg NH_3-N/L.

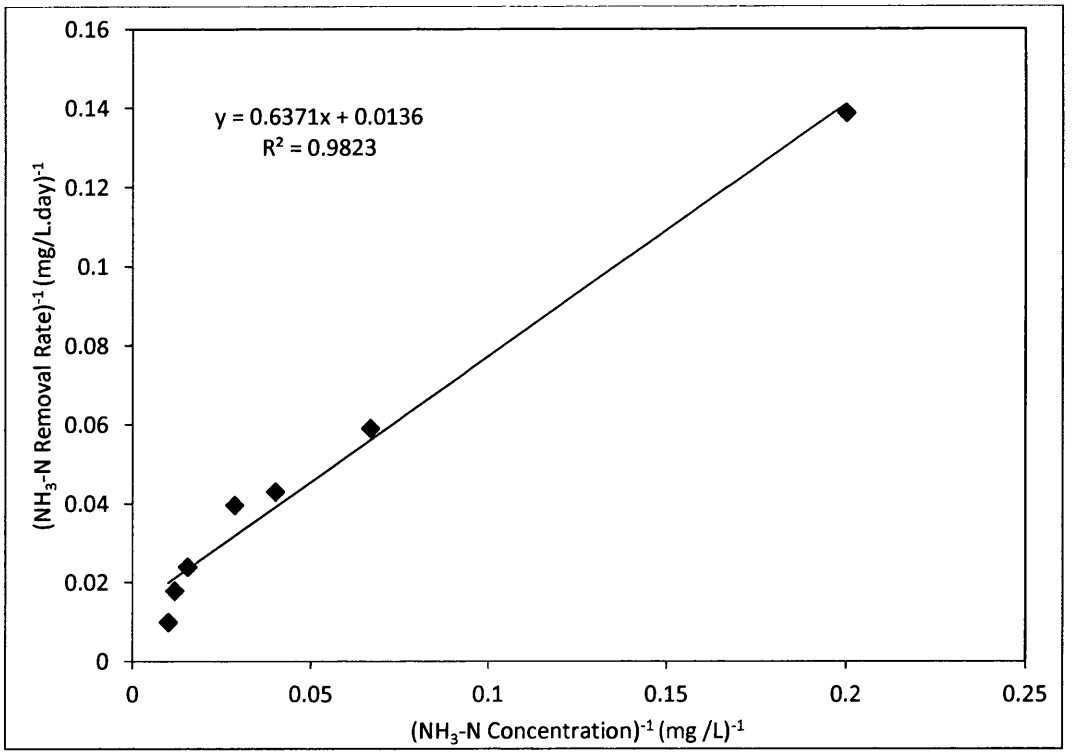


Figure 4.5: A double-reciprocal plot showing the effect of the substrate concentration on the ammonia-nitrogen removal rate (nitrification process).

Based on these results, the inhibition of nitrification in nitrifying bacteria in this experimental investigation was high compared to other reported values obtained from other studies. The value of the half saturation constant for the two-step process of AOB and NOB in aerobic nitrifying granules was found to be 9.1 mg NH_3-N/L (Fang *et al.*, 2009); this low value was achieved using granulation of nitrifying sludge in a sequencing batch reactor (Fang *et al.*, 2009). The nitrifying granule-based sequencing batch reactor is a very complex biological system with numerous internal interactions among process variables and sludge characteristics. Furthermore, the biological processes in the granules are governed by the concentration gradients of oxygen and diverse substrates, and these parameters greatly influence the overall performance of the system (Su and Yu, 2006).

In the activated sludge model proposed by Henze *et al.* (2000), the half saturation constant, *Ks*, had a value of 0.07 mg NH_3-N/L. This value is lower compared to the investigation done by Fang *et al.* (2009) for the nitrifying granular system. This value for the half saturation constant was obtained regardless of the model verification approach used with the activated sludge (Henze *et al.*, 2000). Other studies done on the nitrification kinetics of activated sludge in a biofilm system using a mathematical approach provided a

value of the half saturation constant of 0.27 mg NH_3-N/L (Thalla *et al.,* 2010). However, this activated sludge-biofilm reactor was operated at a high temperature (35 + 2°C) with a low pH value between 4.3 and 4.5 and with biomass recycling back into the system. Moreover, this reactor was a continuous reactor with ammonia-nitrogen feeding in the range of 16 to 17 mg NH_3-N/L (Thalla *et al.,* 2010).

Research was performed by Wiesmann (1994) using a high concentration of ammonia-nitrogen with an initial amount of 100 mg NH_3-N/L in a fluidized bed reactor system and 300 mg NH_3-N/L to 1000 mg NH_3-N/L in a stirred tank reactor system; the value of the half saturation constant, *Ks*, was 540 mg NH_3-N/L. The saturation constant obtained by Wiesmann (1994) is much higher than that found in the present study using a batch culture immobilized system with the K2 AnoxKaldnes packing material. Several other studies on activated sludge enrichment culture obtained a value for the half saturation constant, *Ks*, in the range of 0.3 to 0.7 g NH_4–N/L for the nitrification process at a temperature of 20°C (EPA 2002; Henze *et al.,* 2002).

There is considerable variation in the values of the half saturation constant, *Ks*, for nitrifying bacteria, and there is no clear correlation with either the type of culture for the nitrifying bacteria, the reactor system used, or parameters such as dissolved oxygen, pH and temperature. In spite of that, these factors do contribute to the different of half saturation constant values (Wiesmann *et al.,* 2007).

However, in this experimental study on a batch culture using the immobilized K2 AnoxKaldnes packing material, a possible reason for the high measured value of the half saturation constant, *Ks*, obtained in this investigation is due to the enrichment culture that was used in this research study as the enrichment culture of the nitrifying bacteria was maintained with a non-limiting supply of ammonia-nitrogen at an initial level of 100 mg/L. Hence, the efficiency of the mechanism for the uptake of ammonia-nitrogen by nitrifying bacteria in the reactor may have been reduced. Since these cultures were not permitted time to acclimatise to the experimental ammonia-nitrogen concentrations, the half saturation constant in this experimental study was high.

Figure 4.6 shows the graph of the nitrate-nitrogen production rate for different concentrations of ammonia-nitrogen in the immobilized moving carrier batch reactor with the K2 AnoxKaldnes packing material. Nitrate-nitrogen production increased steadily when the concentration of ammonia-nitrogen went from 5 mg/L to 100 mg/L ammonia-

nitrogen. This showed that the AOB and NOB were heavily populated and attached in the batch reactor with the K2 AnoxKaldnes packing material. These nitrifying bacteria consumed energy from the oxidation of ammonia-nitrogen and the conversion of nitrite-nitrogen to produce nitrate-nitrogen in the complete nitrification process.

The nitrate-nitrogen (NO_3-N) production rate (mg/L.day) was calculated according to the geometric average calculation with respect to time:

$$NO3-N\ production\ rate\ \left(\frac{mg}{L.day}\right) = \frac{(NO3-N\ Production)\left(\frac{mg}{L}\right)}{Sum\ of\ days} \quad (4.5)$$

The percentage maximum value for the nitrate-nitrogen production rate (%) with respect to time was calculated from the following equation:

$$\%\ of\ NO3-N\ production\ rate = \frac{NO3-N\ production\ rate\left(\frac{mg}{L.day}\right)}{The\ highest\ value\ of\ NO3-N\ production\ rate\left(\frac{mg}{L.day}\right)} \times 100 \quad (4.6)$$

The results for nitrate-nitrogen production for the concentration range from 5 mg/L to 100 mg/L ammonia-nitrogen are given in *Appendix N.* The results of using equation 4.5 and equation 4.6 are shown in Figure 4.6. Corresponding to Figure 4.6, the graph in Figure 4.7 was determined using the double-reciprocal plot (Lineweaver & Burk, 1934).

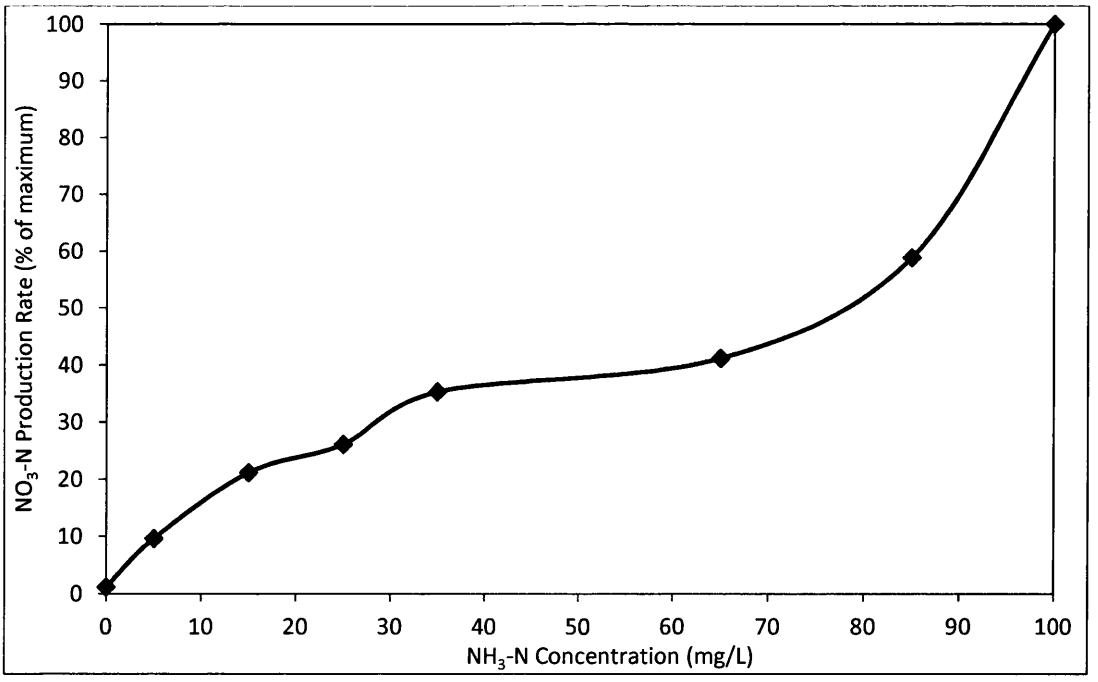


Figure 4.6: Nitrate-nitrogen production rate for the batch culture using the K2 AnoxKaldnes packing material with different concentrations of ammonia-nitrogen.

The rate of nitrite-nitrogen oxidation for the production of nitrate-nitrogen is shown in Figure 4.7. The nitrate-nitrogen production rates followed Michaelis–Menten kinetics for ammonia oxidation based on the results that were re-plotted on a double-reciprocal plot (Lineweaver & Burk, 1934). The values obtained for the maximum rate, V_{max}, and the half saturation constant, *Ks*, were 71.42 mg/L.day and 32.643 mg/L, respectively. The calculation of the intercept for the maximum rate, V_{max}, and slope to verify the half saturation constant, *Ks*, was performed using the linear equation shown in Figure 4.7.

This value was considered to be high, possibly since the nitrifying bacteria in the enrichment culture from the fish effluent used in this study was maintained with a non-limiting supply of ammonia-nitrogen (100 mg/L ammonia-nitrogen initially) in serial batch culture. This factor may have influenced the performance of the nitrifying bacteria in the batch reactor systems, which lowered the maintenance energy of the nitrifiers required to perform ammonia-nitrogen uptake.

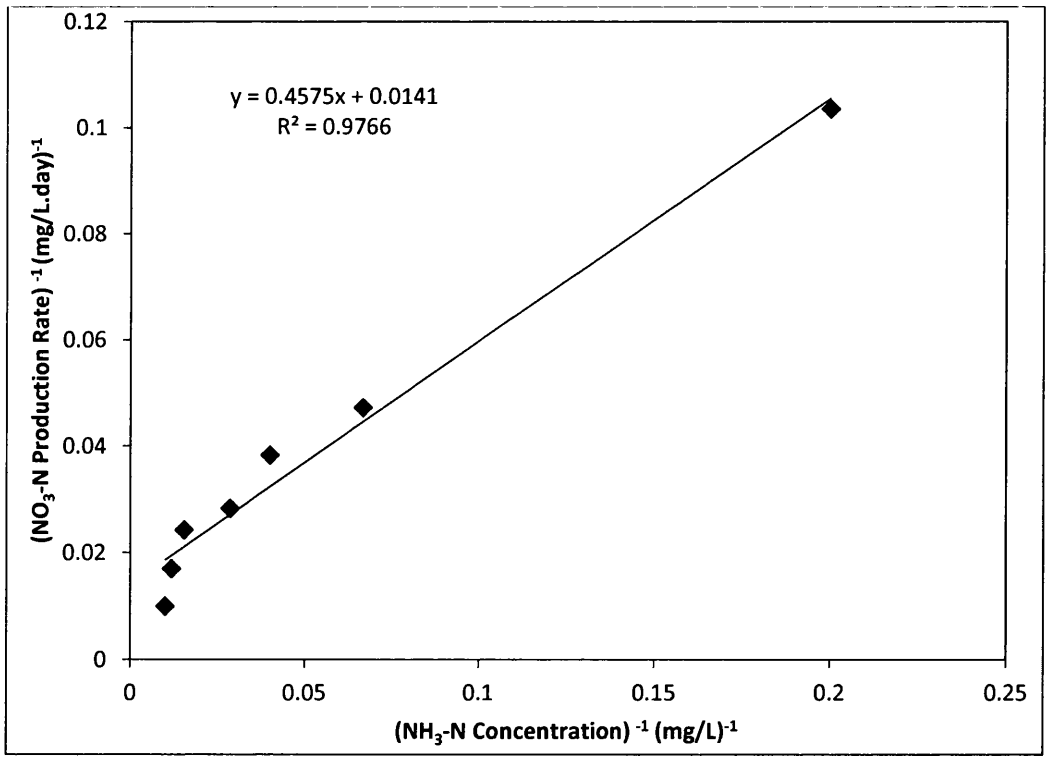


Figure 4.7: Double-reciprocal plot showing the effect of ammonia oxidation on the nitrate-nitrogen production rate based on the substrate concentration of ammonia-nitrogen.

This experimental study using a batch culture reactor demonstrated the degradation process of ammonia-nitrogen at different concentrations. The use of an immobilized system with the K2 AnoxKaldnes packing material could retain a high density of nitrifying bacteria to perform the ammonia oxidation process by converting ammonia-nitrogen to nitrite-nitrogen and nitrate-nitrogen. This microbial community of nitrifying bacteria was capable of adapting to changes in ammonia-nitrogen levels while maintaining nitrification activity.

Based on the results of the experimental study, the AOB and NOB in the batch culture reactor were tolerant to ammonia-nitrogen concentrations from 5 mg/L to 100 mg/L. In the presence of an optimum amount of dissolved oxygen, temperature and pH in the given environment, the nitrifying organisms could survive and attach to the immobilized suspended carrier system.

In the next section, experiments were performed with higher concentrations of ammonia-nitrogen, i.e. up to 500 mg/L. This study was done to analyse the link between a high ammonia-nitrogen concentration to in situ ammonia oxidization limitation by nitrifying bacteria. Hence, by introducing different ammonia-nitrogen concentrations to the batch culture with nitrifying bacteria, the identification of this inhibition could be determined. This experiment was designed to improve the distribution of nitrifiers in the immobilized system with the K2 AnoxKaldnes packing material for further analysis of the degradation of oestrogens in a batch culture system.

4.2.2 High substrate concentration of ammonia-nitrogen (NH_3-N)

An experiment was carried out to investigate ammonia oxidation with high concentrations of ammonia-nitrogen, i.e. from 100 mg/L up to 500 mg/L. The analysis was carried out using the same one litre culture bottles as described in Figure 4.1 with attached growth of the batch culture on the K2 AnoxKaldnes packing material. Medium A was autoclaved and added to the reactor together with 100 mL of fish effluent enrichment culture from the serial batch. The temperature was controlled at 30°C and the pH was set to 8. The aeration was maintained above 6 mg/L, and the values were constantly checked with a dissolved oxygen probe. The cultured was sampled on a daily basis and the samples were analysed for ammonia-nitrogen, nitrate-nitrogen and nitrite-nitrogen. The incubation period for the high concentrations of ammonia-nitrogen was 22 days.

The results of the experiment are shown in Figure 4.8, which shows the concentration of ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen in batch reactors with nitrifying bacteria on the K2 AnoxKaldnes packing material. As shown in Figure 4.8, A) for the ammonia-nitrogen concentration of 200 mg/L, the value for nitrate-nitrogen increased steadily when the ammonia-nitrogen value decreased towards zero and nitrite-nitrogen was consumed by the NOB. In Figure 4.8, B) for the ammonia-nitrogen concentration of 300 mg/L, nitrate-nitrogen slowly built up and the ammonia oxidation process began to slow down toward the end of the incubation period. In Figure 4.8, C) for the ammonia-nitrogen concentration of 400 mg/L and D) for the ammonia-nitrogen concentration of 500 mg/L, the ammonia oxidation process was retarded, possibly due to the high concentration of ammonia-nitrogen present in the batch reactor with the K2 AnoxKaldnes packing material.

According to the experimental analysis, the nitrification process was significantly affected by a high concentration of ammonia-nitrogen in the batch reactor. The ammonium ions were converted to nitrite ions at a faster rate than nitrite ions were converted to nitrate ions. Therefore, excessive ammonium ion discharge or deamination of organic-nitrogen compounds may inhibit nitrification (Gerardi, 2003). Based on these results, the nitrification process of the nitrifying bacteria began to slow down when a high concentration of ammonia-nitrogen was introduced to the reactor system. These high concentrations of ammonia-nitrogen produced a certain amount of free ammonia (FA) that could not be tolerated by the nitrifying bacteria. Following from the results shown in Figure 4.8, a concentration above 400 mg/L ammonia-nitrogen contributed to excessive ammonia discharge or FA present in the batch culture system. This inhibition decreased the microbial activity in the batch reactor with the K2 AnoxKaldnes packing material. In respond to this situation, a longer period of time was needed by the nitrifying bacteria to fully oxidize ammonia-nitrogen.

Several studies have shown that the inhibitory effect of ammonia on nitrification is due not to the ammonia itself, but rather due to FA, which can form with high concentrations of total ammonia (Anthonisen *et al.*, 1976, Kim *et al.*, 2008). Furthermore, the deterioration in nitrogen removal efficiency was mainly due to ammonia accumulation in the reactor system. A possible reason for this depreciation in nitrogen removal performance could be due to the increase in FA and free nitrous acid (FNA). FA

is inhibitory to AOB and NOB. However, FNA, rather than ammonium ions (NH_4^+) and nitrite ions (NO_2^-), is inhibitory only to NOB (Terada *et al.,* 2003).

From this point of view, a long retention time is required for the AOB and NOB to adapt to changes in the environmental conditions when a high level of ammonia-nitrogen is found in the batch reactor system. A novel approach to operating the immobilized system with the K2 AnoxKaldnes packing material resulted to complete nitrogen removal. The suspended carriers in the batch reactor provided intermediate support for the nitrifying bacteria. New colonies of nitrifying bacteria could be developed and attached to the support carrier. High nitrogen transformation could be achieved through the action of nitrifying bacteria on the ammonia-nitrogen accumulated by the filters. This activity plays a role in producing an oxygen diffusion gradient that will create conditions favourable for the nitrification process (Tal *et al.,* 2003).

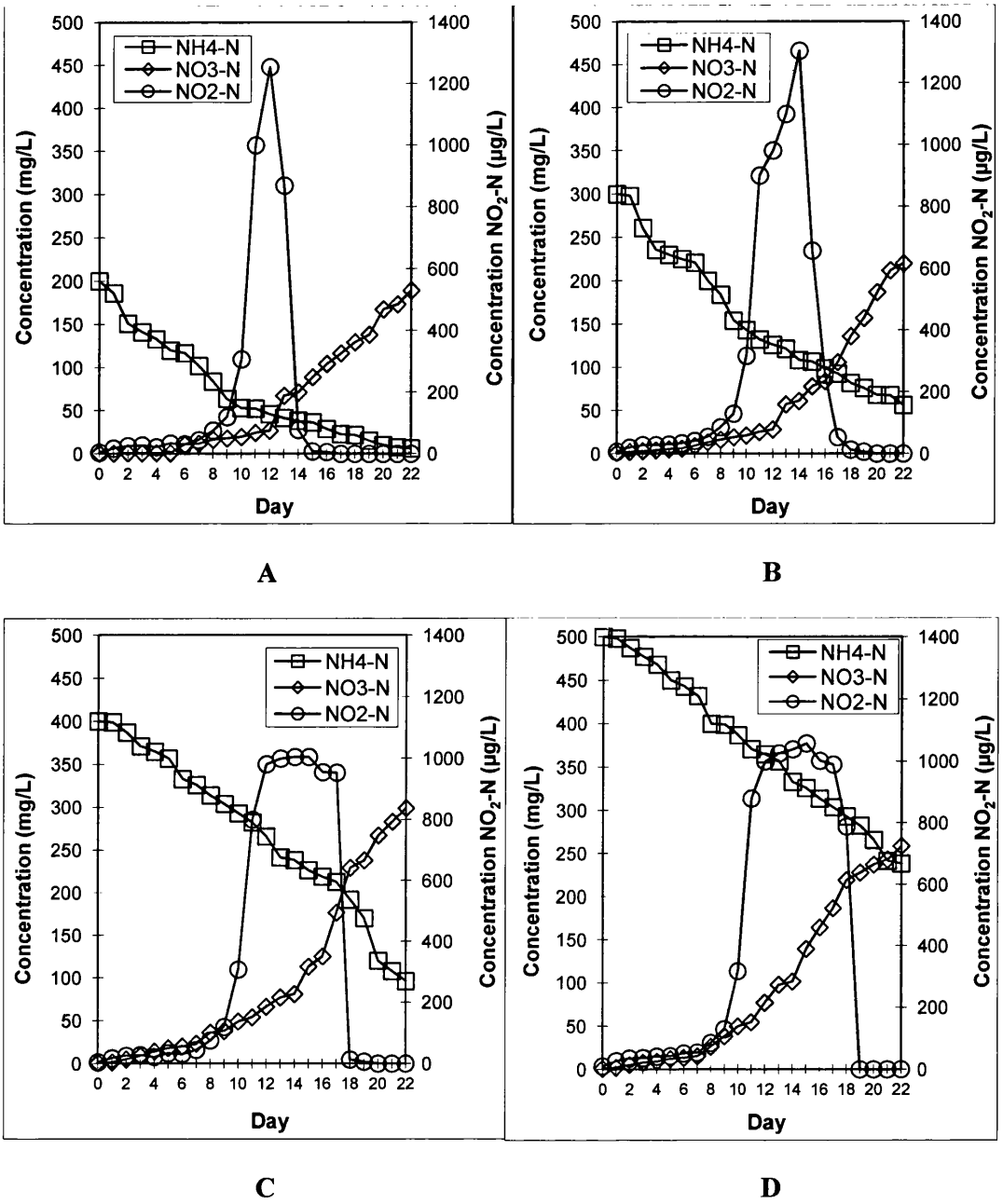


Figure 4.8: Nitrification using the K2 AnoxKaldnes packing material in batch reactor experiments with high concentrations of ammonium sulphate. A) 200 mg/L ammonia –nitrogen, B) 300 mg/L ammonia-nitrogen, C) 400 mg/L ammonia-nitrogen, D) 500 mg/L ammonia-nitrogen.

Figure 4.9 shows the reduced nitrification values at five different concentrations of ammonia-nitrogen with the K2 AnoxKaldnes packing material in the batch reactor. The concentrations of 100 mg/L ammonia-nitrogen, 200 mg/L ammonia-nitrogen, 300 mg/L ammonia-nitrogen, 400 mg/L ammonia-nitrogen and 500 mg/L ammonia-nitrogen were assessed. Figure 4.9 shows decreased amounts of ammonia-nitrogen after 22 days of incubation, with the highest decline in ammonia-nitrogen at the concentration of 100 mg/L ammonia-nitrogen.

The formula for exponential decay was used to describe the limitation of nitrifying bacteria in the presence of different high concentrations of ammonia-nitrogen shown in Figure 4.9 for the experimental study using the batch reactor immobilized system with the K2 AnoxKaldnes packing material.

$$N = N_0 e^{kt}, \quad k \leq 0 \tag{4.7}$$

where

N = concentration of ammonia-nitrogen remain in the system after a certain incubation period.

N_o = initial concentration of ammonia-nitrogen

t = time

k = decay rate

The decay rate, k, for the concentration of 500 mg/L ammonia-nitrogen was calculated as -0.055. This value was the highest decay rate compared to the other decay rates obtained from different concentrations of ammonia-nitrogen that were analysed. The values for the decay rate of nitrifying bacteria at 400 mg/L, 300 mg/L and 200 mg/L ammonia-nitrogen were -0.124, -0.133 and -0.177, respectively.

The decay rate in this study was found to be relatively similar to the decay rate obtained for nitrifying bacteria in activated sludge, with a value in the range of 0.2 to 0.06 in similar conditions (Salem *et al.*, 2006). For all activated sludge models, a default decay rate with a value of 0.15 at 20°C was used under all conditions (Henze *et al.*, 2000). However, the decay rate of nitrifying bacteria is an uncertain parameter (Salem *et al.*, 2006).

The decay process is, in general, a complex process modelled in a relatively simple mathematical manner (Van Loosdrecht and Henze, 1999; Salem *et al.*, 2006). The

decay process comprises maintenance energy requirements, the real decay of cells, protozoa grazing and other important factors. The rate of decay can be expected therefore to vary strongly depending on the conditions in a system. Maintaining the optimum incubation conditions for a certain period of time was also an essential aspect to prevent water evaporation and the loss of biomass. In practice, good pH control and maintaining incubation conditions during the experimental analysis has proven to be a crucial factor to ensure good results (Lee and Oleszkiewicz, 2003).

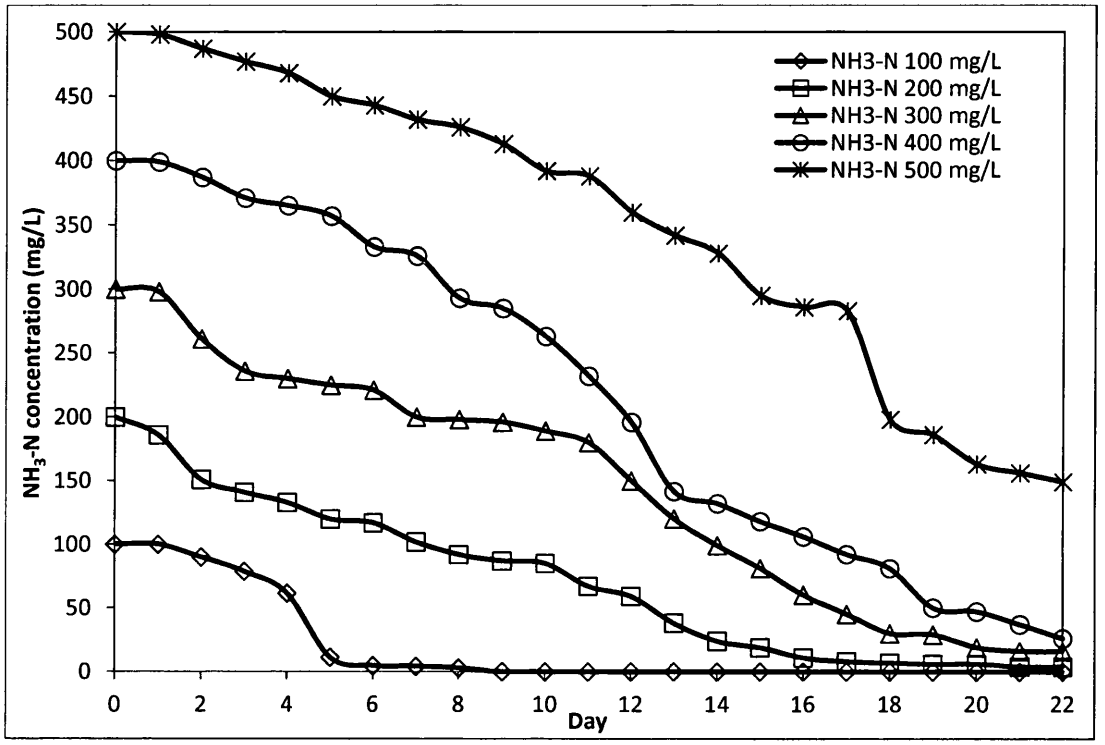


Figure 4.9: The effect of high concentrations of ammonia-nitrogen in the batch culture with the K2 AnoxKaldnes packing material.

This experiment determined the influence of high ammonia-nitrogen concentrations on the growth and/or decay of nitrifying bacteria in the batch culture reactor. The procedure in the batch culture system performed under the optimum conditions was designed to assess the reduction in activity of both AOB and NOB correlated with different concentrations of ammonia-nitrogen.

Here, from the calculation of the decay rate, the reduction in endogenous respiration activity within the AOB and NOB were correlated with high ammonia-nitrogen concentrations. When the ammonia-nitrogen level was high in the reactor

system, the nitrifying bacteria were exposed to extreme conditions (Wiesmann *et al.*, 2007), whereas the slow growth of nitrifying bacteria under high concentrations of free ammonia reduced the activity of nitrifying bacteria. A prolonged incubation time was needed for the nitrifying bacteria to readjust to the surrounding conditions to achieve higher ammonia oxidation for a complete nitrification process.

Nitrifying bacteria use ammonia as a source of energy and electrons. The nitrification reaction can be inhibited by ammonia itself at high concentrations (Kim, 2003). Figure 4.10 shows the percentage of ammonia-nitrogen removal rate at low concentrations to high concentrations of ammonia-nitrogen according to Equation 4.1 and Equation 4.2. The results for the amounts of ammonia-nitrogen are given in *Appendix N*. The ammonia-nitrogen removal rate increased with an increase in the ammonia-nitrogen concentration up to 400 mg/L and then apparently declined with higher amounts of ammonia-nitrogen. This result indicates that an ammonia-nitrogen concentration below 400 mg/L ammonia-nitrogen does not inhibit the microbial activity of nitrifying bacteria in the batch reactor with the K2 AnoxKaldnes packing material.

As the ammonia-nitrogen concentration increased, FA also increased. The inhibitory effect of ammonia on nitrification was due to FA in the system, which can be formed at high concentrations of total ammonia (Anthonisen *et al.*, 1976; Kim *et al.*, 2008). NOB such as *Nitrobacter* will become inhibited (Rosenwinkel and Cornelius, 2005). FA opposed the nitrification process and caused a reduction in ammonia oxidation and a failure of nitrite-nitrogen build-up. From the results shown in Figure 4.10, ammonia-nitrogen concentrations above 400 mg/L contributed to the inhibition of FA present at these high concentrations of ammonia-nitrogen in the batch reactor. This inhibition decreased the microbial activity of nitrifying bacteria in the batch reactor with the K2 AnoxKaldnes packing material.

The reduction of the percentage ammonia-nitrogen removal rate at concentrations higher than 400 mg/L suggested that nitrifying bacteria activity was being obstructed in the presence of high ammonia-nitrogen concentrations. Nitrifying bacteria are sensitive to the changing environment as they are slow-growing bacteria. Implementing the K2 AnoxKaldnes packing material in the batch reactor system allowed the nitrifying bacteria to be cultivated more easily in the attached system. A high population of nitrifiers could be achieved in a certain period of time to carry out the ammonia oxidation process.

Furthermore, the transport mechanisms in immobilized systems with suspended carriers might even enhance nitrite-nitrogen and nitrate-nitrogen accumulation. Dissolved oxygen is normally consumed only in the first 50-100 μm of biofilms due to deficient oxygen transfer (Okabe and Watanabe, 2000). Therefore, biofilm reactors using immobilized suspended carriers such as the K2 AnoxKaldnes packing material are designed to move in the batch reactor system to achieve high mass transfer coefficients at the biofilm/liquid inter-phase, which may be advantageous for the complete nitrification process.

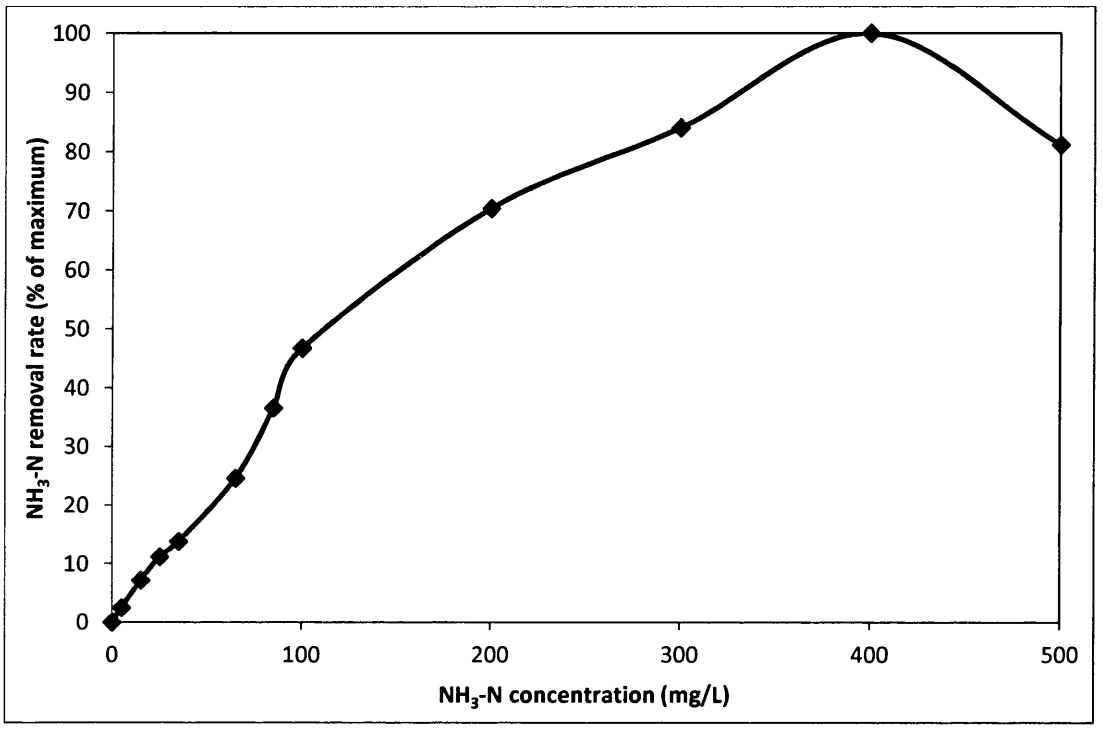


Figure 4.10: The influence of the ammonia-nitrogen concentration on the ammonia-nitrogen removal rate in batch culture using the K2 AnoxKaldnes packing material.

4.3 Batch Culture of Without Packing in One Litre Reactors

Experiments using batch culture without a packing material were also carried out to see the different performance of nitrifying bacteria in ammonia oxidation with suspended growth. The same one litre culture bottles were used as the reactors. One litre of Medium A was prepared and autoclaved and placed in four different culture bottles with 100 mL of fish effluent enrichment from a serial batch. The same temperature of 30°C and pH 8 was used in these reactors. Aeration was supplied through a pumice stone

using an aeration pump connected by silicone tubing through a hole in the cap of the reactor. The dissolved oxygen was closely monitored with a dissolved oxygen probe on a daily basis so that aeration was maintained above 6 mg/L in the reactor. The incubation period for the suspended growth of nitrifying bacteria in the batch culture without packing material was around 15 to 16 days.

The concentrations of ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were determined as stated in Chapter 3 - Preliminary Study of Enrichment Culture, on a daily basis, and the pH for the batch reactor was checked using a pH probe. Figure 4.11 shows the results for the concentrations of ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen for the suspended growth of batch culture without a packing material for different concentrations of ammonia-nitrogen. Figure 4.11 A) shows the concentration of ammonia-nitrogen at 35 mg/L, B) 65 mg/L ammonia-nitrogen, C) 85 mg/L ammonia-nitrogen and D) 100 mg/L ammonia-nitrogen.

The results obtained in Figure 4.11 show that the ammonia oxidation process occurred in the batch reactor without a packing material. The AOB and NOB consumed ammonia-nitrogen and nitrite-nitrogen to produce nitrate-nitrogen. The ammonia-nitrogen value decreased throughout the period of incubation and, simultaneously, the nitrite-nitrogen value increased. At one point of accumulation, the nitrite-nitrogen level decreased sharply with a high build-up of nitrate-nitrogen for all the concentrations in Figure 4.11.

As for the comparison between the batch reactors with the K2 AnoxKaldnes packing material and the batch reactors without the packing material, the nitrate-nitrogen build-up at the ammonia-nitrogen concentrations of 35 mg/L, 65 mg/L, 85 mg/L and 100 mg/L in the batch reactors without the packing material did not increase steadily and the ammonia oxidation process was slower than in the batch reactors with the K2 AnoxKaldnes packing material. These situations occurred because the populations of nitrifying bacteria were lower in the suspended growth culture than in the attached growth culture.

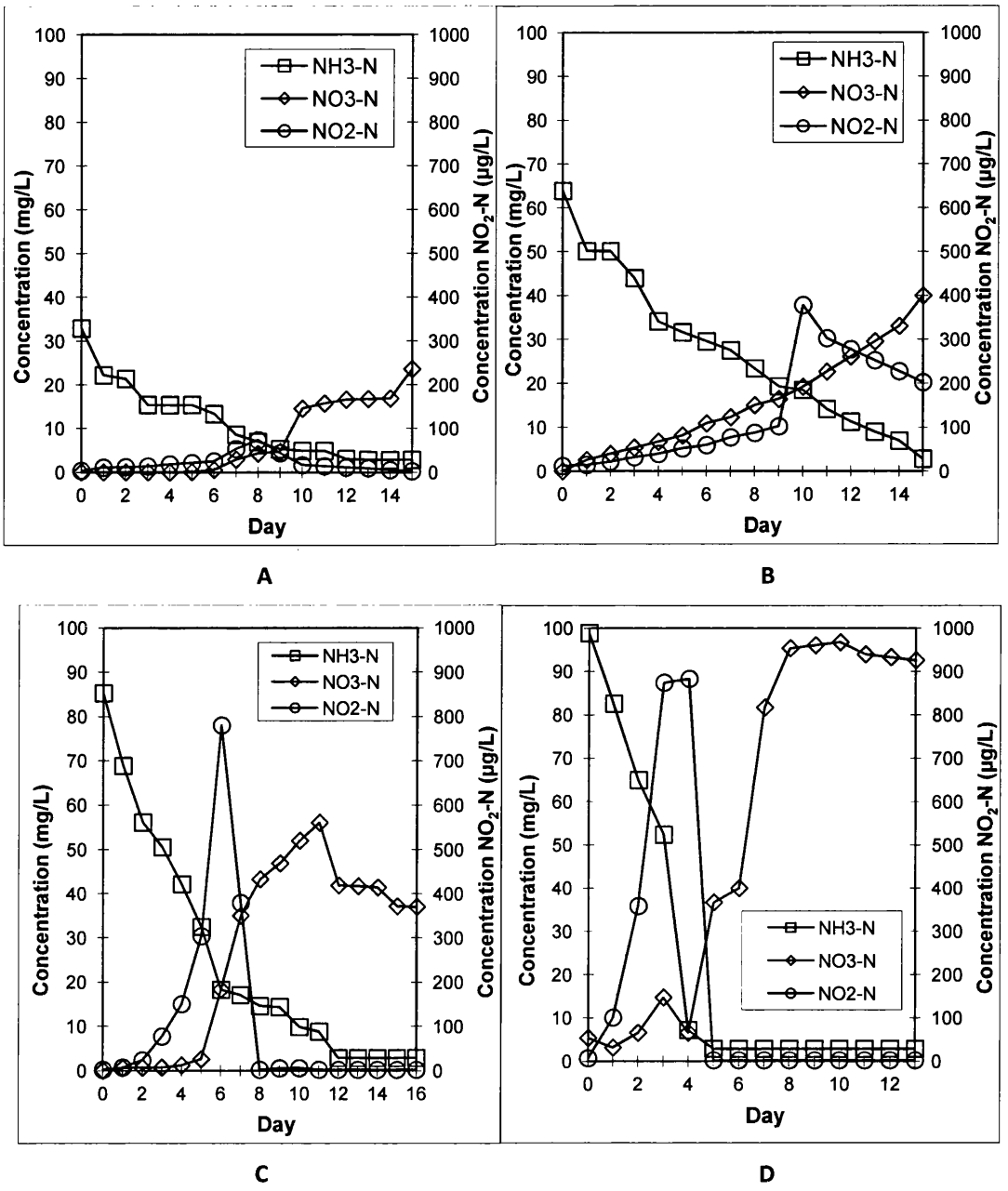


Figure 4.11: The effect of ammonia concentration on the identification processes in the absence packing material for different concentrations of ammonia-nitrogen; A) 35 mg/L, B) 65 mg/L, C) 85 mg/L, D) 100 mg/L.

The experimental analysis of the batch culture in the absence of the packing material was conducted to compare the results from this experiment to the results achieved in the batch culture with the immobilized system using the K2 AnoxKaldnes packing material at different ammonia-nitrogen concentrations. This investigation

provided comprehensive insight into the importance of suspended carriers which combine features of both fixed-growth and activated sludge systems.

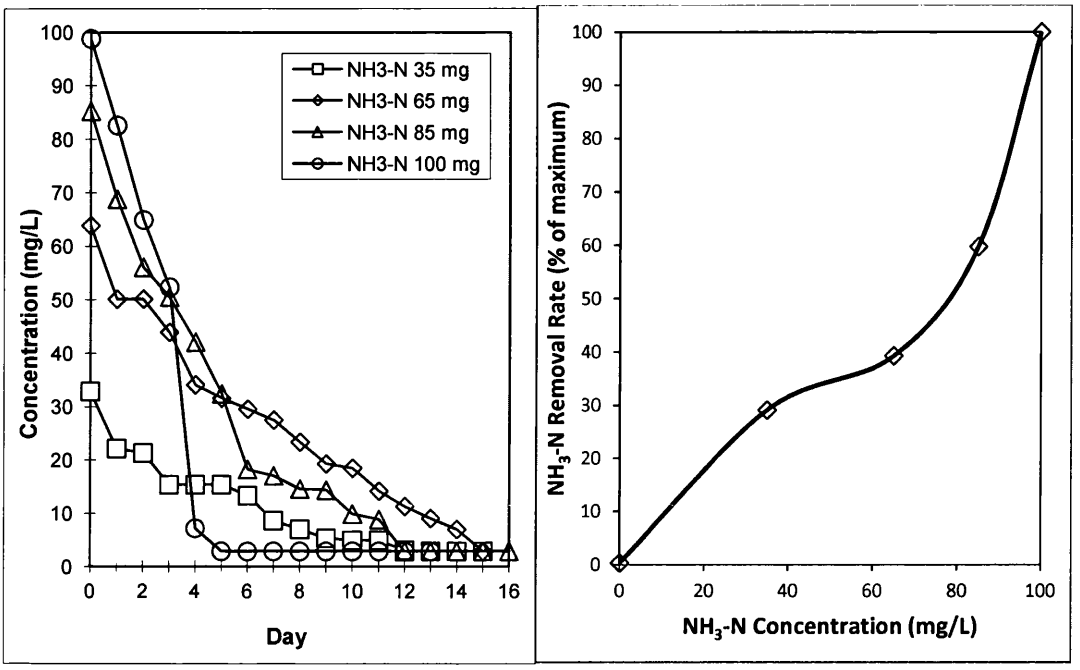
In an immobilized system, the microbial community is largely attached on the surface of supported media and retained within the reactor as a biofilm. This microbial community can flourish and multiply better than when present in a suspension as free-floating cells or small flocs (Biswas and Turner, 2012). This immobilized system of supported media, compared to the absence of the packing material system, could support the development of microbial biofilms of nitrifying bacteria on the surface of the carriers within the microenvironment which occurred in the batch reactor system. The movement of the suspended packing material in the reactor system maintains effective gas and nutrient transfer, since an ideal biofilm is relatively thin and evenly distributed over the carrier surface (Li *et al.,* 2011). Turbulence in the batch reactor system influences substrate and oxygen transfer within the suspended carrier (Zafarzadeh *et al.*, 2010).

In contrast to the absence of the packing material in the batch culture system, the performance of the immobilized system with the K2 AnoxKaldnes packing material was much better. Higher ammonia oxidation was found in the experimental analysis of the immobilized packing material at different concentrations of ammonia-nitrogen compared to the batch culture without the suspended carrier.

Figure 4.12 shows the different concentrations of suspended growth of nitrifying bacteria in batch culture in the absence of the packing material. The ammonia-nitrogen removal rate performance in the batch reactor without the packing material as a function of the ammonia-nitrogen concentration is given in Figure 4.12 B). The amounts of ammonia-nitrogen obtained from the experimental analysis are given in *Appendix O.* The calculation of the removal rate percentage was evaluated according to Equation 4.1 and Equation 4.2, as explained in an earlier section.

The results shown in Figure 4.12 describe how the ammonia-nitrogen removal rate increased with an increase in the ammonia-nitrogen concentration. This shows that the nitrifying bacteria in the suspended growth batch reactor without the packing material behave in the same way as the attached growth in the moving bed batch reactor with the K2 AnoxKaldnes packing material. Both nitrifying bacteria cultures, i.e. in the batch reactor with the K2 AnoxKaldnes packing material and the batch reactor without the packing material, can consume ammonia-nitrogen up to 100 mg /L. Rapid consumption of ammonia-nitrogen was observed at an ammonia-nitrogen concentration of 100 mg/L for

the nitrifying bacteria in the batch culture without the packing material. This occurred since the bacteria in the serial batch enrichment culture had been grown in an initial ammonia-nitrogen concentration of 100 mg/L.



A B

Figure 4.12: The effect of the ammonia-nitrogen concentration in the batch reactor without the packing material. A) Different concentrations of ammonia-nitrogen at certain incubation periods. B) The removal rate of ammonia-nitrogen (nitrification process) against the ammonia-nitrogen concentration.

4.4 Detection of 17α-Ethynylestradiol (EE2) and Mestranol (MeEE2) using Gas Chromatography-Mass Spectrometry (GC-MS)

There are several types of equipment that are suitable to detect this synthetic drug. The most recent and modern equipment employs gas chromatography and mass spectrophotometry (GC-MS). In the beginning of this study, the calibration for 17α–ethynylestradiol (EE2) and mestranol (MeEE2) was performed using GC-MS to detect these compounds in standard stock solutions and to check the retention time of these compounds with changes in concentration in methanol as the solvent. The same calibration was also performed for mestranol (MeEE2) with standard stock solutions prepared in ethanol.

4.4.1 Materials and methods

The chemicals 17α-ethynylestradiol (EE2) and mestranol (MeEE2) were purchased from Sigma Aldrich (purity grade 99%). All solvents were of analytical grade, and the methanol and ethanol for stock solutions were purchased from Merck (Germany). Hexane (Sigma Aldrich) was used for gas chromatography-mass spectrometry.

The stock solution for EE2 at a concentration of 1000 mg/L was prepared in methanol and stored in the refrigerator at 4°C. This stock was designated Stock A. The working solution for GC-MS analysis was prepared from Stock B with a concentration of 100 mg/L in methanol stored at 4°C. Methanol was used to prepare the stock solution of EE2 as this compound is soluble only in methanol, diethyl ether and acetone and very slightly soluble in cold water (MSDS Data Sheet). For the MeEE2 stock solution, the working solution, Stock C, was prepared at a concentration of 100 mg/L MeEE2 in ethanol and stored at 4°C. MeEE2 is soluble in ethanol, acetone, diethyl ether, chloroform and dioxane and slightly soluble in methanol (MSDS Data Sheet).

Serial dilutions of Stock B for EE2 were prepared in methanol. There were two standards prepared with concentration ranges from 1000 ppb to 100 ppb and 100 ppb to 20 ppb. These standards were analysed immediately after dilution to reduce degradation of the materials. The same standard was also prepared for Stock C, i.e. MeEE2 in ethanol. The serial dilutions were inserted in separate vials with a label, and all these vials were put in the numbered casing in the automated sampling port. The calibration curves for EE2 and MeEE2 are illustrated in *Appendix G.*

4.4.2 Procedure for GC separation and MS detection

The gas chromatograph-mass spectrometry equipment (model 7890A, Agilent Technologies) was equipped with a 5975C inert MSD with triple axis detector, shown in Figure 4.13. This apparatus was used for determination of EE2 and MeEE2. The experiments were conducted using a 30 m column with a 0.25 mm internal diameter and 0.25 μm film thickness (AB-5 MS column, Abel Industries, USA) for quantification of both synthetic oestrogens.

The injection port temperature was 250°C for both oestrogens. The oven temperature was held at 100°C for 4 min then programmed at 50°C min^{-1} to 290°C, which was held for 10 minutes to detect EE2. For the quantification of MeEE2, the oven temperature was held at 100°C for 4 min then programmed at 15°C min^{-1} to 290°C, which

was held for 10 minutes. The carrier gas (helium) flow rate was held constant at 1 mL/min. Pulse splitless injections of 1 µL were made by use of a model G 4513 A (Agilent Technologies) autosampler with a splitless period of 2 minutes and a 14 psi pulse.

The MS detector was operated in selected ion monitoring (SIM) mode; m/z values used for quantification and/or identification of the target analytes are summarized in Table 4.1. The total analysis time was 10.338 minutes for EE2 and 17.079 minutes for MeEE2. The limit of detection (LOD) was set to 2 µg/L for both synthetic oestrogens for EE2 and MeEE2. This method for the detection of oestrogens was followed according to Hájková *et al.* (2006) with some modifications.

Figure 4.13: Gas chromatograph-mass spectrometer (GC-MS) model 7890A equipped with a 5975C inert MSD with a triple axis detector and a CN 93901609 autosampler (Agilent Technologies).

Table 4.1: GC-MS analysis of EE2 and MeEE2.

Analyte	Retention time (min)	SIM	
		Quantification ion (m/z)	Confirmation ions (m/z)
17α – ethynylestradiol (EE2)	10.338	296	160, 213
Mestranol (MeEE2)	17.079	227	174,310

4.4.3 Solid phase extraction method

Before the samples were analysed by the gas chromatograph-mass detector (GC-MS), the solid phase extraction (SPE) method was used to separate the oestrogen compounds from the water sample into suitable solvents. Here, a separation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. Analytical laboratories use solid phase extraction to concentrate and purify samples for further analysis.

4.4.3.1 Principle of solid phase extraction (SPE)

Solid phase extraction uses the affinity of solutes dissolved or suspended in a liquid (known as mobile phase) for a solid through which the sample is passed (known as the stationary phase) to separate a mixture into desired and undesired components. The result is that either the desired analytes of interest or undesired impurities in the sample are retained on the stationary phase. The portion that passes through the stationary phase is collected or discarded, depending on whether the portion contains the desired analytes or undesired impurities. If the portion retained on the stationary phase includes the desired analytes, they are then removed from the stationary phase for collection in an additional step, in which the stationary phase is rinsed with an appropriate eluent.

4.4.3.2 Extraction procedure

The procedure for extraction was followed according to the method of Noppe *et al.* (2005). An SPE cartridge (C18, 500 mg, 6 mL, Agilent Technologies) was used for this extraction of the samples in the experiments. The extraction was performed following the manufacturer's guidelines. All samples were filtered first through 0.2 µm Whatman filter syringes with a volume of 10 mL. Before extraction, the cartridges were conditioned with suitable solvents at a flow rate of 10 mL/min. The cartridges were pre-conditioned by passing 20 mL of acetone and 20 mL of methanol and the cartridges were rinsed twice with 10 mL ultrapure water. When the sample had been drawn, the cartridges were vacuumed for at least 30 minutes. The elution was performed using 5 mL of acetone and 15 mL of methanol. The extracts were stored at 4°C in the dark until clean-up before final analysis. Figure 4.14 shows the SPE manifold with C18 (500 mg, 6 mL Agilent Technologies) cartridges on the top of the SPE manifold which is used in the experimental study.

Figure 4.14: Solid phase extraction (SPE) manifold with cartridges and vacuum port gauge to control the vacuum applied to the chamber.

4.4.3.3 Clean-up procedure

The sample extracts were vaporized to dryness; in this study, an oven was used instead of a Rotavapor (Noppe *et al.*, 2005). After the samples were dried, all the samples were reconstituted with 500 µL of chloroform and used for solid-phase extraction. Silica (Si, 500 mg, 6 mL, Agilent Technologies) was used for this clean-up procedure. The cartridges were placed on the manifold and conditioned twice with 2.5 mL n-hexane under vacuum. Before the samples were applied to the cartridges, 5 mL of n-hexane were added to the sample, mixed well and the mixture was transferred on to the cartridges. After the samples had been drawn through the cartridges, another 5 mL of n-hexane were added to the sample flasks and transferred onto the cartridges. Under the Si cartridges, NH_2 (100 mg, 1 mL, Agilent Technologies) cartridges were placed to retain humic acids and other interfering substances. Both cartridges were rinsed with 5 mL of n-hexane. The elution was performed with 5 mL of chloroform-acetone (4:1). Finally, these extracts were then analysed by GC-MS.

Ultrapure water spiked with both oestrogens, i.e. EE2 and MeEE2, were also test with the same extraction and clean-up methods as mention earlier with the recoveries of the ultrapure samples were 90% of recoveries.

4.5 Moving Bed Batch Reactor with the K2 AnoxKaldnes Packing Material for the Removal of EE2 and MeEE2

The experiments using the moving bed batch reactor with the K2 AnoxKaldnes packing material with the addition of synthetic oestrogens, i.e. EE2 and MeEE2, were run in the same way as the previous experimental study using the batch reactor with the K2 AnoxKaldnes packing material.

One litre culture bottles were used as the reactor for the batch culture experiments for the moving bed reactor with the K2 AnoxKaldnes packing material. One litre of Medium A was prepared and autoclaved for 15 minutes and placed in the reactor with 100 mL of fish effluent enrichment culture from the serial batch. The reactor was aerated using a pumice stone with an aeration pump connected by silicone tubing through a hole on the cap of the reactor. The dissolved oxygen was closely monitored with a dissolved oxygen probe on a daily basis such that the dissolved oxygen was maintained above 6 mg/L in the reactor. The bottles were closed to ensure that no spillage of the medium

occurred and that maximum aeration was achieved in the reactor. Aeration allowed the submerged K2 AnoxKaldnes packing material to move constantly in the reactor medium.

The initial concentrations of 100 µg/L EE2 and 100 µg/L MeEE2, prepared as discussed in an earlier section, were added to the moving bed batch reactor with the K2 AnoxKaldnes packing material containing 35 mg/L, 65 mg/L, 85 mg/L and 100 mg/L ammonia-nitrogen. The experiments were run for certain incubation periods at a controlled temperature of 30°C and at pH 8. The detection of oestrogens was performed according to the procedure described in Section 4.4, whereas the results for the experimental analysis are provided in *Appendix P*.

This research was done according to the results of the investigation conducted earlier for the experimental analysis of ammonia oxidation at different concentrations of ammonia-nitrogen in the batch culture system with the K2 AnoxKaldnes packing material. Furthermore, the performance of nitrifying bacteria in the immobilized batch culture system with the K2 AnoxKaldnes packing material was investigated in the degradation of the synthetic oestrogens EE2 and MeEE2. Here, the importance of this experimental analysis was to evaluate the percentage removal rate by the nitrifying bacteria in the immobilized batch culture system in reducing levels of the synthetic pollutants, EE2 and MeEE2.

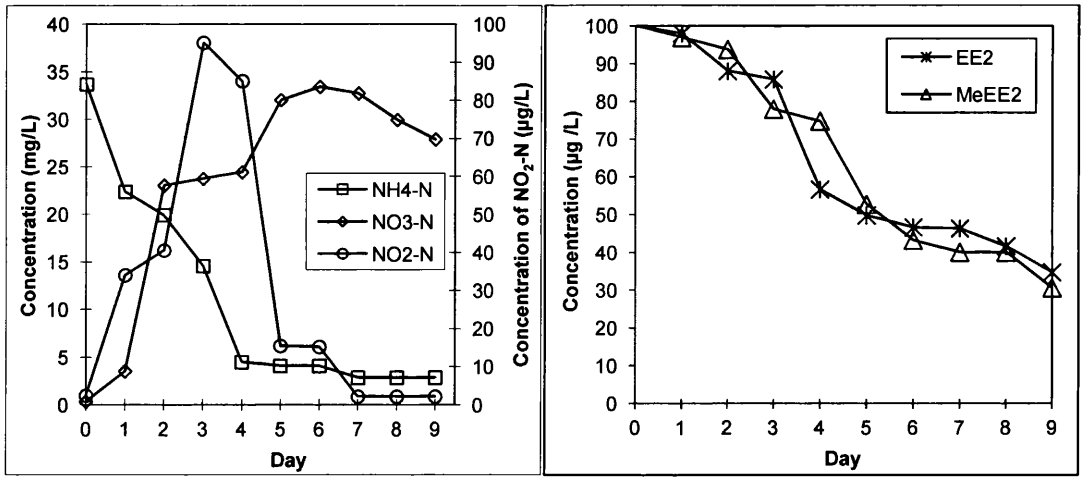
4.5.1 Novelty of the research

This study on the degradation of EE2 and MeEE2 by nitrifying bacteria in a moving bed batch reactor with the K2 AnoxKaldnes packing material was used in this research. This experimental investigation, the performance of the moving bed batch reactor with the K2 AnoxKaldnes packing material with different concentrations of ammonia-nitrogen was evaluated to observe the degradation of the synthetic oestrogens, EE2 and MeEE2.

The levels of ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were determined according to methods outlined in Chapter 3 - Preliminary Study of Enrichment Culture. For the quantification of the synthetic oestrogens EE2 and MeEE2, a gas chromatograph with a mass detector was used. These methods were described in Section 4.4.

4.5.2 Investigation of the removal of EE2 and MeEE2 in the moving bed batch reactor

Figure 4.15 A) shows the results for ammonia oxidation in the moving bed batch reactor with the K2 AnoxKaldnes packing material at 35 mg/L of ammonia-nitrogen. The ammonia-nitrogen levels constantly decreased overtime, with the build-up of nitrate-nitrogen value throughout the incubation period. At the same time, shown in Figure 4.15 B), the results for the removal of the synthetic oestrogens EE2 and MeEE2 show that the concentrations of both oestrogens were significantly reduced. As shown in Figure 4.15 B), the concentration of EE2 and MeEE2 was close to 30 μg/L for both synthetic oestrogens. The removal rate for both synthetic oestrogens in the moving bed batch reactor with the K2 AnoxKaldnes packing material was about 70% removal over the incubation period. By comparing the nitrification profile and that of the oestrogen degradation activity correlates to nitrite-nitrogen reduction.

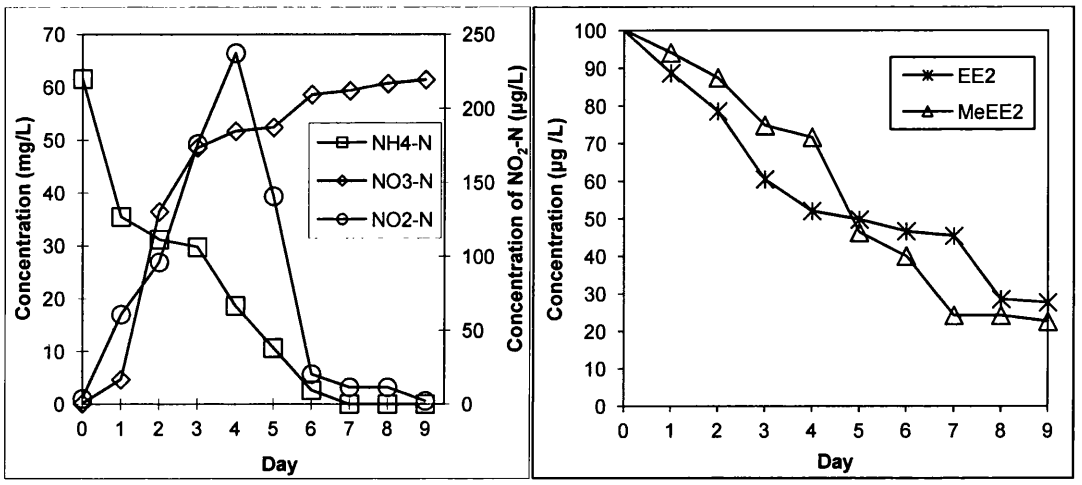


A B

Figure 4.15: A) Results for ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen. B) Degradation of EE2 and MeEE2 at a concentration of 35 mg/L ammonia-nitrogen in the moving bed batch reactor.

Figure 4.16 A) shows the moving bed batch reactor with the K2 AnoxKaldnes packing material at a concentration of 65 mg/L ammonia-nitrogen. As shown in Figure 4.16 A), the value of ammonia-nitrogen declined towards zero with the production of nitrate-nitrogen to the maximum value of nearly 60 mg/L nitrate-nitrogen in an incubation period of 9 days. This shows that the nitrifying bacteria consumed most of the ammonia-nitrogen in the batch reactor due to the ammonia oxidation process.

In Figure 4.16 B), the levels of the synthetic oestrogens EE2 and MeEE2 decreased further compared to the moving bed batch reactor with the K2 AnoxKaldnes packing material at 35 mg/L ammonia-nitrogen. The levels of EE2 and MeEE2 were reduced to 27 µg/L and 22 µg/L, respectively. The removal percentage of EE2 was 73%, whereas the removal percentage for MeEE2 was 78%, which was slightly higher than the removal of EE2.



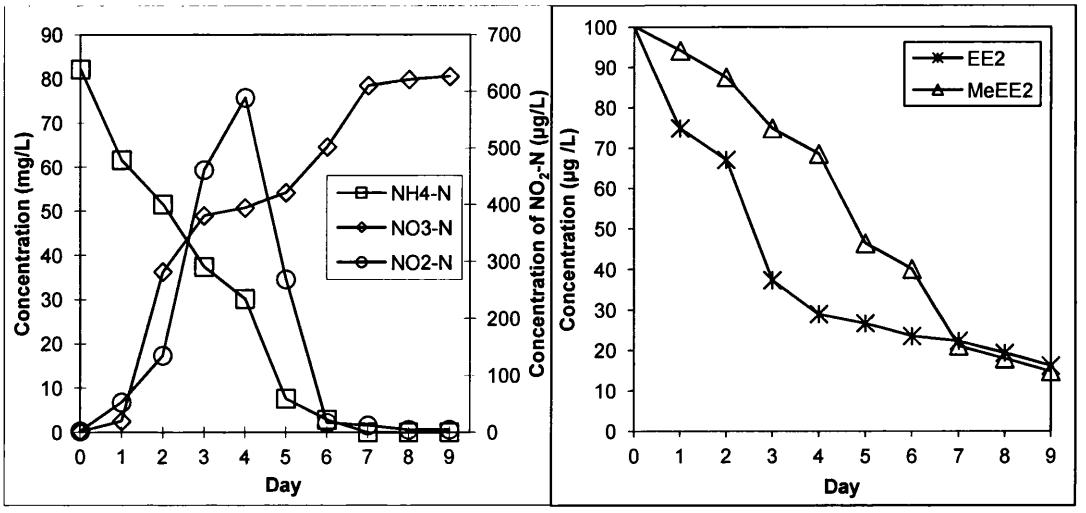
A B

Figure 4.16: Nitrification and oestrogen degradation in a moving bed batch reactor. A) 65 mg/L ammonia-nitrogen. B) Degradation of EE2 and MeEE2.

Figure 4.17 A) shows the results for the moving bed batch reactor with the K2 AnoxKaldnes packing material at 85 mg/L ammonia-nitrogen. The ammonia-nitrogen level decreased rapidly towards zero in the 7 day incubation period. During that time, the nitrate-nitrogen was produced via the ammonia oxidation process from the consumption of ammonia-nitrogen and nitrite-nitrogen by AOB and NOB in the moving bed batch reactor. The level of nitrate-nitrogen produced was nearly 80 mg/L at the end of the incubation period. Again, the most rapid declined in oestrogens was during the nitrite-nitrogen degradation stage.

Figure 4.17 B) shows the results for the degradation of EE2 and MeEE2 at 85 mg/L ammonia-nitrogen in the moving bed batch reactor with the K2 AnoxKaldnes packing material. As shown in Figure 4.17 B), MeEE2 decreased slowly compared to EE2; however, toward the end of the incubation period, the value for both oestrogens was nearly the same, i.e.16 µg/L and 14 µg/L for EE2 and MeEE2, respectively. The percentage removal of both oestrogens was much higher than at 35 mg/L and 65 mg/L

ammonia-nitrogen in the moving bed batch reactor with the K2 AnoxKaldnes packing material, i.e. 84% for EE2 and 86% for MeEE2, respectively.

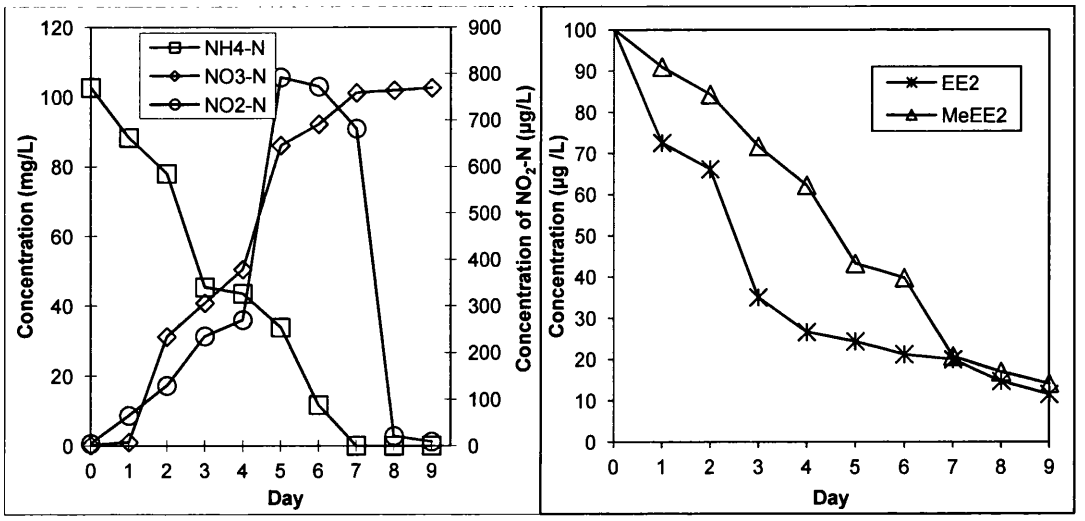


A B

Figure 4.17: Nitrification and oestrogen degradation in the moving bed batch reactor. A) 85 mg/L ammonia-nitrogen. B) Degradation of EE2 and MeEE2.

Figure 4.18 A) shows the concentration of 100 mg/L ammonia-nitrogen in the moving bed batch reactor with the K2 AnoxKaldnes packing material. The concentrations of ammonia-nitrogen reduced rapidly toward the end of the incubation period. The build-up of nitrate-nitrogen was nearly 100 mg/L, demonstrating that the ammonia oxidation process by nitrifying bacteria occurred at the highest rate.

Figure 4.18 B) shows the results for the decreased levels of EE2 and MeEE2. Figure 4.18 B) shows the same pattern of degradation for both synthetic oestrogens as the results shown in Figure 4.17 B). MeEE2 decreased slowly compared to EE2. The levels of EE2 and MeEE2 were 11 µg/L and 14 µg/L, respectively. The percentage removals for the oestrogens were 89% and 86% for EE2 and MeEE2, respectively. This was the highest removal percentage achieved for the oestrogens compared to the other results at different initial concentrations of ammonia-nitrogen in the moving bed batch reactor with the K2 AnoxKaldnes packing material.



A B

Figure 4.18: Nitrification and oestrogen degradation in a moving bed batch reactor for. A) 100 mg/L ammonia-nitrogen. B) Degradation of EE2 and MeEE2.

As can be seen in results shown above, the experimental investigation demonstrated that the enrichment of actively growing nitrifying bacteria in the moving bed batch reactor with the K2 AnoxKaldnes packing material is capable of degrading the synthetic oestrogens EE2 and MeEE2. The degradation of both oestrogens was increased when the concentration of ammonia-nitrogen was increased to 85 mg/L or 100 mg/L in the moving bed batch reactor with the K2 AnoxKaldnes packing material. The percentage removal of EE2 and MeEE2 was nearly 90% at all the ammonia-nitrogen concentrations in the moving bed batch reactor with the K2 AnoxKaldnes packing material.

4.6 Conclusions

These studies on the enrichment of one litre batches were performed on a slightly larger scale than the flask culture. Regarding on this matter, this experimental analysis allowed for more effective samples and the development of time course experiments to observe the dynamics of the ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen transformation within the batch culture system. The K2 AnoxKaldnes packing material was chosen for these studies based on the previous flask culture work described in Chapter 3 - Preliminary Study of Enrichment Culture. These studies show that the rates of transformation were dependent on the ammonia-nitrogen concentration, and the kinetics were typical of those observed by other workers (Chandran and Smets, 2001; Blackburne

et al., 2007; Zielińska, 2011). The saturation constant for ammonia-nitrogen transformation was thought to be slightly high. The formation and disappearance of nitrite-nitrogen was observed and is a classic observation by other workers (Carvallo *et al.,* 2002; Carrera *et al.,* 2004; Jubany *et al,* 2005). The levels of nitrite-nitrogen in the culture were dependent on the amount of ammonia-nitrogen added.

The use of the K2 AnoxKaldnes packing material also showed that the system was more effective than with one without the packing material, producing a more stable and reliable transformation. However, the final outcome of the transformation without packing material was similar to that of the packed batch cultures. In the next chapter, where continuous cultures are investigated, the packing material provided an effective retention method for these slow-growing bacteria.

The cultures of nitrifying bacteria were then investigated for their ability to transform oestrogens in batch culture. These results show that these substances were indeed degraded and that the amount of degradation was influenced by the amount of ammonia-nitrogen added. There was some suggestion that the best degradation rate coincided with the degradation of nitrite-nitrogen and the formation of nitrate-nitrogen (Yi and Harper, 2007). MeEE2 may be degraded preferentially over EE2 at high ammonia-nitrogen concentrations (Ternes et al., 1999b).

The synthetic hormone EE2 is primarily removed in wastewater treatment plants (WWTP) by sorption, and nitrifying biomass has been shown to be capable of EE2 biodegradation (Clouzot *et al.,* 2010). Membrane bioreactor (MBR) technology combined with an activated sludge system was chosen to develop a community of autotrophic, nitrifying micro-organisms. These MBR systems can be achieved and successfully remove more than 90% EE2 (Clouzot *et al.,* 2010). However, the disadvantages of this reactor system were predominantly membrane fouling and the rejection efficiency of the membrane type, which depended on the physicochemical properties of the organic micro-pollutants (Liu *et al.,* 2009).

The adsorption of three estrogenic compounds, i.e. bisphenol A (BPA), 17β-estradiol (E2), and EE2 on several powdered activated carbons (PAC) was investigated by Yoon *et al.* (2003). The removal efficiency of the activated carbon for these estrogenic compounds was found to be nearly 90% (Yoon *et al.,* 2003). However, the removal efficiency of the activated carbon was controlled by several factors including the

physiochemical properties of the organic pollutant and the type of the activated carbon used (Liu *et al.,* 2009). Furthermore, the adsorption capabilities of the activated carbon depended on the activated carbon type. Hence, the service life of the activated carbon could affect the performance of the oestrogen removal rate (Iwasaki *et al.,* 2001). The adsorption capacity of activated carbon decreased with an increase in the adsorbent concentration and the presence of surfactant and humic acids (Zhang and Zhou, 2005).

Here, the results with the moving bed batch reactor using the K2 AnoxKaldnes packing material showed an excellent reduction in EE2 and MeEE2. This study demonstrated that the biodegradation of oestrogens in the batch culture system was approximately 90% for EE2 and MeEE2. Therefore, the K2 AnoxKaldnes packing material can be considered a reliable system based on the usage of small cylindrical carriers which move freely in the reactor system. These cylindrical carriers are specifically designed to provide a large surface area for the growth of attached microbial biofilms (Accinelli *et al.,* 2012).

Furthermore, the microbial growth of the nitrifying bacteria was mainly confined to the surface of the moving K2 AnoxKaldnes packing material, especially within the sheltered internal surfaces (Ritmann and McCarty, 2001). This resulted in the formation of a stable and sheltered biofilm, protecting the microbes from alterations in the wastewater parameter characteristics, in this study attributed to organic pollutants, i.e. oestrogens. Besides promoting the microbial activity of nitrifying bacteria, the major advantages of the moving bed batch reactor using the K2 AnoxKaldnes packing material also include reduced head losses and the elimination of backwashing costs (Ødegaard *et al.,* 1994).

The present study investigating a moving bed batch reactor with the K2 AnoxKaldnes packing material demonstrated the same removal rate for oestrogens compared with other removal techniques. The feasibility of using this immobilized system is considered to be the best alternative method for the removal of oestrogens, as this technique has shown satisfying results. In addition, this immobilized suspended carrier system offers several advantages compared to other treatment method as this treatment technology is considered to have low maintenance and more cost effective.

CHAPTER 5

CONTINUOUS CULTURE

This chapter involves the study of continuous cultures of nitrifying bacteria; the first part introduces the basic theory that will be utilised to determine growth kinetics and provides a comparison of performance under various conditions.

5.1 Background Theory

There are four general types of continuous culture, depending on the control parameters and operation mode. These types are as follows: a) chemostat (steady state conditions under substrate limitations); b) auxostat (steady state conditions under nutrient-sufficient conditions and cell concentration); c) multi-stage continuous culture (in the presence of several vessels, mimicking tubular flow (or plug flow) reactor systems, i.e., steady state conditions with a spatial/temporal distribution of the culture); and d) continuous cell-recycle (an unsteady state system in which biomass accumulates, except where the maintenance energy (substrate consumption rate) for growth equals the feed rate).

The general concept and theory of these types of continuous cultures was described by Zeng (1999). All these cultures have a common feature; that is, they consist of one or more culture vessels into which fresh medium or culture from a preceding vessel is continuously introduced at a rate, F, expressed in litres per hour (L/h), and the culture volume, V, expressed in litres (L), which is kept constant by continuous removal of the culture. The diagram of chemostat type is shown in Figure 5.1.

The chemostat is defined as a continuous culture system in which the feed is set externally and cell growth is limited by a selected nutrient. The second condition means that the specific growth rate, μ (h^{-1}), of the organism is a function of a single growth-limiting nutrient. However, this definition may be relaxed to include continuous cultures simultaneously limited by multiple nutrient components (Gottschalk, 1992). Continuous cultures that are fed with an inhibitory nutrient or that form toxic products can be limited by growth inhibition even when all nutrients are present in excess. This condition cannot be referred to as a chemostat culture.

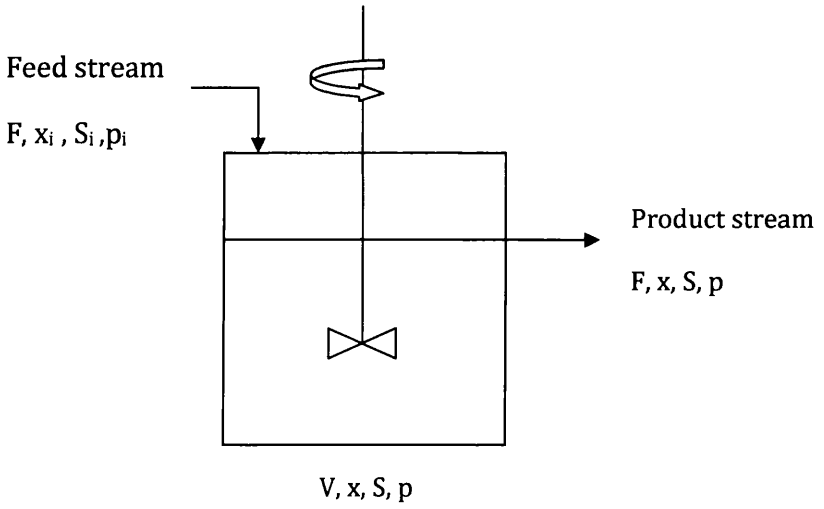


Figure 5.1: Continuous stirred-tank reactor (Chemostat)

A chemostat is usually started as a batch culture. Before a nutrient becomes limiting, the nutrient feed is started. The cells grow until the chosen nutrient becomes limiting and from this point on cell growth is limited by the rate of medium addition. The specific growth rate of a chemostat culture can be determined from the material balance for biomass:

Net increase in biomass = biomass in incoming medium + growth – output - death

In mathematical terms this is:

$$dx/dt = x_i F/V + \mu x - x F/V - k_d x \qquad (5.1)$$

where;

dx/dt is the rate of accumulation of biomass per unit of time and per volume (g cell/L/h);
x_i is the biomass concentration (g cell dry weight/L) in the incoming medium;
x is the biomass concentration (g cell dry weight/L) in the bioreactor;
and k_d is the specific death rate of cells (h^{-1}).

This expression can be simplified if one assumes that the chemostat is at a steady state, that there are no cells in the incoming medium, and that cell death is negligible. The assumption of a steady state implies that there is no net accumulation of cells in the bioreactor and thus the left-hand side of the equation is equal to zero. Furthermore, if the dilution rate, D, is introduced and defined as F/V, equation 5.1 becomes:

$$\mu x = Dx \qquad (5.2)$$

Hence;

$$\mu = D \qquad (5.3)$$

This is one of the most important properties of a chemostat. The specific growth rate of a culture over a range up to the maximum specific growth rate (μ_{max}) can be precisely controlled by the nutrient feed rate if a constant volume is maintained.

In a steady-state chemostat with a sterile feed and negligible cell death, the specific growth rate, μ is equal to the dilution rate, D. This relationship is useful for determining kinetic and yield parameters in the cell culture. Pirt (1975) and Doran (1995) have both discussed these principles in details.

5.1.1 Maximum specific growth rate (μ_{max}) and substrate saturation constant (Ks)

Cell growth can be modelled using the Monod kinetics equation, where, in equation (5.3) $\mu = D$, then in a chemostat culture,

$$D = \mu_{max} S/(Ks + S) \qquad (5.4)$$

where;

μ_{max} is the maximum specific growth rate;

Ks is the substrate saturation constant;

and S is the steady-state substrate concentration in the reactor.

Rearranging Equation (5.4) gives the following linear equation that enables determination of the constants via a reciprocal plot:

$$S/D = Ks/\mu_{max} + S/\mu_{max} \qquad (5.5)$$

Linearization of the data for the reciprocal plot minimizes distortions in experimental error, especially of the substrate (Moser, 1985). Accordingly, Equation (5.5) is used to evaluate μ_{max} and Ks. Therefore, a reciprocal plot of S/D versus S should give a straight line with slope $1/\mu_{max}$ and intercept Ks/μ_{max}.

5.2 Continuous Reactors for Partial Fixed Bed K2 AnoxKaldnes Packing Material (PFBR) and Moving Bed K2 AnoxKaldnes Packing Material (MBBR)

Two continuous systems were investigated in this PhD research study. The first continuous reactor system was a partial fixed bed with K2 AnoxKaldnes packing material (PFBR). The second continuous reactor system was a moving bed of K2 AnoxKaldnes packing material (MBBR). These two systems were used to observe and investigate the performance of these configurations for the removal of ammonia-nitrogen and to compare the nitrifying kinetics of the two systems containing K2 AnoxKaldnes packing material. In addition, the transformation of the synthetic oestrogens 17α – ethynylestradiol (EE2) and mestranol (MeEE2) under chemostat conditions were investigated. K2 AnoxKaldnes packing material was selected for use in these two systems after comparing the results obtained for all the packing materials in Chapter 3-Preliminary Study of the Enrichment Culture.

The aim of this experimental investigation was to examine enrichment cultures of nitrifying bacteria in K2 AnoxKaldnes packing material for the degradation of ammonia-nitrogen and the absorbing oestrogens in a continuously aerated partial fixed bed and/or moving bed reactor. Here, the influence of the oestrogens on both reactors' performance in terms of EE2 and MeEE2 removal would be determined, in which the percentage

removal would be determined and some kinetics analysis will be taken into account to make comparisons between the PFBR and MBBR systems. Furthermore, in Chapter 5, the analysis study for the continuous reactor is a further research approached in a continuation of the study from Chapter 4-Batch Culture.

5.2.1 Continuous Reactor for partial fixed bed of K2 AnoxKaldnes packing material (PFBR)

A laboratory scale plexiglass reactor with an internal diameter of 30 cm and a height of 170 cm as shown in Figure 5.2 was used for the experimental study. The reactor can be filled with a total volume of 35 L and the working volume was 16 L.

For the partial fixed bed system, a perforated plate was used to prevent the packing material from floating freely. Figure 5.3 shows the schematic diagram of the K2 AnoxKaldnes packing material in the partial fixed bed system for the continuous culture reactor. The perforated plate could be adjusted to hold the K2 AnoxKaldnes packing material in the middle of the 16 L working volume in the reactor. The reactor was filled with 50% K2 AnoxKaldnes packing material with specifications as listed in Chapter 3-Preliminary Study of the Enrichment Culture.

The reactor medium was kept at 30° C as the feed medium was heated to the same temperature in the water bath before entering the reactor. Mixing and aeration were provided by a compressor with pressurised air through a thin metal pipe with a circular shape at the end. The circular shape of the thin metal pipe was perforated so that air could be diffused and create a turbulence vortex from the bottom of the reactor. The airflow rate was controlled via a gas flow controller and a flow meter. The dissolved oxygen (DO) was maintained up to 6 mg/L and was routinely checked with a dissolved oxygen probe (Neotek Ponsel, France) that was attached inside the reactor.

The pH of the medium in the reactor was controlled by a pH controller with a dosing pump delivering either acid or base (Hanna Instrument, USA). The influent medium was fed through 15' of Masterflex tubing connected to a digital peristaltic pump (Cole-Parmer, USA). The tubing was calibrated and the peristaltic pump was set according to the dilution rate. The effluent was collected in an overflow sampling port on the top left side of the reactor.

Figure 5.2: Photograph of Continuous Reactor for a Partial Fixed Bed with K2 AnoxKaldnes packing material (PFBR).

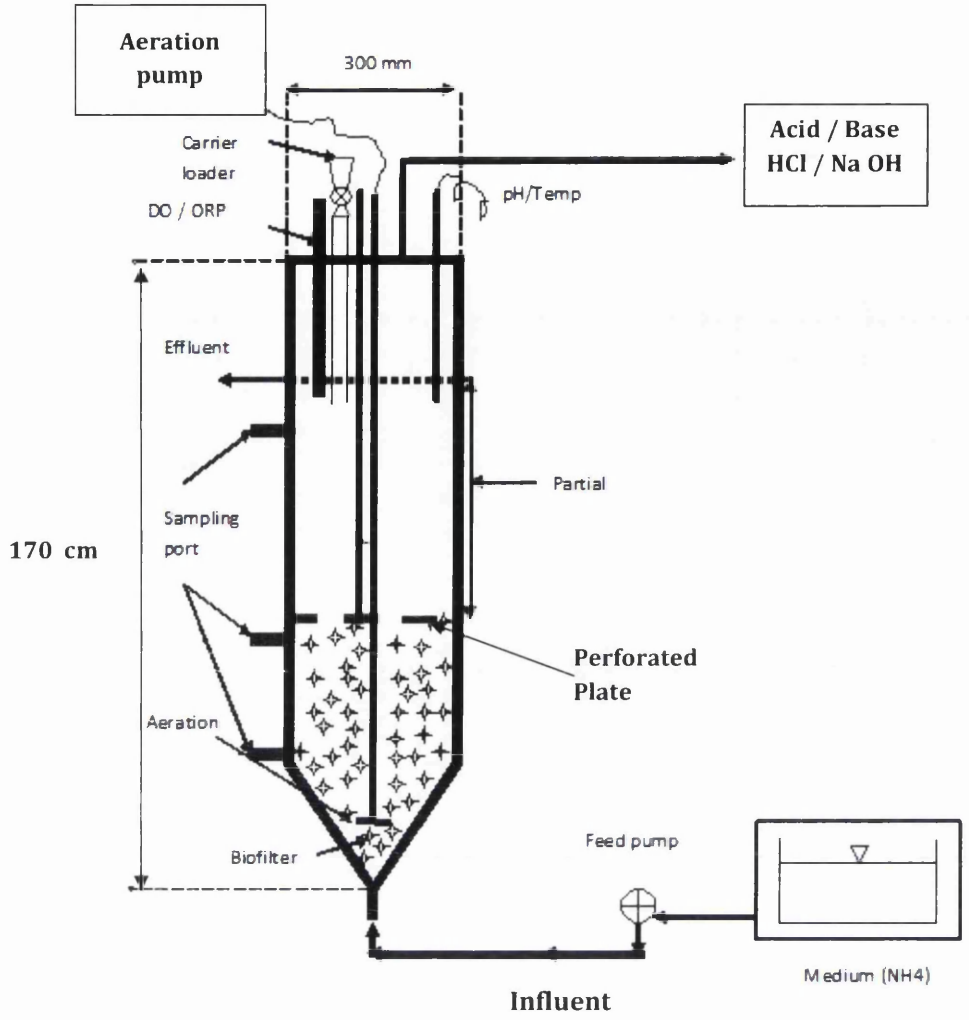


Figure 5.3: Schematic Diagram of PFBR

5.2.1.1 Start–up of the partial fixed bed reactor (PFBR) for the growth enrichment of nitrifying bacteria

The partial fixed bed K2 AnoxKaldnes reactor was set to a working volume of 16 L. An overflow pipe on the side of the tank allowed the effluent from the reactor to overflow to the effluent tank. Medium A was prepared in 30 L tanks every day and was added in a larger tank of 100 L volume to make sure there was sufficient Medium A when the reactor was running in continuous mode. Ammonia-nitrogen at a concentration of 35 mg/L and synthetic oestrogens 17 α- ethynylestradiol (EE2) and mestranol (MeEE2) at concentrations of 100 µg/L were added daily into the reactor. The 35 mg/L ammonia-nitrogen concentration was chosen to reduce the inhibition by free ammonia (FA) that

would occur if higher ammonia-nitrogen concentrations were used. This inhibition factor has already been discussed in Chapter 4 - Batch Culture. Two litres of nitrifying bacteria from the serial batch culture taken from enriched fish effluent were inoculated in the reactor. The temperature and pH were set as explained in a previous section. An 8 L volume or a total weight of 1433.6 gram (1.433 kg) of K2 AnoxKaldnes packing material was added in the reactor. This K2 AnoxKaldnes packing material occupied approximately 50% of the reactor volume.

Nitrifying bacteria require a long retention time in the reactor, meaning that a slow flow rate must be chosen (Ritmann and McCarty, 2001). Since nitrifying bacteria are a slow growing bacterium, with high sensitivity to many environmental factors, nitrification becomes the rate-limiting step in the biological nitrogen removal process (Siripong and Rittmann, 2007). For that reason, a long retention time has been considered to be the main mechanism to ensure the nitrification process is efficient and maintain stable growth of the nitrifying bacteria (Li *et al.*, 2013).

For this purpose, a digital peristaltic pump (Cole-Parmer, USA) was introduced into the reactor. The digital pump could be set to a flow rate as low as 20 mL/min. Before the influent was pumped into the reactor, the pump was calibrated and 15' Masterflex tubing was used to connect the digital pump to the reactor. Table 5.1 shows the various flow rates and dilutions rate that were chosen in this PhD research study.

Table 5.1: Various flow rates and dilution rates for the continuous reactor with K2 AnoxKaldnes packing material

Flow rate (mL/min)[a]	Flow rate (L/day)	Dilution rate (d^{-1})[b]	Hydraulic Retention Time, HRT (day)[c]
20	28.8	1.8	0.56
30	43.2	2.7	0.37
40	57.6	3.6	0.28
50	72.0	4.5	0.22
60	86.4	5.4	0.19

[a]1 mL/min = 1.44 L/day;
[b]Dilution rate (d^{-1}) = Flow rate/Volume;
[c]HRT (day) = Volume/Flow rate.

Periodically, samples from the reactor were taken to monitor the process. The samples were kept at 4°C prior to analyses. The measurements of ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were determined using the methods in Chapter 3-Preliminary Study of the Enrichment Culture. EE2 and MeEE2 were determined using a gas chromatograph mass detector as explained in Chapter 4-Batch Culture. All the measurements were taken daily. For both oestrogens, a solid phase extraction technique was used to separate the samples from water into solvents before analysis in a gas chromatograph mass detector.

5.2.1.2 Results for Partial fixed bed reactor (PFBR)

As ammonia would inhibit the microbial activity of the nitrifying bacteria in the enriched fish effluent in the partial fixed bed reactor, an ammonia-nitrogen concentration of 35 mg/L was selected. A level <100 mg/L NH_3-N for start-up of a nitrification reactor in an activated sludge process was suggested by Li and Zhao (1999). From the results in Chapter 4-Batch Culture, the accumulation of free ammonia (FA) as an inhibitory factor was noted at or above 400 mg/L ammonia-nitrogen. The purpose of the experiments was to check the kinetics of the nitrifying bacteria from the enriched fish effluent in the partial fixed bed reactor in addition to the degradation of the synthetic oestrogens EE2 and MeEE2.

The partial fixed bed continuous reactor with K2 AnoxKaldnes packing material was run for approximately three months. During this time, measurements of ammonia-nitrogen, nitrite-nitrogen, nitrate-nitrogen and both synthetic oestrogens, EE2 and MeEE2, were collected on a daily basis and analysed according to Chapter 3-Preliminary Study of the Enrichment Culture and Chapter 4-Batch Culture. Figure 5.4 shows the results from the influent and effluent of ammonia-nitrogen in the partial fixed bed reactor throughout the continuous substrate feeding of 35 mg/L ammonia-nitrogen at varying dilution rates.

As shown in Figure 5.4, the ammonia-nitrogen was nitrified by ammonia oxidizing bacteria (AOB). The effluent concentration of ammonia-nitrogen was high during the initial start-up period of the PFBR. During the initial run of the reactor, a flow rate of 20 mL/min with a dilution rate of 1.8 d^{-1} was run for 21 days and gave a stable ammonia-nitrogen concentration in the effluent of 0.5 mg/L ammonia-nitrogen. Using a

second flow rate of 30 mL/min with a dilution rate of 2.7 d^{-1}, the concentration of ammonia-nitrogen was elevated at the beginning, attaining 5 mg/L, and then decreased to 1.1 mg/L but increased again on days 30 and 34 with values of 3.5 mg/L and 4.4 mg/L, respectively. The ammonia-nitrogen in the effluent was stable on day 42 at a concentration of 1 mg/L.

When the dilution rate was changed to 3.6 d^{-1} the concentration of ammonia-nitrogen was very high, approximately 9.6 mg/L in the effluent. The concentration of ammonia-nitrogen, however, declined to 3.5 mg/L on the second day and then performed erratically between 2 mg/L and 5 mg/L. The concentration of ammonia-nitrogen then decreased again to 2.5 mg/L in the effluent.

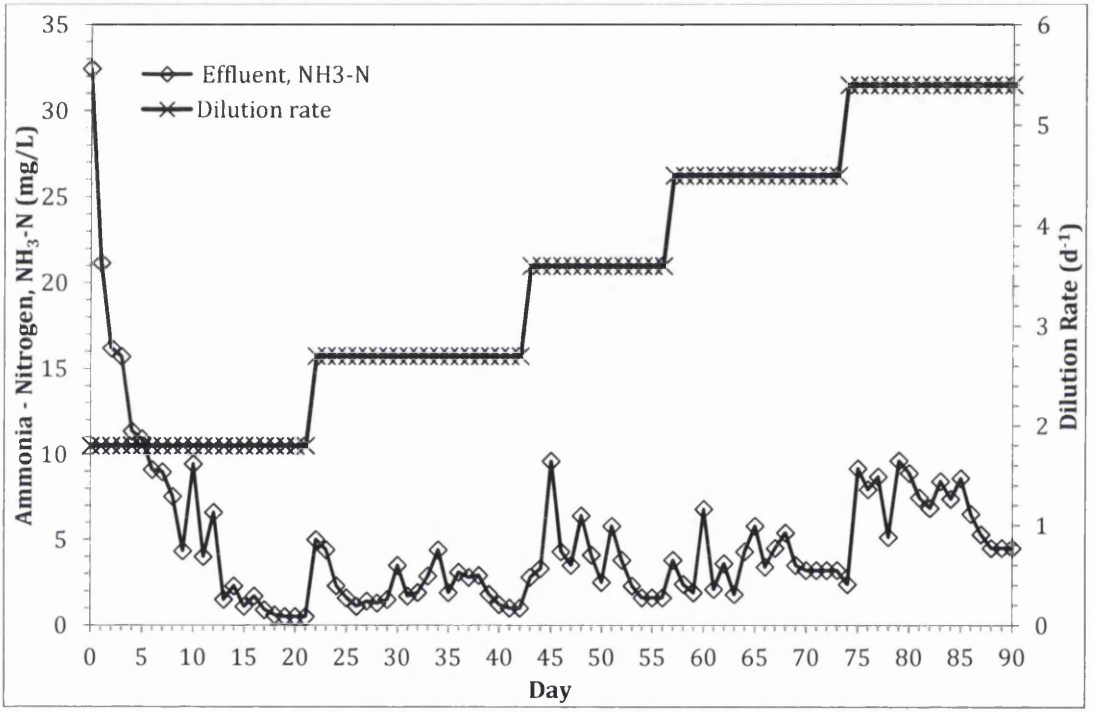


Figure 5.4: The effect of dilution rate on the Partial Fixed Bed K2 AnoxKaldnes packing material (PFBR) for a concentration of 35 mg/L ammonia-nitrogen.

At a dilution rate of 4.5 d^{-1} the effluent concentration of ammonia-nitrogen was slightly higher, 3.8 mg/L, and exhibited erratic behaviour. At the final dilution rate of 5.4 d^{-1}, the concentration of ammonia-nitrogen went higher and reached a peak value of 9.2 mg/L in the effluent. Towards the ends of the trail using a dilution rate of 5.4 d^{-1}, the value of ammonia-nitrogen was stable at 4.5 mg/L.

The concentration of ammonia-nitrogen in the effluent of the continuous reactor for the partial fixed bed with K2 AnoxKaldnes packing material could be reduced to about 2 mg/L ammonia-nitrogen. For the first three dilution periods, the ammonia-nitrogen remained at or below 2 mg/L; however, the concentration increased to 4.5 mg/L during the final dilution rate of 5.4 d^{-1}.

From these results and observations, the ammonia-nitrogen in the effluents is clearly affected by the dilution rate. At a high dilution rate of 5.4 d^{-1}, the hydraulic retention time (HRT) is 0.19 day (see Table 5.1) compared to the low dilution rate of 1.8 d^{-1} with an HRT of 0.56 day. A higher retention time is required for maximum removal efficiency of the ammonia-nitrogen in the effluent concentration (Borghei and Hosseini, 2004). The outcome was related to the dilution rates and HRT, and is reflected in the increased amounts of ammonia-nitrogen in the effluents.

Here, the indication from the experimental investigation demonstrated that the population dynamics of the nitrifying bacteria and the nitrification activities were strongly influenced by the HRT (Borghei and Hosseini, 2004; Azizi *et al.,* 2013). The increase in ammonia-nitrogen in the effluents at a high dilution rate of 5.4 d^{-1} and low HRT of 0.19 day indicated a decrease in the proportion of ammonia oxidizing bacteria (AOB) fractions among the total nitrifying bacteria in the reactor system of the PFBR.

Research conducted by Li *et al.,* (2013) studied the impact of HRT on the nitrification process and the performance of the nitrifying bacteria in a conventional activated sludge system fed with synthetic inorganic wastewater over a long period of time. The results presented by Li *.et al.,* (2013) showed that *Nitrosomonas sp.* was the dominant AOB and adjusting the HRT to a lower value led to biomass washout. The number of AOB is generally several times higher than that of NOB, whereas AOB can acquire more energy than NOB during nitrification (You *et al.,* 2003). Generally, both AOB and NOB prefer to grow in dense clusters (Koop and Pommerening-Roser, 2001). However, the aggregates formed by NOB are normally smaller than those of AOB, and *Nitrobacter sp.* sometimes tend to occur as free-living cells. Therefore, NOBs are more susceptible to washout due to a decrease in HRT (Daims *et al.,* 2001). This suggestion was interpreted to mean that shorter HRTs induced a selective decrease in AOB relative to NOB in the reactor system (Li *et al.,* 2013).

In the experimental investigation of the PFBR, the changes in the operating parameters dilution rate and HRT contributed to differences in the distribution of community structure for nitrifying bacteria with different metabolic activities in the nitrification bioreactor system. A decrease in HRT led to biomass washout in the reactor system (Ritmann and McCarty, 2001). However, the PFBR maintained a relatively high level of AOB despite the decrease in the AOB fraction accompanying the decrease in HRT, whereas the results showed low ammonia-nitrogen concentrations were detected in the effluents at dilution rates between 1.8 d^{-1} to 4.5 d^{-1}, when the HRT value was at 0.56 day to 0.22 day, respectively.

Here, the growth system attached to the suspended carrier of K2 AnoxKaldnes packing material played an important mechanism in retaining the large volume of biomass from being totally washed out from the reactor system. The PFBR reactor is a combined system of attached growth and suspended growth (Grady *et al.*, 2011). In the PFBR reactor, the clusters of nitrifying bacteria were securely affixed on the surface of the K2 AnoxKaldnes packing material. Furthermore, the PFBR reactor tends to be more stable regardless of changes in the dilution rate and HRT, with relatively low ammonia-nitrogen being observed in the effluent concentrations in the reactor system.

5.2.1.2.1 Maximum specific growth rate and substrate saturation constant

The Monod equation for specific growth rate, Equation (5.4), is analogous mathematically to the Michaelis-Menten expression for enzyme kinetics.

$$\mu = \mu_{max} S/(Ks + S) \qquad (5.4)$$

The typical value of *Ks* is very small, on the order of mg per litre for carbohydrates substrates; however the value of *Ks* is depended on the compound itself (Doran, 2012). The amount of growth-limiting substrate in the culture media is normally much greater than *Ks*. As a result, μ provided *S* is greater than about 10 *Ks*. This allows for the measurement of μ_{max} from the batch culture. However, because the values of *Ks* in cell culture are usually very low, accurate determination of this parameter from batch culture is difficult. A better estimation of μ_{max} can be made using a continuous cell culture (Doran, 2012).

In response to the explanation stated earlier, further analysis has been done in addition, corresponding to Chapter 4-Batch Culture, whereas in Chapter 5-Continuous Culture, a research investigation was carried out to evaluate the specific growth rate, μ_{max} of the enrichment culture for nitrifying bacteria This observation was made prior to the difficulty in determining the specific growth rate, μ_{max} of the enriched culture of nitrifying bacteria in Chapter 4-Batch Culture.

If cell growth can be modelled using the Monod kinetics equation, for a partial fixed bed continuous reactor with K2 AnoxKaldnes packing material in chemostat culture, the reciprocal equation, (Equation 5.5) $S/D = S/\mu_{max} + Ks/\mu_{max}$ can be used to graphically determine the specific growth rate and saturation constant.

Table 5.2: Data obtained from ammonia-nitrogen results at different dilution rates for the PFBR.

Dilution rate (d^{-1}), D	NH_3-N concentration (mg/L), S	S/D
1.8	0.5	0.277
2.7	1.0	0.370
3.6	1.6	0.444
4.5	3.2	0.711
5.4	4.5	0.833

The linearization of data taken from Table 5.2 for the reciprocal plotting of the experimental values of ammonia-nitrogen concentration under stable conditions at the end of each dilution rate period is shown in Figure 5.5, with μ_{max} and Ks evaluated according to Equation (5.5). This reciprocal plot of S/D versus S should give a straight line with slope $1/\mu_{max}$ and intercept Ks/μ_{max}.

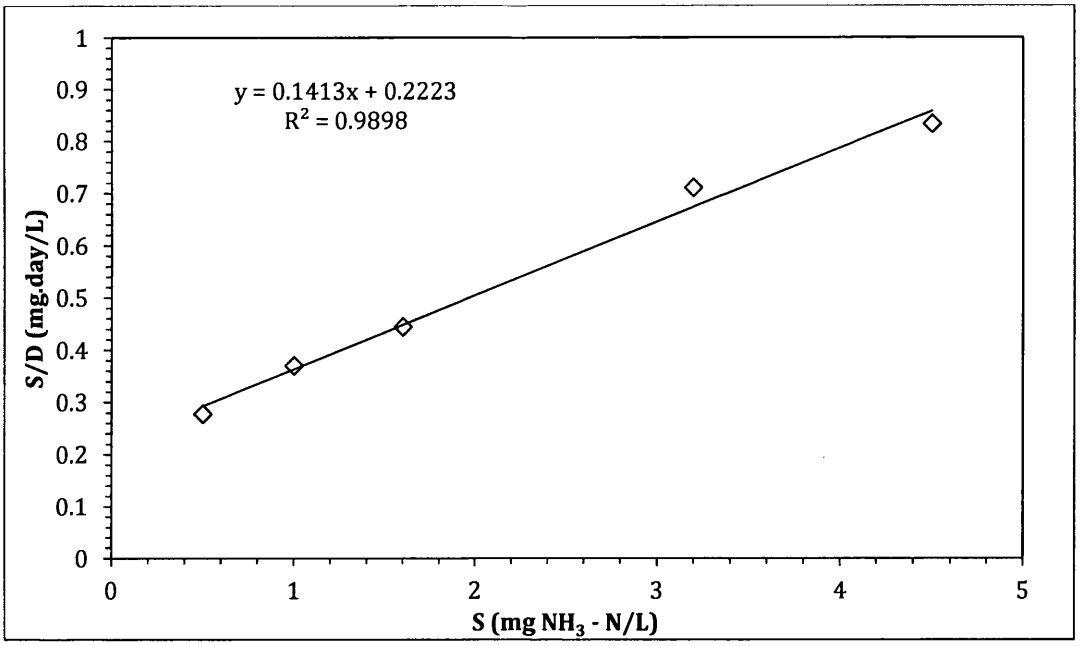


Figure 5.5: Plot of S/D versus S for the PFBR. The slope is 0.141 or μ_{max} = 7.092 d^{-1} (3.3 h). The intercept is 0.222 = K_S/μ_{max} or K_S= 1.574 mg NH_3-N/L at 35 mg/L ammonia-nitrogen at 30°C and pH 8.0

From the reciprocal plot of *S/D* versus *S* for the PFBR, the maximum growth rate, μ_{max} is 7.092 d^{-1} and the saturation constant, *Ks* is 1.574 mg NH_3-N/L. This value of *Ks* is much lower than the result obtained for *Ks* in the batch culture described in Chapter 4.

The maximum growth rate, μ_{max} of 7.092 d^{-1} and the saturation constant, *Ks* of 1.574 mg NH_3-N/L obtained for the PFBR system were compared to those in other research studies. An investigation published by Lin (2008) determined a specific growth rate, μ_{max} of 0.25 d^{-1} and a saturation constant, *Ks* of 6.5 mg NH_3-N/L in a continuous culture of a combined moving-fixed bed of biofilm on polyurethane foam sponge. Here, from the experimental analysis of the PFBR system, a higher specific growth rate, μ_{max} value was calculated compared to the research study of Lin (2008). However, the saturation constant, *Ks* for the PFBR reactor was low compared to that reported by Lin (2008).

Fang *et al.,* (2009) determined a specific growth rate, μ_{max} of 2.16 d^{-1} (0.09 h^{-1}) and a saturation constant, *Ks* of 9.1 mg NH_3-N/L in a sequencing batch reactor with aerobic nitrifying granules. These values obtained by Fang *et al.,* (2009) were also in a different range than those retrieved from the experimental investigation of the PFBR reactor system in the current research study. Distinctive values for the specific growth

rate, μ_{max} and saturation constant, *Ks* have been related to the physiochemical processes of biofilm development, where the most important aspects were the affects of mass transfer, detachment and porosity profiles that were linked to the shear stress and configuration of the reactor itself (Ni and Yu, 2010).

In the PFBR system, operating under steady-state conditions, substrate availability in the biofilm is hindered by two major resistance factors; the near-stagnant liquid film existing near the outer surface of the carrier (external mass transfer) and the biofilm existing on the inner surface of the carrier through which internal diffusion of the substrate takes place (internal mass transfer) (Ni and Yu, 2010). For the external mass transfer, when a liquid flows through a biofilm attached on the outside of the carrier, there exists a region near the biofilm-carrier surface where the liquid velocity is very low, giving rise to a near-stagnant film of liquid that offers maximum resistance to the transfer of substrate through the inner surface of the biofilm-carrier (Mudliar *et al.,* 2008).

However, the investigation of the PFBR system played an important role in understanding the physical mechanisms of biofilm detachment from the immobilized suspended carrier of K2 AnoxKaldnes packing material. This evaluation analysis has far-reaching implications for biological nitrogen removal practices and provides knowledge relevant to related wastewater processes that will facilitate the engineering and optimization of this novel technology.

5.2.1.2.2 Nitrification process for partial fixed bed reactor (PFBR)

Figure 5.6 shows the volumetric nitrification loading rate (NLR) in mg/L.day at varying nitrification loading rates (NLR) in the PFBR. The working volume of the reactor was fixed at 16 L and the continuous dilution rates (d^{-1}) were as shown in Table 5.1. The following equation was used to obtain the values used in Figure 5.6 (Yusof *et al.,* 2010).

The calculations for the nitrification rate (NLR) are defined as follows:

$$NLR = \frac{(\mathrm{NH3-N})in \times flow\ rate}{Reactor\ volume} \quad (5.6)$$

$$NLR\ rate(volumetric) = \frac{(NH3-N)in-(NH3-N)out \times flow\ rate}{Reactor\ volume} \quad (5.7)$$

Where;

$(NH_3\text{-}N)_{in}$ is the influent ammonia-nitrogen concentration;

$(NH_3\text{-}N)_{out}$ is the effluent of ammonia-nitrogen concentration;

V is the reactor volume;

and F is the flow rate.

The results in Figure 5.6 show the NLR in terms of the rate of ammonia-nitrogen removal rate in the PFBR. With the NLR fixed at 63 mg/L.day (21 days) throughout, the removal rate of ammonia-nitrogen (that is, the volumetric NLR) increased by 62.1 mg/L.day. The volumetric NLR increased and was stable at 91.85 mg/L.day throughout the similar NLR of 94.5 mg/L.day. Here, we can see that the increase in the volumetric NLR was contributed to by the consumption of ammonia-nitrogen by ammonia-oxidizing bacteria during the nitrification process, with no inhibition caused by free ammonia (FA) or free nitrous acid (FNA) (Zekker *et al.,* 2011).

Beyond these loading rates, the rate of removal was considerable lower than the NLR, possibly because of the high concentration of ammonia-nitrogen incoming in the PFBR system. A high NLR would produce high concentrations of FA and FNA, inhibiting the ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) and slowing down the nitrification process and reducing the performance of the PFBR reactor (Yusof *et al.,* 2010).

The PFBR system also contributed to the decrease in the volumetric NLR in volumetric terms. The K2 AnoxKaldnes packing material was fixed in the middle of the working volume in the continuous reactor. There, the nitrifying bacteria attached to the inner surface of the K2 AnoxKaldnes packing material, making the situation difficult for the nitrifying bacteria to degrade the ammonia-nitrogen. The process of ammonia oxidation was reduced as the surface area of the K2 packing material was not freely exposed to the medium in the continuous reactor.

As a consequence, a mass transfer limitation occurred in the PFBR system that was related to the condition of the external film diffusion in partial fixed bed reactors with immobilized cultures. This difficulty is connected to the process of transporting substrate from bulk liquid to the immobilized bio-active surface together with the low diffusion and reaction of substrate within the bio-active layer (Kathiravan *et al.,* 2010).

A laminar film exists next to the surface media that is in contact with a moving fluid, whereas the transport of substrate to the biofilm occurs primarily by molecular diffusion (external mass transfer) (Dizge and Tansel, 2010).

The biochemical reactions in the partial fixed bed reactor also played an important role in controlling the metabolic rate of the biofilm in the immobilized system. Biofilm growth has been proposed to occur by a four-step mechanism: 1) formation of a thin layer microorganism, 2) increase in the biofilm's thickness, 3) breakage of the biofilm clusters and release of particles (biomass) due to excess growth, and 4) formation of small pellets by detached particles (Di Iaconi *et al.,* 2006).

Mass transport through the biofilm takes place by several mechanisms such as diffusion of the substrate from the solution to the biofilm, diffusion-reaction through the biofilm, and adsorption-diffusion through the immobilized surface. The micro scale reaction and diffusion phenomena are especially important in biofilms, as these processes control the growth of the microorganisms by affecting the availability of nutrients and oxygen within the bioactive layer. The different layers within the biofilm result in the differentiation of organisms according to the availability of oxygen and substrate, which in turn affect the biofilm's characteristics (i.e., structure, thickness, sub-layers) (Dizge and Tansel, 2010).

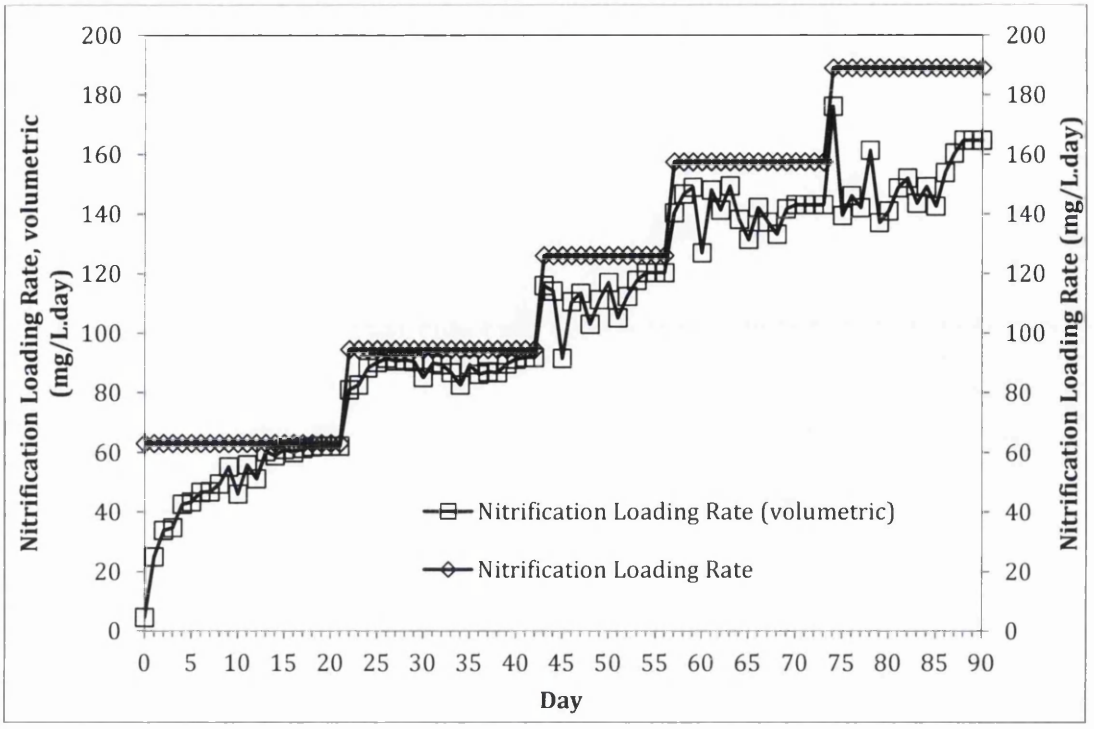


Figure 5.6: Results of ammonia-nitrogen removal rate (volumetric) at different ammonia-nitrogen loading rates in the PFBR.

Figure 5.7 shows the ammonia-nitrogen removal rate in percentage terms, for the volumetric NLR. At the start-up of the continuous reactor, the removal rate of ammonia-nitrogen was only 7%, but the value increased rapidly to nearly 88% for a NLR of 63 mg/L.day after 9 days of operation. With a NLR of 63 mg/L.day, the percentage removal of ammonia-nitrogen remained consistently high throughout, reaching 100% towards the end. For a NLR of 94.5 mg/L.day, the percentage removal of ammonia-nitrogen decreased at the beginning with a value of 86% but then increased to nearly 98%. As the rate was increased further the efficiency was gradually reduced but was still over 90%.

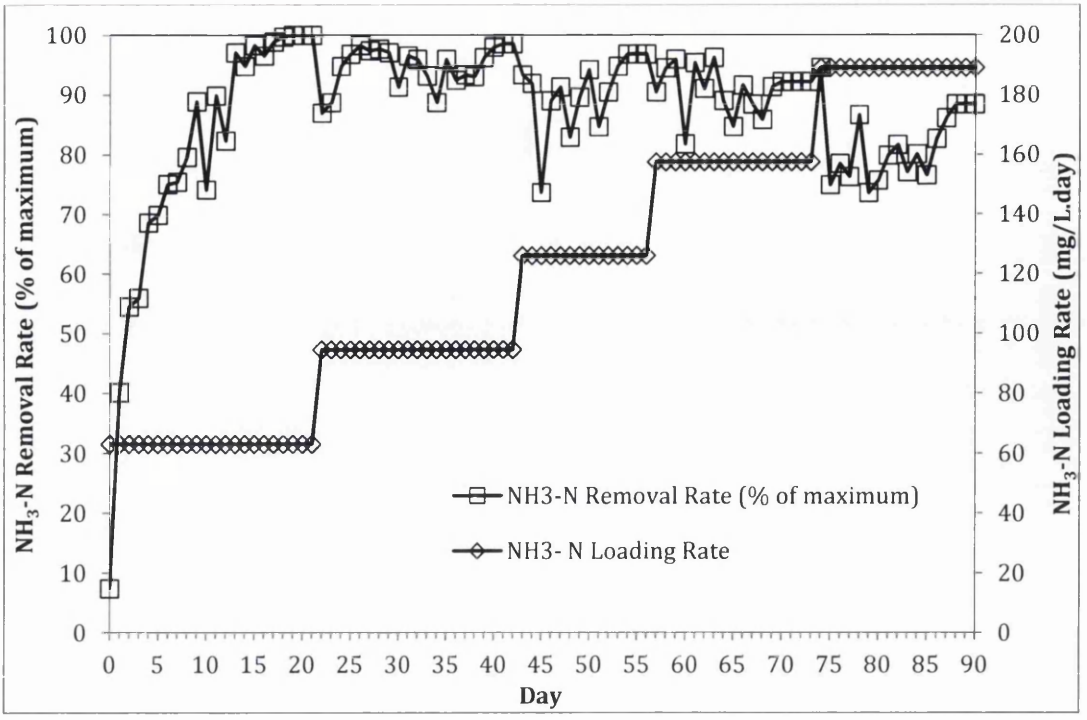


Figure 5.7: Results for ammonia-nitrogen removal rate for the volumetric NLR in % of maximum at different NLRs in the PFBR.

According to the results shown in Figure 5.7 with a NLR of 189 mg/L.day, the percentage removal rate of the ammonia-nitrogen dropped significantly and the value was not fully stable until the end of the incubation period, due to the restricted mass transfer and biochemical reactions in the PFBR system. When the fluid flows through the PFBR reactor, there are regions near the surface of the packing material where the fluid velocity is very low. In such regions around the exterior of the packing media, substrate transport takes place primarily by molecular diffusion. Hence, at a high NLR of 189 mg/L.day, the observed reaction can be significantly decreased by the external film diffusion. The local rate of film diffusion of the substrate from the bulk fluid to the surface of the immobilized cells was controlled by the concentration of the substrate, by the concentration difference between the bulk and the external surface of the immobilized cells (Nath and Chand, 1996).

In this situation, the ammonia-nitrogen concentration is very high with a final NLR of 189 mg/L.day; hence, this condition causes limited mass transfer by the end of the trial. However, the PFBR system recovered shortly after the 88 days of operation, by

which time the percentage removal rate of the ammonia-nitrogen was stabilized and constant at nearly 90%.

5.2.1.2.3 Production of nitrite-nitrogen and nitrate-nitrogen in the PFBR.

Nitrate-nitrogen production in the PFBR was determined as shown in Figure 5.8. The production of nitrate-nitrogen showed that the complete nitrification process occurred in the PFBR. After an initial period where the nitrification process became established, good performance was observed that slowly became less efficient as the process flow rate was increased. The levels of nitrate-nitrogen were about 30 mg/L and slowly decreased to 25 to 27 mg/L. Nitrate-nitrogen production for a dilution rate of 1.8 d^{-1} increased greatly throughout the 21 days of operation; however, the accumulation of nitrate-nitrogen dropped to values of 21 mg/L and 24 mg/L nitrate-nitrogen subsequently, on the 10 and 12 days of operation.

Throughout the entire period with a dilution rate of 1.8 d^{-1}, the concentration of nitrate-nitrogen production was maintained at 30 mg/L. The value of nitrate-nitrogen production dropped to 26 mg/L nitrate-nitrogen when the dilution rate was increased up to 2.7 d^{-1}. The production of nitrate-nitrogen was maintained with the dilution rate of 2.7 d^{-1}, even though the production of nitrate-nitrogen decreased to values of 27 mg/L and 26 mg/L on the 30 and 34 days of operation, respectively. Towards the end at a dilution rate for 2.7 d^{-1}, nitrate-nitrogen production increased and was stable at 30 mg/L nitrate-nitrogen.

From these observations, the PFBR reactor has clearly established a complete nitrification process. Nitrate-nitrogen production has been achieved with respect to co-existence and syntrophy (a phenomenon whereby one species lives off the products of another species; by which the growth of one partner is improved or depends on the nutrients or growth factors or substrate provided by the other partner) (Yusof *et al.*, 2010). In this case, this refers to the association of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), which contributed to the success of the nitrification process in the PFBR reactor system.

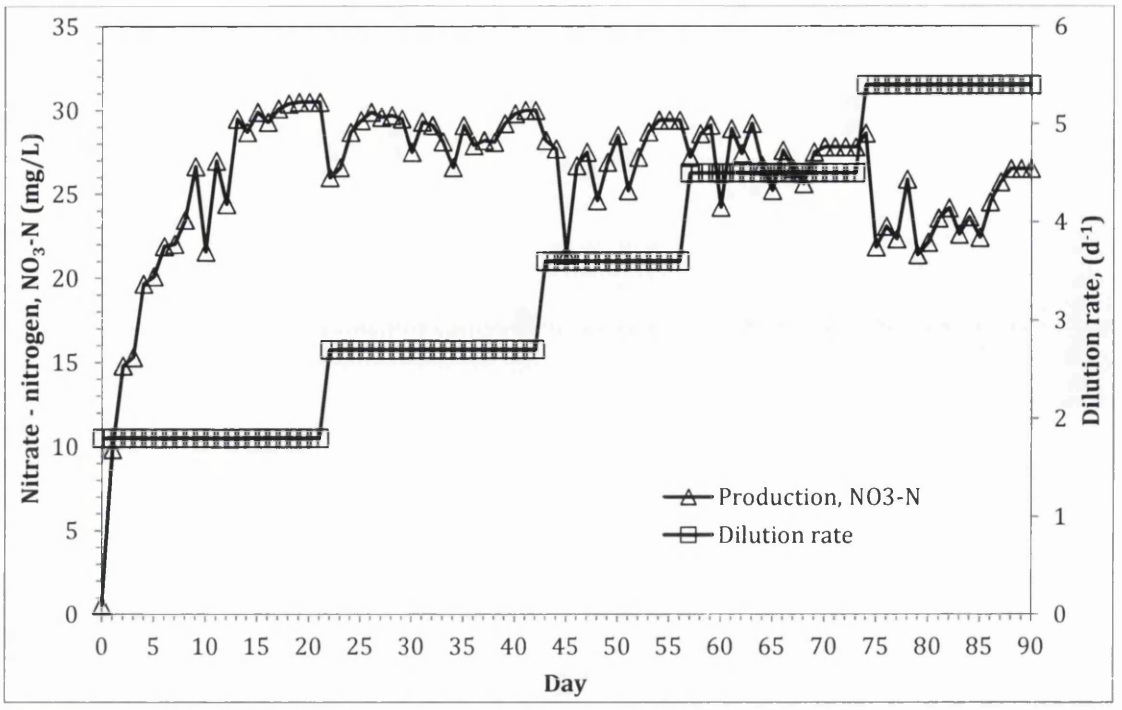


Figure 5.8: The production of nitrate-nitrogen in the effluent in for PFBR at various dilution rates.

Figure 5.9 shows the overall production of nitrite-nitrogen and the concentrations of ammonia-nitrogen effluent in the PFBR at various dilutions throughout the entire 90 days of operation. The production of nitrite-nitrogen was high at the beginning of the start-up of the continuous reactor. This increasing value was due to degradation of the ammonia-nitrogen in the reactor by ammonia-oxidizing bacteria (AOB) that contributed to the high value of nitrite-nitrogen production. However, nitrite-nitrogen production decreased afterwards and the initial nitrite-nitrogen build-up was significantly reduced to low levels. This condition is due to the presence of nitrite oxidizing bacteria (NOB) that consumed the nitrite-nitrogen (substrate) and accumulated nitrate-nitrogen (product). As the dilution rate of the system was increased then the levels of nitrite-nitrogen and ammonia-nitrogen slowly increased with subsequent lowering of the formation of nitrate-nitrogen.

During the first 10 days of operation, a transient accumulation of nitrite-nitrogen was observed in the PFBR system. This condition was due to slow consumption of nitrite-nitrogen by the NOB. Nitrite oxidizing bacteria are relatively slow growing compared to AOB (Villaverde *et al.*, 2000; Blackburne *et al.*, 2007). However, this transient

accumulation slowly fell after several days of operation as a result of acclimatization of the NOB to the high nitrite-nitrogen content (Tan *et al.*, 2008).

From the observations during the 11 days to 74 days of operation, the nitrifying bacteria (AOB and NOB) adapted and became well-established in the PFBR reactor system. This is reflected in the smaller values of ammonia-nitrogen and nitrite-nitrogen in the effluents shown in Figure 5.9. This means the activity of the nitrifying bacteria could tolerate the changes in the ammonia-nitrogen concentration in the influent and a complete nitrification process was achieved with respect to the excess nitrate-nitrogen in the PFBR system (Yusof *et al.*, 2010).

Despite this, towards the 75th day of operation in the PFBR, the rate of ammonia-nitrogen and nitrite-nitrogen concentrations had temporarily increased in the effluents. This circumstance was triggered by the higher concentration of ammonia-nitrogen introduced into the PFBR system, which could not be sustained by the nitrifying bacteria (AOB and NOB). Nevertheless, on the 76th day of operation the nitrification process had recovered, indicating the nitrifying bacteria withstood the extreme environment due to the high ammonia-nitrogen concentration. In addition, from the results shown in Figure 5.9, the concentrations of ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen have all stabilized toward the end of the operation of the PFBR reactor system.

From this experimental investigation of the PFBR system, this has demonstrated that using K2 AnoxKaldnes packing material in the reactor provided high specific surface area, allowing the development of highly active biofilms that are tolerant to changes in feed quality and environmental conditions. The presence of static media with microporous structure as well as the porosity of the packing arrangement (i.e., particle size and shape of the media inside the bioreactor) creates a complex system from both mass transport and reaction kinetics perspectives (Dizge and Tansel, 2010).

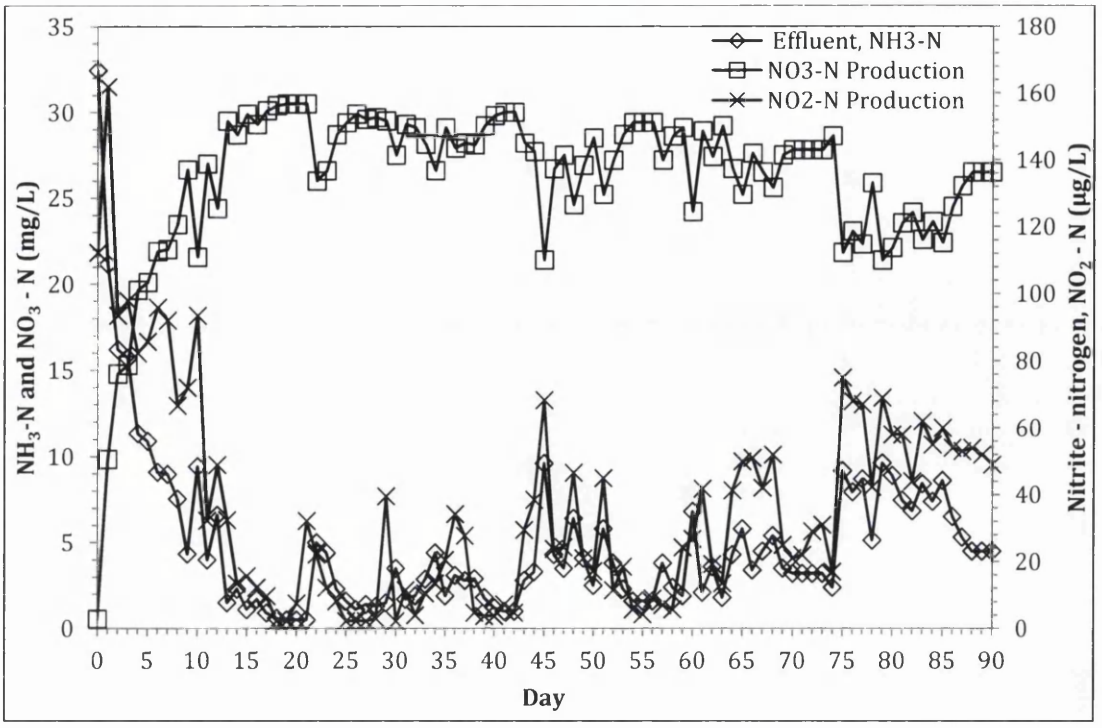


Figure 5.9: The production of nitrite-nitrogen and nitrate-nitrogen and effluent concentration of ammonia-nitrogen in the PFBR reactor with various dilution rates.

In comparison with the activated sludge system, such a system of activated sludge requires higher HRT in order to maintain sufficient numbers of slow growing nitrifying bacteria in the aeration reactor (Arnold *et al.*, 2000). A higher HRT value in the range of 26 to 27 days was applied to ensure 100% ammonia-nitrogen removal in the activated sludge system (Carrera *et al.*, 2003). This is in contrast to the PFBR system using immobilized suspended media that needed a shorter HRT (0.19–0.56 day) to complete the nitrification process. As a consequence, the study of the PFBR system has shown the feasibility of using a simple system that contributed to acclimatizing the nitrifying bacteria in the reactor system, which could then lead to the high nitrification performance.

5.2.1.2.4 The degradation of synthetic estrogens 17α-ethynylestradiol (EE2) and mestranol (MeEE2) in the continuous PFBR reactor.

The synthetic oestrogens 17α-ethynylestradiol (EE2) and mestranol (MeEE2) were added daily simultaneously at a concentration of 100 μg/L to the PFBR system.

5.2.1.2.4.1 17α-ethynylestradiol (EE2) in the PFBR reactor.

Figure 5.10 shows the effluent concentration of EE2 in the PFBR. At the first dilution rate of 1.8 d^{-1}, the concentration of EE2 reduced gradually towards the end of the studied period. The levels were reduced from the feed to 24–30 μg/L. However, as the dilution rates were increased, the degradation levels slowly reduced. At the high dilution rate, only 40% of the oestrogen was removed. Clearly the rate of removal was dependent on residence time in the reactor.

In this research, the sorption and co-metabolism of EE2 by the nitrifying bacteria was confirmed in the PFBR system. The first start-up period of the reactor has stimulated faster and better degradation of the EE2. This was in response to the low dilution rate (1.8 d^{-1}) and long HRT (0.56 day) applied in the system that deliberately supported the EE2-degraders (nitrifying bacteria) in their survival and colonization on the submerged carriers (Forrez *et al.,* 2009).

This nitrifying activity has been already determined to contribute to EE2 removal. A study conducted by Yi and Harper (2007) clearly showed that ammonium monooxygenase (AMO), which is the enzyme that catalyses the first step in nitrification, has a role in the degradation of EE2. The reaction stoichiometry was consistent with a conceptual model involving a binuclear copper site located at the AMO active site (Yi *et al.,* 2006). The most interesting outcome of this work for the investigation of the PFBR system is that the EE2 removal was maintained in the effluents despite the continuous addition of high concentrations of EE2 to the PFBR system at several different dilution rates and HRT values.

This scenario conveyed the considerable amount of nitrifying bacteria still contained and attached in the immobilized suspended carrier of K2 AnoxKaldnes packing material in the PFBR system that was able to remove EE2. This is supporting evidence

that using suspended carrier media is an advantage to preserve the nitrifying bacteria's population density under the given conditions.

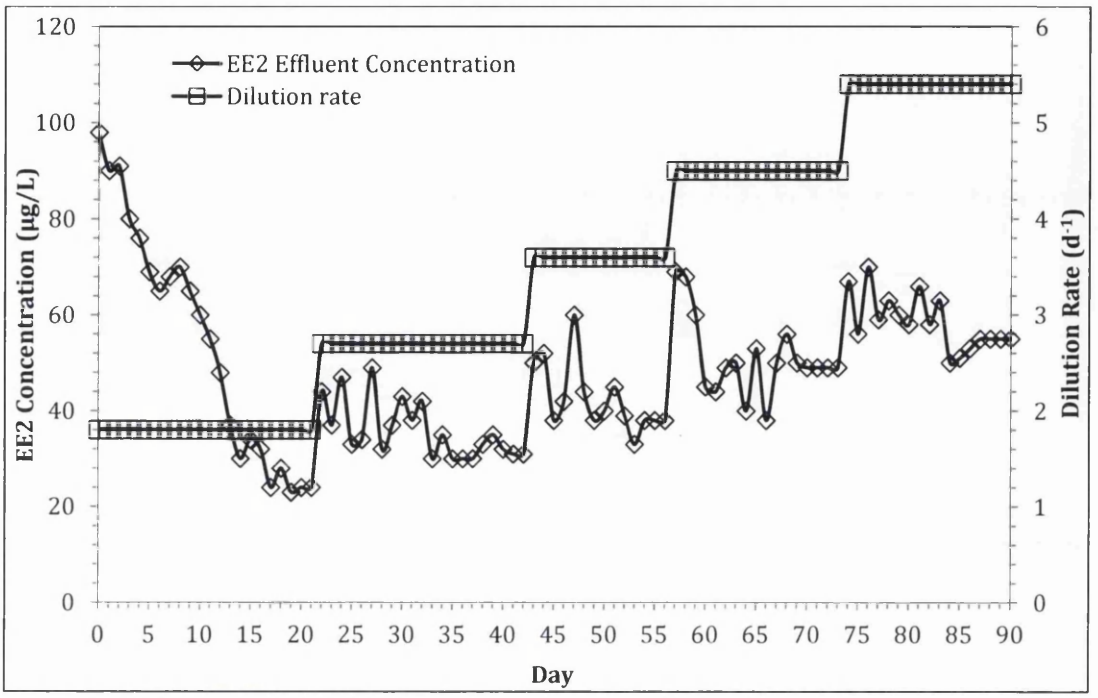


Figure 5.10: The effluent concentration of 17α-ethynylestradiol (EE2) in the PFBR system at various dilution rates.

Figure 5.11 shows the effect of the volumetric EE2 loading rate on the EE2 loading rate in the PFBR system. The removal rates were much higher at low loading rates compared to a high loading rate where substantial quantities of the material were not removed. The results presented in the figure emphasise this observation. Relative to the kinetics of ammonia-nitrogen removal, oestrogen removal was much slower. The system was capable of removing about 200–270 µg/L.day.

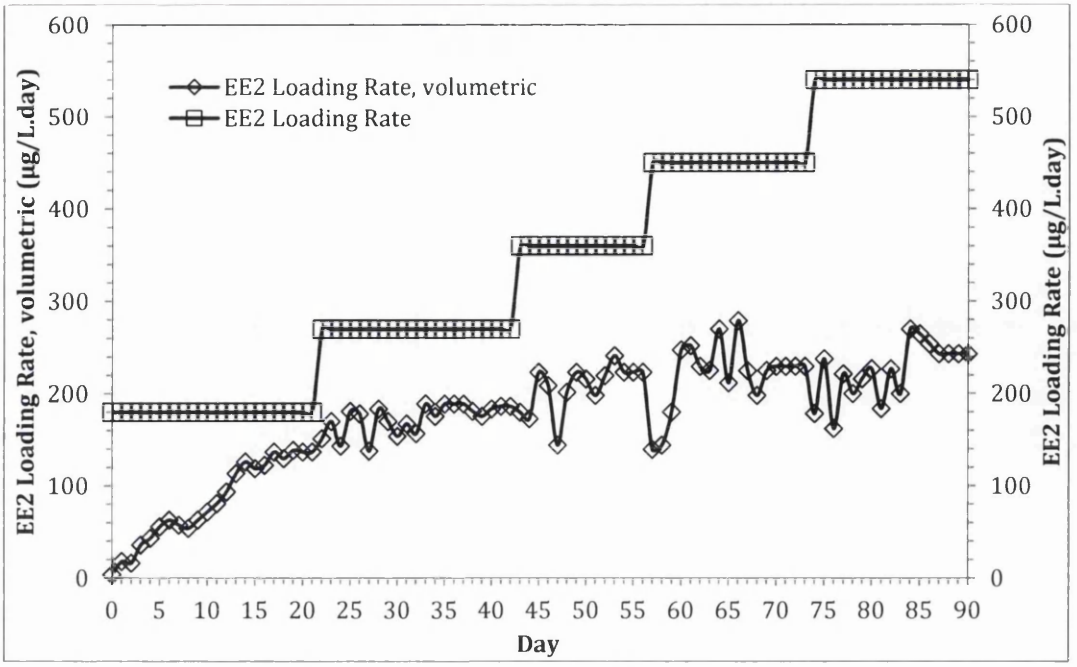


Figure 5.11: The volumetric 17α-ethynylestradiol (EE2) loading rate and the EE2 loading rate in the PFBR system at various dilution rates.

The experiment indicates the PFBR reactor system was enriched with nitrifying bacteria, with a maximum growth rate, μ_{max} of 7.092 d^{-1} and hydraulic retention time (HRT) value of 0.141 day (3.3 h) (see section 5.2.1.2.1). Here, the nitrifying bacteria have successfully removed EE2 that was continuously being added to the PFBR system at an HRT of 0.141 day (3.3 h). This result may be compared to the study by Forrez *et al.*, (2009), which examined the removal of EE2 in an aerated nitrifying fixed bed reactor during ammonia-nitrogen starvation. The HRT obtained by Forrez *et al.*, (2009) was 0.33 day (8 h) for degradation of the EE2 without adding any ammonia-nitrogen to the reactor system of the nitrifying bacteria.

In a study by Forrez *et al.*, (2009), the nitrifying bacteria were able to maintain their population density without supplementation with ammonia-nitrogen in the feed for two months. However, comparing the HRT in the PFBR system and the results obtained by Forrez *et al.*, (2009), the HRT achieved in the PFBR system was less than the HRT obtained by Forrez *et al.*, (2009). This indicates that the PFBR system could accomplish better performance compared to the system evaluated by Forrez *et al.*, (2009). In the PFBR system, a feeding supply of ammonia-nitrogen (35 mg/L) was continuously being added to the system, in contrast to the system applied by Forrez *et al.*, (2009) where no

ammonia-nitrogen was added to the system with only recirculation of effluent back to the system. Ammonia-nitrogen is an important substrate (food) for nitrifying bacteria, so that in the PFBR system a greater activity of nitrifying bacteria is achieved and greater performance can be obtained in removing EE2 compared to the system examined by Forrez *et al.,* (2009).

A research study done by Azizi *et al.,* (2013) on the removal of organic pollutants by reducing chemical oxygen demand (COD) in residential wastewater under given operation and designing conditions evaluated HRT of 0.25 day (6 h) and 0.083 day (2 h) for a conventional activated sludge process (CASP) and a packed-bed biofilm reactor (PBBR), respectively. However, the research conducted by Azizi *et al.,* (2013) did not consider the removal of oestrogens from the treatment systems.

Figure 5.12 shows the percent change in the volumetric EE2 loading rate in the PFBR reactor with various loading rates of EE2. The maximum rate of removal was observed at low dilution rates at the beginning of the process. The efficiency of the process was reduced as the dilution rate was increased, reducing the residence time in the reactor.

Biological removal of EE2 by a nitrifying bacterial enrichment culture was conducted by De Gussame *et al.,* (2009) in a membrane bioreactor. Complete nitrification and removal of EE2 was achieved. The efficiency of removal of EE2 was between 80 and 90%. Here, from the results of Figure 5.12, the PFBR system could attain greater than 90% removal of EE2 with a loading rate of 180–270 μg /L.day. The performance of the membrane reactor (De Gussame *et al.,* 2009) and the PFBR are parallel in successfully reduced the EE2 concentration in their treatment systems. In spite of that, there are several disadvantageous of using a membrane reactor compared to the immobilized carrier media in the PFBR system. A membrane reactor system is prone to membrane fouling and needs a constant frequency of back-washes to prevent clogging by filamentous bacteria (Clouzot *et al.,* 2010). Therefore, use of the immobilized reactor system of the PFBR may create an effective tool to eliminate the disadvantages of choking and clogging with low HRT and contribute to the low overall cost of handling and maintenance (Azizi *et al.,* 2013).

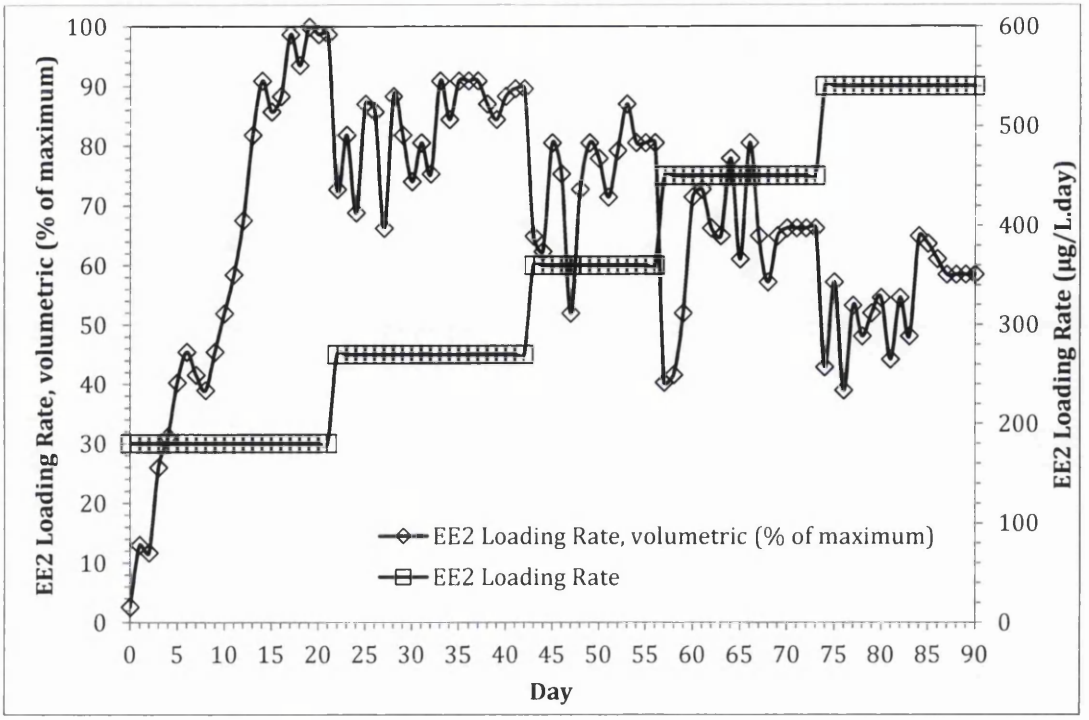


Figure 5.12: The volumetric loading rate of EE2 as a percentage of maximum and the EE2 loading rate in a continuous PFBR system with various dilution rates.

5.2.1.2.4.2 Mestranol (MeEE2) in the PFBR system.

Figure 5.13 shows the MeEE2 effluent concentration at various dilution rates in the PFBR system. The data follow a similar trend to that of oestrogen presented above. The MeEE2 was reduced to about 20 µg/L and this level increased as the dilution rates increased. Nevertheless, the concentration of MeEE2 maintained a value of 52 µg/L during the last 90 days of the operation of the PFBR.

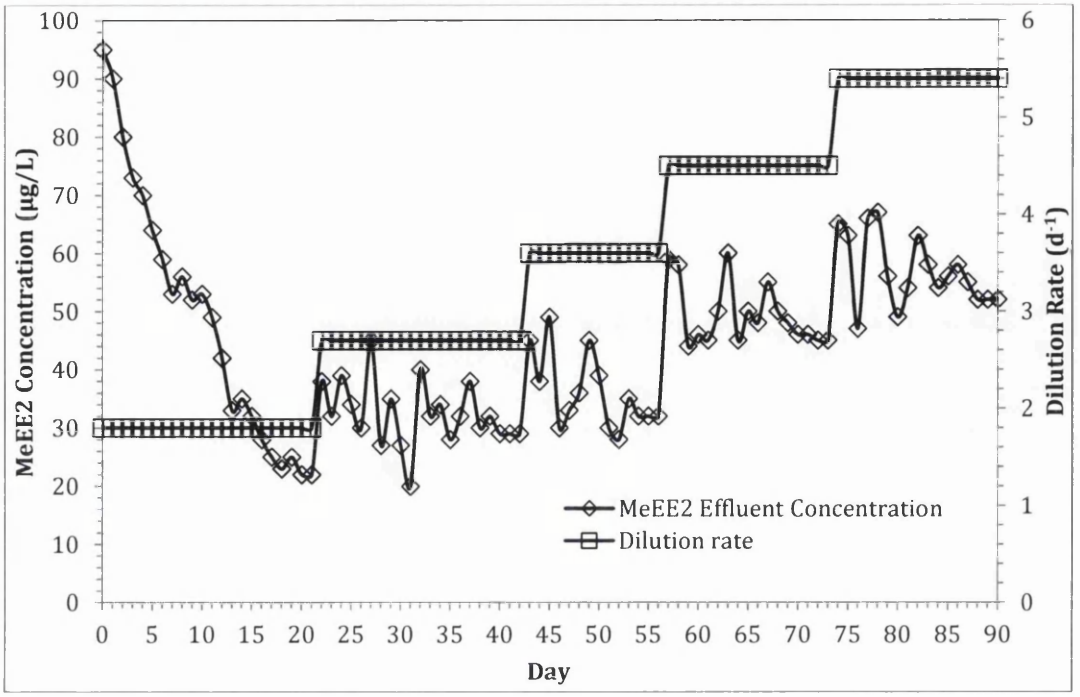


Figure 5.13: The concentration of mestranol (MeEE2) in the PFBR system with various dilution rates.

Figure 5.14 illustrates the volumetric loading rate of MeEE2 against the loading rate of MeEE2 throughout the 90 days of continuous operation of the PFBR system. Again, the removal performance was similar to that of oestrogen in that the maximum removal rates were between 180 and 250 µg/L.day.

Natural estrogenic steroids (estrone (E1); 17β-estradiol (E2); estriol (E3)) and synthetic steroids (EE2, MeEE2) have moderate binding to sediments and are reported to degrade in soil and water. However, little research has been conducted on the fate of synthetic steroids, especially MeEE2 (Ying *et al.*, 2002). A research study conducted by Ren *et al.*, (2007) for a sequencing batch reactor (SBR) indicated that the biodegradation of E1, E2 and EE2 were dependent on the activity of the ammonia oxidizing bacteria (AOB). The investigation (Ren *et al.*, 2007) found that better nitrification performance corresponded to higher degradation of E1, E2 and EE2. Here, these new experimental results show that MeEE2 can be reduced (sorption and degradation) along with EE2 in the PFBR system.

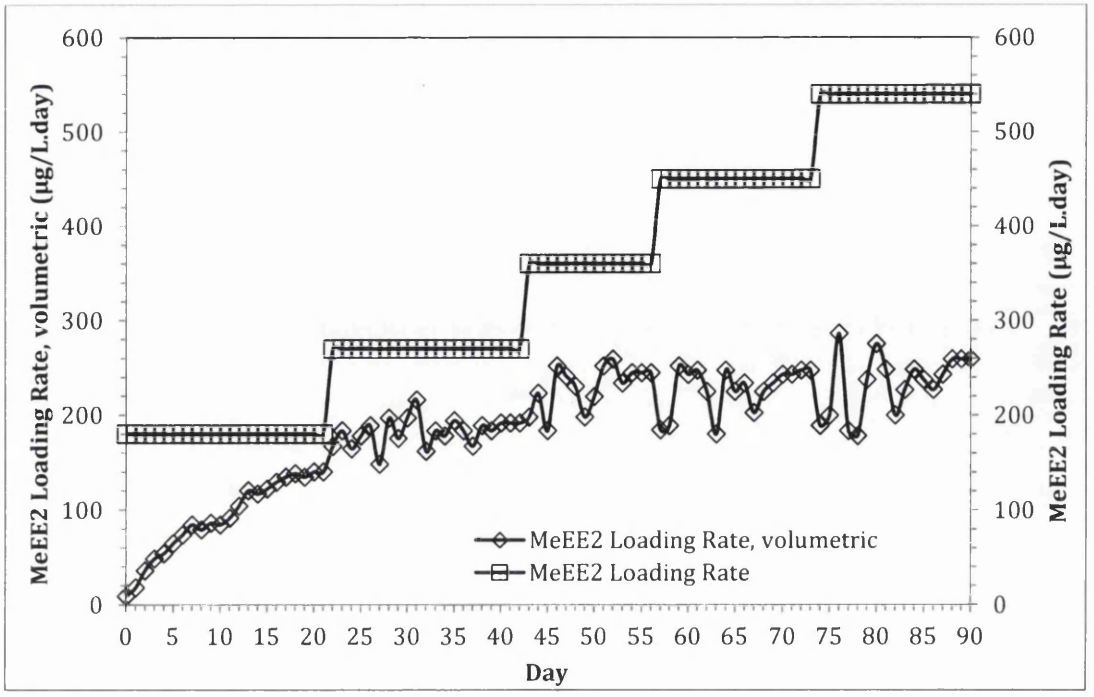


Figure 5.14: The volumetric mestranol (MeEE2) loading rate and mestranol (MeEE2) loading rate in the PFBR system at various dilution rates.

Figure 5.15 shows the percentage of the maximum volumetric loading rate for MeEE2 against the loading rate of MeEE2 in the PFBR system. The percent removal of MeEE2 gradually increased at the initial loading rate of MeEE2 of 180 µg/L.day. The percent removal of MeEE2 had achieved 98% towards the end of 21 days of operation. Above these loading levels, the percent removal gradually declined to below 60% at 540 µg/L.day.

The results determined from Figure 5.15 demonstrated that MeEE2 degradation depended on the activity of nitrifying bacteria in the PFBR system. The co-metabolic degradation and sorption of the MeEE2 was dominated by the nitrification process occurring in PFBR reactor, which correlated with the nitrifying bacteria attached to the immobilized suspended carrier. With the first two MeEE2 loading rates (180 µg/L.day and 270 µg/L.day), a high volumetric loading rate of MeEE2 was achieved. Accordingly, more complete removal of oestrogens can be obtained by securing a highly efficient nitrification process in the treatment system (Ren *et al.*, 2007; Cajthaml *et al.*, 2009).

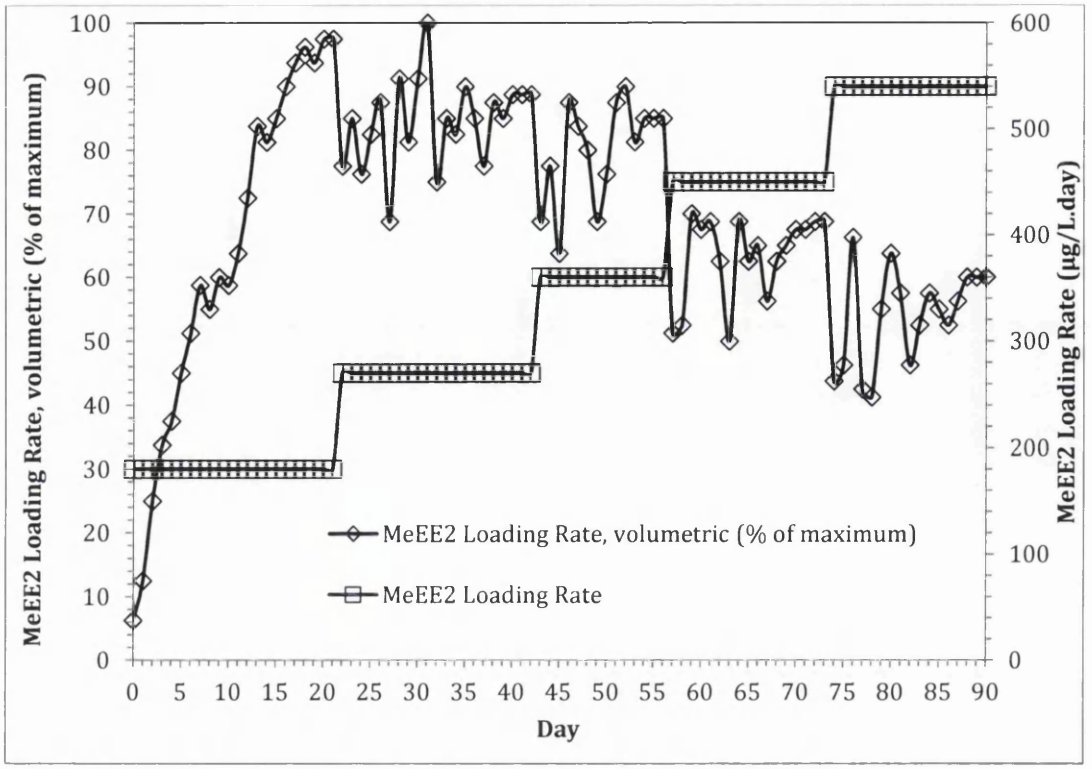


Figure 5.15: The volumetric MeEE2 loading rate as a percent of the maximum and the MeEE2 loading rate in the PFBR system.

5.2.2 Continuous reactor of moving bed for K2 AnoxKaldnes packing material (MBBR)

The continuous reactor system with a moving bed for K2 AnoxKaldnes packing material (MBBR) was exactly the same as the PFBR. A laboratory-scale plexiglass reactor with the same dimensions, as shown in Figure 5.16 (30 cm internal diameter and 170 cm height), was used for the experimental study. The reactor was filled with a working volume of 16 L.

For the moving bed system, the perforated plate was put near the outflow sampling port to retain the packing material on the surface of the working volume of 16 L. The perforated plate was used to retain the K2 AnoxKaldnes packing material so that the packing materials were submerged in the water when air was introduced to the system. The air diffusion from the reactor bottom created turbulence in the medium as the K2 AnoxKaldnes packing material would move freely under the surface; this was in contrast to the partial fixed-bed system, in which a metal perforated plate was used to

retain the packing material in a fixed position in the middle of the working volume inside the reactor.

Figure 5.17 shows the schematic diagram of the K2 AnoxKaldnes packing material in the moving bed system in the continuous culture reactor. The detailed workings of the MBBR have already been explained in the previous section in 5.2.1 for the PFBR.

Figure 5.16: Photograph of continuous reactor for moving bed K2 AnoxKaldnes packing material (MBBR).

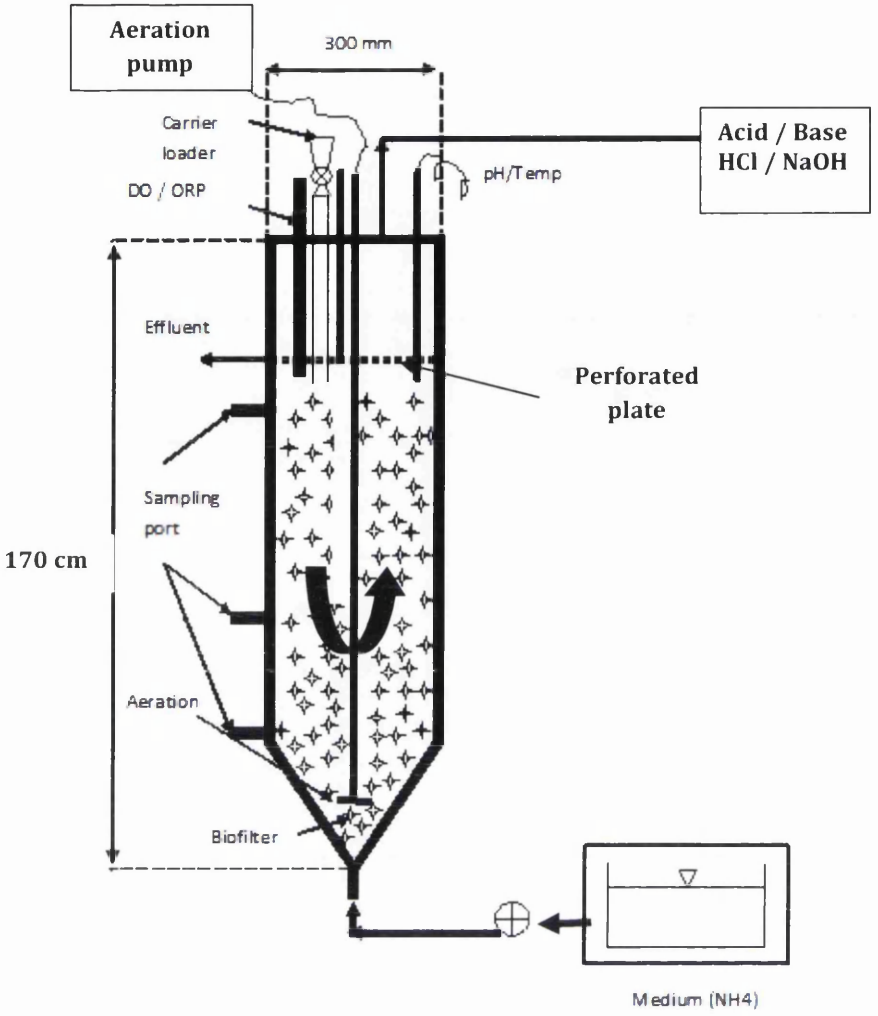


Figure 5.17: Schematic diagram of MBBR.

5.2.2.1 Start-up moving bed reactor (MBBR) for growth enrichment of nitrifying bacteria

The moving bed K2 AnoxKaldnes reactor was set to a working volume of 16 L. An overflow pipe on the side of the tank allowed the effluent from the reactor to overflow to the effluent tank. Medium A was prepared in 30 L tanks every day and was inserted in a larger tank of size 100 L to ensure there was sufficient Medium A when the reactor was run in continuous mode. The reactor was operated at a control temperature of 30°C and at pH 8, the optimum temperature and pH, respectively, for the growth of nitrifying bacteria. A concentration of 35 mg/L of ammonia-nitrogen and 100 µg/L of synthetic oestrogens (EE2 and MeEE2) were added daily into the reactor. Two litres of nitrifying bacteria from the serial batch culture derived from fish effluent was inoculated in the reactor.

Approximately the same amount of K2 AnoxKaldnes packing material used in the partial fixed-bed continuous reactor was inserted in the moving bed continuous reactor (8 L or a total weight of 1434 grams = 1.434 kg). The packing material was allowed to move freely on the submerged stage in the continuous reactor, as shown in Figure 5.16.

The measurements of ammonia-nitrogen, nitrite-nitrogen and nitrate–nitrogen were determined using the methods described in Chapter 3-Preliminary Study for the Enrichment Culture. The synthetic oestrogens EE2 and MeEE2 were determined using a gas chromatograph mass detector as explained in Chapter 4-Batch Culture. All measurements were taken daily. For both oestrogens, a solid phase extraction technique was used to separate the samples from the water and solvents before analysis in the gas chromatograph mass detector. The samples were kept in a 4°C refrigerator prior to analyses.

A digital peristaltic pump (Masterflex L/S, Cole-Parmer, USA) was introduced into the reactor and set to a low rate (20 mL/min).The dilution rates used for the moving bed reactor were the same as those used in the partial fixed-bed reactor shown in Table 5.1. Before the influent was pumped to the reactor, the pump was calibrated, and 15' Masterflex tubing was used to connect the digital pump from the feed tank to the reactor.

5.2.2.2 Results for Moving bed reactor (MBBR)

The moving bed continuous reactor with K2 AnoxKaldnes packing material was operated for over three months, the same duration of operation for the partial fixed bed continuous reactor with K2 AnoxKaldnes packing material. Figure 5.18 shows the results for ammonia-nitrogen effluents in the moving bed continuous reactor.

As shown in Figure 5.18, the ammonia-nitrogen was degraded by the ammonia oxidizing bacteria (AOB) in the nitrification process. The effluent of ammonia-nitrogen was high on the first day of the start-up period of the MBBR, with a concentration of 22 mg/L concentration. After this initial period the levels of ammonia-nitrogen were below 2 mg/L. As the dilution rate increased the levels of ammonia-nitrogen rose slowly until at the highest dilution rate of 5.4 d^{-1} the levels of ammonia-nitrogen were about 3 to 5 mg/L, after which they then stabilized at 3 mg/L.

The MBBR reactor showed better ammonia-nitrogen degradation based on the results in Figure 5.18 compared to the PFBR system. This was primarily because of the freely submerged immobilized media effectively occupying the total working volume of the reactor. This situation corresponded to an improved hydraulic residence time distribution, which has significant effects in terms of the quality control of out-flowing treated water (Azizi *et al.*, 2013).

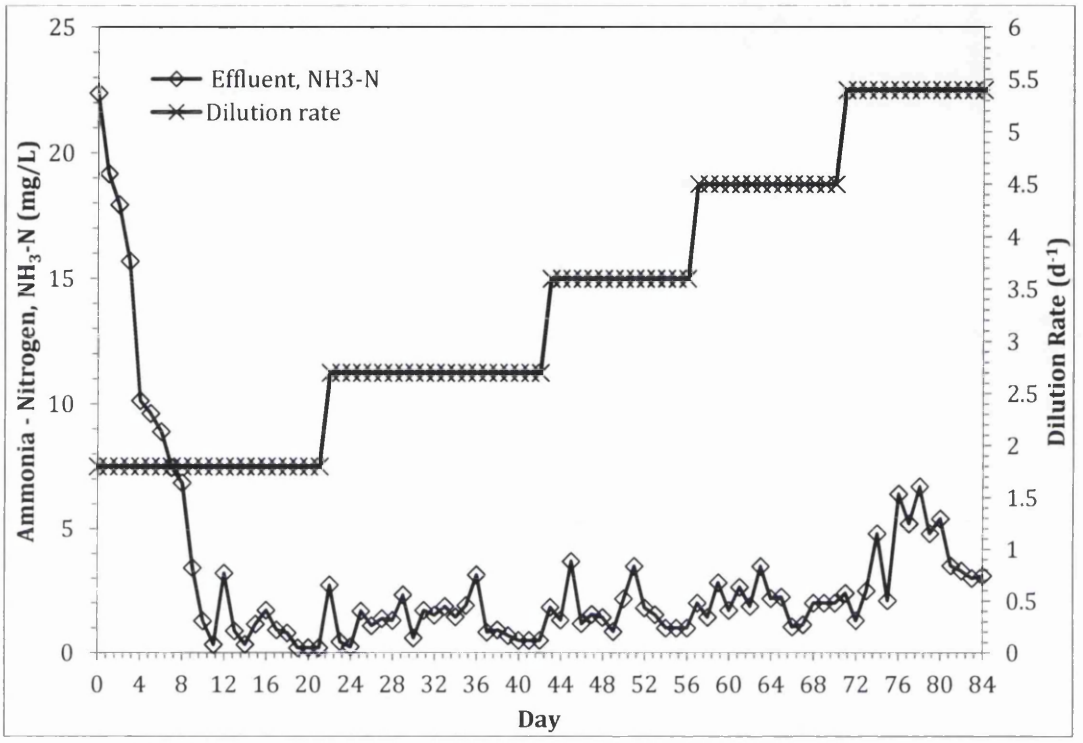


Figure 5.18: Results from the MBBR system with various dilution rates at a concentration of 35 mg/L ammonia-nitrogen.

Clearly, this system operated well over the period of the experiment and gave slightly better performance than the PFBR described in earlier sections, whereas in the MBBR system the effluent concentrations of ammonia-nitrogen were slightly lower compared to the PFBR system and the effluent was more uniformly concentrated. These results are indicative of better mixing within the MBBR system.

5.2.2.3 Maximum specific growth rate and substrate saturation constant

The reciprocal equation $S/D = S/\mu_{max} + Ks/\mu_{max}$ was used for the graphical determination of the maximum specific growth rate and substrate saturation constant. According to the experimental data of S and D for the MBBR from Table 5.3, a plot of S/D versus S is shown in Figure 5.19 with the values:

$$1/\mu_{max} = 0.158,\ Ks/\mu_{max} = 0.103$$

$$\mu_{max} = 6.329\ d^{-1},\ Ks = 0.652\ mg/l$$

Table 5.3: Data on ammonia-nitrogen at different dilution rates for the MBBR system.

Dilution rate (d^{-1}), D	NH_3-N concentration (mg/L), S	S/D
1.8	0.2	0.111
2.7	0.5	0.185
3.6	1.0	0.278
4.5	2.0	0.444
5.4	3.1	0.574

The maximum growth rate and substrate saturation constant for the MBBR system were μ_{max} = 6.329 d^{-1} and Ks = 0.652 mg NH_3-N/L, respectively. The use of synthetic wastewater in this nitrifying biofilm system achieved a higher maximum growth rate compared to the maximum growth rates AOB and NOB at 20°C, which have been reported to be 0.54–0.77 day^{-1} and 0.67–1.08 day^{-1}, respectively (Vadivelu *et al.*, 2006; Blackburne *et al.*, 2007).

This could be due to the K2 AnoxKaldnes packing material used in the moving bed reactor as support material for the biomass, which provided a large surface area for the microorganisms' growth and prevented cell wash-out by the attachment of the nitrifying bacteria. Hence, by using this packing material as a carrier can conduct successful ammonia-nitrogen removal treatments at higher dilution rates (WEF, 2005).

The specific growth rate value determined by this experimental study was in agreement with other reported research for nitrifying biofilm systems (Tijhuis *et al.,* 1995; Bougard *et al.,* 2006).

The substrate saturation constant, *Ks* of 0.652 mg NH_3-N/L that was obtained in the MBBR was lower than that from the PFBR system, whereas the value of substrate saturation constant, *Ks* for the PFBR system is 1.574 mg NH_3-N/L. The research conducted by Chen *et al.,* (2006) for a rotating biological contactor (RBC) in re-circulating aquaculture systems evaluated the substrate saturation constant, *Ks* of 2 mg NH_3-N/L at a working temperature of 27°C. For the consequences, comparing the result achieved in the MBBR system for substrate saturation constant, *Ks* with the PFBR system and the result obtained by Chen *et al.,* (2006), the performance on the MBBR system has accomplished higher efficiency in carry out the nitrification process.

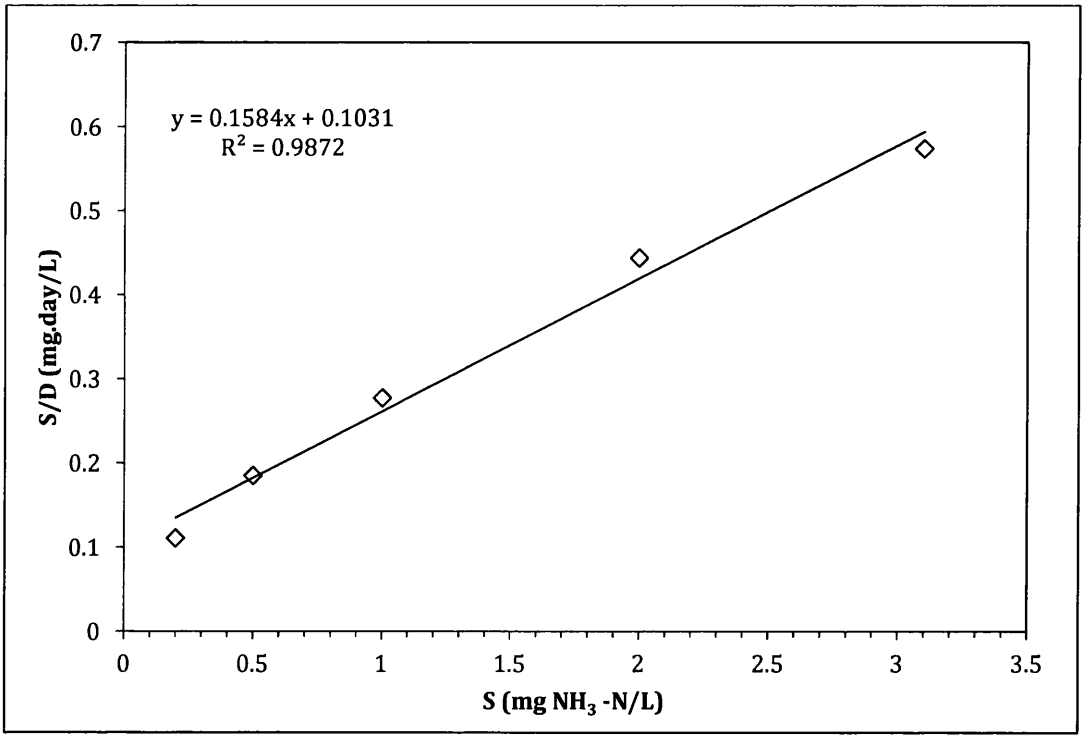


Figure 5.19: Graphical determination of the *Ks* and μ_{max} using experimental data for the MBBR system at 35 mg NH_3-N/L, T = 30°C, pH = 8.0

5.2.2.4 Nitrification Process in the MBBR.

Figure 5.20 shows the volumetric nitrification loading rate (NLR) in mg/L.day at various nitrification loading rates (NLR) in the MBBR. The reactor was fixed with a working volume of 16 L and the continuous dilution rates (d^{-1}) used were as shown in Table 5.1. The calculations obtained from equation (5.6) and equations (5.7) with the results are illustrated in Figure 5.20. The results show that after a start-up period the system showed very good performance over the range of dilution rates tested; however, when the dilution rate was increased to 5.4 d^{-1} there was a substantially increased amount of ammonia-nitrogen in the effluent.

The MBBR achieved a stable and higher volumetric NLR due to the free movements of the K2 AnoxKaldnes packing material, which provide a larger surface area for the nitrifying bacteria that were attached to packing material, which can then consume more ammonia-nitrogen in the nitrification process. From the results in Figure 5.20, the MBBR was more competent in carrying out the nitrification process compared to the PFBR system. The volumetric NLRs shown in Figure 5.20 achieved comparable values coinciding with the amounts of ammonia-nitrogen applied to the MBBR system. Here, the MBBR system is shown to be more stable system with greater resistance to shock loads.

The design behind the use of MBBR system is based on stimulating and localizing the growth of nitrifying bacteria on the surface of freely moving carriers. This system reduces the formation of biofilm clogs, which also cause head losses (pressure drop due to friction as liquid flows through the system) in the MBBR reactor (Accinelli *et al.*, 2012). The present configuration of the MBBR reduced channelling phenomena and effectively activated the surface area of the support media, which helped maintaining better contact with the polluted water (in this case ammonia-nitrogen) and therefore efficient biodegradation was facilitated (Azizi *et al.*, 2013). This also contributed to the presence of extra oxygenation over the whole surface of the support media, so the mass transfer problem could be reduced (Dizge and Tansel, 2010). In the PFBR system, the mass transfer of substrate and nutrients into the biofilm is a limiting factor for the nitrification rate (see full explanation in Section 5.2.1.2.2).

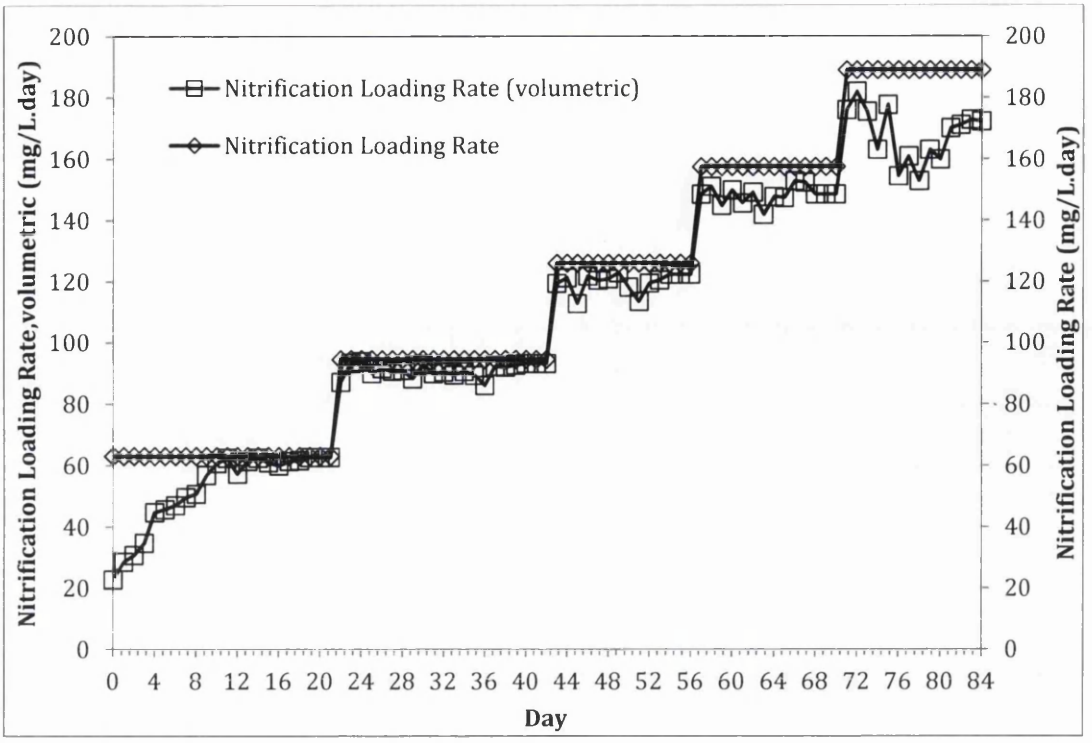


Figure 5.20: Results of ammonia-nitrogen removal rate (volumetric) at different ammonia-nitrogen loading rates in the MBBR system.

Figure 5.21 shows the results of ammonia-nitrogen removal rate for the volumetric NLR in percentage terms in the MBBR. The performance increases up to 11 day, where the removal rate reaches maximum performance. With the exception of the high flow rate at the final dilution rate, the system operated at or near 95% removal, although a slight declined can be noted as the dilution rates (feed rate) are increased.

The K2 AnoxKaldnes packing material is deliberately moving freely in the active volume of the MBBR system. High turbulence from the compressor causes movement of the suspended carriers and affects the thickness of the water film and subsequent high rate of transfer of substrate from the bulk liquid through the biofilm (Chen *et al.*, 2006). In response to this situation, the percent removal rate of ammonia-nitrogen shown in Figure 5.21 achieved remarkably high values against the loading rate of ammonia-nitrogen applied in the MBBR system. These results demonstrate that the MBBR system completes the nitrification process far better than the PFBR system.

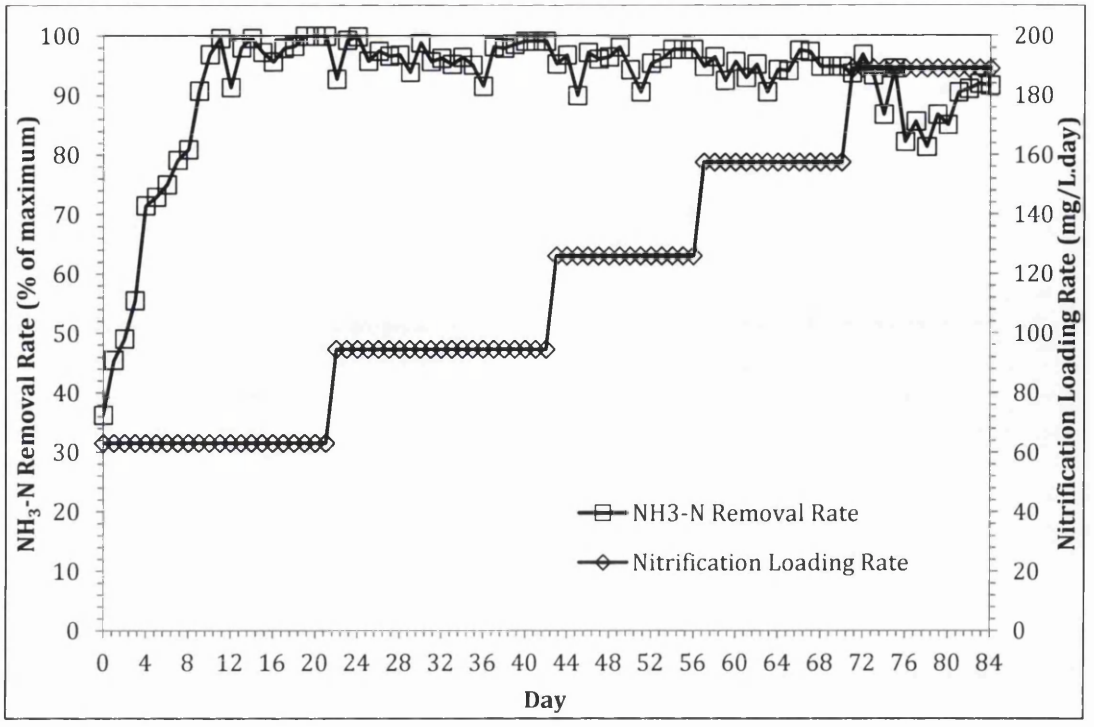


Figure 5.21: Results of ammonia-nitrogen removal for different volumetric NLRs as a percentage of maximum at different NLRs in the MBBR.

5.2.2.5 Formation of nitrite-nitrogen and nitrate-nitrogen in the continuous MBBR reactor

Figure 5.22 shows the production of nitrate-nitrogen in the MBBR. Apart from the start period, good conversion rates were observed with the production of over 32 mg/L of nitrate-nitrogen. However, from the point when the highest feed rate was employed there was a substantial drop in nitrate-nitrogen production. However, this sudden decrease in nitrate-nitrogen was overcome after several days of operation of the MBBR. The concentration of nitrate-nitrogen in the effluents was maintained at the final dilution rates of 5.4 d^{-1}. The low values of nitrate-nitrogen in the effluents were mainly due to the high dilution rates applied to the system, whereas the hydraulic HRT was decreased. The accumulation of nitrate-nitrogen declined with the reduction in the HRT. In spite of this, the nitrification process has recovered after a period of time when the MBBR system was in steady state towards the ends of operation.

The performance of the MBBR system with respect to the nitrification process proved to be more reliable than the PFBR system. The MBBR system responded very

sharply to changes in the influent flow rate and ammonia-nitrogen loading rate in the system (Li *et al.*, 2011). Due to the capacity of the MBBR reactor, this system was able to develop a nitrifying consortium acclimated to the stressful conditions imposed, which promoted very effective nitrification (Bassin *et al.*, 2011).

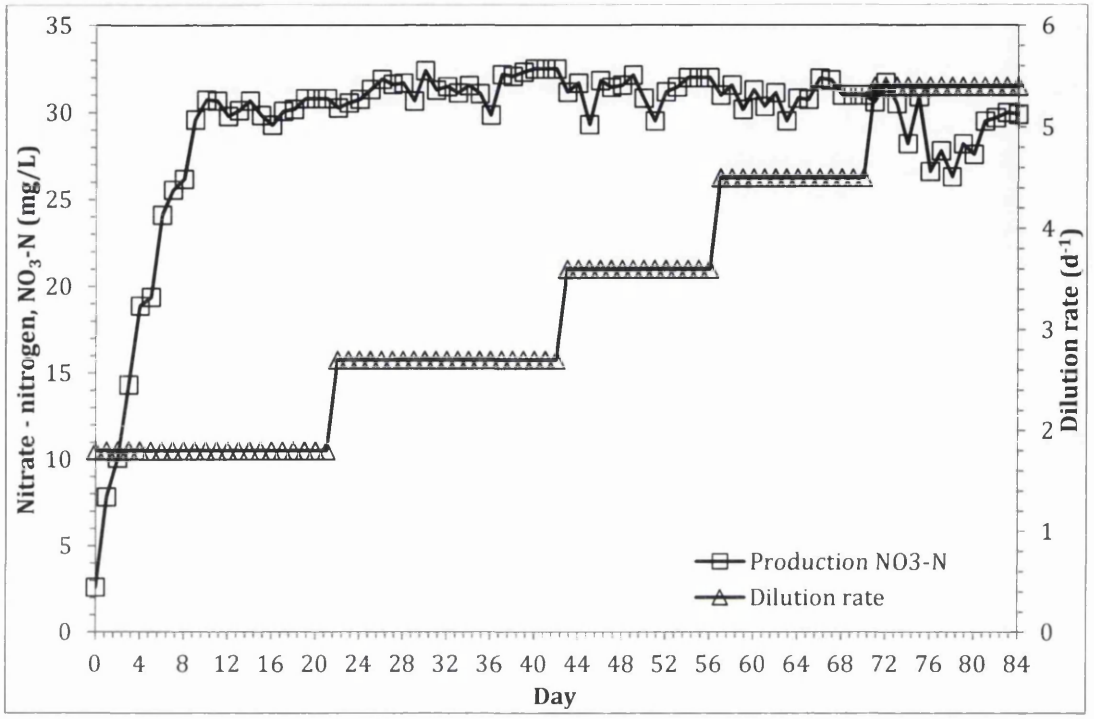


Figure 5.22: The production of nitrate-nitrogen in the MBBR at various dilution rates for a concentration of 35 mg/L ammonia-nitrogen.

The time-course of product formation of nitrite-nitrogen and nitrate-nitrogen and the degradation of ammonia-nitrogen in the effluents of the MBBR system are displayed in Figure 5.23. As with previous observations, the performance of the system was excellent after the start-up period of 12 days. Apart from occasional spikes in nitrite-nitrogen levels, good ammonia-nitrogen removal and nitrate-nitrogen formation were achieved.

The higher diffusion of mass transport in the MBBR system had a significant effect on the consumption of ammonia-nitrogen and nitrite-nitrogen for the formation of nitrate-nitrogen in the MBBR system. The movement of the support media in the MBBR system increases the saturation of dissolved oxygen and results in substrates being distributed over the entire surface area of the carriers (inner and outer surface), so the contact between the bulk-water and the biofilm of nitrifying bacteria attached on the

suspended carriers becomes greater (Zhu and Chen, 2002). Hence, the assimilation of nitrifying bacteria is increased due to the high rate of oxidation of ammonia-nitrogen in the MBBR reactor. From the results of these experiments, a better nitrification process was achieved in the MBBR reactor in contrast to the PFBR system, in which the latter reactor was affected by the restraint of mass transport diffusion in the system (Mudliar *et al.,* 2008).

The MBBR system is self-cleaning and has low head loss, this corresponding to the K2 AnoxKaldnes packing material that moves freely through the water and is stirred hydrodynamically by an air compressor (Rusten *et al.,* 2000). The high shear stress from the water circulation and carrier collisions helps to control biofilm development and stabilize the reactor system (Dupla *et al.,* 2006). This condition is explained by the continuous removal of biofilm from the outer surface of the carriers in conjunction with the abrasion process between carriers. As a result, biofilm thickness was governed by air turbulence, ensuring that the carriers are evenly distributed throughout the working volume of the MBBR system (Maurer *et al.,* 2001).

The jet nozzle allowed increasing shear stress to be generated on the carrier surface in order to detach biomass and degas the carriers. Hence, this contributed to biofilm control, which had significant impact on the nitrification performance of the MBBR system (Welander and Mattiason, 2003; Dupla *et al.,* 2006). The biofilm erosion exerted by the moving fluid is stronger in the inner part of the carriers. Thin biofilms on the carriers of the MBBR system are more dependent on substrate availability than thick biofilms (Melo and Vieira, 1999). Increasing the shear stress on the biofilm would deliberately increase the mass transfer and consequently the nitrification process performance of the carriers, especially for younger biofilms (Dupla *et al.,* 2006). Thus, under the conditions stated, the MBBR system would be the recommended choice for optimizing nitrification performance where advanced nitrogen removal is desired.

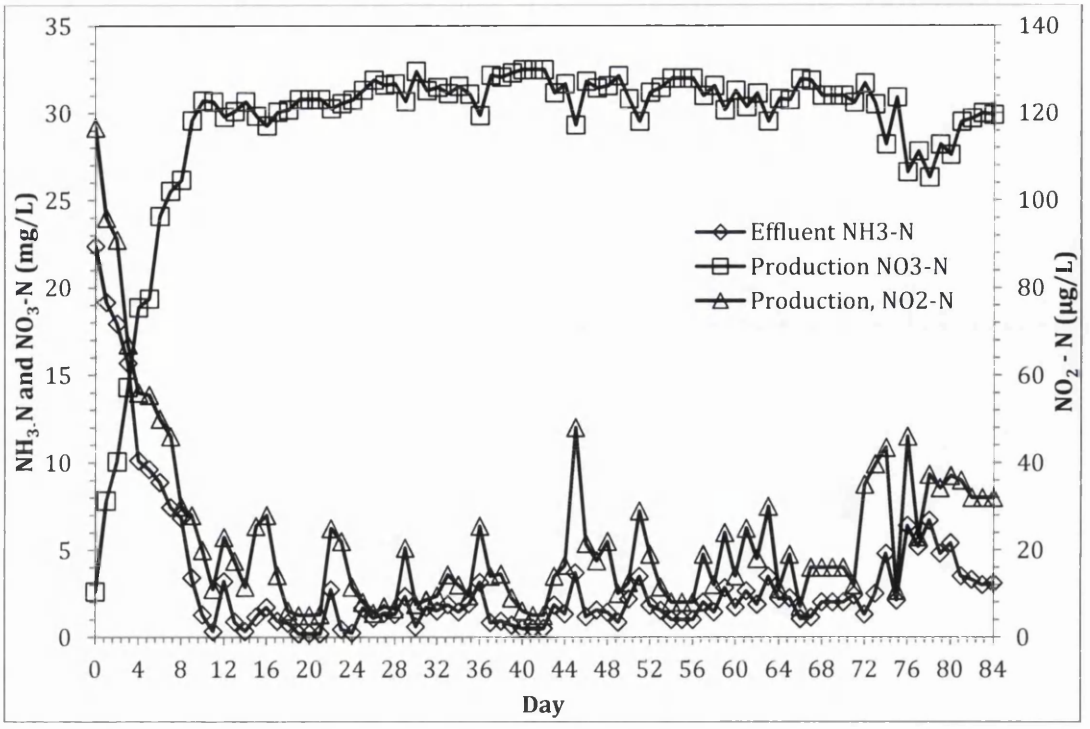


Figure 5.23: The production of nitrite-nitrogen and nitrate-nitrogen and the effluent of ammonia-nitrogen in the MBBR system.

5.2.2.6 The degradation of synthetic oestrogens EE2 and MeEE2 in the MBBR system

The combined removal of EE2 and MeEE2 were studied in the MBBR system. A feed composition of 100 µg/L of EE2 and MeEE2 subsequently were added to the MBBR system under up-flow mode with different flow rates as shown in Table 5.1 in a previous section.

5.2.2.6.1 17α-ethynylestradiol (EE2) in the MBBR system

Figure 5.24 shows the concentration of the effluent of EE2 in the MBBR at various dilution rates. As the results show, the concentration of EE2 declined gradually over the start up period of 12–15 days, after which substantial removal was observed. Levels below 20 µg/L were achieved by the end at the slowest dilution rate (1.8 d^{-1}). However, as the dilution rate increased the concentration in the reactor slowly increased as well. By the end of the experiment at the highest dilution rate (5.4 d^{-1}) the

concentration had increased to about 40 µg/L. Overall the system was able to remove substantial quantities of the hormone for prolonged periods of operation. Gradually, the MBBR system attained good effluent quality in terms of low concentration of EE2 after treatment in the reactor system.

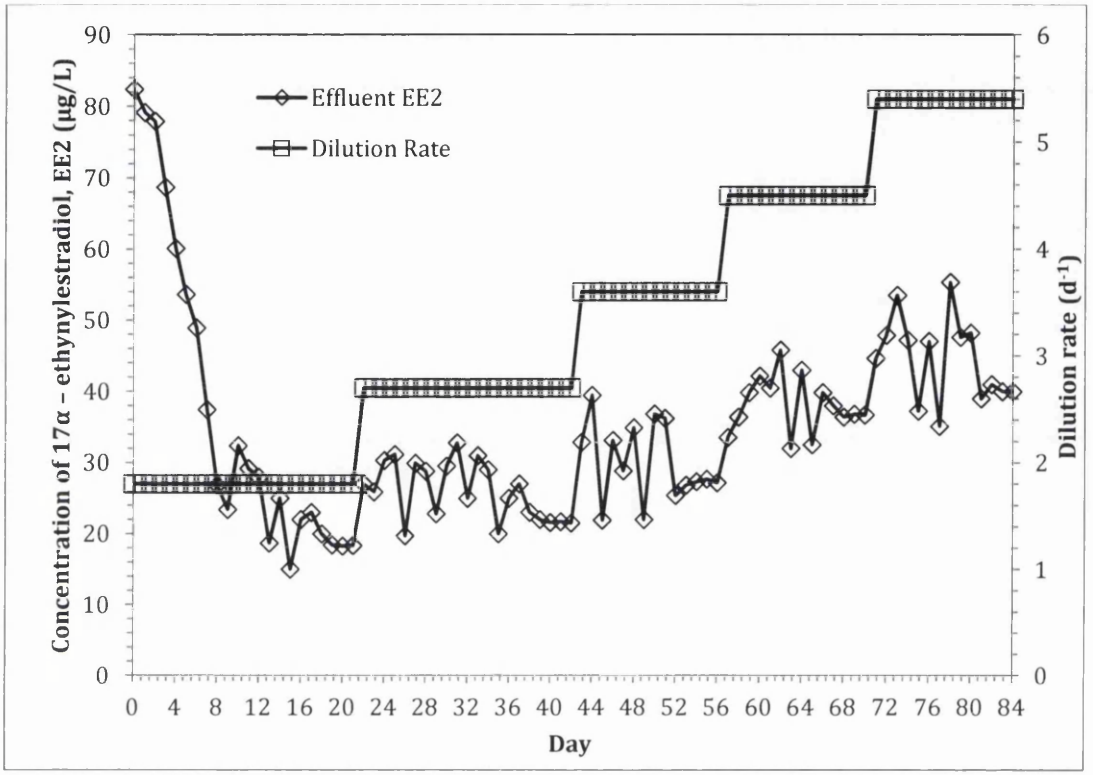


Figure 5.24: The effluent concentration of 17α-ethynylestradiol (EE2) in the MBBR system at various dilution rates.

Figure 5.25 shows the volumetric EE2 loading rate and the EE2 loading rate in the MBBR. The volumetric loading rates of EE2 increased steadily towards values ranging from 137 µg/L.day to 350 µg/L.day over the duration of the experiment. The removal rate becomes saturated and only slowly increases with increased feed rate of the hormone, with a resultant rise in the effluent concentration of EE2.

The feed was supplied to the reactor with a digital peristaltic pump (Masterflex L/S, Cole-Parmer, USA) at different flow rates depending on the operational parameters of dilution rate, HRT and loading rates (NH_3-N, EE2 and MeEE2). During an adaptation period, at a loading rate of 180 µg/L.day, high bacterial enrichment of nitrifying bacteria was determined by operating the MBBR system at a long HRT of 0.56 day (13.44 h). Here, sorption and biodegradation of EE2 has been achieved in the MBBR system with

high removal of EE2. Furthermore, the dynamic biodegradation of EE2 in the MBBR reactor was tested for the maximum EE2 removal capacity by increasing the loading rates and decreasing of HRT.

Respectively, the volumetric loading rates of EE2 exhibited astonishing stabilization after higher loading rates of EE2 and low HRT were applied to the MBBR system. Furthermore, the volumetric loading rates of EE2 improved as the operation of the MBBR reactor system was prolonged, despite the saturation of the EE2 that occurred towards the end with a loading rate of 540 µg/L.day in the MBBR system.

Bacteria that degrade natural hormones (E1 and E2) are able to co-metabolize EE2 (Pauwels *et al.,* 2008). The activity of the nitrifying bacteria has been reported to contribute to EE2 removal (Yi and Harper, 2007). However, at the final loading rate of 540 µg/L.day for the EE2, the nitrifying bacteria in the MBBR system could not tolerate the large amounts of EE2 introduced to the system, resulting in unsteady values for the volumetric loading rate of EE2 at the end of the period of operation.

Figure 5.26 shows the rate of removal as a percentage of the maximum and the EE2 loading rate in the MBBR. After a rapid rise in the rate of removal of EE2, there was steady decline in the removal rate as the experiment proceeded and the feed rate increased. However, from the results shown in Figure 5.26, the percentage removal of EE2 in the MBBR reactor system was relatively dependent on the HRT applied in the system. A higher population of nitrifying bacteria would be available to achieve sorption and biodegradation process of EE2 with a higher HRT applied to the treatment system. A higher HRT would enable sufficient growth of the nitrifying bacteria, as nitrifying bacteria are slow growing microorganisms (Vader *et al.,* 2000). The integration of long HRT at low dilution rate, which is associated with greater capacity of high biomass concentration (nitrifying bacteria), lead to high removal efficiency of steroid oestrogens (in this case; EE2) (Johnson *et al.,* 2005).

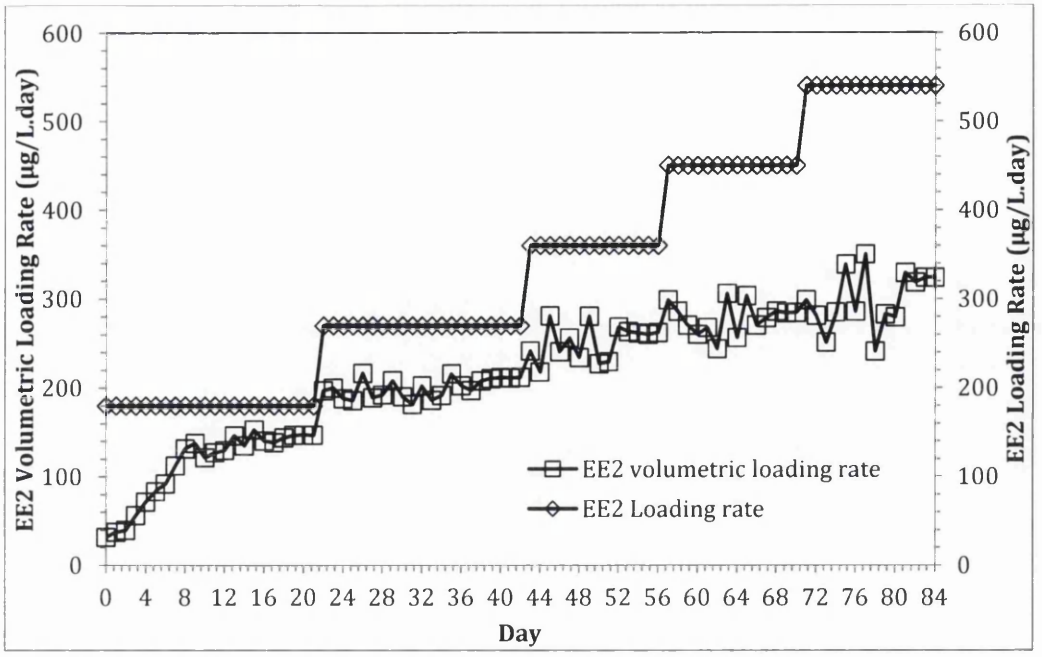


Figure 5.25: The volumetric EE2 loading rate and the (EE2) loading rate in the MBBR system.

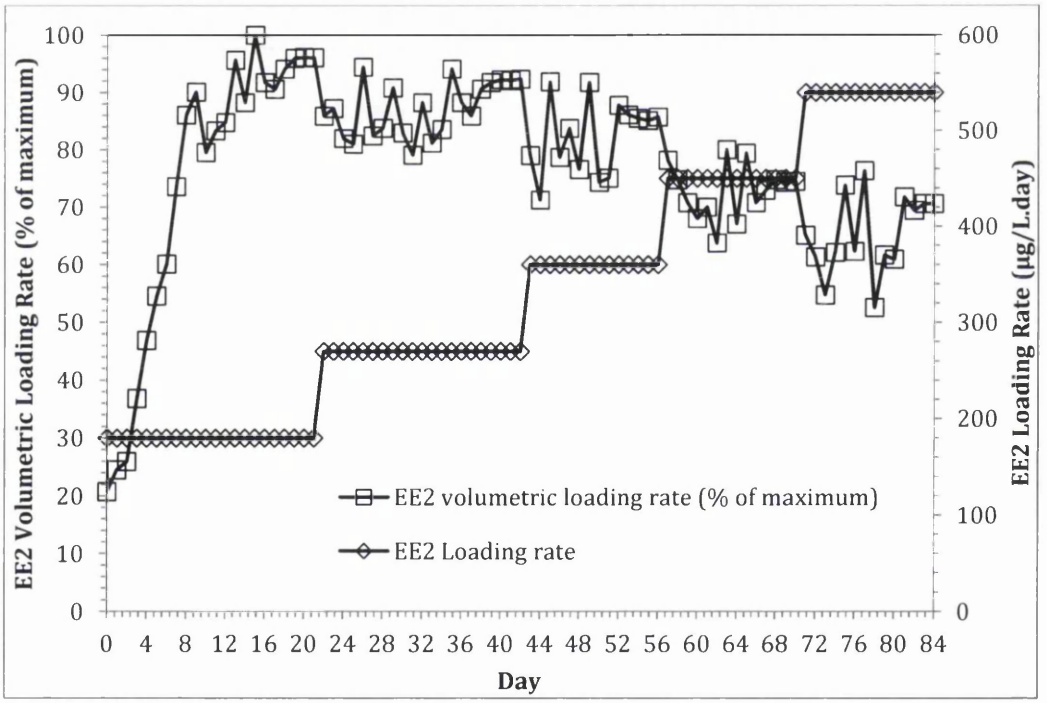


Figure 5.26: The volumetric EE2 loading rate as a percentage of the maximum and the EE2 loading rate in the MBBR system.

5.2.2.6.2 Mestranol (MeEE2) in the MBBR system

Figure 5.27 shows the effluent concentration of MeEE2 in the MBBR at various dilution rates over a period of 84 days. The concentrations of MeEE2 were substantially reduced after the initial start-up period, where the hormone was reduced to less than 20 µg/L. As with the oestrogen, MeEE2 concentrations rose with increasing dilution rate and by the end of the experiment, at a high dilution rate, the concentration had risen to 35 µg/L.

The MBBR system was capable of removing the hormone for prolonged periods of operation. Here, during the low dilution rate (1.8 d^{-1}) and long HRT (0.56 day), MeEE2 biodegradation has begun and the values varied throughout the different dilution rates and HRT introduced into the MBBR system. The relevant data evaluated confirmed that MeEE2 can be degraded simultaneously with EE2 in the MBBR reactor system. However, the important mechanisms that triggered the sorption and biodegradation of the oestrogens required a long HRT in the MBBR system with sufficient supplementation of the nitrifying bacteria in the treatment system (Cirja *et al.*, 2007).

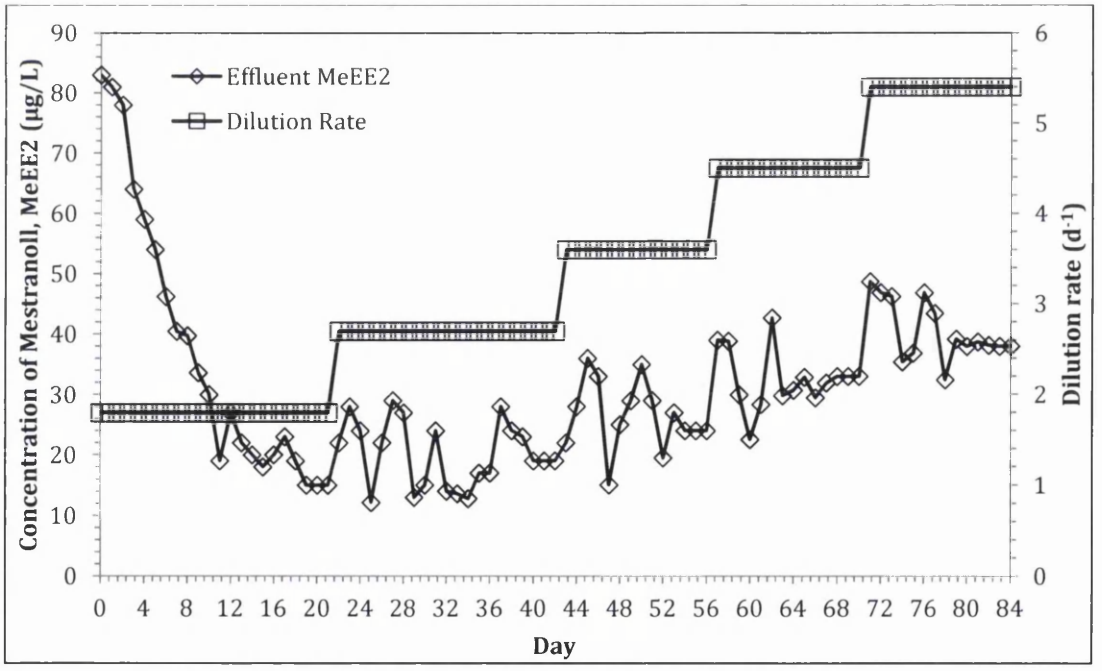


Figure 5.27: The effluent concentration of MeEE2 in the MBBR at various dilution rates.

Figure 5.28 shows the MeEE2 volumetric loading rate and the MeEE2 loading rate in the MBBR, while Figure 5.29 shows the percentage removal of the hormone from the MBBR system. These results follow a similar pattern to the results observed with oestrogen EE2, where after the initial start-up period good degradation was achieved at a low dilution rate, and as the dilution rate (and feed rate) increase so do the residual quantities of the hormone in the effluent stream. The degradation capacity of the MBBR system becomes progressively saturated.

A higher percentage of removal of MeEE2 was noted in the MBBR system compared to in the PFBR system described in the previous section (Section 5.2.1.2.4.2). The elimination of MeEE2 cultivated from the MBBR reactor system increased throughout the operation period, showing that the MBBR system is more competent with high loading of MeEE2 in the system. However, faster degradation of the oestrogens by the nitrifying bacteria occurred after a period of acclimation (Cajthaml *et al.*, 2009).

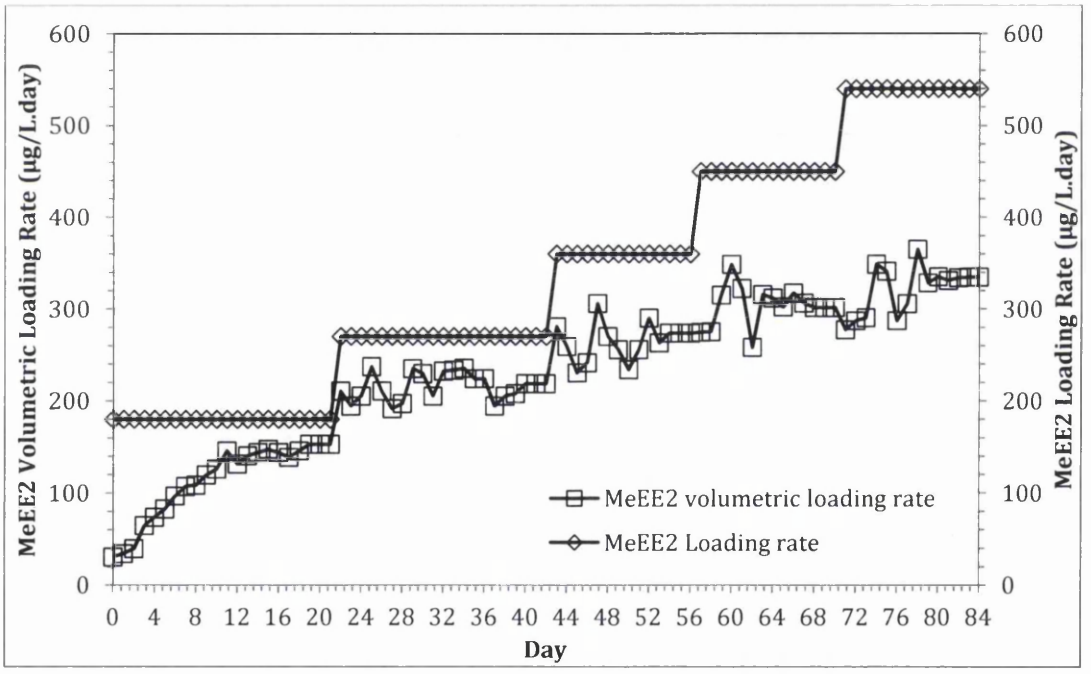


Figure 5.28: The volumetric MeEE2 loading rate and the MeEE2 loading rate in the MBBR system.

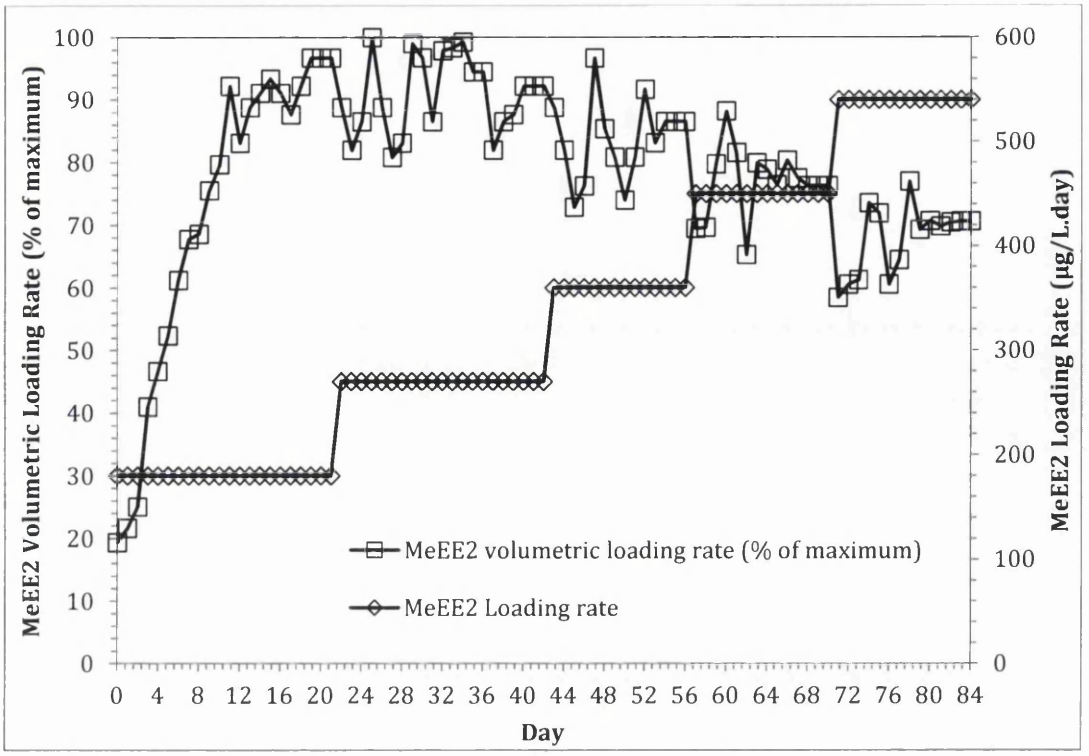


Figure 5.29: The volumetric MeEE2 loading rate as percentage of the maximum and the MeEE2 loading rate in the MBBR system.

5.3 Conclusions

The work in this chapter has investigated nitrification and hormone degradation in two types of film reactor, PFBR and MBBR. The techniques developed here show that the processes can be carried out at a large enough scale to give confidence in further scale-up of nitrification operations. Both reactor types could be run for long periods achieving good nitrification and compared well with other published studies (Kim *et al.*, 2008; Liu *et al.*, 2011; Yusof *et al.*, 2010; Wang *et al.*, 2012). The rates of degradation were good and correlated well with the residence time and the feed rate, but the systems did suffer to some degree from saturation of the degradation kinetics. The systems were capable of removing substantial quantities of ammonia-nitrogen with relatively low residence times (high dilution rates far in access of the growth rate of the nitrifying bacteria).

These MBBR and PFBR systems can be compared with previous batch work reported, and shows that the continuous system works more efficiently with good

conversion of ammonia-nitrogen to nitrate-nitrogen, with only a small amount of nitrate-nitrogen being present in the effluents.

The performance of PFBR and MBBR for the degradation of 17α-ethynylestradiol (EE2) and mestranol (MeEE2) at low concentrations in ammonia-nitrogen nitrifying systems has been evaluated in this research study. The capacity of biofilm reactors to treat toxic wastes in the context of studying the efficiency of the wastewater treatment (PFBR and MBBR) has been fulfilled. This research study reports substantial degradation of hormones in nitrifying systems. Both types of continuous reactor were able to degrade the hormone substrates for prolonged periods.

Apart from that, the choice of dilution rate and HRT is critically important in designing the reactor configuration (Zekker *et al.,* 2011). The choice of longer HRT and shorter dilution rate are important for nurturing the nitrifying bacteria in the PFBR and the MBBR reactors, as the microorganisms are slow growing bacteria (Jonoud *et al.,* 2003). However, with the supported media implemented in the system would give more surface area for the attachments of nitrifying bacteria to develop and flourished (Azizi *et al.,* 2013). The support media in the immobilized treatment system would also prevent wash-out of the biofilm from the system compared to activated sludge system (Biswas and Turner, 2012). This comprehensive method would emphasize higher efficiency of the nitrification process and produced good quality of the effluents.

By implementing long HRT, the achievement for stable nitrification process can be fulfilled as the nitrifying bacteria (AOB and NOB) has been given sufficient time to establish (Zekker *et al.,* 2011). The acclimatization period in very important stage in the reactor treatment systems, where in this progression stage the activity of the nitrifying bacteria is relatively high and huge amounts of hazardous substances is removed from the system (Forrez *et al.,* 2009). Here, the HRT have to be long enough to achieved high biomass concentration (Azizi *et al.,* 2013).

A study conducted by Borghei and Hosseini, (2004) for the treatment of phenolic wastewater using a moving bed biofilm reactor determined that by reducing HRT from 1 day (24 h) to 0.33 day (8 h) the removal efficiency of the chemical oxygen demand (COD) gradually decreased due to the increase in the hydraulic loading rate. Here in the study of MBBR, the HRT used was 0.56 day (13.44 h) to 0.19 day (4.56 h) the same as the HRT used in the PFBR system. From the experimental results retrieved, similar

patterns were observed for the MBBR reactor system and the PFBR reactor system compared to the study of Borghei and Hosseini, (2004). The most crucial parameters of HRTs need to be taken into account for the design of the treatment system. However, the immobilized system of the MBBR and the PFBR is considered to be more relevant in contrast to the activated sludge treatment system which required higher values of HRT, i.e. 26–27 day (1.08 -1.13 h) (Carrera *et al.,* 2003).

In comparing the MBBR and PFBR reactor systems, the MBBR is the more stable system and could stand higher resistant to shock loads after the microbial acclimatisation and the establishment of the steady-state conditions. Furthermore, the nitrification performance of the MBBR achieved greater success in contrast to the PFBR reactor system, where high quality of effluents was retrieved from the MBBR reactor treatment system.

CHAPTER 6

DISCUSSION AND CONCLUSION

This thesis set out to investigate and report on nitrification processes and possible cometabolism of low concentrations of oestrogens (EE2 and MeEE2) using film bioreactors based on specific packing design for this purpose. This area of research is important as the environmental treatment of ammonia-nitrogen and oestrogens are being recognized for their potential environmental impact. The work has shown that pilot scale operation of film reactors is possible and that they can work for prolonged period of operation converting ammonia-nitrogen to nitrate-nitrogen (nitrification) and substantially reducing the concentrations of hormones. As such the thesis has been successful in demonstrating these processes and the potential of this technology for performing these waste treatment operations. The highlights and points of interest are discussed in the proceeding sections.

6.1 Enrichment Culture

Simple serial enrichment processes proved successful in developing nitrifying cultures. The nitrifying bacteria grew more successfully in Medium A compared to Medium B. The enrichment culture from the fish effluents achieved higher ammonia oxidation compared to the soil enrichment culture (refer Figure 6.1). The optimum condition for these transformations by these enrichments was 35° C and pH 8 and was similar to those found by other workers (Groeneweg *et.al.,* 1994; Grunditz and Dalhammar, 2001; Vadivelu *et al.,* 2007; Blackburne *et al.,* 2007). The nitrification in the K2 AnoxKaldnes packing material

was the best material compared to the other packing materials studied. This work set the basis for future work.

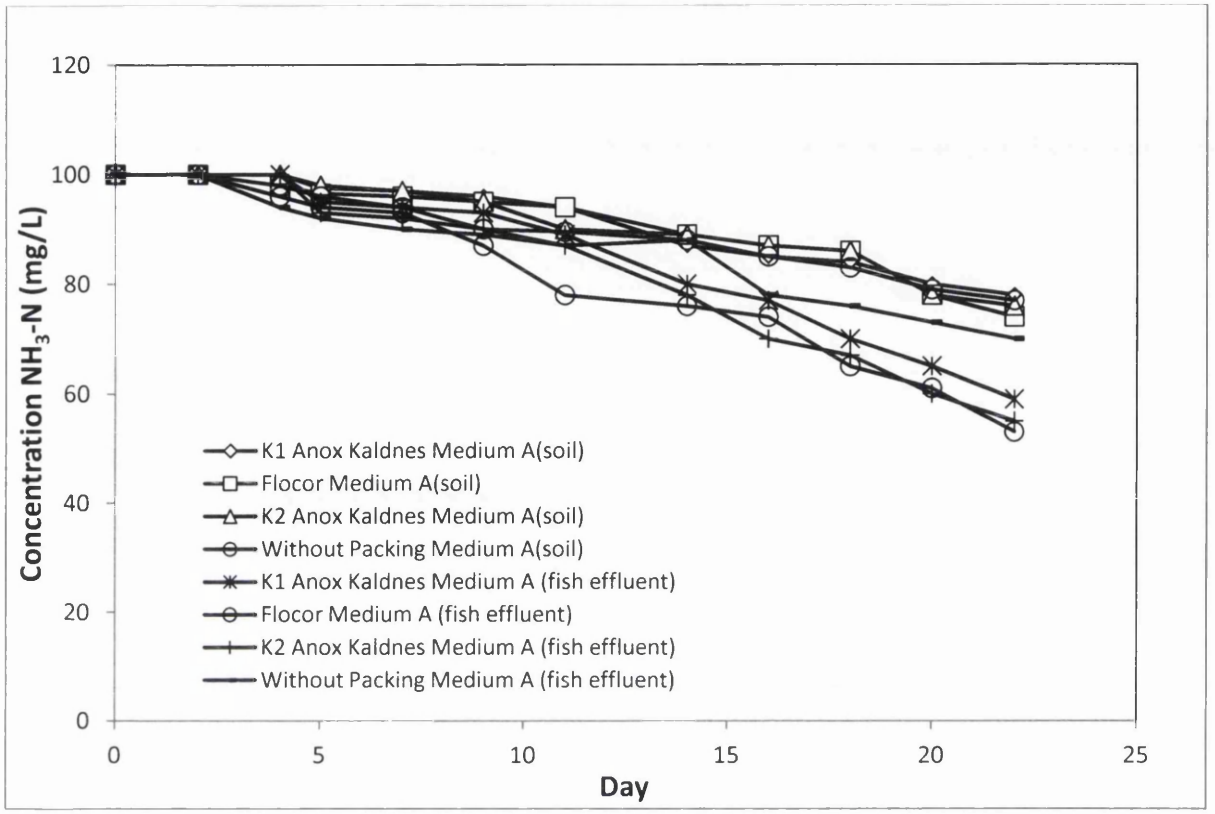


Figure 6.1: The comparison of ammonia – nitrogen degradation in the Medium A for soil and fish effluent in serial batch cultures of packing materials and without packing material.

The investigation of nitrification in batch cultures allowed initial studies of the transformation rates and further assessment of the influence for the packing materials in more detail. The analytical procedures and other methodology were developed further to allow the study of kinetics of nitrifications processes. The highest removal rate of ammonia-nitrogen was obtained in the concentration of 100 mg/L ammonia-nitrogen. Nitrification followed Monod kinetics with a measured value of 49 mg/L of the half-saturation constant, *Ks* in the batch reactor with a moving bed of K2 packing materials. This relatively high value of *Ks* is due to non-limiting ammonia-nitrogen concentrations in serial batches (100 mg/L ammonia-

nitrogen initially) resulting in inefficiency of the bacterial system for the uptake of ammonia-nitrogen.

Inhibition of nitrification was found at high ammonia-nitrogen concentrations (above 400 mg/L), which caused the release of the inhibition factor free ammonia (FA) (Anthonisen *et al.,* 1976; Bae *et al.,* 2001; Kim *et al.,* 2008). This inhibition decreased the microbial activity in the batch reactor of K2 AnoxKaldnes packing materials (Di Iaconi *et al.,* 2006). Above than 400 mg/L ammonia – nitrogen, the decay rate is considerably high (see Chapter 4 – Batch Culture for further explanation). Under these conditions, when a concentration above 400 mg/L of ammonia-nitrogen is applied to the batch reactor system, the nitrifying bacteria capacity would be reduced in the batch reactor of K2 AnoxKaldnes packing materials for the nitrification process (Li and Zhao, 1999; Yusof *et al.,* 2010).

Aerobic batch experiments containing fish effluents enrichment were carried in order to investigate the persistence of two synthetic oestrogen compounds under aerobic conditions with varying concentrations of ammonia-nitrogen. The batch experiments showed that while in contact with nitrifying bacteria that consumed ammonia-nitrogen, the synthetics oestrogens 17α – ethynylestradiol (EE2) and mestranol (MeEE2) were eliminated in the batch experiments with approximately linear time dependence (Ternes *et al.,* 1999b; Johnson *et al.,* 2000 and Cajthaml *et al.,* 2009). However, degradation products from the contraceptives 17α – ethynylestradiol (EE2) and mestranol (MeEE2) were not observed or detected.

The co – metabolism of 17α – ethynylestradiol (EE2) and mestranol (MeEE2) was confirmed to occur in the batch culture of nitrifying bacteria (Yi and Harper, 2007). Conclusively, the degradation of the 17α – ethynylestradiol (EE2) and mestranol (MeEE2) occurred in the batch reactor with the moving bed of K2 AnoxKaldnes packing materials. Furthermore, the removal rates of 17α – ethynylestradiol (EE2) and mestranol (MeEE2) were correlated with the concentration of ammonia-nitrogen in the batch reactor (Forrez *et al.,* 2009). When the ammonia-nitrogen concentration was high in the batch reactor, the percentage removal rates of contraceptives 17α – ethynylestradiol (EE2) and mestranol (MeEE2) were subsequently higher. This could be explained by the large amount of ammonia - nitrogen substrate available to be consumed by the nitrifying bacteria, as the population of nitrifying bacteria gradually multiplied with the increases of the ammonia – nitrogen substrate

(Shi *et al.,* 2004). For the conclusion, the nitrifying bacteria from the fish effluents in the batch reactor of moving bed K2 AnoxKaldnes packing materials were able to degrade the 17α – ethynylestradiol (EE2) and mestranol (MeEE2).

6.2 Comparison between Continuous Reactors PFBR and MBBR

The performances of the continuous reactors with a partial fixed-bed of K2 AnoxKaldnes packing materials (PFBR) and a moving bed of K2 AnoxKaldnes packing materials (MBBR) were evaluated to examine the effects of low ammonia-nitrogen concentrations on the reactor systems in the presence of two synthetic oestrogen compounds, 17α - ethynylestradiol (EE2) and mestranol (MeEE2). The effects on the results of the interference of inhibitory factors and nitrification loading rate were also discussed to find the correlation between those factors in the degradation of the two synthetic oestrogen compounds (Jafari *et al.,* 2009; De Gusseme *et al.,* 2009).

Figure 6.2 shows the correlation between the applied ammonia- nitrogen loading rate and the rate of nitrification in the continuous reactor with a partial fixed-bed of K2 AnoxKaldnes packing materials. From the results displayed in Figure 6.2, the ammonia-nitrogen removal rates were within the range of the ammonia-nitrogen loading rates. However, for the first ammonia-nitrogen loading rate, the removal rate is the lowest at first, before reaching maximal values at higher ammonia-nitrogen loading rates. For the higher range of the ammonia-nitrogen loading rates, the ammonia-nitrogen removal rate varied; however, the rate of ammonia – nitrogen removed was within the loading rate ranges.

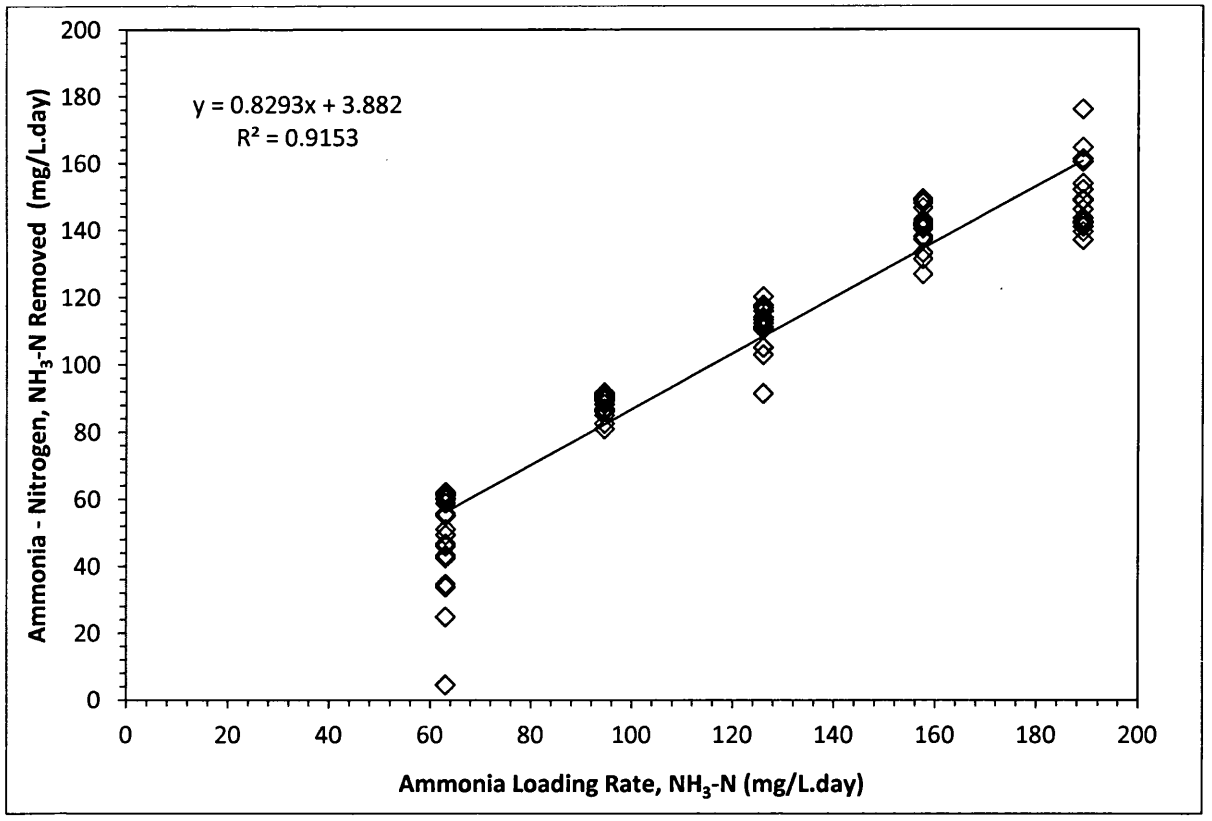


Figure 6.2: Correlation between the applied ammonia-nitrogen loading rate and the rate of nitrification in the PFBR.

Figure 6.3 shows the correlation between the applied ammonia-nitrogen loading rate and the rate of nitrification in the continuous reactor with a moving bed of K2 AnoxKaldnes packing materials. The ammonia-nitrogen removal rates fell within the range of the ammonia-nitrogen loading rates; these correlations are also in agreement with the values shown in Figure 6.2 for the partial fixed-bed system. For the MBBR, the loading rate of ammonia-nitrogen (Figure 6.3), the ammonia-nitrogen removed was better compared to the PFBR. Towards the higher loading rates, the ammonia-nitrogen removed was also at full capacity. Nevertheless, when approaching the highest ammonia-nitrogen loading rate, the ammonia-nitrogen removed was unsteady and had wide ranges. These situations were the same in the PFBR.

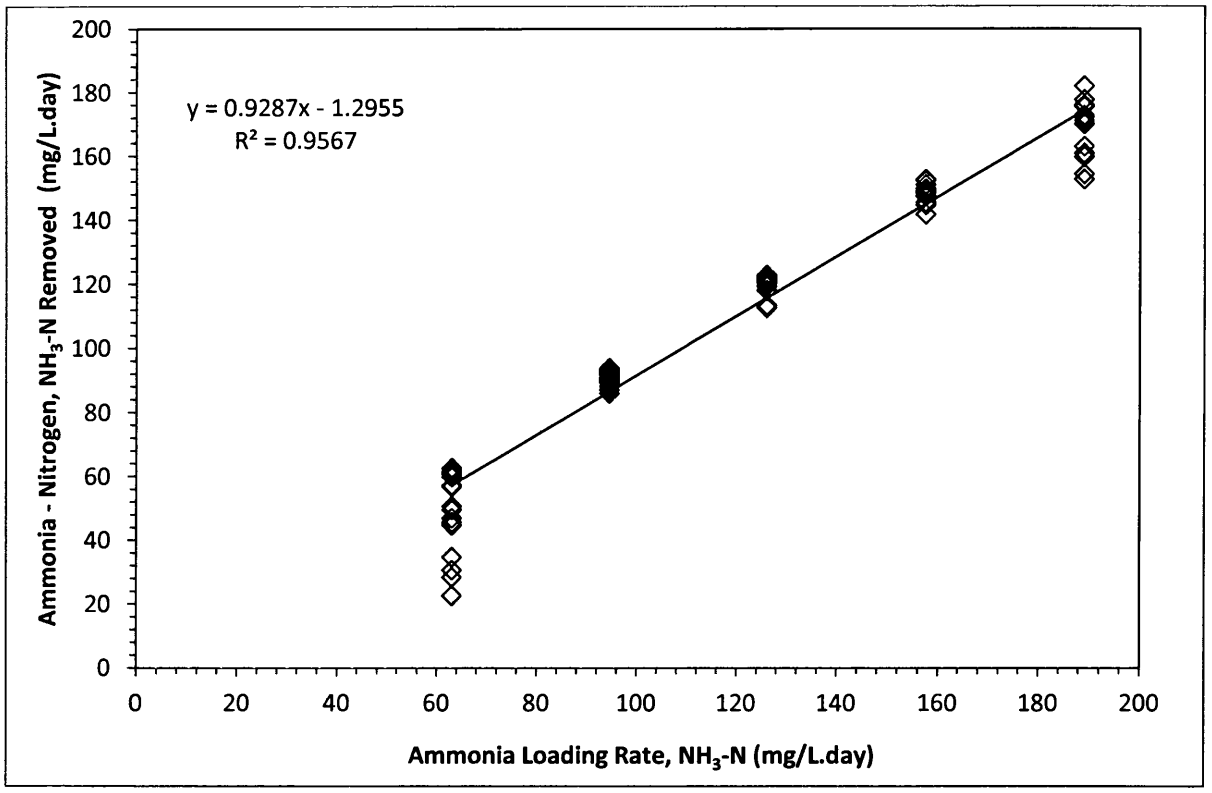


Figure 6.3: Correlation between the applied ammonia-nitrogen loading rate and the rate of nitrification in the continuous reactor for the MBBR.

Figure 6.4 shows the comparison between the PFBR and the MBBR systems for the removal rate of ammonia-nitrogen at different nitrification loading rate. The removal rates of ammonia-nitrogen in the PFBR continuous reactor were similar to the removal rate of the MBBR system. From the observation, the MBBR performed slightly better than the PFBR, however in both cases as the feed rate were increased then the efficiency of the process declined slightly as the capacity of the system became saturated. The MBBR was noticeably better at the high loading rates and more stable to shock loads compared to PFBR. Both systems were demonstrated to have the ability to operate for long period of operation (84 days). Here, the nitrification loading rates has significantly influenced the performance of MBBR and PFBR system. At high loading rates with a short hydraulic retention time (HRT), created situation where insufficient nitrifying bacteria is remained in the reactors system and this consequently reduced the nitrification process efficiency (Borghei and Hosseini, 2004; Yusof *et al.*, 2010 and Biswas and Turner, 2012).

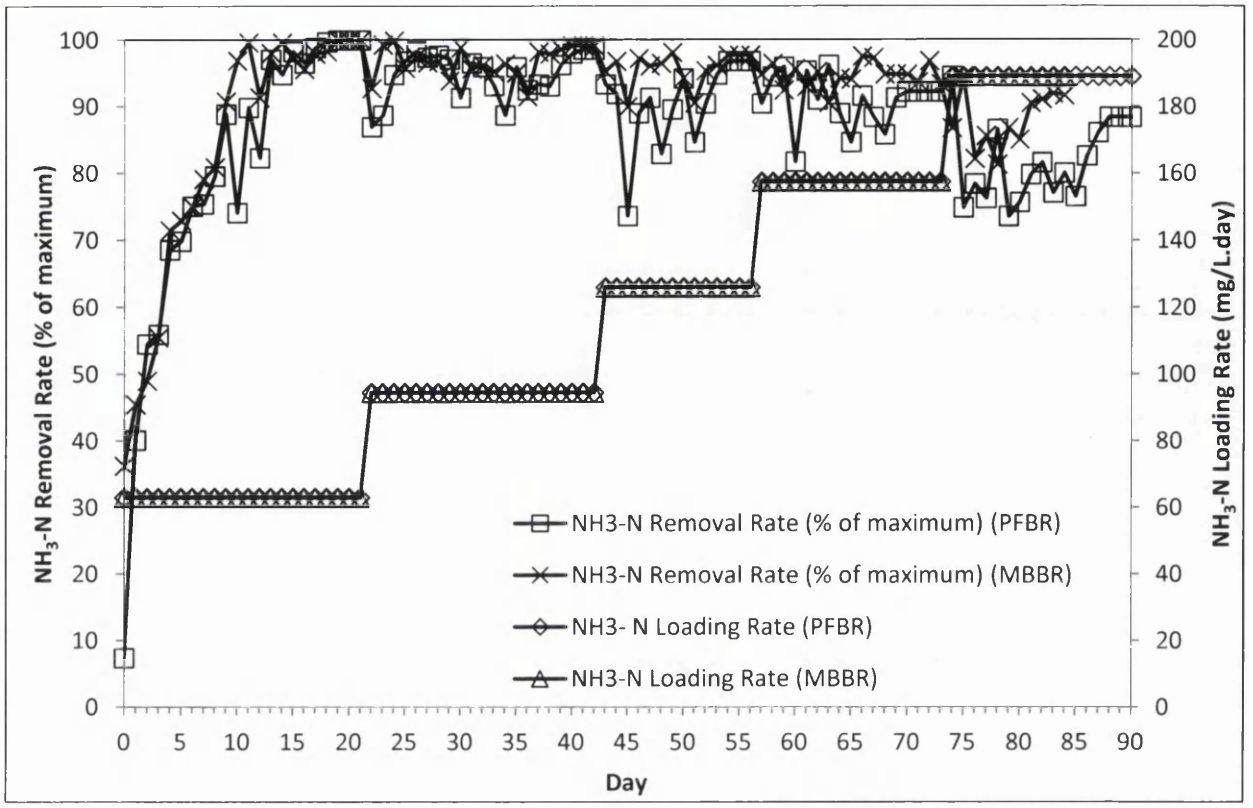


Figure 6.4: The comparison between the percentage removal rate of ammonia-nitrogen at different nitrification loading rate in PFBR and MBBR.

Figure 6.5 shows the comparison between the removal of 17α – ethynylestradiol (EE2) with the 17α – ethynylestradiol (EE2) loading rate in the PFBR and MBBR. After period of start where the films were developed the degradation rate increase rapidly with the MBBR more quickly than the PFBR. Once developed the degradation rates were substantial through whole period of the operation of both reactors. The profiles of the effluent were more erratic for the PFBR than the MBBR.

For the conclusion, the degradation of the 17α – ethynylestradiol (EE2) was better in the MBBR system by virtue of the higher removal rates. From the results retrieved, the MBBR is proven to be more preferred polishing treatment system rather than PFBR when the optimum conditions for the growth of nitrifying bacteria are fulfilled. This may be attributed to the abrading action on the biofilms observed in MBBR compared to the PFBR, so allowing a slightly healthier more productive biofilm (Mudliar *et al.*, 2008; Azizi *et al.*, 2013).

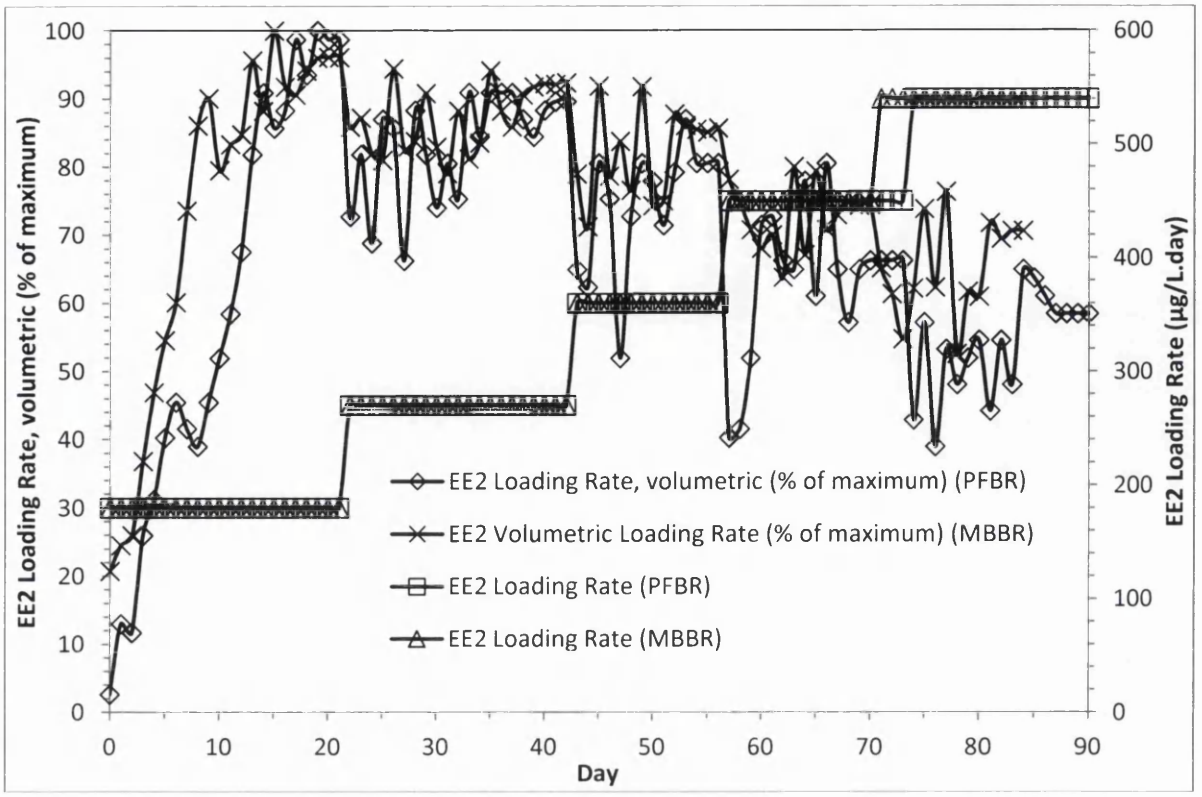


Figure 6.5: The comparison between the percentage removal of 17α – ethynylestradiol (EE2) applied to 17α – ethynylestradiol (EE2) loading rate in PFBR and MBBR.

Figure 6.6 shows the comparison between the percentage removal of mestranol (MeEE2) against the mestranol (MeEE2) loading rate in PFBR and MBBR. The removal rates of mestranol were high in two systems of PFBR and MBBR and large follow similar trends to that of estrogen detailed above. Again both systems could achieve substantial degradation rate for prolonged periods with some loss of performance at high dilution rates.

However from this results investigation showed that the MBBR continuous reactor performance was more suitable tertiary treatment for the reduction of mestranol (MeEE2) in the wastewater compared to the PFBR.

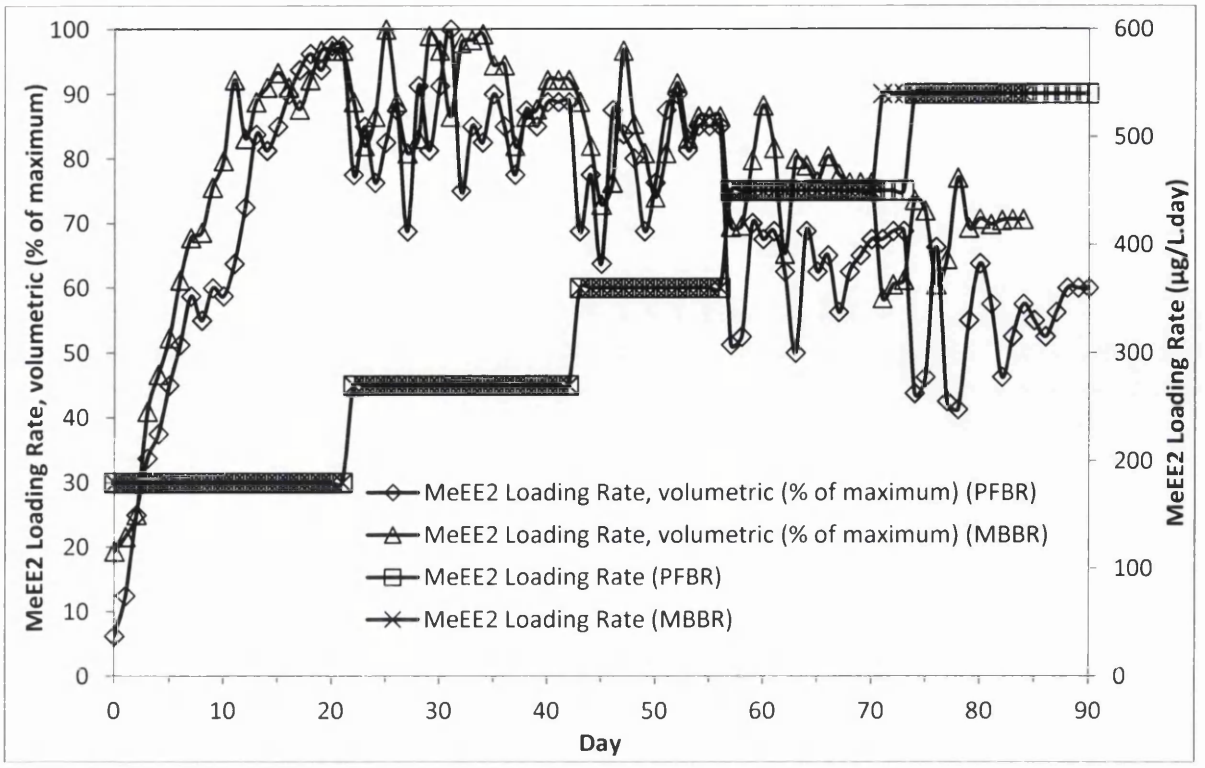


Figure 6.6: The comparison between the percentage removals of mestranol (MeEE2) applied to mestranol (MeEE2) loading rate in PFBR and MBBR.

6.3 Future work

As the objective of the PhD research study has been fulfilled a sound platform for further work has been set. Several areas for further work can be investigated. A deep study of biofilm in the K2 AnoxKaldnes packing materials can be develop using a molecular techniques to estimate the biofilm thickness and the population dynamics of nitrifying bacteria in the PFBR and MBBR systems. This is to understand more clearly the bacterial film attached on the packing materials itself and how this develops.

The data obtained here can be used as a basis to validate kinetic models of the processes involved both for nitrification and the utilization and degradation of contraceptive hormones. Further work on the degradation of the contraceptives could also be informative to help deduce important degradative capacities of nitrifying enrichment populations and how this may enhance contraceptive hormone degradation.

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APPENDIX A

Gram Staining Procedure: Hucker Staining Method (Reddy *et al.*, 2007)

Three reagents were prepared as follows;

Solution A; Crystal violet (certified 90% dry content) 2.0 g, Ethanol, 95% (vol/vol) 20 mL.

Solution B; Ammonium oxalate 0.8 g, distilled water 80 mL.

A and B were mixed together to obtain the crystal violet staining reagent. This was then stored for 24 h, then the solution was filtered (Whatman No.1) and stored until use.

Mordant; Iodine1.0 g, Potassium iodide 2.0 g, distilled water 300 mL.

The mordent was prepared by grinding the iodine and potassium iodide in a mortar, and water was added slowly with continuous grinding until the iodine is dissolved. The mordant is stored in amber bottles until use.

Decolorizing Solvent; Ethanol, 95% (vol/vol).

Counterstain; Safranin O was used as a red counterstain. This contained 0.25g in 10 mL of 95% (vol/vol) ethanol and this was added to 100 mL distilled water.

Procedure:

The sample was prepared by heat fixing the sample on a cleaned glass slide. The slide was then placed in staining rack and the smear of bacteria culture was flooded with the crystal violet staining reagent for 1 min. The smear was then washed in a gentle and indirect stream of tap water for 2 seconds. After this process, the smear was soaked with iodine mordant for 1 min. The same process of washing for 2 seconds is applied and later the slide was dried with absorbent paper. The smear was then flooded with 95% ethanol for 30 seconds with agitation and washed with water. The smear were then soaked with safranin counterstain for 10 seconds and being washed again with a gentle and indirect stream of tap water until no colour appears in the effluent. The slide were then dried with absorbent paper and put under the light microscope by a low – magnification (x 400) for observation.

APPENDIX B

Figure B1 shows the calibration curve for protein of bovine serum albumin (BSA).

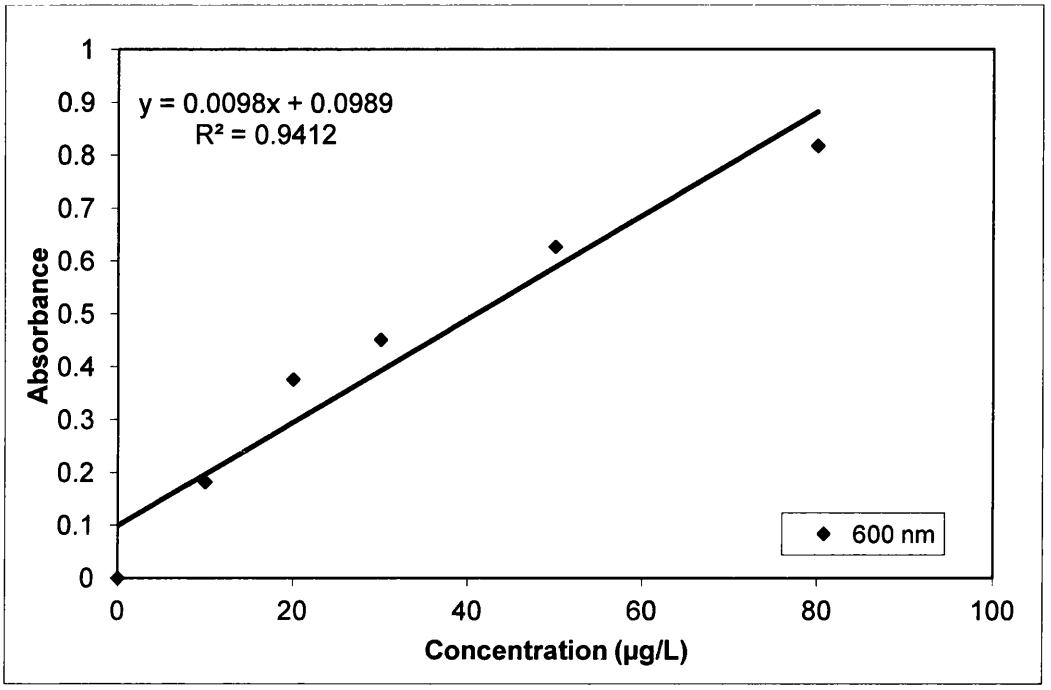

Figure B1: Calibration curve for protein of bovine serum albumin (BSA)

APPENDIX C

Detection of ammonium – nitrogen (NH_4– N)

Colorimetric ammonium - nitrogen analysis (Standard Method, 2005; Alef and Nannipieri, 1995)

Principle of the method:

Ammonium reacts with salicylate and hypochlorite in a buffered alkaline solution in the presence of sodium nitroprusside to form the salicylic acid analogue of indophenol blue. The blue – green colour produced is measured at 660 nm. A complexing agent is added to remove interfering polyvalent cations.

Reagents:

The colourizing reagent was prepared as follows; 34 g sodium salicylate, 25 g sodium citrate and 25 g sodium tartrate were dissolved in 750 mL distilled water, then 0.12 g sodium nitroprusside, was added and dissolved and the final solution was made up to 1000 mL.

Alkaline hypochlorite solution was prepared by dissolved of 30 g sodium hydroxide in 750 mL distilled water. 10 mL sodium hypochlorite solution was added (> 5% available chloride) and the final solution was made up to 1000 mL.

Calibration:

The quantity of 4.719 g dry ammonium sulphate was dissolved in 400 mL distilled water in a 1000 mL volumetric flask and the solution is made up to volume (= 1000 mg/L stock solution). From the stock solutions, the working standards of 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 mg NH_4 – N/L were prepared and used for the calibration. Figure C1 shows the calibration graph for ammonium – nitrogen.

Procedure:

0.1 mL aliquots of sample or standard solution were transferred to test tubes. The amount of 5.0 mL of colour reagent were added, mixed well and then allowed to stand for 15 min. After that, 5.0 mL of alkaline hypochlorite solution were then added to the test tubes and mixed well. The colour was then allowed to develop for 1 h before the absorbance was read at wavelength of 660 nm.

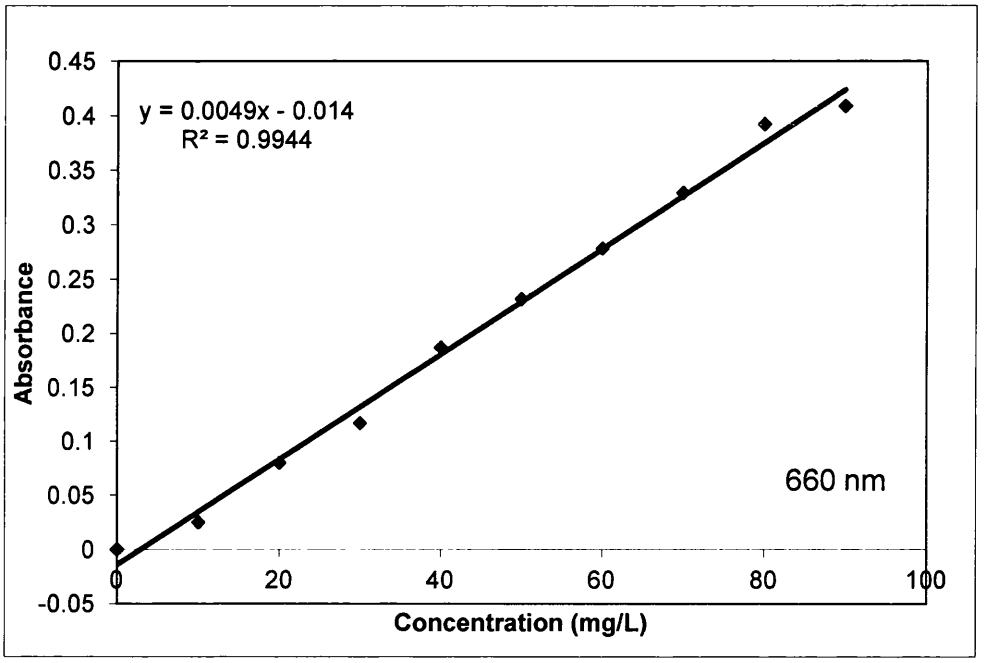

Figure C1: Calibration graph of ammonium – nitrogen ($NH_4 - N$) (slope = 0.004 and intercept = - 0.014)

APPENDIX D

Detection of nitrate – nitrogen (NO_3- N)

Colorimetric nitrate analysis (Standard Method, 2005; Alef and Nannipieri, 1995)

Principle of the method:

The Griess – Ilosvay method for the determination of nitrate involves the reduction to nitrite by cadmium and subsequent staining by the formation of an azo dye. As the method includes nitrite that was present initially, an extra nitrite determination without Cd reduction has to be run if significant amount of NO_2^- are expected; the nitrate concentration was then calculated by the difference between the two determinations.

Material and apparatus:

A glass column, 1 cm in diameter, 30 cm length, fitted with a glass frit and Teflon stopcock at the outlet, and a liquid reservoir (>75 cm^3) on the top. The outlet can be connected to a 100 mL volumetric flask via flexible tubing and a glass tube, which penetrates a two – hole rubber stopper. A vacuum source and regulating valve were connected similarly. Figure D1 shows the cadmium reduction column for nitrate – nitrogen determination.

Chemicals and solutions:

The preparation for copperized cadmium (Cd) reagent was using 25 g of coarse – powdered or granular Cd metal which was pre-treated with 6 M HCl for 1 min; the acid was then decanted and the residue was washed twice with distilled water. Two successive treatments with 2% w/v $CuSO_4.5H_2O$ are followed, with distilled water washings before and, thoroughly, after the second treatment. When the entire blue and light grey colour had disappeared from the washing water, the reagent was then filled into the glass column.

Ammonium chloride – EDTA solution; 13 g NH_4Cl and 1.7 g disodium ethylenediamine tetra acetate are dissolved in 900 mL distilled water and the pH was adjust to 8.5 with concentrated NH_4OH and the solution was made up to 1 L with de-ionized water.

Dilute ammonium chloride – EDTA solution; 300 mL of NH_4Cl – EDTA solution was diluted to 500 mL with de-ionized water.

The diazotizing reagent was prepared by the dissolved of 0.5 g sulphanilamide in 100 mL 2.4 M HCl and stored at 4°C.

0.3 g of (N – (1 – naphthyl – ethylenediamine hydrochloride) was dissolved in 100 mL 0.12 M HCl and stored in an amber bottle at 4°C for the coupling reagent solution.

Calibration:

The nitrate stock standard solution (1000 mg/L) was prepared with 7.218 g dry potassium nitrate (analytical grade) was dissolved in 600 mL distilled water and then were diluted to 1000 mL with de-ionized water and the solution was mixed well.

For the nitrate working standard solutions, the amounts of 0, 0.2, 0.4, 0.6, 1 and 2 mL of the stock standard solution were inserted into six quantity of 100 mL volumetric flasks and the volume were made up with distilled water. The resulting standard concentrations were then; 0, 2, 4, 6, 10 and 20 mg NO_3 - N/L.

Procedure:

The first step was to fill the glass column with diluted NH_4Cl– EDTA to one third of its height followed by Cd granules up to a height of 20 cm. The air bubbles were monitored as their existence was undesirable because of oxidation effects. The column was then washed with diluted NH_4Cl - EDTA by tenfold the pore volume of the Cd column at a flow rate of 8 mL/min.

1 mL concentrated NH_4Cl – EDTA was added before the 25 mL of sample was poured on the top of the Cd column. 75 mL dilute NH_4Cl – EDTA was then passed through the column at the flow rate of 110 mL/min until the top of the column was reached. The first 25 mL of sample effluent was discarded with the remaining then collected in a flask attached to the rubber stopper. The same procedure was carried out for other samples without washing the column between each sample. The column was then washed with diluted NH_4Cl – EDTA solution after finished. The column was then stored in the same solution until further use.

2 mL of the diazotizing solution was added to the contents of the volumetric flask and the same amount of 2 mL of the coupling reagent was added to the flask after 5 minutes. The volume was made up to 100 mL with de-ionized water. The flask was mixed well and was allowed to stand for 20 minutes. The absorbance was then read at 540 nm wavelength against a reagent blank (de-ionized water). The working standard was treated in the same way.

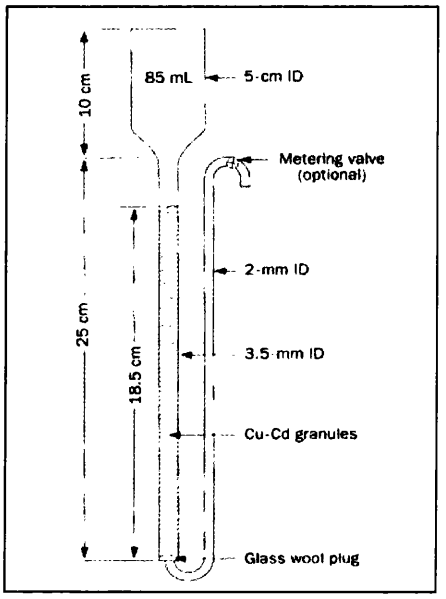

Figure D1: Cadmium Column for nitrate - nitrogen analysis (Standard Methods, 2005)

Colorimetric determination of nitrate – nitrogen (Alef and Nannipieri, 1995)

This method was use to compare with those obtained with the results from cadmium column analysis for nitrate – nitrogen determination.

Principle of the method:

Principle of the method is that salicylic acid is nitrated in an alkaline solution and measured with light absorbance at 410 nm wavelength.

Chemicals and solutions:

4 M solution of sodium hydroxide (NaOH) was prepared by a quantity of 160 g NaOH was dissolved in 600 mL de-ionized water and the solution is made up to 1000 mL and mixed well.

5% of salicylic acid solution was made by the dissolved of 5 g of salicylic acid in 95 mL concentrated sulphuric acid (H_2SO_4) (95 – 97%).

Calibration:

Nitrate stock solution (1000 mg NO_3 – N/L) was made by dissolved of 7.223 g dry potassium nitrate in de-ionized water and the volume was made up to 1000 mL in a volumetric flask. The amounts of 0, 1, 2, 3, 4 and 5 mL of the stock solution were inserted to 6 quantity of 100 mL volumetric flasks and made up to volume to give the concentrations of 0, 10, 20, 30, 40 and 50 mg NO_3 – N/ L working standards. Figure D2 shows the calibration graph of nitrate – nitrogen.

Procedure:

The amount of 500 µL of each standards and samples were inserted into different test tubes. 1 mL of salicylic acid solution was added inside each test tube and the solutions were mixed well immediately and allowed to stand for 30 minutes. Another 10 mL of 4 M NaOH was added in each test tube and the solutions were mixed well and are allowed to stand for 1 hour for colour development. The absorbances were read at 410 nm wavelength on a spectrophotometer.

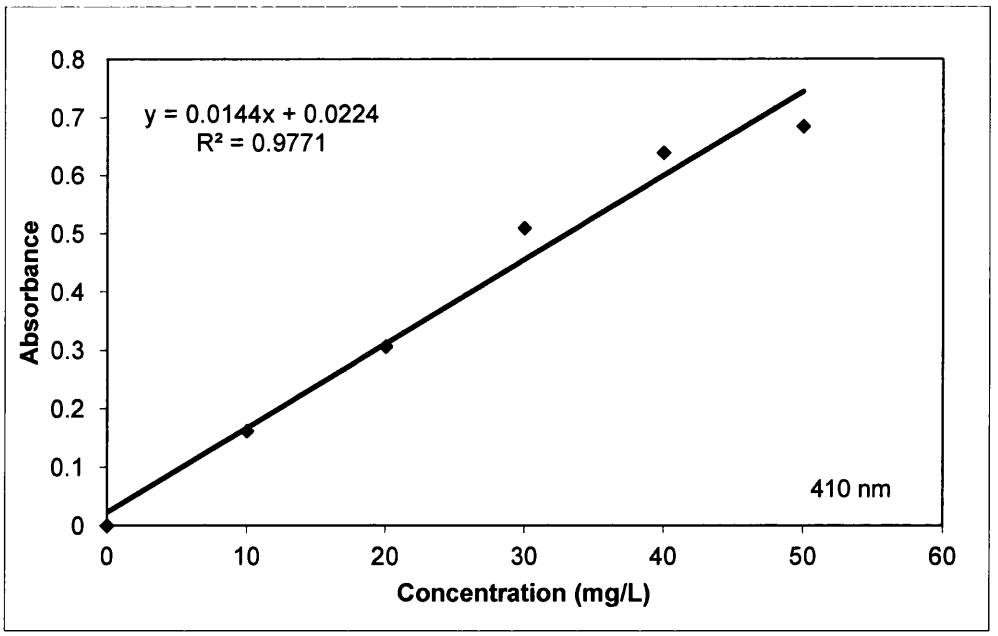

Figure D2: Calibration graph of nitrate – nitrogen (slope = 0.014 and intercept=0.022)

APPENDIX E

Detection of nitrite - nitrogen (NO_2- N)

Colorimetric determination of nitrite - nitrogen (Standard Methods, 2005; Alef and Nannipieri, 1995)

This method is analogous to colorimetric nitrate determination by modified Griess – Ilosvay procedure except that the reduction step is omitted.

Principle of the method:

Nitrite in soil extracts forms a diazonium salt with sulphanilamide, a primary aromatic amine in acidic solution. The absorbance at 540 nm is measured after coupling with N – (1 – naphthyl) – ethylenediamine.

Chemicals and solutions:

The diazotizing reagent was prepared with 0.5 g sulphanilamide are dissolved in 100 mL 2.4 M HCl and stored at 4°C.

The coupling reagent was prepared with 0.3 g of (N – (1 – naphthyl) – ethylenediaminehydrochloride) are dissolved in 100 mL 0.12 M HCl and stored in an amber bottle at 4°C.

Calibration:

The nitrite stock standard solution (1000 mg NO_2-N/L); 4.925 g dry sodium nitrite was dissolved in 600 mL distilled water and the solution was made up to 1000 mL and mixed well. The solution was stable for at least 6 months if stored in refrigerator.

The nitrite intermediate standard solution (10 mg NO_2-N); 10 mL of the stock standard solution was diluted to 1000 mL with distilled water and the solution is mixed well.

The nitrite working standard solution; Amounts of 0, 1, 2, 3, 4 and 5 mL were put into six quantity of 50 mL volumetric flasks and each flasks were diluted with 45 mL distilled water to make the final volume of 50 mL. The final concentration was then; 0, 0.2, 0.4,

0.6, 0.8, 1.0 mg NO_2 – N /L respectively. Figure E1 shows the calibration graph of nitrite – nitrogen.

Procedure:

2 mL of sample with 45 mL distilled water were poured in a 50 mL volumetric flask. 1 mL of sulphanilamide solution was added to the flask and the content was allowed to mix and stand for 5 minutes. 1 mL of the coupling reagent was then added to the flask and the sample was mixed well and allowed to stand for 20 minutes for the colour development. The flask final volume was made up to 50 mL. The result is read with the absorbance at 540 nm wavelength against the reagent blank (de-ionized water). The working standard solutions were treated in the same way as the procedure mention. The nitrite concentration in extracts remains stable for several weeks at 4°C (Keeney and Nelson, 1982).

Most cations and anions, and extracted organic matter do not interfere with the colorimetric determination, while Hg^{2+} and Cu^{2+} lead to higher and lower results, respectively, due to the decomposition of the diazonium salt in the latter case. The series of analyses include a control to correct for the colour of solutions.

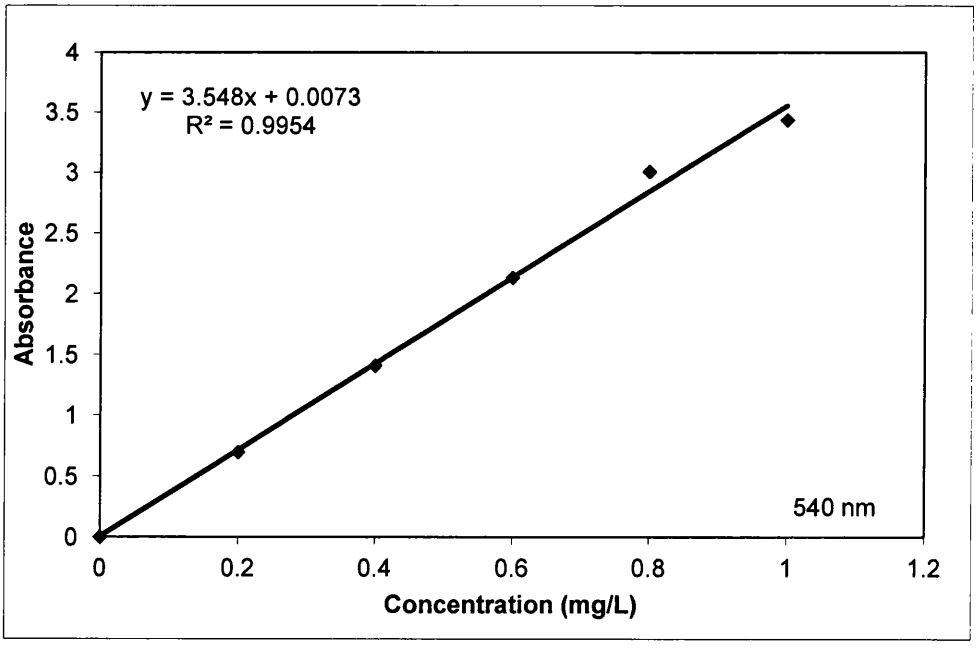

Figure E1: Calibration graph of nitrite – nitrogen (slope = 3.548 and intercept = 0.007)

APPENDIX F

Procedure for Ion Chromatography

Chemical and Solutions:

The preparation of eluent solution was as follows; 2 mL of sodium bicarbonate ($NaHCO_3$) (Dionex) 0.5 M and 16 mL of sodium carbonate (Na_2CO_3) (Dionex) 0.5 M were added into 800 mL de-ionized water in 1 L volumetric flask. The volume of volumetric flask was made up until 1 L with de-ionized water.

The regeneration solution was made using 12.5 mL of sulphuric acid (H_2SO_4) (J.T. Baker) 2.5 M added into 800 mL de-ionized water in 1 L volumetric flask. The volume of volumetric flask was made up to 1 L with de-ionized water.

Calibration:

Three 100 mL of volumetric flask were washed with de-ionized water. 1 mL of Seven Anion Standard II was added to 100 mL volumetric flask and the volume was filled with the de-ionized water until 100 mL to give Standard 1.

2 mL of Seven Anion Standard II is added into the second volumetric flask and the same amount of volume was filled with de-ionized water to 100 mL, to give Standard 2.

4 mL of Seven Anion Standard II was added into the third volumetric flask and the same procedure was repeated as before to give Standard 3.

All the three standards were run and processes by the Chromeleon software. The results of different concentrations of the seven anions are shown in page layout of Chromeleon software.

Samples preparation:

The preparation of the samples was as follows; samples were collected in high density polyethylene containers that have been thoroughly cleaned with de-ionized water (DI). Strong acids or detergents were not use because these will leave traces of ions on the container walls. These ions may interfere with the analysis.

For the samples that are not analysed on the day, the samples is filtered through 0.45 μm filters immediately after collection; otherwise, the bacteria in the samples may cause the ionic concentration to change over time. The samples are refrigerated at 4°C (39°F) to suppress bacterial growth.

The samples containing nitrite or sulphite were run as soon as possible as nitrite oxidizes to nitrate, and sulphite to sulphate, thus increasing the measured concentrations of these ions in the sample. Samples that do not contain nitrite and sulphate can be refrigerated for at least one week with no significant changes in anion concentrations.

For the samples with high concentrations of anions a dilution step was done with the use of eluent or de-ionized water (18.2 megaohm) to dilute the sample. When using carbonate eluent, diluting with eluent minimizes the effect of the "water dip" at the beginning of the chromatograph. For the dilution of samples with eluent, the same lot of eluent were used to prepare for the calibration standards. This is most important for fluoride and chloride, which the elute is neared the water dip.

Procedure:

Liquid flows through the ion chromatograph ICS – 90 along the following flow path which the eluent from the eluent reservoir is pressurized by the gas, forced into the pump, and passes through the pressure transducer. From there, it is pushed through a pulse damper/restrictor assembly, which smooth minor pressure variations from the high – speed pump to minimize baseline noise. The eluent then flows into the injection valve. When sample was loaded into the sample loop, the injection valve toggles to the inject position. This combines the injected sample with the eluent and pushes it through the sample loop.

The eluent/sample mixture was pumped through the guard and separator columns, where the ions are separated by the ion exchange process (that is, different sample ions migrate through the columns at different rates, depending upon their interactions with the ion exchange sites).The eluent/sample mixture flows through the suppressor. There, detection sensitivity was enhanced by suppressing the conductivity of the eluent and enhancing the conductivity of the analyte. Regenerant flows continuously through the suppressor, restoring the ion exchange sites to their original state.

The eluent/sample mixture then flows through the DS5, where the analytes were detected and a signal was produced and sent to Chromeleon software. Finally, the eluent flows out of the cell and is directed into the regenerant reservoir, where it pressurizes the regenerant and forces it into the suppressor. Figure F1 shows the flow path through the ICS – 90 Ion Chromatograph System.

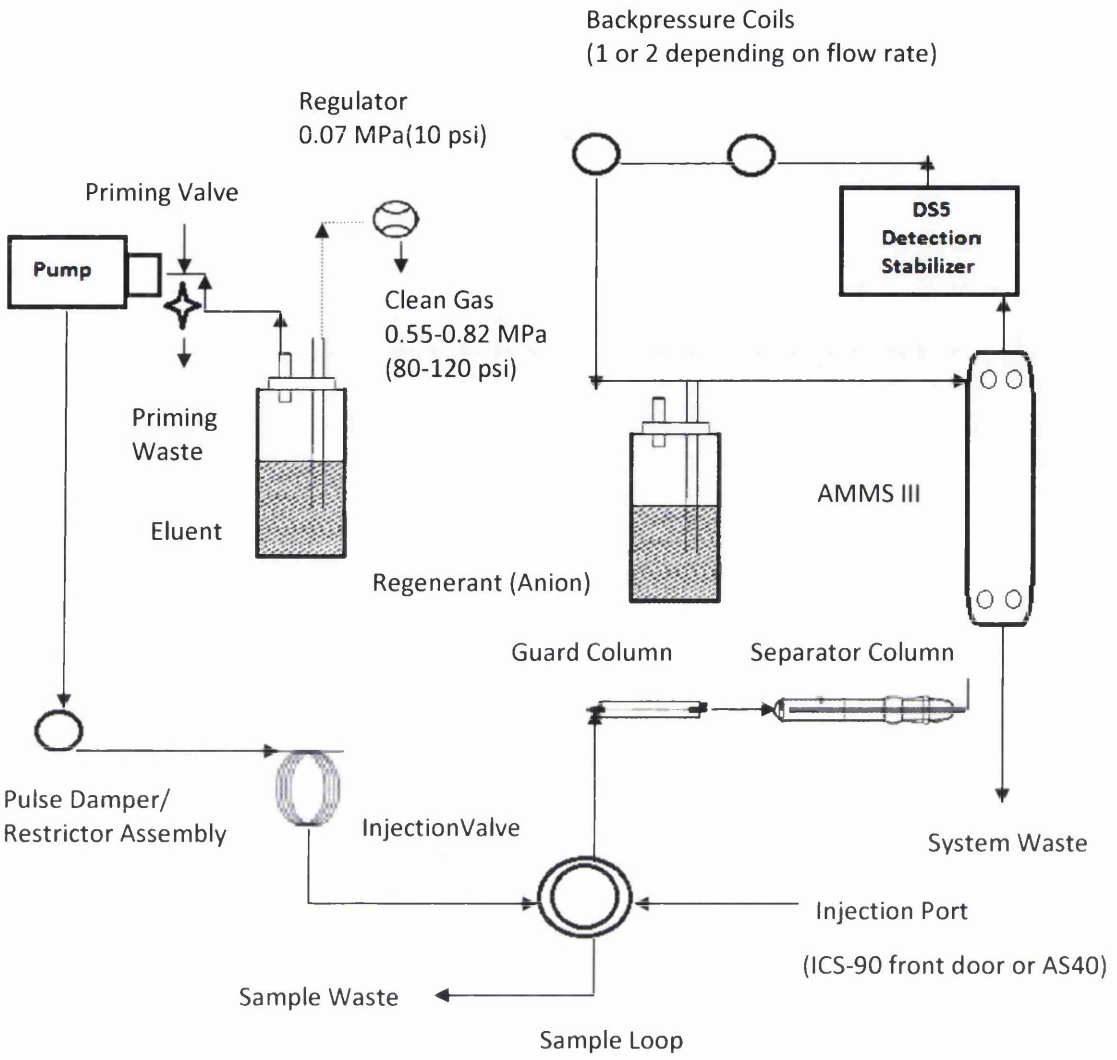

Figure F1: Procedure diagram of ion chromatograph, Dionex (ICS-90).

Table F1: Concentrations of ions and area for Standard 1 Ion Chromatograph

Sample Name:	**std1**	*Inj. Vol.:*	**20.0**
Sample Type:	**standard**	*Dilution Factor:*	**1.0000**
Program:	**AS14A Production Test Chromatogram**	*Operator:*	**n.a.**
Inj. Date/Time:	**20.09.11 13:20**	*Run Time:*	**17.00**

No.	Time min	Peak Name	Type	Area µS*min	Height µS	Amount ppm
1	3.49	Fluoride	BMB	0.053	0.474	0.1967
2	5.12	Chloride	BMB	0.183	1.263	0.9894
3	6.08	Nitrite	BMB	0.114	0.610	0.9993
4	7.77	Bromide	BMB	0.080	0.332	1.0060
5	8.77	Nitrate	BMB	0.091	0.333	1.0013
6	10.63	Phosphate	BMB	0.117	0.240	1.9482
7	12.81	Sulfate	BMB	0.139	0.289	1.0880
		TOTAL:		0.78	3.54	7.23

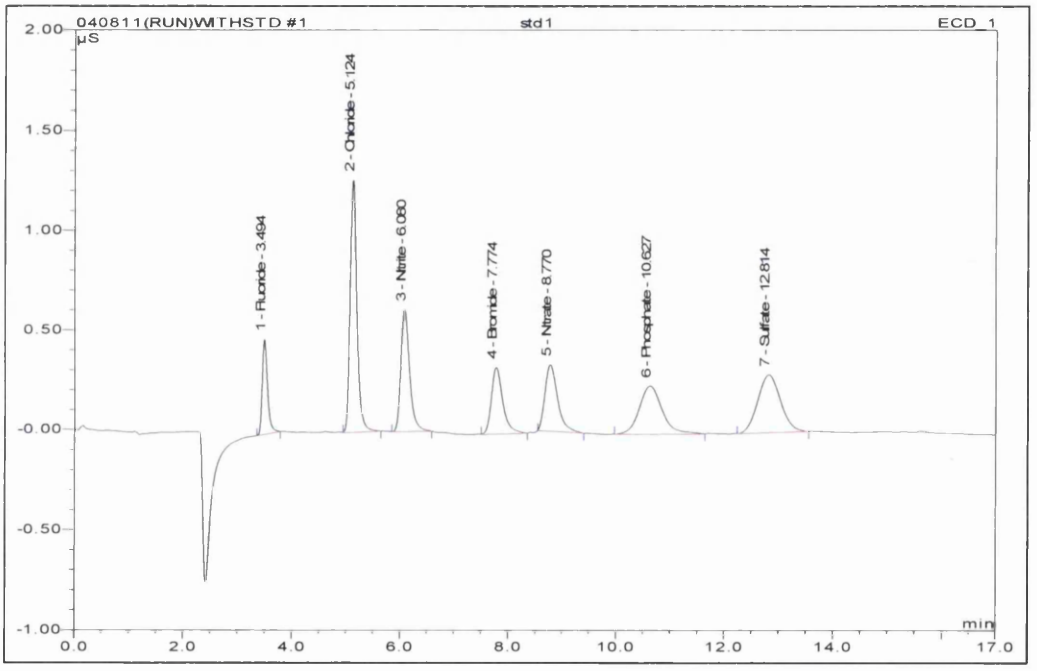

Figure F2: Calibration Graph for Standard 1 Ion Chromatograph.

Table F2: Calibration Graph for Standard 2 Ion Chromatograph

Sample Name:	**std2**	*Inj. Vol.:*	**20.0**
Sample Type:	**standard**	*Dilution Factor:*	**1.0000**
Program:	**AS14A Production Test Chromatogram**	*Operator:*	**n.a.**
Inj. Date/Time:	**20.09.11 13:40**	*Run Time:*	**17.00**

No.	Time min	Peak Name	Type	Area µS*min	Height µS	Amount ppm
1	3.49	Fluoride	BMB	0.083	0.742	0.4050
2	5.13	Chloride	BMB	0.292	1.988	2.0158
3	6.08	Nitrite	BMB	0.179	0.960	2.0011
4	7.78	Bromide	BMB	0.123	0.515	1.9909
5	8.77	Nitrate	BMB	0.146	0.535	1.9980
6	10.62	Phosphate	BMB	0.184	0.382	4.0777
7	12.82	Sulfate	BMB	0.211	0.433	1.8680
		TOTAL:		1.22	5.55	14.36

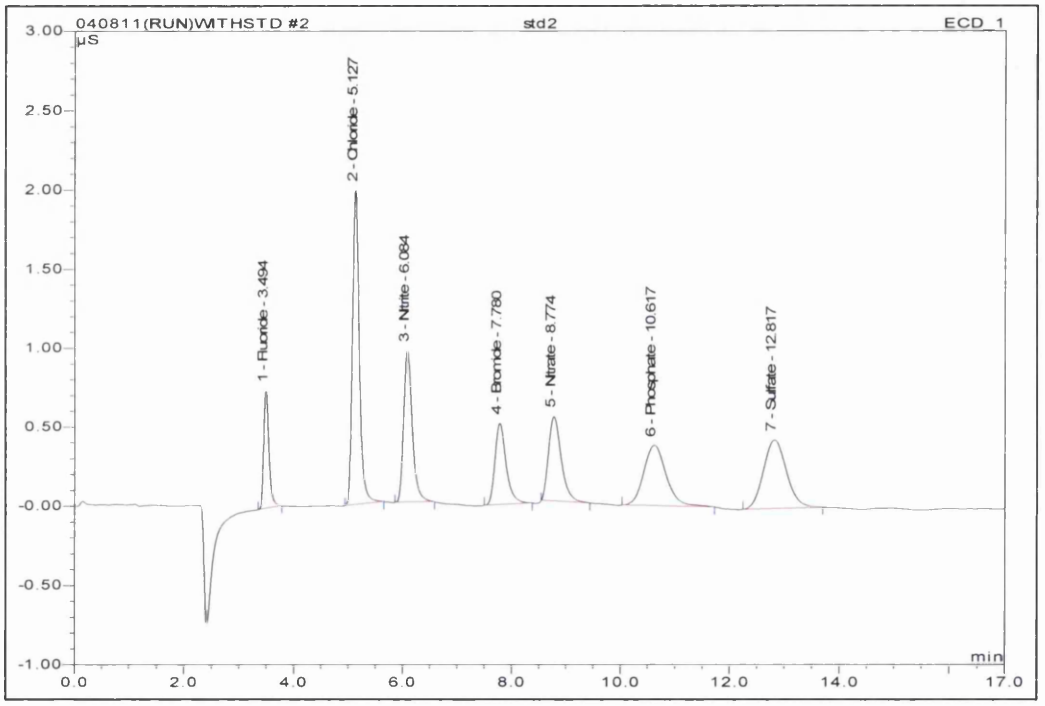

Figure F3: Calibration Graph for Standard 2 Ion Chromatograph.

Table F3: Calibration Graph for Standard 3 Ion Chromatograph

Sample Name:	**std3**	*Inj. Vol.:*	**20.0**
Sample Type:	**standard**	*Dilution Factor:*	**1.0000**
Program:	**AS14A Production Test Chromatogram**	*Operator:*	**n.a.**
Inj. Date/Time:	**20.09.11 13:59**	*Run Time:*	**17.00**

No.	Time min	Peak Name	Type	Area µS*min	Height µS	Amount ppm
1	3.50	Fluoride	BMB	0.140	1.255	0.7983
2	5.13	Chloride	BMB	0.504	3.409	3.9947
3	6.09	Nitrite	BMB	0.310	1.652	3.9996
4	7.79	Bromide	BMB	0.211	0.887	4.0030
5	8.78	Nitrate	BMB	0.257	0.934	4.0007
6	10.63	Phosphate	BMB	0.306	0.645	7.9741
7	12.82	Sulfate	BMB	0.413	0.800	4.0440
		TOTAL:		2.14	9.58	28.81

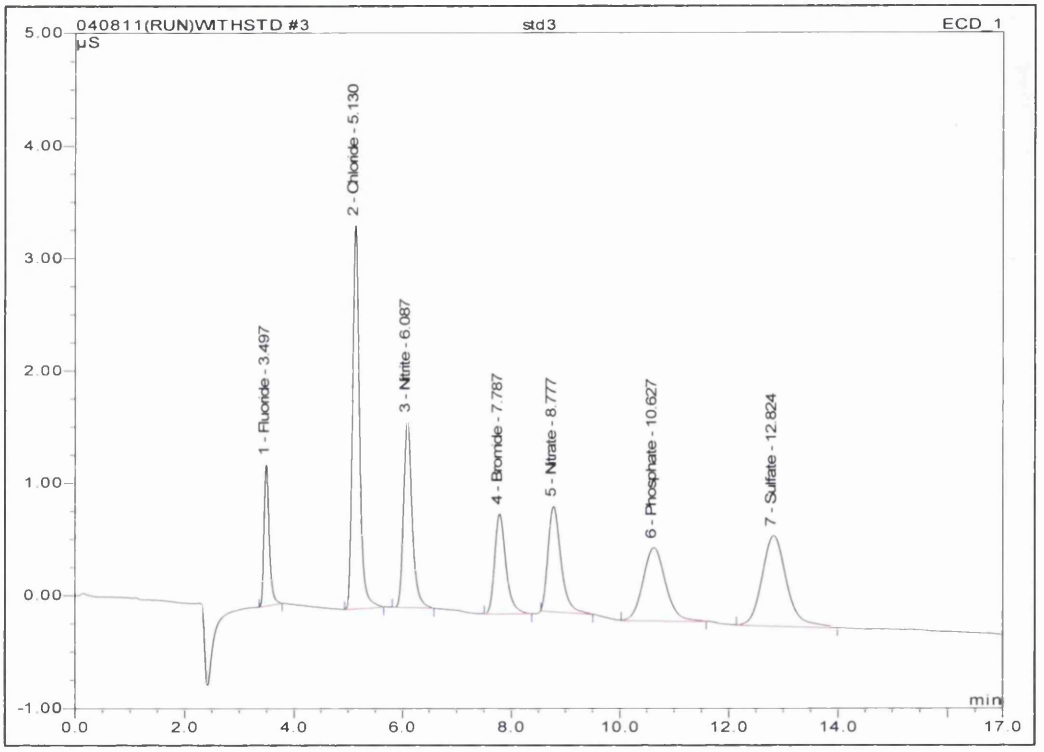

Figure F4: Calibration Graph for Standard 3 Ion Chromatograph.

APPENDIX G

Gas Chromatograph – Mass Spectrometry (GC – MS)

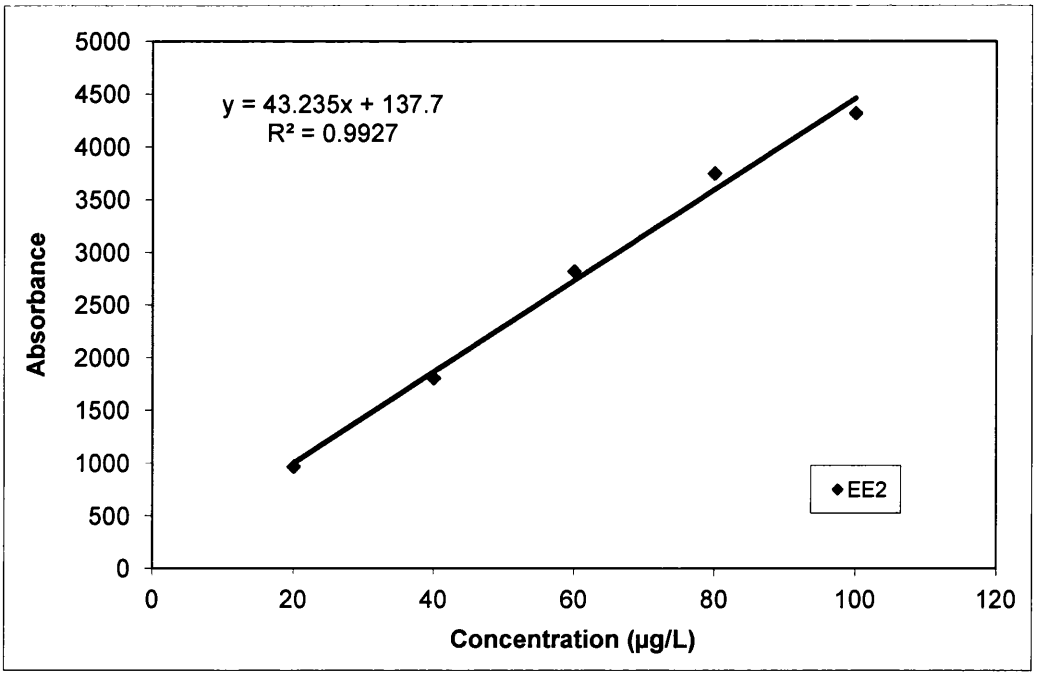

Figure G1: Calibration curve for 17 α – ethynylestradiol (slope = 43.23 and intercept = 137.7)

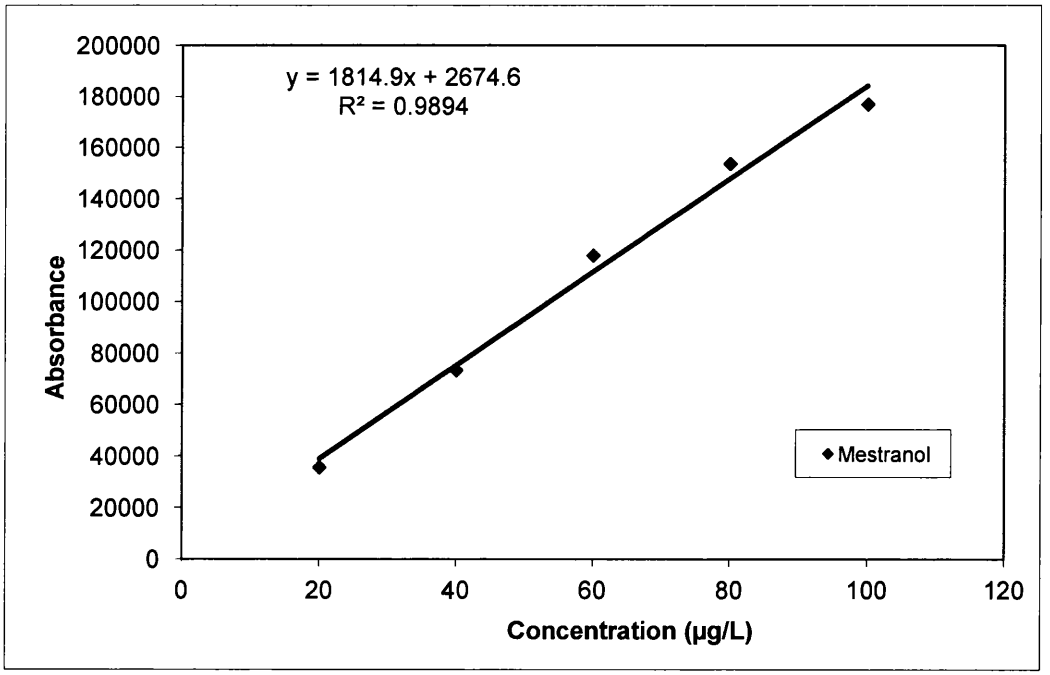

Figure G2: Calibration curve for mestranol (slope = 1814 and intercept = 2674)

APPENDIX H

Table H1: The results of nitrate - nitrogen accumulation in various temperatures for K1 AnoxKaldnes packing materials for concentration of 30 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	NO3 - N Production Rate (mg/L. day)	%
Temperature (° C)	Concentration of NO3 – N (mg/L)								
0	0.1	0	0	0	0	0	0	0.005	0.112
4	0.1	0.5	0.8	1	1.2	1.5	2.2	0.348	8.156
15	0.1	0.8	1.4	2.4	3.8	5.9	7.8	1.057	24.804
20	0.1	1.5	2.8	4.7	6.1	8.1	11.6	1.662	38.994
25	0.1	2	3.3	6.3	8.7	10.5	15	2.186	51.285
30	0.1	3.8	8.6	13.3	15.2	17.4	21	3.781	88.715
35	0.1	4.7	9.7	15.2	17.6	19.1	23.1	4.262	100.000
40	0.1	3.1	7.8	11.7	14.8	17	19.8	3.538	83.017
50	0.1	0	0	0	0	0	0	0.005	0.112

Table H2: The results of nitrate -nitrogen accumulation in various temperatures for Flocor packing materials for concentration of 30 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	NO3 - N Production Rate (mg/L. day)	%
Temperature (° C)	Concentration of NO3 – N (mg/L)								
0	0.1	0	0	0	0	0	0	0.005	0.102
4	0.1	0.7	1.2	1.5	2	2.3	3.2	0.524	11.224
15	0.1	1	1.7	2.9	4.3	6.4	8.3	1.176	25.204
20	0.1	2.1	3.5	5.6	7.3	9.2	12.7	1.929	41.327
25	0.1	2.6	4.3	7.6	9.5	11.8	16.7	2.505	53.673
30	0.1	4.6	9.6	14.8	16.7	18.3	23.8	4.186	89.694
35	0.1	5.2	11.1	16.8	19.8	20.4	24.6	4.667	100.000
40	0.1	3.9	8.2	13.5	15.2	19.5	22.4	3.943	84.490
50	0.1	0	0	0	0	0	0	0.005	0.102

Table H3: The results of nitrate - nitrogen accumulation in various temperatures for K2 AnoxKaldnes packing materials for concentration of 30 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	NO3 - N Production Rate (mg/L. day)	%
Temperature (° C)	Concentration of NO3 – N (mg/L)								
0	0.1	0	0	0	0	0	0	0.005	0.093
4	0.1	0.83	1.5	2	2.3	2.5	3.6	0.611	11.968
15	0.1	1.4	1.9	3.1	4.8	7.1	9.2	1.314	25.746
20	0.1	2.7	4.6	6.1	8.6	10.3	13.2	2.171	42.537
25	0.1	3.7	5.6	8.7	10.1	12.9	17.9	2.810	55.037
30	0.1	5.6	10.7	15.9	17.8	19.7	24.9	4.510	88.340
35	0.1	6.1	12.4	17.9	21.8	22.7	26.2	5.105	100.000
40	0.1	4.2	9.7	14.5	16.3	20.4	23.7	4.233	82.929
50	0.1	0	0	0	0	0	0	0.005	0.093

Table H4: The results of nitrate - nitrogen accumulation in various temperatures for without packing material for concentration of 30 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	NO3 - N Production Rate (mg/L. day)	%
Temperature (° C)	Concentration of NO3 – N (mg/L)								
0	0.1	0	0	0	0	0	0	0.005	0.120
4	0.1	0.2	0.6	0.8	1	1.3	2	0.286	7.177
15	0.1	0.6	1.2	2.1	3.2	5.6	6.2	0.905	22.727
20	0.1	1	1.8	4	5.9	7.8	10.6	1.486	37.321
25	0.1	1.5	2.8	5.7	7.6	9.4	14.2	1.967	49.402
30	0.1	2.9	7.8	12.5	14.3	16.4	19.8	3.514	88.278
35	0.1	3.5	8.6	14.2	16.4	18.3	22.5	3.981	100.000
40	0.1	2.7	6.6	10.3	13.5	16.5	17.8	3.214	80.742
50	0.1	0	0	0	0	0	0	0.005	0.120

APPENDIX I:

Table I1: The results of nitrate - nitrogen accumulation in various temperatures for K1 AnoxKaldnes packing material for concentration of 70 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	7	8	9	10	11	NO3 - N Production Rate (mg/L. day)	%
Temperature (° C)	Concentration of NO3 – N (mg/L)													
0	0.8	0	0	0	0	0	0	0	0	0	0	0	0.012	0.201
4	0.8	1	2.3	3.8	4.6	6.5	8.9	10.7	13.2	15.7	18.5	21.6	1.630	27.076
15	0.8	2.6	4.5	6	10	15.3	19.6	23.1	27.6	30.1	35.7	40.1	3.264	54.202
20	0.8	2.8	5.1	7.5	12.1	17.6	21.4	26.2	30.7	34.4	40.5	45.1	3.700	61.449
25	0.8	3	6.2	9.3	15.3	20.1	25.6	30.7	33.8	40.4	46.5	50.7	4.279	71.062
30	0.8	4.4	6	10	19	25	30	35	40	43	55	61.2	4.991	82.889
35	0.8	5.8	8.7	16.3	21	26	37	43	50.1	57	63	68.7	6.021	100.000
40	0.8	3.9	5.2	8.4	13.3	21.1	25	30.3	37.6	40.6	48.9	53.6	4.374	72.647
50	0.8	0	0	0	0	0	0	0	0	0	0	0	0.012	0.201

Table I2: The results of nitrate - nitrogen accumulation in various temperatures for Flocor packing material for concentration of 70 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	7	8	9	10	11	NO3 - N Production Rate (mg/L. day)	%
Temperature (° C)	Concentration of NO3 – N (mg/L)													
0	0.8	0	0	0	0	0	0	0	0	0	0	0	0.012	0.179
4	0.8	2.1	3.4	4.5	6.3	7.8	10.1	12.3	14.7	17.9	20.3	23.6	1.876	27.634
15	0.8	3	5.5	6.8	12.4	17.8	21.5	26.8	29.3	33	37	42	3.574	52.656
20	0.8	4.4	6.7	8.9	14.3	19.5	24.8	26.3	30.7	35.6	41.2	44.3	3.902	57.478
25	0.8	5.1	7.3	11.4	17.8	23.4	30.2	35.6	40.5	44.3	49.7	52	4.820	71.004
30	0.8	6.3	8.5	12.4	21	28	35	42	47	51	62	66	5.758	84.821
35	0.8	7.6	11	19.6	27	33	47	50	56	60	67	69	6.788	100.000
40	0.8	5.5	7.5	10.5	17.4	25.7	32.8	39.6	43.8	49.6	54.2	60.1	5.265	77.567
50	0.8	0	0	0	0	0	0	0	0	0	0	0	0.012	0.179

Table I3: The results of nitrate - nitrogen accumulation in various temperatures for K2 AnoxKaldnes packing material for concentration of 70 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	7	8	9	10	11	NO3 - N Production Rate (mg/L. day)	%
Temperature (° C)	Concentration of NO3 – N (mg/L)													
0	0.8	0	0	0	0	0	0	0	0	0	0	0	0.012	0.167
4	0.8	3.2	4.2	5.4	7.6	8.9	12.1	14.5	17.8	20.1	24.3	27.5	2.218	30.634
15	0.8	4.5	6.7	7.8	14.3	20.4	24.6	30.5	34.6	36	41	44	4.018	55.493
20	0.8	5.2	7.5	10.1	16.8	23.6	29.7	33.4	37.6	43.2	47.8	52.6	4.671	64.511
25	0.8	5.8	8.6	13.8	20.1	27.6	34.8	40.6	46.5	49.8	52.1	55.7	5.397	74.534
30	0.8	7.9	10.3	14.3	25	30.5	40.8	46.5	50.7	54.8	65.7	68.1	6.294	86.922
35	0.8	8.7	13.2	20.8	30	36.8	50.5	54.2	60.4	65.6	68	68.9	7.241	100.000
40	0.8	6.3	8.5	12.8	19.8	27.8	36.5	42.5	46.9	53.5	60.1	64	5.750	79.410
50	0.8	0	0	0	0	0	0	0	0	0	0	0	0.012	0.167

Table I4: The results of nitrate - nitrogen accumulation in various temperatures for without packing material for concentration of 70 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	7	8	9	10	11	NO3 - N Production Rate (mg/L. day)	%
Temperature (° C)	Concentration of NO3 – N (mg/L)													
0	0.8	0	0	0	0	0	0	0	0	0	0	0	0.012	0.224
4	0.8	0.8	2	3	4	6	8	10	13	14	17	20	1.494	27.557
15	0.8	1.8	3.7	5.6	9.5	14.2	18.6	21.1	23.4	26.5	31.3	36.8	2.929	54.025
20	0.8	2	4.3	6.7	11.9	16.5	20	23	28	31	37	40	3.352	61.822
25	0.8	2.8	5.8	8.7	13.4	18.7	23.1	25.7	30.8	37.5	44.3	46.5	3.911	72.135
30	0.8	4	5.6	9	14	21	27	31	36	40	47	53	4.370	80.604
35	0.8	5	8	14	19	23	32	39	45	51	57	64	5.421	100.000
40	0.8	3	5	8	12	20	21	28	32	39	45	50.1	3.998	73.756
50	0.8	0	0	0	0	0	0	0	0	0	0	0	0.012	0.224

APPENDIX J

Table J1: The results of nitrite - nitrogen accumulation in various pH for K1 Anox Kaldnes packing material for concentration of 30 mg/L ammonia – nitrogen

pH	Time (day)							NO2 – N Production Rate (μg/L. day)	%
	0	1	2	3	4	5	6		
	Concentration of NO2 – N (μg/L)								
6	0.5	2	3	6.5	10	15	15	2.476	22.462
6.5	0.5	3.5	4.8	7.3	16	20	20	3.433	31.145
7	0.5	5	7	11	20	35	40	5.643	51.188
7.5	0.5	6.8	11.8	20	34	45	59	8.433	76.501
8	0.5	10	20	34	42	58	67	11.024	100.000
8.5	0.5	8	13	31	38	51	53	9.262	84.017

Table J2: The results of nitrite - nitrogen accumulation in various pH for Flocor packing material for concentration of 30 mg/L ammonia – nitrogen

pH	Time (day)							NO2 – N Production Rate (μg/L. day)	%
	0	1	2	3	4	5	6		
	Concentration of NO2 – N (μg/L)								
6	0.5	1.5	3.4	6	12	13	16	2.495238	20.03824
6.5	0.5	3.7	5.1	8	18	29	29	4.442857	35.67878
7	0.5	5.2	7.3	10	23	38	48	6.285714	50.47801
7.5	0.5	7.1	12	20	28	37	61	7.885714	63.32696
8	0.5	13	27	36	50	66	69	12.45238	100
8.5	0.5	6.8	11.1	20	30	43	52	7.780952	62.48566

Table J3: The results of nitrite - nitrogen accumulation in various pH for K2 Anox Kaldnes packing material for concentration of 30 mg/L ammonia – nitrogen

pH	Time (day)							NO2 – N Production Rate (μg/L. day)	%
	0	1	2	3	4	5	6		
	Concentration of NO2 – N (μg/L)								
6	0.5	2.5	4.5	7	16	18	18	3.167	22.581
6.5	0.5	4	5.9	10	21	32	36	5.210	37.148
7	0.5	5.8	8	16	30	42	54	7.443	53.073
7.5	0.5	7.6	13.4	24	44	53	67	9.976	71.138
8	0.5	19	30	45	58	67	75	14.024	100.000
8.5	0.5	7	11.3	25	38	49	54	8.800	62.750

Table J4: The results of nitrite - nitrogen accumulation in various pH for without packing material for concentration of 30 mg/L ammonia – nitrogen

pH	Time (day)							NO2 – N Production Rate (µg/L. day)	%
	0	1	2	3	4	5	6		
	Concentration of NO2 – N (µg/L)								
6	0.5	1.2	2.3	3.5	8	13	13	1.976	20.333
6.5	0.5	2.8	3.2	4.8	13	17	17	2.776	28.564
7	0.5	4.1	5.3	8	17	30	36	4.805	49.437
7.5	0.5	5.9	9.7	19	28	41	48	7.243	74.522
8	0.5	9.6	18	30	38	50	58	9.719	100.000
8.5	0.5	7	12	28	30	46	50	8.262	85.007

APPENDIX K

Table K1: The results of nitrate - nitrogen accumulation in various pH for K1 Anox Kaldnes packing material for concentration of 30 mg/L ammonia – nitrogen

pH	Time (day)							NO3 – N Production Rate (mg/L. day)	%
	0	1	2	3	4	5	6		
	Concentration of NO3 – N (mg/L)								
6	0	1	1.5	2	3	4	4	0.738	23.846
6.5	0	1.5	2	3.2	4	7.1	7.5	1.205	38.923
7	0	1.8	2.6	5.7	6.2	9.3	9.8	1.686	54.462
7.5	0	2.6	4.8	7.5	8	12	15	2.376	76.769
8	0	3	6	9	10	17	20	3.095	100.000
8.5	0	2.8	5.6	8.1	9.2	10.8	13	2.357	76.154

Table K2: The results of nitrate - nitrogen accumulation in various pH for Flocor packing material for concentration of 30 mg/L ammonia – nitrogen

pH	Time (day)							NO3 – N Production Rate (mg/L. day)	%
	0	1	2	3	4	5	6		
	Concentration of NO3 – N (mg/L)								
6	0	1.5	2	3	4	5	5	0.976	25.625
6.5	0	1.8	3	4	6	7	8	1.419	37.250
7	0	2	4	5	8	9	11	1.857	48.750
7.5	0	2	5	7	13	17	20	3.048	80.000
8	0	2	7	11	17	20	23	3.810	100.000
8.5	0	2	5.6	9	15	16	19	3.171	83.250

Table K3: The results of nitrate - nitrogen accumulation in various pH for K2 Anox Kaldnes packing material for concentration of 30 mg/L ammonia – nitrogen

pH	Time (day)							NO3 – N Production Rate (mg/L. day)	%
	0	1	2	3	4	5	6		
	Concentration of NO3 – N (mg/L)								
6	0	2	3	4	6	6.5	6.5	1.333	32.407
6.5	0	2.2	3.4	5	7	8	8	1.600	38.889
7	0	2.5	4.6	6	9	10	11	2.052	49.884
7.5	0	3.2	5.6	8	15	18	21	3.371	81.944
8	0	3.4	8	12	18	21	24	4.114	100.000
8.5	0	3	5.8	9	13	16	18	3.086	75.000

Table K4: The results of nitrate - nitrogen accumulation in various pH for without packing material for concentration of 30 mg/L ammonia – nitrogen

pH	Time (day)							NO3 – N Production Rate (mg/L. day)	%
	0	1	2	3	4	5	6		
	Concentration of NO3 – N (mg/L)								
6	0	0.5	1	1	2	3	3	0.500	17.500
6.5	0	1	1	2	3	6	6.3	0.919	32.167
7	0	1	2	4	5	7	7	1.238	43.333
7.5	0	2	3	7	7	12	14	2.143	75.000
8	0	3	6	8	9	16	18	2.857	100.000
8.5	0	2	4	7	9	13	14	2.333	81.667

APPENDIX L:

Table L1: The results of nitrite - nitrogen accumulation in various pH for K1 AnoxKaldnes packing material for concentration of 70 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	7	8	9	NO2 - N Production Rate (µg/L. day)	%
pH	Concentration of NO2 – N (µg/L)											
6	5	8	13	17	20	30	40	50	50	50	6.288889	20.21429
6.5	5	11	20	27	35	48	53	60	70	70	8.866667	28.5
7	5	13	23	39	50	70	80	100	109	120	13.53333	43.5
7.5	5	17	30	52	68	118	200	230	250	280	27.77778	89.28571
8	5	25	40	70	90	150	210	240	270	300	31.11111	100
8.5	5	19	26	50	65	100	148	201	240	270	24.97778	80.28571

Table L2: The results of nitrite - nitrogen accumulation in various pH for Flocor packing material for concentration of 70 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	7	8	9	NO2 - N Production Rate (µg/L. day)	%
pH	Concentration of NO2 – N (µg/L)											
6	5	9	12	15	23	34	42	55	60	60	7	19.12568
6.5	5	13	28	37	46	51	60	62	68	69	9.755556	26.65452
7	5	14	38	52	66	73	90	113	120	132	15.62222	42.68367
7.5	5	20	56	71	98	120	187	221	270	279	29.48889	80.57073
8	5	30	72	90	130	180	220	290	310	320	36.6	100
8.5	5	25	52	68	89	111	154	200	250	260	26.97778	73.70978

Table L3: The results of nitrite - nitrogen accumulation in various pH for K2 AnoxKaldnes packing material for concentration of 70 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	7	8	9	NO2 - N Production Rate (µg/L. day)	%
pH	Concentration of NO2 – N (µg/L)											
6	5	11	17	20	25	45	55	64	70	75	8.6	23.00832
6.5	5	18	23	35	40	59	63	72	84	86	10.77778	28.83472
7	5	23	30	44	58	80	100	117	125	138	16	42.80618
7.5	5	42	59	86	102	134	190	255	284	290	32.15556	86.02854
8	5	50	79	100	120	147	236	300	315	330	37.37778	100
8.5	5	43	64	81	90	113	160	201	251	262	28.22222	75.50535

Table L4: The results of nitrite - nitrogen accumulation in various pH for without packing material for concentration of 70 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	7	8	9	NO2 - N Production Rate (µg/L. day)	%
pH	Concentration of NO2 – N (µg/L)											
6	5	6	10	13	18	28	34	42	44	46	5.466667	18.85057
6.5	5	9	16	18	24	38	47	51	56	56	7.111111	24.52107
7	5	11	19	33	42	65	70	78	86	90	11.08889	38.23755
7.5	5	13	28	49	58	100	180	200	230	230	24.28889	83.75479
8	5	19	35	63	93	120	200	230	260	280	29	100
8.5	5	17	23	40	57	90	150	200	212	220	22.53333	77.70115

APPENDIX M

Table M1: The results of nitrate - nitrogen accumulation in various pH for K1 AnoxKaldnes packing material for concentration of 70 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	7	8	9	NO3 - N Production Rate (mg/L. day)	%
pH	Concentration of NO3 – N (mg/L)											
6	0	0.8	1	2	4	6	7	8.5	9	9	1.0511	22.4171
6.5	0	1.5	2.3	6	10	12	14	15	15	15	2.0178	43.0332
7	0	2	4	8	14	20	23	25	28	28	3.3778	72.0379
7.5	0	2.5	5.3	9	16	23	25	30	32	36	3.9733	84.7393
8	0	3	6	10	19	25	30	35	40	43	4.6889	100.0000
8.5	0	2	4.8	8	12	19	20	23	27	30	3.2400	69.0995

Table M2: The results of nitrate - nitrogen accumulation in various pH for Flocor packing material for concentration of 70 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	7	8	9	NO3 - N Production Rate (mg/L. day)	%
pH	Concentration of NO3 – N (mg/L)											
6	0	1	1.5	2.2	4.3	6.7	8	9	10	10	1.171	23.165
6.5	0	2	3	7	11	13	16	17	17	17	2.289	45.275
7	0	2.5	5	9	15	20	23	25	27	27	3.411	67.473
7.5	0	3	6	10	18	22	26	31	33	38	4.156	82.198
8	0	3.5	8	14	20	27	32	37	41	45	5.056	100.000
8.5	0	3.2	7	9	16	20	23	25	30	34	3.716	73.495

Table M3: The results of nitrate - nitrogen accumulation in various pH for K2 AnoxKaldnes packing material for concentration of 70 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	7	8	9	NO3 - N Production Rate (mg/L. day)	%
pH	Concentration of NO3 – N (mg/L)											
6	0	1.4	2	3	5.6	7	9	10	11	11	1.333	24.804
6.5	0	2.4	3.2	8	12	14	17	18	18	18	2.458	45.721
7	0	3	5.4	10	16	23	26	28	28	28	3.720	69.202
7.5	0	3.5	6.4	11	19	26	28	33	38	40	4.553	84.704
8	0	4	8.9	15	21	28	34	38	43	50	5.376	100.000
8.5	0	3.8	7.4	10	18	21	24	27	31	33	3.893	72.427

Table M4: The results of nitrate - nitrogen accumulation in various pH for without packing material for concentration of 70 mg/L ammonia – nitrogen

Time (day)	0	1	2	3	4	5	6	7	8	9	NO3 - N Production Rate (mg/L. day)	%
pH	Concentration of NO3 – N (mg/L)											
6	0	0.5	0.8	1.5	3	6	6.8	8	8	8	0.947	22.362
6.5	0	1	2	5	9	10	13.7	14	14	14	1.838	43.412
7	0	1.5	3	7	12	18	22	23	25	25	3.033	71.654
7.5	0	2	5	8	14	20	25	30	32	34	3.778	89.239
8	0	2.5	5	9	18	23	26	32	35	40	4.233	100.000
8.5	0	1.5	4	8	15	19	20	23	27	29	3.256	76.903

APPENDIX N :

Table N1: The results of 1 L batch culture for K2 AnoxKaldnes packing materials for concentration of 35 mg/L ammonia – nitrogen

Date	Day	Ph	Temperature	Total Dissolved Solids (TDS), ppt	Conductivity, mS	Salinity, ppt	Dissolved oxygen, mg/L	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
3/7/2012	0	8.95	30	9.35	13.31	7.54	5.6	35	2.201	0.00
4/7/2012	1	7.985	30	9.33	13.27	7.54	5.5	30	37.280	1.00
5/7/2012	2	7.765	30	9.045	12.765	7.265	5.315	28	38.031	3.00
6/7/2012	3	7.7	30		13		5.315	20	40.787	5.00
7/7/2012	4	7.7	30		13		5.315	18	42.291	9.00
8/7/2012	5	7.7	30		13		5.315	15	43.544	12.00
9/7/2012	6	7.735	30	9.45	13.475	7.78	5.115	12	47.051	12.30
10/7/2012	7	7.69	30	9.58	13.525	7.77	5.455	13	48.054	13.00
7/11/2012	8	7.5	30		13.8		5.315	10	48.555	14.00
7/12/2012	9	7.47	30	OR	14.025	8.285	5.4	9	52.814	16.00
7/13/2012	10	7.495	30	OR	15.225	8.815	5.7	8	55.069	18.45
7/14/2012	11	7.445	30	OR	15.385	8.955	5.4	7	15.732	19.21
7/15/2012	12	7.8	30		16		5.315	6	8.716	23.37
7/16/2012	13	7.77	30	OR	16.4	9.57	5.4	5	8.465	26.83
7/17/2012	14	7.815	30	OR	17.36	OR	5.4	4.8	6.962	28.56
7/18/2012	15	8	30	OR	17.91	OR	5.4	3.2	5.459	35.48
7/19/2012	16	7.83	30	OR	19.68	OR	5.4	2.6	5.208	33.20
7/20/2012	17	7.88	30	OR	OR	OR	5.4	1.5	2.201	33.13
7/21/2012	18	7.885	30	OR	OR	OR	5.4	1.2	2.201	33.47

Table N2: The results of 1 L batch culture for K2 AnoxKaldnes packing materials for concentration of 65 mg/L ammonia – nitrogen

Date	Day	Ph	Temperature	Total Dissolved Solids (TDS), ppt	Conductivity, mS	Salinity, ppt	Dissolved oxygen, mg/L	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
12/7/2012	0	8	30				6.28	65.000	16.483	0.000
7/13/2012	1	7.55	30	9.845	13.73	8.015	6.28	50.126	29.262	1.000
7/14/2012	2	7.46	30	9.185	12.975	7.455	6.91	50.126	16.734	3.000
7/15/2012	3	8	30				6.91	44.373	20.743	7.000
7/16/2012	4	7.715	30	9.33	13.14	7.525	6.91	40.264	23.248	11.000
7/17/2012	5	7.83	30	9.335	13.08	7.55	6.91	34.100	34.273	13.000
7/18/2012	6	7.125	30	9.495	13.29	7.695	6.91	33.689	27.758	13.500
7/19/2012	7	7.91	30	9.645	13.535	7.745	5.94	26.703	48.805	14.000
7/20/2012	8	7.025	30	9.55	13.45	7.78	5.94	26.087	89.396	15.000
7/21/2012	9	7.93	30	9.55	13.99	8.145	5.94	25.059	166.067	21.947
7/22/2012	10	7.18	30	9.55	14.36	8.36	5.94	18.074	262.282	23.400
7/23/2012	11	7.86	30	OR	14.435	8.36	5.94	10.471	349.477	30.923
7/24/2012	12	8	30	OR	15.105	8.795	5.94	9.855	310.139	34.992
7/25/2012	13	8	30	OR	15.04	8.72	5.94	8.006	2.201	39.838
7/26/2012	14	8	30	OR	15.945	9.27	5.7	7.184	4.456	41.014
7/27/2012	15	8	30	OR	16.3	9.51	5.295	6.978	20.242	51.568
7/28/2012	16	8	30	OR	17.36	OR	5.05	5.951	16.483	55.098
7/29/2012	17	7.9	30	OR	17.655	OR	5.125	5.540	38.532	60.000
7/30/2012	18	7.8	30	OR	17.63	OR	5.24	2.869	8.966	62.435

Table N3: The results of 1 L batch culture for K2 AnoxKaldnes packing materials for concentration of 85 mg/L ammonia – nitrogen

Date	Day	Ph	Temperature	Total Dissolved Solids (TDS), ppt	Conductivity, mS	Salinity, ppt	Dissolved oxygen, mg/L	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
7/22/2012	0	8	30	9.335	12.955	7.645	5.7	85	2.201342	0
7/23/2012	1	7.14	30	9.8	13.815	7.95	5.7	69.64573	2.201342	1
7/24/2012	2	8	30	9.92	13.99	8.065	5.7	65.12546	27.50783	6
7/25/2012	3	8	30	9.85	13.885	8	5.7	62.65986	87.14094	9
7/26/2012	4	7.245	30	9.995	13.99	8.07	5.7	47.45533	185.1096	12
7/27/2012	5	7.145	30	OR	14.19	8.195	6.47	38.62026	435.6689	22
7/28/2012	6	7.16	28.6	OR	14.11	8.23	5.435	15.81346	640.3758	23
7/29/2012	7	7.27	29.7	OR	14.455	8.39	5.435	14.58066	630.604	30.91236
7/30/2012	8	7.255	30	OR	14.435	8.34	5.435	10.88226	6.711409	49.46274
7/31/2012	9	7.515	30	OR	14.38	8.34	5.435	12.93693	5.208054	49.53196
1/8/2012	10	7.48	30	OR	14.5	8.4	5.435	7.389328	3.955257	57.0767
2/8/2012	11	7.59	30	OR	14.725	8.43	5.435	2.869062	2.201342	76.73457
3/8/2012	12	7.75	30	OR	14.725	8.505	5.435	0	0	53.06207
4/8/2012	13	7.25	28	OR	14.8	8.63	5.435	0	0	51.33162
5/8/2012	14	7.66	28.6	OR	14.97	8.84	5.08	0	0	49.60117
6/8/2012	15	7.75	28.4	OR	15.24	9	5.08	0	0	47.93995
7/8/2012	16	7.81	29	OR	14.18	8.19	5.08	0	0	47.24777
8/8/2012	17	8	29					0	0	40
9/8/2012	18	8	29					0	0	0

Table N4: The results of 1 L batch culture for K2 AnoxKaldnes packing materials for concentration of 100 mg/L ammonia – nitrogen

Date	Day	Ph	Temperature	Total Dissolved Solids (TDS), ppt	Conductivity, mS	Salinity, ppt	Dissolved oxygen, mg/L	NH4-N	NO2-N	NO3-N
7/30/2012	0	7.24	30	9.73	13.735	7.9	4.2	100.8767	9.7181	0.6642
7/31/2012	1	7.56	30	9.6	14.215	8.2	4.9	89.9869	123.7226	4.1943
1/8/2012	2	7.525	30	9.505	13.395	7.69	4.27	78.6863	328.4295	12.0159
2/8/2012	3	8.69	30	9.8	13.98	7.7	4.03	61.4271	824.0358	14.5077
3/8/2012	4	7.75	30	9.91	13.02	8.04	3.4	11.2932	936.5369	15.8921
4/8/2012	5	7.27	30	9.525	13.53	7.78	3.91	4.7183	2.2013	37.7125
5/8/2012	6	7.595	30	9.44	13.235	7.65	4.29	4.3073	0	42.8870
6/8/2012	7	7.69	30	9.83	13.97	7.95	3.75	2.8691	0	51.4661
7/8/2012	8	7.79	30	OR	14.63	8.48	3.79	0	0	54.6135
8/8/2012	9	7.87	30	OR	14.55	8.58	4.3	0	0	64.5443
9/8/2012	10	7.94	30	OR	15.09	8.78	4.17	0	0	87.7125
10/8/2012	11	7.34	30	OR	15.615	8.77	3.92	0	0	42.8870
11/8/2012	12	7.68	30	OR	15.19	8.92	3.87	0	0	31.4661
12/8/2012	13	7.92	30	OR	15.53	9.08	4.33	0	0	24.6135
13/8/2012	14	8	30					0	0	24.5443
14/8/2012	15	8	30					0	0	24.4751
15/8/2012	16	8	30					0	0	14.4385
16/8/2012	17	8	30					0	0	9.9394
17/8/2012	18	8	30					0	0	8.7626

Table N5: The results of 1 L batch culture for K2 AnoxKaldnes packing materials for concentration of 5 mg/L ammonia – nitrogen

Day	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
	5		
0	5	0	0
1	3.5	2	0.5
2	2	7	1
3	1	0	1.5
4	0.2	0	2
5	0	0	3.2

Table N6: The results of 1 L batch culture for K2 AnoxKaldnes packing materials for concentration of 15 mg/L ammonia – nitrogen

Day	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
	15		
0	15	0	0
1	10	8	1
2	8	19	2
3	6	24	3
4	5	0	5
5	3	0	7
6	0	0	12

Table N7: The results of 1 L batch culture for K2 AnoxKaldnes packing materials for concentration of 25 mg/L ammonia – nitrogen

Day	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
	25		
0	25	0	0
1	20	2	0.2
2	15	5	1
3	13	9	4
4	10	15	7
5	8	18	10
6	6	27	10.3
7	5	36	11
8	4	40	16
9	2	0	19
10	1	0	20
11	0	0	23
12	0	0	25

Table N8: The results of 1 L batch culture for K2 AnoxKaldnes packing materials for concentration of 200 mg/L ammonia – nitrogen

Day	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
0	200.000	4.456	0.526
1	186.000	17.485	0.400
2	151.000	25.503	0.872
3	141.000	27.758	0.900
4	133.000	21.745	1.000
5	120.000	33.772	1.000
6	117.000	31.517	10.770
7	102.000	44.546	11.739
8	83.812	75.615	16.271
9	63.949	119.714	18.001
10	53.127	307.383	19.524
11	52.375	999.732	24.369
12	46.008	1254.000	26.829
13	41.559	869.000	67.390
14	38.411	78.000	71.262
15	36.496	8.000	89.125
16	28.895	6.000	104.283
17	22.731	0.000	116.616
18	21.855	0.000	129.291
19	15.060	0.000	137.943
20	10.060	0.000	167.360
21	7.663	0.000	173.207
22	6.000	0.000	189.000

Table N9: The results of 1 L batch culture for K2 AnoxKaldnes packing materials for concentration of 300 mg/L ammonia – nitrogen

Day	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
0	300.000	6.456	1.000
1	298.000	19.485	2.000
2	261.000	27.503	3.000
3	236.000	27.758	4.000
4	230.000	29.745	5.000
5	225.000	33.772	6.000
6	221.000	41.517	9.000
7	200.000	54.546	13.000
8	183.812	85.615	16.000
9	153.949	129.714	19.000
10	143.127	317.383	21.000
11	132.375	899.732	25.000
12	126.008	980.635	28.000
13	121.559	1099.177	57.000
14	108.411	1304.438	61.000
15	106.496	657.438	78.000
16	98.895	254.828	84.000
17	92.731	53.324	106.000
18	81.855	12.975	136.000
19	76.060	6.210	157.000
20	68.060	0.000	187.000
21	67.663	0.000	212.000
22	56.000	0.000	220.000

Table N10: The results of 1 L batch culture for K2 AnoxKaldnes packing materials for concentration of 400 mg/L ammonia – nitrogen

Day	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
0	400.000	4.456	1.000
1	399.000	17.485	1.500
2	387.000	25.503	5.000
3	371.000	27.758	9.000
4	365.000	21.745	14.000
5	357.000	33.772	18.000
6	333.000	31.517	20.000
7	326.000	44.546	23.000
8	313.812	75.615	36.000
9	303.949	119.714	38.000
10	293.127	307.383	49.000
11	282.375	799.732	54.000
12	266.008	980.635	66.000
13	241.559	999.177	77.000
14	238.411	1004.438	81.000
15	226.496	1004.438	113.000
16	218.895	954.828	125.000
17	212.731	953.324	176.616
18	191.855	12.975	229.291
19	170.060	6.210	237.943
20	120.060	0.000	267.360
21	107.663	0.000	283.207
22	96.000	0.000	299.000

Table N11: The results of 1 L batch culture for K2 AnoxKaldnes packing materials for concentration of 500 mg/L ammonia – nitrogen

Day	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
0	500.000	10.000	1.000
1	498.000	27.000	2.000
2	487.000	35.000	5.000
3	477.000	37.000	8.000
4	468.000	41.000	9.000
5	450.000	43.000	12.000
6	443.000	51.000	13.000
7	432.000	54.000	15.000
8	400.000	85.000	26.000
9	399.000	129.000	38.000
10	387.000	317.000	49.524
11	371.000	879.000	54.369
12	365.000	999.000	76.829
13	357.000	1025.000	97.390
14	333.000	1038.000	101.262
15	326.000	1057.000	139.125
16	313.812	1001.000	164.283
17	303.949	989.000	186.616
18	293.127	788.000	219.291
19	282.375	0.000	227.943
20	266.008	0.000	237.360
21	241.559	0.000	243.207
22	238.411	0.000	259.000

APPENDIX O

Table O1: The results of one litre batch culture for without packing materials for concentration of 35 mg/L ammonia – nitrogen

Date	Day	Ph	Temperature	Total Dissolved Solids (TDS), ppt	Conductivity, mS	Salinity, ppt	Dissolved oxygen, mg/L	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
8/7/2012	0	7.89	30	9.96	14.135	8.115	6.04	34.8672	3.7047	0
9/7/2012	1	7.795	30	9.365	13.155	7.565	5.525	22.1829	11.2215	0
10/7/2012	2	7.56	30	9.435	13.29	7.665	6.095	21.3611	12.2237	0
11/7/2012	3	7.4	30	9.6	13.6	7.9	6	15.4025	13.7271	0
12/7/2012	4	7.39	30	9.945	13.99	8.055	6	15.4025	18.7383	0.0412
13/7/2012	5	7.525	30	OR	14.19	8.255	6	15.4025	21.4944	0.1104
14/7/2012	6	7.45	30	OR	14.33	8.27	6.14	13.3479	25.5034	0.5949
15/7/2012	7	8	30	OR	14.4	8.3	6	8.6221	53.8166	2.9484
7/16/2012	8	8.74	30	OR	14.51	8.45	6.14	6.9784	73.6107	4.3327
7/17/2012	9	8.74	30	OR	14.6	8.71	6.14	5.3347	44.2953	4.7480
7/18/2012	10	8	30	OR	15.09	8.995	6.14	4.9237	16.9843	14.5769
7/19/2012	11	6.895	30	OR	15.415	9.415	6.79	4.9237	14.4787	15.7537
7/20/2012	12	6.9	30	OR	16.155	9.675	6.79	3.0745	11.2215	16.5843
7/21/2012	13	6.885	30	OR	16.61	OR	6.79	2.8691	9.7181	16.7227
7/22/2012	14	7.195	30	OR	17.32333	OR	6.79	0	5.7092	16.8611
7/23/2012	15	6.855	30	OR	19.285	OR	6.79	0	2.7025	23.5753

Table O2: The results of one litre batch culture for without packing materials for concentration of 65 mg/L ammonia – nitrogen

Date	Day	Ph	Temperature	Total Dissolved Solids (TDS), ppt	Conductivity, mS	Salinity, ppt	Dissolved oxygen, mg/L	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
8/7/2012	0	7.89	30	9.96	14.135	8.115	6.04	65.000	12.224	0.041
9/7/2012	1	7.795	30	9.365	13.155	7.565	5.525	50.126	14.729	2.602
10/7/2012	2	8	30	9.435	13.29	7.665	6.095	50.126	22.246	3.987
11/7/2012	3	8	30	9.6	13.6	7.9	6	43.962	32.268	5.371
12/7/2012	4	8	30	9.945	13.99	8.055	6	34.100	39.785	6.755
13/7/2012	5	8	30	OR	14.19	8.255	6	31.634	52.313	8.140
14/7/2012	6	7.45	30	OR	14.33	8.27	6.14	29.580	59.830	10.908
15/7/2012	7	8	30	OR	14.4	8.3	6	27.525	77.369	12.293
7/16/2012	8	8	30	OR	14.51	8.45	6.14	23.416	87.391	15.061
7/17/2012	9	8	30	OR	14.6	8.71	6.14	19.306	102.425	16.446
7/18/2012	10	7.095	30	OR	15.09	8.995	6.14	18.485	378.040	19.215
7/19/2012	11	6.895	30	OR	15.415	9.415	6.79	14.170	302.872	22.675
7/20/2012	12	6.9	30	OR	16.155	9.675	6.79	11.293	277.817	26.136
7/21/2012	13	8	30	OR	16.61	OR	6.79	9.033	252.761	29.597
7/22/2012	14	8	30	OR	17.32333	OR	6.79	6.978	227.705	33.058
7/23/2012	15	8	30	OR	19.285	OR	6.79	2.869	202.649	39.980

Table O3: The results of one litre batch culture for without packing materials for concentration of 85 mg/L ammonia – nitrogen

Date	Day	Ph	Temperature	Total Dissolved Solids (TDS), ppt	Conductivity, mS	Salinity, ppt	Dissolved oxygen, mg/L	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
8/7/2012	0	8	30	9.6	13.5	7.79	5.6	85.000	2.201	0.041
9/7/2012	1	8	30	9.686667	13.445	7.795	5.6	68.824	7.463	0.595
10/7/2012	2	8	30	9.915	13.955	8.055	5.6	56.085	23.499	0.664
11/7/2012	3	8	30	OR	14.255	8.24	5.6	50.537	77.119	0.733
12/7/2012	4	8	30	OR	14.585	8.435	5.575	42.113	150.282	1.218
13/7/2012	5	8	30	OR	14.945	8.65	5.16	32.456	303.624	2.602
14/7/2012	6	8	30	OR	15	8.8	5	18.279	780.188	18.315
15/7/2012	7	8	30	OR	15.195	8.93	6	17.046	379.293	34.996
7/16/2012	8	8	30	OR	15.79	9.23	6	14.581	2.201	43.233
7/17/2012	9	8	30	OR	15.99667	9.385	5.09	14.375	5.459	46.902
7/18/2012	10	8	30	OR	16.275	9.46	6	9.855	5.960	52.024
7/19/2012	11	8	30	OR	16.3	9.5	6	8.828	2.201	56.108
7/20/2012	12	8	30	OR	16.58	9.65	6	2.869	0.000	41.849
7/21/2012	13	8	30	OR	15.39	9.83	4.32	0.000	0.000	41.710
7/22/2012	14	8	30	OR	17.28	OR	4.27	0.000	0.000	41.433
7/23/2012	15	8	30	OR	17.59	OR	4.4	0.000	0.000	37.142
7/24/2012	16	8	30	OR	17.91	OR	4.09	0.000	0.000	37.004

Table O4: The results of one litre batch culture for without packing materials for concentration of 100 mg/L ammonia – nitrogen

Date	Day	Ph	Temperature	Total Dissolved Solids (TDS), ppt	Conductivity, mS	Salinity, ppt	Dissolved oxygen, mg/L	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)
7/30/2012	0	7.27	30	9.805	13.815	7.96	4.05	98.82199	6.961969	5.370975
7/31/2012	1	7.6	30	OR	14.1	8.15	4.95	82.59013	100.6711	3.156004
1/8/2012	2	7.51	30	OR	14.115	8.16	4.535	64.91999	358.4966	6.547678
2/8/2012	3	8.61	30	OR	14.01		3.87	52.38653	874.1477	14.71538
3/8/2012	4	7.75	30	9.94	14.16	8.04	4.11	7.183862	882.9172	6.547678
4/8/2012	5	7.335	30	OR	14.61	8.28	3.93	2.869062	2.201342	36.72666
5/8/2012	6	7.6	30	OR	14.61	8.48	4.8	0	0	39.97989
6/8/2012	7	7.695	30	OR	14.63	8.51	4.68	0	0	81.71825
7/8/2012	8	7.79	30	OR	14.86	8.63	4.34	0	0	95.35416
8/8/2012	9	7.89	30	OR	15.15	8.83	4.57	0	0	96.04634
8/9/2012	10	7.94	30	OR	15.29	8.89	5.4	0	0	96.73852
8/10/2012	11	7.53	30	OR	15.64	9.11	5.44	0	0	93.96981
8/11/2012	12	7.695	30	OR	15.935	9.36	5.62	0	0	93.27763
8/12/2012	13	8.025	30	OR	16.47	9.665	3.91	0	0	92.58545

APPENDIX P

Table P1: The results of one litre batch culture for K2 AnoxKaldnes packing materials for concentration of 35 mg/L ammonia – nitrogen with 100 µg/L 17α – ethynylestradiol (EE2) and 100 µg/L mestranol (MeEE2)

Date	Day	Ph	Temperature	Total Dissolved Solids (TDS), ppt	Conductivity, mS	Salinity, ppt	Dissolved oxygen, mg/L	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)	EE2 (µg/L)	MeEE2 (µg/L)
12/9/2012	0	8.52	22.3	9.26	13.06	7.5	5.43	34.689	2.452	0.355	100.019	100.169
9/13/2012	1	8.54	22.7	9.56	13.24	7.73	5.46	22.388	34.022	3.539	98.029	97.007
9/14/2012	2	8.48	27.3	9.54	13.47	7.72	3.51	19.923	40.537	23.058	88.130	93.845
9/15/2012	3	8.45	26.8	9.58	13.44	7.82	4.63	14.581	95.159	23.750	85.933	78.033
9/16/2012	4	8.47	29	9.85	13.9	8.04	4.48	4.513	85.136	24.442	56.697	74.871
9/17/2012	5	8.47	24	9.73	13.5	7.92	5.27	4.102	15.481	32.056	49.828	52.735
9/18/2012	6	8.38	23.1	9.63	13.44	7.77	5.61	4.102	15.230	33.441	46.682	43.248
9/19/2012	7	8.33	23.1	OR	14.14	8.23	5.46	2.869	2.201	32.749	46.381	40.086
9/20/2012	8	8.32	22.9	OR	14.43	8.38	5.58	2.869	2.201	29.980	41.640	40.086
9/21/2012	9	8.31	27	OR	14.88	8.7	4.34	2.869	2.201	27.903	34.701	30.599

Table P2: The results of one litre batch culture for K2 AnoxKaldnes packing materials for concentration of 65 mg/L ammonia – nitrogen with 100 μg/L 17α – ethynylestradiol (EE2) and 100 μg/L mestranol (MeEE2)

Date	Day	Ph	Temperature	Total Dissolved Solids (TDS), ppt	Conductivity, mS	Salinity, ppt	Dissolved oxygen, mg/L	NH3-N (mg/L)	NO2-N (μg/L)	NO3-N (mg/L)	EE2 (μg/L)	MeEE2 (μg/L)
12/9/2012	0	8.52	22	9.29	13.08	7.49	6.33	65.000	3.705	0.109	100.019	100.169
9/13/2012	1	8.52	22.4	9.53	13.4	7.59	5.07	35.546	60.832	4.677	88.778	94.161
9/14/2012	2	8.57	27.3	9.65	13.62	7.85	3.84	31.231	96.161	36.517	78.624	87.520
9/15/2012	3	8.49	26.7	9.94	13.98	8.12	4.63	29.793	176.089	48.561	60.490	74.871
9/16/2012	4	8.46	28.6	OR	14.11	8.23	4.47	18.697	237.477	51.745	52.071	71.709
9/17/2012	5	8.4	23.8	OR	14.39	8.33	5.37	10.684	140.761	52.437	49.828	46.411
9/18/2012	6	8.34	22.8	OR	14.56	8.46	5.35	2.671	20.492	58.667	46.682	40.086
9/19/2012	7	8.29	22.8	OR	15.07	8.62	5.35	0.000	11.472	59.359	45.456	24.275
9/20/2012	8	8.26	22.5	OR	15.58	9.05	5.41	0.000	11.472	60.744	28.595	24.275
9/21/2012	9	8.26	27.5	OR	16.29	9.58	4.61	0.000	2.201	61.436	27.762	22.693

Table P3: The results of one litre batch culture for K2 AnoxKaldnes packing materials for concentration of 85 mg/L ammonia – nitrogen with 100 µg/L 17α – ethynylestradiol (EE2) and 100 µg/L mestranol (MeEE2)

Date	Day	Ph	Temperature	Total Dissolved Solids (TDS), ppt	Conductivity, mS	Salinity, ppt	Dissolved oxygen, mg/L	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)	EE2 (µg/L)	MeEE2 (µg/L)
12/9/2012	0	8.55	21.9	9.66	13.45	7.82	6.27	85.0	2.2	0.2	100.0	100.2
9/13/2012	1	8.55	21.9	9.66	13.45	7.82	6.27	61.6	52.8	2.5	74.9	94.2
9/14/2012	2	8.56	22.5	9.93	13.96	8.05	5.5	51.6	135.0	36.3	67.1	87.5
9/15/2012	3	8.43	27.2	9.89	13.75	8.04	4.28	37.4	461.5	48.9	37.4	74.9
9/16/2012	4	8.39	26.6	9.73	14.14	8.21	4.73	30.2	589.0	50.7	28.9	68.5
9/17/2012	5	8.29	28.7	OR	14.25	8.24	4.37	7.6	269.0	54.2	26.7	46.4
9/18/2012	6	8.28	30	OR	13.64	8.1	5.46	2.9	17.0	64.6	23.6	40.1
9/19/2012	7	8.15	30	OR	14.43	8.28	5.53	0.0	12.2	78.4	22.3	21.1
9/20/2012	8	8.09	30	OR	14.75	8.46	5.55	0.0	4.7	79.8	19.3	17.9
9/21/2012	9	8.08	30	OR	15.22	8.78	5.5	0.0	4.7	80.5	16.2	14.8

Table P4: The results of one litre batch culture for K2 AnoxKaldnes packing materials for concentration of 100 mg/L ammonia – nitrogen with 100 µg/L 17α – ethynylestradiol (EE2) and 100 µg/L mestranol (MeEE2)

Date	Day	Ph	Temperature	Total Dissolved Solids (TDS), ppt	Conductivity, mS	Salinity, ppt	Dissolved oxygen, mg/L	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)	EE2 (µg/L)	MeEE2 (µg/L)
12/9/2012	0	8.61	30	9.21	12.94	7.4	6.05	102.733	4.957	0.209	100.019	100.169
9/13/2012	1	8.61	30	9.21	12.9	7.4	6.05	88.351	65.092	1.040	72.587	90.999
9/14/2012	2	8.55	30	9.8	13.72	7.94	5.42	78.077	129.485	31.218	66.203	84.358
9/15/2012	3	8.46	30	9.98	14.15	8	5.42	45.408	235.221	40.840	35.048	71.709
9/16/2012	4	8.4	30	OR	14.51	8.44	5.42	43.559	270.300	50.530	26.629	62.222
9/17/2012	5	8.29	30	OR	14.64	8.55	5.42	33.902	792.215	86.108	24.385	43.248
9/18/2012	6	8.21	30	OR	14.88	8.63	5.42	11.712	772.421	92.199	21.240	39.770
9/19/2012	7	8.08	30	OR	14.88	8.59	5.42	0.000	681.969	101.198	20.014	20.796
9/20/2012	8	8.01	30	OR	15.07	8.75	5.42	0.000	21.494	101.890	14.717	17.001
9/21/2012	9	7.97	30	OR	15.99	9.35	5.42	0.000	8.966	102.582	11.572	14.155

APPENDIX Q :

Table Q1: The results of effluents for NH_3-N, NO_2-N, NO_3-N, EE2 and MeEE2 for K2 AnoxKaldnes packing materials for concentration of 35 mg/L ammonia – nitrogen with 100 µg/L 17α – ethynylestradiol (EE2) and 100 µg/L mestranol (MeEE2) in partial fixed bed reactor (PFBR)

ml/min	Dilution, D (1/day)	Day	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)	EE2 (µg/L)	MeEE2 (µg/L)
20	1.8	0	32.450	112.250	0.550	98.000	95.000
20	1.8	1	21.160	162.002	9.840	90.000	90.000
20	1.8	2	16.200	93.454	14.800	91.000	80.000
20	1.8	3	15.700	97.937	15.300	80.000	73.000
20	1.8	4	11.340	82.291	19.660	76.000	70.000
20	1.8	5	10.900	85.709	20.100	69.000	64.000
20	1.8	6	9.100	95.888	21.900	65.000	59.000
20	1.8	7	8.980	92.000	22.020	68.000	53.000
20	1.8	8	7.540	66.613	23.460	70.000	56.000
20	1.8	9	4.340	72.000	26.660	65.000	52.000
20	1.8	10	9.430	93.450	21.570	60.000	53.000
20	1.8	11	4.000	32.268	27.000	55.000	49.000
20	1.8	12	6.600	48.966	24.400	48.000	42.000
20	1.8	13	1.500	32.474	29.500	37.000	33.000
20	1.8	14	2.300	13.454	28.700	30.000	35.000
20	1.8	15	1.100	15.732	29.900	34.000	32.000
20	1.8	16	1.700	12.268	29.300	32.000	28.000
20	1.8	17	0.900	9.718	30.100	24.000	25.000
20	1.8	18	0.600	2.201	30.400	28.000	23.000
20	1.8	19	0.500	2.201	30.500	23.000	25.000
20	1.8	20	0.500	7.485	30.500	24.000	22.000
20	1.8	21	0.500	32.201	30.500	24.000	22.000
30	2.7	22	5.000	22.201	26.000	44.000	38.000
30	2.7	23	4.400	12.201	26.600	37.000	32.000
30	2.7	24	2.300	8.201	28.700	47.000	39.000
30	2.7	25	1.600	2.201	29.400	33.000	34.000
30	2.7	26	1.100	2.201	29.900	34.000	30.000
30	2.7	27	1.400	2.201	29.600	49.000	45.000
30	2.7	28	1.300	2.953	29.700	32.000	27.000
30	2.7	29	1.500	39.575	29.500	37.000	35.000
30	2.7	30	3.500	2.201	27.500	43.000	27.000
30	2.7	31	1.700	11.472	29.300	38.000	20.000
30	2.7	32	1.900	3.955	29.100	42.000	40.000
30	2.7	33	2.869	10.470	28.131	30.000	32.000
30	2.7	34	4.400	13.477	26.600	35.000	34.000

30	2.7	35	1.900	20.492	29.100	30.000	28.000
30	2.7	36	3.100	34.273	27.900	30.000	32.000
30	2.7	37	2.800	27.758	28.200	30.000	38.000
30	2.7	38	2.900	4.707	28.100	33.000	30.000
30	2.7	39	1.800	3.705	29.200	35.000	32.000
30	2.7	40	1.200	3.705	29.800	32.000	29.000
30	2.7	41	1.000	7.213	30.000	31.000	29.000
30	2.7	42	1.000	4.707	30.000	31.000	29.000
40	3.6	43	2.800	29.468	28.200	50.000	45.000
40	3.6	44	3.300	38.499	27.700	52.000	38.000
40	3.6	45	9.600	68.282	21.400	38.000	49.000
40	3.6	46	4.300	23.772	26.700	42.000	30.000
40	3.6	47	3.500	24.273	27.500	60.000	33.000
40	3.6	48	6.400	46.595	24.600	44.000	36.000
40	3.6	49	4.100	20.993	26.900	38.000	45.000
40	3.6	50	2.500	17.163	28.500	40.000	39.000
40	3.6	51	5.800	44.980	25.200	45.000	30.000
40	3.6	52	3.800	11.472	27.200	39.000	28.000
40	3.6	53	2.300	18.488	28.700	33.000	35.000
40	3.6	54	1.600	5.709	29.400	38.000	32.000
40	3.6	55	1.600	4.206	29.400	38.000	32.000
40	3.6	56	1.600	8.966	29.400	38.000	32.000
50	4.5	57	3.800	6.962	27.200	69.000	59.000
50	4.5	58	2.400	5.709	28.600	68.000	58.000
50	4.5	59	1.900	24.251	29.100	60.000	44.000
50	4.5	60	6.800	26.756	24.200	45.000	46.000
50	4.5	61	2.100	41.790	28.900	44.000	45.000
50	4.5	62	3.600	19.239	27.400	49.000	50.000
50	4.5	63	1.800	13.477	29.200	50.000	60.000
50	4.5	64	4.300	41.289	26.700	40.000	45.000
50	4.5	65	5.800	50.000	25.200	53.000	50.000
50	4.5	66	3.400	51.000	27.600	38.000	48.000
50	4.5	67	4.500	42.000	26.500	50.000	55.000
50	4.5	68	5.400	52.000	25.600	56.000	50.000
50	4.5	69	3.500	25.000	27.500	50.000	48.000
50	4.5	70	3.200	21.000	27.800	49.000	46.000
50	4.5	71	3.200	22.000	27.800	49.000	46.000
50	4.5	72	3.200	29.000	27.800	49.000	45.000
50	4.5	73	3.200	31.000	27.800	49.000	45.000
60	5.4	74	2.388	17.000	28.612	67.000	65.000
60	5.4	75	9.169	75.000	21.831	56.000	63.000
60	5.4	76	7.936	68.000	23.064	70.000	47.000
60	5.4	77	8.690	67.000	22.310	59.000	66.000
60	5.4	78	5.128	42.291	25.872	63.000	67.000
60	5.4	79	9.621	69.000	21.379	60.000	56.000

60	5.4	80	8.895	58.000	22.105	58.000	49.000
60	5.4	81	7.457	58.000	23.543	66.000	54.000
60	5.4	82	6.841	44.000	24.159	58.000	63.000
60	5.4	83	8.416	62.000	22.584	63.000	58.000
60	5.4	84	7.381	55.000	23.619	50.000	54.000
60	5.4	85	8.600	60.000	22.400	51.000	56.000
60	5.4	86	6.500	54.000	24.500	53.000	58.000
60	5.4	87	5.300	53.000	25.700	55.000	55.000
60	5.4	88	4.500	54.000	26.500	55.000	52.000
60	5.4	89	4.500	52.000	26.500	55.000	52.000
60	5.4	90	4.500	49.000	26.500	55.000	52.000

Table Q2: The results of effluents for NH_3-N, NO_2-N, NO_3-N, EE2 and MeEE2 for K2 AnoxKaldnes packing materials for concentration of 35 mg/L ammonia – nitrogen with 100 µg/L 17α – ethynylestradiol (EE2) and 100 µg/L mestranol (MeEE2) in moving bed reactor (MBBR)

ml/min	Dilution, D (1/day)	Day	NH3-N (mg/L)	NO2-N (µg/L)	NO3-N (mg/L)	EE2 (µg/L)	MeEE2 (µg/L)
20	1.8	0	22.388	116.707	2.612	82.388	83.000
20	1.8	1	19.169	96.000	7.831	79.169	81.000
20	1.8	2	17.936	91.000	10.064	77.936	78.000
20	1.8	3	15.690	67.000	14.310	68.690	64.000
20	1.8	4	10.128	56.000	18.872	60.128	59.000
20	1.8	5	9.621	55.500	19.379	53.621	54.011
20	1.8	6	8.895	50.000	24.105	48.895	46.212
20	1.8	7	7.457	46.000	25.543	37.457	40.469
20	1.8	8	6.841	30.000	26.159	26.841	39.735
20	1.8	9	3.416	28.000	29.584	23.416	33.624
20	1.8	10	1.279	20.000	30.721	32.388	30.000
20	1.8	11	0.334	11.000	30.666	29.169	19.000
20	1.8	12	3.210	23.000	29.790	27.936	27.000
20	1.8	13	0.881	17.500	30.119	18.690	22.000
20	1.8	14	0.334	11.500	30.666	25.000	20.000
20	1.8	15	1.156	25.500	29.844	15.000	18.000
20	1.8	16	1.717	28.000	29.283	22.000	20.000
20	1.8	17	0.923	14.200	30.077	23.000	23.000
20	1.8	18	0.799	6.000	30.201	20.000	19.000
20	1.8	19	0.200	5.000	30.800	18.416	15.000
20	1.8	20	0.200	5.000	30.800	18.279	15.000
20	1.8	21	0.200	5.200	30.800	18.334	15.000
30	2.7	22	2.731	25.000	30.269	27.000	22.000
30	2.7	23	0.444	22.000	30.556	25.881	28.000

30	2.7	24	0.239	11.500	30.761	30.334	24.000
30	2.7	25	1.677	8.000	31.323	31.156	12.157
30	2.7	26	1.088	5.500	31.912	19.717	22.000
30	2.7	27	1.375	7.000	31.625	29.923	29.000
30	2.7	28	1.307	6.500	31.693	28.799	27.000
30	2.7	29	2.335	20.500	30.665	22.799	13.000
30	2.7	30	0.595	7.500	32.405	29.512	15.000
30	2.7	31	1.704	8.500	31.296	32.799	24.000
30	2.7	32	1.512	9.500	31.488	25.000	14.000
30	2.7	33	1.869	14.345	31.131	31.000	13.600
30	2.7	34	1.444	12.000	31.556	29.000	12.800
30	2.7	35	1.910	9.500	31.090	20.000	17.000
30	2.7	36	3.142	25.500	29.858	25.000	17.000
30	2.7	37	0.828	14.000	32.172	27.000	28.000
30	2.7	38	0.924	14.500	32.076	23.000	24.000
30	2.7	39	0.690	9.000	32.310	22.000	23.000
30	2.7	40	0.500	6.000	32.500	21.595	19.000
30	2.7	41	0.500	5.000	32.500	21.704	19.000
30	2.7	42	0.500	5.000	32.500	21.512	19.000
40	3.6	43	1.841	14.000	31.159	32.869	22.000
40	3.6	44	1.321	16.500	31.679	39.444	28.000
40	3.6	45	3.689	48.000	29.311	21.910	36.000
40	3.6	46	1.170	21.500	31.830	33.142	33.000
40	3.6	47	1.553	17.500	31.447	28.828	15.038
40	3.6	48	1.430	22.000	31.570	34.924	25.000
40	3.6	49	0.855	10.000	32.145	22.000	29.000
40	3.6	50	2.183	12.500	30.817	36.841	35.000
40	3.6	51	3.485	29.000	29.515	36.224	29.000
40	3.6	52	1.813	19.000	31.187	25.403	19.470
40	3.6	53	1.533	11.500	31.467	26.841	27.000
40	3.6	54	1.000	8.000	32.000	27.321	24.000
40	3.6	55	1.000	8.000	32.000	27.689	24.000
40	3.6	56	1.000	8.000	32.000	27.170	24.000
50	4.5	57	2.006	19.000	30.994	33.553	39.001
50	4.5	58	1.430	12.000	31.570	36.430	38.854
50	4.5	59	2.827	24.000	30.173	39.855	29.955
50	4.5	60	1.704	14.000	31.296	42.183	22.524
50	4.5	61	2.649	25.000	30.351	40.485	28.341
50	4.5	62	1.868	18.000	31.132	45.813	42.670
50	4.5	63	3.484	30.000	29.516	32.000	29.808
50	4.5	64	2.184	11.000	30.816	43.000	30.689
50	4.5	65	2.252	19.000	30.748	32.526	32.869
50	4.5	66	1.046	7.000	31.954	39.855	29.444
50	4.5	67	1.129	16.000	31.871	38.006	31.910
50	4.5	68	2.000	16.000	31.000	36.430	33.000

50	4.5	69	2.000	16.000	31.000	36.827	33.000
50	4.5	70	2.000	16.000	31.000	36.704	33.000
60	5.4	71	2.400	11.942	30.600	44.649	48.690
60	5.4	72	1.300	35.000	31.700	47.868	46.841
60	5.4	73	2.500	39.680	30.500	53.484	46.224
60	5.4	74	4.800	43.450	28.200	47.184	35.403
60	5.4	75	2.100	10.641	30.900	37.252	36.841
60	5.4	76	6.400	46.000	26.600	47.046	46.841
60	5.4	77	5.200	23.000	27.800	35.129	43.416
60	5.4	78	6.700	37.286	26.300	55.335	32.388
60	5.4	79	4.800	34.204	28.200	47.595	39.169
60	5.4	80	5.400	37.000	27.600	48.211	37.936
60	5.4	81	3.500	36.000	29.500	39.000	38.690
60	5.4	82	3.300	32.000	29.700	41.000	38.128
60	5.4	83	3.000	32.000	30.000	40.000	38.000
60	5.4	84	3.100	32.000	29.900	40.000	38.000