A Novel Aerobic Process for Carbon and Nitrogen Removal from Wastewater using a Biofilm with Passive Aeration

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This thesis is presented for the degree of Doctor of Philosophy in Environmental Engineering

June 2015

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Raphael M G Flavigny

To my family
Abstract

Conventional municipal wastewater treatments use about 50% of their energy for bulk liquid aeration to oxidise dissolved organic carbon (C) to CO$_2$ and ammonium (NH$_4^+$) to nitrite (NO$_2^-$) and nitrate (NO$_3^-$). This thesis aims at reducing the energy requirement for bulk liquid aeration for the oxidation of dissolved carbon and ammonium in wastewater. This was done by developing two separate biofilm reactors that respectively oxidise C and NH$_4^+$ with passive aeration. Thereafter, the combination of the two processes was tested to achieve complete dissolved carbon and total Nitrogen (N) removal without aerating the bulk liquid.

The first biofilm reactor removed dissolved carbon with a sequencing batch mode. The biofilm was flooded with wastewater, and dissolved C was biologically stored as Poly-Hydroxyalkanoates (PHAs) under anaerobic conditions, followed by the oxidation of PHAs to CO$_2$, under aerobic conditions. The aeration was achieved by draining the wastewater, resulting in mere exposure of the biofilm to atmospheric oxygen partial pressure. The storing biomass was developed in 9 weeks from Activated Sludge (AS) and biomass of a storage driven denitrification biofilm, with a strict oscillation of: anaerobic conditions with acetate in solution, and exposure to the atmosphere (i.e. aerobic conditions) without dissolved carbon. The DNA analysis along with the testing of metabolites in biomass and solution demonstrated that the oscillating conditions enriched the biomass with *Candidatus Accumulibacter*, a known Glycogen Accumulating Organism (GAO). The process was operated over 9 months and repeatedly stored acetate as PHAs under anaerobic conditions and oxidised it during air exposure. Overall, > 80% of the acetate added to the biofilm
was removed at a rate of 4 Cmmol/L/h (128 g/m$^3$/h BOD) and the reactor’s Hydraulic Retention Time (HRT) was 3 h. Both the rate and HRT were faster than conventional AS processes.

The second reactor developed aimed at reducing the energy cost for oxygen supply for ammonium oxidation. To reduce the energy use for ammonium oxidation a two-step method was used. The first step was ammonia adsorption onto zeolite used as carrier for nitrifying biomass. The second step was the ammonia oxidation of the adsorbed ammonia using trickle method for oxygen transfer. The zeolite used in this study was an Australian Clinoptilolite zeolite (2 – 3.35 mm) with a maximum ammonium adsorption rate of 0.12 mmol-N/g/h (1.68 mg-N/g/h). Results showed that the nitrifying biomass was capable of oxidising 93 % of adsorbed ammonium on zeolite as nitrate when trickling the whole batch volume (1 bed volume) of wastewater, but the recovery reduced to < 34 % when only 20% of the liquid was recycled for reduced energy expense. To complete the total N removal, nitrate drained from this reactor was denitrified in the first reactor by GAO using PHA stored. The combination of the two reactors achieved 99 % of the acetate removal and 93 % of the nitrogen removal. However, liquid recirculation between two reactors was thought to be an energy cost that could be prevented.

To reduce energy consumption, a single zeolite amended biofilm was synthesised by adding the GAO, nitrifiers and zeolite powder together, with the objective to remove dissolved C and total N within a single biofilm reactor. The operating principle was to simply fill the reactor and keep it anaerobic, so as to let the wastewater in contact with biofilm to biologically store dissolved acetate and adsorb ammonium on zeolite (Stage 1). Then aerobic conditions (Stage 2) were provided by draining the liquid. The liquid was recirculated for mass transfer at 0.4 m$^3$/m$^2$/d,
which is a fraction of that used in trickle filter reactor. The zeolite amended biofilm reactor treated wastewater with a total treatment time of 19 h. Removal efficiencies were > 94 % for C and 80 % for total N. The production of dinitrogen gas under atmospheric oxygen partial pressure demonstrated that Simultaneous Nitrification and Denitrification (SND) occurred in air. SND in air can be explained to be due to an oxygen gradient formed in the biofilm.

As the biofilm had been synthesised from different biomasses, it was tested for medium term sustainability. GAOs and nitrifiers were considered sustained in the synthesised biofilm over an operating period of 30 cycles (3 months), as their removal rates remained similar or improved over time. However, over this period the nitrite oxidizing bacteria (NOB) were washed out of the system, which is advantageous for effective nitrogen removal and known as SND over nitrite. It means that the zeolite amended biofilm reactor can effectively denitrify wastewater with low C/N ratio. On the contrary, conventional wastewater treatment plants are not effective at denitrifying low C/N wastewater.

To optimise the zeolite amended biofilm reactor, its treatment time was shortened from 19 to 5 h and by omitting liquid recirculation in Stage 2. Under these short treatment times the removal efficiencies were > 83 % and 75 % for C and total N respectively over the tested period of 18 cycles. The system operating with 5 h treatment times is promising as a pre-treatment process for C and total N removal with a minimum of energy expense. This work does not propose a new process, it is the novel combination of the processes that achieve the novelty in this thesis.

Overall, the system developed in this work is a novel combination of known biological and chemical steps for carbon and nitrogen removal from wastewater. In
the first reactor, direct atmospheric contact between the carbon storage biomass and oxygen to oxidise PHAs was novel. In the zeolite amended biofilm reactor, the operation of SND in full atmospheric oxygen condition was novel and demonstrated zeolite bio-regeneration as well as PHAs oxidation. This novel biofilm reactor repeatedly removed soluble carbon and total nitrogen without liquid recirculation, over a medium term operation.
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Contributions of Others

The work presented in this thesis was primarily designed, experimentally executed and interpreted by Raphael Flavigny. Individual chapters were also prepared by Raphael Flavigny, and the contributions of others beyond supervisory input are described below.

Chapter 2:

Raphael Flavigny and Dr Ralf Cord-Ruwisch contributed intellectually to the experimental design and concept. The experiments were conducted and analysed by Raphael Flavigny. Dr Lucy Skillman conducted the DNA analysis of the activated sludge and the biofilm samples and helped with the interpretation of the results. Raphael Flavigny and Dr Ralf Cord-Ruwisch interpreted the results and provided intellectual input through discussion. Raphael Flavigny and Dr Ralf Cord-Ruwisch wrote the paper. Raphael Flavigny amended the writing of the paper to fit the chapter format and maintain the overall thesis structure.

Chapter 5

Dr Liang Cheng provided the initial idea to the development of the zeolite amended biofilm reactor. The experimental design was develop with input from Dr Liang Cheng, the experimental execution was conducted by Raphael Flavigny. Raphael Flavigny, Dr Liang Cheng and Dr Ralf Cord-Ruwisch interpreted the results and provided intellectual input through discussion.
Publications

Peer review journal


R.Flavigny 2014 Diabetic glucose meter for the determination of glucose in microbial cultures, *Journal of Microbiological Methods* (100) pp: 91-92

Conference

Abbreviations

Anammox – Anaerobic Ammonium Oxidation
AOB – Ammonium Oxidising Bacteria
AS – Activated Sludge
ATP – Adenosine Triphosphate
BAF – Biological Aerated Filter
BV – Bed Volume
C – Carbon
CANON – Completely Autotrophic Nitrogen Oxidation over Nitrite
CEC – Cation Exchange Capacity
Cmol – Carbon mole
CO₂ – Carbon dioxide
DI – Deionised
DNA – Deoxyribonucleic Acid
DO – Dissolved Oxygen
EC – Effective capacity
ETC – Electron Transport Chain
GAO – Glycogen Accumulating Organism
HLR – Hydraulic Loading Rate
HRT – Hydraulic Retention Time
K⁺ – Potassium
K_{ads} – Adsorption constant
K_{dsp} – Desorption constant
K_{I} – Inhibition constant
K_{O} – Oxygen affinity constant
K_{S} – Substrate affinity constant
Na⁺ – Sodium
NADH/NAD⁺ – Nicotinamide (Oxidised/Reduced)
Adenine – Dinucleotide
NH₄⁺ – Ammonium
NO₂⁻ – Nitrite
NO₃⁻ – Nitrate
NOB – Nitrite Oxidising Bacteria
OD – Optical Density
OHO – Ordinary Heterotrophic Organism
PAO – Phosphate Accumulating Organism
PCR – Polymerase Chain Reaction
PHA – Poly-hydroxy-alkanoates
PH2MV – poly-hydroxy-2-methyl-valerate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>PHB</td>
<td>Poly-hydroxy-butyrate</td>
</tr>
<tr>
<td>PHV</td>
<td>Poly-hydroxy-valerate</td>
</tr>
<tr>
<td>PO$_4^{3-}$</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PVC</td>
<td>PolyVinyl Chloride</td>
</tr>
<tr>
<td>RBC</td>
<td>Rotating Biological Contactor</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotation Per Minute</td>
</tr>
<tr>
<td>SBR</td>
<td>Sequencing Batch Reactor</td>
</tr>
<tr>
<td>SND</td>
<td>Simultaneous Nitrification and Denitrification</td>
</tr>
<tr>
<td>V$_{ads}$</td>
<td>Adsorption rate</td>
</tr>
<tr>
<td>V$_{dsp}$</td>
<td>Desorption rate</td>
</tr>
<tr>
<td>V$_{overall}$</td>
<td>Overall rate</td>
</tr>
<tr>
<td>SS</td>
<td>Suspended Solids</td>
</tr>
<tr>
<td>TCA</td>
<td>Tri-Carboxylic Acid</td>
</tr>
<tr>
<td>TF</td>
<td>Trickle filter</td>
</tr>
<tr>
<td>TN</td>
<td>Total nitrogen</td>
</tr>
<tr>
<td>TP</td>
<td>Total Phosphate</td>
</tr>
<tr>
<td>WW</td>
<td>Wastewater</td>
</tr>
</tbody>
</table>
Chapter 1  Introduction: a review of the aerobic processes for wastewater treatment

1.1  Organic carbon and major nutrients content in municipal wastewater

Wastewater can be divided into two categories, municipal and industrial wastewaters. Industrial wastewaters are characterised by the industry’s process and because of the numerous types of industries the wastewater composition cannot be generalised [1,2]. Most industries are responsible for the treatment and disposal of the wastewater produced in their processes. For this reason, industrial wastewater will not be looked at in this work and the focus will be on municipal wastewater.

Municipal wastewater contains dissolved organic carbon and nutrients that require removal, prior to being disposed of, to prevent environmental pollution (e.g. eutrophication) [2,3]. The major nutrients include nitrogen compounds, such as ammonium (NH$_4^+$), nitrite (NO$_2^-$), nitrate (NO$_3^-$), and phosphate (TP). The remaining pollutants are referred to as micro-pollutants. Micro-pollutants include recalcitrant compounds (e.g. pharmaceutical compounds) and heavy metals, but the fate of micro-pollutants in wastewater treatment processes will not be reviewed in this work, some reviews are available on these topics [e.g. 4,5]. This work reviews different biological aerobic mechanisms for organic carbon and nutrients removal, and the application of biological activities in municipal wastewater treatment plants for effective removal.

1.1.1  Source of organic carbons in municipal wastewater

In municipal wastewater, organic carbon compounds come in suspended and dissolved forms. Suspended organic carbon compounds are insoluble and require
physical processes to be removed from solution, such as screening, filtering or settling [6]. Suspended organic carbon (e.g. fats, carbohydrates) represents about 60-80 % of total organic carbons [1]. Suspended organic carbons are not biologically removed in treatment plants, thus suspended organic carbons are measured as Chemical Oxygen Demand (COD) in mgO\textsubscript{2}/L. The COD is a standard colorimetric measure [7], which uses a chemical compound (potassium dichromate) to oxidise all organic compounds in wastewater. The oxidation process consumes oxygen which is measured as a change in colour (by photo-spectrometry). In municipal wastewater, suspended carbon represents 590 mgO\textsubscript{2}/L COD [2]. Considering that suspended carbon can be removed through physical processes, it will not be further investigated in this work.

Dissolved carbon makes up the remaining 20-40 % of total organic carbons present in wastewater. Dissolved carbon compounds are produced from degraded proteins, suspended carbon and other chemicals present in the wastewater [1]. Dissolved organic carbon compounds can be defined as “complex” such as polysaccharides, or “simple” such as acetate (CH\textsubscript{3}COOH). In fact most complex organic compounds degrade to simple sugars such as acetate, propionate or butyrate, which make up to 90 % of dissolved organic carbons [1]. Dissolved organic carbon compounds are easily degraded by bacteria and are measured as Biological Oxygen Demand (BOD) expressed in mgO\textsubscript{2}/L. The BOD measurement is a standardised method [7] which measures the oxygen required by a consortium of bacteria to degrade dissolved organic carbon in wastewater over 5 days (BOD\textsubscript{5}). A typical wastewater contains 250-350 mg/L BOD [1]. The ratio of COD/BOD reveals the proportion of readily degradable organic carbon. In municipal wastewater this ratio range is from 2.4 [2] to 3.35 [8].
1.1.2 Source of nitrogen in municipal wastewater

In municipal wastewater, the total Nitrogen (N) concentration is around 3 mmol-N/L (42 mg-N/L) ranging from 25 mg/L to 53 mg/L [1]. Ammonium is the most common form of nitrogen (N) compounds in the inflow [1,2]. Ammonium (NH$_4^+$) is produced in the process of urea degradation, or from amine groups of degraded proteins [1]. The process for NH$_4^+$ removal produces nitrite (NO$_2^-$) and nitrate (NO$_3^-$) which are soluble species in the wastewater.

1.1.3 Source of phosphate in municipal wastewater

Phosphorus, as the chemical element (P), is not found in wastewater, however, phosphate (TP) is found in concentrations of approximately 0.48 mmol-P/L (15 mg-P/L)[1,2]. Phosphate comes from proteins in faeces and also from laundry products [1]. Phosphate removal will not be specifically addressed in this thesis, a brief overview of the current methods used for its removal are presented below.

1.2 Biological organic carbon and nutrients removal mechanisms

1.2.1 Biological organic carbon removal

Under aerobic conditions, Ordinary Heterotrophic Organisms (OHOs) degrade simple organic carbons (e.g. acetate) to produce adenosine triphosphate (ATP) as an energy source for growth. Acetate (i.e. electron donor) is oxidised to CO$_2$ in the aerobic respiration process depicted in Figure 1-1.

There is also a respiration mechanism which uses oxidised chemical species, other than oxygen, as electron acceptors (e.g. NO$_2^-$, SO$_4^{2-}$). This respiration is referred to as an anoxic respiration, and it differs from aerobic respiration because the ATP obtained from anoxic respiration is less (e.g. 30 % decrease in ATP for nitrate respiration [9]), which results in less biomass production [9-11]. In order to
stay in line with the literature, the term “anoxic” refers to an environment with electron acceptors other than oxygen [1,8] for example nitrite and nitrate in which denitrification can occur. This differs from the use of “anaerobic” conditions described an environment without oxygen or other electron acceptor [1,8].

Figure 1-1: Schematic diagram of the tri-carboxylic acid (TCA) cycle and electron transport chain in the mitochondria of bacteria. The respiration mechanism produces energy, as adenosine triphosphate (ATP), which permits growth of micro-organisms [12].
1.2.2 Biological storage for dissolved organic carbon removal

Another biological option to remove dissolved organic carbons from wastewater is dissolved carbon storage and the subsequent oxidation of the stored material [13,14]. The enrichment of biomass capable of carbon storage is a process requiring oscillating conditions, such as alternating oxidative (aerobic) and reducing (anaerobic) conditions. In an activated sludge, oscillating conditions are achieved by using feast and famine periods which results in a change in dissolved oxygen in solution [15,16]. Oscillating conditions selectively enrich specialised organisms called Glycogen Accumulating Organisms (GAOs) that effectively store soluble carbon [17]. This storage mechanism is known as a luxury uptake [18] and the aerobic/anaerobic oscillating conditions result in the biological activity as described below:

1. Under anaerobic conditions, GAOs ferment the glycogen present in their cells as an energy source to store dissolved organic carbon as poly-hydroxyalkanoates (PHAs)[18]. The PHAs formed are dependent on the dissolved carbon present [19]. For example, acetate produces poly-hydroxybutyrate (PHB) and/or poly-hydroxyvalerate (PHV) [20], while propionate storage results in the production of poly-hydroxy-2-methylvalerate (PH2MV) [21].

2. Once under aerobic conditions, GAOs oxidise some of the PHAs and use the resulting energy for growth [19]. The remaining PHAs form glycogen, which enables repeated dissolved organic carbon storage in subsequent anaerobic phases.

GAOs have been extensively researched [13,19,20,22,23]. More recently, two different approaches using these organisms have been attempted. One approach aims at maximising PHA production from wastewater. For example Jiang et al. [24] discovered and isolated a specialist bacteria *Plasticumulans acidivorax*, which
achieved 85 % (w/w) of PHB accumulation per gram biomass. However, the optimum growth temperature for this organism is 30 °C which is not applicable in wastewater treatment processes. The other approach has been to make use of GAO without trying to maximise PHA production (≤ 60 % w/w), but just as a means to reduce the energy expense that is associated with treatment processes, such as aeration and pH control [9,16,23,25,26]. In these researches, complete storage of the soluble carbon was reported. Carbon storage was usually combined with N removal and this will be discussed below.

1.2.3 Nitrogen removal: a two-step bacterial mechanism

The biological removal of dissolved nitrogen from wastewater requires the combination of two biological mechanisms: nitrification and denitrification. Nitrification is an aerobic process; while denitrification occurs under anaerobic conditions and requires an electron donor (e.g. organic carbon compounds). These reactions are mutually exclusive and tend to inhibit each other. The biological mechanism for each reaction is described below.

1.2.3.1 Nitrification

Nitrification is an aerobic chemo-autotrophic biological process that oxidises ammonium to nitrite, followed by nitrite to nitrate. It is accepted that there are two reactions that occur in the nitrification process [27]. Ammonium Oxidising Bacteria (AOB) include Nitrosomonas species [28] and catalyse nitrification: \( \text{NH}_4^+ \) oxidation to \( \text{NO}_2^- \) [27] (Eq. 1-1)

\[
\text{Eq. 1-1: } \text{NH}_4^+ + 1.5 \text{O}_2 \rightarrow \text{NO}_2^- + 2 \text{H}^+ + \text{H}_2\text{O}
\]

This reaction would create biomass and could be express as (Eq. 1-1a [29]).
55 NH₄⁺ + 76 O₂ + 109 HCO₃⁻ → C₅H₇NO₂ + 54 NO₂⁻ + 104 H₂CO₃ + 57 H₂O

The second reaction is nitratation which oxidises NO₂⁻ to NO₃⁻ [27] (Eq. 1-2 or Eq1-2a with the biomass produced [29]), this reaction is driven by Nitrite Oxidising Bacteria (NOB). NOBs include *Nitrobacter* and *Nitrococcus* species [28].

Eq. 1-2: \( \text{NO}_2^- + 0.5 \text{O}_2 \rightarrow \text{NO}_3^- \)

Eq. 1-2a: \( 400 \text{NO}_2^- + \text{NH}_4^+ + 195 \text{O}_2 + \text{HCO}_3^- + 4 \text{H}_2\text{CO}_3^- \rightarrow \text{C}_5\text{H}_7\text{NO}_2 + 400 \text{NO}_3^- + 3\text{H}_2\text{O} \)

As it appears from the above equations, the aeration requirement to oxidise NH₄⁺ to NO₃⁻ is 2 moles of O₂ per mole of NH₄⁺. The total air supply for the oxidation of 3 mmol-N/L NH₄⁺ (42 mg-N/L) present in municipal wastewater can be calculated using the following assumptions:

- molar volume constant value of 24.5 L/mol,
- 10 % O₂ transfer efficiency in liquid, and
- 21 % of O₂ in air.

To completely oxidise NH₄⁺ to NO₃⁻, the total volume of air required is 7 L per L of wastewater (L<sub>ww</sub>) (Table 1-1). One can expect to reduce this volume by 1/4 when stopping oxidation to NO₂⁻ (i.e. 5.25 L<sub>air</sub>/L<sub>ww</sub>). The nitrification process also produces acidity (Eq. 1-1) which is detrimental to the treatment process’s activities. However, wastewater has a pH ranging from 6.5 to 7.5 and contains a certain buffer capacity (average 200-300 mg CO₃²⁻/L [8,30]) which prevents excessive acidification of the wastewater.
Table 1-1: Calculation of the total air required for complete nitrification of ammonium (NH$_4^+$) of 3 mmol-N/L in wastewater to nitrate (NO$_3^-$).

*a* is the concentration in the inflow of 1 L of municipal wastewater

<table>
<thead>
<tr>
<th>NH$_4^+$ inflow (mmol/L)</th>
<th>O$_2$ for NO$_3^-$ prod. (mmol/L)</th>
<th>Molar volume (mL/mmol)</th>
<th>V. O$_2$ for NO$_3^-$ prod. (mL/O$_2$/L)</th>
<th>O$_2$ content in air (%)</th>
<th>Transfer efficiency (%)</th>
<th>Total air required (mL/O$_2$/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>6</td>
<td>24.5</td>
<td>147</td>
<td>21</td>
<td>10</td>
<td>7000</td>
</tr>
</tbody>
</table>

1.2.3.2 Denitrification

To complete N removal, nitrite and nitrate must be reduced. Denitrification is the biological process of reducing nitrite and nitrate to N$_2$ gas, using an electron donor under anoxic conditions so that it is used by NO$_2^-$ and/or NO$_3^-$. Commonly, electron donors are dissolved organic carbons and are already present in the wastewater. N$_2$ gas production is a result of multiple biological reactions (Eq. 1-3 to Eq. 1-6) and Eq. 1-7 presents the overall denitrification reaction that occurs with acetate (CH$_3$COOH) as an electron donor (8 electrons/acetate). Eq. 1-8 is an example established from the literature that demonstrate the stoichiometric equation for the denitrifying biomass production when using methanol (CH$_3$OH) as an electron donor [31]. In the absence of electron donors denitrification is inhibited [32].

It is possible to calculate the theoretical electron requirements for complete denitrification by comparing the electrons required to the ones available. To denitrify 3 mmol/L NO$_3^-$ (42 mg-N/L), produced from the ammonium in the inflow, 1.9 mmol/L acetate (15.8 mmol/L electron or 122 mg/L BOD) would be required (Eq. 1-7 and Eq. 1-8). The BOD in the inflow is 4 times higher than the required BOD for complete denitrification; therefore no external carbon source should be required. Note also, that the denitrification process reduces the need for buffering the
ammonium oxidation, because denitrification reactions consume 50% of the acidity produced by nitrification [8].

\[ \text{Eq. 1-3: } 8 \text{NO}_3^- + 2 \text{CH}_3\text{COOH} \rightarrow 8 \text{NO}_2^- + 4 \text{CO}_2 + 4 \text{H}_2\text{O} \]

\[ \text{Eq. 1-4: } 8 \text{NO}_2^- + \text{CH}_3\text{COOH} + 2 \text{H}_2\text{O} \rightarrow 8 \text{NO}_{(g)} + 2 \text{CO}_2 + 8 \text{OH}^- \]

\[ \text{Eq. 1-5: } 8 \text{NO}_{(g)} + \text{CH}_3\text{COOH} \rightarrow 4 \text{N}_2\text{O}_{(g)} + 2 \text{CO}_2 + 2 \text{H}_2\text{O} \]

\[ \text{Eq. 1-6: } 4 \text{N}_2\text{O} + \text{CH}_3\text{COOH} \rightarrow 4 \text{N}_2(g) + 2 \text{CO}_2 + 2 \text{H}_2\text{O} \]

\[ \text{Eq. 1-7: } 8 \text{NO}_3^- + 5 \text{CH}_3\text{COOH} + 2 \text{H}_2\text{O} \rightarrow 4 \text{N}_2 + 10 \text{CO}_2 + 8 \text{H}_2\text{O} + 8 \text{OH}^- \]

\[ \text{Eq. 1-8: } \text{NO}_3^- + 1.08 \text{CH}_3\text{OH} + 0.24 \text{H}_2\text{CO}_3 \rightarrow 0.056 \text{C}_5\text{H}_7\text{NO}_2 + 0.47 \text{N}_2 + 1.68 \text{H}_2\text{O} + \text{HCO}_3^- \]

An alternative to the nitrification and denitrification process was discovered about 20 years ago [33]. It is Anaerobic Ammonium Oxidation (Anammox) which utilises \( \text{NH}_4^+ \) as an electron donor for the denitrification of \( \text{NO}_2^- \) [34]. This is an anaerobic process and will not be discussed further.

1.2.4 Biological storage driven denitrification

In the nitrogen removal process, denitrification is the final step to remove nitrite and nitrate to \( \text{N}_2 \) gas. The denitrification process is not always successful because of excess oxidation of dissolved organic carbon during the ammonium oxidation phase [1,8]. Biological dissolved carbon storage can maintain the carbon in the reactor for longer, because stored carbon oxidation is slower than that of dissolved organic carbon [13,25,26]. The stored carbon is used as an endogenous electron donor source for denitrification, resulting in storage driven denitrification [9,14,35]. Carbon storage organisms include GAOs and Phosphate Accumulating Organisms (PAOs, which are discussed below) and have been used for storage driven denitrification [13,23,36]. Denitrifying GAOs (DGAOs) have been suggested to be different from
GAOs [35], yet Jones et al. [25] reported that the aerobic storage organism (GAOs) were capable of denitrification without acclimatisation time.

1.2.5 Phosphorus removal processes

This work focuses on a novel removal method for dissolved organic carbon and nitrogen and therefore phosphorus (TP) removal will not be investigated in this research. Some basic information on both chemical and biological phosphorus removal mechanisms conventionally used in wastewater treatment plants are presented below. The conventional method for phosphate removal is chemical precipitation with metallic ions, for example iron (Fe$^{3+}$) and aluminium (Al$^{3+}$). Fe$^{3+}$ and Al$^{3+}$ have a similar ability to precipitate phosphate out of solution with 10% of difference between the two metallic salts [37]. However, the precipitated phosphate is added to the sludge, and because of sludge age and bacterial processes, the precipitated phosphate in the sludge can be released back in the wastewater through biological processes [38,39] and makes the wastewater unfit for disposal. Chemical phosphate precipitation is expensive because of chemical purchase and usually there is an excess dosage because dissolved organic carbon tends to reduce the chemical phosphate precipitation efficiency [40,41].

Biological phosphate removal from municipal wastewater with AS without chemical addition was first reported by Srinath et al. [42] (> 90%). The mechanism was explained later as a biological luxury phosphate uptake [43]. The luxury phosphate uptake requires oscillating conditions to develop Phosphate Accumulating Organisms (PAOs) [44,45]. PAOs under anaerobic conditions will oxidise their stored poly-phosphate (poly-P) to gain ATP and store dissolved organic carbon within their cell as PHAs. The poly-P oxidation releases PO$_4^{3-}$ in solution. Thereafter, under aerobic conditions and in the absence of dissolved carbon source,
PAOs oxidise the stored PHA for growth, but also store the dissolved $\text{PO}_4^{3-}$ as poly-P. The overall bacterial mechanism stores either dissolved carbon (anaerobically) or $\text{PO}_4^{3-}$ (aerobically). Figure 1-2 is a schematic diagram of the PAO's cyclic process of dissolved carbon and phosphate storage. It is of interest to note that the storage of poly-P does not remove phosphate; it is the continuous harvesting of the biomass that results in phosphate removal from the system [3]. It is the failure of biological phosphate removal that led to the discovery of the GAOs [46] as described in Section 1.2.2. In fact, PAO's enrichment depends on the Phosphate to Carbon (P/C) ratio of the inflow of wastewater, at a P/C ratio $\geq 0.08$ mol-P/mol-C PAOs are selectively enriched, otherwise GAOs are enriched [46]. Finally, PAOs were also reported to be able to accomplish denitrification in oscillating anoxic and anaerobic conditions [36].

Figure 1-2: Simple schematic diagram of the operation of PAOs. Under anaerobic condition, stored poly-phosphate (poly-P) is oxidised to provide the energy for acetate (Ac) uptake. Acetate is stored as PHA. Under aerobic condition PAOs oxidise PHAs to CO$_2$ and simultaneously store dissolved phosphate to restore the poly-P pool required for dissolved carbon storage.
1.3 Wastewater treatment technologies for removal of dissolved carbon and nitrogen

The biological mechanisms for the removal of dissolved carbon, nitrogen and phosphate in wastewater are presented above. The operation of biological treatment plants will be discussed next. Wastewater treatment technologies are broadly separated in two categories:

1. Suspended biomass or Activated Sludge (AS), which consists of a concentrated biomass (called sludge) in solution to remove dissolved carbon and nutrients. The treated effluent and sludge are separated in a clarifier at the end of the treatment.

2. Biofilms, or attached growth, consist of biomass that grows on surfaces. These surfaces are maximised to increase the biomass concentration. This technology does not require wastewater and biomass separation step.

1.3.1 Suspended biomass technologies

1.3.1.1 Aerobic treatment systems

AS processes simply aerate wastewater and allow biomass to grow in the liquid by using dissolved carbon and nutrients, which result in dissolved carbon and nutrient removal. Thereafter the nutrient-free wastewater can be disposed of. However, the biomass cannot be discharged into the environment. The effluent flows in a clarifier that uses gravity to separate the solids (i.e. biomass) and the treated wastewater [2]. The effluent is disposed of and the retained biomass used for a rapid start-up of the treatment process [6]. The excess sludge produced is usually dewatered, treated for pathogen removal and then landfilled.
1.3.1.2 Combined aerobic and anaerobic systems

AS refers to multiple processes all of which have biomass in suspension. All the processes will not be reviewed in depth here, Grady et al. [6] and Tchobanoglou et al. [8] have effectively discussed and reviewed most of the current treatment processes. Two broad categories of treatment processes are touched upon below:

1. Continuous flow systems, and
2. Batch systems

Continuous flow systems are continuously fed with wastewater and the outflow is perpetually discharged. As wastewater flows through the reactor, different biological reactions occur in the reactor’s space. There are anaerobic and aerobic chambers through which the wastewater is moved until it reaches the outlet. One of the continuous flow systems is a plug flow reactor. Plug flow reactors start with an aeration system oxidising carbon to CO$_2$ by OHOs, and nitrifying biomass oxidising NH$_4^+$ to NO$_2^-$ and then produce NO$_3^-$. This aeration process oxidises the dissolved organic carbon and inhibits the denitrification process in the following compartment. When these systems were installed initially, additional carbon was added in the form of methanol or sugars [1,3]. Plug flow systems achieve 95 % COD removal but limited N removal [2] because of the incomplete denitrification. A reverse plug flow system was developed to optimise denitrification using endogenous dissolved carbon present in the wastewater. Initially, the inflow of wastewater is under anaerobic conditions and in contact with ‘returned’ wastewater containing nitrite and nitrate (Figure 1-3). NO$_2^-$ and NO$_3^-$ are reduced, and dissolved C is oxidised; therefore N removal is increased to > 85 % [47]. Thereafter, the wastewater is aerated and the remaining carbon and the ammonium are oxidised (and can be returned). The aeration step in the AS process represents about 50 % of the total energy used in the
treatment [48] and should be prevented. The clarifier step enables the separation of the biomass from the disposable treated effluent. The clarifying step achieves a reduction in suspended solid by 60 to 90% [2]. Some of the sludge is returned and is in contact with the inflow for rapid treatment, while the remaining sludge is disposed of after dewatering and pathogens removal.

Figure 1-3: Operation of conventional activated sludge. The nitrified wastewater returned to the front of the reactor enables denitrification using endogenous carbons. The subsequent aeration nitrifies the remaining \( \text{NH}_4^+ \).

In batch systems, the biological reactions (i.e. carbon oxidation, nitrogen oxidation and denitrification) are separated by providing different operating conditions (i.e. anaerobic and aerobic) over a given period of time. This is different to a plug flow, which separates the reactions through the reactor’s space. The operation of batch systems enables a better control of the carbon and nitrogen removal and also achieves biomass separation in a single reactor [2,49]. Sequencing Batch Reactors (SBRs) operate a reaction period composed of anaerobic and aerobic phases. The aeration phase can be modulated to achieve dissolved carbon and nitrogen removal. Modulating the aeration period prevents the complete oxidation.
of dissolved carbons, which maximises denitrification with endogenous dissolved carbon. Total carbon and > 98% of the dissolved nitrogen removal from synthetic wastewater were reported by controlling the aeration in an SBR [50]. After the reaction time, the biomass is separated in a settling period, and the supernatant is decanted, and a new batch of wastewater is added (Figure 1-4).

The batch system operation creates periods of feast and famine [51] which favours biomass granulation. Granules are hard conglomerates of different species of bacteria that do not easily break and settle rapidly, and in full scale treatments are referred to as granular sludge process [51,52]. Granular sludge process operates with a low Dissolved Oxygen (DO) concentration in liquid. The formation of oxygen and substrate gradients inside granules characterises this type of biomass [53]. The outer part of the granule is aerobic, and some dissolved carbon and ammonium are oxidised, resulting in a decrease in DO concentration inside the granule [53]. Inside the granule, because of the anoxic environment, denitrification will oxidise the remaining carbon and reduce the nitrite and nitrate formed in the outer part of the granule. The combination of the gradients and multiple micro-organisms results in Simultaneous Nitrification and Denitrification (SND) in the reactor under aerobic conditions. The granular sludge process achieves more than 90 % of the BOD removal and > 87 % (max 97 %) of the total N removal [54,55]. Granular sludge also reduces the operating cost by 7-17 % compared to conventional activated sludge [56]. However, this process still requires aeration in the bulk solution to transfer oxygen in the liquid which is an expensive process [48]. In addition, the process’ operation requires advanced technology and knowledge and cannot be easily implemented [51].
It is of interest to note that granular sludge has a ~93 % water content \[57\] while suspended biomass contains 99 % water. Low water content is beneficial to wastewater treatment operators because most of the cost associated with excess sludge disposal is proportional to its volume \[51,52\].

![Diagram of a sequencing batch reactor](image)

Figure 1-4: Operation of a sequencing batch reactor. The reaction phase includes both aerobic and anaerobic phases to remove nitrogen and carbon.

### 1.3.2 Biofilm reactors technologies

Biofilm processes use the growth of bacterial biomass on surfaces to remove the nutrients dissolved in wastewater. Biofilms for wastewater treatment have developed around the same time as AS processes 100 years ago \[58\]. In biofilm wastewater treatment plants, carriers for bacterial growth can be varied and cheap (e.g. rocks and pebbles) and result in a greater biomass retention. An increase in biomass speeds up the nutrient removal rate \[59\]. The support material for biomass growth can either be available material on site (e.g. rocks) or specially designed polypropylene material.
(e.g. propylene bio-balls, “random” carrier media) which maximises the surface area and maintains the active biomass in the reactor [60]. “Random” carrier materials have been developed in the past couple of decades and have brought biofilm reactors in competition with conventional AS reactors [61]. There are multiple biofilm processes that can be used for municipal wastewater treatment. The processes that will be discussed here are Trickling Filter (TF) and one of its variations, Biological Aerated Filter (BAF) and Rotating Biological Contactor (RBC).

1.3.2.1 Trickle filter

TF process is an example of an old biofilm technology (> 100 years old) that is still in use today [1]. It has the benefit of a low operating cost, reasonable treatment capacity and low maintenance and operator skills [2]. Conventional TFs have a reasonable efficiency to remove soluble carbon (70-85 %)[62], however, total nitrogen removal is usually limited or does not occurring at all [1]. In fact, conventional TFs are constantly aerobic, which completely oxidises dissolved carbon to CO$_2$ and ammonium to nitrite and nitrate. It is understandable that the rapid and complete oxidation of dissolved organic carbon in TF leads to nitrate building up in the effluent. As a consequence, TFs are designed for specific objectives, which usually do not involve the complete dissolved carbon and nitrogen removal. TFs can be used as a preliminary step for rapidly removing dissolved carbons from high strength wastewater [60] and the effluent is then further treated with AS processes. Alternatively, TFs can be used as a separate nitrification stage using specially enriched nitrifying biomass [60,63].

TFs can be operated as a single pass or multi-pass system. The single pass operation is the trickling of wastewater over the biofilm once before its disposal. To maximise nutrient removal, the rate of trickling is slow (Hydraulic Load Rate (HLR)
= 1-3.7 L/m²/d [64]), however, this has the disadvantage of the spatial distribution of the bacteria with respect to the biofilm depth. The organisms’ distribution in the reactor is due to the substrate gradient throughout the reactor. OHOs are enriched at the top because of the high availability of dissolved organic carbon, while the lower part of the biofilm is enriched with nitrifying biomass [8]. As a result of the biomass distribution in the biofilm, nitrate remains in the effluent and this results in total N removal efficiency of < 80 % [8]. The operation of a multi-pass TF allows for increased DO supply to the biofilm [3] such that the HLR can be increased (9.5-37 L/m²/d)[64]. In theory, multi-pass TF energy consumption is one-third to one-quarter of that of an AS process [65], however the application of the process can result in an equivalent consumption of energy to the AS process [65].

Conventional TF use rather coarse material for bacterial growth, making the treatment process quite large. Pebbles and rocks are carrier materials that have a low porosity value which means that to maximise the surface area for the biomass TFs are built on large land surface area. In addition, carrier materials are heavy and make it difficult to increase the treatment plant height (≤ 5 m) and therefore the diameter of the process can reach 10-20 m [64], which is not desirable in urban areas where land availability is scarce.

1.3.2.2 High rate trickle filter

To overcome some of the barriers associated with conventional TF, high rate TFs have been developed in the 1950s [1,2,64] and started with the development of novel carrier media made of plastics. Plastic media could be small plastic cylinders (called “random” [3]) or modular plastic sheets that can result in different flow directions (vertical or cross flow [60]). Some benefits of plastic media are a large surface area and high porosity (> 90 %) [2,3] which maximise biomass growth and reduce reactor
size. In addition, this media is light and results in increased reactor heights. High rate TFs have been successfully operated with a height of 12 m [2]. High rate TFs have reached BOD removal of 60-85 % [64,66] and, when it is a nitrifying trickle filter, a total N removal of > 98 % [63]. However, it is not widely applied in the municipal wastewater treatment industry because of odour problems and unreliable pathogen removal (20-90%) [2,8].

1.3.2.3 Biological aerated filter: a submerged biofilm technology

Biological Aerated Filters (BAFs) are reported to have been used throughout the 20th century, though their complete understanding and use were developed in the 1970s [58]. The main goal of BAFs is to remove organic carbon and/or ammonium and Suspended Solids (SS). The flow of this system can be varied from upflow or downflow [58]. The media used are numerous and have been tested for their efficiencies [67,68] but it will not be looked at here. BAF's reactors are filled with carrier materials that enable high bacterial growth and limit washout. The tight configuration of the carrier materials in BAF reactors results in filtration of SS [8].

One problem is the absorption of SS on the biofilm surface can lead to the inhibition of biological processes; for example 50 % decrease in nitrifying capacity [69]. Another problem is that the biomass oxidises carbon and ammonium using an air supply to the bulk liquid [8] which is expensive and should be avoided. In BAFs, the oxygen transfer is improved compared to an AS process by about 10 %, probably because of the longer residence time of the air due to the tortuosity of the tight packed biofilm [58]. Even though pure oxygen has been used to maximise the BAF's operations, in particular for nitrification [70], the aeration mechanism remains an inefficient process as discussed previously (Table 1-1). Considering the large amount of biomass present, the oxygen supplied can be immediately consumed and this
becomes the limiting step of the process, rather than either the biomass or the substrate availability as is the case in other reactor types [71]. BAF processes have been reported to remove 85-95% of SS [72], BOD removal can reach > 85% [8], and total N removal varies according to the operation setting. BAF can achieve N removal > 85% when using a low C/N ratio of wastewater [8]. The high biomass content in these reactors result in fast rates such that the operating time is significantly reduced by up to 1.5 h [72].

1.3.2.4 Rotating biological contactors

Rotating Biological Contactors (RBCs) are wastewater plants composed of multiple discs on a rotating shaft (Figure 1-5). The disc surface provides the support for biomass growth and the rotation of the disc is maximised 1-2 rpm to increase the DO in wastewater [3,73]. The DO is used to oxidise carbon and ammonium. Some plants add submerge blowers to increase the DO in wastewater [73]. The dissolved carbon removal can vary drastically over a year of operation from 60% to 95% [74] and the N removal efficiency is highly dependent on the dissolved organic carbon added to the reactor [71]. On energy considerations, the disc rotation with biomass represents the majority of the expense associated with this process and therefore presents 50% savings compared to AS processes [75]. However, the implementation of RBCs has been limited in the past 20 years [8].
1.3.3 Simultaneous nitrification and denitrification in biofilms for complete carbon and nitrogen removal

RBCs have been reported to achieve SND [71,76]. The biofilm structure throughout the biofilm thickness is homogenous (equivalent amount of heterotrophs, nitrifiers and denitrifiers) but the biomass activities differ in the biofilm structure [71]. The surface of the biofilm achieves nitrification and the lower part of the biofilm denitrifies the nitrite and nitrate formed [71]. However, the nitrogen removal efficiency remains towards 60 % even though SND occurred in the reactor, because of incomplete nitrification [71].

In generic terms, biofilm processes retain biomass and the continuous growth of the biomass results in a thick layer of biofilms. The thickness of the biomass results in an oxygen and a substrate gradient [59]. The oxygen gradient is widely acknowledged to significantly affect the biomasses in biofilm processes [59,71,73]. Gradient formation in biofilms has been the main reason for SND process in reactors that would normally not be able to achieve complete carbon and total N removals in
technologies such as TFs [77] or processes using plastic materials for the biomass growth [78].

1.3.4 Parallel nitrification and denitrification: a two sludges process to maximise removals efficiencies

Parallel Nitrification and Denitrification (PND) is a technology developed at Murdoch University [79]. This technology separates the carbon oxidising and the nitrifying biomasses in two biofilm reactors. The dissolved carbon removal mechanism uses a carbon storage biomass (GAOs). The resulting carbon-free wastewater is trickled over a nitrifying biofilm to produce nitrite and nitrate. The nitrite and nitrate wastewater is then returned to the carbon storage biofilm for denitrification which results also in the oxidation of the stored carbon (i.e. electron donor). This technology achieved > 80 % carbon storage and close to 100 % nitrogen removal [14]. The use of separate biofilm reactors maximises the rates of the individual processes (Denitrification rate = 2.36 mmol-N/L/h, nitrification rate = 1.1 mmol-N/L/h [14]). The nitrification reaction in this process used rapid recirculation rate and liquid recirculation from one reactor to the other resulting in large amounts of energy consumed, estimated similar to AS processes [80].

1.4 Zeolite: an additional nitrogen removal process

Nitrogen compounds are the limiting factor for algae growth in the marine environment and are to be kept at low concentrations in the discharge effluent of municipal treatment plants. Nitrogen discharge regulations is becoming increasingly stringent (≤ 5mg/L) [81] in particular in areas of high environmental sensitivity such as the Great Barrier Reef in Australia. In order to maintain constant low nitrogen level in the effluent of biological treatment processes, zeolite can be used to remove dissolved ammonium from wastewater [82-84].
1.4.1 Zeolite structural characteristics for ammonium removal

In addition to the biological processes described previously, dissolved ammonium removal can be achieved through an ion-exchange process with a natural material: zeolite [85,86]. Zeolites have also been studied for their ability to remove other chemicals such as heavy metals [86,87] but this will not be looked at here. Zeolites are quarried throughout the world, which makes zeolite an ideal candidate for ammonium removal compared to manufactured ion-exchange materials, such as resins, which require high purity chemicals for their production [88].

Zeolites are natural hydrated aluminosilicate rocks with ion-exchange properties. The ion-exchange property is created by the zeolite’s chemical structure, which is composed of multiple tetrahedrons made of oxygen; and each tetrahedron contains either a silicon (Si) or aluminium (Al) atom in its centre (Figure 1-6). The combination of these atoms creates a negatively charged structure, which adsorbs cations, such as sodium (Na\(^+\)), to maintain the charge balance. Once zeolites are in contact with wastewater, Na\(^+\) in the structure is exchanged with other cations in solution, mainly NH\(_4^+\), resulting in a net adsorption of NH\(_4^+\) [86]. Once the zeolite structures are filled with NH\(_4^+\), it is said to have reached its maximum Cation Exchange Capacity (CEC). However, the maximum CEC is not a practical value, because a large concentration of ammonium would remain in the effluent before reaching this value [85,89].
1.4.2 Zeolite composition impact on adsorption capacity of different cations

Cation adsorption by zeolite differs with respect to the cation species, at equimolar concentrations, some cations are preferentially adsorbed on zeolite. The cations adsorbed more are said to have a higher affinity. Cation affinity can be determined from an isotherm curve [90,91]. The isotherm curve is usually determined by multiple equilibria, and uses only two cation species in solution to determine the ratio in solution and on zeolite after equilibrium is reached (i.e. binary equilibrium experiments). From the isotherm curves, the selectivity coefficient (alpha, $\alpha$) is determined with respect to another cation [90,92,93]. In the case of an isotherm being a straight line from 0 to 1 (Figure 1-7), then $\alpha = 1$, which means that the two cations tested are adsorbed equally on zeolite at equimolar concentrations. An isotherm curve above the unity curve shows $\alpha > 1$ which means that the cation tested is more readily adsorbed on zeolite compared to the other, and the opposite if $\alpha < 1$.

Figure 1-6: Schematic diagrams of the repeating unit in zeolites where $n$ represents the number of repeats. Note that because of the tetrahedron with aluminium, the overall structure has two negative charges.
According to affinity values, one can organise cations in a sequence of the most to the least readily adsorbed, this is called an affinity sequence. However, according to the zeolite’s origin, affinity sequences differ because of the composition in aluminosilicates. The variation in aluminosilicates and therefore affinity resulted in different names for zeolites; for example there are Clinoptilolite, Chabazite and Modernite [86,88]. The most appropriate zeolite for ammonium removal is Clinoptilolite [85] because its affinity sequence has NH$_4^+$ amongst the most preferred cations [82,93]:

$$\text{Cs}^+ > \text{Rb}^+ > \text{K}^+ > \text{NH}_4^+ > \text{H}^+ > \text{Na}^+ > \text{Ca}^{2+} > \text{Fe}^{3+} > \text{Li}^+$$

Clinoptilolite has been used to remove ammonium from municipal wastewater [82,85,94,95]. Dissolved ammonium removals range from 65 to 98% of the ammonium inflow [82,83,95].

![Figure 1-7: Theoretical Langmuir isotherm using two hypothetical cations A\(^+\) and B\(^+\). The α value from the curves are: 0.25 (●), 1 (▲) and 4 (■).](image-url)
1.4.3 Zeolite’s cation adsorption process

The net adsorption rate of ammonium is the sum of the adsorption rate and desorption rate. As the adsorption rate is greater than the desorption rate, this results in a decrease in NH$_4^+$ in solution, which is a net ammonium removal. The rates are dependent on the ammonium molecule diffusion to the cation exchange sites [83,92].

The adsorption rate ($V_{ads}$) is dependent on an empirical adsorption constant ($K_{ads}$) and the concentration of cations in solution ([A$^+$]$_S$ in mol/L) (Eq. 1-9). The desorption rate ($V_{dsp}$) is dependent on an empirical desorption factor ($K_{dsp}$) and the amount of cations on the solid surface ([A$^+$]$_Z$ in meq/g) (Eq. 1-10). Where meq is milli-equivalent, it is the amount of cation (in mmol) multiplied by the electric charge(s) of the cation. The overall rate ($V_{overall}$) is the adsorption rate minus the desorption rate (Eq. 1-11). At equilibrium the adsorption and desorption driving forces are equal, therefore the overall rate is 0 (Figure 1-8).

\begin{align*}
\text{Eq. 1-9:} & \quad V_{ads} = K_{ads} \times [A^+]_S \\
\text{Eq. 1-10:} & \quad V_{dsp} = K_{dsn} \times [A^+]_Z \\
\text{Eq. 1-11:} & \quad V_{overall} = V_{adsorption} - V_{desorption}
\end{align*}
Figure 1-8: Schematic diagram of the ion exchange process of one cation (triangle) in solution with a cation present on the resin (star). At time initial the adsorption ($V_{Ad}$) driving force is high (thick arrow) and the desorption ($V_{Dep}$) driving force is low (thin arrow) resulting in a net adsorption of triangles. At equilibrium both driving forces are equal (same arrows) hence the ion concentration in solution is constant.

1.4.4 Ammonium adsorption in a dynamic flow of wastewater

As mentioned above, adsorption depends on the cation diffusion [83,92]. In a wastewater treatment plant, if the flow rate is faster than the cation diffusion, the adsorption will be limited by the wastewater flow rate. The cation diffusion rate benefits from an increase in surface area of the zeolite particles [85,86]. In fact, Wen et al. [96] reported an increase in ammonium removal rate by 4.1 times, by increasing the surface area while maintaining the same flowrate. Furthermore, zeolite pre-treatment results in better adsorption, for example, washing the zeolite with excess Na$^+$ can result in an immediate ammonium adsorption and increase its removal by 15% [91,97].
1.4.5 Zeolite regeneration mechanisms

Dissolved ammonium removal from wastewater cannot be achieved repeatedly once zeolite is filled with adsorbed ammonium [85,98]. Zeolite requires regeneration, which is the removal of the adsorbed ammonium from the exchange sites in zeolite structure. One method is chemical regeneration, which uses cation (e.g. sodium, Na\(^+\)) rich solution (i.e. regenerant) to desorb adsorbed ammonium on zeolite. The desorption of NH\(_4^+\) by Na\(^+\) requires high concentration to maximise the Na\(^+\) adsorption rate (Eq. 1-8). Sodium is widely available as a chemical from brine and sea water, however, there are two main drawbacks to chemical regeneration. The first is that brine and sea water contain other cations. Some of the other cations (e.g. potassium) will have a higher affinity than ammonium, and will remain in the regenerated zeolite decreasing ammonium adsorption. Thus, the chemicals used for regeneration are more expensive and can reach up to 40\% of the treatment cost of an ion exchange process [99]. The second is that the brine resulting from the regeneration contains ammonium which is still untreated. The treatment of ammonium-rich saline water is not efficient because nitrification and denitrification are inhibited by high salinity [99,100].

Another possible mechanism for removal of adsorbed ammonium is biological regeneration (i.e. bio-regeneration). This mechanism uses the biological nitrification process described previously (Section 1.2.3.1). The principle is the oxidation of adsorbed ammonium, a cation, to nitrite and/or nitrate, an anion, which cannot be adsorbed onto zeolite. After oxidation, the zeolite does not contain ammonium and therefore can resume ammonium adsorption. Nitrite and nitrate produced from the bio-regeneration can be denitrified prior to the wastewater disposal. Repeated zeolite
bio-regeneration has not been reported to significantly change the adsorption of ammonium in subsequent cycles by up to 20% [82].

The nitrifying biomass oxidises dissolved ammonium, therefore adsorbed ammonium must be desorbed to complete bio-regeneration of the zeolite. The desorption mechanism can be maximised by using regenerant solution (less concentrated than chemical regeneration), such as saline solution (NaCl) [82], or buffer solution (NaHCO₃) [101], to prevent the pH drop caused by nitrification (Eq. 1-1). One shortcoming of the bio-regeneration is that nitrifying biomass requires aeration for the ammonium oxidation process. The aeration mechanism is an expensive and inefficient process (Table 1-1) and should preferably be avoided. Overall, clinoptilolite is a type of zeolite that has promising characteristics to remove ammonium from wastewater; however, the regeneration process can be expensive, whether it is chemical or biological.

Electrolysis can be used to oxidise ammonium in solution to N₂ gas [102,103]. In wastewater, ammonium concentration is relatively low and increases the electrolysis time, but ammonium concentration can be increased through adsorption and desorption of ammonium on zeolite [101]. This mechanism can be applied rapidly and can treat ammonium effectively [100]. However, this work focuses on biological activity to achieve carbon and nitrogen removal through biological catalyst pathways, while electrolysis uses electricity produced from fossil fuels, and requires desorption of the ammonium using a regenerant solution.

In summary, dissolved carbon and nitrogen compounds removal rely on different bacterial activities. A mixture of bacteria oxidise dissolved carbon to CO₂, NH₄⁺ to
NO$_2^-$ and NO$_3^-$ and reduce NO$_2^-$ and NO$_3^-$ to N$_2$ gas. Conventional treatment technology design (i.e. AS) has been developed to maximise the bacterial activities to remove the dissolved C and total N. However, the energy cost associated with these methods is usually quite important. At least 50% of the energy consumed is for the oxygen transfer to the liquid for oxidation. In addition, in AS the sludge content is maintained around 5 g/L which is one reason for a relatively lengthy treatment process of 12 h. Biofilm processes maximise the biomass retention and can operate at a faster rate, thus reducing the treatment time. However, the effluent quality is not sufficient for disposal, and some of these biofilm processes (e.g. BAFs) still use liquid aeration of the system to achieve their removal efficiencies. The energy expense, related to the aeration, in these types of pre-treatment processes should be avoided.

1.5 Derivation of the thesis objectives

This thesis aims at using a biofilm grown on packing material to reduce the energy cost associated with the treatment process of ammonium and carbon removal. The research presented here will test for an alternative treatment method with high carbon and total nitrogen removal rates, and reduced aeration costs associated with dissolved carbon and ammonium oxidation. The novel treatment system will be presented as a proof of concept, hence this research will only use synthetic wastewater. The use of real wastewater and the cost estimation of applying the novel treatment are beyond the scope of this work. The objectives of the thesis were developed as follows:

1. Develop a dissolved carbon removal process using biological carbon storage (Chapter 2).
2. Verify the capacity of zeolite to remove ammonium in wastewater and achieve its biological regeneration (Chapter 3 and 4).

3. Test for a combined process of carbon and ammonium removal from wastewater, using a zeolite amended biofilm reactor (Chapter 5).

4. Investigate the synthesised biofilm sustainability and its operation (Chapter 6).

5. Optimise the zeolite amended biofilm process for carbon and ammonium removal from wastewater (Chapter 7).
Chapter 2  Organic carbon removal from wastewater by a PHA storing biofilm using direct atmospheric air contact as oxygen supply

2.1 Introduction

Biological wastewater treatment aims at removing organic carbon (Biological Oxygen Demand, BOD) and nutrients: nitrogen (N) and phosphate (TP). Most recent research has focused on the effective removal of nutrients as they are responsible for eutrophication. However, the biological aerobic removal of the BOD from wastewater is the main energy cost to the activated sludge (AS) treatment plant operator [48]. Significant energy is required for the supply of poorly soluble oxygen from the atmosphere into the bulk solution. For example, the removal of 10 Cm mol/L dissolved organic pollution (320 mg/L BOD) requires 0.245 L of Dissolved Oxygen (DO) available to 1 L of bacterial suspension. Assuming a practical oxygen transfer efficiency of 10 % and an air oxygen content of 21 %, a treatment plant would provide about 50 times the oxygen volume as air (about 11.7 L of air) per L of wastewater. As air has to be provided under sufficient pressure to lift a water column of typically 5 m high, it is understandable that despite advances in air supply technologies, the energy cost for air supply is high (570 J/L-wastewater).

The oxygen supply to the biomass in AS processes also initiates the oxidation of ammonia (NH$_4^+$) to nitrite (NO$_2^-$) or nitrate (NO$_3^-$), which represents an additional oxygen demand [104]. A wastewater with a typical C/N ratio content of 6 g-C/g-N [105] approximately requires an additional 30 % of oxygen for the oxidation of NH$_4^+$ to NO$_2^-$. While this component of air supply may seem unavoidable in the case of traditional N removal by nitrification and denitrification, there are current trends

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1 This Chapter was published in Bioresource Technology 187, pp 182-188
suggesting alternatives for N removal. These include the anaerobic ammonia oxidation (Anamnox) [106] and other forms of nitrogen recovery, for example completely autotrophic nitrification over nitrite (CANON) [107,108].

In principle, bacteria tend to store organic compounds (poly-hydroxy-alkanoates, PHA) if there are limiting growth factors, which prevent organisms from using the BOD as a source for biomass growth and energy [109]. In wastewater, where inorganic nutrients are typically available, the key mechanism that provokes bacteria to store BOD, as PHA, is the short term depletion of oxygen [110,111]. In the literature, conditions of alternating oxygen supply have been demonstrated to encourage the AS biomass to gradually build up reservoirs of reducing power in the form of PHA [13,112]. PHA must be oxidised to restore the storage capacity [112].

In this chapter we describe the use of anaerobic storage of dissolved carbon for removing BOD compounds without the costly transfer of oxygen into the bulk wastewater. The approach is to selectively develop and maintain a biofilm rich in PHA accumulating bacteria and to provide it with oxygen by draining the reactor and thus enabling direct contact of the bacterial biomass (here biofilm) with the atmosphere.

2.2 Materials and methods

2.2.1 Reactor dimensions and set-up

A cylindrical 2 L PVC reactor (12cm Ø and 29cm height) with openings at the top and bottom (Figure 2-1) was filled with packing material (AMB™ Biomedia Bioballs with a specific surface area of 850 m²/m³ and approximate active surface of
500 m²/m³) such that the material filled the entire volume of the reactor. The volume taken by the packing material was 300 mL such that the working volume of the reactor without biomass was 1.7 L.

2.2.2 Reactor operation

Prior to operation, the described reactor was inoculated with AS and biomass from a previously used biofilm reactor for storage driven denitrification [14,113]. After seeding, the reactor was operated automatically by specifically timed phases (Table 2-1). The reactor was filled with synthetic wastewater (180 mL/min) then maintained under anaerobic conditions for 2 hours, with liquid recirculation to maximise the contact of substrate and biomass (50 mL/min). The anaerobic phase was followed by gravity drainage of 10 min. This allowed air penetration of equal volume to the liquid drained. Thereafter, further air intake was prevented using a solenoid, and the volume of air was recirculated for 1 hour. The oxygen in the head space was measured by a DO probe (Mettler Toledo, InPro 6800).

The reactor operated on a sequence of fill and draw, which has been used for different reactor [8] for example sequencing batch reactor (SBR). However, compared to other “fill and draw” reactors, the system of fill and draw used here allows for the oxygen to be in contact with the biomass. This could reduce significantly the energy cost associated with aeration of liquid. This system will be referred to as passive aeration. “Passive aeration” has been used in the literature to describe a process which maximise the transfer of oxygen in the atmosphere to the liquid without using any aeration mechanism [114]. Therefore, in line with this definition, the aeration process tested here is described as a passive aeration mechanism.
Two probes (pH and ORT) were used to measure and continually recorded the values into a spreadsheet, using a LabJack U12 data acquisition card and the process control software LabVIEW<sup>TM</sup> (version 7.1 National Instrument) (Figure 2-1).

![Diagram of operated reactor for BOD to PHA storage using a biofilm](image)

**Figure 2-1**: Schematic diagram of the operated reactor for BOD to PHA storage using a biofilm.

**Table 2-1** Time schedule of operation of the BOD to PHA storage biofilm reactor in sequencing batch mode.

<table>
<thead>
<tr>
<th>Operation time (min)</th>
<th>Phase</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 5</td>
<td>Fill</td>
<td>Replacing air space by synthetic wastewater</td>
</tr>
<tr>
<td>5 - 125</td>
<td>Store</td>
<td>Uptake of soluble BOD as PHA under water circulation</td>
</tr>
<tr>
<td>125 - 135</td>
<td>Drain</td>
<td>Replacing treated wastewater by air</td>
</tr>
<tr>
<td>135 - 195</td>
<td>Vent</td>
<td>Provide air for oxidation of stored organics</td>
</tr>
</tbody>
</table>
2.2.3 Synthetic wastewater and trace element solution composition

A synthetic wastewater was used, which consisted of (mg.L\(^{-1}\)): CH\(_3\)COONa 660, NH\(_4\)Cl 160, NaHCO\(_3\) 125, KH\(_2\)PO\(_4\) 44, MgSO\(_4\).7H\(_2\)O 25, CaCl\(_2\).2H\(_2\)O 300, FeSO\(_4\).7H\(_2\)O 6.25, yeast extract 50, and 1.25 mL.L\(^{-1}\) of trace element solution, which contained (g.L\(^{-1}\)): ethylene-diamine-tetra-acetic acid (EDTA) 15, ZnSO\(_4\).7H\(_2\)O 0.43, CoCl\(_2\).6H\(_2\)O 0.24, MnCl\(_2\).4H\(_2\)O 0.99, CuSO\(_4\).5H\(_2\)O 0.25, NaMoO\(_4\).2H\(_2\)O 0.22, NiCl\(_2\).6H\(_2\)O 0.19, NaSeO\(_4\).10H\(_2\)O 0.21, H\(_3\)BO\(_4\) 0.014 and NaWO\(_4\).2H\(_2\)O 0.050.

2.2.4 Analytical

2.2.4.1 Acetate analysis

An Agilent 7820A Gas Chromatograph (GC) with auto-sampler was used to quantify acetate concentrations. Samples were acidified with formic acid (1 % (v/v)) before 0.4 μL samples were injected onto an Alltech ECONOCAP\textsuperscript{TM} EC\textsuperscript{TM} 1000 column (15 m x 530 μm (i.d.) 0.25 μm). The carrier gas (N\(_2\)) was set at a flow rate of 3 mL/min and at the inlet the sample was split 10:1. The oven temperature was programmed as follows: initial temperature 70 °C; temperature ramp 5 °C/min to 100 °C; held for 2 min; temperature ramp 70 °C/min to 230 °C; held for 2 min (to remove residual). Injector and detector were set at 200 °C and 250 °C respectively. The peak area of the Flame Ionisation Detector (FID) output signal was computed via integration using the EzChrom Elite Compact software (© 2005, V.3.3.2SP2). The detection limit determined was 0.5 μmol/L of acetate.

Chemical Oxygen Demand (COD) was determined by the closed reflux colorimetric method according to the standard method [7]. COD readings were obtained against known concentrations of acetate in wastewater (1 to 10 mmol/L).
2.2.4.2 Poly-hydroxybutyrate analysis

Poly-hydroxybutyrate (PHB) was extracted from the biomass using a method adapted from Smolders et al. [115]. The samples were esterified in 1:4 concentrated HCl:propanol solution for 2 h at 100 °C in a water batch. The culture tubes were sealed with Teflon lids to prevent loss of volatile solvents. Aliquots of 3 mL of DI water were used to clearly separate the organic and the aqueous layers. The organic layer was transferred to a GC vial for analysis. Similarly, standards of 0, 3.3, 6.6, 9.9, 13.2 mmol/L beta-hydroxybutyrate were prepared using a stock solution of 200 mmol/L HB (Sigma-Haldrich).

After the above steps of PHB hydrolysis and esterification of the hydrolysed products, hydroxybutyric acid, the resulting ester (propyl-hydroxy-butanoate), was analysed using the same GC and column as above with the following conditions. The sample was split at the inlet 5:1. The oven temperature program was: initial temperature 80 °C; temperature ramp 70 °C to 152 °C ; temperature ramp 4 °C/min to 160 °C; temperature ramp 70 °C/min to 230 °C held for 2 min.

The PHB chromatogram produced two additional peaks at higher retention times. These peaks were assumed to be hydroxy-valerate (PHV) in two different isomeric forms. The amount of the two additional peaks was estimated from the HB standard curve using benzoic acid as an internal standard.

2.2.4.3 Glycogen analysis

Biomass was collected and freeze-dried (Hetosicc-CD 4) between 10 and 20 hours. Dried biomass was accurately weighed in a digestion tube. The biomass was then digested in a solution of 0.9 mol/L HCl for 4h at 100 °C in a water bath. The insoluble biomass was removed and the pH of a 3 mL supernatant of the digested
solution was adjusted to 7.2 (±0.2) using 0.35 mL of 10 mol/L NaOH and 0.5 mL of 0.9 mol/L KH$_2$PO$_4$. The sample was tested by an enzymatic glucose analyser (AccuCheck) against a linear glucose standard curve (0 to 10 mmol/L glucose) [116].

Both the glycogen and the PHB analysis were conducted in a single experiment to achieve a carbon balance including PHA, glycogen and acetate. The large quantity of the total biomass prevents representative sample, therefore, this experiment measured the change in carbon compounds on a subsample of the total biomass. Under normal operating conditions (i.e. continuous flow) changes in stored products (PHA and glycogen) were difficult to observe, therefore the change was maximised by extended period of time for bacterial storage.

### 2.2.4.4 DNA analysis

DNA from the reactor and from AS were extracted using Power Soil DNA analysis extraction kit (MO-Bio). The DNA was stored at – 20 °C until further analysis. Variable regions of the bacterial 16S rRNA gene were amplified by barcoded pyrosequencing as previously described in Coghlan et al. [117]. Briefly, universal bacterial fusion primers [118] were used to generate PCR amplicons in triplicate and pooled. The forward primer F515 (5´ GTGCCAGCMGCCGCGGTAA 3´) and the reverse primer R806 (5´ GGACTACHVGGGTWTCTAAT 3´) targeted the V4 hypervariable region of the 16S rRNA. PCR was carried out in a 25 µL total volume including 4 µL of template DNA, containing: 2.5 mmol/L MgCl$_2$ (Fisher Biotec, Aus), 1× Taq polymerase buffer (Fisher Biotec, Australia), 0.4 µmol/L dNTPs (Astral Scientific, Australia), 0.4 mg BSA (Fisher Biotec, Australia), 0.4 µmol/L of each primer, and 0.25 µL of AmpliTaq Gold DNA polymerase (ABI). The PCR conditions included: initial denaturation at 95 °C for 5 minutes, followed by 40
cycles of 95 °C for 30 s, 54 °C 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 minutes (Corbett Research, NSW, Aus). Amplicons were purified (AMpure beads, Invitrogen) and DNA concentration estimated by ethidium gel staining to approximate equimolar concentrations for emulsion PCR. Bead:template ratios for the emulsion were determined by qPCR [119]. The Roche GS Junior run set up included an emulsion PCR step, bead recovery, and the sequencing run. All of these procedures were carried out according to the Roche GS Junior protocols [120]. In order to screen for high quality sequences, the sequencing output files were processed as described in Coghlan et al. [117]. This yielded 269 and 165 high quality sequences for the reactor’s biomass and the AS respectively. The resultant BLAST files were imported into the program MEtaGenome ANalyzer (MEGAN version 4.62.1) [121] for taxonomy using the following lowest common ancestor parameters: min score of 65, top percent of 5, and min support of 1.

2.3 Results

2.3.1 Development of a biofilm removing soluble acetate anaerobically

For the purpose of developing a biofilm reactor that specifically selects for bacteria that maximise the BOD (i.e. acetate) storage from the inflow, rather than oxidising the acetate (as observed in AS and in trickling reactors), strict selective conditions were applied after seeding the reactor. The selective conditions entailed the provision of acetate in the absence of oxygen, followed by providing oxygen in the absence of dissolved acetate. To accomplish this, the reactor was operated in a sequencing batch mode for 2 months as follows:
• anaerobic flooding of the biofilm with synthetic wastewater using acetate (16 Cmmol/L 512 mg/L BOD) as the carbon source for 2 hours
• draining of the synthetic wastewater after complete acetate storage as PHA
• passive aeration of the biofilm by keeping it drained in the presence of atmospheric air for 1 hour

The exposure to air was for the purpose of providing oxidative power to the biomass to oxidise the stored dissolved carbon (i.e. PHA) and by-passing the costly transfer of oxygen to the bulk liquid. This oxygen exposure to the biomass was expected to form glycogen from PHA thus enabling carbon uptake (i.e. acetate) in the subsequent anaerobic phase [18]. The continued operation under this scheme of anaerobic storage and biomass exposure to air was expected to selectively enrich the biomass with bacterial species capable of effective anaerobic acetate storage.

The reactor was operated continuously and its anaerobic acetate storage monitored (Figure 2-2). After only partial storage of acetate at the beginning (3 Cmmol/L acetate or 384 mg/L BOD, over 2 hours), the rate and storage capacity of acetate improved after 9 weeks of operation, reaching a storage rate of 10 Cmmol/L/h acetate (320 mg/L/h BOD) and a storage capacity of 40 Cmmol/L (1280 mg/L BOD). The storage mechanism was possible without liquid recirculation and the rate was only decreased by 10 % (Appendix A). The test without liquid recirculation provided an insight on the capacity of the reactor to remove soluble carbon, but was beyond the scope of the chapter. Clearly, the removal rate as well as the mass of acetate taken up exceeded the capacity of typical AS processes achieving < 1 Cmmol/L/h and 128 mg/L/h BOD respectively [122].
To eliminate the possibility of acetate being converted to another soluble organic compound, COD analysis was carried out in parallel to acetate analysis of the effluent. No evidence of organic species other than acetate was found (data not shown).

![Acetate Concentration vs Time](image)

**Figure 2-2**: Acetate storage after 2 (●), 8 (■) and 9 (▲) weeks of operation. The acetate supplied was lowered after 2 weeks, from 12 Cmmol/L to 7.5 Cmmol/L, to prevent the development of non-storing bacteria during the aerobic phase when acetate is present in the water.

### 2.3.2 Specialised biofilm with GAO and its metabolism

In general, the intermittent supply of oxygen is known to lead to BOD storage as PHA by Phosphate Accumulating Organisms (PAOs) [123]. PAOs accumulate phosphate as an energy store under aerobic conditions. This is then hydrolysed and released under anaerobic conditions providing sufficient energy for BOD uptake and its polymerisation as PHA. However, in the present reactor the aerobic phosphate accumulation cannot occur because in the aerobic phase the bulk liquid containing phosphate was drained. As a consequence, an alternative anaerobic energy source to polyphosphate must be used to store dissolved carbon in the biofilm described here.
The known alternative to PAO metabolism is the metabolism of the Glycogen Accumulating Organisms (GAOs). They synthesize glycogen from PHA under aerobic conditions, which serves via fermentation as the energy source for BOD uptake and PHA storage under anaerobic conditions [18]. Clearly, our biofilm operation would be likely to select for GAO rather than the traditional PHA storing PAO. Furthermore, at the low P/C (Pmol/Cmol) ratio of ≤ 0.02 used in our experiments PAO would be outcompeted by GAO [124,125].

After 9 months of operation a biomass sample from the biofilm, and AS from Woodman Point Wastewater Treatment (Perth, Australia) were used for DNA extraction and sequencing. The aim was to compare obvious differences in biomass composition (Figure 2-3). In the biofilm reactor the second largest population was Candidatus competibacter (10.7 %) which is a known GAO [46,126]. In theory, this could be expected because of the selective operation of the system offering anaerobic conditions with acetate followed by an aerobic environment without acetate (and without phosphate which could otherwise lead to PAO). However, the presence of the genus Haliangium is unusual (~40 %), as these belong to the myxobacteria species, which are known as predators to other bacteria [127]. Similar to GAOs, myxobacteria also have their food source in the aerobic phase, while other typical heterotrophs have no organic carbon food supply after draining. This aerobic feeding of predators in the biofilm provides one possible explanation for the low sludge production observed. Representatives of PAO (C. accumulibacter) were not detected. This is because PAO requires oxygen and phosphate together [111,128] whereas in the reactor, after the liquid is drained for aeration, the phosphate has also been drained.
A proper carbon balance including PHA, glycogen and acetate would be able to evaluate whether the biofilm behaviour is in accordance with GAO metabolism. The large volume of the biofilm biomass in the reactor prevents representative sampling which is needed for carbon balance purposes. Therefore, after thorough mixing of the reactor’s biomass, a subsample was used for the carbon balance experiment. An extended aerobic period and an increased dose of acetate was provided to generate changes in the overall storage products (PHA and glycogen) that were sufficiently large to show significant differences against the background storage products. The time provided in this batch experiment was increased compared to the continuous flow experiment to maximise the change in storage products.
To test whether the carbon removal behaviour under anaerobic conditions was consistent with the glycogen metabolism of typical GAOs, a simple carbon balance was established. The biofilm subsample (5.6 g dry biomass) was first aerated for 6 hours. Overnight, it was then suspended anaerobically in a solution of synthetic wastewater (100 mL) with excess acetate (20 Cmmol/L) to record glycogen and acetate conversions to PHA (Figure 2-4). Overall it was found that the carbon balance was maintained throughout this anaerobic phase. The glycogen oxidation (i.e. decrease) provides the ATP source required for acetate uptake, and the resulting production of PHA (Figure 2-4), as expected from the literature [19,111,126]. In the anaerobic period 1.0 Cmol of PHAs was produced per Cmol of the combined reactants, acetate and glycogen. This is in line with the reported anaerobic PHA yield ranging from 0.87 to 0.99 Cmol of PHB produced per Cmol consumed (VFA + glycogen) [129,130].

Under aerobic conditions, the carbon balance was reasonably conserved indicating a glycogen production of 1.1 Cmmol_{glyc}/Cmmol_{PHA}, which is slightly higher than the result of 0.8 obtained by Filipe et al. [126] and similar to the value of 1.0 by Liu et al. [131]. The expected carbon loss as CO₂ originating from carbon respiration could not be accounted for in the carbon balance.

Overall the results show that the biofilm behaviour is in line with the GAO metabolism demonstrated in the literature [19,126]. Anaerobically, the carbon taken up via acetate was accounted for by the combination of PHA gain and glycogen loss. Aerobically, carbon usage for glycogen production was similar to the carbon release from PHA consumption.
2.3.3 Direct passive oxygen supply to the drained biofilm

Once soluble acetate was removed from the wastewater, the liquid was drained by opening the bottom and the top of the reactor (valve operated system) to allow air to fill the void volume. This allowed the microbial cells to be supplied with oxygen while bypassing costly oxygen transfer to the bulk solution. The reactor was then closed to provide a reproducible amount of oxygen for all trials.

Considering the liquid volume contained in the reactor was approximately 1 L, then there is 210 mL or 8.5 mmol of O₂ available for oxidizing 8.5 Cmmol of acetate stored. Mass balance showed that, of the acetate removed from solution (12 Cmmol/L), only about half was oxidised by oxygen (Figure 2-5). Therefore, carbon accumulated within the system either in the form of biomass or alternatively as storage material (e.g. glycogen).
Figure 2-5: Typical behaviour of a single cycle of the storage biofilm reactor during anaerobic acetate storage (●) (0-2 h) and calculated aerobic acetate oxidation (○) (2-3h). Oxygen consumption (■) was used to calculate acetate oxidation.

### 2.3.4 Minimum oxygen requirements

To test whether providing one pore volume of air was sufficient for the long term reactor operation and acetate removal, the reactor was run continuously for 24 cycles. Over 80 hours, it was demonstrated that 14 Cmmol/L acetate (448 mg/L BOD) present in the synthetic wastewater were removed in cycles of 3.5 h (Figure 2-6). Therefore the removal rate of acetate was 4 Cmmol/L/h (123 mg/L/h BOD), which equates to a carbon removal rate that is about 3 times higher than typically observed in AS plants [122]. No significant biomass output was recorded over this time. From the reproducible oxygen uptake curves (data not shown) it could be predicted that approximately 50% of the acetate added was respired (Table 2-2).

Above, the acetate was continuously removed with a single reactor void volume of air. To test for the maximum carbon to oxygen ratio needed to enable sustained
operation, the acetate concentration was incremented to 22 Cmmol/L and 30 Cmmol/L (Figure 2-6). The time provided for acetate uptake was increased from 2 hours to 4 hours and to 7.5 hours for the highest concentration. The oxygen supply was maintained to a single void pore volume, the C/O₂ ratio is therefore increased from 1.3 to 2.1 and 2.9 for the highest concentration.

At a feed concentration of 22 Cmmol/L, > 90% of acetate was continually removed over 18 subsequent cycles, while providing still only 1 pore volume of air (Figure 2-6). When elevating the acetate concentration to 30 Cmmol/L acetate could no longer be stored sustainably, as indicated by 50 % residual acetate being present in the effluent after 5 cycles (Figure 2-6).

Using stoichiometric considerations, the fact that up to 22 Cmmol/L of acetate (26.4 Cmmol of acetate per reactor) could be removed continuously with 1 pore volume of air suggests that sufficient stored acetate is oxidised to allow for repeated BOD uptake. In fact, calculated from the oxygen content, approximately 27 % of the added acetate was oxidised (Table 2-2) with the remainder retained within the biomass. On the contrary, when 30 Cmmol/L were added, the cycles were not sustained (Figure 2-6). In this case, 1 pore volume oxidised 20 % of the added acetate. This suggests that there is a minimum of PHA oxidation required to sustain BOD uptake.

Overall, if at least 27 % of the added acetate is oxidised, 22 Cmmol/L removal was sustained. However, if 20 % or less of the added acetate is oxidised, 30 Cmmol/L removal was not sustained. It seems that if more than a quarter of the added BOD is oxidised then the BOD storage can be sustained (Table 2-2).
Chapter 2

Acetate removal rate was maximised by the presence of a large amount of biomass (50 g/L) that stored and oxidised the acetate in solution during the anaerobic and aerobic condition respectively. Biomass growth consumes carbon and therefore could significantly affect the acetate removal. Under the oscillating conditions provided, the carbon is stored (i.e. anaerobic conditions) and then oxidised under aerobic conditions to achieve growth (ref). Once PHAs is stored its oxidation produces glycogen and biomass. Considering the large amount of biomass in the reactor and assuming half the stored acetate is used for growth, the biomass growth also plays an important role in the repeated acetate storage which was not quantified in this work.

![Graph](image_url)

**Figure 2-6:** The effect of increasing the carbon to oxygen ratio on the continuous removal of acetate. Continuous operation of the storage biofilm reactor under repeated cycles of synthetic wastewater with 1 pore volume of air provided: 24 cycles of 14 Cmmol/L, 18 cycles of 22 Cmmol/L and 5 cycles of 30 Cmmol/L. Example cycles of 14 Cmmol/L (●) and carbon outflow (○), of 22 Cmmol/L (▲) and carbon outflow (Δ), and of 30 Cmmol/L (■) and carbon outflow (□).
Table 2-2: Operation parameters of the reactor to test the effect of acetate to oxygen ratio to sustain continuous acetate removal. From the oxygen consumption, the acetate oxidation was calculated and the acetate storage determined.

<table>
<thead>
<tr>
<th>Ac input rate CmM/h</th>
<th>Ac input /cycle CmM</th>
<th>O₂ input mM</th>
<th>Carbon to O₂ ratio C:O₂</th>
<th>Ac oxidised /cycle CmM</th>
<th>Percentage C oxidised %</th>
<th>Ac remaining in outflow</th>
<th>Sustained removal?</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>14</td>
<td>10.3</td>
<td>1.36</td>
<td>5.9</td>
<td>42</td>
<td>&lt;1%</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>22</td>
<td>10.3</td>
<td>2.1</td>
<td>5.9</td>
<td>27</td>
<td>&lt;1%</td>
<td>Yes</td>
</tr>
<tr>
<td>3.5</td>
<td>30</td>
<td>10.3</td>
<td>2.9</td>
<td>5.9</td>
<td>20</td>
<td>~50%</td>
<td>No</td>
</tr>
</tbody>
</table>

2.4 Discussion

2.4.1 Difference in biomass

The described biofilm reactor is similar to trickle filters used for wastewater treatment in a number of ways, such as the biomass growth on carrier material, the provision of aeration and the BOD removal. However, significant differences can be pointed out both in terms of microbial composition and operational attributes.

Because of the strict cycling of anaerobic acetate storage to PHA followed by aerobic PHA oxidation, only those heterotrophic bacteria that can effectively store BOD as PHA, namely GAO can be sustained in the biofilm. The current sequencing batch operation of the biofilm reactor would thus select for the development of a distinctly different biomass to that in trickle reactors.

2.4.2 Carbon removal rate and the associated energy expense

With an acetate removal rate of 4 Cmmol/L/h (123 g/m³/h BOD), the described biofilm process demonstrated a 10 to 20 times faster volumetric carbon removal rate than that obtained for traditional trickle reactors.
Table 2-3). Possible reasons for the rather high rates of BOD removal could be:

- the high surface area of carrier material used in the bioreactor (850 m$^2$/m$^3$)
- the high biomass content of the biofilm (50 g dry biomass per L of reactor volume)

Assuming a 5 m high reactor and 3.5 h treatment time without recirculation, the energy required is 4 W/m$^3$ (Eq 2.1 and 2.2). Considering that trickling reactors (high rate with plastic media) are recirculating 4 to 7 times, their energy usage is typically 6 to 10 W/m$^3$ [132]. So our biofilm requires 1.5 to 2.5 times less energy expense compared to trickle reactors.

Eq. 2.1: \[ W = H \times m \times g = 5 \times 1,000 \text{ kg/m}^3 \times 9.8 = 49,000 \text{ J/m}^3 \]

Where \( W \) is work in joules per cubic metre of wastewater (J/m$^3$), \( H \) the height in metre (m), \( m \) the mass in kilogram (kg) and \( g \) the gravity constant (9.8 m/s$^2$)

Eq. 2.2: \[ P = W \times t = 49,000 \times 12,600 = 3.89 \text{ W/m}^3 \]

Where \( P \) is the power in watts per cubic metre of wastewater treated (W/m$^3$), \( W \) is the work (Joules) and \( t \) the time (s) here 3.5h or 12,600 seconds.
2.4.3 Removal of other nutrients

The described biofilm reactor removes BOD rapidly and energy-efficiently but does not remove phosphate and nitrogen. These nutrients can be removed by the novel low-energy nitrogen removal processes using Anammox bacteria, such as the completely autotrophic nitrogen removal over nitrite (CANON) which uses limited aeration for partial oxidation of ammonia to nitrite, followed by the Anammox process leading to N\textsubscript{2} formation [108]. However, this process cannot be effectively applied with existing wastewater streams because of the dissolved BOD in the inflow [134]. The currently described process would be a fast and cost-effective way to remove dissolved BOD from wastewater prior to nitrogen removal treatments,

<table>
<thead>
<tr>
<th>System</th>
<th>BOD removal rate g/m\textsuperscript{3}/h</th>
<th>BOD inflow g/m\textsuperscript{3}</th>
<th>HRT h</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trickle reactor for communal wastewater, rock media</td>
<td>11.7</td>
<td>599.5</td>
<td>51.2</td>
<td>Doan et al. [133]</td>
</tr>
<tr>
<td>Trickle reactor for communal wastewater, rock media</td>
<td>4</td>
<td>250</td>
<td>62.5</td>
<td>Gray [1]</td>
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<tr>
<td>Trickle reactor for communal wastewater, rock media</td>
<td>5</td>
<td>250</td>
<td>50</td>
<td>Forster [2]</td>
</tr>
<tr>
<td>Sequencing operation of PHA storing biofilm</td>
<td>128</td>
<td>480</td>
<td>3.5</td>
<td>Present research</td>
</tr>
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</table>
which requires low BOD wastewater. The additional removal of nitrogen by a process linked to the described biofilm reactor has been designed and will be described in Chapter 3 and 4.

2.5 Conclusion:

- A simple sequence of anaerobic condition (filling) and aerobic condition (drainage) selectively enriched the biomass with GAOs from AS. This method is easily applicable for existing biofilm reactors.
- The biofilm was capable to sustain acetate removal from synthetic wastewater without transferring air into the bulk wastewater and hence bypassing the energy expense for oxygen transfer.
- Atmospheric air provides oxidative power via passive aeration to the biofilm for PHA oxidation, hence recovering the biofilm’s ability to store acetate in subsequent cycles. A repeated liquid recycle as needed for trickling reactors was not needed.
Chapter 3  Dissolved ammonium removal with zeolite as an ion-exchange material

3.1  Introduction

The dissolved carbon was biologically stored and oxidised without liquid bulk aeration in the previous chapter. The dissolved ammonium (NH$_4^+$) remaining in wastewater must be removed prior to disposal of the wastewater to the environment. This chapter investigates the ammonium removal using zeolite as an ion-exchange material. Clinoptilolite in particular has been used for ammonium removal [82,94,135]. However, zeolite being a natural product, there are significant variations in clinoptilolite from different geographical locations [86]. In this study, an Australian clinoptilolite is tested for its ammonium removal capacity, following the below objectives:

- Effect of zeolite particle size on Effective Capacity (EC)
- Effect of zeolite particle sizes on the ammonium adsorption rate
- Effect of contact time on ammonium adsorption
- Effect of ammonium loading on its desorption

3.2  Materials and methods

3.2.1  Zeolite preparation

The zeolite used in this study was an Australian clinoptilolite (Werris Creek, New South Wales, Australia), obtained from Zeolite Australia Pty Ltd. The initial preparation was to sieve the zeolite in grain size range of <150 μm, 150 - 250 μm, 250 μm - 1 mm, 1 - 2 mm, 2 - 3.35 mm and 3.35 - 4 mm. The zeolite was washed
with deionised water and dried at 105 °C for at least 24 h. The end product of this preparation was called ‘virgin’ zeolite.

3.2.2 Experimental set-ups

3.2.2.1 Effective capacity experimental set-up

The Effective Capacity (EC) was determined in batch equilibria. In 250 mL volumetric flask, 5 g of zeolite was in contact with 100 mL of ammonium chloride (NH₄Cl, 99.5 % purity) solution. The ammonium solution concentration ranged from 0 – 16 mmol/L by doubling the concentrations (6 tests). The volumetric flasks were shaken so that the liquid was in continuous movement, but the particles did not collide and break. After 72 h the ammonium concentration was determined in the liquid. In addition, two particle sizes of zeolite were tested: 250μm - 1mm and 2 - 3.35 mm. In total 12 batch equilibria were tested, and after equilibrium was reached the ammonium adsorbed on zeolite was determined as follows:

\[
\text{Eq. 3-1: } Z_{\text{NH}_4^+} = (\text{[NH}_4^+\text{]}_i - \text{[NH}_4^+\text{]}_e)/W_z
\]

Where \( Z_{\text{NH}_4^+} \) is the ammonium on zeolite in meq/g, \([\text{NH}_4^+]\) is the concentration of ammonium in solution (mmol/L), the subscript \( i \) and \( e \) correspond to the time initial and at equilibrium respectively, and \( W_z \) is the zeolite weight in g. The term meq (milli-equivalent) refers to the amount of substrate (in mmol) timed by its electrical charge(s).

3.2.2.2 Ammonium adsorption rate experimental set-up

The ammonium adsorption rate was determined for three different zeolite particle sizes, using the same ammonium concentration solution (4.5 mmol/L or 63 mg-N/L). The particle sizes tested were: 150-250 μm, 250 μm – 1.0 mm, 2.0 – 3.35 mm.
In a 250 mL volumetric flask, 10 ±0.2 g of zeolite was added to 100 mL of 4.5 mmol/L \( \text{NH}_4\text{Cl} \) and the dissolved \( \text{NH}_4^+ \) concentration monitored. The samples were centrifuged at 1350 rpm for 5 min to remove possible zeolite particles. If \( \text{NH}_4^+ \) concentration was not immediately determined, the samples were stored at -20 °C. The adsorption rate was determined from Eq. 3.2:

\[
V_{\text{Ads}} = \frac{Z_{\text{NH}_4^+}}{(t_f - t_i)}
\]

Where \( V_{\text{Ads}} \) is the adsorption rate of ammonium in mmol/g/h (or meq/g/h), \( Z_{\text{NH}_4^+} \) is the ammonium adsorbed on zeolite as calculated in Eq. 3.1, \( t \) is the time (h), and the subscripts \( i \) and \( f \) stand for initial and final.

### 3.2.2.3 Flow through experimental set-up

The flow through experiments (i.e. breakthrough) used two identical cylindrical reactors (10.5 cm height and 3 cm \( \Theta \)) of total volume 75 mL. The zeolite weight in the reactor was maintained at 69.0 ±1.0 g, and the reactor working volume was 30 mL. The experiments were run with two zeolite particle size: 0.15 - 0.25 mm and 2 - 3.35 mm. The flow rates were varied for the experiments and are provided with the results. The flow rate is expressed in bed volume per h (BV/h), where BV is the liquid volume required to fill the void volume between the zeolite particles (i.e. working volume). The ammonium concentration in the effluent was measured after centrifuging the sample at 1350 rpm for 5 min. Samples were stored at -20 °C if the concentration was not determined immediately.

### 3.2.2.4 Desorption rate experimental set-up

The desorption rate was to be measured against the amount of ammonium adsorbed on zeolite. Before measuring the desorption rate, different amounts of ammonium were loaded on zeolite (2 - 3.35 mm). The ammonium loading on zeolite
was done in 5 batches in Schott bottles. The same zeolite weight (10 ± 0.2 g) was in contact with an increasing volume (0.1 to 1.5 L) of the same ammonium concentration solution (3 mmol-N/L or 42 mg-N/L). The bottles were shaken so that the liquid was moving, but the particles did not collide and break. The ammonium concentration was monitored daily for 89 h, and the ammonium amount on zeolite was determined as per Eq. 3-1 (Table 3-1).

Table 3-1: Ammonium loaded on zeolite after 89 h contact between solution and 10 g zeolite.

<table>
<thead>
<tr>
<th>Volume (mL)</th>
<th>Ammonium on zeolite</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol-N/g</td>
<td>mg-N/g</td>
</tr>
<tr>
<td>100</td>
<td>31</td>
<td>0.43</td>
</tr>
<tr>
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<td>55</td>
<td>0.77</td>
</tr>
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</tr>
<tr>
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</tbody>
</table>

The desorption tests were conducted on each zeolite load by placing a subsample (5.0 ± 0.1 g) of each loaded zeolite in 10 mmol/L NaCl (99.5 % purity) and monitoring the ammonium concentration, and the time course enables calculating the desorption rates.

### 3.2.3 Ammonium analysis

The ammonium analysis was done according to the Nesslerization method [7]. In 4 mL cuvettes 2 mL of samples were pre-treated with Mineral stabilizer (10 µL, Hatch) and polyvinyl alcohol-dispersing agent (10 µL, Hatch), to inhibit the precipitation of calcium, magnesium, iron and sulphide when treated with the Nessler reagent. Then, 100 µL Nessler reagent (10 % (W/V), Hatch) was added to the mixture. The samples were mixed by rotating the cuvettes 180 ° three times and
measuring the absorbance at 425 nm after exactly 1 min of reaction. A standard absorbance curve was determined for ammonium concentrations of 0 to 0.3 mmol/L, and the samples’ concentrations were determined using the samples’ absorbance against the standard curve. The samples were diluted as required to fit the standard curve.

3.3 Results

3.3.1 Effective ammonium capacity at different particle sizes

To start, the effective capacity (EC) is explained. EC is the amount of cation exchanged on the zeolite that will be dependent on the experimental conditions [89]. In our experimental conditions, the EC is the value obtained at the ammonium concentration in wastewater (3 mmol/L). The ammonium adsorption was measured for 2 zeolite particle sizes (0.25 mm – 1 mm and 2 – 3.35 mm) in batch tests with 5 g of zeolite in contact with 100 mL ammonium concentration ranging from 0 – 16 mmol/L. At equilibrium, when the concentration in solution is 3 mmol/L then the adsorbed ammonium on zeolite corresponds to the EC of the tested Australian clinoptilolite.

The EC for the Australian clinoptilolite, at the ammonium concentration in wastewater, was 0.12 meq/g (1.68 mg-N/g) (Figure 3-1), and the difference in zeolite EC for ammonium removal did not differ significantly for the particle size. This is in line with the results using clinoptilolite of different origins reviewed by Wang and Peng [86].
3.3.2 Rate of ammonium adsorption

The previous experiment demonstrated that the zeolite’s particle size did not significantly affect the EC at equilibrium. However, the use of zeolite in a full scale plant would depend on the ammonium adsorption rate. The aim of this experiment is to quantify the ammonium adsorption rate for different Australian clinoptilolite particle sizes. The same zeolite weight of different sizes (150-250 μm, 250 μm – 1 mm and 2 – 3.35 mm) was suspended in an ammonium solution (4.5 mmol/L or 63 mg-N/L) (Section 3.2.2.2). It was found that the initial adsorption rate over the first 3 min was most indicative of the ammonium adsorption rate that can be achieved by virgin zeolite (Figure 3-2). The initial ammonium adsorption rate increased 4.4 times when the particle size decreased 12.5 times (Figure 3-3). This demonstrated that zeolite with the finest particles increased the ammonium adsorption rate. This
suggested that small zeolite particles would be beneficial to adsorb $\text{NH}_4^+$ from wastewater [83,97].

Figure 3-2: The effect of zeolite particles size on the ammonium adsorption kinetics. The sizes of particles were 150 $\mu$m - 250 $\mu$m (▲), 250$\mu$m - 1mm (■) and 2mm - 3.35 mm (♦).

Figure 3-3: The effect of zeolite’s particle size on the initial ammonium adsorption rate. The values were determined from Figure 3-2, as the ammonium adsorption over 3 minutes.
3.3.3 Repeated ammonium adsorption

The above experiments have shown ammonium adsorption during a single batch. However, zeolite in an operating reactor would be in contact with ammonium repeatedly. The following experiment was designed to test whether ammonium can be repeatedly adsorbed onto the zeolite. In a column reactor, zeolite (69.4 g, 2 – 3.35 mm) was in contact with a repeated batch of ammonium solution for 30 minutes (2 BV/h), which was drained before a new batch was added, and this sequence was repeated 5 times. The ammonium concentration in the drained solution was measured to evaluate the ammonium removed. The outflow concentration in the 5th batch was 4 times higher than in batch 1 (Figure 3-4). As expected, the ammonium concentration after each batch increased. Considering the Australian guidelines [81,136] value of 0.3 mmol-N/L (< 5 mg-N/L) for maximum nitrogen concentration in treatment plant effluent, the results show that up to 4 repeat treatments can be done without going over the ANZECC guidelines. Overall, zeolite seemed a promising mechanism for an immediate ammonium removal.
3.3.4 Effect of contact time on ammonium adsorption

Previously, repeated batches of wastewater were added to the column containing zeolite. Instead of repeated batches, a continuous flow of synthetic wastewater over the zeolite bed would be more convenient because such system provides a standardized amount of wastewater. A flow-through system operates on the principles of a continuous supply of ammonium solution to zeolite, and the recording of ammonia in the outflow over time. The ammonium concentration can then be plotted as a function of BV that had passed through the column reactor. At a flow rate of 3.6 BV/h and 0.6 BV/h up to 1.5 and 5.5 BV of ammonium solution could be treated and yet remain under the ANZEEC guidelines value (Figure 3-5). This experiment demonstrated that in a flow-through system, a decrease in flow rate resulted in an ammonium adsorption improvement. The improved adsorption infers that an increase in contact time between ammonium and zeolite particle maximised the diffusion of ammonium to the cation exchange sites.

Figure 3-4: The effect of the repeated batch treatment of zeolite (2 – 3.35 mm) on the ammonium concentration in the outflow. Ammonium concentration in the inflow was 3.0 ±0.2 mmol/L (42 mg-N/L). The dashed line represents the ANZECC guideline value of 0.3 mmol/L (5 mg/L) of maximum ammonium concentration discharge.
3.3.5 The combined effect of zeolite particle size and contact time

From the previous experiment, a decrease in flow rate increased the ammonium adsorption rate. The aim is now to test the effect of zeolite particle size on ammonium breakthrough in the flow-through system. Two zeolite columns with two particle sizes (< 150 μm and 0.25 – 1 mm) were provided the same ammonium solution of 3 mmol/L NH₄⁺ (42 mg-N/L) at the same flow rate 0.6 BV/h. As expected, decreasing the particle size delayed the breakthrough of ammonium in the effluent (Figure 3-6). In fact, 15 BV of ammonium solution passed through the small particle reactor before breaching the ANZEEC guideline value. The coarser zeolite reactor only passed 5 BV. The difference in surface area between the two reactors can explain the difference in adsorption. Fine zeolite has a larger surface area which enables maximising the ammonium adsorption rate, thus delaying the breakthrough point (Appendix B).
Figure 3-6: The effect of zeolite size on the breakthrough curves with an inflow of wastewater containing $3.0 \pm 0.1$ mmol/L of ammonium. Both curves were established with the same flow rate (0.6 BV/h), the zeolite diameter size was <0.15 mm (♦) and 0.25 - 1 mm (●). The dashed line represents the ANZEEC guidelines for N concentration (0.3 mmol/L or 5 mg/L).

### 3.3.6 The effect of ammonium adsorbed on zeolite on ammonium desorption rate

When the ammonium concentration in the outflow is greater than the ANZEEC guidelines, this demonstrates that the zeolite does not remove ammonium sufficiently. Thus zeolite requires regeneration to resume ammonium adsorption to a level less than the value set in the ANZEEC guidelines. The rate of desorption is important to ensure that the zeolite can be reused in an applied process. To verify whether the ammonium desorption rate depends on the amount of ammonium adsorbed on zeolite, different ammonium loads were added to the zeolite: 31 μmol/g, 55 μmol/g, 84 μmol/g, 130 μmol/g, and 153 μmol/g. Desorption was tested by having zeolite (5 g) in contact with a solution of NaCl (10 mmol/L) and monitoring the ammonium concentration in solution. The desorption rate was 8 times faster when zeolite contained 2.1 mg-N/g (153 μmol/g) compared to zeolite containing 0.43 mg-
N/g (31 μmol/g) (Figure 3-7). Ammonium desorption rate accelerated with respect to the ammonium content on zeolite (Figure 3-8), which is in line with other zeolite studies [100,137]. In an applied process, the desorption of the last amount of adsorbed ammonium on zeolite can limit the regeneration speed.

Figure 3-7: Time course of the ammonium release in solution from zeolite loaded with varying NH$_4^+$ amounts. The loads were: 31 μmol/g (0.43 mg-N/g) (×), 55 μmol/g (0.77 mg-N/g) (▲), 84 μmol/g (1.1 mg-N/g) (●), 130 μmol/g (1.8 mg-N/g) (♦), and 153 μmol/g (2.1 mg-N/g) (■). To the same zeolite weight an increasing volume of a 3 mmol/L ammonium solution was added such that the ammonium adsorbed increase.
3.4 Discussion

3.4.1 Chemical regeneration of zeolite: an undesirable process

Zeolite cannot adsorb ammonium indefinitely, and in order to have a viable process, zeolite needs to be regenerated and stripped of its ammonium load. Chemical regeneration uses cation rich solution called regenerant solution. The regenerant solution needs to have a high cation concentration to achieve a rapid adsorption rate [100,138], and consequently a rapid ammonium desorption. It is most common to use NaCl or NaHCO₃ salts [82,98,139]. The lesser affinity of Na⁺ makes regenerated zeolite (i.e. Na⁺ on exchange sites) more effective at ammonium adsorption subsequently, in fact a pre-treated zeolite with Na⁺ can increase the ammonium adsorption by 15 % [91]. However, the brine containing NH₄⁺ produced by chemical regeneration is a problem because nitrifying biomasses tend to be inhibited in the presence of sodium [137]. In addition, even though NaCl is widely available, it is preferable to limit the use of chemicals because of the transport and

Figure 3-8: The effect of the amount of ammonium loaded onto zeolite on the release rate. The release rates were obtained from Fig 3-7.
extraction costs. Instead of a chemical regeneration of zeolite, a biological regeneration with a nitrifying biomass will be tested in the following chapter.

3.4.2 Effective zeolite particle size to use

The tested Australian clinoptilolite of small particles (150 – 250 μm) had a 4.4 times faster ammonium adsorption rate than the same zeolite with coarse particles (2 – 3.35 mm). Despite this benefit, some problems are associated with fine zeolite particles to achieve biological regeneration. One of the problems is that in a reactor with a fine zeolite, oxygen supply to the nitrifiers requires zeolite fluidisation. Bed fluidisation is an energy intensive process which is undesirable.

To avoid fluidisation of the zeolite bed, it was decided that coarse zeolite could be used, and air supply could be provided by mere exposure of the nitrifier to the atmospheric air. Coarse zeolite (2 – 3.35 mm) has a viable ammonium removal speed. It adsorbed 4 BV (3 mmol-N/L/BV) before breaching the ANZEEC guideline value of 0.3 mmol/L in the effluent, with a contact time of 30 min per BV (2 BV/h). Thus the ammonium removal rate was 5.7 mmol/L/h, which is faster than the AS process at 1 mmol/L/h [50]. However, the AS process removes total nitrogen (i.e. nitrification and denitrification) while zeolite adsorbs ammonium which is yet to be oxidised and denitrified, which will be studied in the next chapters.

One drawback of coarse zeolite is that it reduces the wastewater volume contained in a reactor and hence affects the reactor’s design. The volume occupied by zeolite is referred as porosity. Nguyen and Tanner [95] reported 61 % porosity for New Zealand clinoptilolite particle size of 2 - 2.8 mm. If a treatment reactor was designed to treat 1 L of wastewater and used a 61 % porous zeolite, then the actual size of the reactor would need to be increased proportionally to the porosity of the
material (61 %). Therefore, a zeolite reactor treating 1 L of wastewater would have an actual volume of 1.6 L. This is a drawback associated with coarse zeolite, but it has the benefit of preventing energy usage for fluidisation.

3.4.3 The effect of wastewater cations on the adsorption and desorption rates

The presence in wastewater of cations other than ammonium affects its net adsorption. The other cations that are present in higher concentration are: Na\(^+\) and Ca\(^{2+}\) (Table 3-2). However, clinoptilolite has generic affinity sequences which demonstrate which cations are preferentially adsorbed [82,90,91,140]:

\[
Cs^+ > K^+ > NH_4^+ > H^+ > Na^+ > Ca^{2+}
\]

On the basis of this affinity constant, ammonium will be preferentially adsorbed onto ammonium compared to most cations in wastewater, with the exception of K\(^+\). Overall, the Australian clinoptilolite used in this study seemed to be a desirable material to be tested further for complete nitrogen removal.

Table 3-2: Cations concentration (mmol/L) and equivalent (meq/L) in the synthetic wastewater used in this study.

<table>
<thead>
<tr>
<th>Cation in synthetic wastewater</th>
<th>mmol/L</th>
<th>meq/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+)</td>
<td>9.5</td>
<td>9.5</td>
</tr>
<tr>
<td>NH(_4^+)</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>K(^+)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>0.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>
3.5 Conclusion

- Fine and coarse zeolite had similar effective capacity, for ammonium removal in wastewater, of 0.12 meq/g (1.68 mg-N/g) when providing a contact time of 72 h.

- The ammonium adsorption rate benefited significantly from finer particles because of the increase in surface area. The adsorption rate was approximately 4 times faster with the finer particle size of 150 μm compared to the particle size 2 – 3.35 mm. However, there are drawbacks associated with continuous use of fine particles, in particular the fluidization for regeneration.

- In a flow-through system, ammonium adsorption benefited from the longer contact time. At a flow rate of 0.6 BV/h, the zeolite adsorbed ammonium from 15 BV before breaching the ANZEC guideline value of 0.3 mmol/L (5 mg-N/L). Increasing the flow rate to 3.6 BV/h resulted in a breakthrough after 5 BV.

- Ammonium desorption is necessary for zeolite regeneration. The desorption rate increased up to 8 times when the adsorbed ammonium increased 5 times. Thus the complete zeolite regeneration to the last ammonium amount might become a limiting factor.

- The place of ammonium in the affinity sequence of clinoptilolite makes the ammonium preferentially adsorbed compared to most other cations in wastewater. The Australian clinoptilolite tested in this study is a promising material to develop a process for total nitrogen removal in wastewater.
Chapter 4  Nitrifying biofilm on zeolite for bio-regeneration using a trickle aeration system

4.1 Introduction

Australian clinoptilolite zeolite was demonstrated to be a promising material for ammonium (NH$_4^+$) removal from wastewater (Chapter 3). Zeolite biological regeneration is preferred over chemical regeneration. Bio-regeneration requires supplying oxygen to the nitrifiers to oxidise the adsorbed ammonium on zeolite to nitrite (NO$_2^-$) and nitrate (NO$_3^-$). The use of coarse particle zeolite (2 – 3.35 mm) enables draining and trickling wastewater, which can be used for passive aeration. In this chapter, the aim is to test the biological oxidation of the ammonium adsorbed on zeolite without liquid aeration. The approach to this study was to develop a nitrifying biofilm on coarse zeolite particles as carriers. The reactor operating sequence consisted of flooding the reactor for anaerobic ammonium adsorption on zeolite, followed by trickling the liquid over the drained nitrifying biofilm to oxidise the adsorbed ammonium without liquid aeration.

4.2 Materials and methods

4.2.1 Reactor operations and set-up

4.2.1.1 Reactor dimension

A cylindrical 2 L PVC reactor (12cm Ø and 29cm height) with openings at the top and bottom was filled with 1790 ± 2 g clinoptilolite zeolite (Zeolite Australia Pty. Ltd.) of size 2 - 3.35 mm (0.53 m$^2$/L$_{reactor}$). The zeolite size was chosen so that air could diffuse throughout the reactor [141]. Zeolite occupied 1 L of the total volume,
therefore the reactor’s working volume without biomass was 1 L. The working volume of the reactor (1 L) will be referred to as Bed Volume (BV) throughout this chapter. A nitrifying biomass culture (Section 4.2.2) was used to inoculate the reactor. The inoculation entailed the trickling recirculation of 2 L nitrifying biomass (5.1 g/L) over the zeolite for 24 h. The recirculation, with a trickling method (20 L/h), was done after the zeolite had adsorbed ammonium (6 mmol) to favour nitrifier attachment.

4.2.1.2 Reactor operation

In the experiments the reactor operated in the following manner:

- Synthetic wastewater (Section 4.2.3) filled the reactor using an upflow peristaltic pump (180 mL/min). The reactor was under anaerobic conditions to adsorb ammonium on zeolite, thus preventing immediate oxidation by the nitrifiers.
- The ammonium-free wastewater was drained and recirculated with a trickling mechanism over the zeolite biofilm (0.2 m³/m²/d). This washed the metabolites formed by the nitrifiers, and provided oxygen, which maintained the nitrification of the adsorbed ammonium by the biofilm (Figure 4-1).

Two probes (pH and ORT) were used to measure and the values were continually record into a spreadsheet, using a LabJack U12 data acquisition card and the process control software LabVIEW™ (version 7.1 National Instrument).

The recirculation was used to washout the built up of metabolites. The oxygen provision was not necessarily achieved by the recirculation (Chapter 2), but it was used here because the aim of this chapter is to provide a proof of concept and improvements will attempt to stop the recirculation of liquid in the next chapters.
Figure 4-1: Schematic diagram of the biological oxidation of ammonium in a single reactor system. A. Anaerobic ammonium adsorption on zeolite. B Nitrification using the biofilm grown on zeolite particles.

4.2.2 Nitrifying culture

The nitrifying culture was developed over four years from an Activated Sludge (AS) sample from a local Sequencing Batch Reactor (SBR) wastewater treatment plant (Woodman Point, Perth, Western Australia). The operation to develop the nitrifying culture was a sequencing batch mode of feast and famine to favour rapid settlers. A 10 L reactor was fed every second day and the reactor operation timing was as follows: 0.5 h settling (no air, no stirring), 0.5 h decant (no air, no stirring and 4 L (40 %) liquid removal), 48 h reaction (feed added with aeration and stirring to create famine conditions).

The nitrifying biomass feed was prepared so that the concentration in the reactor was (g/L): 1 NaHCO₃, 0.3 (NH₄)₂SO₄, 0.11 KH₂PO₄, 0.05 CaCl₂, 0.01 MgCl₂, 0.01
FeSO$_4$.7H$_2$O, 0.02 yeast extract. Once weekly, 1.5 mL of trace elements solution (Section 2.2.3) was added.

### 4.2.3 Synthetic wastewater composition

The synthetic wastewater used in this work was described in Chapter 2 (Section 2.2.3). However, these experiments required a carbon-free synthetic wastewater, therefore the CH$_3$COONa (in mmol/L) was replaced with the same concentration of NaCl (mmol/L) in order to maintain the ionic strength.

### 4.2.4 Experimental set-up

- **Zeolite**

The zeolite used was described in Section 3.2.1. This was the zeolite used in all experiments unless otherwise stated.

- **Experimental set-up to test nitrate recovery in a small volume**

To test the ability to recover nitrate species in a small volume, the reactor was operated as mentioned in Section 4.2.1.2, but the recirculation volume was changed from 1 BV (i.e. 1 L) to 200 mL and 50 mL.

### 4.2.5 Analysis

Ammonium analysis was conducted according to Section 3.2.3

#### 4.2.5.1 Nitrite

NO$_2^-$ was measured by colorimetric method, measuring the absorbance light at 540 nm [7]. In a 4 mL cuvette, 1 mL of sample was in contact with 1 mL of 1 % (w/v) sulphanilic acid in 1 mol/L HCl. Thereafter 1 mL of 0.1 % N-1-Naphthylethyldiamine was added to the mix and the mixture was left to react for at least 20 min but no more than 30 min. A new standard curve was prepared when any
of the reagent solutions were replaced. The standard curve was measured for a standard nitrite solution of concentration comprised between 0.00 and 0.05 mmol/L. The wastewater samples were diluted to fit in the standard curve if required.

4.2.5.2 Nitrate

$\text{NO}_3^-$ was measured by colorimetric method at 420 nm [7]. In a 4 mL cuvette, 40 $\mu$L of sample reacted with 10 $\mu$L of freshly prepared saturated ammonium amidosuphonate (to remove nitrite) for 10 min. Thereafter, 0.2 mL of 5 % sodium salicylate in 98 % sulphuric acid followed by 2 mL of cold (4 ℃) freshly prepared 4 mol/L NaOH were added to the mixture, and left to react for 30 min. Before the absorbance measurement was taken, the cuvettes were rotated 180 ° to remove air bubbles formed and released by the reaction heat.

4.3 Results

4.3.1 Nitrification of adsorbed ammonium on zeolite by suspended nitrifiers

Bio-regeneration of zeolite nitrifies the adsorbed ammonium on zeolite, in this study on the Australian clinoptilolite zeolite. The purpose of this experiment is to verify that suspended nitrifiers can oxidise the adsorbed ammonium. Initially, ammonium in wastewater was adsorbed onto zeolite, then nitrifying biomass was added and finally the solution was aerated. The nitrifying biomass oxidised about 0.5 mmol/L of the ammonium that was adsorbed on the zeolite (Figure 4-2). It was also noted that the solution acidified, which is expected and confirms the nitrification reaction.
Figure 4-2: Time course of sum of the nitrite and nitrate (▲) production after ammonium (●) adsorption on zeolite. The nitrification process decreases pH (♦) and the initial ammonium concentration added was 3.7 mmol/L.

4.3.2 Nitrification rate from adsorbed ammonium on zeolite

Previously, it was confirmed that suspended nitrifiers oxidised the ammonium adsorbed on zeolite. The rate of nitrification of ammonium adsorbed on zeolite is critical for the process to operate at a practical speed. The aim is to quantify the speed of adsorbed ammonium oxidation relative to that of dissolved ammonium oxidation under saturated conditions. The same ammonium concentration was added to two reactors containing the same suspended nitrifying biomass concentration, with the test reactor containing zeolite (5 g) and the control no zeolite. The ammonium concentration throughout the experiment stayed below 0.5 mmol/L in the test reactor. The presence of zeolite halved the nitrification rate. Nitrification rates were 1.5 mmol/L/h in the control and 0.75 mmol/L/h in the test experiment (Figure 4-3). The decrease in nitrification rate is important, however the nitrification rate of
adsorbed ammonium is still viable when it is compared to a conventional AS process with a nitrification rate of 1 mmol/L/h [50].

![Figure 4-3: Effect of zeolite on the biomass nitrification rate. Sum of NO\textsubscript{2}^- and NO\textsubscript{3}^- without zeolite (■), NH\textsubscript{4}^+ without zeolite (●). Sum of NO\textsubscript{2}^- and NO\textsubscript{3}^- in presence of zeolite (□), NH\textsubscript{4}^+ in the presence of zeolite (○). The same ammonium concentration of 3.4 mmol/L and biomass concentration (5 g/L) were added to both reactors. When aerated, the pH was manually maintained above 7.2.](image)

### 4.3.3 Ammonium adsorption on zeolite with a nitrifying biofilm

The above experiments used suspended biomass and zeolite. In the applied process, the zeolite in the reactor will be bio-regenerated by a nitrifying biofilm grown on zeolite particles. Briefly, the nitrifying biofilm development consisted of trickling a nitrifying biomass solution over zeolite particles and operating the reactor for 2 weeks (Section 4.2.1.2). The presence of the nitrifying biofilm on top of the zeolite might be acting as a barrier to ammonium adsorption. The aim of this experiment is to test the effect of the nitrifying biofilm on the zeolite’s ammonium adsorption rate. The test reactor contained zeolite coated with nitrifiers and the control reactor contained abiotic zeolite. Under anaerobic conditions, ammonium
was added to both reactors and the ammonium concentration was monitored. The ammonium adsorption rates were 80% similar, with (2.55 mmol/L/h) and without (3.08 mmol/L/h) nitrifying biomass (Figure 4-4). The presence of the nitrifying biofilm decreased the ammonium adsorption rate on zeolite but not sufficiently to question the viability of the process. In the presence of biofilm on zeolite, a similar drop in ammonium adsorption has been reported in the literature: 22% [96] and 25% [101]. Without biofilm, the ammonium molecule diffusion is controlled by the molecule’s travel through the zeolite’s matrix. With a biofilm, ammonium molecules must, in addition, diffuse through the biofilm layer which decreased the adsorption rate [101].

![Figure 4-4: Ammonium uptake on zeolite with (□) and without (■) a nitrifying biofilm. The feed concentration was 3.0 ±0.2 mmol/L ammonium, nitrate and nitrite were not detectable in the experiment. The wet weight of both zeolite was 5.0 ±0.2 g.](image)

**4.3.4 Nitrification of the adsorbed ammonium**

The nitrifying biofilm had a marginal effect on the ammonium adsorption, but it might not be able to oxidise all of the adsorbed ammonium. The goal is to test
whether the nitrifying biofilm can recover the adsorbed ammonium, which can be accounted for in the form of nitrite and nitrate in solution. The wastewater flooded the zeolite in the reactor, and dissolved ammonium was adsorbed (2.9 mmol/L). Thereafter, the liquid was drained and trickled over the zeolite for mass transfer of the metabolites produced and oxygen supply. The adsorbed ammonium recovered was 93 % in the form nitrate, and no nitrite was measurable (Figure 4-5). This demonstrates that the nitrifying biofilm present on the zeolite is capable of oxidising most of the adsorbed ammonium. This is important as it shows that the zeolite can be regenerated biologically, which will enable repeated ammonium storage.

![Figure 4-5: The effect of passive aeration on the nitrate concentration in liquid. The nitrate concentration (2.6 mmol/L) achieved was 93 % of the added ammonium (2.9 mmol/L) (dashed line).](image)

**4.3.5 Ammonium recovery in small volumes for energy savings**

The previous experiment showed that the studied zeolite can be bio-regenerated with a nitrifying biofilm. However, the bio-regenerated solution contains nitrate that must be removed before disposal into the environment to prevent eutrophication. For the total N removal process to be complete, the nitrate will be denitrified in a
separate reactor. This will be investigated in the next chapter (Chapter 5). To provide the nitrate solution to the denitrifying reactor, there will be a liquid recirculation between the nitrifying biofilm reactor and the denitrifying reactor. Liquid transfer requires energy which can be assumed to be proportional to the liquid volume. In order to reduce the energy expense associated with the full liquid recirculation, the aim is to reduce the trickling wastewater volume over the nitrifying biofilm.

This experiment tests whether, by having a small volume of recirculation, adsorbed ammonium recovery is achievable. Carbon-free wastewater (1 L) was added to the zeolite (3 mmol) for each experiment. Two nitrification volumes were tested for recovery of stored ammonium on zeolite. The trickled volume was reduced from 1 L to 200 mL and 50 mL. If complete nitrogen recovery is assumed, the expected nitrate concentrations in the trickle solution are 15 mmol/L and 60 mmol/L for the respective volume solution recirculated. However, it was found that the nitrogen recovery was 34 % (5.1 mmol/L) and 10 % (5.9 mmol/L) respectively (Figure 4-6). Reducing the liquid volume did not achieve a full ammonium recovery. The poor recovery is probably due to the increase in proton concentration caused by the nitrification process and is investigated below.
4.3.6 Nitrifying biomass inhibition by pH decrease during nitrification

The nitrifying biofilm on zeolite oxidised ammonium to nitrate and produced protons. Reducing the trickling volume increased the proton concentration, which acidified the solution. To verify that protons produced inhibit the active biomass, the pH, DO and nitrate concentrations were monitored during the zeolite bio-regeneration with 200 mL trickling volume. Over the first 3 h, the pH dropped to 5.3, nitrate stopped being produced and the oxygen consumption decreased as can be observed from the DO increase (Figure 4-7). After 3 h, the pH was manually adjusted and maintained at 7.0. The nitrate production resumed and the DO decreased. This demonstrated that the nitrifying biomass recovered its nitrification capacity (Figure 4-7). Overall, when the trickling volume was decreased, the solution acidified and inhibited the nitrifying biofilm, which could explain the poor recovery with a decrease in volume.

Figure 4-6: The effect of decreasing the recirculation volume from 1 L to 200 mL (○) and 50 mL (Δ), on nitrate concentration in solution. The ammonium in wastewater (---) was adsorbed on zeolite and considering the volumes recirculated the expected concentrations were 15 and 60 mmol/L for the respective solutions.
Figure 4-7: Time course of the nitrate concentration (Δ) produced from adsorbed ammonium. pH (□) control was achieved with an aliquot addition of 1 mol/L NaOH. The increase in DO (○) demonstrated a lack of nitrifier activity, while its decrease demonstrated an increase in nitrifying activity.

4.3.7 pH control to recover the adsorbed ammonium in a small liquid volume

To maximise the recovery of adsorbed ammonium, pH was controlled above 7.5 with NaOH addition, while 50 mL of liquid was recirculated over the zeolite with nitrifying biofilm. The nitrate concentration (7.1 mmol/L) was only 12% of the expected 60 mmol/L (Figure 4-8). The pH control did not lead to an increased recovery of nitrate in the recirculation liquid. The consequence of the poor recovery despite the pH control on the viability of the process is discussed below.
Chapter 4

Figure 4-8: The effect of pH controlled above 7.5 on nitrate (Δ) concentration in solution. The ammonium concentration in wastewater (—) was adsorbed on zeolite and considering 200 mL of volume was recirculated, 60 mmol/L of nitrate recovery was expected.

4.4 Discussion

4.4.1 Complete recovery of adsorbed ammonium with bio-regeneration of zeolite and no air pumping to the bulk liquid

The Australian clinoptilolite used in this study adsorbed 1 BV of ammonium from wastewater and was completely bio-regeneration when trickling 1 BV over the zeolite. The bio-regeneration process used a nitrifying biofilm grown on zeolite, and 93% ammonium adsorbed on zeolite was recovered as nitrate. Zeolite bio-regeneration has been researched previously [94,99-101,137,138,141-143] and most of these researchers have used regenerants solution to desorb the ammonium from zeolite, for example NaCl or NaHCO₃. Lahav and Green [138] have successfully used zeolite has a carrier for nitrifying biomass and have achieved consistent 95% ammonium removal but used liquid aeration and NaHCO₃ regenerant solution (50 meq/L), which has a significant energy input. Park et al. [143] tested Korean clinoptilolite in an AS process and were able to fully bio-regenerate zeolite without
regenerant solution but used air pumping. In this chapter the nitrogen recovery (93 %) was possible without regenerant and using a trickling system.

4.4.2 Decreasing trickled liquid volume: Incomplete ammonium recovery

The nitrogen recovery was possible when using 1 BV for nitrification. Reducing the recirculation liquid volume was tested to minimise the energy required to transfer liquid from the nitrifying biofilm reactor to the denitrifying reactor. The volume was reduced to 200 mL (1/5th of a BV) and the ammonium recovery was only 34 % and 10 % with 50 mL recirculated. The less than optimum recovery reflected that the zeolite was not regenerated. Incomplete regeneration makes the proposed reactor impracticable. The wastewater acidification during the nitrification process was partly responsible for the poor recovery. However, even with a pH control, the recovery test only marginally improved the nitrogen recovery (12 %). The use of a regenerant solution would probably facilitate ammonium desorption in solution and improve nitrogen recovery in a small volume [144] (Appendix C). For example, Malovanyy et al. [144] have concentrated NH₄⁺ from synthetic wastewater (40 mg-N/L) to a level of 94 mg/L with concentrated NaCl regenerant (30 g/L).

However, in an effort to keep costs as low as possible, it was decided that the total N removal process would use 1 BV and avoid the use of a regenerant solution. In effect, once ammonium is in brine its treatment becomes more difficult because nitrifiers are inhibited by salt concentration [100]. Overall, it was assumed that it would be more costly to run a system with chemical addition for regenerant, than operating a system with a large recirculation volume (i.e. 1 BV).
4.4.3 Wastewater alkalinity to maximise nitrification

As nitrification occurs the pH in wastewater decreases, and a drop in pH inhibits nitrifying biomass. Wastewater contains a buffer capacity called alkalinity, which neutralizes protons formed, preventing inhibition. In general wastewater contains 200-300 mg CO$_3^{2-}$/L [8,29], and the synthetic wastewater used in this work has a similar carbonate content of total CO$_3^{2-}$ 213 mg/L (calculated from the chemical concentrations). During the zeolite bio-regeneration, nitrification produces acidity and consumes the alkalinity from wastewater, such that the nitrification rate slows down [29]. As a consequence zeolite bio-regeneration takes longer and hence increases the reactor operation time. The effect of alkalinity on the zeolite regeneration could provide an insight on minimising the regeneration. In future research, the alkalinity range and the chemical buffer species (e.g. phosphate or carbonate) should be tested.

It is interesting to note that the biological storage of soluble carbon uptake increased the pH and seemed to increase the buffer capacity of the wastewater (data not shown). This side effect of the storage carbon would benefit to be studied to minimise the zeolite bio-regeneration time, however testing the effect of alkalinity was beyond the scope of this study.

4.4.4 Selective enrichment of the nitrifying biomass on zeolite carrier

In this chapter, the zeolite reactor with nitrifying biofilm treated a carbon-free synthetic wastewater, because it was assumed that the carbon was removed in the initial step developed in Chapter 2. The organic carbon-free wastewater added to the zeolite reactor will enrich the nitrifying biomass [141], thus will increase the nitrification rate. This would be beneficial because the zeolite was demonstrated to halve the nitrification rate (0.75 mmol/L/h) compared to a biomass in suspension and
therefore, increasing the biomass can counterbalance the effect of zeolite on nitrification rate.

In activated sludge process, nitrifying biomass has a relatively slow growth rate compared to heterotrophic biomass and can be removed [1]. On the contrary, biofilms are resilient to strong changes (e.g. shock loads) [8]. Thus developing a nitrifying biofilm on zeolite takes advantage of biofilms’ robustness [59] and enhances the biomass growth.

4.5 Conclusion

- The nitrification rate was halved in the presence of zeolite (0.75 mmol/L/h). But the nitrification rate was still reasonable compared to that of an AS process 1 mmol/L/h. This could be compensated by the selective enrichment of the nitrifying population because of the absence of dissolved carbon in the inflow (i.e. no heterotrophs will grow).

- Adsorbed ammonium from 1 BV was recovered at 93 % as nitrate by trickling 1 BV mechanism. This demonstrates the capacity of the zeolite to bio-regenerate and the possibility of repeatedly adsorbing ammonium. However, the recovery was 34 % when using 200 mL (1/5th of 1 BV), thus reactor operation for multiple BVs treatment is unlikely to occur.

- The absence of enough cations to desorb ammonium was probably the main reason for the poor recovery in a small volume. However, the use of regenerant to maximise the nitrification was deemed undesirable and it was decided that the reactor would operate to remove ammonium from 1 BV.
Chapter 5  Combination of the two processes for carbon and total nitrogen removal from wastewater

5.1  Introduction

In this study, dissolved carbon has been stored by Glycogen Accumulating Organisms (GAOs) as Poly-Hydroxy-Alkanoates (PHAs) in one biofilm reactor (Chapter 2). In a separate biofilm reactor, ammonium was adsorbed on zeolite (Chapter 3), and zeolite bio-regeneration was achieved by nitrifying adsorbed ammonium producing a nitrate solution (Chapter 4). The next step to achieve total Nitrogen (N) removal is denitrification, and it requires dissolved carbon, which is unavailable as it is stored as PHA in GAO in a separate reactor. However, PHAs are electron rich species that have been used for denitrification in previous studies [13,14].

In this chapter, storage driven denitrification will be tested in the carbon storage reactor. If it is verified, the overall process for complete carbon (C) and total N removals would have been demonstrated as individual steps. Yet the combination of the processes in continuous operation requires testing to ensure that each individual process behaves in the same fashion when operated in sequence.

Two operating modes of these steps will be evaluated in this chapter:

1. Operating the two storage processes, of dissolved carbon and ammonium, in sequence with separate reactors, and achieving denitrification by linking the effluent of the second to the first reactor.

2. Combining and operating the two processes in one single biofilm reactor to achieve both dissolved carbon and total nitrogen removal.
5.2 Materials and methods

5.2.1 Operation of two biofilm reactors for carbon and nitrogen removal

The biofilm reactor for carbon storage and its oxidation is the same as the one described in Chapter 2. The nitrifying biofilm reactor with zeolite was described in Chapter 4. The operation of the process for the carbon and nitrogen removal was as follows, and Figure 5-1 provides a visual aid to comprehend the operation:

- Under anaerobic conditions, wastewater filled the carbon biofilm reactor from bottom to top (0.18 L/min) and was recirculated (50 mL/min) maximising contact of wastewater and biomass which stores dissolved carbon (2h).

- The carbon free wastewater was drained (30 min) from the carbon biofilm reactor with N₂ gas for pressure equalisation. The second reactor was filled with this liquid from bottom to top (0.12 L/min) and left in contact with the zeolite to permit ammonium storage (30min).

- The ammonium free liquid was pressure drained and recirculated (7h), by trickling from top to bottom (0.2 m³/m²/d), to permit nitrification and zeolite bio-regeneration.

- The nitrate rich liquid was recirculated to the carbon storage reactor and left in contact with the carbon biofilm for denitrification (2h).

- The carbon and nitrogen free effluent was disposed of.
Figure 5-1: Schematic diagram of the two reactors’ operations in sequence. 1. Initial wastewater filling in GAO biofilm reactor (R1) to achieve anaerobic carbon storage. 2. Once acetate was removed, the wastewater was transferred to the nitrifying biomass reactor with zeolite (R2) to achieve ammonium storage on zeolite. 3. Trickling of the liquid for nitrification in R2. 4. Recirculation of the nitrified solution in R1 to achieve denitrification.

5.2.2 Zeolite amended biofilm reactor development

The zeolite amended biofilm reactor was a cylindrical reactor 14 cm high and 6.8 cm diameter. The total volume of the reactor was 400 mL. GAO (5.1 g dry weight) on its carrier was obtained from the carbon storage biofilm and was added to the reactor. Then, 15 g powder (50 - 80 μm) of Australian clinoptilolite (Zeolite Australia Pty. Ltd.) was suspended in a synthetic wastewater solution and trickled over the biofilm for 24 h. No Optical Density (OD) was measurable in the solution, which meant that all the zeolite was in contact with the GAO biofilm. This zeolite size was selected to overcome the shortcomings identified previously. Considering that the Australian clinoptilolite in this study has an effective capacity of 1.68 mg-N/gz (Section 3.3.1), the total reactor capacity is 25.2 mg-N (1.8 mmol-N). Thereafter, a 2 L solution of nitrifying biomass (Section 4.2.2) at a concentration of
5.4 g/L (OD$_{600} = 1.2$) was trickled over the existing biomass and zeolite of the reactor for 24 h at 120 mL/min. OD measurement confirmed that the biomass was removed from solution over this period of time (OD$_{600} \sim 0.1$). This single biofilm reactor is hereafter referred to as the zeolite amended biofilm reactor.

5.2.3 Zeolite amended biofilm operation

The zeolite amended biofilm reactor was operated in cycles of 48 h as follows (Figure 5-2):

- **Stage 1: Anaerobic (24 h)**
  1. Dissolved carbon storage in GAO biofilm
  2. Ammonium adsorption onto zeolite
- **Stage 2: Aerobic (24 h)**
  3. Liquid drainage and recirculation (15 mL/min) for nitrification (Table 5-1)
  4. Storage driven denitrification, which reduces nitrite and nitrate, and oxidises carbon

In the reactor volume (400 mL), the combination of the biomasses, zeolite and carrier material filled half of the reactor (200 mL), and this material’s pore void volume was 130 mL. This pore void volume corresponds to 1 Bed Volume (BV), which is the volume of liquid that is in contact with the biomasses and zeolite. So the working volume of the reactor is equal to the remaining 200 mL of reactor in addition to the void volume (130 mL), which equates to 330 mL. Each cycle operation therefore treated 2.5 BV (330 mL) of wastewater. On this consideration, each working volume contains about 1 mmol of ammonium (3 mmol/L per 0.33 L), and considering the effective capacity (Section 3.3.1), zeolite can adsorb 1.8 mmol-
N without regeneration. The zeolite amended biofilm could operate about 2 cycles without requiring regeneration.

Figure 5-2: Schematic diagram of the reactor operation. During Stage 1, the inflow of wastewater was anaerobic, GAOs stored acetate and at the same time, zeolite adsorbed ammonium. Once carbon and ammonium were removed from solution, the liquid was drained. In Stage 2 the wastewater was recirculated at a slow rate and the air intake allowed for simultaneous nitrification and denitrification. The air intake valve was continuously open to maintain the air inflow throughout the biofilm.

Table 5-1: Flow rate calculation to determine the hydraulic loading rate in the zeolite amended biofilm during the aeration period (Stage 2).

<table>
<thead>
<tr>
<th>Flow rate</th>
<th>Biomass</th>
<th>Bioballs</th>
<th>Reactor</th>
<th>HLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>mL/min</td>
<td>m3</td>
<td>m2/m3</td>
<td>m2</td>
<td>L/m2/h</td>
</tr>
<tr>
<td>15</td>
<td>0.9</td>
<td>1.5 x 10^-4</td>
<td>350</td>
<td>0.05</td>
</tr>
</tbody>
</table>

5.2.4 Experimental set-up for N₂ production rate from ammonium adsorbed on zeolite

To test the effect of the amount of adsorbed ammonium on the N₂ production rate, three ammonium amounts were added to the zeolite (0, 0.02 and 0.13 meq/g_zoelite).
Firstly, an ammonium-free synthetic wastewater was added to the zeolite amended biofilm reactor in Stage 1 (0 meq/g), and the \( \text{N}_2 \) production measured in Stage 2 (Section 5.2.5.2).

To add more ammonium on zeolite, one cycle operation was done with synthetic wastewater (3 mmol-N/L). In Stage 1, synthetic wastewater (3 mmol/L) was added to the reactor and the ammonium removed measured to calculate the ammonium adsorbed on zeolite (0.02 meq/g, or total 0.3 meq). Subsequently the \( \text{N}_2 \) production in Stage 2 was measured.

Finally, three subsequent working volumes of synthetic wastewater were added without Stage 2. The ammonium adsorbed on zeolite was measured from the ammonium concentration in the outflow of each BV, and the ammonium adsorbed on zeolite was 0.13 meq/g. Thereafter \( \text{N}_2 \) production was tested as per Section 5.2.5.2.

5.2.5 Analysis

5.2.5.1 Carbon and nitrogen analysis

All the analysis of the dissolved nitrogen species and organic carbon species were done as described in the previous chapters (Section 2.2.4.1, 3.2.3, 4.2.5.1 and 4.2.5.2). The synthetic wastewater was prepared according Section 2.2.3 unless otherwise stated.

5.2.5.2 \( \text{N}_2 \) gas analysis

\( \text{N}_2 \) production due to denitrification was measured in Schott bottles of 150 mL closed with a rubber stopper to make them air-tight. The measurement required the biomass to be in a condition equivalent to atmospheric air without \( \text{N}_2 \) gas. After
placing a known weight of biomass (± 30 mg) in the air-tight Schott bottle, the air in the Schott bottle was purged with Helium (high purity 99 %) for 2 minutes. O₂ was added by an air-tight syringe through the rubber stopper to achieve atmospheric oxygen partial pressure in the reactor (20 %). The O₂ volume to be added (to make 20 %) was determined by weighing the maximum volume of DI water contained in the Schott bottle and adding 20 % of it as O₂. This method assumes that water has a density of 1.0 g/L, so its weight is equal to its volume. At the same time, a Schott bottle (150 mL) without biomass was prepared in the same manner and was used as a control. The control was to verify that the sampling method did not introduce air.

The sampling method was made with an air-tight syringe (500 μL) that perforated the rubber stopper. The sample volume analysed was 100 μL. All samples were done in duplicate and an average of the value was used for determining the N₂, O₂ and CO₂ content. The gas determination was done through a Gas Chromatograph (Shimadzu 2014 GC) fitted with an Alltech concentric column (CTR CAT number 8700, 6 ft) that has a molecular sieve 13x 80/100 washed outer column (outer diameter 1/4"), which separates the oxygen and the nitrogen compounds and gives clear peaks. The inner column (outer diameter 1/8") was filled with Porapak Q (silanated silica), which separates the CO₂ from all compounds. The analysis time was 3.5 min, the column temperature was constant at 40 °C, the injection and detector temperatures set at 150 °C and the Helium carrier gas flow was 50 mL/min (He high purity).
5.3 Results

5.3.1 Operation of two biofilm reactors in sequences

5.3.1.1 Denitrification using stored carbon

Zeolite bio-regeneration using a nitrifying biofilm produced a nitrate solution (chapter 4), which must be removed. Conventional treatment plants remove nitrate by denitrification. To test whether the previously described GAO biofilm (chapter 2) was capable of denitrification using its stored carbon (PHA) as electron donor, a subsample of the GAO biomass on its carrier (5 g dry biomass) that had stored carbon anaerobically (8 mmol/L acetate) was exposed to a solution of 2 mmol/L NO$_3^-$, which was recirculated over the GAO biofilm. Under anaerobic conditions, NO$_3^-$ was fully removed, at a rate of 0.3 mmol/L/h, and the pH increased from 6 to 7.2 (Figure 5-3). This shows that the carbon storage biomass was capable of reducing nitrate.

![Figure 5-3: Denitrification test of the reactor without soluble acetate and ammonium. pH (●) and NO$_3^-$ concentration (■).](image_url)
5.3.1.2 Combination of processes for C and total N removal

The processes of C and total N removals have been demonstrated in separate reactors (chapters 2, 3 and 4), and the capability of the GAO biofilm reactor to denitrify has been documented above. It is now of interest to test the operation of the process in a continuous run. To combine all processes, the wastewater was added to both reactors in the sequence described in Section 5.2.1 (Figure 5-1). Briefly, the carbon storage biofilm reactor was filled and soluble carbon was stored. The carbon-free wastewater was drained and transferred to the nitrifying reactor. Ammonium was adsorbed onto the zeolite. The nitrification of the adsorbed ammonium was done by trickling the liquid over the zeolite coated with nitrifiers. The nitrate wastewater (~ 2.8 mmol/L) was returned to the carbon storage reactor for denitrification.

The dissolved carbon and nitrogen removal from wastewater was achieved in a continuous run with a combination of two processes in sequence (Figure 5-4). About 99 % of COD and ammonium were removed from solution as PHAs and adsorbed on zeolite respectively. The subsequent nitrification step converted 93 % of the adsorbed ammonium as nitrate, demonstrating the bio-regeneration of the zeolite. The nitrate wastewater was fully denitrified while in contact with GAOs. The overall process resulted in a wastewater without dissolved carbon or nitrogen, which makes it safe for disposal. This experiment demonstrated the proof of concept that C and total N removal can be achieved in a storage process and an oxidation process without bulk liquid aeration. Limiting liquid recirculation will be investigated further in Chapter 7.
Figure 5-4: Time course of the processes used in a single run to remove dissolved carbon and nitrogen from wastewater. The carbon as acetate (♦) was biologically stored, and the ammonium (●) was adsorbed onto the zeolite with nitrifying biomass. In the aeration period, nitrate (■) was produced. Returning the nitrate wastewater to the carbon storage biofilm denitrified the solution and produced a wastewater without carbon or nitrogen.

5.3.1.3 Consideration on the viability of a two-reactor process for C and N removal

The combined processes described above require transferring the full reactor’s volume between the nitrification reactor and the carbon storage/denitrification reactor. Assuming that the two reactors are 5 m in height and are separated by 5 m, the theoretical energy used to move the liquid from one reactor to the next can be calculated at 98 J/L. The energy expense for the liquid transfer can be assumed to be proportional to the liquid volume, so to minimise the expense the liquid circulation should be reduced. Reducing the volume in the nitrifying biofilm reactor was tested (Section 4.3.5). However, it was found that decreasing the liquid volume from 1 L to 200 mL caused the build-up of metabolites leading to decreased nitrification and nitrogen recovery of only 30 % (i.e. no zeolite regeneration). Furthermore, using
small volumes required chemical dosage for pH control. Therefore, it was concluded that volume reduction was not ideal to achieve the complete carbon and nitrogen removal process.

One could argue that recirculating a small liquid volume between the two reactors multiple times would avoid metabolite accumulation from nitrification and the adverse pH drop, and hence enable the complete nitrification and zeolite bio-regeneration. However, the transfer of liquid from one reactor to the next would have to be done multiple times which also represents an energy expense. In addition, nitrification via recirculated liquid trickling requires extra land surface area to build a recirculation vessel, which reduces the viability of the process.

In order to improve the applicability of the process, we hypothesised that combining the two biomasses (GAO and nitrifying biomass with zeolite) in a single reactor could achieve carbon and nitrogen removal, while allowing for energy savings (no chemicals, less space, and no air pumping required). The organisation of the biomasses in a combined biofilm reactor could be approximated as two separate bioreactor compartments, with one nitrifying biofilm on zeolite and one GAO biofilm (Figure 5-5). However, this would still cause the problems found with two separate bioreactors, as recirculating small volumes of liquid would decrease the pH and prevent the zeolite bio-regeneration. Therefore, the distance separating the components: GAOs, zeolite and nitrifiers should be minimised (Figure 5-5). The short distance between the components would enable the zeolite to regenerate and produce nitrate and almost immediately denitrify it and thus control the pH. In order to achieve the minimum distance between each component, a biofilm was ‘synthesised’. The synthesis was done by adding the three components separately
one after the other: GAOs, powdered zeolite and nitrifying biomass as described in the method Section 5.2.2.

![Diagram](image.png)

**Figure 5-5**: Schematic diagram to distinguish the potential operation of two separate bio-compartment in a single reactor and the mixing of two components throughout the reactor to minimise the distance between the reactions.

### 5.3.2 Zeolite amended biofilm testing

#### 5.3.2.1 Carbon and nitrogen removal in a synthesised biofilm

The zeolite amended biofilm reactor was ‘synthesised’ (Section 5.2.2) and the reactor contained 2.5 BV per cycle to be treated. The reactor was prepared in order to remove both dissolved carbon and ammonium with minimum liquid recirculation and increase the process’ viability. However, mixing the components together, for synthesising the biofilm, might prevent one or all of the following processes to operate: the GAOs to store carbon, the zeolite to adsorb ammonium, the nitrifying biomass to oxidise ammonium and the denitrification to reduce nitrate. Therefore, the dissolved carbon and nitrogen removals from wastewater were tested with this
newly synthesised biofilm reactor. Immediately after seeding, the biofilm reactor was operated for one cycle (48 h) comprising two stages of 24 h:

- Anaerobic Stage 1 for carbon storage and ammonium adsorption, and
- Aerobic Stage 2 for zeolite bio-regeneration and denitrification.

Stage 2 still required some liquid recirculation, but it was recirculated at 0.4 $m^3/m^2/d$ which is 2 to 10 times lower than low rate trickling filters [8]. The top and bottom openings of the reactor allowed air diffusion throughout the reactor and exposed the biofilm to oxygen. In the anaerobic phase (Stage 1), the biomass achieved complete acetate storage and zeolite adsorbed 82 % of the ammonium (Figure 5-6 A.). In Stage 2, the sum of nitrite and nitrate in solution increased to 1.0 mmol/L, showing that nitrification occurred. After 3 h, the oxidised N species concentrations decreased (Figure 5-6 B.), suggesting that denitrification occurred. The synthesised biofilm seemed to achieve complete carbon and nitrogen removal by integrating the nitrification and denitrification processes. It is important to note that the presence of oxygen is known to inhibit denitrification, hence the decrease in oxidised N-species concentrations requires further investigation to explain the seemingly “aerobic denitrification”.
Figure 5-6: A. Time course of the anaerobic acetate (♦) and ammonium (●) removal. B. Time course of the aerobic phase (Stage 2). The ammonium (●) was less than the cumulative nitrite (▲) and nitrate (■) after 2 hours, suggesting “aerobic denitrification”.

5.3.2.2 Investigation of the biofilm layout to explain “aerobic denitrification”

5.3.2.2.1 Nitrifying biomass’ oxygen consumption favours “aerobic denitrification”

In the synthesised biofilm, nitrifying biomass oxidised the adsorbed ammonium on zeolite and consumed oxygen; hence creating an oxygen gradient in the biofilm. This is similar to published observations of SND in Activated Sludge (AS) [145]. It
was postulated that without nitrification there is no oxygen gradient in the biofilm and therefore denitrification is inhibited. The effect of oxygen consumption by the nitrification process on the denitrification rate was tested in two separate reactors with and without ammonium under aerobic conditions. The control had ammonium adsorbed on zeolite, and the test did not. To ensure that the test had no remaining ammonium adsorbed on zeolite, an aeration period of 48 h was provided for oxidation. Therefore, when the reactors were operated, only the control reactor had an active nitrifying biomass. In both reactors, a wastewater with excess nitrate was trickled on their respective biofilms. The denitrification rate of the reactor with active nitrifiers was 8.4 mmol/L/h, while in the absence of nitrification to consume oxygen the denitrification rate was slower at 5.5 mmol/L/h. (Figure 5-7) at DO > 4 mg/L. The slower denitrification rate can be explained by deeper oxygen penetration in the biofilm. Without nitrification, the oxygen penetrates in the biofilm and there is more biomass exposed to air, resulting in less biomass under anaerobic conditions, hence the denitrification rate is slowed down. Overall, the effect of oxygen consumption by the nitrifying biomass partly explained the “aerobic denitrification”.

It is worth mentioning that in the test reactor, with active nitrification, the aeration enabled the nitrification of approximately 2 mmol/L adsorbed ammonium. The oxidised N produced was not measurable because of the excess nitrate added at time 0, and therefore the measured denitrification rate did not consider the additional ammonium adsorbed. This suggests that the denitrification rate with active nitrifying biomass was probably greater than the one reported above.
5.3.2.2.2 Biofilm in suspension inhibits “aerobic denitrification”

The nitrification process does not fully explain the “aerobic denitrification”. The biomass’ maintenance reactions might create the oxygen gradient throughout the biomass even in the absence of nitrification. The aim is to test whether “aerobic denitrification” was possible if the biomass was in suspension; that is when the entire biomass is exposed to oxygen (no gradient). After carbon storage, a subsample of the biofilm was separated from its carriers and was suspended in an aerated nitrate solution. The suspension process did not affect the biomass viability because the oxygen uptake was maintained (data not shown). The nitrate concentration in solution remained constant demonstrating the inhibition of the denitrification (Figure 5-8). Acetate was added after 2 hours to ensure that denitrification was not limited by the absence of electron donor. Even in the presence of soluble electron donors, denitrification did not occur. The biofilm did not denitrify when in suspension, hence no “aerobic denitrification” process or micro-organisms were shown to exist in the
synthesised biofilm. In the proposed zeolite amended biofilm process, as the oxygen penetrates the biofilm, it is consumed by the aerobic processes (e.g. maintenance), and creates anoxic conditions in the deeper layers of the biofilm. The seemingly “aerobic denitrification” observed did not actually occur in air. Denitrification could occur because of the lack of oxygen penetration to the biofilm, rather than denitrifying biomass being tolerant to oxygen [32].

Figure 5-8: The effect of removing the biofilm structure on the denitrification, in the presence of high NO$_2^-$ (▲), under saturated DO (●) conditions, and at constant pH (■). The arrow indicates the addition of 8 mmol/L acetate that ensured electron donors were not limiting.

5.3.2.3 Nitrification rate in the zeolite amended biofilm

So far, the process using the zeolite amended biofilm reactor was capable of removing soluble carbon and nitrogen in wastewater. The process involves the biological carbon storage and the ammonium adsorption on zeolite in the anaerobic Stage 1 (Section 5.3.2). The bio-regeneration of zeolite, through nitrification, was suggested to occur by a small accumulation of nitrite and nitrate in solution, and because of an oxygen gradient in the biofilm, NO$_2^-$ and NO$_3^-$ were denitrified. The
nitrification rate is important for the viability of the process, however, because nitrification and denitrification occur simultaneously, the nitrification rate cannot be simply measured. The synthesised biomass was first starved of carbon for 48 h to prevent denitrification. The oxidation rate of the adsorbed ammonium on zeolite was measured by monitoring the oxidised N species in solution. The initial nitrification rate was 0.7 mmol/L/h over 3 h and then decreased (Figure 5-9). This nitrification rate is somewhat lower than what is obtained by AS cultures (1.0 mmol/L/h) [50]. It is of interest to outline that the denitrification rate was 8.4 mmol/L/h under DO > 4 mg/L (Section 5.3.2.2.1) while the nitrification is about 10 times slower, which explains the reasons for no nitrite and nitrate build up in Stage 2.

After 3 h, the solution’s pH dropped to 6.3 which can explain the decreased nitrification rate. However, under the normal reactor operation (i.e. no carbon starvation), denitrification will occur simultaneously and will consume the acidity. Without liquid acidification, the biofilm is expected to maintain its nitrification rate. The results from the combination of denitrification and nitrification experiments imply that the biofilm achieved a Simultaneous Nitrification and Denitrification (SND) process under atmospheric air conditions.
Figure 5-9: In the absence of stored carbon, sum of nitrite and nitrate $\left(\text{NO}_2^- + \text{NO}_3^-\right)$ $\bullet$ concentration increased in solution and the nitrification decreased the pH $\times$ in solution. Ammonium $\bullet$ was removed at 80%.

5.3.3 Repeated operation of the zeolite amended biofilm

The synthesised biofilm made of GAOs, nitrifying culture and zeolite achieved dissolved carbon and TN removal through SND. Considering that the biofilm has been synthesised rather than selectively enriched, the achievements demonstrated above might not be sustained over time. The aim is to test for the carbon and nitrogen removal in the reactor over a longer period of time. During the operation of 22 cycles (6 weeks), representative cycles were tested for acetate and total N removal. In Stage 1, 82% of ammonium in the inflow was removed consistently, and in Stage 2 only 0.1 mmol/L (1.4 mg-N/L) nitrate concentration remained (Figure 5-10 A.). In Stage 1, the carbon was continuously stored to 99% (Figure 5-10 B.). The result demonstrated that the synthesised biofilm could sustain carbon and ammonium removal with a simple aeration process of atmospheric exposure, and mass transfer through a recirculation of the liquid during Stage 2 at a Hydraulic
Loading Rate (HLR) of 0.4 m$^3$/m$^2$/d, which is at least 4 times less than a slow rate trickling filter [8].

![Graph A](image1.png) ![Graph B](image2.png)

Figure 5-10: A. Total nitrogen, sum of ammonium, nitrite and nitrate, in the inflow of Stage 1 (black), at the outflow of Stage 1 (hatched) and in the outflow of Stage 2 (white). B. Acetate concentration in the inflow (black) and outflow (white) of Stage 1.

### 5.3.4 Nitrogen gas production in the biofilm under atmospheric conditions

The long-term operation of the zeolite amended biofilm reactor demonstrated consistent N removal from the wastewater. Under atmospheric oxygen partial pressure, the N removal was achieved by oxidising the adsorbed ammonium on
zeolite, and denitrification using the stored carbon by GAOs. However, N_2 gas still needs to be quantified to ensure that ammonium removal is not simply due to continuous adsorption by zeolite but results in N_2 emission. The denitrification of the ammonium adsorbed on zeolite in a single cycle and then in three subsequent cycles was tested by placing a sample of the biofilm (3.12 g dry weight) in an atmosphere of Helium (He = 80 %) and oxygen (O_2 = 20 %), and monitoring the N_2 gas produced (Section 5.2.5.2). Over a period of 24 h Stage 2, 29.7 mL of N_2 was produced per litre of reactor (Figure 5-11 A). The N_2 production increased to 42.3 mL/L when three subsequent wastewater cycles were added to the zeolite amended biofilm reactor in Stage 1 (Figure 5-11 B). These experiments showed that denitrification by the synthesised biofilm was indeed occurring in the presence of full atmospheric oxygen partial pressure (Appendix D). To account for the N_2 that could have inadvertently entered the system, an abiotic control (no zeolite and no biofilm in the vessel) was run in each experiment with Helium (80 %) and Oxygen (20 %) and sampled similarly to the experiment. It showed that no N_2 was introduced by the sampling method.

A nitrogen mass balance was not possible because the oxygen supplied decreased over time and limited the nitrification, therefore the synthesised biofilm could not completely nitrify the adsorbed ammonium on zeolite. In fact, the nitrogen recovery was less than 60 %. On the contrary, under normal operation of the zeolite amended reactor, the oxygen supply would be the same continuously because of oxygen diffusion from the top and bottom aperture of the reactor. It is expected that the recovery would be maximised. It was also observed that some N_2 production in the zeolite amended biofilm reactor even without added ammonium in Stage 1 (Figure 5-
this suggested that not all of the adsorbed ammonium was oxidised under normal operating conditions.

Figure 5-11: Time course of the nitrogen gas (■) produced in zeolite amended biofilm in Stage 2. A. When 1 load of wastewater was added in Stage 1. B. When 3 subsequent loads of wastewater were added in Stage 1. C. Without added ammonium in Stage 1. Legend: Oxygen (●), CO₂ (▲). The expected nitrogen production (-----) was calculated from the ammonium added to the reactor in Stage 1. The abiotic control that tested for air introduction by the sampling method showed constant nitrogen (□) and oxygen (○) therefore the method did not introduce air in any of the experiments.
5.4 Discussion

5.4.1 Two reactors process compared to parallel nitrification denitrification

The operation of the two reactor process in sequences was successful and removed > 99 % of the dissolved organic carbon and 93 % of the total N. This is a good performance compared to an AS sludge. However, the reactor operation is more comparable to the Parallel Nitrification Denitrification (PND) process developed at Murdoch university [79]. The difference lies in the nitrifying reactor whereby the present study uses zeolite as a carrier material for the nitrifiers. Thus the aeration for oxidation of ammonium is possible by air exposure instead of liquid aeration. The removal efficiencies are similar to that of PND that were > 95 % of dissolved carbon and 98 % of nitrogen [14]. However, PND had a treatment time of 8 h which has the advantage of a more compact treatment process compared to the treatment time 11 h presented for zeolite amended biofilm reactor.

5.4.2 Oxygen gradient in zeolite amended biofilm and the apparent “aerobic denitrification”

The operation of the zeolite amended reactor to reduce the cost of a two reactor operation resulted in the observation of an apparent “aerobic denitrification”. The “aerobic denitrification” was investigated and it was determined that the oxygen gradient formed within the biofilm enabled to achieved carbon storage driven denitrification (Figure 5-8). “Aerobic denitrification” has been reported in suspended biomass [53,146] and the authors have suggested that the oxygen gradient in the floc is responsible for an anoxic zone, and creating an apparent “aerobic denitrification”. In these studies, the DO was carefully controlled to achieve SND in air (DO ± 1 mg/L). In biofilms, “aerobic denitrification” was also reported and it was achieved in higher DO concentrations. Masuda et al. [71] reported SND in a rotating biological
contactor with DO in liquid up to 2 mg/L, which was due to the partial oxygen pressure drop, as their reactor was covered. Our proposed biofilm achieved SND in full atmospheric air. It is known that the biofilm development results in gradients in oxygen and in substrate [59], but it is the first time that SND in full air is reported.

5.4.3 Comparison of the HRT for the zeolite amended biofilm reactor and AS

The hydraulic retention time (HRT in h) is the time required to treat one reactor’s volume. It is conventionally calculated as the reactor volume over the flowrate. In the zeolite amended reactor, the working volume is 330 mL (2.5 BV), and it is replaced every 48 h. Using the conventional calculation, the HRT is 48 h. However, we argue here that the HRT of this reactor is less than 48 h. The reactor working volume is 330 mL. Yet, the pore void volume of the zeolite amended biofilm is 130 mL that is equal to 1 Bed Volume (BV), thus the reactor’s working volume contains 2.5 BV (Figure 5-12). Therefore, in 48 h 2.5 BV are treated, that is an HRT of 19 h per BV.

Conventional AS processes using a Sequencing Batch Reactors (SBR) have HRT of 12 h [147,148]. This HRT is smaller than that of the zeolite amended biofilm reactor proposed in this study. Considering that the HRT is proportional to the reactor land surface area used [6], then the zeolite amended biofilm reactor would have a total surface area greater than that of an AS process. This is a significant drawback for the applicability of the zeolite amended biofilm reactor described here. Optimisation of the zeolite amended biofilm is investigated further in Chapter 7.
5.5 Conclusion

- The combination of all processes developed in previous chapters enabled complete removal of dissolved carbon and total N, by linking the reactors together in a continuous run. After carbon storage, the same biomass could operate a storage driven denitrification, which completed the total N removal process. The process achieved similar efficiencies compared to PND without liquid aeration but used longer treatment time.

- The operation of a synthesised biofilm with GAOs, nitrifying biomass and zeolite in a single biofilm (i.e. Zeolite amended biofilm reactor) was immediately able to remove 99% of dissolved carbon and 80% nitrogen. Even though the biofilm was synthesised, it was capable of operating over 21 cycles without loss in efficiency.

- Considering that the zeolite amended biofilm treated 2.5 BV in 48 h, the treatment time for 1 BV was 19 h, which is longer than that of a traditional
AS process (12 h). Thus the zeolite amended reactor would be less viable if the treatment time is not reduced.

- The biofilm achieved SND under atmospheric oxygen partial pressure, which was reported here for the first time.
Chapter 6  Sustainability of the synthesised biofilm in the single reactor over its long-term operation

6.1 Introduction

The zeolite amended biofilm reactor was developed to prevent energy expense associated with liquid recirculation between two reactors. The biofilm was synthesised by simply adding two biomasses together with zeolite. The aim of this chapter is to test for the sustainability of the GAOs and the nitrifying biofilm in the zeolite amended biofilm reactor after its long-term operation.

6.2 Materials and methods

The zeolite amended biofilm reactor development (Section 5.2.2) and its operation (Section 5.2.3) were described in Chapter 5. The synthetic wastewater was made according to the previous description (Section 2.2.3) unless otherwise stated. Analyses of dissolved organic carbon (Section 2.2.4.1), ammonium (Section 3.2.3), nitrite (section 4.2.5.1) and nitrate (Section 4.2.5.2) were done according the previously described sections.

6.3 Results

6.3.1 Biological carbon storage capacity

In the zeolite amended biofilm reactor that operates with cycle lengths of 48 h, with a Simultaneous Nitrification and Denitrification (SND) process in Stage 2, the synthesised biofilm might not be sustained. To evaluate whether the GAOs’ storage capacity remained sufficiently high, the carbon storage rates were compared after 1
and 14 cycles. The carbon storage rate over the first hour of each test was used to compare the rates. The carbon storage rate increased from 2.7 mmol/L/h to 3.8 mmol/L/h that is an increase of 41%, after operating the reactor for 14 consecutive cycles (Figure 6-1). This demonstrated that the GAOs’ storage capacity did not diminish and appeared to be sustained as shown in Chapter 2.

![Time course of acetate removal](image)

Figure 6-1: Time course of acetate removal in cycle 1 (○) and after 14 cycles (1 month) of operation (●). When looking at the maximum speed of the acetate uptake in the first hour, the rate increased from 2.7 mmol/L/h to 3.8 mmol/L/h.

It was assumed that the 41% rate improvement was due to a 41% GAO biomass increase. Assuming that all the 16 Cmmol/L of acetate per cycle (8 mmol/L of acetate or 512 mg/L BOD) was stored and used for growth, and that biomass molecular weight is 24.6 gX/Cmol [16,23], then the biomass growth was estimated to be 0.33 CmolX/CmolAc (Figure 6-1). This value is in line with the carbon storage biomass growth yield of 0.30 CmolX/CmolAc reported by Beun et al. [23].
Table 6-1: Calculation of GAO yield in the present condition. $T_0$ was the biomass that was added in the reactor (Section 5.2.2) and assuming that the water content was 90%.

<table>
<thead>
<tr>
<th>Number of Cycle</th>
<th>Carbon /cycle Cmmol/L</th>
<th>Volume /cycle L</th>
<th>Total Carbon Cmmol</th>
<th>$T_0$ dried biomass $g_x$</th>
<th>Biomass molecular weight $g_x$/Cmol$_x$</th>
<th>41 % Increase in biomass $g_x$/Cmmol$_x$</th>
<th>Yield Cmmol$_x$/Cmmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>16</td>
<td>1</td>
<td>224</td>
<td>4.5</td>
<td>24.6</td>
<td>1.84</td>
<td>75.0</td>
</tr>
</tbody>
</table>

6.3.2 Nitrifying biomass sustainability

6.3.2.1 Nitrification rate over time

The zeolite amended biofilm reactor had its biofilm structure synthesised by adding an excess of nitrifying biomass (10.2 g) to the GAO biofilm coated with zeolite. In conventional wastewater treatment plants, the ratio of heterotrophs to autotrophs is about 10/1 [8], while in our synthesised biomass it was 1/2. The high proportion of nitrifiers may not be sustained over a long operating time. The aim of this experiment is to test whether nitrifiers were maintained during 30 cycles of the reactor operation. This was done by comparing the reactor’s nitrification capacity after seeding, and 30 cycles of reactor operation. For each test, the nitrification capacity was tested after starving the reactor of carbon for at least one cycle so that denitrification was inhibited. A carbon-free wastewater was trickled over the biofilm, and the nitrite and nitrate concentrations monitored. After seeding, the nitrification rate was 1.1 mmol/L/h and it was similar after 30 cycles of operation with a rate of 1.0 mmol/L/h (Figure 6-2), suggesting that a medium term operation of 3 months was feasible.

It is also noteworthy that in the first two months (15 cycles) of operation, NO$_3^-$ accumulated in the effluent, however, during the last nitrification test only NO$_2^-$ built up (data not shown). Denitrification was excluded because of the carbon starvation...
process prior to the experiments. The \( \text{NO}_2^- \) build-up suggested that Nitrite Oxidizing Bacteria (NOB) had been lost from the nitrifying population over time. This could be of benefit for the reactor performance and is discussed in the next section.

![Figure 6-2: Time course of the sum of nitrite and nitrate (oxidised N species) produced in the zeolite amended biofilm reactor, starved of acetate, after seeding (■) and after 30 cycles (▲).](image)

### 6.3.2.2 Nitrite oxidizing bacteria removal

After 3 months of operation (30 cycles), the biofilm reactor’s effluent contained nitrite while nitrate was not detected. This suggests that NOBs were removed from the biofilm. To test whether prolonged operation eliminated NOBs from the reactor’s biomass, two biomasses were tested:

1. The nitrifying biomass used for seeding and
2. The biomass from the reactor, after 30 cycles (3 months) of operation.

Ammonium (3 mmol/L) was added to both biomasses and the nitrite and nitrate formation was monitored. To ensure that no denitrification could take place, no carbon source was added to both biomasses, and the reactor’s biomass was starved.
for two cycles to exhaust all the stored carbon. The seed culture produced mostly
NO$_3^-$ (2.2 mmol-N/L), while the biomass from the reactor produced exclusively NO$_2^-$
(1.4 mmol-N/L) (Figure 6-3). This supports the previous observation that the
operation of the biofilm reactor may have removed the NOBs initially present in the
seed culture, hence shifting the nitrifying population to almost pure Ammonium
Oxidising Bacteria (AOB).

There is a difference in total N produced after 4 h (Figure 6-3), between the
seeding culture (3 mmol/L) and the biomass from the zeolite amended biofilm
reactor (1.5 mmol/L). This might be due to the fact that in the biofilm reactor there is
some ammonium adsorbed on zeolite.

![Figure 6-3: Effect of time on NOBs present in the reactor. Time course of the seed culture production of NO$_2^-$ (Δ) and NO$_3^-$ (□) and the reactor biomass production of NO$_3^-$ (■) and NO$_2^-$ (▲) after 3 months of operation in a sequencing batch biofilm with SND.](image-url)
6.4 Discussion

6.4.1 Operation of GAO biomass to benefit nitrifying biomass development

In AS processes, the proportion of AOB in overall population will depend on the relative growth yield of AOB and heterotrophic organism. It can be derived from yield values from the literature (Table 6-2) that a biofilm reactor with GAO to store acetate will result in 50 % less heterotrophic biomass production compared to a conventional AS process with heterotrophic organisms. Accordingly the zeolite amended biofilm reactor described, which relies exclusively on GAO, is likely to contain a biomass ratio with 2 times more AOB than in an AS process. The ratio for each process can be calculated using the value of Table 6-2. In the AS process the ratio of OHO/AOB equates to 52.3, and in the biofilm reactor with pure GAO for carbon storage, the ratio of GAO/AOB equates to 26.8.

Table 6-2: Micro-organisms’ yield as Cmolx per mol of substrate based on the carbon molecular weight of 12 g/mol. The biomass production was based on an assumption that in wastewater the dissolved organic carbon was 16 Cmmol/L (8 mmol/L acetate or 512 mg/L BOD) and the nitrogen content was 3 mmol-N/L (42 mg-N/L).

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Yield</th>
<th>Expected biomass produced per L wastewater (mg/L)(g/m³)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Oxidizing Bacteria</td>
<td>0.06 Cmolx/Nmol</td>
<td>2.2</td>
<td>Strous et al. [149] Third et al. [108]</td>
</tr>
<tr>
<td>Glycogen Accumulating Organism</td>
<td>0.3 Cmolx/CmolAc</td>
<td>57.6</td>
<td>Beun et al. [23]</td>
</tr>
<tr>
<td>Ordinary Heterotrophic Organism</td>
<td>0.5 Cmolx/CmolAc, 0.67 Cmolx/CmolAc</td>
<td>96.0, 128.6</td>
<td>Lessard et al. [147] McClintock et al. [10]</td>
</tr>
</tbody>
</table>
6.4.2 Removal of biomass produced

In the zeolite amended biofilm reactor, the biomass growth was less (Section 6.4.1) than that of conventional AS treatment plant. For this reason, the biomass could be left to build up in the reactor over the duration of the experiments. However, the harvesting of excess biomass produced will diminish the zeolite over time leading to lower ammonium adsorption capacity. This will require continuous addition of new zeolite at a rate equal to the biomass harvesting.

For example, in the zeolite amended biofilm reactor, 15 g of zeolite was added to the biomass (approx. 6 g dry weight). The zeolite was 71% of the total weight (biomass + zeolite). From the Table 6-2 the total dry biomass produced is 59.8 g/dm³ of treated wastewater (16 Cmmol/L and 3 mmol-N/L). To estimate the zeolite replacement, the zeolite distribution on the biomass was assumed to be equal, such that it was assumed that the zeolite was 2.5 g/dm³ (15 g/6 g). Hence, the zeolite wasted is roughly 150 g/dm³ treated wastewater. As an example, Subiaco wastewater treatment plant, in Perth (Western Australia) treat 61 000 m³/d, therefore about 9 tons of zeolite need to be added per day. This zeolite wastage increases the excess sludge production, which counterbalance the lower biomass production. Even though the price of zeolite is not high (~ US $ 100/t), there is the overall cost of mining and transport of the material, which must be considered for the viability of this novel process. Zeolite recovery is one option to prevent excess costs of continuous zeolite addition.
6.4.3 Nitrifying biomass population in zeolite amended biofilm reactor

6.4.3.1 Benefit of NOB wash-out to treat wastewater

The NOB wash-out resulted in the production of nitrite as a final product during the nitrification step. One benefit of nitrite production is that it requires less organic carbon (3 electrons or ~1/3 of acetate) for denitrification than nitrate (5 electrons or ~2/3 of acetate)[13,23]. Thus, wastewater containing low organic carbon content can be entirely denitrified with endogenous dissolved organic carbon using the proposed zeolite amended biofilm reactor, while denitrification would be incomplete in a conventional AS.

6.4.3.2 Possible reason for NOBs washed out

In conventional AS processes, NOB can be diminished by limiting the oxygen supply [150,151]. However, in the zeolite amended biofilm reactor studied, the oxygen was saturating because the biofilm is exposed to atmospheric oxygen partial pressure. The NOB could have been washed out because of the lack of NO$_2^-$ available. The denitrification rate (DO = 4mg/L & NO$_3^-$= 2 mmol/L) was about 10 times faster than the nitrification rate (DO > 4 mg/L and 3 mmol/L ammonium) (Chapter 5, Section 5.3.2.3). The denitrification process will reduce the dissolved NO$_2^-$ and prevent the accumulation of NO$_2^-$ resulting in NOB growth inhibition and consequently their washout.

6.4.3.3 Biomass analysis for further insight

The synthesised biofilm was a combination of nitrifying population and soluble carbon storage biomass. The operation over time of the biofilm might have change the biological populations. Comparing the biomass before and after the operation of the biofilm might have shown the mechanism of the combined soluble carbon and
ammonium removal. In addition, it would provide an insight on the population competitions in the biofilm, hence it is potential to operate over long period of time. The analysis was not conducted in this research; however it is being conducted in our research group. Such analysis will provide great information on the competition and the viability of the process.

### 6.4.4 Denitrification: incomplete oxidation of stored carbon

In the zeolite amended biofilm reactor operated with the particular synthetic wastewater used in this study, denitrification cannot completely oxidise stored carbon in the biomass, because of insufficient dissolved nitrate available (Table 6-3). In chapter 2 (Figure 2-6), the partial oxidation (< 20 %) of stored carbon resulted in < 50 % carbon storage during the subsequent anaerobic phase instead of 99 % with complete oxidation (≥ 27 %). However, this could be addressed by providing oxygen as an electron acceptor via extended exposure to air after drainage, similarly to the operation of the reactor in Chapter 2, additional aeration will oxidise the remaining stored carbon when denitrification is insufficient. Jones et al. [25] have previously reported that both nitrate and oxygen could be used as electron acceptors for a biomass that stored organic carbon.
Table 6-3: Calculation of the electron remaining in the storage biomass after complete denitrification of the total N available in wastewater. Assuming that all the ammonium in wastewater (3 mmol/L) was oxidised to NO$_3^-$.

$^1$ the abbreviation $el$ stand for electron,

$^a$ this is assuming that the NO$_3^-$ to N$_2$,

$^b$ this assume full acetate oxidation to CO$_2$

<table>
<thead>
<tr>
<th>Total NO$_3^-$</th>
<th>Electron required for denitrification</th>
<th>Acetate stored</th>
<th>Electron available</th>
<th>Electron in biomass after denitrification</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol/L</td>
<td>$el^1$/NO$_3^-$ mmol/L</td>
<td>mmol/L</td>
<td>mmol/L</td>
<td>mmol/L</td>
</tr>
<tr>
<td>3</td>
<td>5$^a$</td>
<td>15</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8$^b$</td>
<td>49</td>
</tr>
</tbody>
</table>

6.5 Conclusion

- GAOs activity was improved by the operating of the biofilm.
- The nitrifying biomass was capable of a similar nitrification rate after 30 cycles of operation.
- NOBs were washout from the biomass, probably because of the rapid denitrification rate which reduced the nitrite availability.
- The excess biomass removal will remove zeolite simultaneously creating a significant additional cost to the treatment plant operation.
Chapter 7 Optimisation of carbon and nitrogen removal in the zeolite amended biofilm reactor with passive aeration

7.1 Introduction

The above described zeolite amended biofilm reactor was capable of removing dissolved organic carbon and total Nitrogen (N). During the aeration stage, liquid recirculation was used to provide adequate mass transfer, such as nitrite transfer from AOB to denitrifiers. However, liquid recirculation costs energy and requires a tank to hold and recirculate the wastewater over the biofilm. Therefore, one of the aims of this chapter is to operate the zeolite amended biofilm reactor without liquid recirculation in Stage 2 by using a mere exposure to atmospheric air.

The capacity of the zeolite amended biofilm reactor to remove organic carbon and nitrogen via Simultaneous Nitrification and Denitrification (SND) was possible with a Hydraulic Retention Time (HRT) of 19 h (chapter 5, Section 5.4.3). This HRT was longer than that of traditional activated sludge (AS) processes of about 12 h. Considering that the HRT determines the reactor size [6], reducing the operating HRT of the zeolite amended biofilm reactor would benefit the potential viability of this novel process. Another aim of this chapter is to test whether the HRT can be reduced to less than that of AS.

7.2 Materials and methods

The preparation of the synthetic wastewater and trace element solution (Section 2.2.3), and all the chemical compounds analysis were done according to the previously described methods unless otherwise stated: acetate (Section 2.2.4.1), \( \text{NH}_4^+ \) (Section 3.2.3), \( \text{NO}_2^- \) (Section 4.2.5.1) and \( \text{NO}_3^- \) (section 4.2.5.2).
7.2.1 Reactor operation without liquid recirculation

The zeolite amended biofilm reactor (dimensions and description in Section 5.2.2) treated 2.5 bed volume (BV) in cycle of 48 h (19 h hydraulic retention time), here the hydraulic retention time (HRT) takes into account the total treatment time that includes the aeration time necessary for the successful operation of the reactor. Each cycle was operated in two 24 h stages:

- Stage 1: Anaerobic phase enabling biological carbon storage and ammonium adsorption on zeolite (capacity 25 mg-N, Section 5.2.2)
- Stage 2: Aerobic period without liquid recirculation

In Stage 1, the reactor was filled with synthetic wastewater from the bottom in 10 min (~130 mL/min), and the liquid recirculated to maximise the contact between substrate and biomass (50 mL/min). After 24 h the liquid was removed and disposed of. In Stage 2, the reactor apertures (top and bottom) were open so that continuous air diffusion was possible. The reactor was operated for 8 consecutive cycles and the ammonium concentration was tested in the outflow of Stage 1 after each cycle.

7.2.2 Operation of zeolite amended biofilm reactor with 5 h HRT

The zeolite amended biofilm was transferred to a cylindrical reactor 8 cm high and 6.9 cm in diameter with a total volume 250 mL. Because of the volume occupied by the biomass and its carriers, the reactor’s working volume was thus 130 mL that is 1 Bed Volume (BV). Similarly to the previous operation of the reactor, two stages were used. The difference lay in the operating times of each of these stages, such that the HRT was 5 h:

- Stage 1: Anaerobic period of 1 h,
- Stage 2: Aerobic period of 4 h without liquid recirculation,
As previously stated the anaerobic period was necessary for ammonium adsorption on zeolite and biological carbon storage. Thereafter wastewater was gravity drained, by opening the top and bottom of the reactor, and disposed of. The apertures remained open throughout Stage 2 to sustain air diffusion to the biofilm (Figure 7-1). The sustainability of the system was tested over 18 subsequent cycles. The Stage 2 timing was also varied in later tests, and this will be described in the results section.

Samples were taken in Stage 1 through an airtight sampling port on top of the reactor. A peristaltic pump recirculated the liquid in Stage 1 (0.2 m³/m²/d) to maximise the liquid contact with the biomass, and to allow for a representative sample of the liquid to be analysed.

![Diagram of reactor operation](image)

**Figure 7-1:** Schematic diagram of the reactor operation with a 5 h HRT. Stage 1 operated for 1 h and Stage 2 for 4 h.
7.3 Results

7.3.1 Zeolite amended biofilm reactor operation without liquid in Stage 2

7.3.1.1 Sustainable ammonium removal without liquid recirculation

In Chapter 5, zeolite regeneration in Stage 2 was accomplished by continuous recycling of the total liquid over the drained zeolite coated biofilm. This operation would amount to significant energy expense in a treatment plant application. Rather than minimising recirculation this experiment tests the reactor performance without any recycling of liquid in Stage 2. This means that after uptake of ammonia by zeolite and dissolved carbon by the GAO biofilm in Stage 1, and after draining the solution, the zeolite amended biofilm was merely exposed to air. The ammonium concentration in Stage 1 was monitored over 8 repeated cycles. The ammonium removed per cycle was on average 13.5 mg-N (1.0 mmol-N) (Figure 7-2) that is a removal efficiency of 80 % in each cycle, compared with 82 % removal in the previous experiments with liquid recycling (Figure 5-10), the results show that liquid recycling was not needed for the successful operation of the zeolite amended biofilm reactor.

The cumulative ammonium adsorbed on zeolite (99.4 mg-N or 7.1 mmol-N) over the 8 cycles was about four times the total reactor’s ammonium adsorption capacity (25.2 mg-N or 1.8 mmol-N). This shows that the zeolite was being bio-regenerated.
Figure 7-2: Time course of the ammonium removal in the reactor without liquid recirculation. $\text{NH}_4^+$ concentration in individual cycles (■). Cumulative ammonium adsorbed (●) and the zeolite (15 g) maximum capacity (---). Total treatment time duration of Stage 1 and 2 was 24 h respectively resulting in an overall HRT 48 h / 2.5 BV = 19 h.

7.3.1.2 Effect of omitting the aerobic phase

To verify that Stage 2 truly regenerated zeolite, the effect of omitting Stage 2 on the ammonium adsorption in the subsequent cycle was recorded over 5 h of the 24 h Stage 1 length. Omitting Stage 2 resulted in only 1.3 mmol/L ammonia adsorption in the subsequent cycle compared with 2.4 and 3 mmol/L when a Stage 2 duration of 24 h and 48 h was used respectively (Figure 7-3). This experiment suggests that the aeration provided in Stage 2 regenerates the zeolite by enabling nitrifiers to oxidise the ammonia adsorbed on the zeolite.

After the significant decrease in ammonium adsorption by omitting Stage 2 of the previous cycle, the effect of resuming 24 h aeration on ammonium adsorption in the following cycle was tested. After resuming the aeration period, the zeolite could adsorb the usual amount of ammonium in the next cycle (Figure 7-3). This
demonstrated that resuming the aeration recovered the zeolite ammonium adsorption capacity.

Figure 7-3: Time course of the ammonium adsorption during the 5 h of 24 h of Stage 1, after no aeration (▲), 24 h aeration (●) and 48 h aeration (■). To test whether the zeolite would recover its adsorption capacity, a period without aeration was immediately followed by a 24 h aeration period (○). The volume of synthetic wastewater treated was 2.5 void volumes (BV) of the packed bed biofilm reactor.

## 7.3.2 Operation of reactor with 1 bed volume of wastewater

### 7.3.2.1 Sustained ammonium removal with 5 h HRT

Given that the majority of the ammonium adsorbance occurred in the first hour of Stage 1 the operation of the reactor would be possible over a shorter HRT. To test the dissolved ammonium removal the reactor was operated with 5 h HRT, and without liquid recirculation. The volume of synthetic wastewater treated per cycle was decreased from 2.5 to 1 BV per cycle, and the cycle’s treatment time was shortened from 19 h/ BV to 5 h/ BV (Section 7.2.2). Each cycle consisted of 1 h Stage 1 and 4 h Stage 2 and the reactor was monitored for ammonium removal for 18 cycles. On average, 2.1 mmol/L of the ammonium in the inflow was removed
(Figure 7-4). This represented an ammonium removal efficiency of 73 %. This ammonium removal was sustained over 18 cycles operation. An ammonium removal of 73 % in 5 h HRT compares favourably with the ammonium removal of 80 % achieved previously at HRT of 19 h. However, it is significantly lower that what can be obtained in the laboratory by AS processes optimised for SND operation of 93 % [13].

Figure 7-4: Sustained ammonium removal by the zeolite amended biofilm reactor with a treatment time of 5 h. The dashed line represents the average ammonium removal (2.1 mmol/L) over the cycles measured (12). With the ammonium feed concentration being 3.0 mmol/L this corresponds to 73 % ammonium removal from the influent.

7.3.2.2 Effect of omitting the aerobic phase on the ammonium adsorption in subsequent cycle at 5 h HRT

The sustained ammonium removal over repeated cycles in the zeolite amended biofilm reactor with HRT 5 h is shown above. In a similar experiment to the previous one, the omission of Stage 2 was tested. After 18 cycles of normal operation of 1 h Stage 1 and 4 h Stage 2, the reactor was operated for 3 cycles (cycle
19, 20 and 21) without Stage 2, resulting in a drop of ammonia removal from the average of 2.1 to 1.1 mmol/L over the three cycles tested (Figure 7-5). In cycle 21 the ammonium removal was 1.1 mmol/L which was almost 2 times lower than the reactor’s operation with 4 h Stage 2. This demonstrated that Stage 2 plays a crucial role to enable the ammonium removal in Stage 1 of the subsequent cycle.

To test the effect of resuming aeration on the zeolite ammonium adsorption capacity, the dissolved ammonium removal in Stage 1 was measured after resuming the Stage 2 aeration. Aeration was provided to the biofilm in two ways: firstly by passive aeration through simple air diffusion for 4 h and then for 24 h, and secondly by active aeration using a pump to force air from the bottom to the top of the reactor for 24 h. The passive aeration mechanism did not seem to achieve zeolite regeneration, whether it was provided for 4 h or 24 h, because the ammonium removed from solution was 1.3 mmol/L (41 %) and 1.1 mmol/L (34 %) respectively (Figure 7-5). On the contrary, when Stage 2 was operated with a mechanical ventilation process for 24 h, in the subsequent Stage 1, zeolite adsorbed 2.4 mmol/L of ammonium in the inflow (75 % efficiency). It is possible that the pumping process facilitates air supply throughout the reactor, while with diffusion, the oxygen is consumed rapidly and is not in contact with the whole biomass, thus reducing the zeolite bio-regeneration. This experiment demonstrated that an overloaded zeolite with ammonium could be regenerated with an appropriate aeration mechanism. Such ventilation process is already used in trickle filters [152], so it is not expected to be a problem for the viability of the zeolite amended biofilm reactor.
7.3.3 Effect of 5 h HRT and no liquid recirculation on acetate removal

7.3.3.1 Sustained acetate removal with 5 h HRT

Biological dissolved carbon storage efficiency decreased by 40% when stored Poly-hydroxyalkanoates (PHAs) were insufficiently oxidised during the aerobic period (Chapter 2, Figure 2-6). With the zeolite amended biofilm reactor operating at a HRT of 5 h, Stage 2 decreased from 24 h to 4 h, which might limit PHA oxidation and thus reduce the biological carbon storage capacity in Stage 1. To test whether the dissolved carbon storage could be sustained when operating Stage 2 for 4 h and without liquid, the carbon removal in Stage 1 was monitored in representative cycles of the 18 cycles shown above. The biological acetate removal in Stage 1 was on average 7.5 mmol/L (>80% efficiency) (Figure 7-6). Dissolved carbon was removed sustainably in Stage 1, despite the shortened HRT of 5 h.
Figure 7-6: Removal of acetate in the reactor after 1 h anaerobic Stage 1. The average acetate removed was 7.5 mmol/L (dashed line).

7.3.3.2 Effect of absence of Stage 2 on biological acetate storage

The mechanism for continuous acetate storage depends on Stage 2 for PHA oxidation. After 3 cycles without aeration, the average acetate removal decreased from 7.5 mmol/L to 3.4 mmol/L (Figure 7-7). This is a drop of 63% carbon removal efficiency, compared to the normal operation of the reactor with Stage 2 of 4 h aeration. This experiment demonstrated that without aeration, the reactor could not sustain carbon removal. The operation of Stage 2 is essential for PHA oxidation, presumably by restoring the glycogen pool in GAO available for repeated acetate uptake.
Figure 7-7: Comparison of acetate removal (mmol/L) in Stage 1 during the operation of 18 cycles with aeration (1-18) and without aeration (19-21). The dashed line (- -) is the average acetate removal when operating the zeolite amended reactor with aeration.

7.4 Discussion

7.4.1 Energy consumption of the zeolite amended biofilm reactor compared to the activated sludge process

The Stage 2 aeration period was operated with simple air diffusion through the pore space of the reactor and mere exposure of the biofilm to air. The energy consumption for the operation of the proposed zeolite amended biofilm reactor would be for simply filling the reactor during Stage 1. Besides the filling process, no energy is required as the drainage system would be achieved by gravity. The energy required to fill the zeolite amended biofilm reactor would drastically reduce the treatment cost compared to a conventional treatment plant.

For example, assuming that the zeolite amended biofilm reactor had the same height as a conventional AS process of 5 m, the theoretical energy (in Joules) to lift the wastewater can be calculated. Considering that wastewater is filled from bottom
to top, the energy required to lift the liquid differs whether the liquid is shifted to the top or simply transferred to the bottom of the reactor. The liquid lifted to the reactor’s top requires maximum energy, while no energy is required to transfer liquid at the bottom; therefore, it was assumed that the average energy would be equivalent to lifting the liquid to half of the reactor height (i.e. 2.5 m). The energy required to fill the zeolite amended biofilm can be calculated to be 24.5 J/L<sub>ww</sub>.

In comparison, in the activated sludge process, the theoretical energy required was calculated to be 570 J/L of wastewater. This was calculated from the total air volume required to oxidised carbon in wastewater assuming: 10 mmolC/L BOD to be oxidised, 21 % O<sub>2</sub> in air, 10 % O<sub>2</sub> transfer efficiency and the total air volume required to lift a 5 m column of water (Section 2.1). For the same type of reactor design of 5 m height, the zeolite amended biofilm theoretical energy expense would be > 95 % less energy.

**7.4.2 Decrease in ammonium adsorption rate at 5 h HRT in Stage 1**

It is worth noting that during the operation of the reactor with an HRT of 5 h, the ammonium concentration in the effluent increased over the operation of 18 cycles. In fact, the removal efficiency decreased from 80% to 70 %. The decrease might be due to insufficient zeolite bio-regeneration, because with insufficient aeration time ammonium adsorption in Stage 1 is reduced (Figure 7-5). The decrease in ammonium removal under 5 h HRT operation suggested that the 4 h aeration period might be insufficient to fully bio-regenerate the zeolite. It is possible to recover the ammonium removal in Stage 1 by increasing the Stage 2 timing (Figure 7-3) however, it will result in a longer HRT, which reduces the viability of the zeolite amended biofilm reactor.
When omitting the aeration period (Stage 2) the ammonium removal was not completely stopped, instead the removal simply decreased to 50 % of its adsorption with the normal Stage 2 of 4 h aeration. This can be due to the fact that the total ammonium adsorption capacity of the zeolite amended reactor with the wastewater used in this study was 1.8 mmol-N, and each cycle adds 0.38 mmol (1 BV = 130 mL of 3 mmol-N/L) to the zeolite. Each cycle fills ~20 % of the total zeolite capacity in the reactor; then the reactor adsorbed up to 5 BV of wastewater before the ammonium adsorption stops completely.

### 7.4.3 Combination of two zeolite amended biofilm reactors to maximise removal efficiencies of dissolved carbon and nitrogen

The zeolite amended biofilm reactor has a relatively low energy expense compared to AS (Section 7.4.1), but at 5 h HRT the removal efficiencies were 80 % and 75 % for dissolved carbon and nitrogen removal respectively. The remaining dissolved carbon and ammonium in the effluent are not appropriate for disposal in the environment. To improve the removal efficiencies, it is possible to think that two zeolite amended biofilm reactors in sequence could be operated. Assuming that the removal efficiencies for dissolved carbon and total nitrogen are the same in each reactor, then the total dissolved carbon removal would reach 96 % and the nitrogen removal 93 % (Table 7-1). The HRT would be doubled from 5 h to 10 h, but this is still lower than for an AS process [147,148].
Table 7-1: Calculation of the total efficiency of the two zeolite amended biofilm reactors operating one after the other, to improve the removal of dissolved carbon and nitrogen.

\(a\) is the concentration, Cmmol/L for carbon and Nmmol/L for nitrogen.

<table>
<thead>
<tr>
<th>Content</th>
<th>Inflow Conc. Reactor 1 mmol/L(^a)</th>
<th>Efficiency Reactor 1 %</th>
<th>Removal mmol/L(^a)</th>
<th>Efficiency Reactor 2 %</th>
<th>Removal mmol/L(^a)</th>
<th>Final Outflow conc. mmol/L(^a)</th>
<th>Total efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>16.0</td>
<td>80.0</td>
<td>12.8</td>
<td>80.0</td>
<td>2.6</td>
<td>0.6</td>
<td>96.0</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>3.0</td>
<td>75.0</td>
<td>2.3</td>
<td>75.0</td>
<td>0.6</td>
<td>0.2</td>
<td>93.8</td>
</tr>
</tbody>
</table>

7.5 Conclusion

Reactor operation without liquid recirculation in Stage 2 (48 h HRT):

- The zeolite amended biofilm reactor was able to sustainably remove 80 % of the ammonium in the inflow with mere exposure of the biofilm to atmospheric air. The cumulative ammonium removal was greater than the total zeolite ammonium adsorption, which suggested that zeolite was bioregenerated by mere exposure of the biofilm to air during Stage 2. This was confirmed by the fact that in the absence of aeration (Stage 2), ammonium adsorption in Stage 1 would be drastically decreased to less than 40 %.

Reactor operation with HRT 5 h and no liquid recirculation in Stage 2:

- Dissolved carbon and total nitrogen were removed at 80 % and 73 % of the inflow concentration respectively, by operating the zeolite amended biofilm reactor with 5 h HRT, comprised 1 h anaerobic Stage 1 and 4 h aerobic Stage 2 without liquid.
- Stopping aeration prevented:
o The bio-regeneration of the zeolite in the reactor as was observed by the decrease in ammonium adsorption by 30% in Stage 1, and

o The stored carbon oxidation, because the carbon storage in Stage 1 without aeration decreased by 63%.

- Both zeolite bio-regeneration and stored carbon oxidation could be recovered when aeration was resumed. The reactor was operated with a 5 h HRT and a very limited energy input for the reactor filling. It was estimated that the zeolite amended biofilm reactor would consume in theory 5 times the amount of energy that an AS process would. However, because of residual carbon and nitrogen in the effluent, it requires further treatment to increase removal efficiencies prior to disposal.
Chapter 8  Concluding comments and further research opportunities

8.1  Summary of results

The aim of this chapter is to summarise the results of the development of a novel biofilm process for dissolved carbon and nitrogen removal using passive aeration. The summary will be followed by discussing some distinct benefits but also further research that needs addressing. Finally, the potential retrofit of current treatment plants is proposed to achieve similar results to the ones described in this work.

Conventional wastewater treatment plants remove dissolved organic carbon and nitrogen with aerobic processes that use biomass in suspension. Suspended biomass treatment operation consumes a significant amount of energy to provide oxygen to the bulk liquid in order to sustain the bacterial activities that remove nutrients. Biofilm treatment processes have biomass grown on surfaces enabling high biomass retention. Their energy expense can be reduced to some extent but most of the current biofilms still require air supply to the bulk liquid. Therefore, the present research idea was to develop a novel biofilm treatment process without aeration of the bulk liquid to remove both dissolved carbon and nitrogen. Below are the conclusions from each of the objectives addressed in this thesis.

8.1.1 Objective 1: Develop a process for dissolved carbon removal

A novel process for dissolved carbon removal without liquid aeration was developed. The process relied on Glycogen Accumulating Organism (GAOs) to store dissolved carbon under anaerobic conditions, and oxidise the stored carbon (i.e. poly-hydroxy-alkanoate (PHA)), by mere exposure of the biofilm to atmospheric air, thus preventing the aeration cost associated with bulk liquid aeration. This is a
significant improvement because in the conventional Activated Sludge (AS) process, the energy associated with aeration for the dissolved organic carbon oxidation is at least 570 J/Lww. Our novel process would prevent the energy cost associated with liquid aeration for dissolved carbon oxidation to CO₂.

8.1.2 Objective 2: Quantify that zeolite can adsorb ammonium and can be regenerated with a nitrifying biofilm

To develop a nitrogen removal process, an Australian clinoptilolite was used to store ammonium as an initial step. Large particles (2 – 3.35 mm) was preferred because it allows for air diffusion through the void volume of the particle, and had an ammonium removal rate of 0.12 mmol/g/h. In a reactor with large amount of zeolite resulted in a removal rate faster than that of conventional AS processes (0.1 mmol/L/h). Zeolite was used as a ‘storage’ mechanism prior to biologically regenerating it.

A nitrifying biofilm was grown on zeolite as a carrier. After ammonium adsorption, the wastewater was recirculated for oxygen transfer and achieved nitrification of the adsorbed ammonium. Nitrification allowed 93 % nitrogen recovery as nitrate, which regenerated the zeolite’s adsorption capacity.

8.1.3 Objective 3: Combination of the processes for carbon and total N removal

After successfully testing the capacity of two separate reactors in sequence, a single biofilm reactor was synthesised to reduce the energy expense associated with the recirculation of liquid between two separate reactors. The single biofilm reactor was synthesised by mixing: GAOs, nitrifying biomass and zeolite powder. The zeolite amended biofilm reactor operated with two stages. Stage 1 was anaerobic,
and biological carbon storage and ammonium adsorption was achieved. In Stage 2, a liquid recirculation of 0.4 m$^3$/m$^2$/d was used to keep the biomass wet, supplied oxygen to the biomass. The reactor removed dissolved carbon and nitrogen through Simultaneous Nitrification and Denitrification (SND) process in full atmospheric air, which was possible because of an oxygen gradient in the biofilm. A medium-term operation of the system demonstrated that the synthesised biofilm was capable of sustained operation. The zeolite amended reactor removal efficiencies were > 95 % for dissolved carbon and 82 % for total nitrogen, with an HRT of 19 h/BV which is longer than with a conventional AS treatment plant.

8.1.4 Objective 4: Sustainability of the synthesised biomass

In the zeolite amended biofilm, the nitrifying biofilm maintained its nitrification rate after 30 cycles at approximately 1 mmol/L/h, which demonstrates the sustainability of the nitrifiers. GAOS were also sustained in the biofilm, as observed from the increase in the storage rate. In addition, the Nitrite Oxidizing Bacteria (NOB) were removed from the synthesised biofilm. The rapid denitrification rate (8.4 mmol/L/h) of the GAO in the biofilm was probably responsible for the NOB wash-out from the biofilm. The benefit of removing the NOB from the reactor is that the zeolite amended biofilm reactor can achieve denitrification with endogenous dissolved carbon when treating wastewater with a relatively low C/N ratio.

8.1.5 Objective 5: Optimisation of the zeolite amended biofilm reactor

The zeolite amended biofilm operation optimisation was tested by having no liquid recirculation in Stage 2, and a 5 h HRT. The removal of dissolved organic carbon and total nitrogen was > 80 % and 75 % respectively. It was calculated that the theoretical energy involved in the operation of the zeolite amended biofilm would be 90 % of that of the aeration energy required to oxidise dissolved carbon in
an AS process. Such removal capacities with a minimum of energy input is promising for the application of the process. However, the remaining ammonium (25%) and other nutrients (e.g. TP) require removal. The proposed biofilm treatment process can be a preliminary step to an AS sludge, which will reduce the aeration costs associated with dissolved organic carbon and ammonium oxidation.

8.2 Benefits of the novel process

8.2.1 Benefits of biofilm compared to suspended biomass

Biofilm reactors can accumulate large biomass concentrations, up to 50 g/L [59], and drastically reduce the wastewater treatment time to 1-2 hours [59], which means the reactor’s land surface area can be decreased. Biofilms have other inherent benefits compared to suspended biomass treatment processes. One is the absence of a decanting phase to separate biomass and wastewater. Biofilms are more resistant to shock loads and can recover faster than other systems [153]. The biomass is denser [154] and has less water content (90% water content) [155,156], which decreases the cost of excess sludge disposal, because no dewatering pre-treatment is required, and also reduces the sludge volume.

8.2.2 Benefit of short HRT on treatment plant land surface area

The studied reactor HRT was 2.4 times shorter than used for AS, therefore the reactor surface area would be 2.4 times smaller than that of AS. In addition, Biological Aerated Filters (BAFs) use a similar type of packing media to the one used in this study and, to reduce the land surface area, are operated as high towers of up to 12 m [59,157]. The biofilm reactor developed here could be operated in this manner, which would reduce the land surface area used for treatment plant operation,
but slightly increase the energy associated with treatment for the wastewater displacement to the highest part of the reactor.

### 8.2.3 Comparison to parallel nitrification denitrification process

The reactor was a follow-up work on a patent developed at Murdoch University, which uses two separate biofilm reactors for parallel nitrification and denitrification (PND) [79]. The energy expense associated with the liquid recirculation between two reactors and for nitrification has been acknowledged as one of the drawbacks of the PND system [80]. In the operation of two biofilm reactors, nitrifying biomass was grown on zeolite to reduce the energy cost of aeration for nitrification. The performance of the treatment was similar to that of PND, but the treatment time was 2 times longer. To further reduce the cost associated with liquid transfer between two reactors, the process was improved by synthesising a biofilm that combines the GAO and nitrifying biomasses, and zeolite in a novel single zeolite amended biofilm reactor.

It is of interest to note that the PND process is currently being tested at a full scale wastewater treatment plant by operating a conventional plug flow system in a different way. The results achieved by this treatment plant are promising in terms of improving the nitrogen removal capacity. In comparison to the PND process tested in laboratory, which removes > 99 % of the total nitrogen [80], the proposed zeolite amended biofilm reactor achieves merely 75 % of the TN, however, the studied zeolite amended biofilm reactor here was operated to minimise the cost associated with carbon and nutrients removal. On the contrary, PND required a significant energy input that was comparable to an AS process [80] but achieved much greater removal efficiencies.
8.3 Limitations of the research conducted

8.3.1 Tests on synthetic wastewater instead of municipal wastewater

The biofilm reactors tested throughout this work was done with synthetic wastewater in order to obtain reproducible results, and because of time constraints, testing the reactor with municipal wastewater was not done. Municipal wastewater has some variables that would affect the performance of the process, and it would therefore be important to investigate this. One such variable is the dissolved organic carbon available. It would significantly differ from simple acetate organic carbon provided in this study [113], and would change the organic storage rate and the GAO development. However, Parallel Nitrification and Denitrification (PND) which used GAO, was capable of storing dissolved organic carbon from a municipal wastewater obtained from the Subiaco treatment plant facility (Perth, Western Australia)[80]. Therefore, it is probable that the proposed GAO biomass operated in all the biofilm reactors could achieve dissolved carbon removal from municipal wastewater.

The types of biologically stored carbon vary greatly according to the source of the soluble organic present in wastewater [19,109]. As an example, long chain organic carbons do not always breakdown to acetate, some alternative products are propionate and butyrate which produce PHV [20] or PH2MV [21]. This work demonstrated the proof of concept for simple organic carbon removal, and further investigations are required to remove complex organics present in municipal wastewater. Biological storage of complex organic was demonstrated to vary compared to simple ones such as acetate [113], in addition, the different form of stored carbon might affect the denitrification rate and capacity [158]. In future research, the novel process developed in this work should be tested with real wastewater. The effect of real wastewater on the HRT and the stored carbon
compound are of interest to test the applicability of the process on a large scale. It is important to note that PND process has been applied to in a real wastewater treatment plant and the results showed that the biomass was capable of effectively removing complex soluble carbon in wastewater [80].

Another variable is the presence of Suspended Solids (SS), which are colloids that are difficult to settle and therefore tend to flow in with the wastewater inflow. SS could coat the biomass and affect the biofilm reactor’s removal capacity. For example, in Biological Aerated Filters (BAFs) the SS adsorbed on the biomass reduced the nitrification efficiency by up to 50 % [69]. BAF systems use backwashing, a vigorous aeration and/or high-rate liquid flow through the filter, which dislodge and remove excess biomass and SS [58]. As such BAFs recover their removal capacities. The proposed zeolite amended biofilm could use the same principle to remove SS if the reactor loses its removal capacity. However, in our zeolite amended biofilm reactor, the backwashing would result in biomass removal and also in the loss of zeolite that was attached to the synthesised biofilm. The loss of zeolite would be a problem because of the cost of replacing it, and also because it requires to be added again to the biofilm which results in a shut down time longer than that required for backwashing only. Furthermore, backwashing represents a significant energy input; in fact it is between 15 to 20 % of BAFs’ energy usage [58].

It is possible to think that zeolite could be recovered from the backwashed effluent using a gravity settler. Zeolite is heavier than biomass and therefore its settling velocity is faster than that of biomass, which might be used to recover the zeolite at the bottom of the settler before biomass settling.
8.3.2 Phosphate removal to complete wastewater treatment

The phosphate present in wastewater was not considered in this study. However, it still requires to be removed. One simple removal mechanism is chemical precipitation. The precipitation of phosphate can be achieved with Iron (FeCl$_3$), Aluminium (AlCl$_3$) or calcium carbonate (CaCO$_3$). FeCl$_3$ and AlCl$_3$ have a similar removal efficiency with a difference of not more than 10% [37]. However, phosphate precipitation with Fe$^{3+}$ and Al$^{3+}$ requires acidic solution (pH 3.6 to 6.2) [37], while calcium can achieve phosphate removal at a pH of 8 [41]. Chemical precipitation would be an efficient process for the effluent of the proposed reactor because the absence of organic carbon improves phosphate precipitation [41].

On the contrary, biological phosphate removal cannot be applied to the effluent of the proposed reactor because this mechanism requires dissolved carbon, which is not present in the effluent of this process.

8.3.3 Biofilm synthesis compared to enrichment

In this study, GAO and nitrifier populations were enriched separately before being combined to form a synthesised biofilm. The synthesised biofilm was not developed through a selective enrichment process from AS, and this could jeopardise the application of the process to the treatment of wastewater. It is important to study whether operating a biofilm seeded with AS could develop into a biomass similar to the one achieved from the synthesis. The outcome of such study would reveal whether the reactor could be developed from AS for full scale operation.

The repeated operation of the system will produce biomass over time, which will result in a clogging of the bed reactor. The reason why clogging did not occur during the operation of the process could lie in the presence of predatory organisms on the
biofilm, namely myxobacteria (Chapter 2) and amoeba. Further research on the clogging potential would provide insight on the applicability of the system using real wastewater and development of a biofilm from activated sludge.

8.4 Retro-fit of current technology to develop the novel treatment process

8.4.1 Biological Aerated Filters

BAFs are biofilm reactors that remove both dissolved organic carbon and suspended solids. These reactors use plastic media for biomass growth and aerate the wastewater with blowers to provide oxygen to the biomass. Some BAFs use zeolite as a growth media for bacteria [67,159,160], resulting in better nitrogen removal efficiency of up to 90 % [67]. It is possible that changing the operation of a BAF system with zeolite could result in a single biofilm reactor similar to the one described in this study. The new operation would be to fill the BAF with wastewater under anaerobic conditions and monitor the carbon storage and the ammonium adsorption on zeolite. Once carbon and ammonium are removed, the wastewater would be drained and disposed of, or further treated (e.g. phosphate removal), and the BAF reactor would be open to air diffusion. The aeration process would result in sustained SND in atmospheric air.

8.4.2 Rotating Biological Contactors

Rotating Biological Contactors (RBCs) have biomass developed on discs that are placed on a rotating shaft. The rotation of the discs alternatively exposes the biomass to air and to the liquid. RBCs aim at maximising the liquid aeration and mass transfer by operating the disc rotation at a speed of 1-2 rpm [3,73]. RBCs could be operated in such a way as to develop the biomass described in our biofilm, which is capable of complete dissolved organic carbon and N removal through SND in air.
Simple changes to the RBC operation could result in the proposed biofilm. The changes are:

1. Adding zeolite to the biofilm and
2. Reducing the disc rotation speed.

Adding zeolite to the biofilm results in ammonium adsorption from wastewater, and is known to favour nitrifiers’ growth on zeolite in AS [161,162]. Reducing the discs’ rotation speed would result in providing anaerobic and aerobic conditions alternatively to the biomass. For example, at a speed of 1 rotation per hour, the biomass would be exposed to the wastewater under anaerobic conditions for 30 min and thereafter to atmospheric air conditions for 30 min. It is of interest to note that no literature was found on the operation of an RBC system with zeolite as a catalyst for ammonium removal.

### 8.4.3 Comparison of removal efficiencies with other passively aerated treatment plants

In the literature, passively aerated biofilm reactors have achieved a wide range of dissolved carbon removal efficiency for municipal wastewater from 34 % [163] to 97 % [164]. The removal of total N in most passively aerated systems has a lower range from 22 % [165] to 60 % [166]. Higher removal efficiencies were achieved in a pond system with passive aeration, where 90 % and 95 % of BOD and total N were removed respectively, however, the HRT of such a system was 7.5 days and the efficiency varied widely with the seasonal changes [167]. The zeolite amended biofilm reactor has achieved a dissolved carbon removal of > 83 % and total N of 75 % with an HRT of 5 h which compares relatively well with other passively aerated biofilm reactors. To our knowledge, this research is the first to report a system
capable of high organic and N removal with SND using a passive aerated biofilm reactor with a short HRT.

In conclusion, the zeolite amended biofilm reactor achieved somewhat lower dissolved carbon and nitrogen removal compared to most conventional treatment processes. However, the process consumes only 5% of the energy compared to the aeration required for an AS process, and operated in a relatively short treatment time of 5 h. The effluent of the zeolite amended biofilm contains compounds (e.g., phosphate, remaining ammonium...) that require treatment prior to disposal. In addition, as excess biomass is removed from the reactor, the zeolite would be removed which will result in an increase in operation costs for continuous purchase of the raw material. The zeolite amended biofilm reactor could be used as a pre-treatment prior to an AS process. This would significantly decrease the energy required for the bulk liquid aeration for dissolved organic carbon and ammonium oxidation.
References

References


References


References


Y. Gendel and O. Lahav, "A novel approach for ammonia removal from fresh-water recirculated aquaculture systems, comprising ion exchange and
References


[117] M. L. Coghlan, J. Haile, J. Houston, D. C. Murray, N. E. White, P. Moolhuijzen, M. I. Bellgard, and M. Bunce, "Deep sequencing of plant and


Appendices

Appendix A: Acetate removal without liquid recirculation.

In the previous experiments, soluble acetate was removed using liquid recirculation over the biomass during the anaerobic period. Here, the aim was to determine whether the acetate removal was possible without liquid recirculation in the anaerobic phase. The reactor was operated as described in the method section, but without liquid recirculation. The acetate concentration was monitored and compared to the acetate concentration when liquid was recirculated. The acetate removal rate was 10% lower without liquid recirculation (Figure A-1). This demonstrates that the acetate removal is possible without liquid recirculation but might require a slightly longer hydraulic retention time. The processes tested thereafter will use recirculation because they are proof of concepts, however, in Chapter 7 the system will be tested without recirculation.

![Figure A-1: The effect of recirculation (●) and no recirculation (○) on acetate during the anaerobic phase of the reactor operation](image-url)
Appendix B: Theoretical determination of the surface area of a reactor containing zeolite

The adsorption rate is dependent on the zeolite surface area. However, it is not clear whether it is a linear relationship. The aim is to determine the change in surface area with respect to the zeolite particle size in a fixed reactor volume. The zeolite surface area can be theoretically determined by a mathematical calculation. For the whole exercise the following assumptions were made:

- A reactor volume \( V_R \) of 1 L (1000 cm\(^3\)) is filled with zeolite,
- Zeolite particles are spheres

Firstly the volume for each zeolite sphere was determined using the equation Eq. B-1:

\[
V_S = \left(\frac{4}{3}\right) \pi r^3
\]

Where \( V_S \) is the volume of sphere of radius \( r \) (dm).

The volume of each particle was determined for the zeolite particle size ranging from 0.05 cm to 10 cm (Table B-1). The maximum number of particles \( N_P \) can be determined from the reactor volume \( V_R \) and the sphere volume \( V_S \). However because the particles are spheres, the packing of sphere cannot fill the whole reactor volume. The tight packing of spheres is called hexagonal close packing (HPC) where each sphere is surrounding by 12 other spheres. The maximum reactor volume filled with tightly organised sphere can be 74 % of the total reactor volume (i.e. void ratio 0.26). The volume filled by sphere decreases to 65 % (void ratio = 0.35) or less when spheres are randomly organised. Eq. B-2 was used to determine the number \( N_P \) of tightly packed sphere present in the reactor volume:
Eq. B-2: \[ N_P = \frac{(74\% \times V_R)}{V_S} \]

Where \( N_P \) is the maximum number of particle filling the reactor volume, \( V_R \) is the total volume of the reactor (1000 cm\(^3\)) and \( V_S \) (cm\(^3\)) is the sphere volume per particle.

The sphere surface area was established for each sphere as follows:

Eq. B-3: \[ SA = 4\pi r^2 \]

Where \( SA \) is the surface area (cm\(^2\)) per particle and \( r \) is the radius (cm) of the particle.

The reactor specific surface area is then calculated:

Eq. B-4: \[ SA_R = \frac{(SA \times N_P)}{(V_R)} \]

Where \( SA_R \) is the specific surface area of the reactor (cm\(^2\)/cm\(^3\))

Table B-1: Determination of the surface area based on the zeolite grain size diameter. The sphere volume was determined, then surface area.

<table>
<thead>
<tr>
<th>Particle Diameter cm</th>
<th>Reactor Volume (( V_R )) cm(^3)</th>
<th>Particle Volume (( V_S )) cm(^3)</th>
<th>Amount Particles (( N_P )) number</th>
<th>Particle Surface Area (( SA )) cm(^2)</th>
<th>Reactor Surface Area (( SA_R )) cm(^2)/cm(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>1000</td>
<td>6.54 x10(^{-5})</td>
<td>1.1 x10(^{\times -7})</td>
<td>7.85 x10(^{-3})</td>
<td>88.8</td>
</tr>
<tr>
<td>0.1</td>
<td>1000</td>
<td>5.24 x10(^{-4})</td>
<td>1.4 x10(^{\times -6})</td>
<td>3.14 x10(^{-2})</td>
<td>44.3</td>
</tr>
<tr>
<td>0.5</td>
<td>1000</td>
<td>6.54 x10(^{-2})</td>
<td>1.1 x10(^{\times -4})</td>
<td>7.85 x10(^{-1})</td>
<td>8.8</td>
</tr>
<tr>
<td>1</td>
<td>1000</td>
<td>5.24 x10(^{-1})</td>
<td>1.4 x10(^{\times -3})</td>
<td>3.14 x10(^{0})</td>
<td>4.4</td>
</tr>
<tr>
<td>2</td>
<td>1000</td>
<td>4.19 x10(^{0})</td>
<td>1.8 x10(^{\times 1})</td>
<td>1.26 x10(^{1})</td>
<td>2.2</td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
<td>6.54 x10(^{1})</td>
<td>1.1 x10(^{\times 1})</td>
<td>7.85 x10(^{1})</td>
<td>0.8</td>
</tr>
<tr>
<td>10</td>
<td>1000</td>
<td>5.24 x10(^{2})</td>
<td>1.4 x10(^{0})</td>
<td>3.14 x10(^{2})</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The total surface area of 1 dm\(^3\) reactor containing the zeolite particle can be determined by multiplying the surface area for one particle by the number of particles. The surface area per volume is not a linear relationship (Figure B-1)
because of the calculation for surface area of a sphere is a power equation \((4\pi r^2)\) (Eq. B-3). If the grain size diameter increases 10 times the resulting surface area increases 100 times. This explains why the ammonium adsorption is not linear with respect to the size.

Figure B-1: The effect of the zeolite diameter on the total surface area per litre. The zeolite was assumed to be a sphere and the diameter was varied to calculate its surface area.

Increasing the surface area decreases the distance for ammonium to travel to the exchange sites. Figure B-2 provides a visual aid to picture that increasing the surface area provides more “opportunities” for the soluble ammonium to contact with the zeolite. When water passes between zeolite grains there is a channel of water that is not in contact with the zeolite’s surface (“inactive volume”) therefore the soluble ammonium in this inactive zone cannot be adsorbed. The aim is to reduce the “inactive” volume of water. For example, small zeolite grain (high surface area) creates small channels for the water to go through. Small channels reduce the volume of water that is not in contact with the zeolite’s surface. Therefore, a small grain size reduces the “inactive” water volume and increases the ammonium adsorption rate on
zeolite (Figure B-2). When comparing two flow-through systems with the same flow rate but different particle size, the breakthrough is delayed with small particles and high surface area.

Figure B-2: The effect of a high surface area enables a small channels of water, hence increases the turbulence, which means that the ammonium has more chance of being in contact with zeolite ion exchange sites.
Appendix C: Improving the nitrogen recovery from zeolite

Nitrogen recovery in a small recirculated volumes over the biofilm was unsuccessful (Chapter 4). This is probably because of the low ammonium desorption rate. The low desorption rate can be increased by two mechanisms:

1. Increase the cations concentration in the solution,
2. Decrease the zeolite size.

The presence of high cation concentration in particular Na\(^+\) has been beneficial for bio-regeneration. However, in the present study one of the aims is to reduce the cost of operation, therefore the use of regenerant solution is avoided. The regenerant solution has two drawbacks. The first is that high salinity reduces the nitrification capacity. Therefore the regeneration rate could be reduced. The second is that once ammonium is oxidised, the nitrite and nitrate are present in a brine solution. Brine solution with nitrate still requires treatment and cannot be readily disposed of. Therefore, in this study increasing the cation concentration to desorb ammonium in solution was not considered a viable option.

Now we look into the reason for the increase in desorption rate with a small particle. Adsorption and desorption rates increased as surface area increases. The desorption mechanism requires a counter cation to be adsorbed. Assuming that a Na\(^+\) regenerant solution was used, the Na\(^+\) adsorption is maximised by decreasing the particle size (i.e. greater surface area). Because zeolite is an ion exchange then the ammonium desorbed from the exchange with Na\(^+\) maximises the rate of ammonium desorption (Figure C-1). The decrease in grain size in the present experiment would increase the sodium adsorption rate and the ammonium desorption rate hence increase the nitrification rate. The decrease in particle size can be practically
achieved by a modification of carrier’s material in the reactor, using the polypropylene carriers and sand blasting powdered zeolite as one example.

Figure C-1: Conceptual visualisation of the desorption rate of ammonium (NH$_4^+$) and sodium (Na$^+$) associated with the surface area of the zeolite. Thin arrows reflect a smaller desorption/adsorption rate than thick.
Appendix D: Quantifying the O\textsubscript{2} consumption according to the nitrification and PHB oxidation

In an effort to crosscheck that N\textsubscript{2} production was due to the biological activity in the synthesised biofilm, the oxygen and CO\textsubscript{2} data obtained from the experiment were used to calculate whether the N\textsubscript{2} production was explainable. In order to ease the calculations and the understanding the data are presented as mL of gas produced as per Figure D-1.

The N\textsubscript{2} produced must be coming from nitrite or nitrate reduction, however, only NH\textsubscript{4}\textsuperscript{+} was added in Stage 1. The aim is to calculate the O\textsubscript{2} consumed from the NH\textsubscript{4}\textsuperscript{+} oxidised. First, the NH\textsubscript{4}\textsuperscript{+} oxidised was calculate from the N\textsubscript{2} produced in the experiment (Table D-1). Assuming that all the N\textsubscript{2} produced (2.9 mL) was due to the reduction of NO\textsubscript{3}\textsuperscript{-} then we can calculate the NO\textsubscript{3}\textsuperscript{-} that was reduced (0.24 mmol-N), then the NH\textsubscript{4}\textsuperscript{+} oxidised is 0.24 mmol-N.

Table D-1: Calculation of the NH\textsubscript{4}\textsuperscript{+} oxidised and NO\textsubscript{3}\textsuperscript{-} reduced from the measured N\textsubscript{2} produced in the head space.

<table>
<thead>
<tr>
<th>N\textsubscript{2} produced mL</th>
<th>Molar volume mL/mmol</th>
<th>N\textsubscript{2} produced mmol</th>
<th>NO\textsubscript{3}\textsuperscript{-} reduced mmol</th>
<th>NH\textsubscript{4}\textsuperscript{+} oxidised mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9</td>
<td>24.5</td>
<td>0.12</td>
<td>0.24</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Now oxygen consumed for the nitrification of the NH\textsubscript{4}\textsuperscript{+} was calculated and compared the value to the measured oxygen consumed. The NO\textsubscript{3}\textsuperscript{-} was produced from the nitrification of NH\textsubscript{4}\textsuperscript{+} adsorbed on zeolite. Ammonium oxidation to NO\textsubscript{3}\textsuperscript{-} consumes 2 oxygen atoms therefore 0.48 mmol O\textsubscript{2} was consumed from the calculated NH\textsubscript{4}\textsuperscript{+} oxidised. This is compared to the total O\textsubscript{2} consumed in the reactor
of 0.87 mmol (Table D-2). The oxygen consumed was greater than that can be explained by the nitrification process, and can explain the nitrification required for the N\textsubscript{2} produced.

Table D-2 Calculation of the oxygen consumed and NH\textsubscript{4}\textsuperscript{+} oxidised from the NO\textsubscript{3}\textsuperscript{-} reduced (calculated above).

<table>
<thead>
<tr>
<th>NH\textsubscript{4}\textsuperscript{+} oxidised mmol</th>
<th>Calculated O\textsubscript{2} demand mmol</th>
<th>Experimental O\textsubscript{2} removed mL</th>
<th>mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.24</td>
<td>0.48</td>
<td>21.3</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Assuming that the remaining O\textsubscript{2} consumed (0.39 mmol) was due to PHB oxidation, then 0.08 mmol PHB was oxidised (Table D-3 and Table D-4). The PHB oxidation estimates 0.32 mmol CO\textsubscript{2}; this differs from the actual measure of CO\textsubscript{2} by 16% (Table D-4). This suggests that the measured oxygen consumption in this experiment explains both the nitrification and the oxidation of PHB to CO\textsubscript{2}.

Table D-3: Calculation of the Oxygen consumption required to reduce PHB to CO\textsubscript{2}. Where \textit{elec} stands for electrons.

<table>
<thead>
<tr>
<th>O\textsubscript{2} available for PHB oxidation mmol\textsubscript{O\textsubscript{2}}</th>
<th>\textit{O\textsubscript{2}} accepts elec/O\textsubscript{2}</th>
<th>Electron available mmol\textsubscript{elec}</th>
<th>PHB reduced to CO\textsubscript{2} elec/phb</th>
<th>O\textsubscript{2} oxidised mmol\textsubscript{O\textsubscript{2}/PHB}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.39</td>
<td>4</td>
<td>1.36</td>
<td>20</td>
<td>5</td>
</tr>
</tbody>
</table>
Appendix D

Table D-4: Calculation of the CO₂ production from the PHB oxidation. The PHB oxidation was determined from the O₂ consumed in the experiment that could not be accounted for by the nitrification. Where prod. stands for production and exp. for experiment.

<table>
<thead>
<tr>
<th>O₂ for PHB oxidation mmol O₂</th>
<th>O₂ consumed / PHB</th>
<th>PHB oxidised mmol PHB</th>
<th>CO₂ prod / PHB</th>
<th>CO₂ prod. mmol</th>
<th>Exp. CO₂ prod. mmol</th>
<th>Diff. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.39</td>
<td>5</td>
<td>0.08</td>
<td>4</td>
<td>0.32</td>
<td>0.38</td>
<td>16</td>
</tr>
</tbody>
</table>

In order to crosscheck that the PHB oxidised is actually a realistic value, we aim at comparing the calculated oxidised PHB with the PHB that would be stored in Stage 1. The expected PHB produced comes from 8 mmol/L acetate stored (3.1 mmol). Considering that only half the biomass is present in the N₂ testing reactor then the total acetate present is 1.6 mmol. That corresponds to 0.6 mmol PHB stored in the biomass of the test reactor (Table D-5). Now from the O₂ consumption we have calculated that 0.08 mmol PHB has been oxidised, in addition, the NO₃⁻ denitrification consumed 0.06 mmol PHB. In total 0.14 mmol PHB has been oxidised. That corresponds to 22 % of the added acetate and stored as PHB (Table D-6). Considering that CO₂ is a soluble gas, then there is probably a bit more CO₂ in the solution. In Chapter 2 to continuously store carbon, the stored carbon needed to be oxidised at least 27 %. The percentage of stored carbon oxidised calculated here, is in line with the value obtained in Chapter 2 and can explain the continuous operation of the zeolite amended biofilm reactor, because the zeolite is bio-regenerated and the stored carbon oxidised.
Table D-5: PHB formed in the reactor from the acetate stored in Stage 1 for the biomass used in this experiment. Where elec stands for electrons, Ac for acetate.

<table>
<thead>
<tr>
<th>Acetate in reactor mmol</th>
<th>Electron available mmolelec</th>
<th>PHB Elec./PHB</th>
<th>PHB in the biomass mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6</td>
<td>8</td>
<td>12.8</td>
<td>20</td>
</tr>
</tbody>
</table>

Table D-6: PHB consumed through the denitrification of NO$_3^-$ to N$_2$ (calculated from the N$_2$ gas produced). Where elec stands for electrons.

<table>
<thead>
<tr>
<th>NO$_3^-$ consumed mmol</th>
<th>Electron available mmolelec</th>
<th>PHB elec/PHB</th>
<th>PHB oxidised mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.24</td>
<td>5</td>
<td>1.2</td>
<td>20</td>
</tr>
</tbody>
</table>

Figure D-1: The effect of oxygen (●) on the N$_2$ production (■). The CO$_2$ (▲) production demonstrated that the carbon was oxidised. Simultaneously, an abiotic control demonstrated that the sampling method did not introduce air in the vessel (data not shown).
Appendix E: Diabetic Glucose Meter for the Determination of Glucose in Microbial Cultures

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ABSTRACT:

In wastewater, biological phosphate removal can fail because of the presence of glycogen accumulating organism (GAO), therefore measuring glycogen stored in microbial cultures provides information on the bacterial population type. Once glycogen is hydrolysed to glucose it is accurately measured using a human glucose meter. The standard curves demonstrate linearity regardless of the pre-treatment of the glucose solution at neutral pH.

Keywords: Novel Method, PHA, Glucose, Glycogen Accumulating Organisms, Diabetic Glucose Meter

Enhanced biological phosphate removal (EBPR) sludge uses phosphate accumulating organisms (PAO) to store poly-phosphate under anaerobic conditions. In aerobic conditions poly-phosphate is lysed, resulting in an ATP source for soluble carbon uptake in the form of poly-hydroxy-alkanoate (PHA)\textsuperscript{1}. However PAO can be outcompeted by glycogen accumulating organisms (GAO) resulting in poor phosphate removal in wastewater treatment plants\textsuperscript{2}. GAO use glycogen anaerobically as an ATP source to store organic carbon, producing PHA\textsuperscript{3}. Measuring glycogen in

\textsuperscript{2} Paper published in the *Journal of Microbial Method* (100) pp: 91-92
microbial cultures reveals the presence of the GAO. Measuring glycogen requires it to be split into two glucose molecules. This paper demonstrates that a diabetic glucose meter can be used to measure the glucose issued from GAO glycogen.

Glycogen quantification relies on its separation, by a strong acid or base, to glucose molecules which can be measured. Glucose is measured using three different methods. Once glucose is turned into a volatile compound, it is quantified with high pressure liquid chromatography (HPLC) or gas chromatography (GC). Alternatively enzymatic assays are used for their reliability, but require the purchase of expensive chemicals which expire rapidly.

Blood glucose analysis is required for 347 million diabetic people in the world. Glucose meters were developed in 1962, and are now cheap and readily available. The glucose meter uses an enzyme immobilised onto a strip, which produces a current proportional to the blood glucose concentration. Strips containing the enzymes have been developed for accuracy and extended duration. Wang extensively reviewed each component required for accurate blood glucose measurements. The proposed method uses the widely available diabetic glucose meter.

In this paper, glucose was analysed, in duplicate, using an AccuCheck Active (Roche) glucose meter with the corresponding test strips. The linear trend lines were fitted using Excel™ 2010 and based on the average of the duplicate readings. Note that the solutions without glucose did not produce a usable reading and therefore were not included in the results.

The initial test was to ensure that a glucose solution could be detected by the glucose meter. The following concentration of 0, 2, 3, 4, 5, 6, 7, 8, 9, 10 mmol/L
Appendix E

glucose (AR grade, Merck) were prepared and 10 μL of solution applied on a strip. The linear relationship until 10 mmol/L of the standard solution demonstrates that glucose can be accurately read (□ in Figure 8). The effect of volume applied to the strip (5, 10, 20 μL) and solution temperature did not affect the reading’s linear relationship (R² value > 0.98).

The glucose extracted from biomass would be in acidic solution; therefore the glucose solution was digested with 0.9 mol/L HCl. Given it is an enzymatic method, the pH was neutralised using two aliquots: one of 0.350 mL of 10 mol/L NaOH, and the second of 0.5 mL of 0.9 mol/L KH₂PO₄. The effect of acidity on the linearity was negligible (○ in Figure 8). Separately, the effect of boiling was tested by putting the acid solution in a water bath at 100 °C for 5 h (Δ in Figure 8). The linearity of the standard curve demonstrates that the both effects of acid and boiling are negligible (Figure 8).

*Figure 8: Linear relationship of the glucose meter readings and the standard solutions of glucose solution (□) with acid treatment prior to boiling (○) and after boiling (Δ). All solutions were adjusted to neutral pH (7.5>pH>7.0) before the analysis was conducted.*
The previous results were conducted using a glucose solution. The method required to be tested for detecting glucose extracted from GAO biomass. The GAO was obtained from a laboratory reactor from Murdoch University and seeded with activated sludge (Woodman Point, Perth, Western Australia). The reactor biomass was selectively enriched in GAO to store soluble acetate anaerobically. This was achieved by alternating aerobic (1 h) and anaerobic conditions (2 h); given the low level of phosphate in the aerobic phase, the GAO were grown preferentially over PAO.  

The reactor was provided with synthetic wastewater consisting of (mg.L⁻¹): CH₃COONa 660, NH₄Cl 160, NaHCO₃ 125, KH₂PO₄ 44, MgSO₄.7H₂O 25, yeast extract 50, and 1.25 mL.L⁻¹ of trace element solution, which contained (g.L⁻¹): ethylene-diamine-tetra-acetic acid (EDTA) 15, ZnSO₄.7H₂O 0.43, CoCl₂.6H₂O 0.24, MnCl₂.4H₂O 0.99, CuSO₄.5H₂O 0.25, NaMoO₄.2H₂O 0.22, NiCl₂.6H₂O 0.19, NaSeO₄.10H₂O 0.21, H₃BO₄ 0.014 and NaWO₄.2H₂O 0.050.  

Prior to analysis, the GAO was spun at 2500 rpm for 5 minutes and then was freeze-dried (Hetosicc-CD 4) at -50 °C for a minimum of 6 hours. The glycogen was extracted and lysed from the two samples of GAO dried biomass (sample 1: 27.1 mg and sample 2: 28.3 mg) using the acid method. An aliquot of 3 mL of 0.9 mol/L HCl was added to the dried biomass in culture tubes. The culture tubes were capped and the content digested at 100 °C in a water bath for 5 hours. The solution was then centrifuged at 5000 rpm for 5 minutes to remove the suspended solids resulting from the digestion process, the supernatant contained the glucose to be analysed. The pH was neutralised as explained previously.
To sample 1 an aliquot of glucose solution (2 mmol/L) was added, therefore the readings of both samples are expected to differ by 2 mmol/L. The glucose concentrations obtained from the GAO samples were 4.9 and 3.1 mmol/L respectively (Figure 9). In sample 1, considering the additional glucose, the GAO glucose content was calculated to be 2.9 mmol/L, which is close to the glucose concentration in sample 2.

**Figure 9:** Measurement of glucose concentration in dried biomass sample (black) after acid digestion and boiling. Sample 1 had an additional 2 mmol/L glucose (white) to demonstrate the capacity of the method to measure all glucose.

In conclusion, diabetic glucose measurements of biological glucose were accurate for both laboratory prepared solution and lysed GAO biomass. This method is simple and cheap compared to HPLC, GC. Enzymatic assays are an alternative method, but their lifespan is minimal (< 2weeks) and is expensive. On the other hand the proposed method requires enzymatic strips which last for a year and glucose meters which are readily available at any pharmacy because they are widely used by diabetic people and consequently are significantly cheaper than conventional methods.
ACKNOWLEDGMENT:

The author would like to thank the help and support from Dr Ralf Cord-Ruwisch, and the financial support provided by Murdoch University.

ABBREVIATIONS:

EBRP enhanced biological phosphate removal, PAO phosphate accumulating organisms, GAO glycogen accumulating organism, PHA poly-hydroxy-alkanoate
References:

Appendix F: Organic Carbon Removal from Wastewater by a PHA Storing Biofilm using Direct Atmospheric Air Contact as Oxygen Supply

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Abstract:

The principal reason for the high energy costs for biological wastewater treatment is the poor transfer efficiency of oxygen to the bulk water phase. The current paper describes a biofilm reactor in which oxygen transfer to the bulk solution is avoided by alternating anaerobic submersed (2h) and drained (1 h) operation of the biofilm. During the submersed phase the biofilm enriched for glycogen accumulating organism (GAO) stored the organic carbon (acetate) as poly-hydroxy-alkanoate (PHA). After draining the reactor, this carbon stored as PHA was biologically oxidised, using oxygen directly from the atmosphere. The 12 Cmmol/L (384 mg/L BOD) of acetate was completely removed during long term automated operation of the reactor for 9 months with a cycle length of 3.3 hours. As the process specifically removes dissolved organic carbon but not N or P it could possibly be coupled with novel processes such as Anammox or nutrient recovery.

3 Paper published in Bioresource technology (187) pp:182-188
1. Introduction

Next to the removal of organic carbon (biological oxygen demand, BOD), biological wastewater treatment aims at removing nutrients: nitrogen (N), phosphate (TP). Most recent research has focused on the effective removal of nutrients as they are responsible for eutrophication. However, the biological aerobic removal of the BOD from wastewater is the main energy cost to the treatment plant operator (Young & Koopman, 1991). Significant energy is required for the supply of poorly soluble oxygen from the atmosphere into the bulk solution. For example, the removal of 10 Cmmol/L dissolved organic pollution (320 mg/L BOD) requires 0.245 L of dissolved oxygen (DO) available to 1 L of bacterial suspension. Assuming a practical oxygen transfer efficiency of 10% and an air oxygen content of 21%, a treatment plant would provide about 50 times this volume of air (about 11.7 L of air) per L of wastewater. As this air has to be provided under sufficient pressure to lift a water column of typically 5 m height, it is understandable that despite advances in air supply technologies, the energy cost for air supply is high (570 J/L_{wastewater}).

The oxygen supply to the biomass (activated sludge) also initiates the oxidation of ammonia to nitrite or nitrate, which represents an additional oxygen demand (Marcos, 2007). A wastewater with a typical C:N ratio content of 6 g-C/g-N (Sheng-Peng et al., 2010) approximately requires an additional 30% of oxygen for the oxidation of NH_{4}^{+} to NO_{2}^{-}. While this component of air supply may seem unavoidable in the case of traditional N removal by nitrification and denitrification, there are current trends suggesting alternatives for N removal. These include the
anaerobic ammonia oxidation (Anammox (Kuenen, 2008)) and other forms of nitrogen recovery, for example completely autotrophic nitrification over nitrite (CANON) (Schmidt et al., 2003; Third et al., 2001).

In principle, bacteria tend to store organic compounds (poly-hydroxy-alkanoates, PHA) if there are limiting growth factors, which prevent organisms to use the BOD as a source for biomass growth and energy (Lenz & Marchessault, 2005). In wastewater, where inorganic nutrients are typically available, the key mechanism that provokes bacteria to store BOD, as PHA, is the short term depletion of oxygen (Mino et al., 1998; Smolders et al., 1995). In the literature, conditions of alternating oxygen supply have been demonstrated to encourage activated sludge biomass to gradually build up increasing reservoirs of reducing power in the form of PHA (Satoh et al., 1999; Third et al., 2003). In biofilms this reducing power could be used to subsequently drive denitrification in the form of storage driven denitrification (Hughes et al., 2006; Krasnits et al., 2013).

In the current paper we describe the use of the above principle of anaerobic storage of soluble carbon for removing BOD compounds without the costly transfer of oxygen into the bulk wastewater. The approach is to selectively develop and maintain a biofilm rich in PHA accumulating bacteria and to provide it with oxygen by draining the reactor and thus enabling direct contact of the bacterial biomass (here biofilm) with the atmosphere.
2. Materials and methods

2.1 Reactor dimensions and set-up

A cylindrical 2 L PVC reactor (12cm Ø and 29cm height) with openings at the top and bottom (Figure 1) was filled with packing material (AMB™ Bioballs with a specific surface area of 850 m²/m³) and approximate active surface of 500 m²/m³, such that the material filled the entire volume of the reactor. The volume taken by the packing material was 300 mL such that the working volume of the reactor without biomass was 1.7 L.

Figure 1: Schematic diagram of the operated reactor.

2.2 Reactor operation

Prior to operation, the described reactor was inoculated with activated sludge and biomass from a previously used biofilm reactor for storage driven denitrification (Hughes et al., 2006). After seeding, the reactor was operated automatically by...
specifically timed phases (Table 1). The reactor was filled with synthetic wastewater (within 5 min through a peristaltic pump) then maintained under anaerobic conditions for about 2 hours. The anaerobic phase was followed by gravity drainage of 10 min. This allowed air penetration within the reactor of equal volume to the liquid drained. Thereafter further air intake was prevented, using a solenoid, and the volume of air was recirculated for 1 hours. The oxygen in the head space was measured by a dissolved oxygen probe (Mettler Toledo, InPro 6800).

A pH and ORT probe were used to measure and record the values continually into a spreadsheet, using a LabJack U12 data acquisition card and the process control software LabVIEW™ (version 7.1 National Instrument).

**Table 1:** Time schedule of operation of the BOD to PHA storage biofilm reactor in sequencing batch mode.

<table>
<thead>
<tr>
<th>Operation time (min)</th>
<th>Phase</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 5</td>
<td>Fill</td>
<td>Replacing air space by synthetic wastewater</td>
</tr>
<tr>
<td>5 - 120</td>
<td>Store</td>
<td>Uptake of soluble BOD as PHA under water circulation</td>
</tr>
<tr>
<td>120 - 130</td>
<td>Drain</td>
<td>Replacing treated wastewater by air</td>
</tr>
<tr>
<td>130 - 260</td>
<td>Vent</td>
<td>Provide air for oxidation of stored organics</td>
</tr>
</tbody>
</table>

2.3 Synthetic wastewater composition

A synthetic wastewater was used and consisted of (mg.L⁻¹): CH₃COONa 660, NH₄Cl 160, NaHCO₃ 125, KH₂PO₄ 44, MgSO₄.7H₂O 25, yeast extract 50, and 1.25
mL.\textsuperscript{-1} of trace element solution, which contained (g.L\textsuperscript{-1}): ethylene-diamine-tetra-acetic acid (EDTA) 15, ZnSO\textsubscript{4}.7H\textsubscript{2}O 0.43, CoCl\textsubscript{2}.6H\textsubscript{2}O 0.24, MnCl\textsubscript{2}.4H\textsubscript{2}O 0.99, CuSO\textsubscript{4}.5H\textsubscript{2}O 0.25, NaMoO\textsubscript{4}.2H\textsubscript{2}O 0.22, NiCl\textsubscript{2}.6H\textsubscript{2}O 0.19, NaSeO\textsubscript{4}.10H2O 0.21, H\textsubscript{3}BO\textsubscript{4} 0.014 and NaWO\textsubscript{4}.2H\textsubscript{2}O 0.050.

2.4 Analytical

2.4.1 Acetate analysis

An Agilent 7820A gas chromatograph (GC) with auto-sampler was used to quantify acetate concentrations. Samples were acidified with formic acid (1 % (v/v)) before 0.4 μL samples were injected onto an Alltech ECONOCAP\textsuperscript{TM} ECT\textsuperscript{TM} 1000 column (15 m x 530 um (i.d.) 0.25 μm). The carrier gas (N\textsubscript{2}) was set at a flow rate of 3 mL/min and at the inlet the sample was split 10:1. The oven temperature was programmed as follows: initial temperature 70 °C; temperature ramp 5 °C/min to 100 °C; held for 2 min; temperature ramp 70 °C/min to 230 °C; held for 2 min. Injector and detector were set at 200 and 250 °C respectively. The peak area of the Flame Ionisation Detector (FID) output signal was computed via integration using the EzChrom Elite Compact software (© 2005, V.3.3.2SP2). The detection limit determined was 0.5 μmol/L of acetate.

Chemical oxygen demand (COD) was determined by the closed reflux, colorimetric method according to the standard method (Rice et al., 2012). The COD readings were obtained against known concentration of acetate in wastewater (1 to 10 mmol/L).

2.4.2 PHB analysis
The poly-hydroxybutyrate was extracted from the biomass using a method adapted from Smolders et al. (1995). Dichloromethane was used instead of dichloroethane. The samples were esterified in 1:4 concentrated HCl:propanol solution for 2 h at 150 °C in a Hach COD reactor. The culture tubes were sealed with Teflon lids to prevent loss of volatile solvents. Aliquots of 3 mL of DI water were used to clearly separate the organic and the aqueous layers. The organic layer was transferred to a GC vial for analysis. Similarly standards of 0, 3.3, 6.6, 9.9, 13.2 mmol/L beta-hydroxybutyrate were prepared using a stock solution of 200 mmol/L HB (Sigma-Haldrich).

After the above steps of PHB hydrolysis and esterification of the hydrolysed product hydroxybutyric acid, the resulting ester (propyl-hydroxy-butanoate) was analysed using the same GC and column as above with the following conditions. The sample was split at the inlet 5:1. The oven temperature program was: initial temperature 80 °C; temperature ramp 70 °C to 152 °C; temperature ramp 4 °C/min to 160 °C; temperature ramp 70 °C/min to 230 °C hold for 2 min.

The chromatogram of the PHB produced two additional peaks at higher retention times. These peaks were assumed to be hydroxy-valerate (PHV) in two different isomeric forms. The amount of the two additional peaks was estimated from the HB standard curve using benzoic acid as an internal standard.

2.4.3 Glycogen analysis

Biomass was collected and freeze dried (Hetosicc-CD 4) between 10 and 20 hours. Dried biomass was accurately weighed in a digestion tube. The biomass was then digested in a solution of 0.9 mol/L HCl for 4h at 100 °C in a water bath. The insoluble biomass was removed and the pH of a 3 mL supernant of the digested
solution was adjusted to 7.2 (±0.2) using 0.35 mL of 10 mol/L NaOH and 0.5 mL of 0.9 mol/L KH$_2$PO$_4$. The sample was tested by an enzymatic glucose analyser (AccuCheck) against a linear glucose standard curve (0 to 10 mmol/L glucose) (Flavigny, 2014).

### 2.4.4 DNA analysis

DNA from the reactor and from activated sludge were extracted using Power Soil DNA analysis extraction kit (MO-Bio). The DNA was stored at – 20 °C until further analysis. Variable regions of the bacterial 16S rRNA gene were amplified by barcoded pyrosequencing as previously described in Coghlan et al. (2012). Briefly, universal bacterial fusion primers (Hamady et al., 2008) were used to generate PCR amplicons in triplicate and pooled. The forward primer F515 (5’ GTGCCAGCMGCGCCGCGGTAA 3’) and the reverse primer R806 (5’ GGACTACHVGGGTWTCTAAT 3’) targeted the V4 hypervariable region of the 16S rRNA. PCR was carried out in a 25 µL total volume including 4 µL of template DNA, containing: 2.5 mmol/L MgCl$_2$ (Fisher Biotec, Aus), 1x Taq polymerase buffer (Fisher Biotec, Australia), 0.4 µM dNTPs (Astral Scientific, Australia), 0.4 mg BSA (Fisher Biotec, Australia), 0.4 µM of each primer, and 0.25 µL of AmpliTaq Gold DNA polymerase (ABI). The PCR conditions included: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 s, 54°C 30 s, 72°C for 30 s, and a final extension at 72°C for 10 minutes (Corbett Research, NSW, Aus). Amplicons were purified (AMpure beads, Invitrogen) and DNA concentration estimated by ethidium gel staining to approximate equimolar concentrations for emulsion PCR. Bead: template rations for the emulsion were determined by qPCR (Bunce et al., 2012). The Roche GS Junior run set up included an emulsion PCR step, bead recovery, and the sequencing run. All of these
Appendix F

procedures were carried out according to the Roche GS Junior protocols (http://www.454.com). In order to screen for high quality sequences, the sequencing output files were processed as described in Coghlan et al. (2012). This yielded 269 and 165 high quality sequences for the reactor’s biomass and the activated sludge respectively. The resultant BLAST files were imported into the program-MEtaGenome ANalyzer (MEGAN version 4.62.1) (Huson et al., 2007) for taxonomy using the following lowest common ancestor parameters: min score of 65, top percent of 5, and min support of 1.

3. Results and Discussion

3.1 Development of a biofilm that can remove soluble acetate anaerobically

For the purpose of developing a biofilm reactor that specifically selects for bacteria that maximise the BOD (i.e. acetate) storage from the inflow, rather than oxidising the acetate, as observed in activated sludge and in trickling reactors, strict selective conditions were applied after seeding the reactor. The selective conditions entailed the provision of acetate in the absence of oxygen followed by providing oxygen in the absence of soluble acetate. To accomplish this, the reactor was operated in a sequencing batch mode for 2 months as follows:

- anaerobic flooding of the biofilm with synthetic wastewater using acetate (16 mmolC/L 512 mg/L BOD) as the carbon source for 2 hours
- draining of the synthetic wastewater after completed acetate storage as PHA
- passive aeration of the biofilm by merely keeping it drained in the presence of atmospheric air for 1 hour.
The exposure to air was for the purpose of providing oxidative power to the biomass to oxidise the stored soluble carbon (i.e. PHA) and by-passing the costly transfer of oxygen to the bulk liquid. This oxygen exposure to the biomass was expected to form glycogen from PHA thus enabling carbon uptake (i.e. acetate) in the subsequent anaerobic phase (Liu et al., 1996). The continued operation under this scheme of anaerobic storage and biomass exposure to air was expected to selectively enrich for bacterial species capable of effective anaerobic storage of acetate (i.e. GAO).

The reactor was operated continuously and its anaerobic acetate storage monitored (Figure 2). After only partial storage of acetate at the beginning (3 Cmmol/L or 384 mg/L, over 2 hours), the rate and storage capacity of acetate improved after 9 weeks of operation, reaching a rate of acetate storage of 10 Cmmol/L/h acetate (320 mg/L/h BOD) and a storage capacity of 40 Cmmol/L (1280 mg/L BOD). Clearly, the removal rate as well as the mass of acetate taken up now exceeded the capacity of typical activated sludge process achieving < 1 Cmmol/L/h and 128 mg/L/h BOD respectively (Tandukar et al., 2007).

To eliminate the possibility of acetate being converted to another soluble organic compound, COD analysis was carried out in parallel to acetate analysis of the effluent. No evidence of organic species other than acetate was found (data not shown).
Figure 2: Acetate storage after 2 (●), 8 (■) and 9 (▲) weeks of operations. The acetate supplied was lower, from 12Cmmol/L to 7.5 Cmmol/L, after the week 2 to prevent the development of non-storing bacteria during the aerobic phase when acetate is present in the water.

3.2 Specialised biofilm with GAO and its metabolism

In general the intermittent supply of oxygen is known to lead to BOD storage as PHA by phosphate accumulating organisms (PAO) (Hesselmann et al., 1999). PAO bacteria accumulate phosphate as an energy store under aerobic conditions. This is then hydrolysed and released under anaerobic conditions providing sufficient energy for anaerobic BOD uptake and its polymerisation as PHA. However, in the present reactor the aerobic phosphate accumulation cannot occur because in the aerobic phase the phosphate containing bulk liquid has been drained. As a consequence an alternative anaerobic energy source to polyphosphate must be used in the biofilm described here.

The known alternative to PAO metabolism is the metabolism of the glycogen accumulating organisms (GAO). These synthesize glycogen from PHA under
aerobic conditions, which serves via fermentation as the energy source for BOD uptake and PHA storage under anaerobic conditions (Liu et al., 1996). So clearly our biofilm operation would be likely to select for GAO rather than the traditional PHA storing PAO. Furthermore, at the low P/C (Pmol/Cmol) ratio of ≤ 0.02 used in our experiments PAO would be outcompeted by GAO (Liu et al., 2000; López-Vázquez et al., 2007).

After 9 months of operation a biomass sample from the biofilm and from activated sludge from Woodman point wastewater treatment (Perth, Australia) were used for DNA extraction and sequencing. The aim was to compare obvious differences in biomass composition (Figure 3).

![Diagram](image-url)

**Figure 3:** Comparison of the microbial population of biofilm described (white) with that of activated sludge (grey). Size of the node labels is proportional to the number of sequence reads at each taxonomic level. The pie slices are proportional to differences in sequence reads at the taxonomic level.
In the biofilm reactor the second largest population was *Candidatus competibacter* (10.7 %) which is a known GAO (Filipe et al., 2001; Lopez-Vazquez et al., 2009). In theory, this could be expected because of the selective operation of the system offering anaerobic condition with acetate followed by an aerobic environment without acetate (and without phosphate which could otherwise lead to PAO). However, the presence of the genus *Haliangium* is unusual, as these belong to the myxobacteria which are known as predators to other bacteria (Ivanova et al. (2010). Similar to GAO, which oxidise stored PHA in the aerobic phase, myxobacteria also have their food source in the aerobic phase, while other typical heterotrophs have no organic feed supply after draining. This aerobic feeding of predators in the biofilm provides one possible explanation for the low sludge production observed. Representatives of PAO (*C. accumulibacter*) were not detected. This is because PAO require oxygen and phosphate together (Cech & Hartman, 1993; Smolders et al., 1995) whereas in the reactor, after the liquid is drained for aeration, the phosphate has also been drained.

A proper carbon balance including PHA, glycogen and acetate would be able to evaluate whether the biofilm behaviour is in accordance with GAO metabolism. The large volume of the biofilm biomass in the reactor prevents representative sampling which is needed for carbon balance purposes. Therefore, after thorough mixing of the reactor’s biomass, a subsample was used for the carbon balance experiment. An extended aerobic period and an increased dose of acetate was provided to generate changes in the overall storage products (PHA and glycogen) that were sufficiently large to show significant differences against the background storage products.

To test whether the carbon removal behaviour under anaerobic conditions was consistent with the glycogen metabolism of typical GAO a simple carbon balance
was established. The biofilm subsample (5.6 g dry biomass) was first aerated for 6 hours. It was then suspended anaerobically in a solution of synthetic wastewater (100 mL) with excess acetate (20 Cmmol/L) overnight, to record glycogen and acetate conversion to PHA (Figure 4). Overall the carbon balance was maintained throughout this anaerobic phase. During the anaerobic phase, the glycogen oxidation (i.e. decrease) provides the ATP source for acetate uptake and the resulting production of PHA (Figure 4) as expected from the literature (Bengtsson, 2009; Filipe et al., 2001; Smolders et al., 1995). In the anaerobic period 1.0 Cmol of PHAs was produced per Cmol of the combined reactants, acetate and glycogen. This is in line with the reported anaerobic PHA yield ranging from 0.87 to 0.99 Cmol of PHB produced per Cmol consumed (VFA + glycogen) (Dai et al., 2007; Pisco et al., 2009).

Also under aerobic conditions the carbon balance was reasonably conserved indicating a glycogen production of 1.1 Cmmol_{glyc}/Cmmol_{PHA} which is slightly higher than the result of 0.8 obtained by Filipe et al. (2001) and similar to the value of 1.0 by (Liu et al., 1994). The expected carbon loss as CO₂ originating from carbon respiration could not be accounted for in the carbon balance.
Overall the results show that the biofilm behaviour is in line with the GAO metabolism demonstrated in the literature (Bengtsson, 2009; Filipe et al., 2001). Anaerobically, the carbon taken up via acetate was accounted for by the combination of PHA gain and glycogen loss. Aerobically, carbon usage for glycogen production was similar to carbon release from PHA consumption.

3.3 Direct passive oxygen supply to the drained biofilm

Once the soluble acetate was removed from the wastewater, the liquid was drained by opening the bottom and the top of reactor (valve operated system) to allow air to fill the void volume. This allowed the supply of oxygen to the microbial cells while by-passing costly oxygen transfer to the bulk solution. Then the reactor was closed to provide a reproducible amount of oxygen for all trials.

Considering the volume of the liquid contained in the reactor was approximately 1 L then there is 210 mL or 8.5 mmol of O₂ available for the oxidation of the stored...
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Carbon (i.e. 8.5 Cmmol of acetate). Mass balance showed that, of the acetate removed from solution (12 Cmmol/L), only about half was oxidised by oxygen (Figure 5). Therefore carbon accumulated within the system, either in the form of biomass or alternatively as storage material.

Figure 5: Typical behaviour of a single cycle of the storage biofilm reactor during anaerobic acetate storage (●) (0-2 h) and calculated aerobic acetate oxidation (○) (2-3h). Oxygen consumption (■) was used to calculate acetate oxidised.

3.4 Minimum oxygen requirements

To test whether providing one pore volume of air was sufficient for the long term operation and acetate removal, the reactor was run continuously for 24 cycles. Over 80 hours it was demonstrated that 14 Cmmol/L acetate (448 mg/L BOD) present in the synthetic wastewater were removed in cycles of 3.5h (Figure 6). Therefore the removal rate of acetate was 4 Cmmol/L/h (123 mg/L/h BOD), which is a carbon removal rate that is about 3 times higher than typically observed in activated sludge plants (Tandukar et al., 2007). No significant biomass output was recorded over this
time. From the reproducible oxygen uptake curves (data not shown) it could be predicted that approximately 50% of the acetate added was resired (Table 2).

Above, the acetate was continuously removed with a single reactor void volume of air. To test for the maximum carbon to oxygen ratio needed to enable sustained operation, the acetate concentration was incremented to 22 Cmmol/L and 30 Cmmol/L (Figure 6). The time provided for acetate uptake was increased from 2 hours to 4 hours and to 7.5 hours for the highest concentration. The oxygen supply was maintained to a single void pore volume, the C:O$_2$ ratio is therefore increased from 1.3 to 2.1 and 2.9 for the highest concentration.

At a feed concentration of 22 Cmmol/L > 90% of acetate was continually removed over 18 subsequent cycles with providing still only 1 pore volume of air (Figure 6). When elevating the acetate concentration to 30 Cmmol/L acetate could no longer be stored sustainably, as indicated by 50% residual acetate being present in the effluent after 5 cycles (Figure 6).

Using stoichiometric considerations, the fact that up to 22 Cmmol/L of acetate (26.4 Cmmol of acetate per reactor) could be removed continuously with 1 pore volume of air continuously suggests that sufficient stored acetate is oxidised. In fact, calculated from the oxygen content, approximately 27% of the added acetate was oxidised (Table 2) with the remainder retained within the biomass. On the contrary, when 30 Cmmol/L were added the cycles were not sustained (Figure 6). In this case, the 1 pore volume oxidised 20% of the added acetate. This suggests that there is a minimum of PHA oxidation required to sustain BOD uptake.

Overall, if 27% of the added acetate oxidised, 22 Cmmol/L removal was sustained. However, if 20% or less of the added acetate, 30 Cmmol/L removal was
not sustained. It seems that if more than a quarter of the added BOD is oxidised then
the BOD storage can be sustained (Table 2).

Figure 6: The effect of increasing the carbon to oxygen ratio, on the continuous removal of acetate.
Continuous operation of the storage biofilm reactor under repeated cycles of synthetic wastewater
with 1 pore volume of air provided, 24 cycles of 14 Cmmol/L, 18 cycles of 22Cmmol/L and 5 cycles
of 30 Cmmol/L. Example cycles of 14 Cmmol/L (●) and carbon outflow (○), of 22 Cmmol/L (▲) and
carbon outflow (Δ), and of 30 Cmmol/L (■) and carbon outflow (□).

Table 2: Operation parameters of the reactor to test the effect of acetate to oxygen ratio to sustain
continuous acetate removal. From the oxygen consumption, the acetate oxidised was calculated and
the acetate storage determined.

<table>
<thead>
<tr>
<th>Ac input rate CmM/h</th>
<th>Ac input / cycle CmM</th>
<th>O₂ input CmM</th>
<th>Carbon to O₂ ratio C:O₂</th>
<th>Ac oxidised / cycle CmM</th>
<th>Percentage C oxidised %</th>
<th>Ac remaining in outflow</th>
<th>Sustained removal?</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>14</td>
<td>10.3</td>
<td>1.36</td>
<td>5.9</td>
<td>42</td>
<td>&lt;1%</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>22</td>
<td>10.3</td>
<td>2.1</td>
<td>5.9</td>
<td>27</td>
<td>&lt;1%</td>
<td>Yes</td>
</tr>
<tr>
<td>3.5</td>
<td>30</td>
<td>10.3</td>
<td>2.9</td>
<td>5.9</td>
<td>20</td>
<td>~50%</td>
<td>No</td>
</tr>
</tbody>
</table>
3.5 Operational considerations:

In a number of ways the described biofilm reactor is similar to trickle filters used for wastewater treatment. However, significant differences can be pointed out both in terms of microbial composition and operational attributes.

Because of the strict cycling of anaerobic acetate storage to PHA followed by aerobic PHA oxidation, only those heterotrophic bacteria than can effectively store BOD as PHA, namely GAO can be sustained in the biofilm. The current sequencing batch operation of the biofilm reactor would hence select for the development of a distinctly different biomass to that in trickle reactors.

With an acetate removal rate of 4 Cmmol/L/h (123 g/m³/h) the described biofilm process demonstrated a 10 to 20 times faster volumetric carbon removal rate than that obtained for traditional trickle reactors (Table 3). Possible reasons for the rather high rates of BOD removal could be:

- the high surface area of carrier material used in the bioreactor (850 m²/m³)
- the high biomass content of the biofilm (50 g dry biomass per L of reactor volume)

Assuming a 5 m high reactor and 3.5 h treatment time, the energy required is 4 W/m³. Considering that trickling reactors (high rate with plastic media) are recirculating 4 to 7 times, their energy usage is typically 6 to 10 W/m³ (Metcalf et al., 1972), our biofilm requires 1.5 to 2.5 times less energy expense compared to trickle reactors.
Table 3: Comparison of the hydraulic retention time (HRT) for trickle filters and proposed biofilm.

<table>
<thead>
<tr>
<th>System</th>
<th>BOD removal rate g/m³/h</th>
<th>BOD inflow g/m³</th>
<th>HRT h</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trickle reactor for communal wastewater, rock media</td>
<td>11.7</td>
<td>599.5</td>
<td>51.2</td>
<td>Doan et al. (2008)</td>
</tr>
<tr>
<td>Trickle reactor for communal wastewater, rock media</td>
<td>4</td>
<td>250</td>
<td>62.5</td>
<td>Gray (2004)</td>
</tr>
<tr>
<td>Trickle reactor for communal wastewater, rock media</td>
<td>5</td>
<td>250</td>
<td>50</td>
<td>Forster (2003)</td>
</tr>
<tr>
<td>Sequencing operation of PHA storing biofilm</td>
<td>128</td>
<td>480</td>
<td>3.5</td>
<td>Present research</td>
</tr>
</tbody>
</table>

The biofilm reactor described removes BOD rapidly and energy-efficiently but does not remove phosphorus and nitrogen. The nutrient removal can be achieved by the novel low-energy nitrogen removal processes that include the Anammox bacteria, such as the completely autotrophic nitrogen removal over nitrite (CANON) which uses limited aeration for partial oxidation of ammonia to nitrite followed by the Anammox process leading to N₂ formation (Third et al., 2001). However, this process cannot be effectively applied with existing wastewater streams because of the BOD (Kartal et al., 2010). The currently described process would be a fast and cost effective way to remove soluble BOD from wastewater prior to nitrogen removal treatments, which requires low BOD wastewater. The additional removal of nitrogen by a process linked to the described biofilm reactor has been designed and will be described later.
4. Conclusion:

- A simple sequence of anaerobic conditions (filling) and aerobic condition (drainage) selectively enriched for glycogen accumulating organisms (GAO) from activated sludge. Such method is easily applicable for existing biofilm reactors.
- The biofilm was able of sustained acetate removal from synthetic wastewater without transferring air into the bulk wastewater and hence by-passing the majority of energy expense for oxygen transfer.
- Atmospheric air provides oxidative power via passive aeration to the biofilm for PHA oxidation, hence recovering the biofilm’s ability to store acetate in subsequent cycles. A repeated liquid recycle as needed for trickling reactors was not needed.

ACKNOWLEDGEMENT:

We would like to thank Murdoch University for the financial support of this project (scholarship to R. Flavigny). In addition, the authors would like to acknowledge the help of Dr Lucy Skillman for DNA analysis of the biomass.
References:


Flavigny, R. 2014. Diabetic glucose meter for the determination of glucose in microbial cultures. *Journal of Microbiological Methods*, 100(0), 91-92.


Appendix F


Acknowledgments

First I would like to thank my principal supervisor Dr Ralf Cord-Ruwisch for his novel ideas throughout my work. He provided me with guidance, critical thinking and always pushed me one step further to clear up my research ideas. His ability to think outside the box brings new and challenging ideas which are good to discuss. I would like to thank Murdoch University for providing me with a Murdoch International Ph.D. Student scholarship.

I am ever so grateful to Dr Wipa Charles, my co-supervisor, who has been available for very direct and constructive guidance. Her experience has been invaluable and her rigorous methods helped me develop my critical thinking and thrive for my own research passions.

Thanks to Dr Liang Cheng for his constructive ideas on conducting science experiments and achieving rapid and meaningful results. His novel ideas are greatly appreciated and I look forward to future constructive research output with him.

In addition, I am very thankful to Andrew Foreman and Marc Hampton for their help to set up and operate the gas chromatography (GC) to measure the nitrogen gas.

I would also like to thank my colleagues Emily Quek and Rabbani Ayaz, who have shared my pain and anger, but also mainly great laughs and endless discussions. We have shared so much in these three years that it seems that more time has passed. Thanks for your friendship. Thank you to the new Ph.D. recruits Iqbal Hossain, Mohamed Hassan, Si Ying Lee, Darwin Darwin and Negar Vakilifard for their endless questions on all aspects of Ph.D. life. I look forward to seeing your work.
I also thank my parents and family who have understood me in stressful situations. They have offered me words of comfort when needed. Thanks to my friends who have given me breaks on my journey to share some great times outside the lab. I would like to thank in particular Chris Olney and Chris Oakeley for the admirable and thorough job of proofreading my thesis.

Last but not least, I will never thank my love, Noemie Legendre, enough for her support throughout this journey. She has never looked back. I am now looking forward to us both travelling the same road.