Evaluation and Validation of Passive Aeration Simultaneous Nitrification and Denitrification (PASND) in a Biofilm Reactor for Low-Energy Wastewater Treatment

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement has been made in the text.

Md Iqbal Hossain

November 2017

This Dissertation is Dedicated to My Parents

Abstract

In today's rapidly urbanized and industrialized society, there is an increasing need for cost-effective and environmentally sustainable technologies for the removal of nutrients from the polluted water. Wastewater treatment principally relies on microorganisms for their ability to take up organic substrate and nutrients (i.e., nitrogen) from wastewater. The need for active aeration of the bulk wastewater to provide oxidation power to microorganisms makes the wastewater treatment process extremely energy intensive. However, a recent study suggests that by exposing the biomass directly to air (passive aeration), the cost associated with aeration could be reduced. This thesis aimed to establish such a passively aerated biofilm (to remove organics and nitrogen from wastewater) from activated sludge in order to investigate its real-world feasibility.

To enable cost-effective removal of organic compounds, a biofilm enriched with glycogen accumulating organism (GAO) was developed by selective enrichment from activated sludge using sequences of anaerobic flooding followed by aerobic exposure of the biofilm directly to the atmosphere. The transition of activated sludge to the GAO biofilm was completed within eight weeks of continuous selective operation. The GAO biofilm enabled anaerobic removal of organic carbon (biochemical oxygen demand or BOD) from wastewater which was stored intracellularly as poly-hydroxyalkanoate (PHA). The PHA was oxidized in the subsequent aerobic stage to regenerate the biofilm's BOD storage capacity. With using acetate as synthetic BOD, the biofilm demonstrated efficient (>99%) and stable removal of organic carbon at an average rate of 256 mg BOD L^{-1} h^{-1}.

Long-term operation of the established GAO biofilm leads to the very low amount of excess sludge produced. This is of particular interest since sludge disposal cost is the second greatest operational expense in traditional wastewater treatment facilities. The average excess sludge (volatile suspended solids or VSS) production rate was found to be 0.05 g VSS g^{-1} BOD removed which is about 10-times lower than that of activated sludge process. Factors such as the high biomass content (21.41 g VSS L^{-1} of reactor) and the low growth yield of GAO were found to be associated with little sludge production. In addition, a high number of a predatory protozoan (*Tetramitus*) was found inhabiting the biofilm that minimized sludge production by effectively grazing on cells.

In order to allow next to organic carbon also nitrogen removal from wastewater, a hybrid biofilm system was developed by incorporating zeolite (an ion-exchange material) into the GAO biofilm and activated sludge as the sole source of nitrifying bacteria. During the anaerobic phase, zeolite adsorbed ammonium which was removed in the subsequent aerobic stage by the combined action of nitrifying and GAO bacteria via simultaneous nitrification and denitrification (SND). The occurrence of SND under full atmospheric partial pressure was confirmed by trickling nitrate solution over the biofilm system which resulted in nitrate reduction in full atmospheric condition. Over four months of continuous operation, the biofilm reactor demonstrated sustained BOD (>90%) and nitrogen (about 70%) removal performance with a short hydraulic retention time (HRT) of 5 h (2 h anaerobic and 3 h aerobic phase). The inadequate nitrogen removal efficiency was attributed to the limited capacity (1.474 mg NH_4^+-N g^{-1}) of the zeolite used in this study. However, a subsequent repeat treatment of the effluent in the same biofilm reactor resulted in about 96 % ammonium removal from wastewater.

The capability of the GAO biofilm to treat high-strength (up to 4-times) wastewater was evaluated while keeping the same anaerobic duration (2 h). The amount of ammonium adsorbed onto zeolite was found to increase proportionally with influent feed concentration. However, the aerobic time required for zeolite regeneration was longer. Compared to single (1x) and double (2x) strength wastewater, the quadruple (4x) strength synthetic wastewater resulted in nitrite accumulation which took about 5 h (aerobic phase) for complete reduction. Similarly, the BOD removal rate of the biofilm system increased from 543 to 2308 mg L^{-1} h^{-1} for 1x and 4x strength wastewater, respectively. The increased uptake of BOD by GAO biofilm resulted in the improved storage of PHA (5.02 and 18.6 mmol L^{-1} for 1x and 4x wastewater, respectively) which contributed to the efficient regeneration of zeolite. The biofilm system showed its stability for the treatment of different strength wastewater over a period of 2-months operation suggesting the feasibility of 4x or more concentrated wastewater treatment using the proposed biofilm technology with low aeration energy input.

To further optimize nitrogen removal performance in the zeolite amended GAO biofilm, several anaerobic and aerobic phases were used while keeping the total treatment time the same (8 h). An increase in the treatment cycles from 2 to 8, increased the nitrogen removal efficiency from 79% to >99%. A simple numerical model was developed that could effectively explain the trends of nitrogen removal in multiple treatment cycles on the basis of the Langmuir ion-exchange isotherm.

The main conclusion drawn from the study is that passively aerated GAO biofilm system can be established from standard activated sludge within a reasonable time. The amendment of the GAO biofilm by the addition of zeolite as an ammonium adsorbent enables nitrogen removal from wastewater. The proposed biofilm technology has the potential to reduce the energy cost associated with aeration while significantly improving nitrogen removal from high-strength wastewater.

Publications and Conference

The following publications were derived from this thesis:

1. Patent

"Water treatment method". Australian Provisional Patent. Application No.: 2017901640 (Part of Chapter 5)

2. Peer reviewed publications

Hossain, M.I., Paparini, A., Cord-Ruwisch, R. 2017. Rapid adaptation of activated sludge bacteria into a glycogen accumulating biofilm enabling anaerobic BOD uptake. *Bioresource Technology*, **228**, 1-8. (Chapter 2)

Hossain, M.I., Paparini, A., Cord-Ruwisch, R. Direct oxygen uptake from air by novel glycogen accumulating organism dominated biofilm minimizes excess sludge production. *Science of the Total Environment*, **640–641**, 80-88 (Chapter 3)

Hossain, M.I., Cheng, L., Cord-Ruwisch, R. Characterization and optimization of passive aeration SND as novel low-energy wastewater treatment system. To be Submitted (Chapter 5)

Hossain, M.I., Cheng, L., Cord-Ruwisch, R. Treatment of high-strength wastewater in a zeolite amended GAO biofilm. To be Submitted (Chapter 6)

Hossain, M.I., Cheng, L., Cord-Ruwisch, R. Optimization of nitrogen removal by shortening and repeating cycles. To be Submitted (Chapter 7)

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3. Conference presentation

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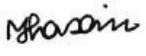
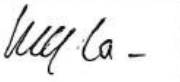
Hossain, M.I., Cheng, L., Flavigny, R.M.G., Cord-Ruwisch, R. Proof of concept of removal of carbon and nitrogen from wastewater through a novel process of biofilm SND (Oral presentation).

Statement of Authors Contribution

Hossain, M.I., Paparini, A., Cord-Ruwisch, R. 2017. Rapid adaptation of activated sludge bacteria into a glycogen accumulating biofilm enabling anaerobic BOD uptake. *Bioresource Technology*, **228**, 1-8. (Chapter 2)

Contributor	Statement of Contribution	Signature
Md Iqbal Hossain	Designed and conducted all experiments, analyzed and processed the data and composed the content of manuscript from initial draft to final submission (75 %)	
Andrea Paparini	Conducted next generation sequencing of microbial communities, analyzed the data and made comments on the manuscript (15 %)	
Ralf Cord-Ruwisch	Advised on experimental design, supervised the project and critically reviewed the paper (10 %)	

Hossain, M.I., Paparini, A., Cord-Ruwisch, R. Direct oxygen uptake from air by novel glycogen accumulating organism dominated biofilm minimizes excess sludge production. *Science of the Total Environment*, **640–641**, 80-88 (Chapter 3)

Contributor	Statement of Contribution	Signature
Md Iqbal Hossain	Designed and conducted all experiments, analyzed and processed the data and composed the content of manuscript from initial draft to final submission (80 %)	
Andrea Paparini	Conducted next generation sequencing of microbial communities, analyzed the data and made comments on the manuscript (10 %)	
Ralf Cord-Ruwisch	Advised on experimental design, supervised the project and critically reviewed the paper (10 %)	

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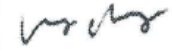
Md Iqbal Hossain	Designed and conducted all experiments, analyzed and processed the data and composed the content of manuscript from initial draft to final submission (80 %)	
Liang Cheng	Participated in the analysis and discussion of experimental results and made comments on the manuscript (10 %)	
Ralf Cord-Ruwisch	Advised on experimental design, supervised the project and critically reviewed the paper (10 %)	

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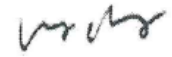
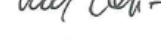
Md Iqbal Hossain	Designed and conducted all experiments, analyzed and processed the data and composed the content of manuscript from initial draft to final submission (80 %)	
Liang Cheng	Participated in the analysis and discussion of experimental results and made comments on the manuscript (10 %)	
Ralf Cord-Ruwisch	Advised on experimental design, supervised the project and critically reviewed the paper (10 %)	

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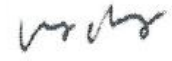
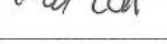
Liang Cheng	Participated in the analysis and discussion of experimental results and made comments on the manuscript (10 %)	
Ralf Cord - Ruwisch	Advised on experimental design, supervised the project and critically reviewed the paper (10 %)	

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Chapter 1
Introduction and Aim

1.1 Wastewater treatment

In our rapidly urbanized and industrialized society, there is an increasing need for cost-effective and environmentally sustainable technologies for wastewater treatment (van Loosdrecht & Brdjanovic, 2014). Wastewater treatment has two fundamental reasons: to prevent pollution by reducing the nutrient content in water and, to enable the reuse of the water as a recycled resource (Gibbs et al., 2004). Wastewater treatment facilities typically employ a number of physical, chemical and biological processes to meet strict nutrient levels in discharged water, set by regulatory authorities (Foley et al., 2010). Among the treatment options, biological wastewater treatment is the most efficient and economical way to remove pollutants from wastewaters. Biological treatment processes principally rely on microorganisms for their ability to take up organic substrate and nutrients such as nitrogen and phosphorus present in the wastewater for their growth, maintenance, and energy generation (Tchobanoglous et al., 2014).

Conventional biological wastewater treatment plants are primarily based on the activated sludge process which was developed more than 100 years ago in England (Ardern & Lockett, 1914). This process utilizes a mixed microbial culture including bacteria, protozoa, and fungi to enhance the decomposition of organic and inorganic compounds (van Loosdrecht & Brdjanovic, 2014). Activated sludge process mainly consists of two steps: an aerated basin and a secondary clarifier (Figure 1.1).

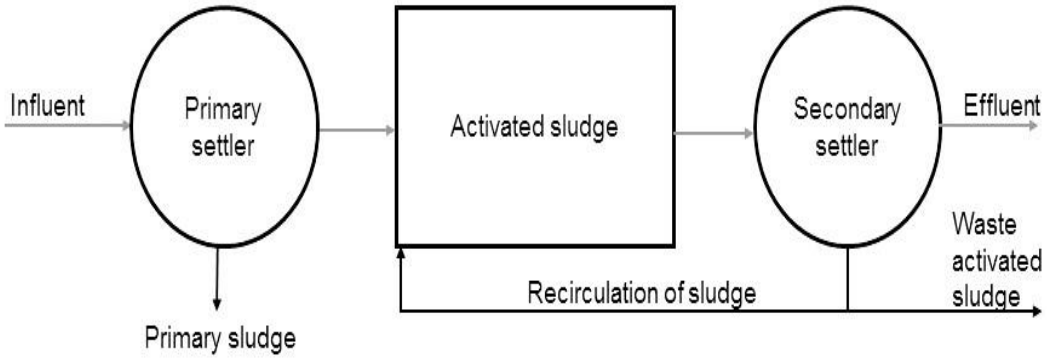

Figure 1.1: A simplified diagram of a traditional activated sludge wastewater treatment plant.

In the aerated basin, air is introduced to mix the wastewater with the activated sludge (which are usually present as flocs) and to provide the oxygen needed for the microorganisms to oxidize the contaminants. Addition of fresh wastewater to the basin and removal of treated wastewater from the basin occur continuously. The treated effluent flows to a secondary settling tank (clarifier), where activated sludge is separated from the treated wastewater (effluent) by gravity. Part of the settled sludge is recycled to the aerated basin to keep a viable concentration of biomass inside the reactor. The remainder of the sludge is dewatered on sludge drying beds, further digested in a sludge digester to remove pathogens, prior to its final disposal.

1.2 Biological carbon removal

The major constituent of municipal wastewater is the organic matter which can be categorized into two different forms: suspended (60-80%) and dissolved (20-40%) (Henze et al., 2008). Suspended organic compounds (e.g., carbohydrates, fats, etc.) are insoluble, and a number of different physical treatment processes such as screening, filtering or settling are used to remove this non-biodegradable component from wastewater. On the other hand, dissolved organic carbons are easily degraded by microorganisms. The biodegradable organic matter is traditionally removed from wastewater through microbial

degradation (oxidative and non-oxidative) by the growth of heterotrophic bacteria using organic carbon as the source of energy and electrons (Tchobanoglous et al., 2014). Factors which are critical to the biological removal of organic carbon includes the availability of sufficient nutrients (nitrogen and phosphorus), O_2 (>0.50 mg L^{-1}), temperature and pH (6.0 to 9.0) (Henze et al., 2008; Tchobanoglous et al., 2014).

1.2.1 Removal of organic carbon by oxidative process

The oxidative removal of the biodegradable organic matter is carried out by mixed cultures of microorganisms. The process requires adequate contact time between the wastewater and heterotrophic microorganisms, and sufficient O_2 and nutrients (Tchobanoglous et al., 2014). During the oxidation process, more than half of the organic carbon is converted to CO_2, and the remainder is assimilated into new (active) biomass. The active biomass is further oxidized by endogenous respiration, resulting in the generation of additional CO_2 and water, along with inactive biomass (i.e., sludge). The excess sludge is removed by sedimentation, leaving the wastewater-free of the original organic matter.

The stoichiometry involved in the oxidation process can be represented by the following equations (Tchobanoglous et al., 2014):

Oxidation and synthesis:

$$COHNS + O_2 + \text{nutrients} \rightarrow CO_2 + NH_3 + C_5H_7NO_2 + \text{other end products} \quad (1.1)$$

Endogenous respiration:

$$C_5H_7NO_2 + O_2 \rightarrow 5CO_2 + 2H_2O + NH_3 + \text{energy} \quad (1.2)$$

In equation (1.1) COHNS is used to represent the organic matter in wastewater, which serves as the electron donor while the O_2 serves as an electron acceptor and $C_5H_7NO_2$ represent new cells.

1.2.2 Non-oxidative removal of organic carbon

The non-oxidative removal of organic carbon from wastewater by microorganisms involves several mechanisms such as assimilation, sorption, and storage (Modin et al., 2016).

1.2.2.1 Assimilation

During the growth of microorganisms present in wastewater, a portion of the soluble, biodegradable organic compounds is assimilated into microbial cells resulting in the removal of organics from the liquid phase. Aerobic heterotrophs growing on organic compounds present in municipal wastewater typically have a yield coefficient of about 0.42 g volatile suspended solids (VSS) g^{-1} BOD removed (Rittmann & McCarty, 2012).

1.2.2.2 Storage

Many microorganisms present in the activated sludge process can store soluble organic carbon as insoluble storage polymers which form intracellular inclusions resulting in the removal of biodegradable organic carbon from wastewater. These polymers exert negligible osmotic pressure on the cell when synthesized due to their high molecular weight (Dawes & Senior, 1973). Dynamic (unbalanced) wastewater plant configurations in activated sludge processes, such as the sequencing batch reactor (SBR) in which the biomass is alternately exposed to feast (anaerobic) and famine (aerobic) conditions, induce the storage behavior (Majone et al., 1999). Microorganisms capable of rapidly storing large amounts of organics in the feast stage have a competitive advantage for growth over other heterotrophic bacteria during the famine phase (van Loosdrecht et al., 1997b).

There are three main types of microbial storage polymers that play a major role in the activated sludge process, namely polysaccharides (e.g., glycogen), polyhydroxyalkanoates (PHAs, e.g., PHB) and polyphosphates (Third, 2003; Van Loosdrecht et al.,

1997a). Polyphosphate (poly P) can fulfill the role of ATP in some microorganisms (Dawes & Senior, 1973). Although microbial cells do not have the capacity for directly storing large amounts of ATP, some cells have developed the capability to store polyphosphate, which produces ATP upon hydrolysis (Third, 2003). Glycogen and PHB are reduced organic compounds, consisting of glucose and hydroxy-acid monomers, respectively. The accumulation of PHB and glycogen depends on the type of carbon substrate and the conditions of microbial growth. When substrates are metabolized via acetyl-CoA, without the intermediate formation of pyruvate, PHB accumulation predominates. On the other hand, the metabolism of the substrate via pyruvate leads to the formation of glycogen (Dawes & Senior, 1973). Therefore, glycogen is usually formed within microbial cells when carbohydrates (e.g., glucose) are present in the liquid, and PHAs when volatile fatty acids (e.g., acetate) are the substrate.

1.2.2.3 Sorption

The sorption process where organic compounds (both biodegradable and inert fractions) and activated sludge flocs first collide and then adhere to each other, plays an important role in many existing and emerging activated sludge based wastewater treatment systems for removal of organic contaminants from the aqueous phase of wastewater (Modin et al., 2016). Existing process configurations that rely on sorption as the sole removal mechanism include contact-stabilization and adsorption-biooxidation (AB) process (Modin et al., 2015).

1.3 Biological nitrogen removal

Nitrogen (N) is an essential element required for life as it is a constituent of biological macromolecules including nucleic acids and proteins. In municipal and industrial wastewater, the nitrogenous matter is mainly composed of inorganic ammonium nitrogen,

which can be present in gaseous (NH_3) and ionic form (NH_4^+), and organic nitrogen (urea, amino acids, and other organic compounds with an amino group). Sometimes wastewaters also contain traces of oxidized forms of nitrogen, mainly nitrite (NO_2^-) and nitrate (NO_3^-) (van Haandel & van der Lubbe, 2012). These reactive species have been found to be associated with numerous environmental problems such as eutrophication (Conley et al., 2009), loss of biodiversity (Dodds et al., 2009; Scherer & Pfister, 2016) and dysfunction of aquatic systems (Dodds et al., 2009). To protect the environment from these adverse effects, the reactive forms of nitrogen need to be removed from the water bodies, which is traditionally done by biological processes. The biological removal of nitrogen from wastewater involves two main reactions: nitrification and denitrification.

1.3.1 Nitrification

Nitrification is the process by which ammonium (NH_4^+) is oxidized to nitrate (NO_3^-) catalyzed by ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea (AOA) (Daims et al., 2016). Nitrification involves two sequential oxidation reactions: nitritation and nitratation. In nitritation, ammonium is first oxidized to hydroxyl-amine (NH_2OH) which is further oxidized to nitrite (NO_2^-) (equation 1.3) (Wiesmann & Libra, 1999). These reactions are catalyzed by the enzyme ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO), respectively.

$$NH_4^+ + 1.3O_2 + 1.98HCO_3^- \rightarrow$$
$$0.0182C_5H_7O_2N + 0.98NO_2^- + 1.04H_2O + 1.89H_2CO_3 \qquad (1.3)$$

In nitratation, nitrite (NO_2^-) is oxidized in the presence of oxygen by nitrite-oxidizing bacteria (NOB) to nitrate (NO_3^-), and the reaction is catalyzed by nitrite oxidoreductase (equation 1.4) (Wiesmann & Libra, 1999).

$$NO_2^- + 0.005NH_4^+ + 0.48O_2 + 0.005HCO_3^- + 0.02H_2CO_3^- \rightarrow$$
$$0.005C_5H_7O_2N + NO_3^- + 0.015H_2O \quad (1.4)$$

Both AOB and NOB are chemo-autotrophs (i.e., use CO_2 as a carbon source for biomass synthesis; ammonium and nitrite for ATP generation). They have lower growth rates (2 d^{-1} and 1.4 d^{-1} for AOB and NOB, respectively) compared to heterotrophic bacteria performing organic carbon removal and denitrification. Therefore, these slow-growing organisms require relatively long sludge retention time (SRT) to be maintained in the bioreactor (Tchobanoglous et al., 2014). Most of the AOB present in wastewater treatment plants are affiliated to the Betaproteobacteria class (*Nitrosomonas* and *Nitrosospira*) (Schmidt et al., 2003), while two known species belonging to the class Gammaproteobacteria (*Nitrosococcus halophilus* and *N. oceani*). On the other hand, NOB belongs to diverse groups such as Alphaproteobacateria class (i.e., *Nitrobacter* spp.), Betaproteobacteria class (i.e., *Nitrotoga* spp.), and *Nitrospira* phylum (i.e., *Nitrospira* spp.) (Daims et al., 2016; Sorokin et al., 2012).

Nitrification process is influenced by several environmental factors such as pH, dissolved oxygen concentration, temperature, the presence of wide range of organic compounds and heavy metals (Bassin et al., 2015). The optimum pH and temperature values for nitrification are 7.5-8.0 and 30-35°C, respectively (Henze et al., 2008; Tchobanoglous et al., 2014). Typically, high dissolved oxygen (DO) concentration (above 2 mg O_2 L^{-1}) is applied to achieve efficient ammonium oxidation (Henze et al., 2008). The concentration of ammonium and nitrite also influence the nitrification process. If their concentrations are too low, substrate limitation will occur. On the other hand, high concentrations of these substrates and their different forms such as unionized NH_3 (at higher pH), undissociated

HNO_2 (at lower pH) inhibit the process (Rittmann & McCarty, 2012; Tchobanoglous et al., 2014).

In wastewater treatment plants, nitrification process is typically performed either in a single-sludge configuration (where heterotrophic and nitrifying bacteria coexist in a single tank to simultaneously remove ammonium and organic carbon) or in a two-stage configuration (where the first stage remove mainly carbon and the second ammonium) (Rittmann & McCarty, 2012).

1.3.2 Denitrification

During denitrification, heterotrophic bacteria sequentially reduce nitrate (equation 1.5) or nitrite (equation 1.6) to dinitrogen gas under anoxic condition. In this process, biodegradable organic carbon (such as methanol, ethanol, acetate and glucose) acts as energy source (electron donor) for ATP generation and nitrate (or nitrite) acts as electron acceptor instead of oxygen. The denitrification reaction using methanol as an electron donor are as follows (Ahn, 2006):

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow$$
$$0.056C_5H_7O_2N + HCO_3^- + 1.68H_2O + 0.47N_2 \quad (1.5)$$

$$NO_2^- + 0.67CH_3OH + 0.53H_2CO_3 \rightarrow$$
$$0.04C_5H_7O_2N + HCO_3^- + 1.23H_2O + 0.48N_2 \quad (1.6)$$

A diverse group of both autotrophic and heterotrophic bacteria (mostly belong to alpha- and betaproteobacteria) capable of using a wide range of electron donors are associated with the denitrification process (Tchobanoglous et al., 2014). Denitrification can also be accomplished by chemolithotrophic bacteria, which use H_2, elemental sulfur or sulfide as electron donors. Moreover, polyphosphate accumulating organism (PAO) and glycogen

accumulating organism (GAO) have been reported to possess denitrifying capability (Carvalho et al., 2007; Saad et al., 2016; Zeng et al., 2003a). These bacteria use their intracellular storage material (PHA and/or glycogen) to reduce nitrate or nitrite (Alzate Marin et al., 2016; Coats et al., 2011; Miao et al., 2015; Miao et al., 2016).

In wastewater treatment plants, denitrification is usually performed in several configurations: pre-anoxic (substrate driven) denitrification, and post-anoxic (endogenous driven) denitrification (Tchobanoglous et al., 2014).

Pre-anoxic denitrification

Organic carbon present in the influent wastewater serves as a carbon source in this process where aerobically produced nitrate is recycled to the pre-anoxic zone for denitrification to occur (Figure 1.2A). The factors that influence the denitrification rate in the pre-anoxic zone include: concentration of readily biodegradable organic substrate in the influent wastewater, the MLSS concentration, and temperature (Tchobanoglous et al., 2014).

Post-anoxic denitrification

In post-anoxic denitrification process (Figure 1.2B), either externally added carbon (exogenous) or internally-stored (endogenous) organic substrates acts as an electron donor to reduce nitrate. Compared with the pre-anoxic systems, the denitrification rate is 3 to 6 times slower in the post-anoxic configurations (Tchobanoglous et al., 2014).

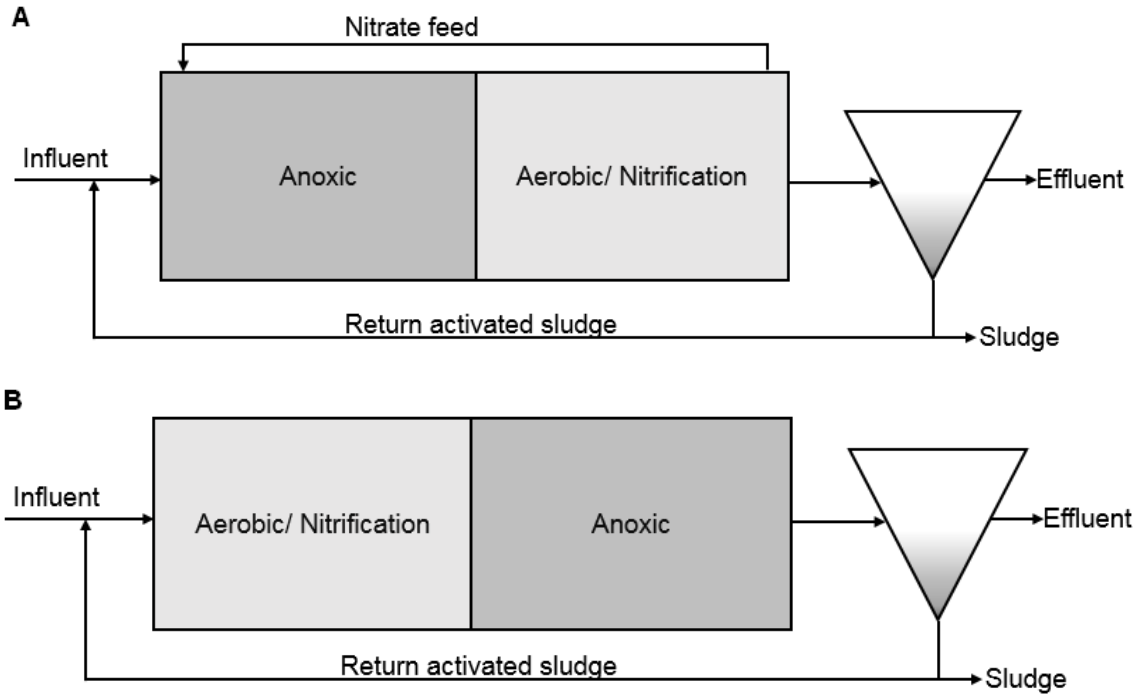

Figure 1.2: Types of denitrification processes and the reactors used for their implementation: (A) Pre-anoxic denitrification, and (B) Post-anoxic denitrification. Adapted from Tchobanoglous et al. (2014).

1.3.3 The Anammox process for nitrogen removal

In conventional wastewater treatment process, the removal of nitrogen which involves nitrification followed by denitrification can be limited due to insufficient organic substrate concentration that acts as an electron donor. For efficient nitrogen removal, the carbon to nitrogen ratio should be at least 7:1 (Pochana et al., 1999). However, different types of wastewater contain a high ammonium concentration and a very low carbon content, resulting in low C: N ratio. The treatment of wastewater with such low C: N ratio by the conventional process is uneconomical, due to the requirement for large amounts of carbon supplements (Third et al., 2005b). As an alternative to the costly addition of carbon substrate, anammox based technology such as CANON (Completely Autotrophic Nitrogen-removal Over Nitrite) process offers an opportunity for complete removal of ammonium in a single reactor (Sliekers et al., 2002; Third et al., 2005b; Third et al., 2001).

The CANON process is based on the conversion of approximately half of the ammonium in the wastewater to nitrite under oxygen-limiting condition. The ammonium oxidizing bacteria simultaneously uses the produced nitrite as the electron acceptor to oxidize ammonium (equation 1.7). The combination of aerobic and anaerobic ammonium-oxidizing activity results in about 87% nitrogen removal, using 63% less oxygen and 100% less organic carbon compared with conventional processes (Third et al., 2005b; Verstraete & Philips, 1998).

$$1NH_4^+ + 0.85O_2 \rightarrow 0.435N_2 + 0.13NO_3^- + 1.3H_2O + 1.4H^+ \quad (1.7)$$

The anammox process is more suitable for high-strength ammonium wastewater (e.g., industrial wastewater, sludge digester effluent, and groundwater with considerably high ammonium concentration and low amount of organic carbon) (Lackner et al., 2014; Li et al., 2017; Winkler et al., 2012). The relatively high C: N ratio limits its application in mainstream domestic wastewater treatment (Ali & Okabe, 2015). By removing organic carbon in a pre-treatment process, anammox system can be used in the mainstream wastewater treatment plant (Winkler et al., 2012), but requires further studies.

1.4 Non-biological Nitrogen removal

In addition to the biological processes described above (Section 1.3), nitrogen can also be removed from waste streams using a process called ion-exchange (a reversible chemical process). In wastewater treatment, ion-exchange is considered to be a simple and cost-effective technique for the removal of inorganic ions especially NH_4^+ (Wang & Peng, 2010). Natural zeolite is the most commonly used ion-exchange material in wastewater treatment plants due to its low cost. There are more than 50 different types of zeolite (e.g.,

clinoptilolite, mordenite, phillipsite, chabazite, etc.), but clinoptilolite is the most abundant natural zeolite and is widely used in the world. However, zeolite has a lower NH_4^+ adsorption capacity compared with chabazite (Wang & Peng, 2010)

1.4.1 Structural characteristics

Zeolite is mainly composed of aluminosilicates with a three-dimensional structure bearing AlO_4 and SiO_4 tetrahedra, where substitution of each aluminum (Al^{3+}) for silicon (Si^{4+}) provides a negative charge on the zeolite framework (Englert & Rubio, 2005). The negative charge within the pores is balanced by positively charged ions such as Na^+, K^+, Ca^{2+}, and Mg^{2+} which are bound with the aluminosilicate structure by weaker electrostatic bonds (Rožić et al., 2000). Thus, these cations can be easily exchanged with certain cations in solution such as ammonium ions (Rožić et al., 2000).

Zeolites are capable of exchanging ions with the external aqueous medium. When zeolites come in contact with wastewater, the cations such as Na^+ present in the structure is exchanged with other cations in solution, principally NH_4^+ resulting in the removal of NH_4^+ from wastewater. Once the zeolite structures are filled with NH_4^+, it is said to have reached its maximum cation-exchange capacity (CEC). The CEC of zeolite depends on its formation environment (Wang & Peng, 2010). For example, the cation-exchange capacity of Australian and Chinese clinoptilolite was found to be 16.8 and 28.7 mg NH_4^+-N g^{-1} of zeolite, respectively (Englert & Rubio, 2005; Wang & Zhu, 2006).

1.4.2 Use of zeolite in wastewater treatment

The removal of ammonium from wastewater using zeolite have been extensively explored in recent years (Wang & Peng, 2010; Widiastuti et al., 2011). However, the limited cation exchange capacity of zeolite limits its application in large-scale process. Once the zeolite is saturated with ammonium, it needs to be regenerated which is traditionally done by

using concentrated salt solutions. Nevertheless, waste brine produced from the regeneration of zeolite cause a disposal problem because of its high salt and total ammonia nitrogen content (Aponte-Morales et al., 2016). As an alternative to the chemical regeneration, microorganisms can oxidize the adsorbed ammonium onto zeolite, and in this way, they regenerate the zeolite (bioregeneration) (Semmens et al., 1977).

The combination of zeolite and biological process have been explored to remove nitrogen from wastewater. Zeolite amendment has been shown to effectively enhance the nitrification rate (He et al., 2007; Miazga-Rodriguez et al., 2012; Park et al., 2002), alleviate the effect of toxic shock load (He et al., 2007; Park et al., 2002), and promote biomass retention by forming bio-flocculated zeolite (Meng et al., 2014; Park et al., 2002) in conventional activated sludge systems. Although zeolite was efficiently regenerated, this ion-exchange material was not completely reused in that suspended growth system. A fraction of zeolite was lost during biomass wasting to control solid retention time. In order to compensate, a dose of zeolite was added after the feeding stage in every cycle (He et al., 2007; Jung et al., 2004; Jung et al., 1999) leading to additional operational cost for wastewater treatment systems.

Nitrogen removal by adding zeolite into biofilm was also evaluated by several researchers. Zeolite provides a surface for microbial growth as a biofilm (Bai et al., 2011; Guerrero et al., 2016) which has been reported to have little influence on the exchange property of this material (Park et al., 2002). In addition, zeolite has been found to promote the growth of AOB (Li et al., 2013) and repress NOB (Yang et al., 2017) resulting in better nitrogen removal efficiency. However, these processes generated nitrite/nitrate which required further treatment by adding organic carbon for subsequent denitrification.

1.5 Biological phosphorus removal

Removal of phosphorus from wastewater is done to avoid eutrophication since phosphorus is also a limiting nutrient is most aquatic systems. The location of the wastewater treatment plant and the potential impact on the receiving water bodies determine the discharge permit limits for phosphorus removal which have ranged from 0.05 to 1.0 mg L^{-1} of phosphorus (Foley et al., 2010; Tchobanoglous et al., 2014). Phosphorus can be removed from wastewater either by chemical or biological process or a combination of both.

Biological phosphorus removal involves the incorporation of phosphorus (P) in the biomass produced in the treatment process followed by removal of the biomass in sludge wasting. This process is very inefficient since only 10-20 % phosphorus removal can be accomplished by this method. However, a modified process configuration has been found to remove more than 80% phosphorus from wastewater, and the process is known as enhanced biological phosphorus removal (EBPR) (Mino et al., 1998; van Loosdrecht et al., 1997b). This process has several advantages over chemical phosphorus removal such as reduced sludge production, lower chemical costs, and potential for recovery of phosphorus (Tchobanoglous et al., 2014).

A group of bacteria commonly called polyphosphate accumulating organisms (PAOs) are principally involved in the EBPR process (van Loosdrecht et al., 1997b). These bacteria uptake and store phosphate intracellularly as a polyphosphate, resulting in the removal of P from the wastewater (Figure 1.3). Unlike ordinary heterotrophic bacteria, PAOs can anaerobically (feast phase) take up organic carbon (e.g., volatile fatty acids) and intracellularly accumulate them as PHA. The ATP for these bioconversions is mainly generated by the oxidation of stored polyphosphate and release of phosphate into wastewater. The

formation of PHA also required reducing power which is produced through the glycolysis of internally stored glycogen (Mino et al., 1998). In the subsequent aerobic (famine) condition, PAOs used their stored PHA as the source of energy for biomass growth, glycogen replenishment, phosphate uptake and subsequent storage (Figure 1.3) (Oehmen et al., 2007).

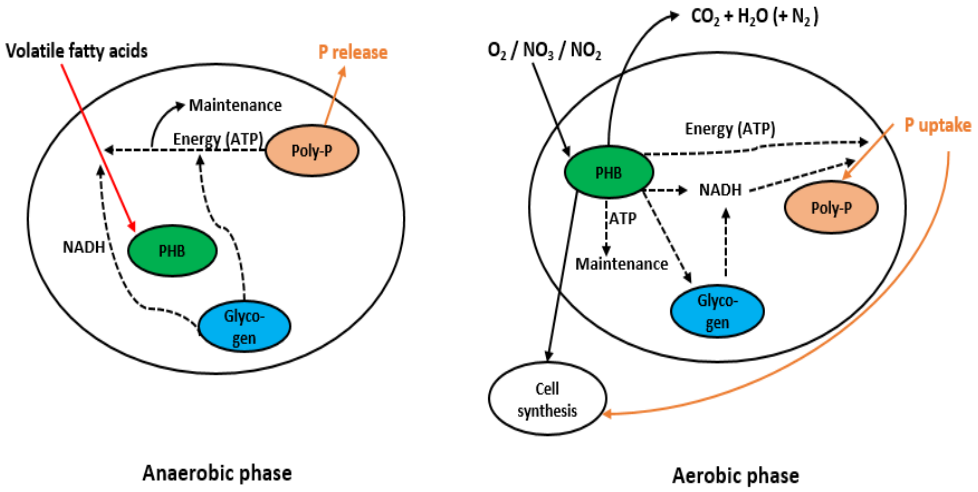

Figure 1.3: Metabolic processes of the polyphosphate accumulating organism under anaerobic and aerobic (anoxic) conditions. Adapted from van Loosdrecht et al. (1997b).

The oscillating conditions of the EBPR system are also known to be favorable for the growth of another type of bacteria called glycogen accumulating organisms (GAOs) (Mino et al., 1995). During anaerobic condition, GAOs take up organics and convert them as PHA with the expense the glycogen which acts as energy and reducing power. Under aerobic stage, they oxidize PHA for the replenishment of glycogen and growth of biomass (Liu et al., 1996). Since the metabolism of GAOs does not involve phosphorus, they do not contribute to phosphorus removal from wastewater (Wang et al., 2008).

Two main groups of GAOs have been described so far (Albertsen et al., 2016). One group belongs to the *Gammaproteobacteria* which were named as "*Candidatus Competibacter phosphatis*" (*Competibacter*) (Crocetti et al., 2002). This group of bacteria forms tetrads, which are large cells in groupings of four. The other putative GAOs are *Defluvicoccus vanus* which belong to the class *Alphaproteobacateria* (Wong et al., 2004). In EBPR wastewater treatment plants, *Competibacter* is the most frequently found GAOs. All of the GAOs identified so far can use nitrate as an electron acceptor in addition to oxygen, but only *Competibacter* Type I can use nitrite as well (Nielsen et al., 2010).

1.6 Biofilm wastewater treatment technologies

In conventional activated sludge process, a number of reactors are needed for complete removal of organic carbon and nutrients (nitrogen, phosphorus). The necessity of several tanks and particularly the need for settling tank (Figure 1.1 and 1.2), increases the foot-print of wastewater treatment plant. This is the reason why the biofilm-based wastewater treatment systems were developed over the years to deal with the space limitation without compromising the effluent quality. Compared with the suspended-growth activated sludge system, biofilm technologies offer numerous advantages such as lower space requirement, high biomass content, operational simplicity, increased energy efficiency, low sludge yield, better recovery from toxic shock loadings, etc. (O'Reilly et al., 2011; Rodgers & Zhan, 2003; Tchobanoglous et al., 2014).

Biofilm based wastewater treatment processes are mainly of two types: (i) fixed bed systems such as trickling filter (TF), membrane aerated biofilm reactor (MABR); and (ii) moving bed systems such as rotating biological contactors (RBCs), moving bed biofilm reactors (MBBRs), fluidised bed reactors (FBRs). A brief explanation of few biofilms

based wastewater treatment process (such as SBBR, TF, and RBC) is described as follows.

1.6.1 Sequencing batch biofilm reactor

The sequencing batch biofilm reactor (SBBR) is a combination of biofilm technology with an activated sludge process. In SBBR, either a fixed or moving carrier material provides the support for biomass growth and development. The large surface area and longer sludge age in SBBR favors the proliferation of slow-growing microorganisms such as nitrifying bacteria (Bassin et al., 2012b) and anammox (Tsushima et al., 2007) bacteria. Moreover, SBBR enhances the accumulation of high biomass content leading to short hydraulic retention time (HRT). The short HRT can result in smaller reactor size, but greater treatment capacity at the same reactor size.

SBBR system has been reported to be a suitable technology for pollutants removal from wastewater. In biofilms, oxygen diffusion limitation results in oxygen concentration gradient which leads to the formation of anoxic micro-zones in the inner parts of biofilms (Ma et al., 2017). The presence of both oxic and anoxic zones in the same biofilm structure promotes nitrogen removal by a process called simultaneous nitrification and denitrification (SND) (Ma et al., 2017; Rahimi et al., 2011). Compared with the conventional two-stage nitrogen removal systems (Figure 1.2), this process requires significantly lower construction cost and energy consumption due to simple processing units (Deng & Ezyske, 2011). Moreover, SND in the biofilm system helps to remove phosphorus (Rahimi et al., 2011) which is driven by intracellularly stored organic substrates (i.e., PHA).

1.6.2 Trickling filter (TF)

Trickling filter (a three-phase system) is the oldest form of biofilm reactors that have been used for more than 60 years (Henze et al., 2008; Naz et al., 2015). In TF systems, large rocks (5-20 cm) or plastic carrier material is used to provide support for the growth of microorganisms as biofilm. Influent wastewater enters the reactor through a distribution zone, and then trickles downward over the biofilm surface. The large support material provides adequate pore spaces to allow air to ventilate and avoids clogging of the filter medium as well (Henze et al., 2008). The partially treated wastewater (effluent) exits from the bottom of the filter and recirculated to ensure suitable hydraulic loading of the trickling filter. The treatment of wastewater in TF results in the generation of suspended solids which is separated from the liquid effluent in a circular or rectangular secondary settling tank (clarifier) (Daigger & Boltz, 2011).

Conventional trickling filters have a moderate (70 to 85%) carbon removal efficiency with very poor or no nitrogen removal performance. Since trickling filters are constantly aerobic; it completely oxidizes soluble organic carbon to CO_2 and ammonium to nitrite and nitrate. Therefore, the denitrification process cannot occur due to lack of electron donor and lead to accumulation of nitrate. As a consequence, TFs are designed for specific purposes, where complete soluble carbon and nitrogen removal is not required. For example, TFs can be used as a pre-treatment step for rapid removal of organics from high-strength wastewater. The effluent can be further treated in the subsequent activated sludge process (Daigger & Boltz, 2011).

The TF can also be used as a post-treatment option. Almeida et al. (2013) integrated a sponge-based trickling filter next to an up-flow anaerobic sludge blanket (UASB) reactor and reported about 97% BOD and 80-95% nitrogen removal. The use of different types

of carrier material could also enhance the nitrifying bacteria which would result in better nitrogen removal efficiency. For example, Sánchez Guillén et al. (2015) used sponge as a carrier to immobilize anammox bacteria which resulted in 80% nitrogen removal with a very short HRT (1 h).

Compared with the activated sludge process, trickling filter has several advantages such as lower energy consumption, relatively shorter treatment time, better sludge thickening properties, less equipment maintenance, and higher efficiency in removing the microbial loads (Daigger & Boltz, 2011; Lamba & Ahammad, 2017; Naz et al., 2015; Tchobanoglous et al., 2014). However, trickling filter has large treatment plant footprint which makes the process unattractive, especially in urban areas where land availability is often limited.

1.6.3 Rotating biological contactors (RBC)

The rotating biological contactor (RBC) is an attached growth bioreactor which consists of multiple circular discs that are mounted on a common horizontal shaft (Singh & Mittal, 2012). The discs are partially submerged (usually 40%) in a tank through which wastewater flows (Cortez et al., 2008) and the shaft is continuously rotated (1-2 rpm) by a mechanical motor or air drive. The rotation facilitates the mixing of bulk liquid, substrate diffusion to the film and subsequent product exchange with the reactor and surroundings (Rittmann & McCarty, 2012).

The RBC disc (usually made of plastic) provides the support for the growth of microorganism as microbial films (biofilm). The biofilm which contains components of active/inactive biomass, biofilm extracellular matrix, and debris (Hassard et al., 2015), is responsible for the degradation of a wide range of pollutants present in the wastewater (Singh et al., 2006). As the biofilm attached to the disk is exposed to the atmospheric air, diffusion

of oxygen into the biofilm takes place. In contrast, when the biofilm is immersed into wastewater, the soluble organic compounds and nutrients diffuse into the biofilm, and the microorganisms metabolize these pollutants for their growth and respiration (Singh & Mittal, 2012). Excess sludge is removed by shear as the disks rotate. A clarifier is often placed after the RBC to remove the detached solids.

There are two main types of RBC: integral and modular (Hassard et al., 2015) (Figure 1.4). Integral units have a treatment capacity of ≤ 250 population equivalents (PE) and usually consists of a single unit combining primary settler, RBC tank and either a contained or separate final clarifier (Hassard et al., 2015). Modular systems (usually >1000 PE treatment capacity) have separate reactors for primary, secondary and solids treatment (Griffin & Findlay, 2000), and allows flexible process operation. Modular RBCs can be operated using parallel flow separation between units allowing operation within acceptable loading limits (Hassard et al., 2015). On the other hand, the operation of several units in series enhances the process performance (Hassard et al., 2015). When multiple RBCs are available, the initial units receive the highest concentration of organic substrate that stimulates the development of heterotrophic microorganisms. As the concentration of organic matter decreases in the distal stages of the configuration, autotrophic nitrifiers start to colonize the biofilm. Therefore, RBC system can undertake both organic carbon and nitrogen removal from domestic and high-strength wastewater (Hiras et al., 2004).

Low energy requirements (50% and 35% lower than that of activated sludge and trickling filters, respectively), small land footprint, resistance to toxic shock loadings, removal of pollutants, high volumetric removal, solids retention and no need for sludge recycle are some advantages of RBC systems (Hassard et al., 2015; Hiras et al., 2004; Najafpour et

al., 2006; Singh & Mittal, 2012). However, mechanical problems frequently occur in the rotating system, which requires frequent maintenance (Mba et al., 1999).

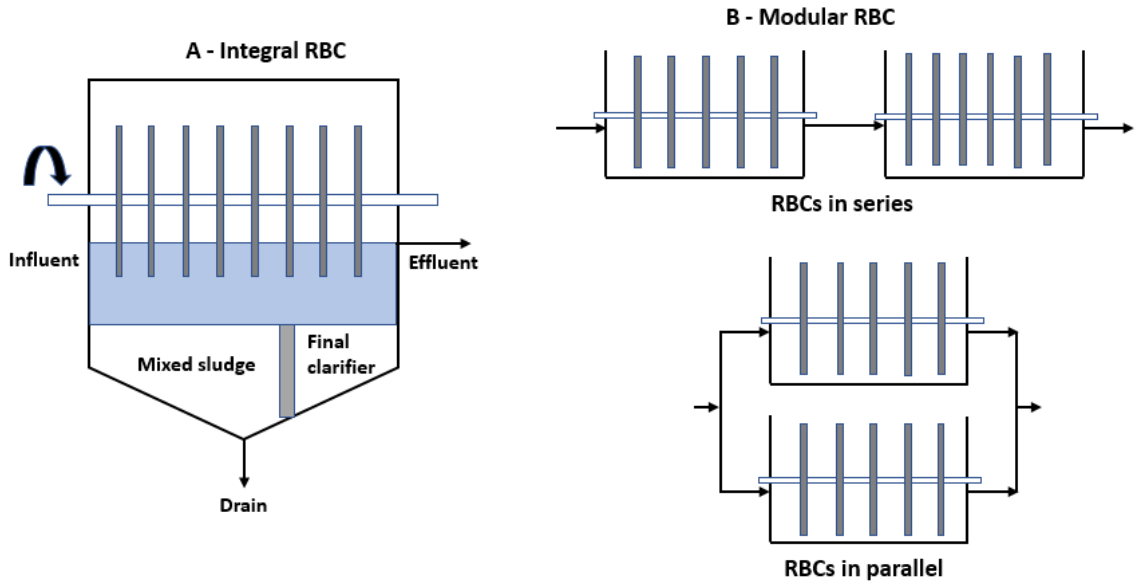

Figure 1.4: Process configurations of RBC technology. Adapted from Hassard et al. (2015).

1.7 Background of the thesis

1.7.1 High energy requirements of wastewater treatment process

Conventional wastewater treatment plants (WWTPs) principally rely on activated sludge (AS) process to remove organic compounds and nutrients from waste streams. However, activated sludge is an energy-intensive process in which air (oxygen) is actively transported from the gas-phase (atmosphere) into the liquid-phase (wastewater) to maintain the wastewater aerobic and the activated sludge in suspension. It has been estimated that about 8 m^3 of air is needed to treat 1 m^3 of wastewater (Tchobanoglous et al., 2014). Such active aeration consumes a significant amount of energy (about 0.6 kWh m^{-3} of wastewater treated) (McCarty et al., 2011), which makes the aeration process the highest energy-consuming component in conventional wastewater treatment plants (Figure 1.5). In most WWTPs based on activated sludge process, active aeration leads to about 40-

60% of the total operational cost of the entire wastewater treatment process (Foley et al., 2010; Tchobanoglous et al., 2014).

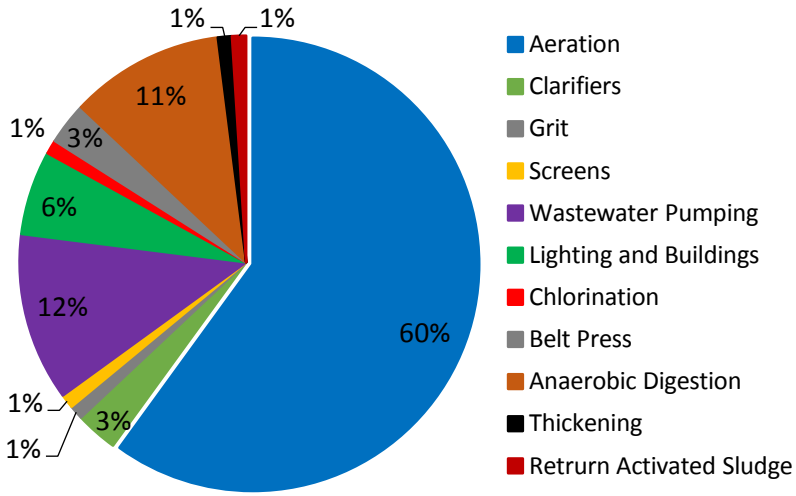

Figure 1.5: The proportion of energy use associated with different components in wastewater treatment system. Adapted from Gu et al. (2017).

The energy demand of aeration principally depends on the mass transfer of air into the water. The energy required for mass transfer is directly proportional to the height of the aeration basin, and therefore, the depth of the wastewater. However, microorganisms present in wastewater only have access to a very slight proportion of oxygen in the transferred air due to limited oxygen transfer efficiency (OTE). In case of diffused aeration, only about 30% (Table 1.1) oxygen is transferred to the liquid phase, with the rest evolving as bubbles (Tchobanoglous et al., 2014). Such inevitable loss represents a waste of energy in the wastewater treatment process. Therefore, it is highly desirable to develop a wastewater treatment technology with improved oxygen transfer efficiency which could decrease the overall energy consumption. A reduction in the energy used will also assist in the reduction of the carbon footprint of the wastewater treatment plant.

Table 1.1: Standard oxygen transfer efficiency (OTE) and oxygenation efficiency (OE) in activated sludge process.

Bubble size	Bubble diameter (mm)	Average standard OTE (%)	Standard OE (Kg O_2 / kWh)
Fine	< 3	10 – 30	1.2 – 2.0
Medium	3 – 6	6 – 15	1.0 – 1.6
Coarse	> 6	4 - 8	0.6 – 1.2

Adapted from von Sperling and de Lemos Chernicharo (2005).

1.7.2 Oxidation of stored PHA by direct oxygen transfer from air to GAO biofilm

In a conventional wastewater treatment plant, up to 60% of the energy is devoted to aeration, usually by mechanical or diffused air devices (Tchobanoglous et al., 2014). However, in diffused aeration, only 4-30% of oxygen supplied by blowers is transferred to the liquid phase due to the limited oxygen transfer efficiency (Table 1.1). Therefore, continuous research is undergoing around the world to find alternative solutions where oxygen is efficiently transferred to the microorganisms to reduce the aeration cost.

Passive aeration where oxygen is provided directly to the biomass could be a suitable treatment option. Recently, Flavigny and Cord-Ruwisch (2015) reported a passively aerated biofilm system to remove organic carbon from wastewater. The process involved a biofilm enriched with glycogen accumulating organism (GAO) which can uptake readily biodegradable organics (BOD) and store as poly-hydroxyalkanoate (PHA) under anaerobic condition. Since oxygen was provided by direct exposure of the biofilm to atmospheric air (to regenerate the biofilms PHA storage capacity), the cost associated with aeration energy was calculated to be substantially reduced. However, the study used cultures that were adapted for years in the laboratory and provided was no indication about the likelihood of GAO biofilms to develop from activated sludge and about the duration of establishing such biofilm from standard activated sludge. This impedes plant operators

intending to make use of this technology. Therefore, one of the goals of the current research was to investigate the feasibility and time required to establish a biofilm enriched with the glycogen accumulating organism from activated sludge.

1.7.3 Nitrogen removal via simultaneous nitrification and denitrification (SND) by a zeolite amended biofilm with direct oxygen uptake from air

The above-described long-term operated GAO biofilm promised substantial energy savings for BOD removal by avoiding oxygen transfer from air to the bulk wastewater solution. By incorporating zeolite (an ion-exchange material) onto the above mentioned GAO biofilm, as well as concentrated highly enriched nitrifying bacteria, this synthetic laboratory culture could demonstrate the effective removal of nitrogen via SND (Flavigny, 2015). However, in large-scale wastewater treatment plant, it is not likely to selectively enrich different types of biomass separately and then to integrate these specialized biomasses together with zeolite into one structure. This gap might limit the application of this novel technology in the real-world. The current research, hence, aimed at developing a biofilm system (by addition of zeolite) from purely activated sludge to test its real-world feasibility.

Thesis objectives

The specific objectives of this thesis are:

- Investigate the feasibility of developing a GAO biofilm from standard activated sludge using sequences of anaerobic flooding followed by direct exposure of the biofilm to atmospheric air (Chapter 2).
- Quantify and explore the underlying reasons for reduced excess sludge production in the GAO biofilm system (Chapter 3).
- Develop a GAO biofilm-based nitrogen removal system using activated sludge as the sole start-up inoculum and to test its real-world practicality (Chapter 4, 5).
- Explore the possibility of treating highly concentrated waste streams (that usually cannot be done by traditional treatment processes because of high costs) by the developed biofilm technology (Chapter 6).
- Optimize the system's nitrogen removal efficiency and treatment time (Chapter 7).

All the chapters of this thesis are presented in the form of published (chapter 2 & 3) and draft (chapter 5, 6 & 7) manuscripts which may be modified later for publication.

Chapter 2

Rapid Adaptation of Activated Sludge Bacteria Into a Glycogen Accumulating Biofilm Enabling Anaerobic BOD Uptake[1]

Abstract

Glycogen accumulating organisms (GAO) are known to allow anaerobic uptake of biological oxygen demand (BOD) in activated sludge wastewater treatment systems. In this study, we report a rapid transition of suspended activated sludge biomass to a GAO dominated biofilm by selective enrichment using sequences of anaerobic loading followed by aerobic exposure of the biofilm to air. The study showed that within eight weeks, a fully operational, GAO dominated biofilm had developed, enabling complete anaerobic BOD uptake at a rate of 256 mg L^{-1} h^{-1}. The oxygen uptake by the biofilm directly from the atmosphere had been calculated to provide significant energy savings. This study suggests that wastewater treatment plant operators can convert activated sludge systems readily into a "passive aeration" biofilm that avoids costly oxygen transfer to bulk wastewater solution. The described energy efficient BOD removal system provides an opportunity to be coupled with novel nitrogen removal processes such as anammox.

[1] This chapter is published in Bioresource Technology (2017) 228: 1-8

2.1 Introduction

Conventional suspended growth activated sludge process, the core part of sewage treatment technology, have been extensively used for wastewater treatment since its introduction 100 years ago and have contributed greatly to our society in terms of environment protection and public health benefits (van Loosdrecht & Brdjanovic, 2014). Activated sludge is a mixture of inactive sewage solids combined with microbial populations, which facilitates the degradation and conversion of pollutants in wastewater treatment plants. The current activated sludge paradigm for wastewater treatment is characterized by relatively high-energy consumption and waste biomass production, which leads to high operational cost (Foley et al., 2010; Tchobanoglous et al., 2014). To overcome these problems, extensive research was undertaken that led to the development of new technologies, which have shown promise to treat wastewater more efficiently.

The sequencing batch reactor (SBR), a modification of the activated sludge process, where all nutrients (nitrogen, phosphorus, and organic carbon) are removed in a single reactor, have gained a great deal of attention due to their improved nutrient removal capacity. This process uses discrete phases regarding nutrient availability (e.g., feast/famine regime with respect to carbon source). Microbial populations normally exposed to this feast/famine condition results in the accumulation of large fraction of the soluble substrate, when available, as internal storage polymers such as poly-β-hydroxyalkanoates (PHAs) (Ciggin et al., 2013; Van Loosdrecht et al., 1997a). The storage polymers act as an electron donor for respiration if electron acceptors (such as oxygen or nitrite) become available. This principle is used in "storage driven denitrification" process such as simultaneous nitrification and denitrification (SND) where heterotrophic bacteria rapidly store soluble substrate as storage polymer (PHAs) that degrades slowly to provide the

reducing power for the process to remove nitrogen from wastewater (Krasnits et al., 2013; Third et al., 2003a).

In much the same way as PHA build-up by the bacterial biomass is advantageous for nitrogen removal, it is also critically involved in biological phosphorus removal as in enhanced biological phosphorus removal (EBPR) by poly-phosphate accumulating organisms (PAOs) (Oehmen et al., 2007). Polyphosphate accumulating organisms can take up organic BOD (e.g. in the form of acetate) and intracellularly store them as PHAs under anaerobic (feast) conditions (Mino et al., 1998). Energy for this biotransformation is generated by the cleavage of intracellular polyphosphate (poly-P) which they previously accumulated during the famine (aerobic) period (Mino et al., 1998), thus removing phosphorus from wastewater.

The dynamic feast-famine (anaerobic-aerobic) regime used in EBPR is also known to favor development of a different phenotypic group of bacteria called glycogen accumulating organisms (GAOs) (Liu et al., 1996; Satoh et al., 1992). Like PAO, these organisms are also able to store volatile fatty acids (VFA) as PHA anaerobically which they use in the subsequent aerobic phase as carbon and energy source. The energy and reducing power required for the anaerobic storage of PHA is provided by the hydrolysis of intracellularly stored glycogen. In aerobic conditions, PHA is oxidized for glycogen replenishment, biomass growth, and aerobic maintenance purposes. Since GAO competes with PAO for anaerobic uptake of VFA without contributing to the phosphorus removal process, they are considered undesirable and a major cause of EBPR failure (Kong et al., 2006; Zhou et al., 2008b). However, conventional EBPR processes generate excess sludge which increases the sludge disposal cost. To mitigate this problem, new technology such as biofilm based processes have been researched and developed.

In recent years, different forms of biofilm-based technology have been used around the world to remove nutrients and pollutants from wastewater. However, biofilm reactors have not been reported much in literature to be capable of developing bacteria that store biological oxygen demand (BOD) as PHA. Hughes et al. (2006) reported that storage driven biofilm reactor could be used to remove nitrogen from waste streams with high nitrogen relative to carbon effectively and efficiently. Moreover, in a recent report, Flavigny and Cord-Ruwisch (2015) described a biofilm reactor enriched in glycogen accumulating organisms that had been operated at very high biomass densities (50 g L^{-1}) for several years under alternating anaerobic/aerobic conditions. The biofilm was able to take up BOD anaerobically. After the biomass had removed the BOD and the treated, largely BOD (acetate) free synthetic wastewater was drained; the biomass could regenerate its biological storage capacity by oxidizing the stored PHA using oxygen directly from the atmosphere. However, it is not known how long it would take to develop such a biofilm from standard activated sludge and how effectively anaerobic biofilm BOD uptake will work. This is an impediment for plant operators intending to make use this technology.

The aim of the current chapter is to describe the transition from activated sludge to GAO dominated biofilm by using selective conditions. The significance of the study is that the results give operators of wastewater treatment plants and design engineers a time estimate for the conversion of a traditional activated sludge biomass to a GAO dominated biofilm reactor that enables low-cost BOD removal via passive aeration.

2.2 Materials and Methods

2.2.1 Experimental setup and operation

Two reactors were constructed and operated in parallel; a sequencing batch biofilm reactor (test reactor) and a trickling filter reactor (control reactor) (Figure 2.1). The sequencing batch biofilm reactor (SBBR) (4 cm diameter and 23 cm height) with a working volume of 0.255 L was equipped with dissolved oxygen (DO), pH and oxidation-reduction potential (ORP) probes. The reactor was completely automated; with all pumps, airflow valves and phase lengths controlled by National Instruments Instrumentation Control Software LabVIEW^{TM} (version 9.1). The trickling filter reactor (TFR) (dimension and working volume as of the sequencing batch biofilm reactor) was set up with a recycle vessel. Both reactors were filled with packing material (AMB^{TM} Biomedia Bioballs), whose specific surface area for biofilm growth and support is 500 m^2/m^3. These carrier materials have a cylindrical shape with 7 mm height and 11 mm diameter. The volume occupied by the empty carrier material was 20% ($V_{carrier}$ /$V_{reactor}$).

Prior to operation, described reactors were inoculated with activated sludge from local wastewater treatment plant (Subiaco, Western Australia). After seeding, the sequencing batch biofilm reactor was operated automatically by specifically timed phases. The reactor was filled with synthetic wastewater (within 5 min through a peristaltic pump), then maintained under anaerobic condition for about 2 hours, followed by gravity drainage (10 min) and exposure of the biofilm directly to air, which was recirculated within the reactor for 1 hour. In contrast, the control reactor was operated in trickling reactor mode at all time where feed (synthetic wastewater) was trickled by recycling over the carrier material. Both the reactors were operated at room temperature (25ºC).

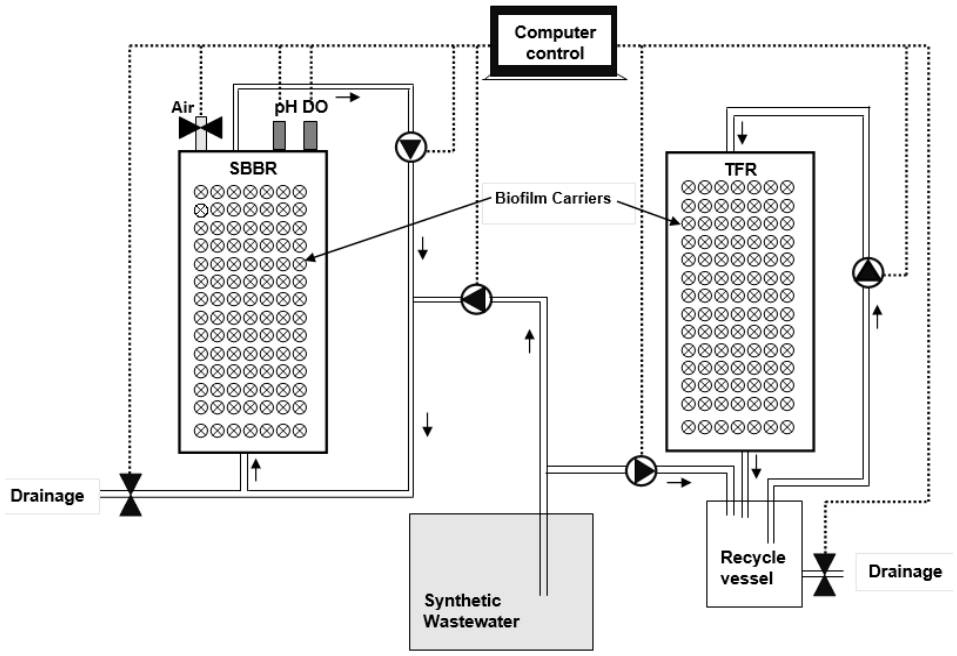

Figure 2.1: Diagrammatic representation of the experimental setup. The biofilm in the SBBR was alternately exposed to synthetic wastewater to facilitate BOD uptake (under anaerobic conditions) and to atmospheric air to regenerate biofilm's storage capacity. In TFR, synthetic wastewater was trickled by recycling over the carrier material containing the biofilm (SBBR = sequencing batch biofilm reactor, TFR = trickling filter reactor).

2.2.2 Synthetic wastewater

Synthetic wastewater was used throughout the experimental period. The standard composition of the synthetic wastewater was (mg L^{-1}): CH_3COONa 660, NH_4Cl 160, KH_2PO_4 44, $NaHCO_3$ 125, $MgSO_4$. $7H_2O$ 25, $CaCl_2$. $2H_2O$ 300, $FeSO_4$. $7H_2O$ 6.25, yeast extract 50, and 1.25 ml L^{-1} of trace element solution, which contained (g L^{-1}): EDTA 15, $ZnSO_4$. $5H_2O$ 0.43, $CoCl_2$. $6H_2O$ 0.24, $MnCl_2$. $4H_2O$ 0.99, $CuSO_4$. $5H_2O$ 0.25, $NaMoO_4$. $2H_2O$ 0.22, $NiCl_2$. $6H_2O$ 0.19, $NaSeO_4$. $10H_2O$ 0.21, H_3BO_4 0.014 and $NaWO_4$. $2H_2O$ 0.050 (Third et al., 2003b).

2.2.3 Histochemical staining

The ability of biofilm material of both reactors to accumulate PHA was determined using chemical staining with Sudan Black B (Jenkins et al., 2004). Smears of biofilm materials

deposited on a glass slide were stained with a 0.3% (w/v in 60% ethanol) Sudan Black B solution for 10 minutes and rinsed with water for 1 second. Slides were then counter-stained for 10 seconds with 0.5% safranin (w/v in deionized water), rinsed well with water and blotted dry. An Olympus BX51 microscope equipped with a charge-couple device (CCD) camera (PanasonicWV-CL830) was used for the observation of the biomass.

2.2.4 Analytical procedures

2.2.4.1 Chemical analysis

Total suspended solids (TSS), volatile suspended solids (VSS) and orthophosphate analysis were carried out according to Standard Methods (APHA, 2012). Five representative plastic carriers were taken from each reactor, and the biomass was detached from them for determination of the TSS and VSS. The total amount of TSS and VSS was calculated on the basis of total number of plastic carriers in the bioreactors.

2.2.4.2 Acetate analysis

Acetate was analysed using an Agilent 7820A gas chromatography (GC) with auto-sampler. Samples were acidified with formic acid (10% v/v) before 0.4 µL samples were injected onto an Altech Econo-Cap™ EC™-1000 column (30 m length × 0.250 mm internal diameter × 0.25 µm film thickness). The carrier gas (N_2) was set at a flow rate of 3 mL min^{-1} and the sample was split 10:1 at the inlet. The oven temperature was programmed as follows: initial temperature 70°C, increased at 5°C min^{-1} to 100°C, held for 2.0 min, increased at 70°C min^{-1} to 250°C, held for 2.0 min. Injector and detector were set at 250 and 300°C respectively. The peak area of the Flame Ionization Detector (FID) output signal was computed via integration using the EzChrome Elite Compact Software© (V.3.3.2 SP2). The detection limit determined as 0.5 µmol L^{-1} of acetate.

2.2.4.3 Poly-β-hydroxyalkanoate (PHA) analysis

Poly-β-hydroxyalkanoate (PHA) including poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV), was measured according to a method adapted from Smolders et al. (1994b). Briefly, approximately 20 mg freeze-dried samples of biomass were put into screw-topped glass tubes, and 1.45 mL of a mixture of 1-propanol and concentrated HCl (4:1), 1.5 mL dichloromethane and 50 μL benzoic acid solution as internal standard (2 g benzoic acid dissolved in 100 mL of 1-propanol) were subsequently added. The tubes were sealed with Teflon lids to prevent loss of volatile solvents. The samples were then digested for 4 hours at 100°C. After cooling, the organic phase was extracted with 3 mL distilled water; 1 mL of the organic phase was dried over Na_2SO_4 and transferred to the GC vials for analysis. 4.5μL of the sample was injected into an Agilent 7820A gas chromatograph (Agilent, USA) equipped with a FID detector and an Altech Econo-Cap™ EC™-1000 column (30 m length × 0.250 mm internal diameter × 0.25 μm film thickness). Nitrogen was used as a carrier gas (3 mL min^{-1}), and the sample was split 1:5 at the inlet. The temperature of injection was 250°C, the temperature of Flame Ionisation Detector (FID) was 300°C, and the temperature ramp of the column started at 80°C, then increased at a rate of 70°C min^{-1} until 152°C, further increased at a rate of 4°C min^{-1} until 160°C, and finally increased again at 70°C min^{-1} until 230°C and held for 2 min, to ensure a cleaning of the column after each injection.

2.2.4.4 Glycogen analysis

Biomass glycogen was analyzed as glucose after acidic hydrolysis, according to the method used by Wang et al. (2015c). Approximately 1-2 mg freeze-dried biomass was weighed into air-tight Pyrex tubes, to which 5 mL of 0.6 M HCl was added and heated at 100°C for 3 h. After cooling to room temperature, samples were sheared by a vortex mixer

for 1 min, and transferred to 10-mL tubes, followed by centrifugation at 2600g for 10 min. About 1 mL supernatant was added to 4 mL of anthrone-H_2SO_4 reagent (0.2% anthrone (w/v) in 80% (v/v) H_2SO_4) in 10-mL colorimetric tubes. All tubes were placed in a water bath at 100 °C for 10 min. After cooling at 4°C for 5 min in cold water, samples were measured by a UV/VIS spectrophotometer (UVmini-1240, Shimadzu, Japan) at 625 nm. Glucose was used as the standard.

Three individual replicates of all experiments were performed. All the data were subjected to analysis of variance (ANOVA) using PAST software (Version 3.14). Statistical significance was tested using the least significant difference (LSD) at the $p<0.05$ level.

2.2.5 Microbial community structure analysis

Biofilm was scraped off the carriers, and genomic DNA was extracted using the Power Soil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instruction and using DNA-free reagents and consumables. A mock-extraction was also carried out, in parallel, using the same reagents and consumables, but no biofilm (extraction blank). The V_4 hypervariable region of the 16S rRNA gene was amplified with the modified version (Apprill et al., 2015) of 515F – 806R primers (Caporaso et al., 2012). Briefly, for each sample, polymerase chain reaction was carried out in a 25 µL total volume including 2.5 µL of normalized total genomic DNA (5 ng/µL), 0.2 µM of each primer and 12.5 µL of 2x KAPA HiFi HotStart Ready Mix (Kappa Biosystems, USA). The PCR cycling protocol consisted of an initial denaturation step of 95°C for 3 min, followed by 35 cycles of DNA denaturation at 95°C for 30s, primer annealing at 55°C for 30s, strand elongation at 72°C for 30s, and a final elongation step at 72°C for 5 min. Extraction blanks and no-template control were always included in all PCR amplifications. For each sample or control, the PCR products from the three replicates were then pooled, checked by gel

electrophoresis and purified using AMPure XP beads (Beckman Coulter, USA). After quantification with the Qubit dsDNA Assay Kit (Thermo Fisher Scientific, USA), amplicons were pooled at equimolar ratios, prior to index PCR using Nextera XT Index Kit V_2-V_5 indexes (Illumina, USA). Products were purified again using AMPure XP beads (Beckman Coulter, USA), quantified with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA) and pooled at the approximately equimolar ratio. The pool was then further concentrated and purified by a QIAquick PCR Purification Kit (Qiagen, USA) and quantified by Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA), prior to dilution to 4 nM and paired-end sequencing (2 x 250 bp; 500 cycle V2) on the MiSeq platform (Illumina, USA).

Sequences were first processed in Geneious 8.0.4 (Kearse et al., 2012). Sequences were then quality filtered using USERARCH (Edgar, 2010), allowing only reads with a <1% error rate to remain and singletons were removed. To identify bacterial genera present in samples, operational taxonomic units (OTUs) were selected by clustering sequencing at 97% similarity with the UPARSE algorithm (Edgar, 2013) and filtered by UCHIME to ensure OTUs were not the result of chimeric reads. Genus level taxonomy was assigned to OTUs against the Greengenes 16S database (August 2013 release) (DeSantis et al., 2006) in QIIME 1.8.0 (Caporaso et al., 2010) using the UCLUST algorithm (Edgar, 2010) with default parameters. Bacterial genera that were identified in extraction reagent blanks and no-template control were removed from the dataset to eliminate background bacterial sequences. Some sequences were manually cross-checked using the National Centre for Biotechnological Information nr collection of databases with the Basic Local Alignment Search Tool (BLAST) for nucleotides, including some sequences that were not identified at the genus level by QIIME.

2.3 Results and Discussion

The sequencing batch biofilm reactor (SBBR) and trickling filter reactor (TFR) were operated continuously under standard conditions by feeding synthetic wastewater for eight weeks after inoculation with activated sludge biomass. As expected, biofilms developed in both reactors over time. In order to quantify to what extent, the alternating exposure of biofilm to anaerobic submerged conditions and to air (after draining) develops specialized storage bacteria such as the GAO in sequencing batch biofilm reactor, a number of parameters were studied that are indicative of the existence and predominance of GAO bacteria in the biomass. Amongst these parameters are anaerobic acetate storage capacity, microscopic observation, intracellular glycogen and PHA levels and microbial community structure analysis.

2.3.1 Development of anaerobic acetate storage capacity

A key indicator for a PHA accumulating organism-rich biofilm is its ability to take up acetate in the absence of oxygen or other electron acceptors such as nitrate. Such acetate uptake can only be explained by storage if methanogenesis is excluded. To test for such anaerobic acetate uptake and storage, both biofilms were submerged with synthetic wastewater and the decrease in acetate monitored over time.

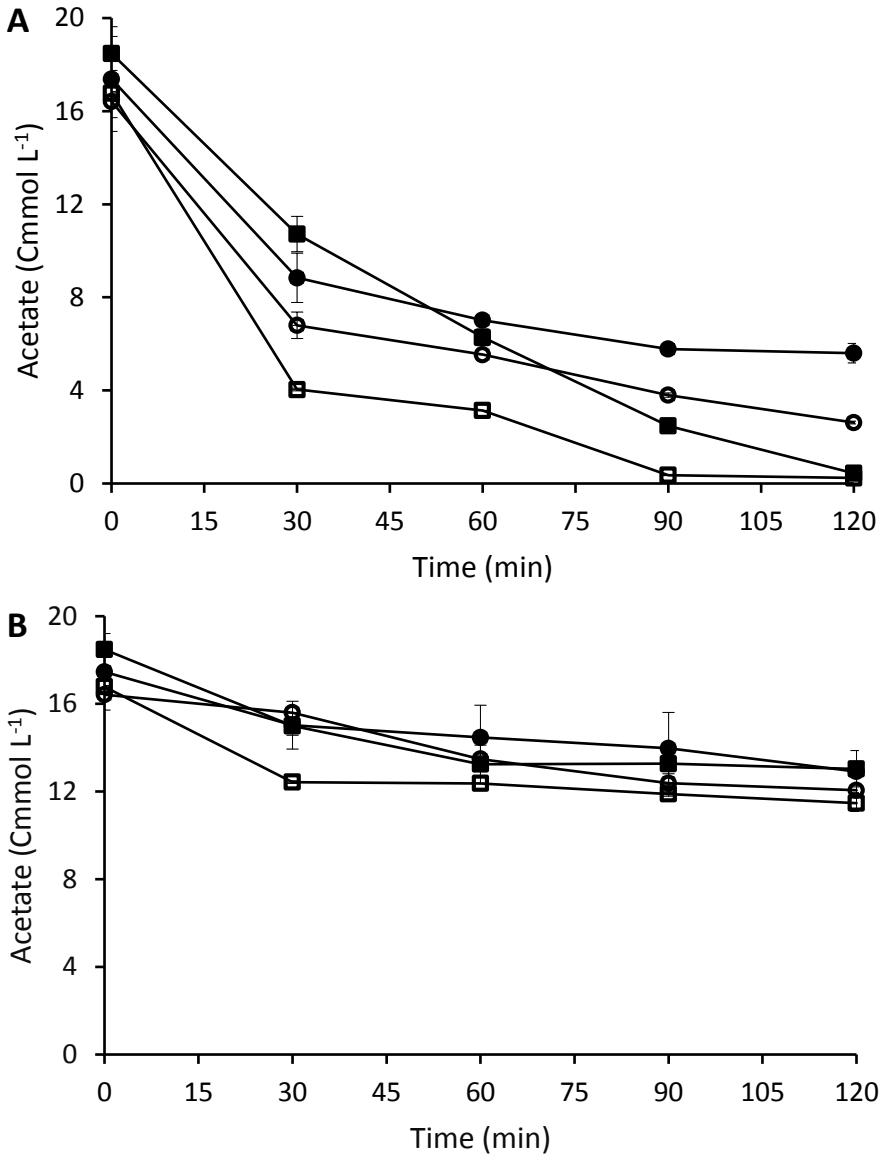

Figure 2.2: Anaerobic acetate storage of the sequencing batch biofilm reactor (A) and trickling filter reactor (B) after 2 (●), 4 (○), 6 (■) and 8 (□) weeks of operation. All values are represented as mean ± standard deviation of three replicates.

The sequencing batch biofilm reactor (SBBR) that was operated under sequential anaerobic storage and subsequent biomass exposure to air conditions demonstrated the increasing capacity of anaerobic acetate storage over time (Figure 2.2A). Already after two weeks of continuous operation, the SBBR showed a clear tendency of acetate storage at a rate of about 9 Cmmol L^{-1} h^{-1} (compared to 4.5 Cmmol L^{-1} h^{-1} in the trickling filter

reactor), suggesting the selection of storage bacteria. The maximum speed of anaerobic acetate storage (initial 30 min) was 24 Cmmol L^{-1} h^{-1} (768 mg BOD L^{-1} h^{-1}) after eight weeks of operation. By comparison, the trickling filter reactor reached a maximum of 5.3 Cmmol L^{-1} h^{-1} at the end of the experimental period (Figure 2.2B) which is due the 40% lower biomass concentration than that of the SBBR. The measured acetate removal rate in sequencing batch biofilm reactor was significantly higher than the rates reported in the literature. Flavigny and Cord-Ruwisch (2015) reported an acetate removal rate of 10 Cmmol L^{-1} h^{-1} (320 mg BOD L^{-1} h^{-1}) in a similar SBBR operated with substantially higher biomass levels. The SBBR showed

Interestingly, despite the fact that the SBBR underwent sequential anaerobic and aerobic phases, it developed a biofilm faster than the TFR. This is evident from microscopic observations and from the difference of aerobic acetate uptake rates after six weeks by both biofilms, which were 7.7 and 4.6 Cmmol L^{-1} h^{-1} for the SBBR and the TFR, respectively. Also, normal time curves of acetate uptake showed that anaerobic acetate uptake by the SBBR was faster than aerobic acetate uptake by the TFR (Figure 2.3), which is likely due to the higher biomass level in the SBBR (volatile solids concentration of the SBBR and TFR were 20.6 and 12.2 g L^{-1} of reactor, respectively). This result suggests that sequencing batch mode operation enhances biomass accumulation in a carrier material which is in accordance with the observation reported by Bassin et al. (2012b).

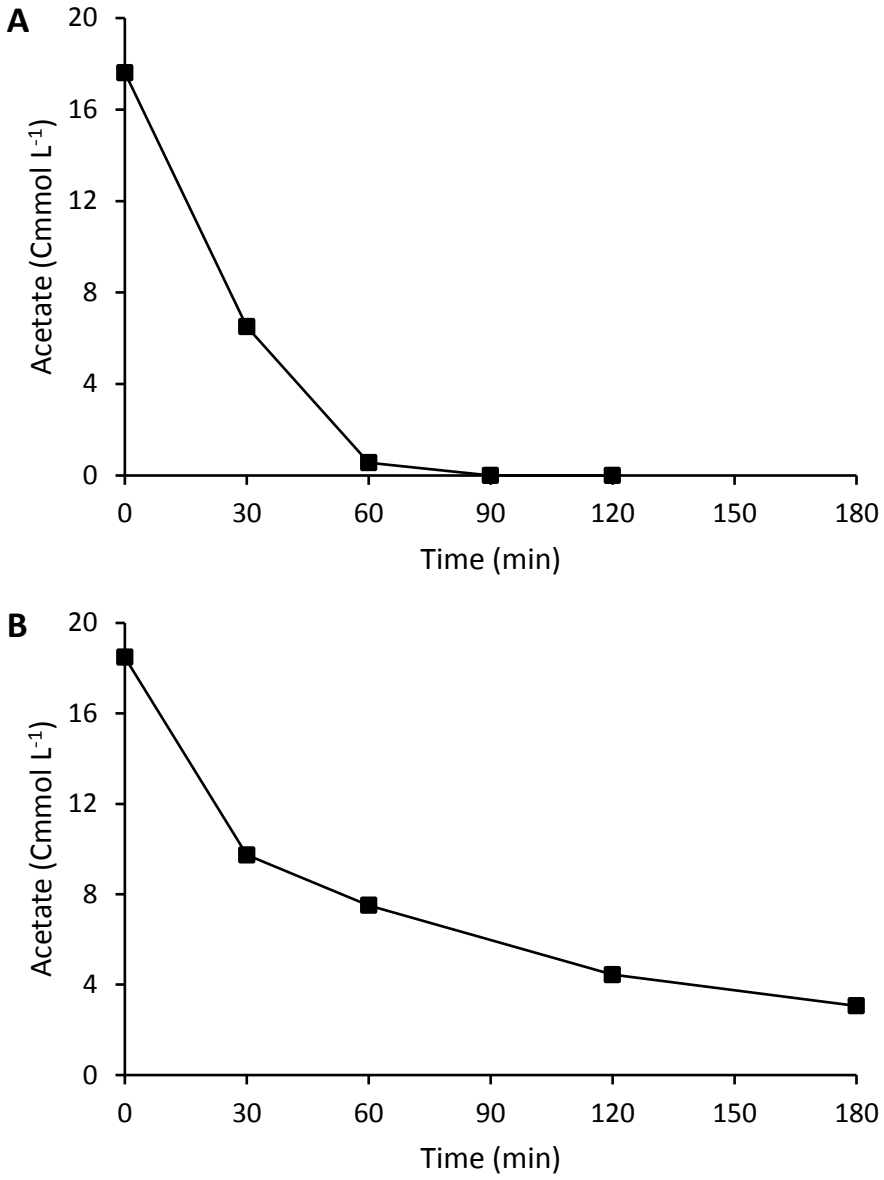

Figure 2.3: Typical operation (anaerobic/aerobic for SBBR and aerobic for TFR) acetate profile of reactors at 6 weeks of operation: SBBR (A) and TFR (B). All values are represented as mean ± standard deviation of three replicates.

2.3.2 Removal of carbon source without release of phosphorus

Two groups of microorganisms can store carbon source anaerobically: the polyphosphate accumulating organisms (PAO) and the glycogen accumulating organisms (GAO). To determine which types of microorganism enriched in this experiment, a batch experiment was done to study the anaerobic acetate (carbon source) uptake and P release profiles of both reactors (Figure 2.4). In sequencing batch biofilm reactor, biomass removed 17

Cmmol L^{-1} acetate while the phosphorus content remained almost equivalent in synthetic wastewater throughout the anaerobic phase (Figure 2.4 A). As expected, the biofilm in the control reactor, in which PHA storage bacteria were not enriched, also did not release phosphate (Figure 2.4 B).

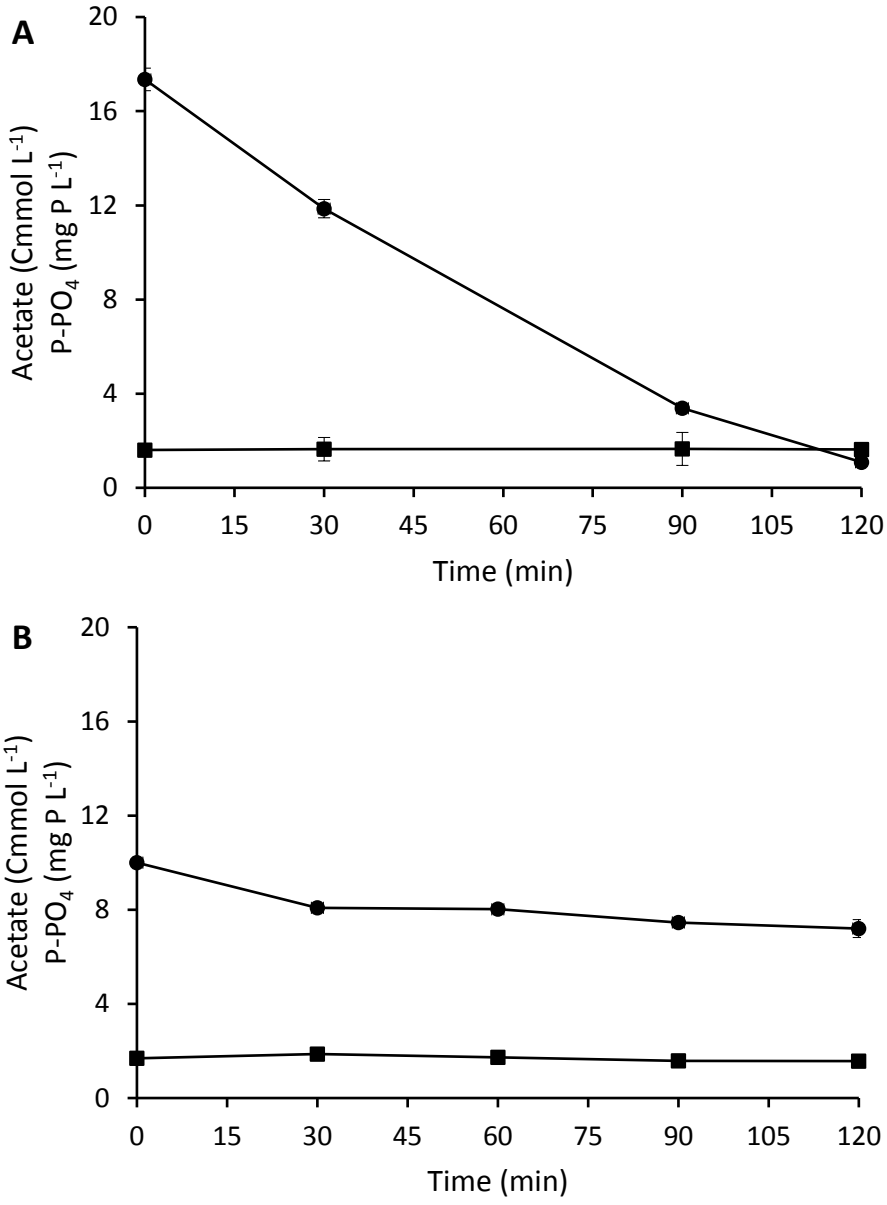

Figure 2.4: Anaerobic acetate and phosphate profiles of batch tests of the SBBR (A) and the TFR (B) biomass after 6 weeks of enrichment: acetate (●) and $P\text{-}PO_4$ (■). All values are represented as mean ± standard deviation of three replicates.

The sequential anaerobic and aerobic condition is known to be favorable for acetate uptake and storage as PHA by phosphate accumulating organisms (PAO). These organisms take up phosphate aerobically as an energy source, followed by hydrolysis and release of phosphate in the anaerobic phase, which provides energy for anaerobic acetate uptake and its polymerization as PHA. However, in this experiment, there is little chance for PAOs to develop in the sequencing batch biofilm reactor because aerobic phosphate accumulation cannot occur as phosphate containing synthetic wastewater has been drained just before the aerobic phase. As a consequence, it is expected that an alternative mechanism of acetate storage as PHA is used which is the mechanism of glycogen-accumulating organisms (GAOs). These organisms use aerobically stored glycogen to enable anaerobic acetate uptake, which is subsequently accumulated as PHAs (Liu et al., 1996). Hence, reactor operation in SBBR would be likely to select for GAO rather than PAO.

From the established understanding of the physiology of GAO bacteria, the reasons why the described operating conditions (anaerobic loading followed by aerobic exposure of the biofilm to air) leads to the selective enrichment of GAO bacteria are as follows: During the initial establishment on the carrier material of an aerobic, acetate fed biofilm from activated sludge, various types of bacteria may attach to the carrier (e.g. via producing expo-polymers). However, after anaerobic loading of the reactor with synthetic wastewater, only those bacteria that can store acetate as storage material (i.e. as PHA) will be able to profit from the subsequent aerobic phase to produce ATP via aerobic respiration (electron transport phosphorylation), hence allowing them to proliferate as a biofilm.

Other bacteria, including PAO, cannot profit from the oxygen. To PHA storing GAO bacteria, the aerobic phase not only provides energy in the form of ATP but also generates glycogen which, in the next anaerobic phase serves as the energy source (ATP from substrate level phosphorylation) for continued anaerobic storage of acetate. Again, only GAO bacteria are expected to absorb significant acetate in the next anaerobic phase as it requires a suitable anaerobic ATP source such as stored glycogen. A chance to store phosphate was not provided and hence the sequential anaerobic and aerobic operation specifically encourages GAO bacteria over PAO to develop the biofilm in SBBR which is in accordance with literature (Crocetti et al., 2002; Dai et al., 2007; Zeng et al., 2003d). Moreover, the high acetate content available at the beginning of the anaerobic phase, favours GAO development via their ability to uptake acetate by diffusion (López-Vázquez et al., 2008).

2.3.3 Microscopic observation

Microscopic investigation of the biomass of SBBR showed that it was dominated by one morphological cell type, large coccobacilli (Figure 2.5). These cells positively stained with Sudan Black B, showing intracellular lipid granules, suggesting the accumulation of PHA. In samples taken after the aerobic period, this cell type did not show the characteristic stain. This finding is similar to the observation reported by Crocetti et al. (2002) who described the abundance of PHA storing spherical cells in an anaerobic and aerobically operated sludge. In contrast, only few lipid containing cells and a majority of rod shaped bacteria was observed in the trickling filter reactor.

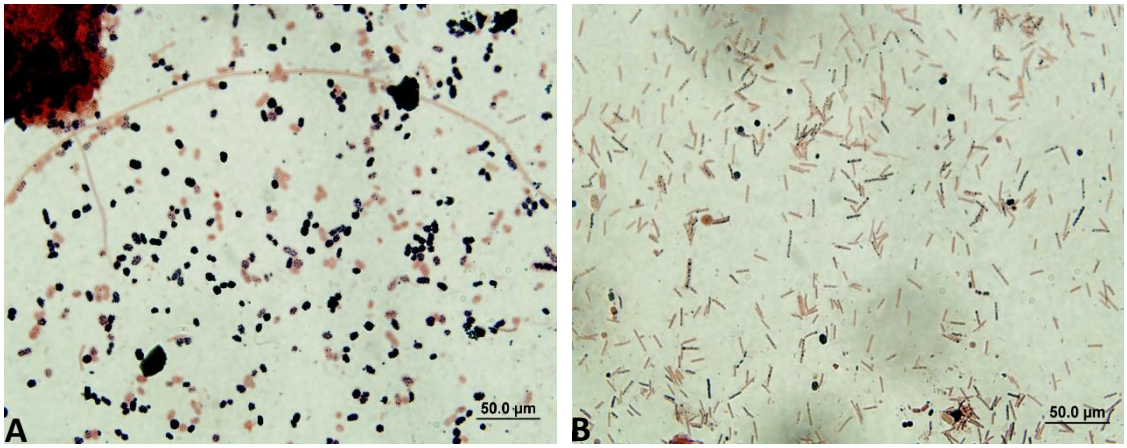

Figure 2.5: Sudan Black B stained sample of biofilm material (A: sequencing batch biofilm reactor, B: trickling filter reactor) after 6 weeks of operation. Black inclusions indicate lipophilic cellular material (i.e., PHAs), and pink areas show non-lipophilic cellular material.

2.3.4 Intracellular glycogen and PHA transformation

After 6 weeks of operation, the sequencing batch biofilm reactor reached a steady state as indicated by nearly identical cycle profiles. The results of one of these cycle studies are depicted in Figure 2.6. Under anaerobic condition, acetate was taken up, with concomitant consumption of glycogen and accumulation of PHA (PHB + PHV) and without the release of phosphorus. In the subsequent aerobic condition, anaerobically accumulated PHA was oxidized to provide energy for glycogen replenishment and biomass growth. This observation clearly shows that the enriched culture in the SBBR demonstrated the GAO behavior, confirming the selective enrichment of this functional group of microorganisms under the used operating conditions.

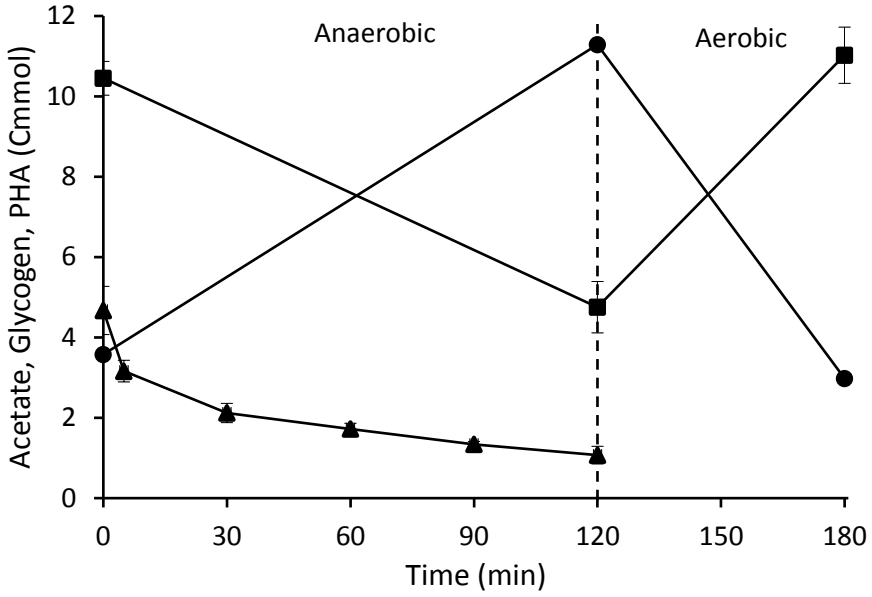

Figure 2.6: Typical carbon transformation during an anaerobic-aerobic cycle of the sequencing batch biofilm reactor: concentrations of acetate (▲), glycogen (■) and PHA (●). All values are represented as mean ± standard deviation of three replicates.

The anaerobic and aerobic stoichiometric data was compared with other reports carried out with GAO and PAO enriched cultures (Table 2.1). The anaerobic $Gly_{degraded}/Ac_{uptake}$ ratio of SBBR was comparatively high with 1.58 (Cmol $Cmol^{-1}$). As glycogen accumulating organisms rely on glycogen as their sole energy source, their $Gly_{degraded}/Ac_{uptake}$ ratio is known to be as high as 1.68 (Cmol $Cmol^{-1}$) (Lopez-Vazquez et al., 2009a). Since the reactor was fed with acetate based synthetic wastewater which was phosphorous limited, the high $Gly_{degraded}/Ac_{uptake}$ ratio in SBBR indicates that the energy required for acetate uptake was mainly derived from glycogen metabolism.

Table 2.1: Stoichiometric parameters observed in this study in comparison with literature values, for processes that based on anaerobic acetate storage.

	Anaerobic			**Aerobic**
	$P_{released}$ / Ac_{uptake}	**$Gly_{degraded}$ / Ac_{uptake}**	**$PHA_{synthesized}$ / Ac_{uptake}**	**$Gly_{synthesized}$ / $PHA_{degraded}$**
This study	0	1.58	2.14	0.75
Enriched GAO cultures				
Lopez-Vazquez et al. (2009a)	-	1.68	2.33	0.96
Oehmen et al. (2005)	0	1.17	1.85	-
Zeng et al. (2003d)	0	1.12	1.86	0.65
Filipe et al. (2001)	0.020	0.92	1.53	0.80
Jeon et al. (2001)	0.015	1.21	2.04	-
Enriched PAO cultures				
Welles et al. (2015)	0.22	0.96	1.47	0.51
Acevedo et al. (2012)	0.73	0.35	1.36	0.39
Zhou et al. (2008b)	0.58	0.45	1.22	-
Smolders et al. (1994a)	0.50	0.50	1.33	0.42

All units expressed in Cmol $Cmol^{-1}$, apart from $P_{released}/Ac_{uptake}$, which is expressed in Pmol $Cmol^{-1}$.

Also, the $PHA_{synthesized}/Ac_{uptake}$ ratio in SBBR was high with 2.14 (Cmol $Cmol^{-1}$) which is close to the value (2.33 Cmol $Cmol^{-1}$) reported by Lopez-Vazquez et al. (2009a) for glycogen accumulating organisms (GAOs). Further, the aerobic $Gly_{synthesized}$ /$PHA_{degraded}$ ratio for SBBR was 0.75 (Cmol $Cmol^{-1}$), which is similar to the ratio obtained by Filipe et al. (2001) for GAO enriched cultures. These results agree with the glycogen accumulating metabolisms and suggest that the enriched culture in SBBR was dominated by GAO.

2.3.5 Microbial community structure analysis

To investigate further the key constituents of the described biofilms, 16S rRNA amplicon sequencing analysis was carried out at week 8. The initial bioinformatic analysis (de-noising, filtering out chimeras) yielded 11747 and 12283 high quality reads for SBBR and TFR respectively, which were assigned to different taxonomic levels (from genus to

family). A portion of the effective bacterial sequences could not be assigned to any taxon, suggesting that some bacteria were novel which was present in both reactors.

The relative abundances of different phyla and classes in *Proteobacteria* for both reactors are shown in Figure 2.7. The most abundant phylum in sequencing batch biofilm reactor was *Proteobacteria* (Figure 2.7A), which accounted for 64.7% of the total bacterial 16S rRNA gene sequences. In contrast, *Bacteroidetes* (42.4%) was the largest component of the total OTUs in trickling filter reactor, followed by *Proteobacteria* (37.6%). The predominance of *Proteobacteria* is in line with previous studies of activated sludge (AS) communities (Zhang et al., 2012). This group is considered important for wastewater treatment because of their role in carbon, phosphorous and nitrogen removal (Yang et al., 2014). On the other hand, *Bacteroidetes* the dominant phylum in the TFR is responsible for sludge foaming and bulking which leads to increased operational cost of wastewater treatment plants (Yang et al., 2014).

Regarding relative abundances of different classes within *Proteobacteria*, there was a significant difference between SBBR and TFR reactor (Figure 2.7B). While *Gamma-proteobactetia* (50.6%) was the most abundant class in the SBBR, the TFR reactor was dominated by *Beta-proteobacteria* (16.3%). This observation suggests that the sequential anaerobic and aerobic phase promotes the proliferation of *Gamma-proteobacteria* which is considered as the chief competitors of PAOs for anaerobic substrate uptake and has shown to be capable of PHA accumulation but lacks the ability to remove phosphorus. On the other hand, *Beta-proteobacteria* normally exists in aerobic bio-systems (Esplugas et al., 2013).

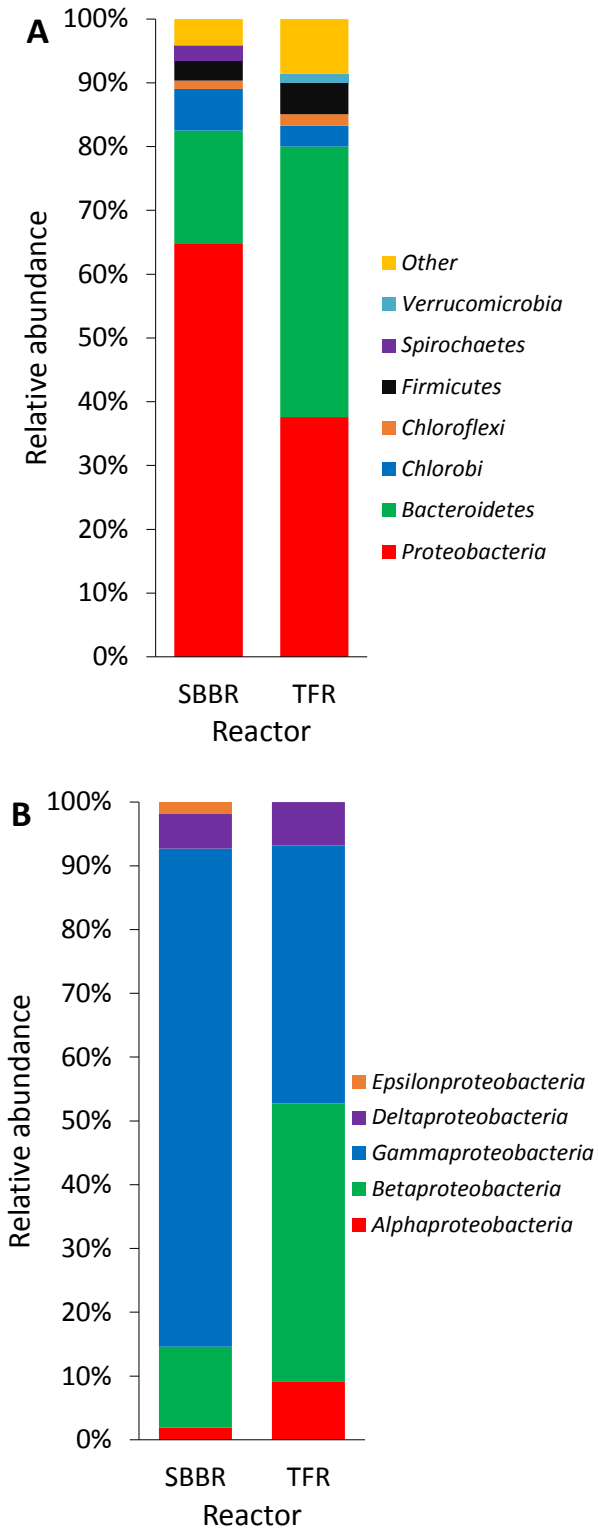

Figure 2.7: Relative abundances of different phyla (A) and classes in Proteobacteria (B). Taxa comprising at least 1% of the total OTUs are listed; others are included in the "other" category.

Bacterial community composition at the genus level (>1% relative abundance) is represented in Figure 2.8 and it can be shown that the sequencing batch biofilm reactor and trickling filter reactor had different predominant bacterial groups. The SBBR is dominated by *Candidatus competibacter* (48.7%) belonging to *Gamma-proteobacteria*, followed by *Bacteroides* (11.17%). This observation is in line with the findings reported in the literature which has shown that *Candidatus competibacter* can be enriched in an anaerobic/aerobic system using acetate as the sole carbon source (Crocetti et al., 2002; Dai et al., 2007; Zeng et al., 2003d). Moreover, other conditions such as increased temperature (about 25°C), low P/C (≤0.02 Pmol/Cmol) ratio used in this study may have contributed to GAOs enrichment and predominance in the SBBR (López-Vázquez et al., 2008; Lopez-Vazquez et al., 2009b).

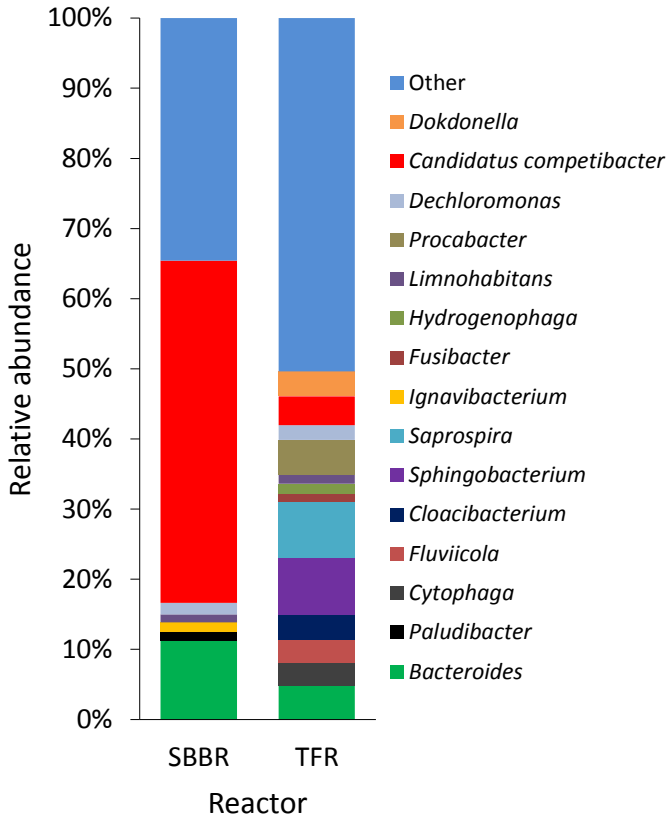

Figure 2.8: Relative abundance of OTUs at genus-level taxonomy. Only genuses comprising at least 1% of the total OTUs are listed; others are included in the "other" category, which also includes some OTUs that could not be classified at the genus level.

On the other hand, the most abundant genus in trickling filter reactor is *Sphingobacterium* (8.1%), followed by *Saprospira* (7.7%) and *Bacteroides* (4.7%). *Sphingobacterium can* rapidly break down organic compounds (Yang et al., 2014) which might be responsible for the predominance of *Bacteroidetes* phylum in trickling filter reactor as they can utilize these available substances instantly (Acosta-Martínez et al., 2008). The low level of acetate storage capacity observed in the TFR could be attributed to the combined actions of *Candidatus competibacter* (4.04%), *Bacteroides* (4.74%), *Hydrogenophaga* (1.37%) present in the reactor.

2.3.6 Practical implications of this study

The current study shows activated sludge suspended biomass can be readily converted to a biofilm reactor that rapidly stores BOD as PHA under anaerobic conditions and subsequently oxidizes PHA to glycogen and CO_2 when exposed to oxygen by mere drainage of the bioreactor. The selective enrichment of the responsible GAO bacteria can be accomplished within a few weeks. This observation suggests that wastewater treatment plant operators can readily implement a fixed bed reactor system that can remove a major proportion of BOD of wastewater. Moreover, the present study demonstrated that GAO dominated biofilm reactor completely removes BOD at a rate of 8 Cmmol L^{-1} h^{-1} (256 mg BOD L^{-1} h^{-1}) which is 20 times faster than that of traditional wastewater treatment system such as trickle reactor (Table 2.2). The efficient and high BOD removal is due to the configuration (anaerobic/aerobic) of the reactor, high surface area (500 m^2/m^3) of carrier material and high biomass content (45 g dry biomass L^{-1} of the reactor) of the biofilm (Ahammad et al., 2013; Flavigny & Cord-Ruwisch, 2015). In addition to superior carbon removal performance, the proposed biofilm reactor is cost effective because it avoids the

energy-expensive transfer of oxygen to the bulk wastewater as observed in typical activated sludge-based processes. The energy requirement of the proposed biofilm reactor (with 3 m height and 3.25 h treatment time) is 2.5 W m^{-3} which is about 60 - 75% less than that of trickling reactors (6 - 10 W m^{-3}) (Tchobanoglous et al., 2014). The energy efficiency phenomena could be attributed largely to the passive aeration of the biofilm and partly to the anaerobic-aerobic operation of the biofilm reactor (Ahammad et al., 2013; Flavigny & Cord-Ruwisch, 2015).

Table 2.2: Comparison of the BOD removal rate of different systems.

System	**HRT (h)**	**BOD inflow (mg L^{-1})**	**BOD removal rate (mg L^{-1} h^{-1})**	**References**
Trickle reactor	51.2	599.5	11.7	Doan et al. (2008)
Trickle reactor	50	250	5	Forster (2003)
Sequencing batch reactor	6.0	500	85.22	Zhao et al. (2016)
GAO biofilm	3.25*	512	256	Present study

* HRT refers to the total treatment time including anaerobic and aerobic phase durations

The described energy-efficient biofilm reactor lends itself to treat wastewaters rich in organic material in combination with a separate nitrification step to remove nitrogen as published for nutrients (carbon, nitrogen) removal using two reactors and three (anaerobic/anoxic/aerobic) stages (Cord-Ruwisch & Hughes, 2012; Zhou et al., 2008a). Alternatively, the proposed biofilm system could also be integrated upstream of low-energy required anammox based process to remove nitrogen.

Anammox is a biological process capable of anaerobic transformation of NH_4^+ to dinitrogen (N_2) gas using NO_2^- as an electron acceptor (Kartal et al., 2013) and has been successfully implemented in sidestream wastewater treatment system. Recently research

focus has moved to the possible application of anammox based processes to mainstream wastewater treatment. However, one of the main challenges for applying anammox process to the main wastewater stream is high C/N ratio. Anammox bacteria cannot compete with heterotrophic denitrifying bacteria at high organic content, which results in low levels of anammox bacteria in the population. Moreover, some organic compounds which are added to the wastewater to improve the nitrogen removal efficiency such as methanol, have been found to cause partial/complete inactivation of anammox activity (Ali & Okabe, 2015). In contrast, low concentration of organic matter does not affect anammox activity significantly but improves total nitrogen removal via heterotrophic denitrification. Since the currently described biofilm reactor is capable of removing soluble organic substances from wastewater rapidly and cost-effectively, it could represent an ideal partner process for subsequent anammox processing, resulting overall in one of the least energy consuming wastewater treatment options.

2.4 Conclusions

The following conclusions could be drawn:

- Within eight weeks, suspended activated sludge biomass was converted to a glycogen accumulating organism (GAO) dominated biofilm reactor.
- The biofilm removed all BOD in the form of storage energy, which enables direct oxygen uptake from the atmosphere (passive aeration) and associated energy savings.
- The biofilm reactor could be integrated with nitrogen removal systems such as parallel nitrification-denitrification (PND) or other anammox based methods which could facilitate the application of this technology to the mainstream wastewater treatment processes.

Chapter 3

Direct Oxygen Uptake from Air by Novel Glycogen Accumulating Organism Dominated Biofilm Minimizes Excess Sludge Production[2]

Abstract

The cost associated with treatment and disposal of excess sludge produced is one of the greatest operational expenses in wastewater treatment plants. In this study, we quantify and explain greatly reduced excess sludge production in the novel glycogen accumulating organism (GAO) dominated drained biofilm system previously shown to be capable of extremely energy efficient removal of organic carbon from wastewater. The average excess sludge production rate was 0.05 g VSS g^{-1} BOD (acetate) removed, which is about 9-times lower than that of comparative studies using the same acetate based synthetic wastewater. The substantially lower sludge yield was attributed to a number of features such as the high oxygen consumption facilitated by direct oxygen uptake from air, high biomass content (21.41 g VSS L^{-1} of reactor), the predominance of the GAO (*Candidatus competibacter*) with a low growth yield and presence of the predatory protozoa (*Tetramitus*) in the biofilm. Overall, the combination of low energy requirement for air supply (no compressed air supply) and the low excess sludge production rate, could make this novel "GAO drained biofilm" process one of the most economical ways of biological organic carbon removal from wastewater.

[2] This chapter is published in Science of the Total Environment (2018) 640–641: 80-88

3.1 Introduction

The conventional activated sludge process is by far the most widely used system to treat municipal and industrial wastewater. Despite its high organic carbon and nutrient removal efficiency, the activated sludge process has a major drawback such as generation of excessive sludge which contains active and inactive microorganisms and must be treated prior to its disposal to prevent adverse impacts on public health and the environment (Guo et al., 2013). Currently, excess sludge management is a raising concern for wastewater treatment plants around the world due to the increasing costs and restrictions associated with sludge treatment and disposal (Huang et al., 2014). The treatment of excess sludge is expensive and may take up to 60% of the plant's total operational cost (Campos et al., 2009). Therefore, the interest in sludge minimization is steadily increasing.

Minimization of excess sludge volume can be done by several dewatering techniques. However, these processes do not reduce the actual solids content of the sludge. Sludge solids minimization strategies can be classified into two major categories: (i) sludge reduction through post-treatment by processes such as anaerobic or aerobic digestion and (ii) *in-situ* reduction of excess sludge during the wastewater treatment (Mahmood & Elliott, 2006; Wang et al., 2017). A number of different approaches, relying on single or a combination of mechanical, physical, chemical and biological methods have been employed to minimize the sludge generated in wastewater treatment facilities (Semblante et al., 2017; Wang et al., 2017). Mechanical sludge treatment methods such as high-pressure homogenizer (150-600 bar) and ultrasonic treatment (at 9-41 kHz for several seconds to 2.5 h), enhances the sludge degradation rate, but the amount of sludge reduced is often limited to less than 20% (Boehler & Siegrist, 2006; Wang et al., 2017). Thermal treatment (carried out at 165-180º C for 30 min) of sludge improves both the sludge volume and its

anaerobic digestibility (Wang et al., 2017). In addition to these technologies, chemical treatment methods such as ozone (O_3), hydrogen peroxide (H_2O_2) and alkali (NaOH) treatment can also enhance sludge degradability, thereby leading to improved sludge minimization. However, these sludge disintegration technologies significantly increase operating costs (Guo et al., 2013; Mahmood & Elliott, 2006; Semblante et al., 2017), and energy footprint of the wastewater treatment plant.

Anaerobic and aerobic digestions are the most common biological post-treatment methods which are implemented between the activated sludge and dewatering processes. Due to high operational complexity and expenses, anaerobic digestion is usually used in large wastewater treatment facilities where biogas co-production can recover the energy used. In contrast, aerobic digestion is typically applied in smaller treatment plants because of its operational simplicity. However, both treatment options suffer from disadvantages such as high initial investments and operational costs (Khursheed & Kazmi, 2011).

To save costs associated with the excess sludge management, it is preferable to reduce sludge production during the wastewater treatment processes (*in-situ*) rather than relying on post-treatment of the sludge produced. One approach that leads to lower sludge production is achieved by extending the solids retention time (SRT, also known as the sludge age) (Ghyoot & Verstraete, 2000; Tandukar et al., 2007). Several studies have shown that at longer SRT conditions, microorganisms use oxygen mainly for cell maintenance (endogenous respiration) rather than cell growth. Such endogenous respiration leads to sludge reduction (Sun et al., 2007). The SRT in biofilms is particularly high, which explains the lower sludge production in biofilm-based wastewater treatment systems compared with activated sludge processes. Furthermore, the long SRT of biofilms enables the growth of slow-growing microorganisms such as nitrifying bacteria (Bassin et al., 2015),

anaerobic ammonia oxidizing (Anammox) bacteria (Tsushima et al., 2007). Additionally, biofilms have also been found to encourage the increased abundance of higher organisms such as eukaryotic predators in active biomass components (Hao et al., 2010).

The presence of predators in the activated sludge and on biofilms has been known since the beginning of activated sludge technology. Predatory microorganisms are at the top of the food chain in the ecological system of wastewater treatment plants, and their concentration depends on the sludge retention time, food sources and wastewater composition. (Revilla et al., 2016). Among the predators commonly found in wastewater treatment plants, protozoa are the most abundant types which may constitute approximately 5% of the total dry-weight of activated sludge (Curds, 1982) and their abundance and diversity are considered as an indicator of process performance (Madoni, 2011). Protozoa help to shape the bacterial community within the niche by releasing mineral nutrients (carbon mineralization) and growth-stimulating compounds that can promote bacterial activity (Ratsak et al., 1996). In addition to these indirect effects, protozoa effectively graze on bacteria and inert particles; in this way, they have a significant role in sludge reduction and improving the wastewater treatment efficiency (Miyaoka et al., 2017; Ratsak et al., 1996).

The improved nutrient removal performance and stability of the biofilm-based wastewater treatment process such as the sequencing batch biofilm reactor (SBBR) are associated with its higher biomass concentration and increased SRT. The alternating anaerobic and aerobic phases used in a typical SBBR promotes the development of storage bacteria (e.g., polyphosphate accumulating organism, glycogen accumulating organism) which are able to uptake organic carbon (biological oxygen demand or BOD) from wastewater under anaerobic conditions and store intracellularly as poly-hydroxyalkanoates (PHAs). In a previous study, a passively aerated glycogen accumulating organism (GAO) dominated biofilm process was developed for energy efficient removal of organic carbon from wastewater (Hossain et al., 2017). In addition to stable performance, the described biofilm system showed little excess sludge production. However, it is not clear what factors contributed to this reduced sludge production.

The objective of this study is to quantify and explain the low excess sludge production observed in a GAO dominated, drained biofilm which is operated sequentially with anaerobic conditions (submersed biofilm) followed by aerobic exposure of the biofilm directly to air (passive aeration). The low sludge yield is verified by measuring the oxygen utilization by the biomass during the aerobic stage. Moreover, microbial community structure analysis and their potential role in reduced sludge production are also discussed.

3.2 Materials and Methods

3.2.1 Experimental setup and operation

A cylindrical reactor with a working volume of 0.255 L was operated in this study (Figure 3.1). The reactor was completely automated; with all pumps, airflow valves and phase lengths controlled by National Instruments Instrumentation Control Software Lab-VIEW^{TM} (version 9.1). The reactor was filled with packing material (AMB^{TM} Biomedia Bioballs), whose specific surface area for biofilm growth and support is 500 m^2/m^3. The carrier material is made from polyethylene - a non-porous polymer. These carrier materials have a cylindrical shape with 7 mm height and 11 mm diameter. The volume occupied by the empty carrier materials was about 20% ($V_{carrier}$ / $V_{reactor}$).

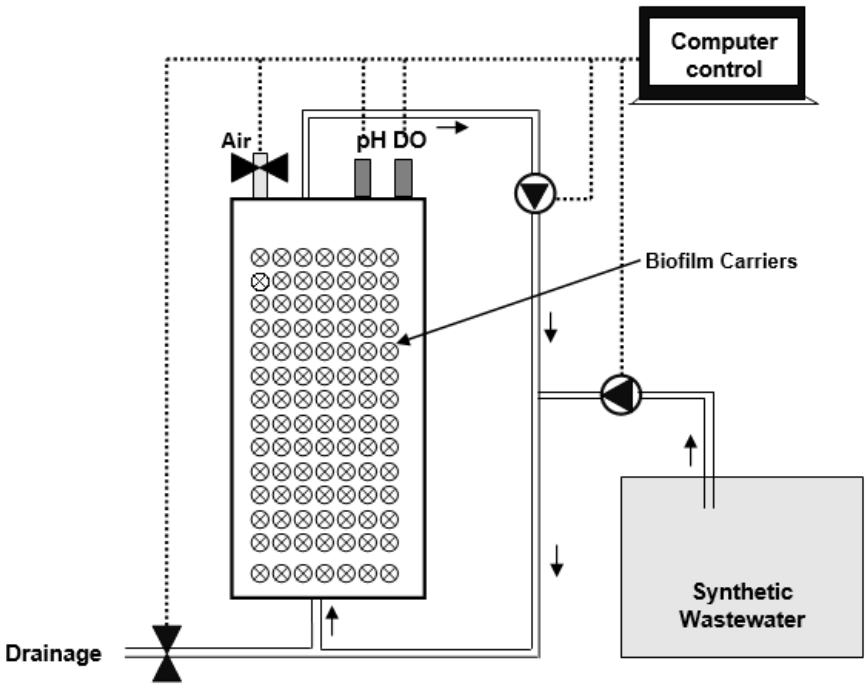

Figure 3.1: Schematic diagram of the experimental setup. The biofilm grown on carrier materials was alternately exposed to synthetic wastewater to facilitate organic carbon uptake and storage as polyhydroxyalkanoates (under anaerobic conditions) and to atmospheric air after draining the liquid by gravity to regenerate biofilm's carbon storage capacity.

Prior to operation, the described biofilm reactor was inoculated with activated sludge from a local wastewater treatment plant (Subiaco, Western Australia). After seeding, the biofilm reactor was operated automatically in a sequencing batch mode by specifically timed phases. The reactor was filled with synthetic wastewater (within 5 min through a peristaltic pump), then maintained under anaerobic condition for about 2 hours. The anaerobic phase was followed by gravity drainage (10 min), which allowed air penetration within the reactor of equal volume to the liquid drained. The air within the reactor was recirculated during the 1 h of aerobic phase to ensure the uniform distribution of air throughout the reactor.

3.2.2 Synthetic wastewater

Synthetic wastewater was used to maintain reproducibility and enable direct comparison with many studies using the same wastewater. The use of this common synthetic wastewater has not been shown to result in lower sludge production. On the contrary, as all its BOD is readily bio-degradable and its composition provides all elements necessary for biomass growth a potentially higher cell yield than with real wastewater could be expected. The standard composition of the synthetic wastewater was (mg L^{-1}): CH_3COONa 660, NH_4Cl 160, KH_2PO_4 44, $NaHCO_3$ 125, $MgSO_4$. $7H_2O$ 25, $CaCl_2$. $2H_2O$ 300, $FeSO_4$. $7H_2O$ 6.25, yeast extract 50, and 1.25 ml L^{-1} of trace element solution, which contained (gL^{-1}): EDTA 15, $ZnSO_4$. $5H_2O$ 0.43, $CoCl_2$. $6H_2O$ 0.24, $MnCl_2$. $4H_2O$ 0.99, $CuSO_4$. $5H_2O$ 0.25, $NaMoO_4$. $2H_2O$ 0.22, $NiCl_2$. $6H_2O$ 0.19, $NaSeO_4$. $10H_2O$ 0.21, H_3BO_4 0.014 and $NaWO_4$. $2H_2O$ 0.050 (Third et al., 2003b).

3.2.3 Analytical procedures

Total suspended solids (TSS), volatile suspended solids (VSS), chemical oxygen demand (COD) and oxygen uptake rate (OUR) analysis were carried out according to Standard Methods (APHA, 2012). Acetate was analyzed using an Agilent 7820A gas chromatography (GC) with auto-sampler. Samples were acidified with formic acid (10% v/v) before 0.4 µL samples were injected onto an Altech Econo-Cap™ EC™-1000 column (30 m length × 0.250 mm internal diameter × 0.25 µm film thickness). The carrier gas (N_2) was set at a flow rate of 3 mL min^{-1} and the sample was split 10:1 at the inlet. The oven temperature was programmed as follows: initial temperature 70°C, increased at 5°C min^{-1} to 100°C, held for 2.0 min, increased at 70°C min^{-1} to 250°C, held for 2.0 min. Injector and detector were set at 250 and 300°C respectively. The peak area of the Flame Ionization Detector (FID) output signal was computed via integration using the EzChrome Elite Compact Software© (V.3.3.2 SP2). The detection limit determined as 0.5 µmol L^{-1} of acetate. The acetate was converted to BOD by considering that one carbon mole of acetate equals 32 mg BOD.

3.2.4 Calculation of sludge retention time and sludge production

The observed sludge yield (Y_{obs}) was calculated to evaluate the excess sludge (solids concentration in reactor effluent) production. Since solids concentrations in the system changed, cumulative terms during the period of the study were used. The observed sludge yield was estimated according to Equation (3.1) (Brosseau et al., 2016).

$$Y_{obs} = \frac{VSS_{effluent}}{BOD_{influent} - BOD_{effluent}} \quad (3.1)$$

The observed sludge yield (Y_{obs}) was determined by the regression of the cumulative sludge production versus the cumulative organic matter removal. The slope of the regression line is considered as the mean sludge yield.

The sludge retention time (SRT) was calculated according to equation (3.2) (Henze et al., 2008):

$$SRT = \frac{\text{Mass of sludge in reactor}}{\text{Mass of sludge wasted per day}} \quad (3.2)$$

3.2.5 Biofilm morphology

Morphology of the biofilm was examined by using scanning electron microscope (SEM). A piece of the plastic carrier was cut carefully with a razor blade to keep the original biofilm structure. The preparation of the sample involved: fixation with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h; three washing steps (10 min each) with 0.1 M cacodylate buffer; post-fixation with 1% osmium tetraoxide (OsO_4) in 0.1 M cacodylate buffer for 1 h; three washing steps with 0.1 M cacodylate buffer (10 min each); gradual dehydration with successive immersions (10 min each) in increasingly concentrated ethanol solution (30%, 50%, 70%, 90% and 100%) and critical point drying. The dried specimen in the carrier material was attached to support with silver glue and coated with gold powder for observation in a Philips XL 20 Scanning Electron Microscope.

3.2.6 Microbial community analysis

For microbial community analysis by amplicon sequencing, biofilm was scraped off triplicate carriers, and genomic DNA was extracted using the Power Soil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions and using

DNA-free reagents and consumables. The V_4-V_5 fragment of the 16S rRNA gene was amplified with 515F-806R primers and the V_9 region of the 18S rRNA was amplified with 1391f-EukBr primers with the addition of mammal blocking primer. All primers and protocols for amplification and sequencing are accessible at the Earth Microbiome Project (EMP) webpage (http://www.earthmicrobiome.org/protocols-and-standards/). Negative controls included extraction blanks for DNA purification and no template controls for PCR amplification. Following amplification, PCR triplicates were pooled into a single sample, checked by agarose gel electrophoresis and purified using AMPure XP beads (Beckman Coulter, USA). After quantification with the Qubit dsDNA Assay Kit (Thermo Fisher Scientific, USA), amplicons were pooled at equimolar ratios, prior to index PCR using Nextera XT Index Kit V_2-V_5 indexes (Illumina, USA). Products were purified again using AMPure XP beads (Beckman Coulter, USA), quantified with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA) and pooled at the approximately equimolar ratio. The pool was then further concentrated and purified by a QIAquick PCR Purification Kit (Qiagen, USA) and quantified by Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA), prior to dilution to 4 nM and paired-end sequencing (2 x 250 bp; 500 cycle V2) on the MiSeq platform (Illumina, USA).

Bacterial and eukaryotic sequences were first processed in Geneious 8.0.4 (Kearse et al., 2012). Sequences were then quality filtered using USEARCH (Edgar, 2010), allowing only reads with a <1% error rate to remain and singletons were removed. Quality-filtered sequences were clustered into operational taxonomic units (OTUs) according to sequence similarity using a 97% similarity threshold with the UPARSE algorithm (Edgar, 2013). Genus level taxonomy was assigned to OTUs against the Greengenes reference database (May 2013 release) for 16S data (DeSantis et al., 2006) and SILVA 108 (Pruesse et al., 2007) for 18S data in QIIME 1.9.1 (Caporaso et al., 2010) using the UCLUST algorithm

(Edgar, 2010). In order to compute the alpha diversity, QIIME was used to calculate three diversity metrics: the Chao 1 metric (estimating the richness of species), the observed OTUs metric (the count of unique OTUs observed in the sample), and the Shannon Index (estimating the evenness of species). Beta diversity metrics were applied to evaluate the difference between microbial communities and reflect the dissimilarity between biomass samples. To measure beta diversity phylogenetically, both weighted and unweighted variants of UniFrac were calculated using QIIME.

3.2.7 Statistical analysis

Statistical analysis of the experimental data was conducted using the SPSS software package (IBM-SPSS v22). A one-way analysis of variance (ANOVA) was used to test whether a certain factor impacted an observed variable. Pearson's correlation coefficient was applied to quantify the relationship between two parameters. An $\alpha = 0.05$ and $p < 0.05$ indicated statistical significance.

3.3 Results and Discussion

3.3.1 Performance of the biofilm reactor

In this study, a lab-scale sequencing batch biofilm reactor was operated to establish a biofilm which can remove soluble organic carbon (BOD) from wastewater anaerobically and store inside the cells as poly-hydroxyalkanoate by providing a strict selective condition. The selective conditions involved the provision of soluble carbon in the absence of oxygen to facilitate carbon storage followed by providing oxygen in the absence of carbon to enable energy (ATP) conservation for growth from the oxidation of the stored polymers. The later step regenerates the BOD storage capability of the biofilm. The continuous operation of this scheme was found to selectively enrich for bacteria capable of effective anaerobic removal of BOD from wastewater.

The anaerobic BOD removal performance of the specialized biofilm for treating synthetic wastewater is shown Figure 3.2. The BOD removal capacity of the biofilm increased gradually and after about 40 days of sequential anaerobic and aerobic operation of the biofilm reactor, more than 98% of BOD as determined by acetate analysis was achieved. The BOD removal performance remained steady for about five months of continuous operation with about 7 cycles per day. This result is in accordance with the observation reported by Flavigny and Cord-Ruwisch (2015).

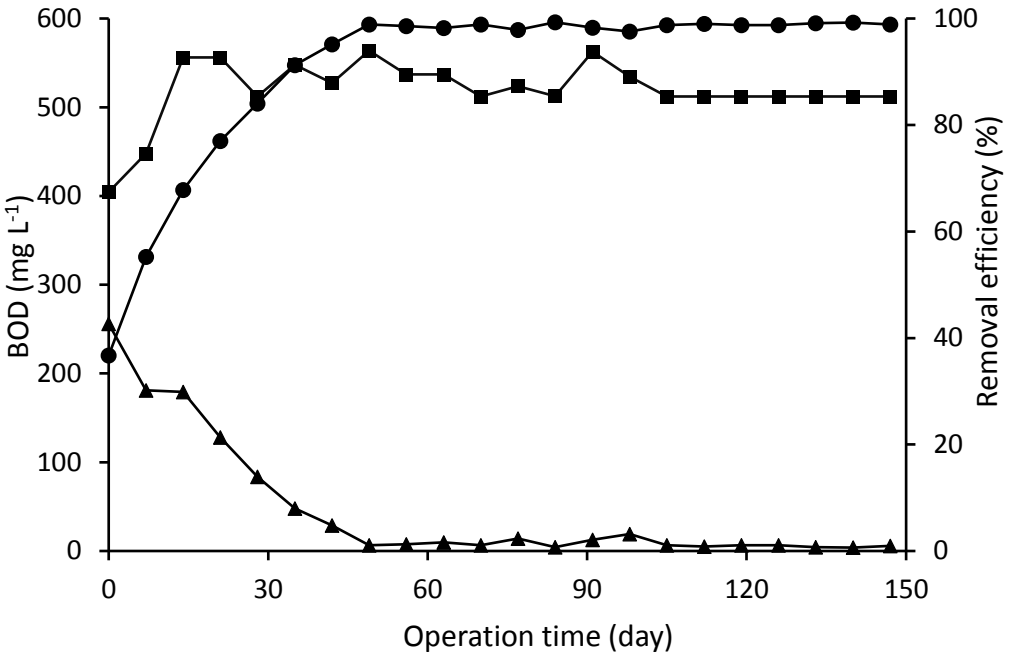

Figure 3.2: Long-term organic carbon (BOD) removal performance of the biofilm reactor calculated from acetate analysis: influent (■); effluent (▲) and removal efficiency (●).

3.3.2 Sludge production from the biofilm reactor

The biofilm reactor showed persistent performance in terms of BOD removal from synthetic wastewater under steady-state conditions. However, no apparent biomass loss was recorded during the experimental period. Occasional slough-off of small pieces of biomass into the effluent was observed only. The TSS and VSS in the effluent increased gradually until day 38 (Figure 3.3 A) and stabilized for the rest of the operation period. The average TSS and VSS concentration in the effluent (between day 40 and day 140 of operation) was 32.18 ± 1.81 and 22.04 ± 1.10 mg L^{-1}, respectively.

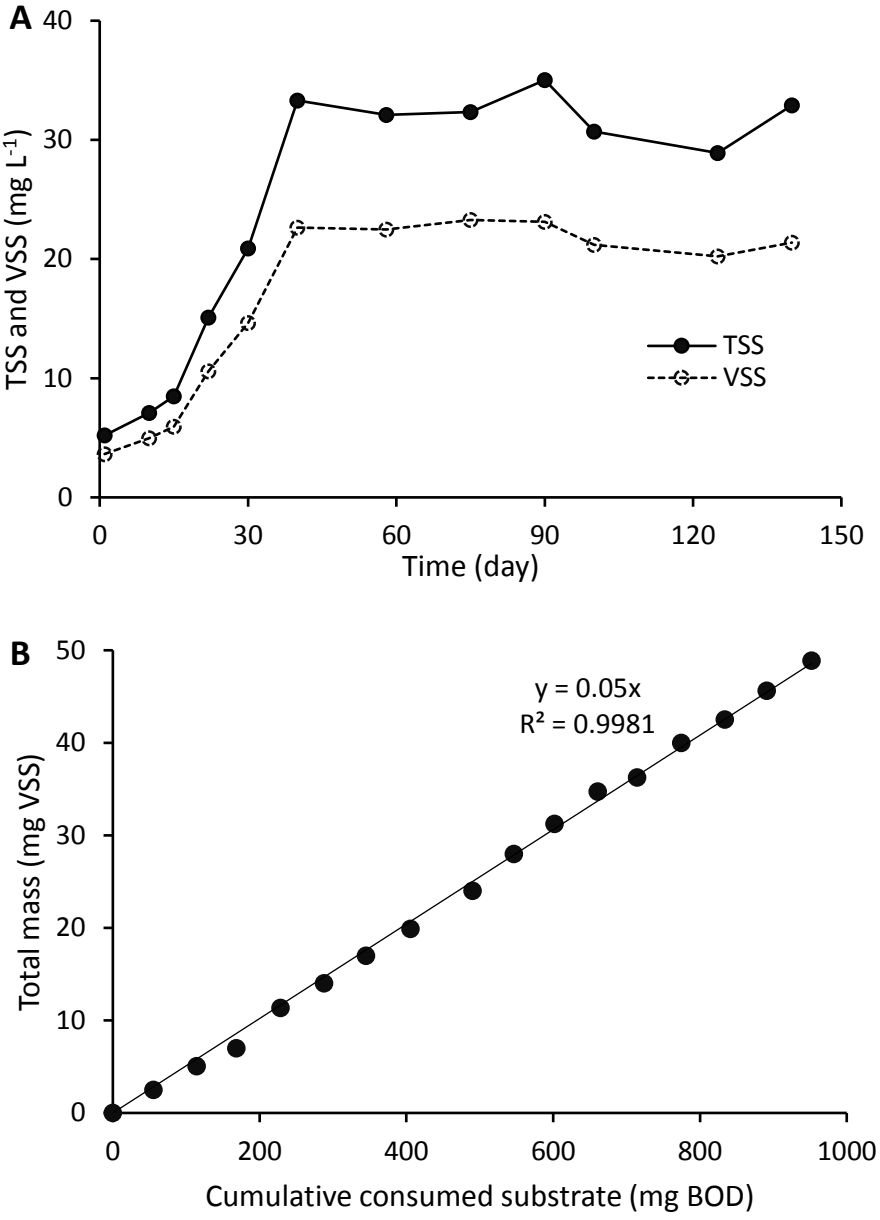

Figure 3.3: (A) The variations of total (TSS) and volatile (VSS) solids in the effluent of the biofilm process; (B) the overall sludge yield in the biofilm system.

To evaluate the excess sludge production in the described biofilm system, the observed sludge yield (Y_{obs}) was determined by the ratio of the cumulative sludge produced and the substrate (BOD) consumed (Figure 3.3 B). The sludge yield in the biofilm process was observed to be 0.05 g VSS g^{-1} BOD removed, which is only about 12% of that of conventional activated sludge process (0.4 g dry biomass g^{-1} substrate removed) (Tchobanoglous et al., 2014). The significantly lower sludge production may be attributed

to the high solids retention time (132 days) in the described biofilm as it has been shown that sludge reduction is associated with an increased sludge age (Habermacher et al., 2015; van Loosdrecht & Henze, 1999). Under prolonged sludge retention time, microorganisms use most of their energy mainly for cell maintenance rather than cell synthesis (Khursheed et al., 2015; Sun et al., 2007), resulting in reduced sludge generation. Moreover, substrate and oxygen gradient formed in biofilm enhances endogenous respiration and thus contributed to the lower sludge production.

In addition to prolonged SRT, the biomass concentration in the biofilm reactor was rather high, with 21.41 ± 1.10 g VSS L^{-1} of the reactor, which is about 4 times higher than that of conventional activated sludge process. With an average volumetric organic loading rate (OLR) of the reactor of 0.96 Kg BOD m^{-3} day^{-1}, the high biomass content of the biofilm reactor resulted in a low food to microorganisms (F/M) ratio of 0.045 g BOD g^{-1} VSS day^{-1}. Such low F: M ratio has been found to be associated with reduced excess sludge generation (Chang et al., 2011).

3.3.3 Biomass yield mass calculations

The biofilm system described in this study showed little sludge production. Generally, low sludge yield is associated with consumption of more oxygen (Huang et al., 2014). Hence, if the operating conditions (direct exposure to air) of the biofilm studied result in less biomass formed there should be a higher than usual oxygen consumption.

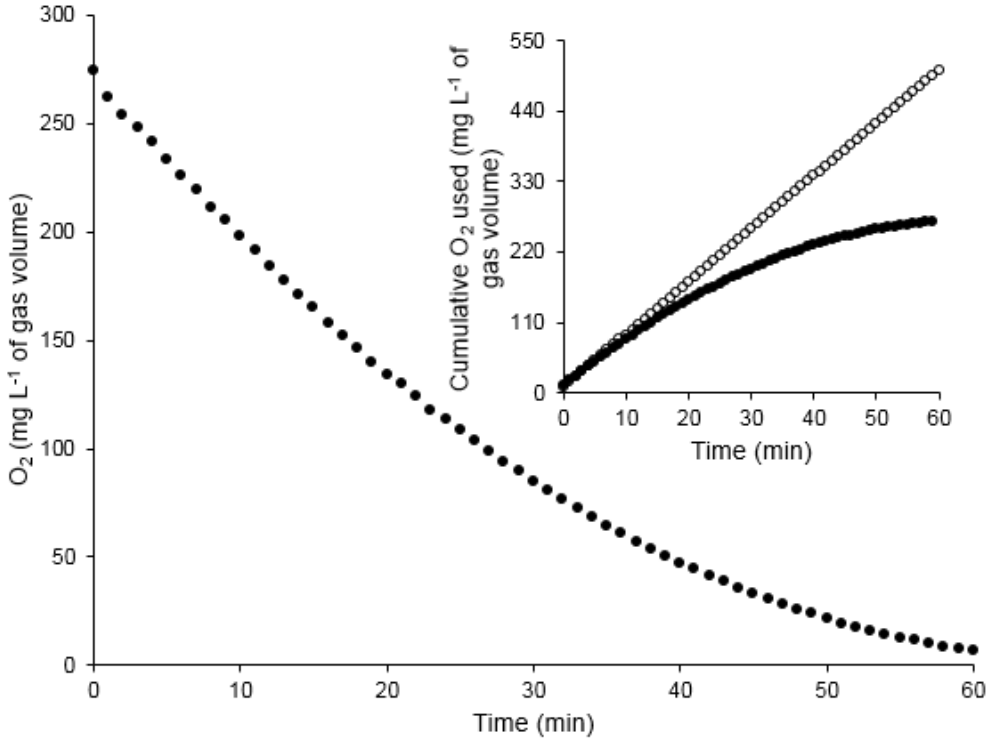

Figure 3.4: Oxygen profile in the headspace of the biofilm reactor after draining during the oxygen uptake batch test (closed vessel). Inset: cumulative oxygen consumed by the biomass in batch test (closed circle), and expected oxygen consumption in open vessel (open circle).

In order to verify this, oxygen consumption by the biofilm was recorded during the aerobic stage (Figure 3.4). All oxygen available in the gas phase was completely used within 60 min. Noticeably the oxygen uptake rate diminished, which was found to be due to oxygen limitation, not substrate exhaustion because re-aeration at the end of the experiment resulted reproducibly in the originally high OUR (data not shown). Considering that during normal operation (no oxygen recording) the reactor was left open to air (no oxygen limitation), it is fair to estimate the total amount of oxygen used by assuming a constant OUR over the entire aerobic phase. This OUR was calculated to be about 495 mg O_2 L^{-1} h^{-1} (here the volume of one L relates both the the void air volume as well as the liquid volume that had been used to supply BOD in the anaerobic phase). Over one hour of duration this extremely high OUR amounts to 495 mg L^{-1} of oxygen consumed (Figure 3.4, inset) for a total BOD removal of 539 mg L^{-1}. This only leaves 44

mg L^{-1} of BOD for assimilation and yield coefficient of 0.083 which is substantially lower than the 0.5 quoted for activated sludge (Tchobanoglous et al., 2014) and similar to the value obtained in the current study. The higher oxygen utilization by the biomass explains the low excess sludge production observed in the present study.

3.3.4 Microscopic observation of biofilm

The morphology of the biofilm developed on the carrier materials was examined using scanning electron microscope and are shown in Figure 3.5. SEM images showed that the biofilm comprised diverse morphotypes with coccoid and filamentous bacteria as the dominant prokaryotic forms (Figure 3.5 A, B). The presence of filamentous bacteria in biofilm reactors operated under sequential anaerobic and aerobic conditions have been reported in several reports (Li et al., 2014; Ma et al., 2018; Zhu et al., 2015) where it has been found that the abundance of filamentous bacteria increased at the early stage of biofilm formation. Filamentous bacteria excrete extracellular polymeric substance (EPS) and other soluble microbial products to form a tightly fibrous structure (Zhu et al., 2015). The fibrous matrix provides a stable spatial structure for the development of the biofilm and resist biomass sloughing (Li et al., 2014; Zhu et al., 2015). Also, the biofilm was found to be colonized by different eukaryotic life forms such as protozoa. For example, the presence of cysts with the pore (typical for amoeba) (Figure 3.5 C) and *Vorticella* (a sessile ciliate) (Figure 3.5 D) was observed as part of the biofilm.

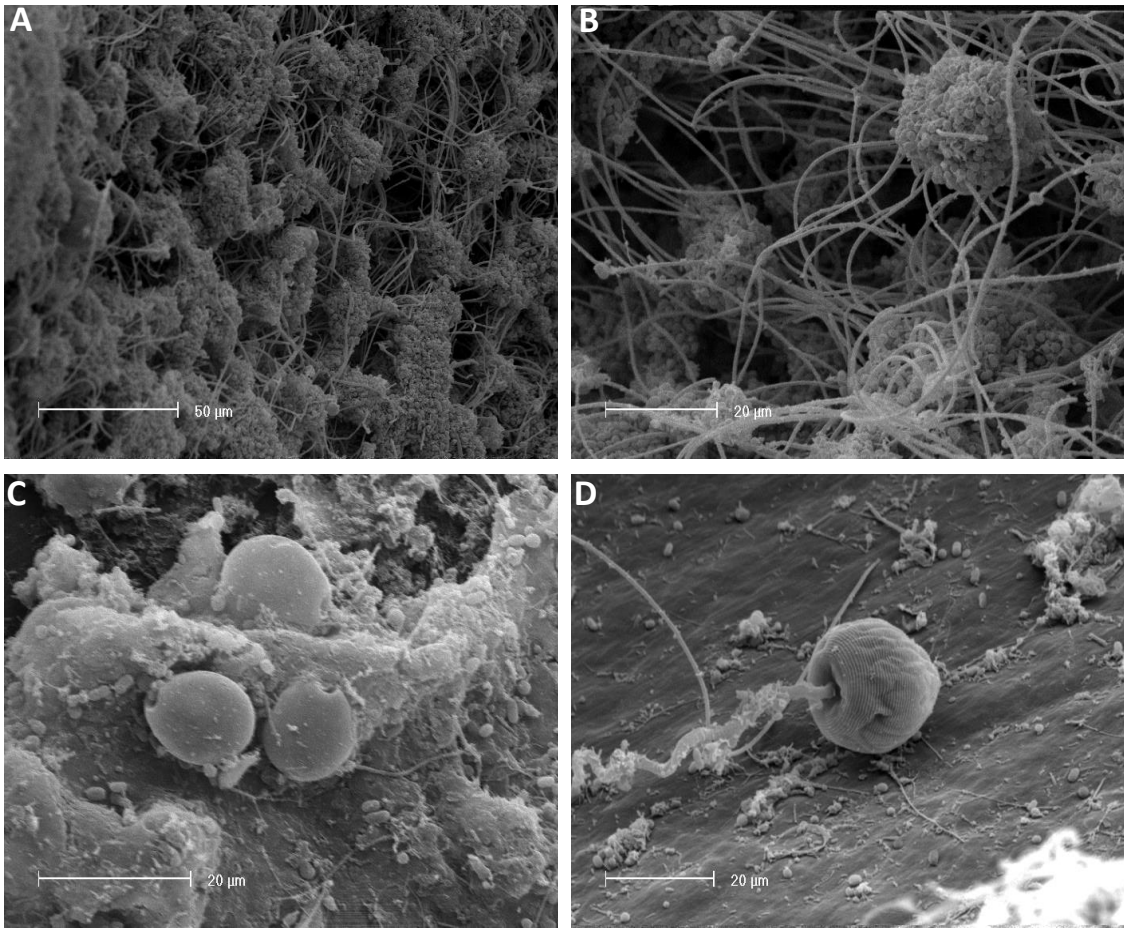

Figure 3.5: Scanning electron microscope images of the microstructure of the biofilm: (A) and (B) typical coccoid and filamentous bacteria; (C) cysts of amoeba; (D) *Vorticella* spp.

3.3.5 Dynamics of microbial community

To reveal the microbial community (bacteria and protozoa) of the biofilm, amplicon sequencing analysis was carried out at different time points over the experimental period. After initial processing, 56185 and 40112 sequences of bacteria and protozoa were found respectively, which were assigned to different taxonomic levels. Moreover, changes in microbial community composition were determined using the Shannon diversity index which reflects both species richness and evenness. The Shannon diversity index of the bacterial population stayed relatively constant between 3.51 ± 0.30 and 2.7 ± 0.26. In contrast, the Shannon diversity of protozoan community decreased from 2.20 ± 0.14 to 0.38 ± 0.26 over 140 days (Figure 3.6).

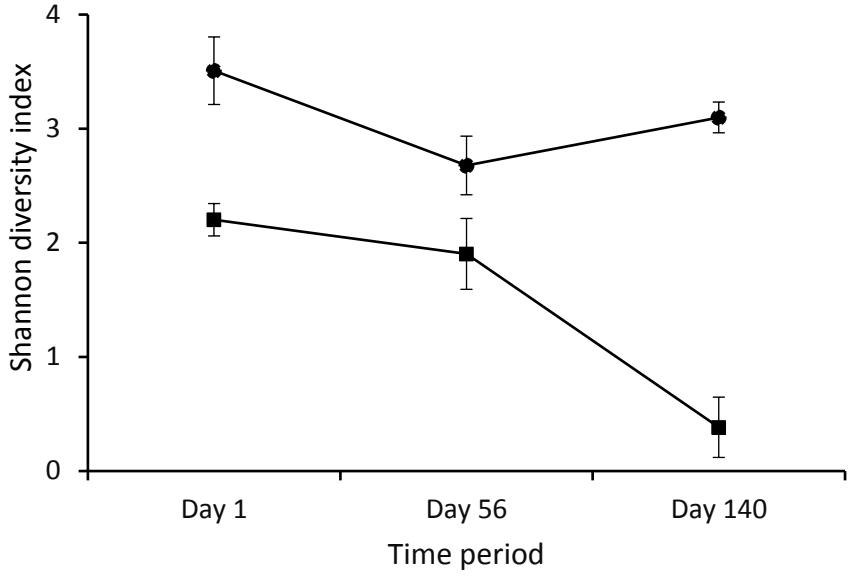

Figure 3.6: Plot of Shannon diversity index for bacteria (●) and protozoa (■) communities over the course of study. Error bars indicate one standard deviation from the mean Shannon diversity index.

3.3.5.1 Bacterial community

The relative abundances of different genera of bacteria present in the biofilm samples are shown in Figure 3.7A. There was a clear shift in the bacterial community composition from initially (day 1) 10 genera with each more than 5% abundance to only two such dominant genera which were *Candidatus competibacter* and *Bacteroides* and together they represented more than 50% of microbial communities in the biofilm samples at day 56 and day 140. The presence of the obligate anaerobe *Bacteroides* suggests that anaerobic fermentation of organic material, presumably during the anaerobic phase played a significant role. Since the organic substrate provided (acetate) is essentially not fermentable, *Bacteroides* may have developed on the conversion of biofilm material such as the extracellular polymeric substances (EPS) produced by other bacteria as *Bacteroides* are known polysaccharide fermenting organisms (Fitamo et al., 2017). On the other hand, *Candidatus competibacter* the most abundant genus in the matured biofilm samples is a

known glycogen accumulating organism (GAO). The stable organic carbon removal performance of the described biofilm system is attributed to the predominance of GAO in the biofilm which is in accordance with previous reports (Flavigny & Cord-Ruwisch, 2015; Hossain et al., 2017). Although the relative abundance of *Candidatus competibacter* decreased from 48.7% (day 56) to 35.1% (day 140), there was no significant effect of their reduction on the carbon removal performance of the biofilm system (Figure 3.2). In addition to the stable carbon removal performance, the glycogen accumulating organism may be associated with the low excess sludge production by the biofilm system, since their biomass growth yield is 17% lower than that of ordinary heterotrophic bacteria (Zeng et al., 2003b).

3.3.5.2 Protozoa community

The protozoa community in the biofilm samples is shown in Figure 3.7B. At the beginning (day 1), the biofilm was co-dominated by *Cryomonadida* (26.21%), *Cercomonas* (13.44%) and *Rotifera* (12.76%). These species of protozoa comprises the typical eukaryotic communities in activated sludge (Madoni, 2011). As the biofilm matured, the diversity of protozoa inhabiting the biofilm decreased and the biofilm was eventually predominated by the amoeboflagellate *Tetramitus*. The relative abundance of *Tetramitus* increased from 2% at day 1 to 10% at day 56 and finally to 77% at day 140. To the best of our knowledge, the dominance of *Tetramitus* in such biofilm has never been reported before.

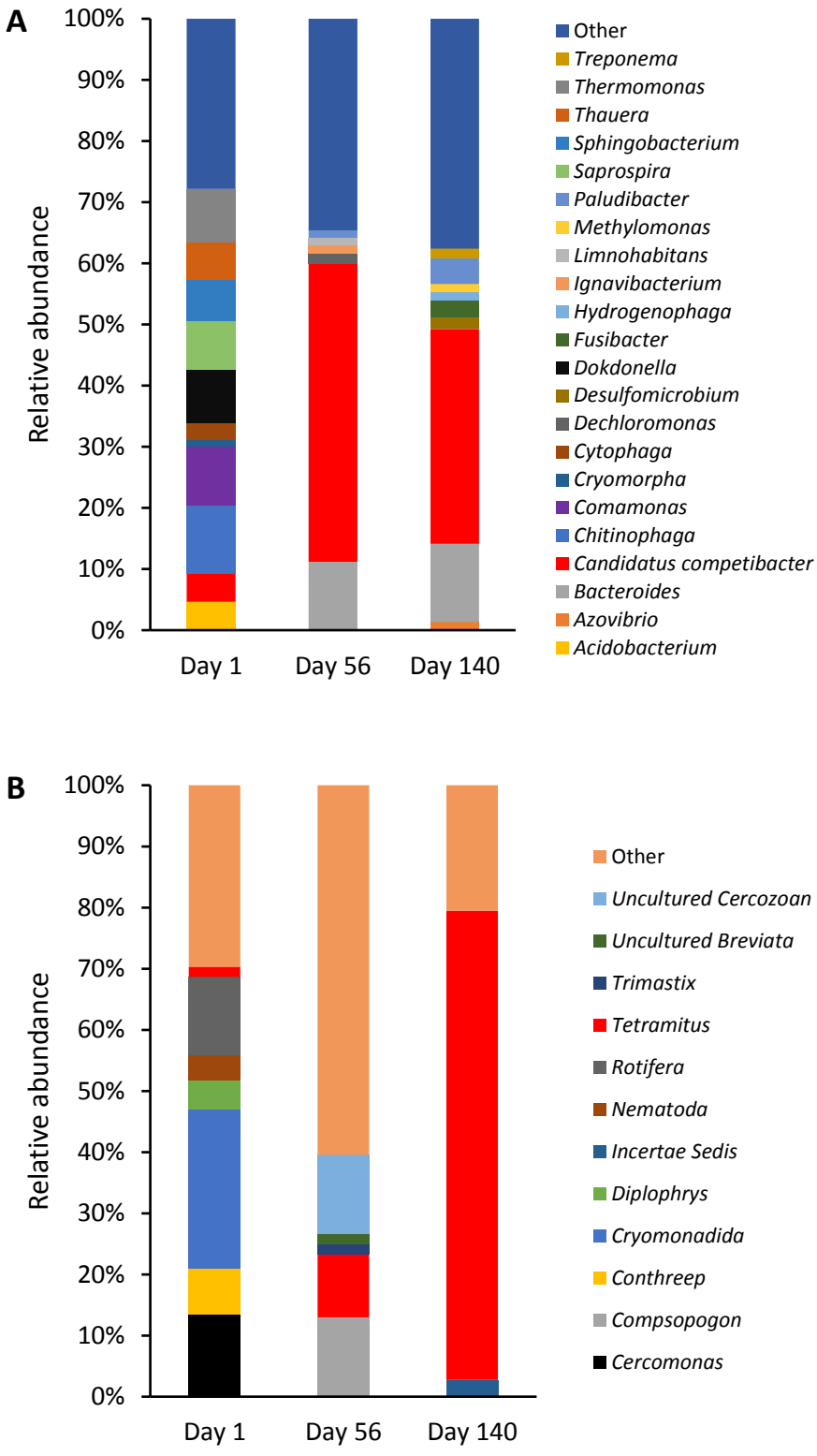

Figure 3.7: Relative abundances of different genus of Bacteria (A) and Protozoa (B) present in the biofilm samples across different time points. Taxa comprising at least 1% of the total OTUs are listed; others are included in the "other" category.

The protozoa community in the described biofilm system is different from that of the conventional wastewater treatment plants. In conventional processes, ciliated protozoa are the predominant species. They reside in the mixed liquor suspended solids and play a significant role in improving effluent water quality by preferentially grazing on dispersed bacteria (Miyaoka et al., 2017). Since the wastewater is completely drained after the anaerobic phase, it is assumed that the ciliated protozoa had little chance to grow in this GAO dominated biofilm system.

The described biofilm process operated continuously under sequential anaerobic and aerobic phases. Very few protozoa species tolerate anaerobic conditions (Dubber & Gray, 2011; Maurines-Carboneill et al., 1998), which could explain the relatively low protozoa diversity in the matured biofilm samples (Figure 3.6). The predominance of *Tetramitus* in the described GAO dominated biofilm may be attributed to its capability of adaptation to anoxic/oxic (A/O) environments as previously observed for A/O moving bed biofilm system (Canals et al., 2013).

Protozoa are known to play an important role in sludge minimization by effectively grazing on bacteria (Hao et al., 2011; Hao et al., 2010). The high numbers of *Tetramitus* in the biofilm may be associated with the reduced excess sludge production since predation by protozoa contributes to decreased overall biomass production. Chabaud et al. (2006) reported that biofilm enhances the efficiency of protozoan grazing by 60% due to the retention of microorganisms in biofilms through adsorption and adhesion, which makes the prey available to protozoa at higher concentrations.

3.3.6 Implications of this study

The cost associated with the treatment and disposal of excess sludge produced is the second greatest operational expense in wastewater treatment plants. Therefore, reducing sludge production during the treatment process provides an obvious economic benefit. In the present research, treatment of synthetic wastewater with the GAO dominated biofilm resulted in about 9-times less sludge production than that of conventional activated sludge process (Tchobanoglous et al., 2014). Further, the observed sludge yield described in this study is significantly lower than that of other biofilm processes found in literature (Table 3.1). Hence, next to the previously described benefits in saving 60-75% aeration energy (Flavigny & Cord-Ruwisch, 2015; Hossain et al., 2017), the described GAO biofilm system also offers saving in sludge disposal costs.

Table 3.1: Comparison of the excess sludge production in different wastewater treatment processes.

Treatment system	**HRT (h)**	**BOD removal efficiency (%)**	**Excess sludge production (g VSS g^{-1} BOD removed)**	**References**
CASP	12	85-95	0.40	Tchobanoglous et al. (2014)
HR-MBBR[a]	0.90	88	0.14[b]	Brosseau et al. (2016)
HFBR[a]	<0.5	97.4	0.13[b]	Clifford et al. (2010)
MBBR[a]	31.2	>95	0.09[b]	Li et al. (2014)
MBBR[a]	8	>90	0.22[b]	Aygun et al. (2008)
SBBR[a]	3.25	>98	0.05	This study

CASP: conventional activated sludge process, HR-MBBR: high-rate moving bed biofilm reactor, HFBR: horizontal flow biofilm reactor, MBBR: moving bed biofilm reactor, SBBR: sequencing batch biofilm reactor

[a] Synthetic wastewater used in all these studies.

[b] Calculated

Traditionally, aerobic digestion or extended aeration is used to minimize the excess sludge. By utilizing extremely long SRT in the described biofilm, aerobic digestion of sludge has become a part of the process and being one reason for the low sludge volume produced. Aerobic sludge minimization by aerobic digestion usually results in additional energy costs due to oxygen transfer to the sludge (Ghyoot & Verstraete, 2000). However, because of the direct oxygen transfer from the air, such aerobic digestion or extended aeration does not generate additional energy costs in the described biofilm. Thus, the GAO dominated biofilm process described in this research promises to lower the operating costs of wastewater treatment plant further.

Due to the modular design of biofilm systems, the biofilm process described in this study could be readily up-scaled and, for example, integrated upstream of innovative biological nitrogen removal steps such as the anammox process which is known to be impeded by the presence of BOD (Li et al., 2017). Such a combination of the GAO dominated biofilm with Anammox could be a promising low-energy approach in organic carbon and nitrogen removal from communal wastewater.

The present study was performed with synthetic wastewater with acetate being used as the sole BOD source, which makes it comparable to other proof-of concept studies using this widely used synthetic wastewater. While the synthetic wastewater used in the current study provides reproducible conditions it does not represent the complexity of the substrate that is present in real wastewater. Real wastewater is known to contain complex organic compounds and suspended material which can influence biofilm sludge yield. Therefore, future efforts verify the performance of this biofilm technology at pilot scale and under various real-world conditions using real primary effluent.

3.4 Conclusion

The idea of using a GAO enriched biofilm that after draining can use oxygen directly from the air does not only enable low energy air supply (no compressed air requirement and side-lining inefficient oxygen transfer via air bubbles) but also results in surprisingly high oxygen consumption leading to a very little excess biomass sludge production. The majority of bacteria (GAO) are nutritionally of higher food value (PHA and glycogen content) than ordinary heterotrophic bacteria. This could be the reason for the peculiar selective enrichment of high numbers of *Tetramitus* as the predator, that contributes to keeping overall biomass growth and excess sludge at a minimum. This biomass reduction by predation is similar to the "aerobic sludge digestion" obtained under extended aeration but with the difference that here no significant additional energy is required as the described novel "aerobic biomass digestion" uses oxygen directly from the air.

Chapter 4

Removal of Ammonium from Wastewater using Zeolite as an Ion-Exchange Material

Abstract

The present study investigates the effectiveness of ammonium removal from wastewater using untreated natural zeolites. Results showed that the rate of ammonium adsorption onto zeolite was very fast (> 70% removal) in the first 20 min and the maximum adsorption occurred at pH 5 when the ammonium ion was the predominant species in aqueous solution. The adsorption kinetics was best approximated by the pseudo-second order model, whereas the adsorption isotherm results indicated that Langmuir model provides the best fit for the equilibrium data. The maximum adsorption capacity of the studied zeolite was found to be 1.47 mg NH_4^+-N g^{-1} of zeolite.

4.1 Introduction

Wastewater originated from different types of industry and household contains various forms of organic and inorganic nitrogen species such as ammonium (NH_4^+-N). Ammonium is a growth limiting nutrient for algae, and the increasing amount of ammonium in water bodies contribute to the occurrence of several environmental problems such as eutrophication (Conley et al., 2009) resulting in the loss of biodiversity and dysfunction of aquatic ecosystem (Scherer & Pfister, 2016). Therefore, in order to protect the environment from these adverse effects, ammonium need to be removed from the wastewater.

Different processes such as biological nitrogen removal (BNR), air-stripping and ion-exchange are available for the removal of ammonium from wastewater. Compared with air-stripping and BNR, ion-exchange is a more promising technique because of its operational simplicity, little space requirement, less by-product formation and robustness to operation environment (Choi et al., 2016; Du et al., 2005; Widiastuti et al., 2011). Hence, ion-exchange seems to be an attractive method for ammonium removal especially when a low-cost ion-exchange material such as zeolite is used.

Recently zeolites have been actively explored in wastewater treatment plants due to its high adsorption capacity for ammonium in aqueous environments (Choi et al., 2016; Green et al., 1996; Hedström & Rastas Amofah, 2008; Jung et al., 2004; Weatherley & Miladinovic, 2004). The ammonium removal property of zeolite is attributed to the fact that zeolite is mainly composed of aluminosilicates with a three-dimensional structure bearing AlO_4 and SiO_4 tetrahedra, where substitution of each aluminum (Al^{3+}) for silicon (Si^{4+}) provides a negative charge on the zeolite framework (Englert & Rubio, 2005). The negative charge within the pores is balanced by positively charged ions such as Na^+, K^+, Ca^{2+}, and Mg^{2+} which are bound with the aluminosilicate structure by weaker electrostatic

bonds (Rožić et al., 2000). Thus, these cations can be easily exchanged with certain cations in solution such as ammonium ions (Rožić et al., 2000).

The cation exchange capacity of natural zeolite to remove ammonium from aqueous solution depends on its structural characteristics (Wang & Peng, 2010). Moreover, ammonium concentration in solution, contact time, zeolite loading and pH of the solution also affect the ion-exchange process (Englert & Rubio, 2005; Rožić et al., 2000). Therefore, a lab-scale study is needed to investigate the influence of these variables on zeolite ion-exchange performance when it is used for practical application.

The objective of the present chapter is to assess the adsorption capacity of ammonium by zeolite and to evaluate the effects of pH, contact time and initial ammonium concentration on the adsorption process. Furthermore, kinetic and equilibrium models are used to evaluate experimental data against theory. Physical and chemical tests are carried out to determine the mechanisms involved in the adsorption process.

4.2 Materials and Methods

4.2.1 Zeolite

The natural zeolite used in this study was obtained from a local supplier (Zeolite Australia Pty. Ltd.) and composed mainly of clinoptilolite and quartz. The zeolites were repeatedly washed with deionized water to remove adhering dirt and soluble impurities, dried at 105°C for 24 h. The dried zeolite was ground in a milling machine to a fine powder and passed through British Standard Sieves (BSS).

4.2.2 Batch adsorption tests

Adsorption batch tests were carried out using zeolite. Two types of batch experiments were conducted: one varying the ammonium concentration and keeping zeolite mass constant and the other varying zeolite mass and keeping the initial ammonium concentration the same. In the experiments with a constant zeolite mass, 100 mL flasks were filled with a fixed amount of zeolite. At the beginning of the experiment, pulses of ammonium were added to the flasks to have different initial ammonium concentrations. For the second type of experiment (same initial concentration), the flasks were filled with different amounts of zeolite. An ammonium pulse was added to have a similar final concentration in each flask. In both cases, the flasks were capped and agitated with a magnetic stirrer at 200 rpm. Samples were taken at different intervals to have an overview of the adsorption kinetics and maximum capacity of zeolite particles used in the present study.

The amount of ammonium adsorbed from the aqueous solution was expressed as ammonium removal capacity per unit mass of the zeolite (Q) as:

$$Q = \frac{(C_o - C_e)\,V}{m} \qquad (4.1)$$

where, C_o (mg L^{-1}) and C_e (mg L^{-1}) are the initial and equilibrium ammonium concentration in solution, respectively; m (g) is the mass of zeolite used; V (L) is the solution volume.

The percentage of ammonium removal efficiency from the aqueous solution was then calculated according to the following equation:

$$\text{Removal efficiency (\%)} = \frac{(C_o - C_e)}{C_o} \times 100 \quad (4.2)$$

4.2.3 Adsorption kinetics

To investigate the mechanism of adsorption, particularly the rate controlling step, the transient behaviour of the ammonium adsorption process was analyzed using the pseudo-first order, pseudo-second order and intra-particle diffusion model. Experimental data were processed and fitted to the kinetic equations.

4.2.3.1 Pseudo-first order model

The following equation determines the pseudo-first order model:

$$\frac{dQ_t}{dt} = K_1 (Q_e - Q_t) \quad (4.3)$$

Where, Q_t (mg g^{-1}) is the amount of ammonium adsorbed at time t (min), Q_e (mg g^{-1}) is the equilibrium adsorption capacity and K_1 (min^{-1}) is the rate constant of pseudo-first order model. The equation can be integrated by applying boundary conditions $Q_t = 0$ at $t = 0$ and $Q_t = Q_t$ at $t = t$, and the equation becomes

$$\ln (Q_e - Q_t) = \ln Q_e - K_1 t \quad (4.4)$$

The plot of $\ln (Q_e - Q_t)$ versus t for an adsorbent will yield a straight line if the adsorption process follows a pseudo-first order kinetic behaviour.

4.2.3.2 Pseudo-second order model

The pseudo-second order model can be expressed in the form of:

$$\frac{dQ_t}{dt} = K_2 (Q_e - Q_t)^2 \quad (4.5)$$

Where K_2 is the pseudo-second order rate constant (g mg^{-1} min^{-1}). After integrating and applying boundary conditions $Q_t = 0$ at $t = 0$ and $Q_t = Q_t$ at $t = t$, linear equation form can be obtained:

$$\frac{t}{Q_t} = \frac{1}{K_2 Q_e 2} + \frac{1}{Q_e} t \quad (4.6)$$

The initial sorption rate, h (mg g^{-1} min^{-1}) at $t \rightarrow 0$ can be defined as:

$$h = K_2 Q_e^2 \quad (4.7)$$

The initial adsorption rate (h), the equilibrium adsorption capacity (Q_e) and the pseudo-second order constant (K_2) can be determined experimentally from the slope and intercept of a plot of t/Q_t versus t (Ho & McKay, 1998; Reddad et al., 2002).

4.2.3.3 Intra-particle diffusion model

The intra-particle diffusion model is commonly used for identifying the adsorption mechanism for design purposes. According to Weber and Morris (1963), for most adsorption processes, the uptake varies almost proportionately with $t^{1/2}$ rather than with contact time and can be expressed as follows:

$$Q_t = K_{id} t^{0.5} + C \quad (4.8)$$

Where Q_t (mg g^{-1}) is the amount adsorbed at time t, and $t^{0.5}$ is the square root of the time and k_{id} (mg g^{-1} $min^{-0.5}$) is the intra-particle diffusion rate constant. A plot of Q_t against $t^{0.5}$

should give a straight line with a slope K_{id} and intercept C when adsorption mechanism follows the intra-particle diffusion process. The values of the intercepts indicate the thickness of the boundary layer, i.e., the larger the intercept, the greater is the boundary layer effect.

4.2.4 Adsorption isotherms

To simulate the adsorption isotherm, two most commonly used models, the Langmuir (Langmuir, 1918), and Freundlich was selected to elucidate the ammonium-zeolite interactions.

4.2.4.1 Langmuir isotherm

Langmuir adsorption isotherm model suggests that sorption takes place at specific homogenous sites within the adsorbent and a uniform distribution of energetic adsorption sites. As a consequence, once the adsorbate molecule occupies a site, no more sorption can take place. Langmuir model is valid for monolayer adsorption onto a surface with a finite number of identical sites. Langmuir parameters were estimated using the following formula:

$$\frac{Q_e}{Q_m} = \frac{bC_e}{1 + bC_e} \tag{4.9}$$

Where Q_e (mg g^{-1}) is the amount of ammonium adsorbed on the zeolite at equilibrium, Q_m (mg g^{-1}) is the maximum adsorption capacity, C_e (mg L^{-1}) is the aqueous phase concentration of the ammonium at equilibrium and b (L mg^{-1}) is the Langmuir constant related to the energy of adsorption. The linear form of Langmuir isotherm equation is represented by the following equation:

$$\frac{C_e}{Q_e} = \frac{1}{bQ_m} + \frac{C_e}{Q_m} \tag{4.10}$$

The maximum adsorption capacity and Langmuir constant were determined from the slopes and intercepts of the plots of C_e/Q_e against C_e. The essential features of Langmuir adsorption isotherm can be expressed as a dimensionless separation factor (R_L), which is derived as follows:

$$R_L = \frac{1}{1+bC_o} \qquad (4.11)$$

The R_L value indicates the shape of the isotherm to be favorable for adsorption ($0 < R_L < 1$) or unfavorable ($R_L > 1$).

4.2.4.2 Freundlich isotherm

Freundlich isotherm model assumes a heterogeneous surface with a non-uniform distribution of heat of adsorption over the surface and binding sites are not equivalent and/ or independent. Freundlich parameters were determined by the formula:

$$Q_e = K_F C_e^{1/n} \qquad (4.12)$$

Where K_F is a constant indicative of the adsorption capacity, and 1/n is the adsorption intensity, Q_e (mg g^{-1}) is the amount of ammonium adsorbed on the zeolite at equilibrium, C_e (mg L^{-1}) is the aqueous phase concentration of the ammonium at equilibrium. The linearization of equation (4.12) is given by equation (4.13) for Freundlich data fitting.

$$\text{Ln } Q_e = \text{Ln } K_F + \frac{1}{n} \text{Ln } C_e \qquad (4.13)$$

In general, when K_F value increases, the adsorption capacity of the bio-sorbent for a given adsorbate increases. Furthermore, the value of Freundlich exponent (n) in the range of 1-10, indicates a favorable adsorption. Linear plots of Ln Q_e versus Ln C_e allow the estimation of K_F and n values from the intercepts and the slope of the plot, respectively.

4.2.5 Desorption batch test

In order to investigate the regeneration of zeolite, desorption batch test was conducted. Firstly, an adsorption test was performed in which a certain amount of zeolite (10 g) was allowed to adsorb ammonium from synthetic wastewater. The equilibrium ammonium concentration was measured after the test, and the amount of ammonium adsorbed onto the zeolite was estimated based on the removed ammonium. The zeolite was sieved to remove all the bulk solution and transferred to flasks (100 mL) filled with 1% NaCl solution. The ammonium concentration was measured at regular time intervals to determine the regeneration efficiency.

4.2.6 Chemical analysis

The ammonium analysis was done according to the Nesslerization method (APHA, 2012). In 4 mL cuvette, 2 mL of samples were pre-treated with Mineral stabilizer and poly-vinyl alcohol-dispersing agent to inhibit the precipitation of calcium, magnesium, iron, and sulfide when treated with Nessler reagent. Then, 100 µL Nessler reagent (10%) was added to the mixture. The samples were mixed by rotating the cuvettes 180º three times and measuring the absorbance at 425 nm exactly after 1 min of reaction. A standard absorbance curve was determined for ammonium concentrations of 0 to 0.40 mmol L^{-1}, and the sample's concentration was determined using the sample's absorbance against the standard curve. The samples were diluted as required to fit the standard curve.

4.3 Results and Discussion

4.3.1 Adsorption batch tests

To investigate the effectiveness of zeolite used in the present study to remove ammonium from wastewater, the effect of contact time, zeolite mass, initial ammonium concentration, and pH was examined using a synthetic wastewater solution.

4.3.1.1 Effect of pH

The pH of the solution has an obvious impact on the removal of ammonium by the zeolite since it can influence both the character of the exchanging ions and the character of the zeolite itself (Du et al., 2005). Therefore, the effect of pH on the ammonium adsorption capacity of zeolite was evaluated over the pH range from 2 to 10 with an initial ammonium concentration of 42 mg L^{-1} (Figure 4.1A). The ammonium adsorption capacity of zeolite increased from 0.40 mg g^{-1} (pH 2) to 0.98 mg g^{-1} (pH 5), and further increase of pH decreased the adsorption capacity. This result is in accordance with those reported by Widiastuti et al. (2011) but in contradict with Du et al. (2005) who reported the highest ammonium removal at pH 6. At low pH, the ammonium ions compete with hydrogen ions for the exchange sites on the zeolite; while at high pH the ammonium ions are transformed to ammonia gas (Figure 4.1B). Therefore, it makes sense that the highest adsorption capacity of zeolite obtained in this study was at pH 5.

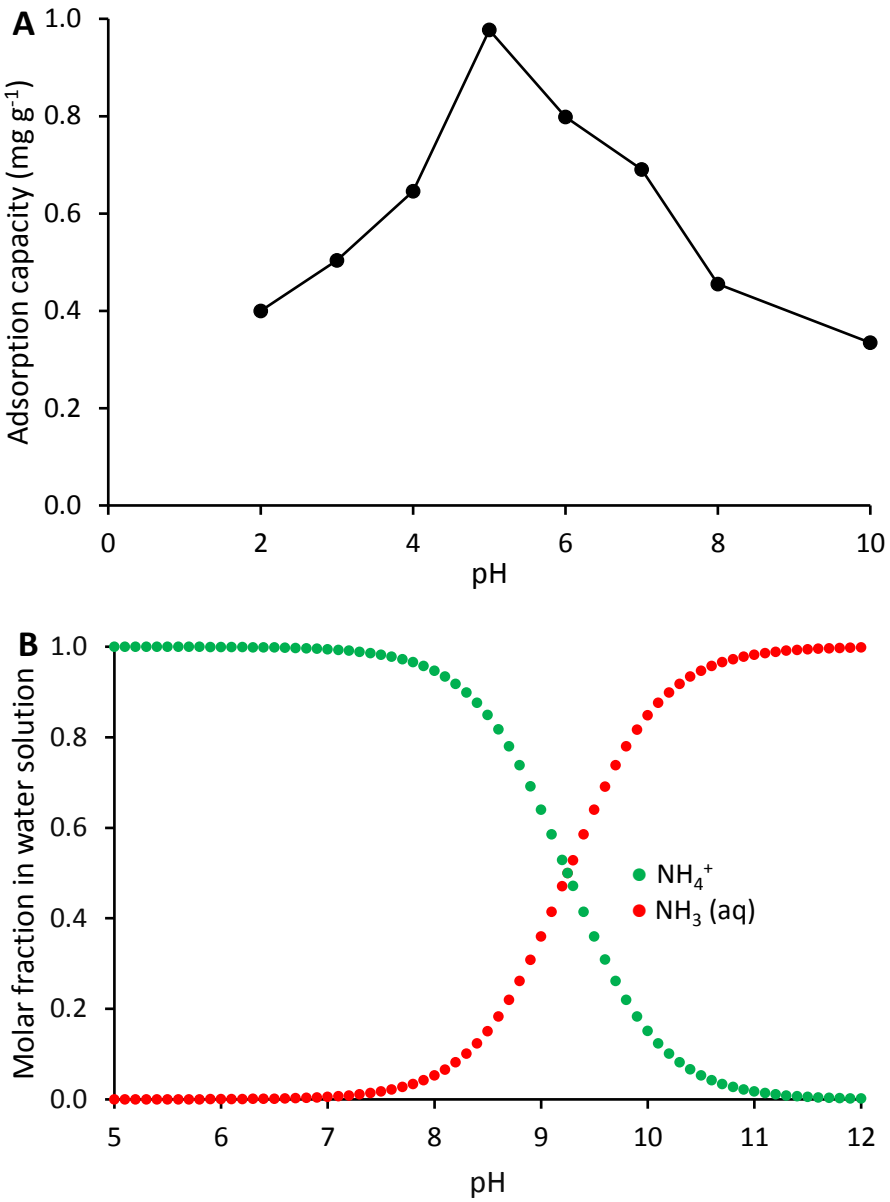

Figure 4.1: Effect of pH on ammonium removal capacity of zeolite (A) (initial ammonium concentration = 42 mg L^{-1}, mass of zeolite = 1 g, volume of solution = 100 mL, Temperature = 25°C, shaker speed = 200 rpm), and impact of pH on ammonium-ammonia equilibrium (B).

4.3.1.2 Effect of contact time

The effect of contact time on the adsorption of ammonium was investigated at four different initial ammonium concentrations, and results are presented in Figure 4.2. It was found that the adsorption capacity (mg of adsorbate per g of adsorbent) increases with increasing contact time. It was also found that the removal of ammonium by zeolite was

very fast with >70% of equilibrium reached in the initial 20 min and then its rate slowed down to approach equilibrium. The rapid ammonium removal has significant practical importance, as it enables the use of smaller reactor volumes, ensuring high efficiency and economy (Loukidou et al., 2004) and offers a much faster way to remove ammonium from wastewater (Lee et al., 2016).

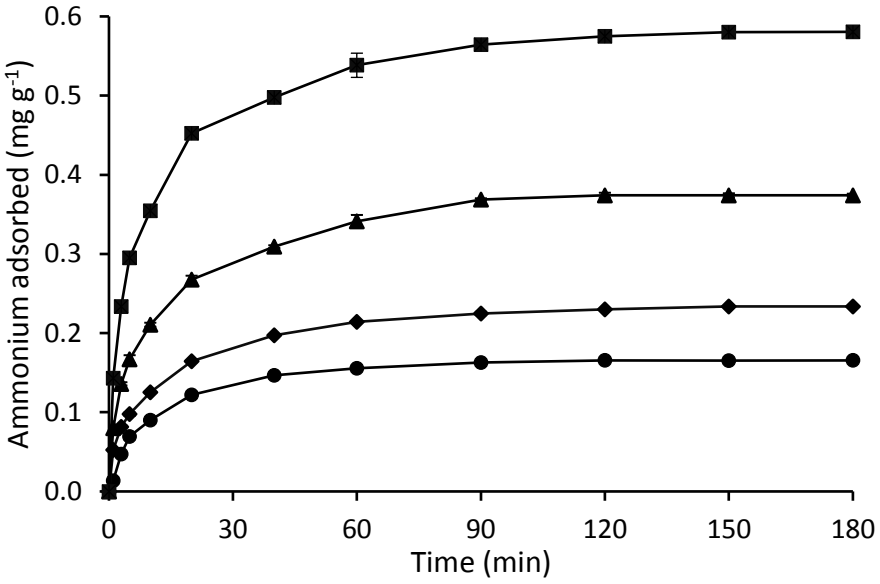

Figure 4.2: Effect of contact time on the adsorption process of ammonium onto zeolite at different initial ammonium concentration: (●) 10 mg L^{-1}, (♦) 20 mg L^{-1}, (▲) 40 mg L^{-1} and (■) 60 mg L^{-1} ammonium (mass of zeolite = 5g, volume of solution = 100 mL, Temperature = 25°C, shaker speed = 200 rpm).

4.3.1.3 Effect of zeolite dosage

The effect of zeolite dose on ammonium removal performance was investigated, and the results are shown in Figure 4.3. Results showed that at equilibrium the percentage of ammonium removal was increased from 14.4% to 94.2% with the increase of zeolite mass from 1 g to 50 g. The better removal of ammonium is because of an increase in zeolite mass results in the increase of available adsorption sites on the zeolite surface (Widiastuti et al., 2011). The adsorption of ammonium increased significantly with increasing zeolite until an equilibrium is reached. This is because the initial ammonium concentration was

constant at various amounts of zeolite loading. Hence, when the ammonium exchanged completely with cations on the zeolite surface at a certain amount of zeolite loading, the ammonium removal reached equilibrium (Widiastuti et al., 2011).

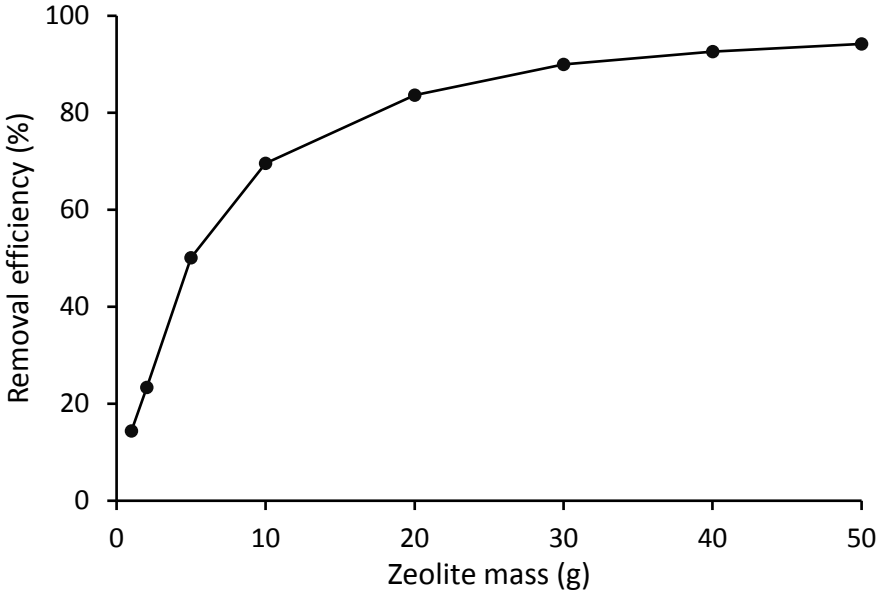

Figure 4.3: Effect of zeolite mass on ammonium removal capacity: initial ammonium concentration = 40 mg L^{-1}, volume of solution = 100 mL, Temperature = 25°C, shaker speed = 200 rpm.

4.3.1.4 Effect of initial ammonium concentration

The removal of ammonium from aqueous solutions largely depends on the initial ammonium concentration. The effect of ammonium removal performance by zeolite was investigated at different initial ammonium concentrations and results are shown in Figure 4.4. It was found that the amount of ammonium adsorbed increased from 0.19 to 1.33 mg g^{-1} with increasing initial ammonium concentration from 10 to 500 mg L^{-1}, respectively. It is because higher initial ammonium concentration provides the driving force to overcome the resistance of the mass transfer of the cation between the aqueous and the solid phase (Dawood & Sen, 2012). This driving force promotes the migration of ammonium ion from the external surface to the internal micro-pores of the zeolite within a given contact time (Widiastuti et al., 2011). The ammonium ion could exchange with cations

not only on the external surface of the zeolite but also on the internal surface of the zeolite. The equilibrium was reached when all the exchangeable ammonium and cation on the external and internal surfaces of the zeolite were reached.

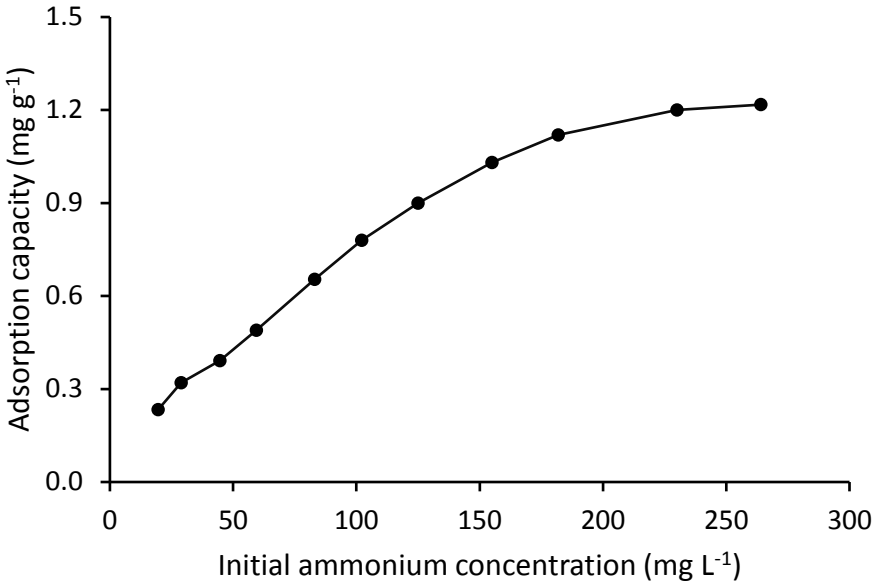

Figure 4.4: Effect of initial ammonium concentration on the amount of ammonium adsorption (removal) onto zeolite: mass of zeolite = 5 g, volume of solution = 100 mL, Temperature = 25°C, shaker speed = 200 rpm.

4.3.2 Adsorption kinetic modeling

The prediction of adsorption kinetics of ammonium onto zeolite is of critical importance for the design of industrial treatment systems. In the present study, three kinetic models, pseudo-first order, pseudo-second order and intra-particle diffusion models were examined to fit the experimental data and to investigate the mechanism of adsorption, which depends not only on the physical or chemical properties of the adsorbent but also on the mass transport process.

4.3.2.1 Pseudo-first and pseudo-second order model

Both the pseudo-first and pseudo-second order model was fitted with experimental data in the present study. The best-fit kinetic model was selected based on both linear regression correlation coefficient (R^2) and the calculated Q_e values. According to the kinetic model results, the correlation coefficients for Lagergren pseudo-first order model obtained at all studied initial ammonium concentrations were low, and the calculated Q_e did not give acceptable values when compared to the experimental ones (data not shown). Hence, the reaction involved in the adsorption of ammonium onto zeolite particles is not the first-order.

The pseudo-second order model, in contrast, showed the best-fit to the experimental data with the highest squared correlation coefficient. Moreover, as shown in Table 4.1, the predicted Q_e values were closer to the experimental results suggesting that the pseudo-second order model provides the best correlation for the adsorption of ammonium onto zeolite. This observation is in line with Widiastuti et al. (2011) who reported the best prediction of ammonium biosorption onto zeolites by pseudo-second order model.

Table 4.1: Pseudo-second order kinetic parameter for ammonium adsorption onto zeolite.

Initial NH_4^+-N conc. (mg L^{-1})	Experimental Q_e (mg g^{-1})	K_2 (g mg^{-1} min^{-1})	Calculated Q_e (mg g^{-1})	h (mg g^{-1} min^{-1})	R^2
10	0.195	1.184	0.201	0.048	0.999
20	0.234	0.624	0.242	0.037	0.999
30	0.304	0.333	0.322	0.034	0.996
40	0.397	0.342	0.403	0.056	0.997
50	0.581	0.350	0.595	0.124	0.999

4.3.2.2 Intra-particle diffusion model

To design and control an adsorption system, it is necessary to elucidate the underlying mechanisms that result in the apparent dynamic behaviour of the system. The experimental data were fitted to an intra-particle diffusion model (Eq. 4.8) to identify the mechanism involved in adsorption. The plot of adsorption capacity, Q_t (mg g^{-1}) of zeolite against the square root of time ($t^{0.5}$) is shown in Figure 4.5 for different initial ammonium concentration.

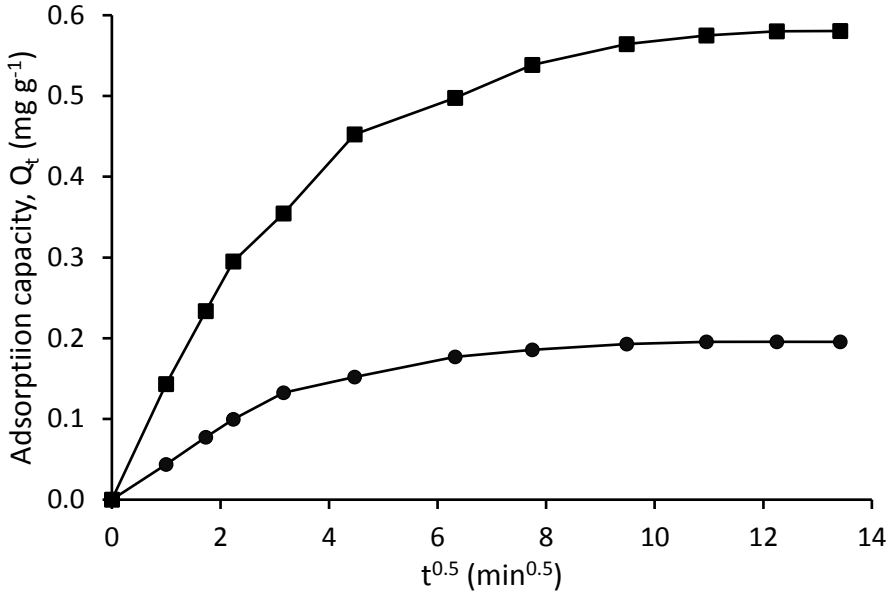

Figure 4.5: Intra-particle diffusion model on different initial ammonium concentration: (●) 10 mg L^{-1}, and (■) 60 mg L^{-1} ammonium.

Figure 4.5 shows that the adsorption of ammonium ions onto zeolite particles is not linear over the whole-time range and can be separated into three regions which confirm that adsorption was a multi-step process. At the beginning of the experiment (first phase), ammonium molecules were transported to the external surface of the zeolite particles through film diffusion at a very fast rate. As the time progress, ammonium molecules adsorbed onto zeolite particles by intra-particle diffusion through pores (second phase) followed by the final equilibrium stage (third phase).

4.3.3 Adsorption isotherms

Adsorption isotherm describes how the adsorbate interacts with the adsorbent and gives an idea of the adsorption capacity of the adsorbent. In the present study, two isotherms (Langmuir and Freundlich) were tested to find the most appropriate correlation of the experimental equilibrium curves. The Langmuir and Freundlich adsorption constants and the corresponding correlation coefficients are listed in Table 4.2.

Table 4.2: Adsorption isotherms tested.

Isotherm models	**Parameters**		
Langmuir	Q_m (mg g^{-1})	b (L mg^{-1})	R^2
	1.474	0.023	0.9911
Freundlich	n	K_F	R^2
	2.749	0.162	0.9173

The higher correlation coefficient of the Langmuir isotherm (Table 4.2) than that of Freundlich isotherm suggest that Langmuir model better described the biosorption equilibrium of ammonium onto zeolite particles. This result indicates that removal of ammonium ions from synthetic wastewater occurred on homogenous zeolite surfaces by monolayer sorption without interactions between the adsorbed ammonium molecules and is in accordance with previous reports (Wahab et al., 2010; Wang et al., 2006). Also, the calculated R_L value for initial ammonium concentration of 10 and 50 mg L^{-1} was estimated to 0.812 and 0.024, respectively, which confirms that the zeolite used was favorable for ammonium adsorption.

The maximum adsorption capacity (Q_m) determined from the Langmuir isotherm was calculated to be 1.47 mg g^{-1} of zeolite (Figure 4.6). This relatively high adsorption capacity indicates that the studied zeolite is more efficient than some natural zeolites (0.9 mg g^{-1}) (Wu et al., 2006), (1.27 mg g^{-1}) (Wang et al., 2006) and could be considered as a

promising adsorbent for the removal of ammonium from wastewater. It should be noted that the present study used raw and untreated zeolites which would be expected to have a lower adsorption capacity than the modified ones. Treatment of zeolites with chemicals (e.g., acids, alkali, and salts of alkaline metals, etc.) improves the ion-exchange and adsorption properties as well as purity of zeolite (Li et al., 2011).

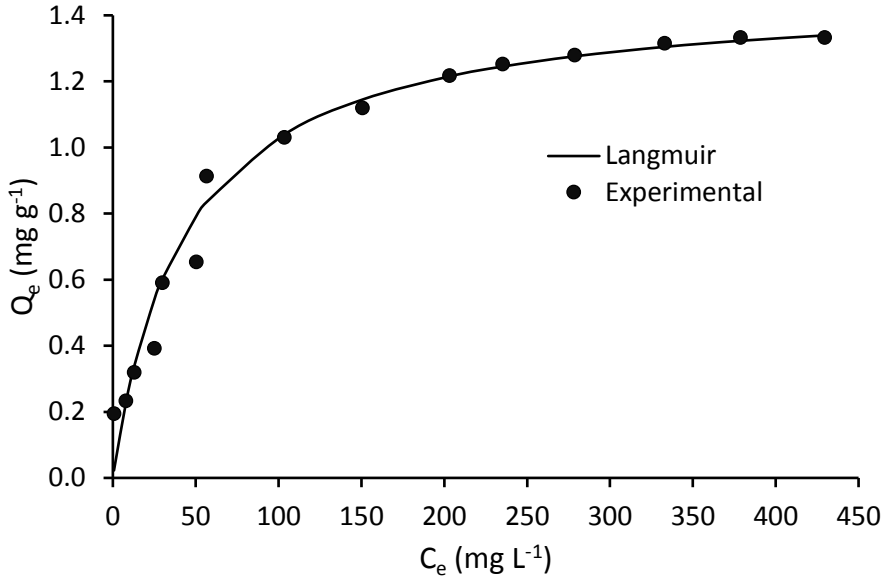

Figure 4.6: The Langmuir adsorption isotherm of ammonium curve.

4.3.4 Desorption batch test

The regeneration of ammonium spent zeolite was investigated by performing desorption batch experiment. First, ammonium was allowed to adsorb to the zeolite (10 g) by incubation at an initial ammonium concentration of 42 mg L^{-1}. After adsorption 11.74 mg L^{-1} remained, giving an adsorbed amount of 0.3 mg NH_4^+-N g^{-1} zeolite. In the subsequent desorption test, the zeolite was incubated in 1% NaCl solution. During desorption process, the ammonium exchanged on the zeolite was released from the zeolite into the solution and the sodium ion in the other direction as shown in the following equation:

$$NH_4 - Zeolite + Na^+ \longrightarrow Na - Zeolite + NH_4^+ \quad (4.14)$$

The ammonium concentration in the bulk solution increased to 27.24 mg L^{-1} during 2 h of the experiment (Figure 4.7). From this test it was concluded that about 90% of the adsorbed ammonium was desorbed, indicating efficient regeneration of zeolite. This observation is in line with Widiastuti et al. (2011) who also reported similar zeolite regeneration efficiencies.

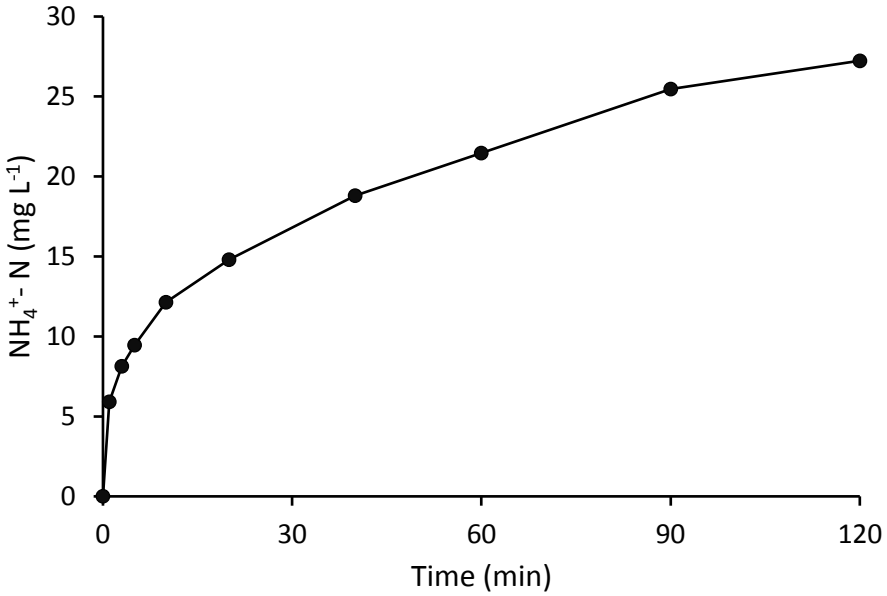

Figure 4.7: Desorption batch test using 1% NaCl solution.

4.3.5 Effect of competing cations on ammonium adsorption

Wastewater contains different cations which might affect the net adsorption of ammonium ion onto zeolite. The cations which are present in higher concentration in synthetic wastewater are: Na^+ and Ca^{2+} (Table 4.3). However, the selective affinity of zeolite determines which cations are selectively adsorbed onto zeolite (Sarioglu, 2005; Wang & Peng, 2010; Widiastuti et al., 2011).

$Cs^+ > K^+ > NH_4^+ > H^+ > Na^+ > Ca^{2+} > Fe^{3+} > Al^{3+} > Mg^{2+}$

Based on affinity constant (as shown above), ammonium is preferentially adsorbed onto zeolite compared with most cations present in wastewater, with the exception of K^+. Hence, the zeolite used in this study seems to be a suitable ion-exchange material for efficient removal of ammonium from wastewater.

Table 4.3: Concentration of different cations present in synthetic wastewater used in the current study.

Cations	Concentrations	
	mmol L^{-1}	**meq L^{-1}**
Na^+	9.5	9.5
NH_4^+	3.0	3.0
K^+	0.3	0.3
Ca_2^+	2.0	4.0
Mg_2^+	0.1	0.2

4.3.6 Practical implications

The present study showed that zeolite is very efficient to remove ammonium from aqueous solution. Nevertheless, the zeolite cannot adsorb ammonium indefinitely, and the sustained ammonium removal depends on the successful regeneration of zeolite which can be done either by biological or chemical methods. Chemical regeneration often involves the use of concentrated salt solutions (regenerant solution) to achieve a rapid desorption of ammonium from zeolite (Lahav & Green, 1998). However, waste bine produced during chemical regeneration of zeolite presents a disposal problem due to its very high salt concentrations (Aponte-Morales et al., 2016; Choi et al., 2016; Doula, 2009; Faghihian & Kabiri-Tadi, 2010). On the other hand, zeolites can be efficiently regenerated using nitrifying biomass in a process called bio-regeneration (Semmens et al., 1977). Nitrifying bacteria oxidize the adsorbed ammonium to nitrate, resulting in zeolite regeneration. However, the nitrate produced need to be further treated before its disposal. The biological reduction of nitrate to nitrogen gas required carbon source supplementation which

would increase the total treatment cost. Therefore, it is highly desirable to develop alternative treatment technology that requires no carbon addition and which removes ammonium efficiently from wastewater.

4.4 Conclusions

The following conclusions could be drawn based on the results presented in the current study:

- The studied zeolite showed the maximum adsorption capacity at a pH of 5 when the ammonium ion was the dominant species in aqueous solution.
- The removal of ammonium by zeolite was fast in the initial 20 min and reached an equilibrium state within 120 min.
- The ammonium removal efficiency increased with an increase in the initial ammonium concentrations and zeolite mass.
- The adsorption kinetics was best fitted with the pseudo-second order model.
- Equilibrium data were very well represented by Langmuir isotherm, and the maximum adsorption capacity was found to be 1.47 mg NH_4^+-N g^{-1} of zeolite.

Chapter 5

Characterization and Optimization of Passive Aeration SND as Novel Low-Energy Wastewater Treatment System

Abstract

In this chapter, a novel biofilm system was developed by incorporating zeolite (an ion-exchange material) particles and activated sludge (source of nitrifying bacteria) into the previously established GAO biofilm. Zeolite and GAO biofilm achieved the simultaneous NH_4^+-N and organic carbon (BOD) removal, respectively under anaerobic condition. The exposure of the biofilm directly to atmospheric air in the subsequent aerobic stage enabled the regeneration of zeolite via simultaneous nitrification and PHA based denitrification. The biofilm process showed a stable >90% BOD and about 70% nitrogen removal efficiency which were attributed to the increased abundances of GAOs and nitrifying bacteria, respectively in the biofilm during the experimental period. Overall, the proposed biofilm process demonstrated a long-term soluble organic carbon and total nitrogen removal efficiency at a constant rate of 137.13 g BOD m^{-3} d^{-1} and 17.26 g NH_4^+-N m^{-3} d^{-1}, respectively. Since oxygen was provided by exposing the biofilm directly to air, the proposed technology could significantly reduce the energy cost associated with aeration leading to a high energy-efficient wastewater treatment process.

5.1 Introduction

The discharge of municipal and industrial wastewater containing reactive nitrogen species into natural water bodies is responsible for environmental problems including eutrophication, loss of biodiversity and dysfunction of aquatic ecosystem (Conley et al., 2009; Dodds et al., 2009; Scherer & Pfister, 2016). Hence, to protect the environment from these adverse effects, nitrogen needs to be removed from the wastewater prior to its disposal. The biological nitrogen removal (BNR) principally involves two conversion process: nitrification and denitrification. During the aerobic nitrification process, nitrifying bacteria including ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) oxidize ammonium to nitrite and nitrite to nitrate, respectively. These oxidized nitrogen components are then reduced to nitrogen gas in the anoxic denitrification stage (Tchobanoglous et al., 2014). However, the energy-intensive transfer of oxygen into the bulk wastewater to enable nitrification and carbon supplementation for denitrification is a key operational cost of a wastewater treatment plants (Xie et al., 2017). Therefore, it is highly desirable to find alternative nitrogen removal technologies with reduced energy and organic carbon requirements.

Simultaneous nitrification and denitrification (SND), an attractive alternative technology for efficient removal of nitrogen from wastewater, has the potential of significantly reducing both energy and carbon requirement for BNR (Camejo et al., 2016; Third et al., 2003a; Wang et al., 2015b). SND requires a slowly degradable carbon substrate which provides the reducing power during the denitrification process. Bacterial intracellular storage polymers such as poly-hydroxyalkanoate (PHA) and glycogen are degraded at much slower rate than the soluble organic substrate and can be used as the electron source for denitrification when no external substrate is available (Third et al., 2003a). There are

two groups of bacteria which can accumulate PHA intracellularly: polyphosphate accumulating organism (PAO) and glycogen accumulating organism (GAO).

The PAO bacteria are responsible for the removal of phosphorus in enhanced biological phosphorus removal (EBPR) process with alternating anaerobic and aerobic stages. PAOs anaerobically take up organic carbon and convert into PHA, using the energy obtained from the hydrolysis of polyphosphate and glycogen. In the subsequent aerobic phase, PAOs oxidize stored PHA to generate energy for phosphorus uptake, glycogen replenishment, and growth (Mino et al., 1995; Mino et al., 1998; Smolders et al., 1994a). GAOs can also uptake and store soluble organic carbon as PHA under anaerobic condition, and when oxygen or nitrate becomes available to use the accumulated PHA for glycogen replenishment and growth (Zeng et al., 2002). Since GAOs do not contribute to the phosphorus removal and compete with PAOs for the substrate, they are considered as undesirable microorganisms in EBPR systems (Oehmen et al., 2006).

Recently, GAOs were found to play an important role in energy efficient removal of organic carbon from wastewater. Flavigny and Cord-Ruwisch (2015) described a passively aerated GAO enriched biofilm system which has shown the stable and rapid removal of organic compounds (BOD) with 60-75% less aeration requirement than that of conventional activated sludge process. The incorporation of an ion-exchange material (i.e., zeolite) and concentrated nitrifying bacteria into the described GAO biofilm enabled simultaneous BOD and nitrogen removal by Passive Aeration Simultaneous Nitrification and Denitrification (PASND) from wastewater (Appendix 1). However, the study used high concentrations of previously enriched cultures, and there was no indication of the likelihood of such biofilm development from standard activated sludge. This gap might limit the application of this novel technology in the real world.

This current study, therefore, aims to develop a zeolite amended GAO biofilm system using activated sludge as the sole inoculumn to investigate its real-world feasibility of enabling extremely low-energy oxygen supply and nitrogen removal via PASND. Using widely used synthetic wastewater, the organic carbon and nitrogen removal performance of this system was investigated over 120-days of operation in a laboratory-scale biofilm reactor. The study also explores the nutrient removal mechanisms of the biofilm system and, the succession of microbial population that resulted in a stable performance of carbon and nitrogen removal.

5.2 Materials and Methods

5.2.1 Zeolite

The natural zeolite used in this study was obtained from a local supplier (Zeolite Australia Pty. Ltd.) and composed mainly of clinoptilolite and quartz. The zeolites were repeatedly washed with deionized water to remove adhering dirt and soluble impurities, dried at 105°C for 24 h. The dried zeolite was ground in a milling machine to a fine powder and passed through British Standard Sieves (BSS). Zeolite particles with size of 75 μm was used in the current study.

5.2.2 Experimental setup and operation process

A tubular laboratory-scale reactor made up of methyl methacrylate with a working volume of 0.755 L was used in this study (Figure 5.1). The reactor was filled (20% $V_{carrier}/V_{reactor}$) with packing material (AMB^{TM} Biomedia Bioballs) containing biofilm enriched with glycogen accumulating organism (GAO) as described in Hossain et al. (2017). Then, 10 g of the zeolite powder (75 μm) was suspended in synthetic wastewater solution and trickled over the GAO biofilm coated packing materials for 24 h until most of the suspended zeolite powder (>99%) was adsorbed onto the biofilm (indicated by the optical density). In order to introduce nitrifying bacteria into the biofilm that could be emulated in real plants, 100 mL of activated sludge (collected from Subiaco Wastewater Treatment Plant, Western Australia) was trickled over the biofilm for 24 h after 5 cycles of initial testing. This zeolite amended hybrid biofilm reactor which aims at enabling simultaneous nitrification and denitrification by using oxygen directly from the air (passive aeration) is hereafter referred to as passive aeration SND (PASND) reactor. The temperature of the reactor was kept at around 25 ± 2°C.

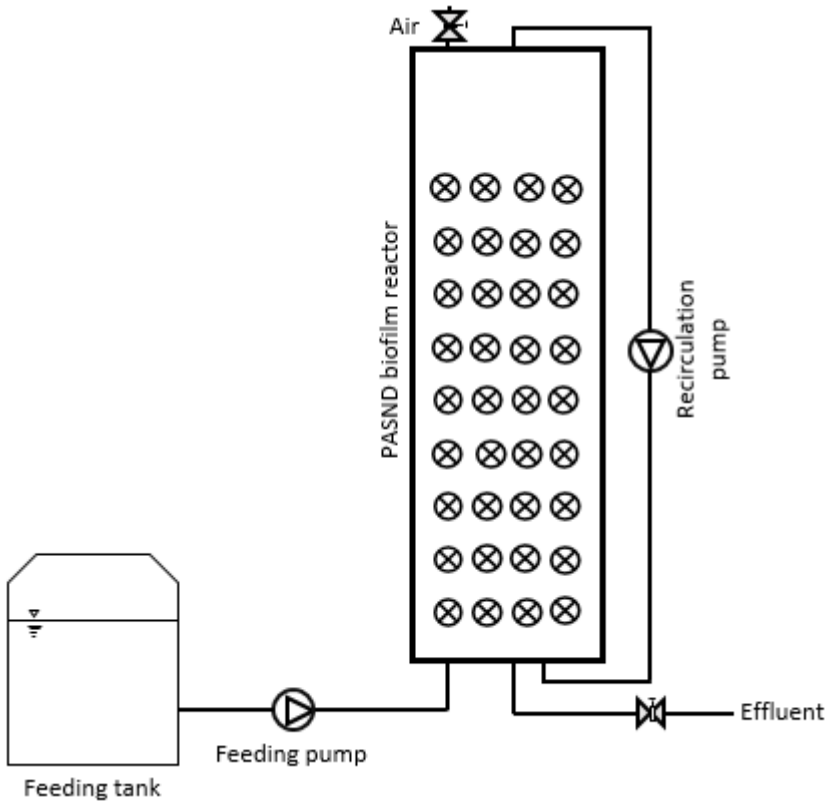

Figure 5.1: The schematic of the passive aeration simultaneous nitrification and denitrification (PASND) biofilm reactor.

The zeolite amended PASND reactor was operated under sequential anaerobic and aerobic two-stage operation mode. During the anaerobic (feast) phase, the reactor was fully loaded with synthetic wastewater (full void volume) and remained in the anaerobic condition for a specific period to adsorb organic carbon and ammonium. The anaerobically treated wastewater was drained out of the reactor by gravity to begin the aerobic (famine) stage. The top of the reactor was open to enable oxygen passively entering the reactor, whereby the biofilm was exposed to atmospheric oxygen. To enable monitoring of nitrogenous compounds in the aerobic stage (after draining), a small volume of the residual liquid (about 20 mL) was slowly recirculated through the biofilm reactor from the top to the bottom.

In the current study, the zeolite amended PASND reactor was operated under decreasing hydraulic retention time (HRT) over a period of 120 days, and the parameters are summarized in Table 5.1.

Table 5.1: Operational conditions of the biofilm reactor system.

Items	**Phase I (0 - 30 d)**	**Phase II (31 - 55 d)**	**Phase III (56 - 90 d)**	**Phase IV (91 - 120 d)**
Anaerobic time (min)	360	180	180	120
Aerobic time (min)	360	360	180	180
Treatment time (h)	12	9	6	5
OLR (g BOD m^{-3} d^{-1})	120-143	154-172	240-264	284-324
NLR (g N m^{-3} d^{-1})	10-11	13-14	19-21	23-25

OLR: organic loading rate; NLR: nitrogen loading rate

The reactor was operated twice (Phase I) to about 5 times (Phase IV) per day during the experimental period

5.2.3 Synthetic wastewater

In this study, synthetic wastewater (Third et al., 2003b) was used in order to maintain tight control and complete knowledge of influent constituents so as to facilitate testing of feasibility (proof-of-concept) of combining biological organic carbon and nitrogen removal. The concentrated synthetic wastewater was prepared in two separate solutions using deionized (DI) water. The first solution contained acetate as carbon source, while the other contained the required nutrients, minerals and trace elements. After dilution with DI water, the influent wastewater contained (mg L^{-1}): CH_3COONa 660, NH_4Cl 160, KH_2PO_4 44, $NaHCO_3$ 125, $MgSO_4$. $7H_2O$ 25, $CaCl_2$. $2H_2O$ 300, $FeSO_4$. $7H_2O$ 6.25, yeast extract 50, and 1.25 ml L^{-1} of trace element solution, which contained (g L^{-1}): EDTA 15, $ZnSO_4$. $5H_2O$ 0.43, $CoCl_2$. $6H_2O$ 0.24, $MnCl_2$. $4H_2O$ 0.99, $CuSO_4$. $5H_2O$ 0.25, $NaMoO_4$. $2H_2O$ 0.22, $NiCl_2$. $6H_2O$ 0.19, $NaSeO_4$. $10H_2O$ 0.21, H_3BO_4 0.014 and $NaWO_4$. $2H_2O$ 0.050.

5.2.4 Anoxic batch tests

To determine the denitrification ability and pathway of the GAO bacteria present in the biofilm, anoxic batch experiments were conducted with nitrite and nitrate according to Bassin et al. (2012a). A small number of carriers coated with the biofilm was taken from the reactor immediately after the anaerobic phase (i.e., after the accumulation of intracellular PHA) using wastewater devoid of NH_4^+-N. The carriers were washed with deionized water. Equal amounts of biomass (5 carriers) were placed in a series of 50 ml flasks filled with the same synthetic media fed to the reactor (except acetate). Nitrogen gas was supplied to each flask to maintain anaerobic conditions. A spike of either a concentrated nitrite or a nitrate solution was dosed to obtain a final concentration of 14 mg L^{-1}. From this point forward, samples were taken at regular intervals for determination of nitrite and nitrate concentration. The flasks containing biomass were maintained at the same operating temperature (i.e., room temperature).

5.2.5 Analytical parameters of interest

The concentrations of chemical oxygen demand (COD), ammonium (NH_4^+-N), nitrite (NO_2^--N) nitrate (NO_3^--N), and orthophosphate (PO_4^{3-}-P) in the influent and effluent of the biofilm reactor were regularly measured to monitor system performance. Ammonium, nitrite, nitrate, orthophosphate, and COD concentrations were determined in accordance with Standard Methods (APHA, 2012). The acetate was measured by Gas Chromatography (Agilent, USA) according to the method used in Hossain et al. (2017). Freeze dried biomass was used to measure polyhydroxyalkanoates (PHAs) and glycogen. PHAs were determined by the sum of poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV), which were analysed as previously reported (Smolders et al., 1994a). Glycogen was analyzed according to the method used by Hossain et al. (2017). Total suspended

solids (TSS), and volatile suspended solids (VSS) were measured according to the Standard Methods (APHA, 2012). pH and DO were measured using a pH meter and a DO meter (Mettler-Toledo, USA), respectively.

5.2.6 Microbial community investigation

Biofilm samples were collected at a different time during the operational period to reveal the evolution of the microbial community of the biofilm. Genomic DNA was extracted from these samples using the Power Soil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instruction, and quantified by spectroscopic methods (NanoDrop 2000, Thermo Fisher Scientific, USA). Bacterial 16S rRNA genes were PCR-amplified with barcoded forward primer 515F and reverse primer 806R (Caporaso et al., 2012). For each sample, polymerase chain reaction was carried out in a 25 μL total volume including 2.5 μL of normalized total genomic DNA (5 ng/μL), 0.2 μM of each primer and 12.5 μL of 2x KAPA HiFi HotStart Ready Mix (Kappa Biosystems, USA). The PCR cycling protocol consisted of an initial denaturation step of 95°C for 3 min, followed by 35 cycles of DNA denaturation at 95°C for 30s, primer annealing at 55°C for 30s, strand elongation at 72°C for 30s, and a final elongation step at 72°C for 5 min. All samples were amplified in triplicate, pooled and visualized in agarose gel. Combined PCR products were purified using the AMPure XP beads (Beckman Coulter, USA) and final amplicon concentrations were quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). Amplicons from all sample were pooled in equimolar ratios and sequenced on the MiSeq platform (Illumina, USA).

The forward and reverse reads were merged and filtered based on minimum lengths and expected errors, as specified in the USEARCH pipeline (Edgar, 2010). To identify bacterial genera present in samples, operational taxonomic units (OTUs) were selected by clustering sequencing at 97% similarity with the UPARSE algorithm and chimeric sequences were removed using UCHIME (Edgar, 2013). Taxonomy was assigned to OTUs against the Greengenes 16S database (August 2013 release) (DeSantis et al., 2006) in QIIME 1.9.1 (Caporaso et al., 2010) using the UCLUST algorithm (Edgar, 2010) with default parameters.

Statistical analysis of the experimental data was conducted using the SPSS software package (IBM-SPSS v22). A one-way analysis of variance (ANOVA) was used to test whether a certain factor had an impact on the observed variable. Pearson's correlation coefficient was applied to quantify the relationship between two parameters. An $\alpha = 0.05$ and $p < 0.05$ indicated statistically significance.

5.3 Results and Discussion

5.3.1 Addition of zeolite to GAO biofilm enables N removal

A glycogen accumulating organism (GAO) biofilm enriched from activated sludge had been demonstrated previously to uptake organic carbon (BOD) anaerobically from wastewater and store it as poly-hydroxyalkanoate (PHA) (Hossain et al., 2018; Hossain et al., 2017). The same GAO biofilm was used in the current study as the start-up. The biofilm reactor with GAO enriched biofilm was operated by exposing the biofilm to the wastewater in an initial anaerobic period of 2 hours. The subsequent aerobic phase was initiated by draining of the liquid to expose the biofilm to air and provide passive aeration. This enabled the GAOs to respire oxygen directly from the air and oxidize the intra-cellular organic reserves (i.e., PHA) stored in the anaerobic period resulting cell growth and the renewal of the anaerobic BOD storage capacity. However, the developed biofilm could not remove nitrogen components (i.e., NH_4^+-N) from the wastewater (Figure 5.2A).

To enable anaerobic nitrogen removal next to the soluble BOD storage by the GAO biofilm, an ammonium adsorbing ion-exchange material (zeolite) known for its ammonium adsorption capacity was incorporated into the biofilm by adding a defined quantity (10 g) and grain size (75 µm) of the material to the biofilm until it was adsorbed into the matrix of the biofilm. During anaerobic exposure of this zeolite amended GAO biofilm, significant ammonium was spontaneously adsorbed by the zeolite as expected from Langmuir kinetics. The zeolite amended GAO biofilm removed 26.8 mg L^{-1} NH_4^+-N within 2 h by adsorption of NH_4^+-N onto the incorporated zeolite particles (Figure 5.2A). In comparison, the GAO biofilm without zeolite removed only 3.1 mg NH_4^+-N L^{-1}. It is worth to note that, the addition of zeolite compromised the BOD uptake by only about 6 percent in this first experimental run (Figure 5.2B).

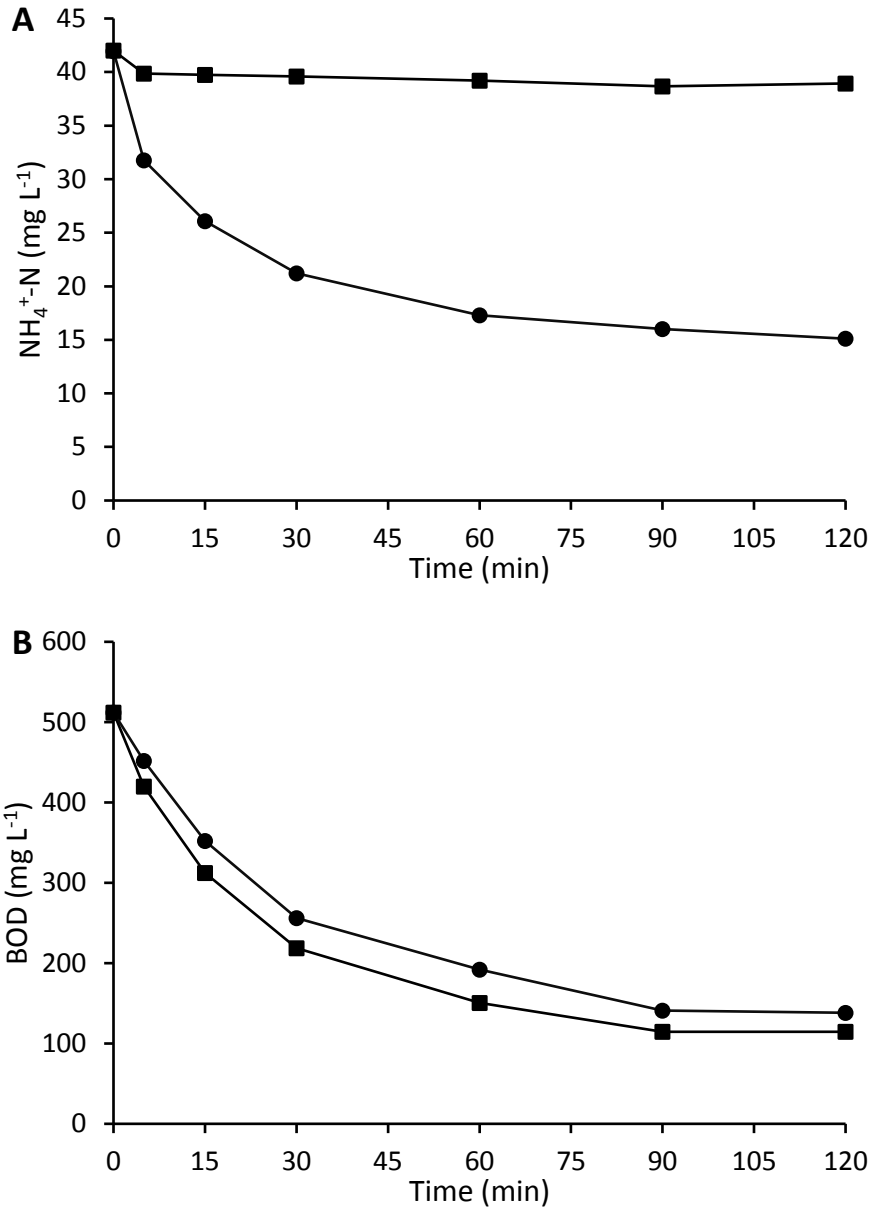

Figure 5.2: Removal of (A) NH_4^+-N and (B) organic carbon (BOD) by a biofilm enriched with GAO bacteria before (■) and after (●) zeolite powder addition.

5.3.2 Bio-regeneration of immobilized zeolite using activated sludge enables sustained N removal

While immediately upon incorporating zeolite within the GAO biofilm, about 67% of NH_4^+-N was adsorbed, in subsequent cycles the percentage of ammonium adsorbed gradually decreased (Figure 5.3, Cycles 1-5) which is in line with Langmuir predictions of exhausting the zeolite's adsorption capacity. Insufficient zeolite regeneration during the aerobic phase can explain the reduced ammonium removal during the anaerobic phase. Therefore, improved aerobic zeolite regeneration was necessary to maintain efficient ammonium adsorption from wastewater.

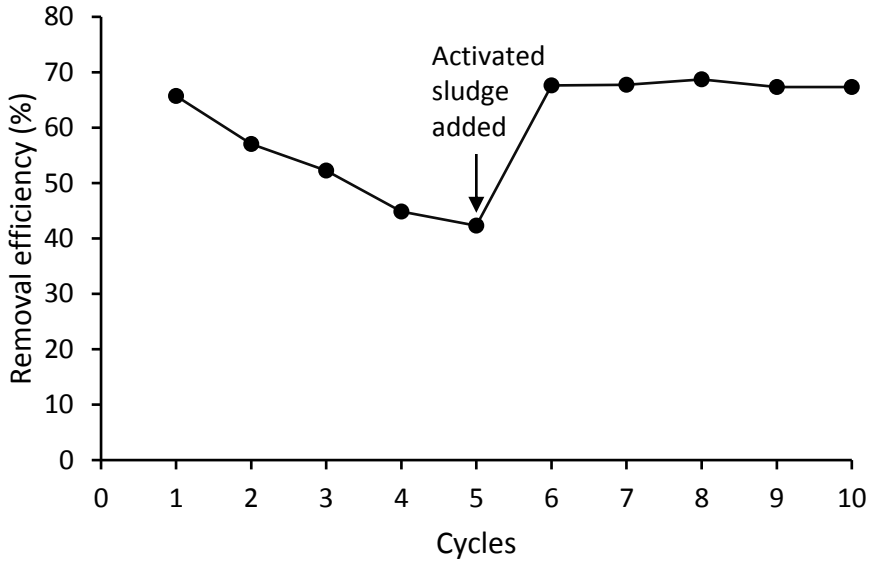

Figure 5.3: NH_4^+-N removal efficiency by the zeolite particles before and after addition of activated sludge into the zeolite amended GAO biofilm.

It is well known that the regeneration of zeolite can be accomplished by biological ammonium oxidation via nitrifying bacteria (Aponte-Morales et al., 2018; Aponte-Morales et al., 2016; Jung et al., 2004; Jung et al., 1999; Park et al., 2002). However, it is

also known that ammonium oxidation by itself results in a pH drop which adversely affects nitrification (Tchobanoglous et al., 2014). To provide nitrification capacity to the biofilm under realistic real-world implementable conditions, 100 mL of fresh activated sludge containing nitrifying bacteria (NH_4^+-N oxidation capacity of 6.73 mg L^{-1} h^{-1}) was added to the zeolite amended GAO biofilm reactor by continued trickling over the existing biofilm for 24 h at which the activated sludge suspension had cleared up to less than OD_{600} 0.10 suggesting all activated sludg biomass had been adsorbed into the biofilm. Immediate exposure of this newly amended biofilm to a new anaerobic phase showed that the anaerobic NH_4^+-N adsorption efficiency had been recovered (Figure 5.3, Cycles 6). In subsequent cycles this NH_4^+-N adsorption efficiency stayed stable (Figure 5.3, Cycles 6-10) suggesting a sustained bioregeneration of the zeolite during the aerobic phase was accomplished by bacterial ammonium oxidation. This behaviour is similar to that observed for a synthetic GAO dominated biofilm with zeolite as ion-exchanger, for which helium gas based studies have shown the occurrence of simultaneous nitrification and denitrification during the aerobic phase (Flavigny, 2015).

5.3.3 Long-term stability of the process

A long-term performance of the described system for BOD and nitrogen removal was tested to examine the process stability with varied organic and ammonium loading rates (Figure 5.4).

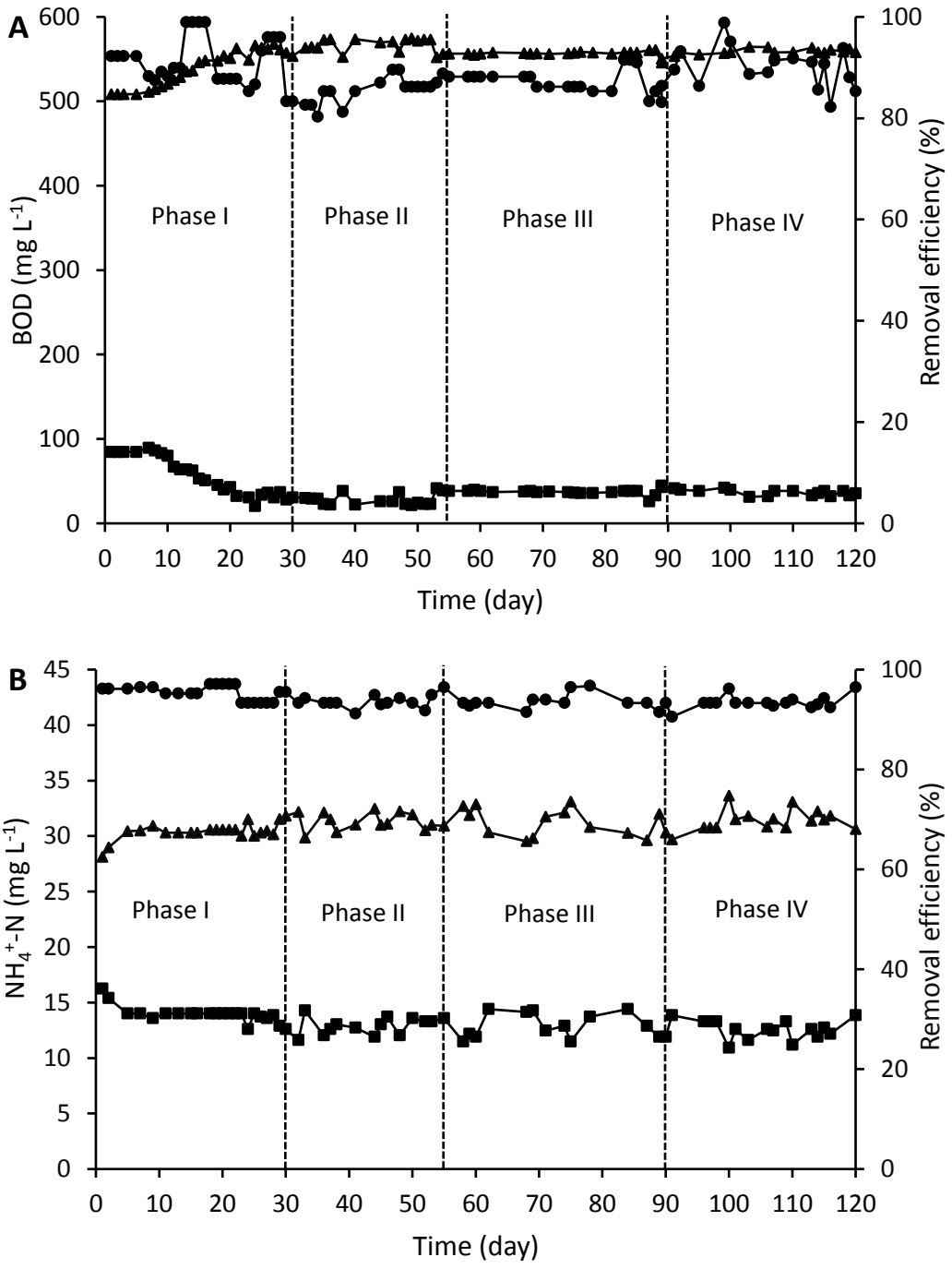

Figure 5.4: Long-term operation of the biofilm system: (A) organic carbon (BOD) removal performance; (B) NH_4^+-N removal performance: (●) Influent; (■) Effluent and (▲) Removal efficiency.

In Phase I (day 0 to 30), the zeolite amended PASND reactor was operated at an HRT (refers to the total treatment time including anaerobic and aerobic phase durations) of 12h with the synthetic wastewater containing BOD (i.e., acetate) and NH_4^+-N at concentrations of 512 and 42 mg L^{-1}, respectively. During this phase, the BOD removal efficiency increased gradually to about 93% until day 21 and remained stable for the rest of the period, whereas the NH_4^+-N removal efficiency stayed almost stable at around 70%. The average BOD and NH_4^+-N removal rate during this phase was 57.8 and 7.3 gm^{-3} d^{-1}, respectively. No significant amount of phosphorus was found to be released during the anaerobic stage, suggesting that anaerobic BOD removal was mainly attributed to the presence of GAOs in the biofilm.

In phase II (day 31 to 55), the PASND reactor was operated at 9 h HRT. The decrease in HRT did not affect the total BOD and NH_4^+-N removal efficiency. Accordingly, the average BOD and NH_4^+-N removal rate increased to 78.5 and 10.6 g m^{-3} d^{-1}, respectively. When the HRT was further decreased to 6 h and 5 h in phase III and phase IV, respectively, the biofilm system still maintained relatively constant removal efficiency of BOD and NH_4^+-N from the synthetic wastewater. Compared to the phase I, the organic carbon and nitrogen removal rates in phase IV were more than doubled to about 137.1 and 17.3 g m^{-3} d^{-1}, respectively.

Overall, the results of long-term operation indicate that an efficient and sustained removal of nitrogen and organic carbon from wastewater was achieved using the novel wastewater treatment system. The average BOD removal efficiency was 93.3 % which is comparable to that of previous studies of organic carbon removal using PHA storing biofilm (Flavigny & Cord-Ruwisch, 2015). In addition, the current zeolite amended GAO biofilm also

showed a stable nitrogen removal efficiency of about 70%, which is similar to the performance found in the biofilm process reported by Lo et al. (2010). It is interesting to note that the increase in nitrogen loading rate (i.e., shorter HRTs) did not adversely affect the nitrogen removal efficiency suggesting that the adsorption capacity of the zeolite was not exhausted and the shorter aerobic phases were sufficient to regenerate the zeolite completely. The latter one will be further proved by the increased number of nitrifying bacteria through microbial community analysis presented below.

5.3.4 Microbial community analysis

To reveal the dynamic changes of the microbial community structure during the start-up of this passive aeration SND process, biofilm samples taken at day 6, 54 and 120 were analyzed with high-throughput 16S rRNA amplicon sequencing. The initial bioinformatic analysis yielded a total of 205290 sequences which were assigned to different taxonomic levels. Overall, the richness of the bacterial community in the biofilm reactor increased over the experimental period, as evidenced by the increase of both alpha diversity (Chao 1 and Shannon index) and numbers of observed OTUs (Table 5.2).

Table 5.2: Community diversity indexes in different biofilm samples.

Samples	No of sequences	Chao 1	Observed OTUs	Shannon index
Day 6	58119	975.67	572	3.97
Day 54	64940	1077.36	648	4.15
Day 120	82231	1213.14	693	4.22

The relative abundance of *Nitrosomonas* a known ammonia-oxidizing bacteria (AOB) increased from 0.12% (day 6) to 0.24% (day 120) during the experimental period (Figure 5.5). Similarly, the abundance of *Nitrospira* increased from 0.28% to 0.51% which is attributed to the increase in the influent NH_4^+-N concentration in different phases of operation as reported by Tian et al. (2017). While most members of *Nitrospira* are considered as nitrite-oxidizing bacteria (NOB), a recent report suggests that a member of the genus *Nitrospira* (*Candidatus Nitrospira defluvii*) can act as both AOB and NOB (Daims et al., 2015). The increase in the concentrations of nitrifying bacteria (i.e., *Nitrosomonas, Nitrospira*) in the biofilm samples explains the trend of improved nitrogen removal performance (faster biological regeneration of zeolite) at higher nitrogen loading rates (Figure 5.4B) observed in the present study.

In addition to nitrifying bacteria, *Candidatus competibacter* (*Ca. competibacter*) - a known glycogen accumulating organism (GAO) - was the most dominant bacterial genus in all the biofilm samples. The relative abundance of 16S rRNA sequence associated with *Ca. competibacter* gradually increased from 6.7% to 20.3% during the four months of continuous operation. Therefore, the improved BOD removal rate described before (Section 5.3.3) is attributed to the increase of relative proportions of the GAO in the biofilm. The augmentation of GAOs in the sequential anaerobic and aerobic biofilm reactor is in line with previous reports (Flavigny & Cord-Ruwisch, 2015; Hossain et al., 2017).

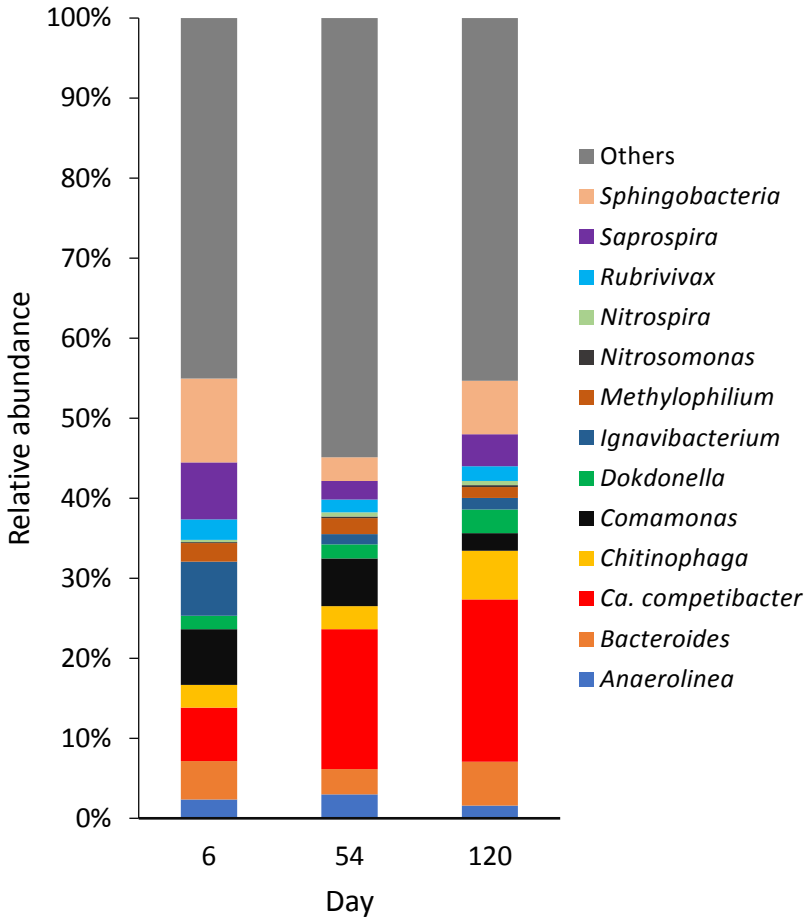

Figure 5.5: Microbial community structure in the biofilm samples over 4 months of continuous operation. The unidentified phylotypes with low relative abundance were added up to "others" to make up to 100% in all cases.

The glycogen accumulating organisms are able to uptake organic carbon anaerobically and accumulate as PHA. GAOs use this intracellularly stored PHA as an electron source for the reduction of nitrate to nitrite and/ or to nitrogen (Bassin et al., 2012a; Coats et al., 2011; McIlroy et al., 2014). In order to compare the denitrifying capability of the GAO present in the biofilm, batch tests (with nitrate and nitrite) were conducted (as per Section 5.2.4.), and results are shown in Figure 5.6. The GAO dominated biofilm reduced nitrate (20.9 mg L^{-1} h^{-1}) about 2-times faster than nitrite (11.8 mg L^{-1} h^{-1}) suggesting that GAOs have a preference to reduce nitrate than to nitrite. This observation is in agreement with

Bassin et al. (2012a) who reported that denitrification occurs mainly via nitrate in GAO dominated systems.

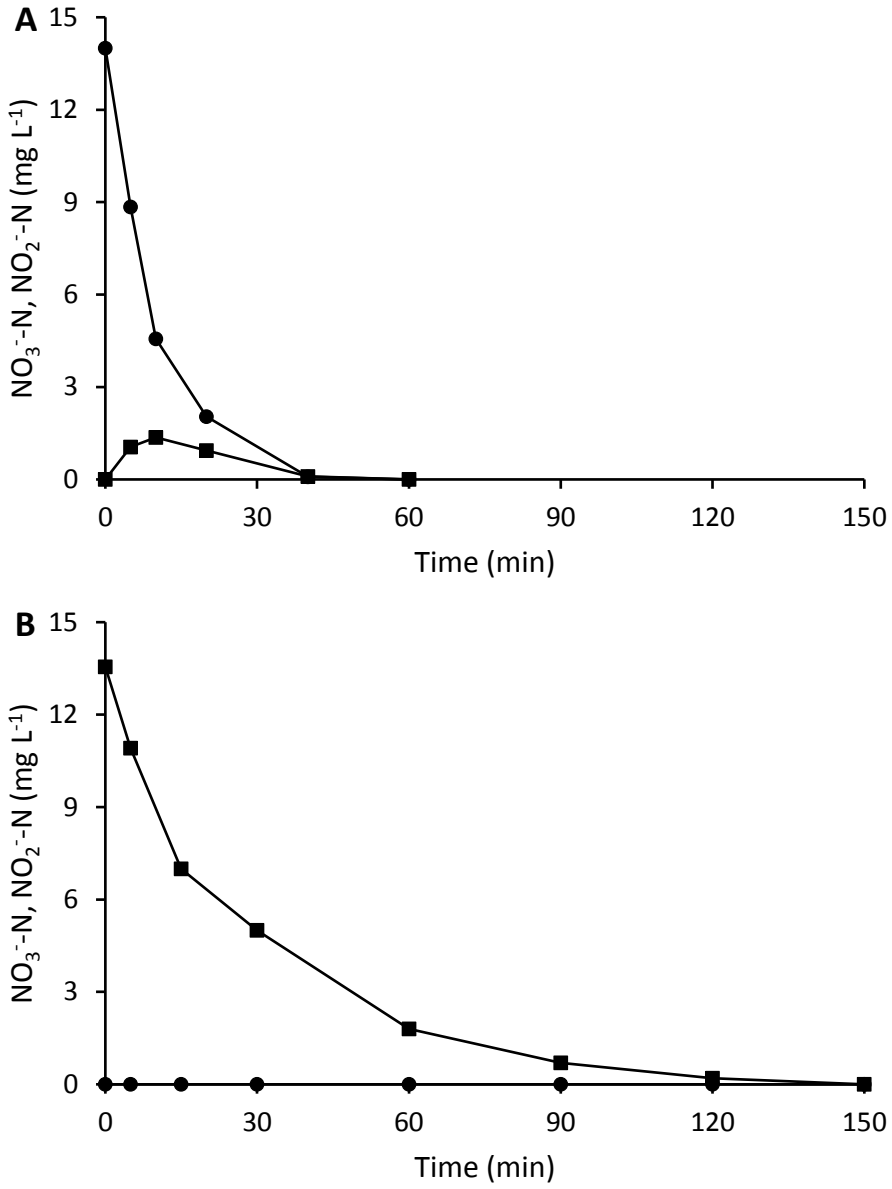

Figure 5.6: Anoxic batch experiments with nitrate (A) and nitrite (B): nitrate (●), nitrite (■).

5.3.5 Mechanisms of sustained nutrient removal of the hybrid biofilm system

To explore in more detail, the mechanisms of the nutrient removal, the variation of nitrogen, extracellular and intracellular carbon sources in a typical cycle (day 119) were analyzed (Figure 5.7). The BOD (i.e., acetate) and NH_4^+-N concentrations in the influent wastewater were 500.5 and 41.2 mg L^{-1} respectively, and the initial PHAs and glycogen content of the biofilm was 7.9 and 14.9 Cmmol L^{-1} respectively.

During the anaerobic stage, the concentrations of BOD and NH_4^+-N gradually decreased to 17.2 and 9.7 mg L^{-1} respectively, followed by PHAs increasing to 30.7 Cmmol L^{-1}, and glycogen decreasing to 3.4 Cmmol L^{-1} (Figure 5.7A, B). Because of the insignificant concentrations of NO_3^--N and NO_2^--N in the influent, BOD utilized for exogenous denitrification could be neglected. Therefore, the soluble BOD in the influent was mostly converted to intracellular carbon sources (i.e., PHAs). It was also found that the PO_4^{3-}-P content of the wastewater almost remained unchanged throughout the anaerobic phase (data not shown), suggesting that anaerobic BOD removal was mainly attributed to the presence of GAOs through glycogen degradation pathway. In addition, during the anaerobic period, the concentration of NH_4^+-N gradually decreased from 41.1 to 9.65 mg L^{-1} (Figure 5.7A). This observation demonstrated a chemical process of NH_4^+-N adsorption by the zeolite.

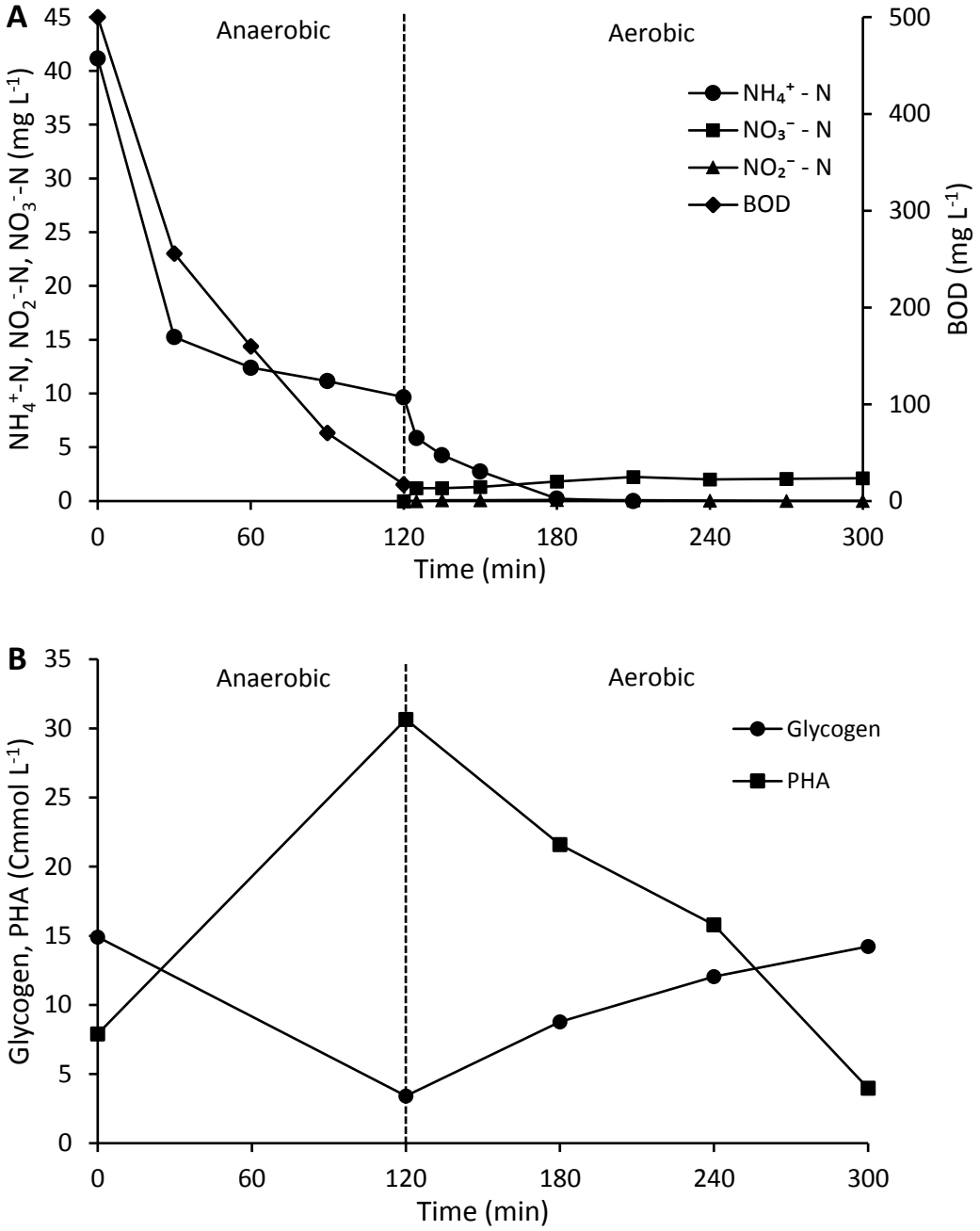

Figure 5.7: Variations of nitrogen and organic carbon concentrations in a typical cycle of phase IV (day 119).

The intracellular carbon transformation stoichiometry in the anaerobic stage was calculated and compared with literature data. The ΔGly/BOD ratio of 0.95 is higher than the reported PAO model value (0.5 Cmol $Cmol^{-1}$) by Smolders et al. (1994a) but similar to the reported GAO model (0.92 Cmol $Cmol^{-1}$) by Filipe et al. (2001). Moreover, the ΔPHA/BOD ratio was 1.87 Cmol $Cmol^{-1}$, which is also similar to the GAO model value of 1.85 and 1.86 Cmol $Cmol^{-1}$ reported by Oehmen et al. (2005) and Zeng et al. (2003d), respectively. Overall, the anaerobic stoichiometric results demonstrated that the activity of GAOs principally drove the removal of BOD and storage as PHA.

During the aerobic period, the direct exposure of the biofilm to air caused the nitrification of the NH_4^+-N as evidenced by the rapid decrease of NH_4^+-N and a slight increase of NO_3^--N concentration (Figure 5.7A). The observed low concentration of nitrogenous compounds (i.e., NO_2^--N and NO_3^--N) was likely due to the occurrence of simultaneous nitrification and denitrification (SND), which has been known to be favoured by the presence of anoxic zones in biofilms (Gibbs et al., 2004; Rahimi et al., 2011). In the aerobic stage, PHAs decreased by 26.7 Cmmol L^{-1} and glycogen increased by 10.8 Cmmol L^{-1}. The PHAs depletion was probably due to both aerobic oxidation using oxygen as an electron acceptor and anaerobic denitrification whereby the nitrite or nitrate provides the electron sink, which is known as storage carbon-driven denitrification (Miao et al., 2015; Zeng et al., 2003a).

The concentration of oxygen is a crucial factor for the occurrence of SND since high oxygen is known to inhibit the denitrification process (Helmer & Kunst, 1998; Ma et al., 2017; Wang et al., 2015b). In the current study, the biofilm system was exposed directly to the atmosphere during the aerobic period. However, high atmospheric oxygen did not seem to lead to nitrite or nitrate build-up from nitrification (Figure 5.7A). To demonstrate

denitrification of the biofilm during ongoing nitrification (regeneration of zeolite), nitrate was added at the beginning of the aerobic phase and ammonium, nitrate and nitrite recorded while recirculating a minimum volume of 20 mL (representing about 15% of the void volume) to enable sampling (Figure 5.8).

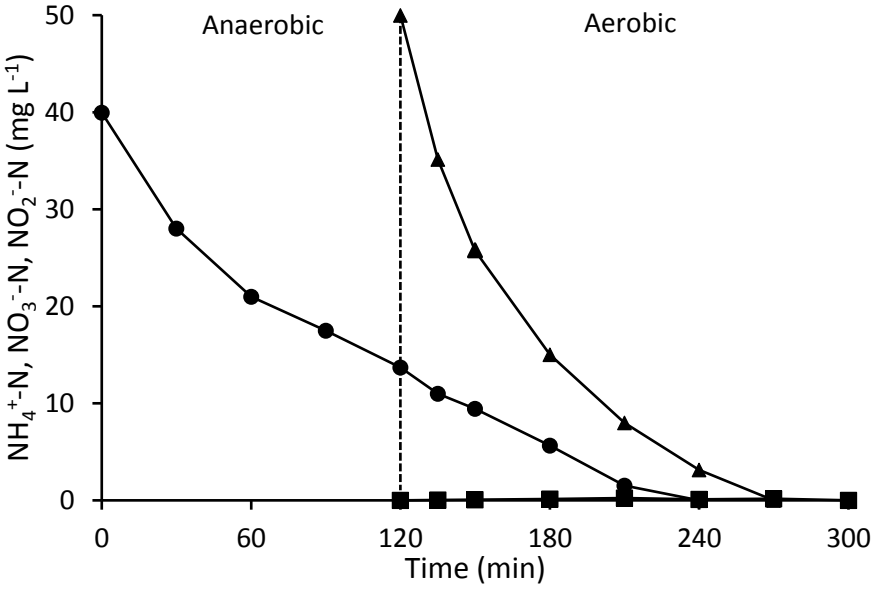

Figure 5.8: Simultaneous nitrification and denitrification (SND) of the described biofilm after draining of the solution and exposure to open air and addition of a nitrate spike (day 119 of operation): (●) NH_4^+-N, (▲) NO_3^--N, (■) NO_2^--N.

The nitrate concentration diminished concomitantly with the ammonium concentration and can only be explained by denitrification (Figure 5.8). Since the recirculated effluent contained a negligible amount of organic carbon, the electron donor for denitrification can be explained by intracellular storage polymer (e.g., PHA). This result confirms the removal of nitrogen via SND during the direct air exposure (passive aeration) of the biofilm which is in accordance with previous reports (Biesterfeld et al., 2003; Tian et al., 2017). The thick and uneven growth of the biofilm on the packing material together with the high respiration rates may be responsible for incomplete penetration of oxygen deeper

into the biofilm, thus forming the oxygen gradient inside the biofilm which led to efficient denitrification.

5.3.6 Implications of this study for wastewater treatment

5.3.6.1 Improvement of ammonium removal by double treatment

The zeolite amended PASND system has demonstrated its ability to stable organic carbon and nitrogen removal from wastewater. However, the nitrogen removal efficiency was found to be only about 70%. The remaining high concentration of NH_4^+-N (around 30%) doesn't meet typical standard limits for discharge (<3 mg N L^{-1}) of the effluent into the environment (Foley et al., 2010). To improve the nitrogen removal efficiency, repeated treatment with a PASND reactor is advisable. However, as the effluent from the first treatment contains no residual BOD, the subsequent treatment in a second reactor is not feasible as BOD is necessary to help driving denitrification during SND. To solve this problem, the effluent was returned to the same reactor in which residual stored PHA is still available. The result shows that after filling the treated wastewater containing 30% residual NH_4^+-N back to the reactor after the aerobic phase, further 70% of NH_4^+-N removal was achieved (Figure 5.9). This increased the total NH_4^+-N removal efficiency to about 96%, suggesting that a high NH_4^+-N removal efficiency is feasible using repeated treatment. This repeat filling and draining of wastewater will incur additional energy costs. Nevertheless, this approach avoids the costly and energy-intensive process of oxygen aeration in the bulk wastewater; thus, the total energy consumption should remain notably lower than the conventional activated sludge process.

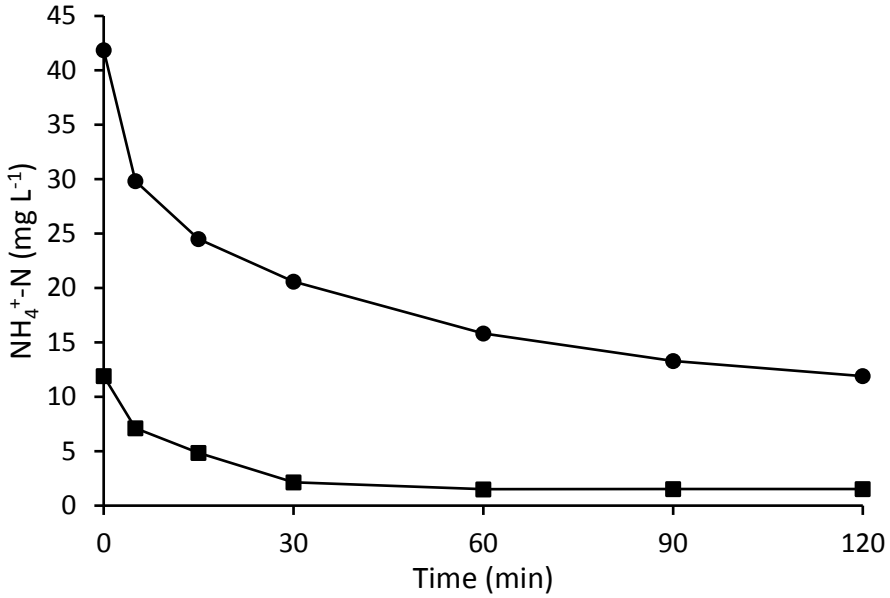

Figure 5.9: Treatment of wastewater in two successive cycles: synthetic wastewater was treated twice in zeolite containing GAO dominated PASND biofilm reactor, resulting in 96% nitrogen removal: (●) treatment 1, (■) treatment 2.

5.3.6.2 Comparison with other nutrient removal systems

The organic carbon and nitrogen removal efficiency of the described process were compared with other relevant systems where nitrogen removal was achieved principally by SND (Table 5.3). The organic carbon removal efficiency of the described biofilm system was higher than that of granular sludge (Rahimi et al., 2011; Wang et al., 2009) and biofilm (Ma et al., 2017; Rahimi et al., 2011) systems. The average nitrogen removal efficiency was due to the single adsorption step by zeolite limited to less than 70% (68.9%). While this is comparable with other N removal efficiency described (Table 5.3), it is not adequate for many municipal applications. However, after two subsequent treatments, the proposed system attained efficient (96%) removal of nitrogen which complies with discharge limits set by regulatory authorities.

Table 5.3: Comparison of the zeolite amended PASND system with related works.

Process	C/N	A/O stage (min)	Removal efficiency (%)		References
			C	N	
Aerobic granular SBR	10	90/240	63.1	52	Wang et al. (2009)
Biofilm SBR	8	120/600	90	70.23	Ma et al. (2017)
Hybrid SBR	10	60/270	-	71.5	Lo et al. (2010)
Biofilm SBR	10	60/330	-	49.5	Lo et al. (2010)
Suspended sludge SBR	10	60/240	-	36.7	Lo et al. (2010)
Fixed bed SBR	3.3-6.7	60/180-430	90.64	70	Rahimi et al. (2011)
SBR	3.3-6.7	60/180-430	85.6	43.8	Rahimi et al. (2011)
SBR	3.5	180/150	81	77.7	Wang et al. (2015b)
UASB + DHS	-	360/150	94.3	59.9	Tandukar et al. (2007)
PASND Biofilm					
Single treatment	8	120/180	93.30	68.90	This study
Double treatment	8	240/360	>99	96	This study

A: anaerobic phase; O: aerobic phase; SBR: sequencing batch reactor; UASB: up-flow anaerobic sludge blanket reactor; DHS: down-flow hanging sponge reactor

5.3.6.3 Sludge production

No significant sludge production was recorded from the PASND biofilm reactor during the experimental period except for occasional slough-off of small pieces of biomass into the effluent. The average VSS concentration in the effluent was about 27 mg $L^{-1,}$ and the observed sludge yield (Y_S) was found to be 0.049 g VSS g^{-1} BOD removed, assuming no net growth of biofilm (Figure 5.10). The biomass growth yield of the current system is 10% of that of the conventional activated sludge process which typically produces 0.5 g dry biomass per g BOD removed (Mahmood & Elliott, 2006). The significantly lower sludge production may lead to reduced sludge treatment and disposal cost which is the second greatest operational expense for the traditional WWTPs.

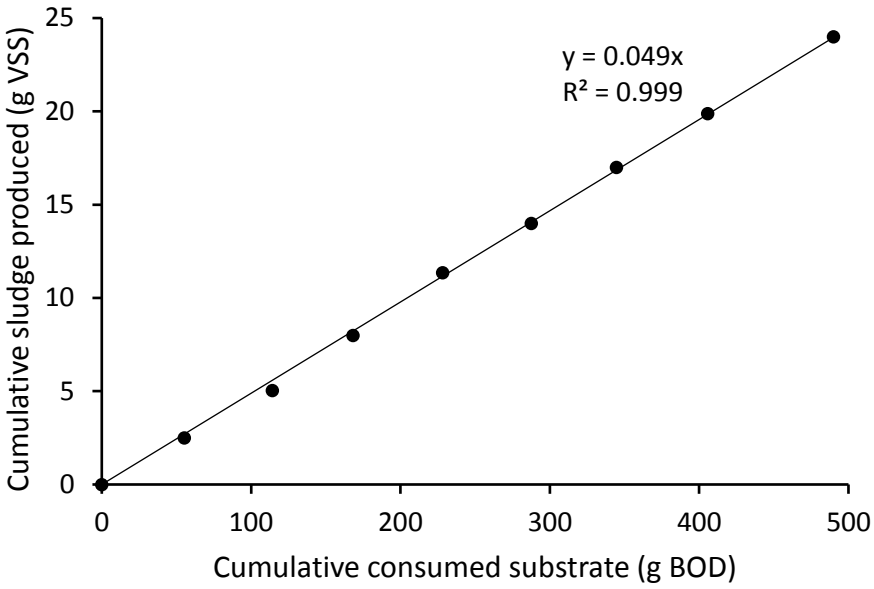

Figure 5.10: Observed sludge yield (Y_S) in the biofilm system.

5.3.6.4 Low-energy cost by avoiding aggressive aeration in bulk wastewater

The major energy consumption of the current system was utilized for driving the motion of the wastewater, such as filling the reactor with wastewater during the anaerobic stage. During the aerobic period, oxygen supply was achieved by passive aeration via air diffusion from the pore space of the reactor to the biofilm. Therefore, the energy required to ventilate the reactor for oxygen supply is significantly lower than the traditional aeration approach. In a typical wastewater treatment plant (WWTP) with an activated sludge (AS) process, the largest energy usage comes from the activated sludge aeration (30 to 75%) (Tchobanoglous et al., 2014). This is because the oxygen supply in bulk wastewater through the aerators is an extremely low efficient and energy-intensive process due to the poor solubility of oxygen in the water as well as the energy needed to against the hydraulic pressure (i.e., diffuser depth). Furthermore, the air bubbles escape rapidly (i.e., few seconds) from the conventional aeration tank, resulting in a short oxygen retention time (ORT) and low standard oxygen transfer efficiency (SOTE) ranging from 4.8% to 34.1%

(Groves et al., 1992). The current system allows a high ORT, enabling almost 100% oxygen consumption efficiency; thus, achieving significant energy savings.

Potentially the wastewater treatment with the PASND system needs a higher capital input (i.e., carrier and zeolite) compared to the traditional activated sludge process. However, it can somehow be offset by the low energy consumption for oxygen supply. Further study on detailed analysis of capital and operational cost of the current system is needed.

5.4 Conclusions

The successful development of zeolite amended PASND biofilm system in this study from activated sludge proved its feasibility in real-world. The established technology efficiently removed soluble organic carbon (by GAOs) and nitrogen (adsorbed onto zeolite) simultaneously from wastewater under anaerobic conditions. The simultaneous nitrification and denitrification (responsible for zeolite regeneration) were achieved in the subsequent aerobic stage by exposing the biofilm directly to the atmospheric oxygen. The proposed biofilm technology demonstrated long-term organic compounds and total nitrogen removal without the need for extensive aeration of the bulk wastewater, which could significantly reduce the energy cost associated with the aeration leading to a high energy efficient wastewater treatment process.

Chapter 6

Treatment of High-Strength Wastewater in a Zeolite Amended GAO Biofilm

Abstract

The present study evaluates the capability of the zeolite amended GAO biofilm system (as described in Chapter 5) to treat high-strength wastewater. The GAO biofilm was found to be able to remove organic carbon (BOD) from a standard-strength (1x) wastewater at a rate of 543 mg L^{-1} h^{-1}, which increased significantly to 2308 mg L^{-1} h^{-1} when the influent concentration was increased by 4-times. The enhanced BOD uptake resulted in an increase in the accumulation of intracellular storage substrate (i.e., PHA) from 5.02 to 18.60 mmol L^{-1} for 1x and 4x wastewater, respectively. A similar trend was also found for nitrogen removal as the result of ammonium adsorption onto zeolite. The regeneration of zeolite achieved via PHA based simultaneous nitrification and denitrification took a different length of aerobic phase for the different strength of wastewater. The established biofilm system also showed a long-term stability over a period of 60 days for effective treatment of different strength wastewater. The biomass community analysis reveals that the improved BOD and nitrogen removal efficiency during the experimental period was attributed to the increased proportions of GAOs and nitrifying bacteria. These results have demonstrated the capability of the proposed biofilm system to treat wastewater with elevated concentrations of organic compounds and nutrients.

6.1 Introduction

High-strength industrial wastewater is a major source of water pollution because of its elevated concentration of organic compounds and nutrients (Mutamim et al., 2012). In order to protect the environment, the high-strength wastewater needs to undergo pre-treatment followed by biological treatment to remove pollutants (Hamza et al., 2016). Conventional biological wastewater treatment technologies principally rely on aerobic processes in which microorganisms degrade the soluble and colloidal organic materials and reclaim the water (Tchobanoglous et al., 2014). However, despite its high organic carbon and nutrient removal efficiency, aerobic activated sludge treatment processes have major limitations such as generation of excessive amounts of sludge and high energy requirement which makes the process energy-intensive with a relatively large carbon footprint (McCarty et al., 2011).

As an alternative to the aerobic wastewater treatment technologies, anaerobic digestion (AD) provides a more suitable option for the treatment of high-strength wastewater that requires no oxygen and produces less excess sludge (Hamza et al., 2016). Moreover, the AD has the potential to recover the used energy by generating biogas; hence, making the treatment process energy neutral or even energy positive (McCarty et al., 2011). Nevertheless, the low growth rate of anaerobic microorganisms, a low settling rate of biomass, high sensitivity to toxic shock loadings and fluctuation in environmental conditions often lead to failure of the AD treatment process (Bustamante & Liao, 2017; Chan et al., 2009). In addition, complete removal of high-strength organic compounds and nutrients (e.g., phosphorus, nitrogen) cannot be achieved by the anaerobic process, and this results in effluent quality that usually fails to comply with the standards (Ahammad et al., 2013).

In recent years, biofilm-based wastewater treatment technology has shown great potential for the treatment of both municipal and industrial wastewater (Barwal & Chaudhary, 2014). Compared with the conventional activated sludge based systems, biofilm processes have several advantages such as operational flexibility, high biomass content, low sludge production and resistance to the toxic shock loading (Ødegaard, 2016; Rodgers & Zhan, 2003). A number of biofilm-based wastewater treatment technology has been developed so far, such as the sequencing batch biofilm reactor (SBBR) which can remove organics and nutrients in a single bioreactor (Ma et al., 2017). The dynamic anaerobic and aerobic conditions of SBBR favors the development of microorganisms (such as polyphosphate accumulating organism and glycogen accumulating organism) which can uptake organic carbon anaerobically and store intracellularly as polyhydroxyalkanoate (PHA) (Flavigny & Cord-Ruwisch, 2015; Hossain et al., 2017; Mino et al., 1998; Van Loosdrecht et al., 1997a).

In our previous study (Chapter 5), we have demonstrated efficient removal of organic carbon and nitrogen from wastewater by incorporating an ion-exchange material (i.e., zeolite) in a GAO biofilm. The process avoids the energy-intensive transfer of oxygen into the bulk wastewater; thus, reducing the aeration energy cost of a wastewater treatment plant. Since the previously described biofilm successfully treated wastewater of municipal strength, investigation of its capability to treat even more concentrated wastewaters is highly desirable.

In response to this need, the feasibility of the proposed zeolite amended GAO biofilm system to treat high-strength wastewater is evaluated in the current study. The stability of the process and the response of microbial communities to the increased organic and nitrogen concentrations are also assessed.

6.2 Materials and Methods

6.2.1 Zeolite

The natural zeolite used in this study was obtained from a local supplier (Zeolite Australia Pty. Ltd.) and composed mainly of clinoptilolite and quartz. The zeolites were repeatedly washed with deionized water to remove adhering dirt and soluble impurities, dried at 105°C for 24 h. The dried zeolite was ground in a milling machine to a fine powder and passed through British Standard Sieves (BSS). Zeolite particles with size of 75 μm was used in the current study.

6.2.2 Experimental setup and operation

A tubular laboratory-scale reactor made up of methyl methacrylate with a working volume of 0.755 L was used in this study (Figure 5.1). The reactor was filled (20% $V_{carrier}/V_{reactor}$) with packing material (AMB^{TM} Biomedia Bioballs) containing biofilm enriched with glycogen accumulating organism (GAO) as described in Hossain et al. (2017). Then, 10 g of the zeolite powder (75 μm) was suspended in synthetic wastewater solution and trickled over the GAO biofilm coated packing materials for 24 h until most of the suspended zeolite powder (>99%) was adsorbed onto the biofilm (indicated by the optical density). In order to introduce nitrifying bacteria into the biofilm that could be emulated in real plants, 100 mL of activated sludge (collected from Subiaco Wastewater Treatment Plant, Western Australia) was trickled over the biofilm for 24 h after 5 cycles of initial testing. This zeolite amended hybrid biofilm reactor which aims at enabling simultaneous nitrification and denitrification by using oxygen directly from the air (passive aeration) is hereafter referred to as passive aeration SND (PASND) reactor. The temperature of the reactor was kept at around 25 ± 2°C.

The zeolite amended PASND reactor was operated under sequential anaerobic and aerobic two-stage operation mode. During the anaerobic (feast) phase, the reactor was fully loaded with synthetic wastewater (full void volume) and remained in the anaerobic condition for a specific period to adsorb organic carbon and ammonium. The anaerobically treated wastewater was drained out of the reactor by gravity to begin the aerobic (famine) stage. The top of the reactor was open to enable oxygen passively entering the reactor, whereby the biofilm was exposed to atmospheric oxygen. To enable monitoring of nitrogenous compounds in the aerobic stage (after draining), a small volume of the residual liquid (about 20 mL) was slowly recirculated through the biofilm reactor from the top to the bottom.

6.2.3 Synthetic wastewater

In this study, synthetic wastewater (Third et al., 2003b) was used to maintain tight control of influent constituents and enable full reproducibility for the establishment of a proof-of-concept study of low energy, passive aeration biological organic carbon and nitrogen removal. The concentrated synthetic wastewater was prepared in two separate solutions using deionized (DI) water. The first solution contained acetate as carbon source, while the other contained the required nutrients, minerals and trace elements. After dilution with DI water, the influent wastewater contained (mg L^{-1}): CH_3COONa 660 – 2640 (1x – 4x wastewater), NH_4Cl 160 – 640 (1x – 4x wastewater), KH_2PO_4 44, $NaHCO_3$ 125, $MgSO_4$. $7H_2O$ 25, $CaCl_2$. $2H_2O$ 300, $FeSO_4$. $7H_2O$ 6.25, yeast extract 50, and 1.25 ml L^{-1} of trace element solution, which contained (g L^{-1}): EDTA 15, $ZnSO_4$. $5H_2O$ 0.43, $CoCl_2$. $6H_2O$ 0.24, $MnCl_2$. $4H_2O$ 0.99, $CuSO_4$. $5H_2O$ 0.25, $NaMoO_4$. $2H_2O$ 0.22, $NiCl_2$. $6H_2O$ 0.19, $NaSeO_4$. $10H_2O$ 0.21, H_3BO_4 0.014 and $NaWO_4$. $2H_2O$ 0.050.

6.2.4 Analytical parameters of interest

The concentrations of chemical oxygen demand (COD), ammonium (NH_4^+-N), nitrite (NO_2^--N) nitrate (NO_3^--N), and orthophosphate (PO_4^{3-}-P) in the influent and effluent of the biofilm reactor were regularly measured to monitor system performance. Ammonium, nitrite, nitrate, orthophosphate, and COD concentrations were determined in accordance with Standard Methods (APHA, 2012). The acetate was measured by Gas Chromatography (Agilent, USA) according to the method used in Hossain et al. (2017). Freeze dried biomass was used to measure poly-hydroxyalkanoates (PHAs) and glycogen. PHAs were determined by the sum of poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV), which were analyzed as previously reported (Smolders et al., 1994a). Glycogen was analyzed according to the method used by Hossain et al. (2017). Total suspended solids (TSS), and volatile suspended solids (VSS) were measured according to Standard Methods (APHA, 2012). pH and DO were measured using a pH meter and a DO meter (Mettler-Toledo, USA), respectively.

6.2.5 Microbial community analysis

Biofilm samples were collected at different time points during the operational period to reveal the microbial community of the biofilm. Genomic DNA was extracted from these samples using the Power Soil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instruction, and quantified by spectroscopic methods (NanoDrop 2000, Thermo Fisher Scientific, USA). Bacterial 16S rRNA genes were PCR-amplified with barcoded forward primer 515F and reverse primer 806R (Caporaso et al., 2012). For each sample, polymerase chain reaction was carried out in a 25 µL total volume including 2.5 µL of normalized total genomic DNA (5 ng/µL), 0.2 µM of each primer and 12.5 µL of 2x KAPA HiFi HotStart Ready Mix (Kappa Biosystems, USA). The PCR cycling protocol consisted of an initial denaturation step of 95°C for 3 min, followed by

35 cycles of DNA denaturation at 95°C for 30s, primer annealing at 55°C for 30s, strand elongation at 72°C for 30s, and a final elongation step at 72°C for 5 min. All samples were amplified in triplicate, pooled and visualized in agarose gel. Combined PCR products were purified using the AMPure XP beads (Beckman Coulter, USA) and final amplicon concentrations were quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). Amplicons from all sample were pooled in equimolar ratios and sequenced on the MiSeq platform (Illumina, USA).

The forward and reverse reads were merged and filtered based on minimum lengths and expected errors, as specified in the USEARCH pipeline (Edgar, 2010). To identify bacterial genera present in samples, operational taxonomic units (OTUs) were selected by clustering sequencing at 97% similarity with the UPARSE algorithm and chimeric sequences were removed using UCHIME (Edgar, 2013). Taxonomy was assigned to OTUs against the Greengenes 16S database (August 2013 release) (DeSantis et al., 2006) in QIIME 1.9.1 (Caporaso et al., 2010) using the UCLUST algorithm (Edgar, 2010) with default parameters.

6.2.6 Statistical analysis

Statistical analysis of the experimental data was conducted using the SPSS software package (IBM-SPSS v22). A one-way analysis of variance (ANOVA) was used to test whether a certain factor impacted an observed variable. Pearson's correlation coefficient was applied to quantify the relationship between two parameters. An $\alpha = 0.05$ and $p < 0.05$ indicated statistically significance.

6.3 Results and Discussion

6.3.1 Treatment of different strength wastewater

A zeolite amended glycogen accumulating organism (GAO) dominated biofilm system was established and operated under sequential anaerobic and aerobic schemes as described before (Chapter 5). This biofilm reactor was shown to remove organic carbon (BOD) and nitrogen from wastewater with low aeration energy expense. To evaluate the capacity of the described biofilm to treat more concentrated wastewater, a baseline trial with standard strength (hereafter referred to as single strength or 1x) synthetic wastewater was tested (Figure 6.1). During the anaerobic period, the biofilm removed about 492 (93%) and 31 (71%) mg L^{-1}, BOD, and NH_4^+-N, respectively. In the subsequent aerobic drained condition, the NH_4^+-N adsorbed onto the zeolite particles was removed due to the occurrence of simultaneous nitrification and denitrification (as described in Section 5.3.5).

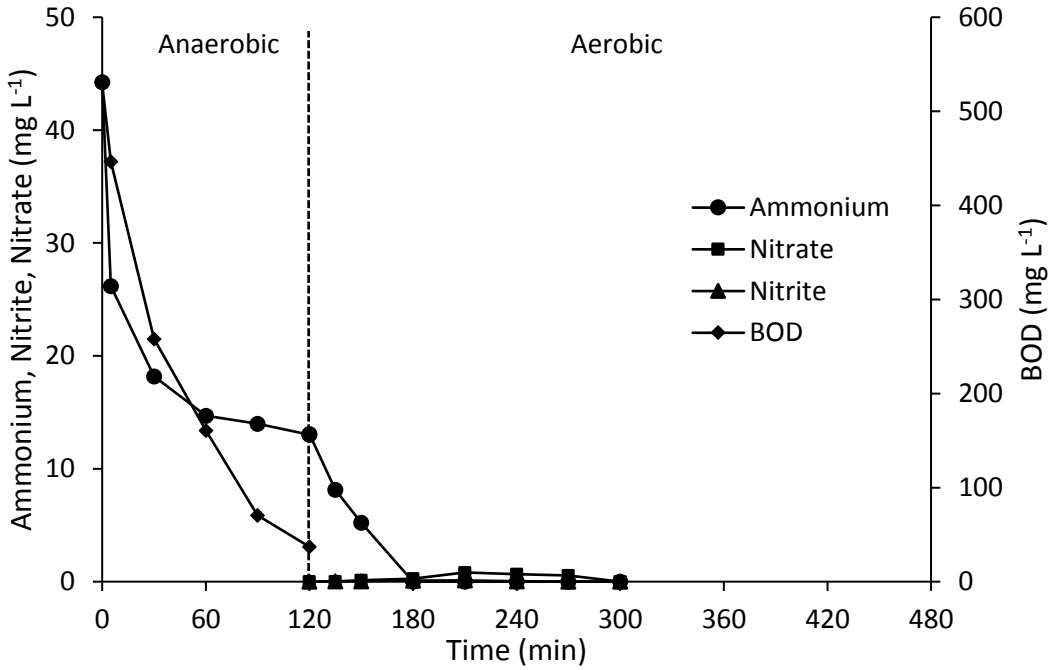

Figure 6.1: Observed conversions in zeolite amended GAO biofilm system with standard-strength (1x) synthetic wastewater.

The ability of the biofilm system to treat double (2x) strength wastewater was also evaluated (Figure 6.2). The doubling of feed concentration resulted in the doubling of the BOD and NH_4^+-N load, as the anaerobic period was kept the same (2 h). Interestingly, the total amount of BOD and NH_4^+-N removed was higher than that of single-strength wastewater. Overall, the doubling of wastewater strength did not require a much longer anaerobic period leading to a total BOD and NH_4^+-N removal of about 874 and 57 mg L^{-1}, respectively. The organic carbon (89%) and nitrogen (69%) removal efficiency were quite similar to that of single-strength wastewater despite the fact that the loading rate was double.

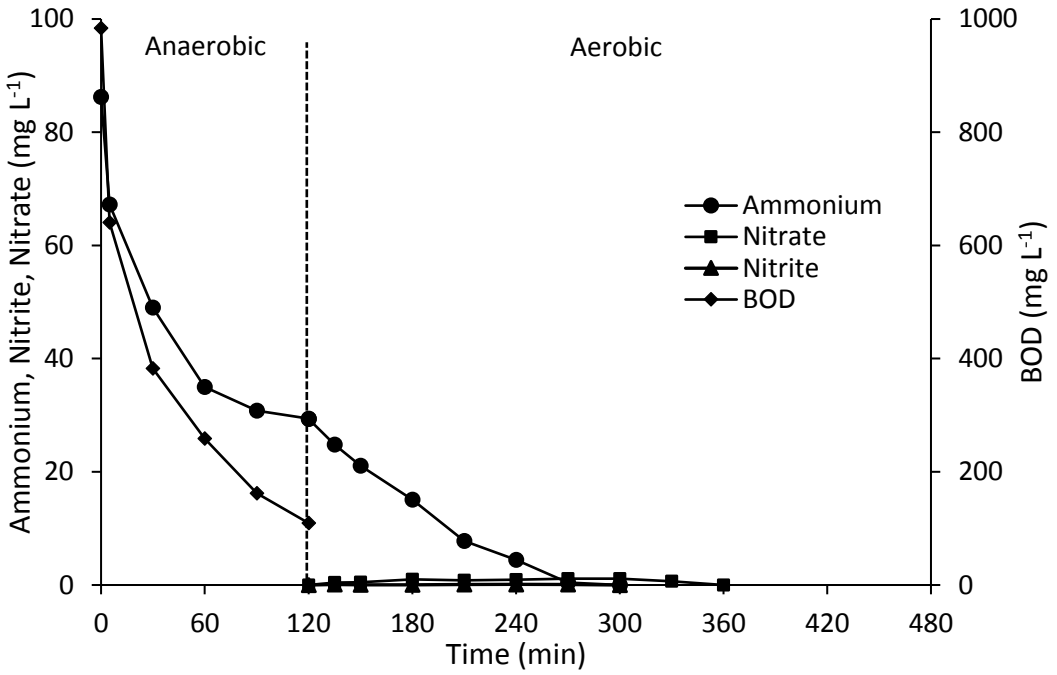

Figure 6.2: Observed conversions in zeolite amended GAO biofilm system with double strength (2x) synthetic wastewater.

The BOD uptake rate of the GAO dominated biofilm system increased when concentrated (2x) wastewater was used. The BOD uptake rate (considering the first 30 min of the anaerobic period) for 2x wastewater was about 1202 mg L^{-1} h^{-1} which was more than two times higher than that of 1x wastewater (543 mg L^{-1} h^{-1}). Such BOD removal rate is exceptionally high (up to 50-times) compared to conventional treatment processes such as trickling bed filter (11.7 mg L^{-1} h^{-1}) (Doan et al., 2008), up-flow anaerobic sludge blanket (UASB) reactor (26 mg L^{-1} h^{-1}) (Tandukar et al., 2007), and activated sludge process (23 mg L^{-1} h^{-1}) (Tandukar et al., 2007).

The total treatment time for different strength wastewater should also take the aerobic regeneration phase into account. The required duration of the aerobic period can be determined from the depletion of the NH_4^+-N adsorbed onto zeolite and consumption of the stored intracellular organic carbon (i.e., PHA). Any longer aeration could lead to the exhaustion of PHA, resulting in poor denitrification capability (Third et al., 2005a). The nitrogen profiles during the aerobic period demonstrated that NH_4^+-N was completely oxidized within 1 h and 2.5 h for single and double strength wastewater, respectively with a negligible amount of nitrite and nitrate accumulation (Figure 6.1 and 6. 2).

The fact that a similar BOD and nitrogen removal efficiencies were obtained irrespective of the strength of wastewater suggesting that the GAO biofilm system studied has the capability of treating even stronger wastewater. When quadruple strength (4x) wastewater was used, the biofilm system was found to remove about 1745 (81%) mg BOD L^{-1} and 117 (68%) mg NH_4^+-N L^{-1} within 2 h of anaerobic phase (Figure 6.3). However, compared with single and double strength feed, the complete NH_4^+-N removal during the aerobic stage required substantially longer time (5 h) and resulted in measurable concentrations of nitrite accumulation. The accumulation of nitrite (11.7 mg N L^{-1}) can be explained

by the observation that denitrifying glycogen accumulating organism shows preference to reduce nitrate rather than nitrite (Section 5.3.4, Figure 5.5) and this observation is in accordance with previous reports (Bassin et al., 2012a; Ribera-Guardia et al., 2016). However, all of the accumulated nitrite was reduced eventually demonstrating the full capability of the biofilm system for the treatment of 4x wastewater.

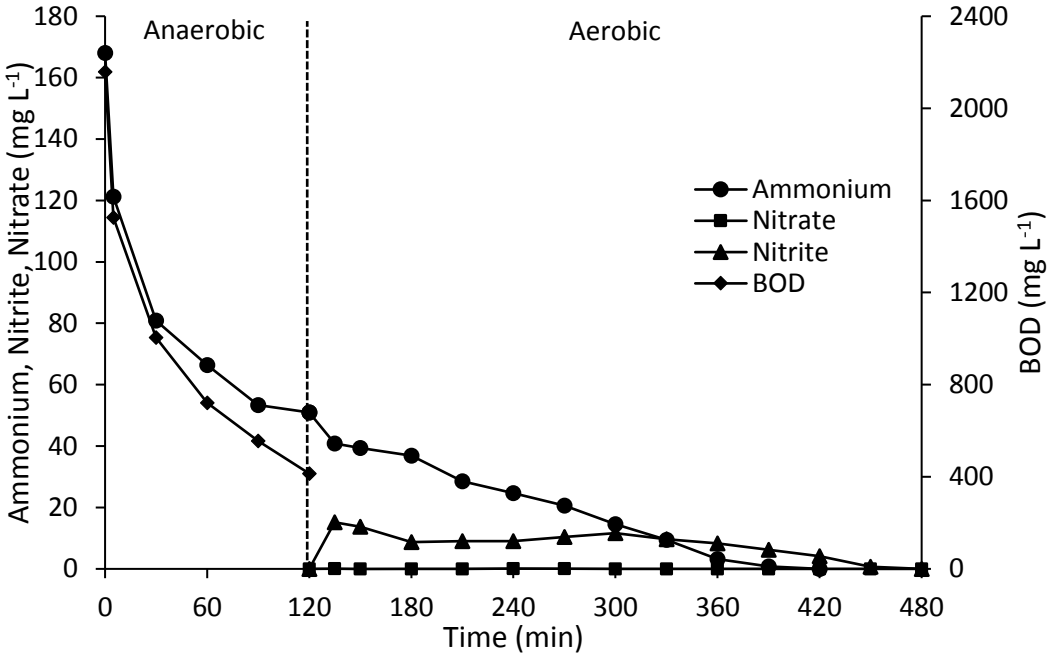

Figure 6.3: Observed conversions in zeolite amended GAO biofilm system with quadruple strength (4x) synthetic wastewater.

Overall, the above results demonstrated that higher strength wastewater could be treated in the zeolite amended GAO biofilm to similar BOD and N removal efficiencies but did not require a proportionally longer treatment time (Table 6.1). Since the aerobic time required for zeolite regeneration increases with feed strength, the aerobic phase length should be increased for stronger feeds. However, excessive aerobic phase lengths would lead to undesired PHA oxidation.

Table 6.1: BOD and nitrogen removal performance of the studied GAO biofilm with different strength wastewater.

Wastewater concentration	BOD removed		NH_4^+-N removed		Treatment time		
	(%)	(mg L^{-1})	(%)	(mg L^{-1})	Anaerobic (min)	Aerobic* (min)	Total cycle (h)
1x	93	492	71	31	120	60	3.0
2x	89	874	69	57	120	150	5.5
4x	81	1745	68	117	120	300	7.0

* Estimated based on ammonium disappearance

6.3.2 Increased accumulation of intracellular PHA

Although the same anaerobic period (2 h) was used for different strength wastewater, the increase in the influent carbon concentration resulted in the increased accumulation rate of the intracellular PHA. For the single-strength wastewater, the biofilm accumulated PHA at a rate of about 2.51 mmol L^{-1} h^{-1} which increased significantly to 9.3 mmol L^{-1} h^{-1} when 4x wastewater was used (Figure 6.4). This observation of enhanced carbon source storage in the presence of increased organic loading is in line with the findings of Tian et al. (2017) who found that increased carbon concentration of the feed caused increased levels of PHA in a biofilm that underwent sequential aerobic and anaerobic conditions.

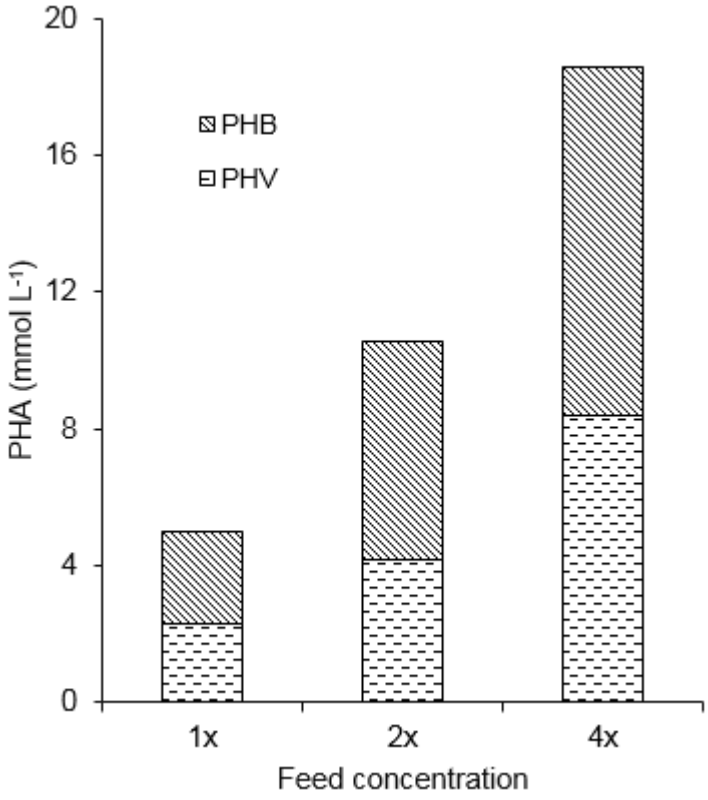

Figure 6.4: Accumulation of PHA in the biomass at different influent feed concentrations.

As described above (Section 6.3.1), the organic carbon (BOD) uptake and hence the PHA accumulation rate gradually slowed down during the anaerobic phase. This could be due to either decreased diffusion rate of the substrate to the biofilm or exhaustion of PHA storage capacity. The fact that the studied biofilm adsorbed BOD at a faster rate and more PHA (10.54 mmol L^{-1}) accumulated in the presence of 2x concentrated feed than that of single-strength wastewater, the substrate (i.e., organic carbon) diffusion limitation could be the reason for the slowing down of the PHA accumulation rate. This possibility of diffusion limitation is in agreement with a previous report (Morgenroth & Wilderer, 1999). The BOD removal rate in a biofilm system is suggested to be principally controlled by diffusion (Fan et al., 2017). This explains why the high organic loading rate (in case of 2x and 4x wastewater) correspondingly increased the BOD uptake rate by the GAO biofilm and ultimately resulted in higher accumulation of intracellular PHA at a faster

rate. The greater availability of reducing material (i.e., PHA) (Figure 6.4) improved the SND during the aerobic phase when high-strength (2x and 4x) feed was used (Figure 6.2 and 6.3) in the current experiment.

6.3.3 Oxygen consumption

As shown above, the use of concentrated wastewater resulted in increased accumulation of intracellular storage material (i.e., PHA) (Figure 6.4). This suggests that during the aerobic phase the biomass which has a higher amount of intracellular stored PHA should use oxygen at a proportionally faster rate because of the first order rate of PHA degradation (Third et al., 2003a). In order to investigate PHA degradation, the oxygen uptake by the biomass was measured (Figure 6.5). Interestingly, there was no significant increase in oxygen uptake rate (OUR) by biomass with a higher level of PHA concentration. In fact, PHA degradation rate was almost identical within the first 30 min (Figure 6.5) for biomass PHA levels with up to 400% difference (Figure 6.4). However, the biomass with the highest concentration of PHA maintained the initial high oxygen uptake rate for longer leading to more rapid oxygen depletion.

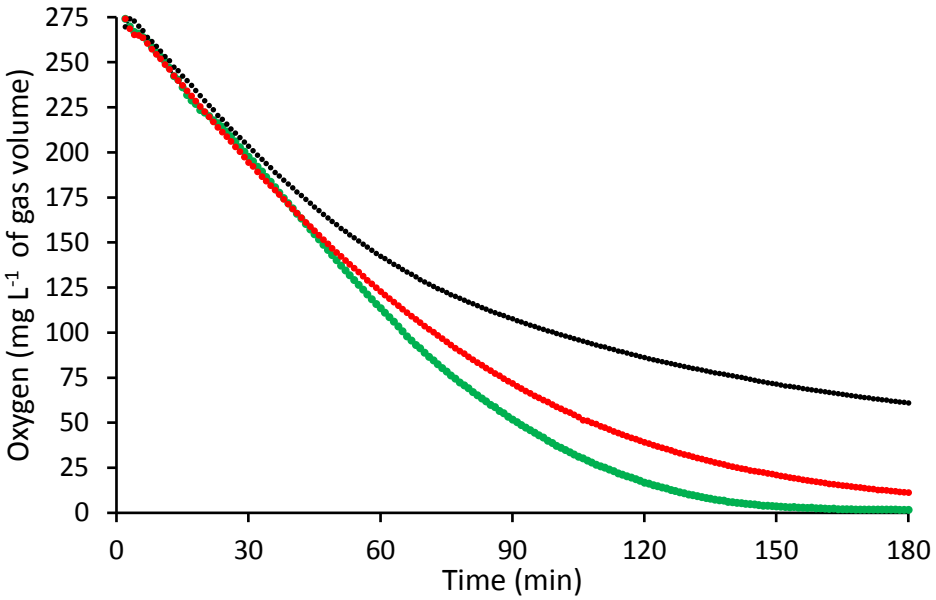

Figure 6.5: Oxygen consumption by the biomass at different feed concentrations: 1x feed (black), 2x feed (red), 4x feed (green).

6.3.4 Long-term performance of the biofilm system

In order to assess the stability of the process, the performance of the zeolite amended GAO dominated biofilm system was evaluated over a period of 60 days for sustained removal of organic carbon and nitrogen from different strength wastewater (Figure 6.6). The duration of the anaerobic period was maintained the same (2 h) to investigate the performance of the system without proportionally increasing the reactor size. However, the length of the aerobic period varied based on previous results (Section 6.3.1) to enable complete regeneration of zeolite.

Using standard (1x) strength wastewater, the biofilm system showed constant performance with an average BOD removal efficiency of 93.4% (Figure 6.6A). Such removal efficiency is considerably higher compared with the traditional anaerobic processes which have shown BOD removal in the range of 70 to 80% (Aiyuk et al., 2004; Li et al., 2017; van Haandel et al., 2006). When the concentrated feed was used, the BOD removal effi-

ciency slightly decreased to about 88% and 81% for the 2x and 4x wastewater, respectively. In contrast, the average organic removal rate (ORR) increased from 0.29 Kg BOD m^{-3} d^{-1} (1x) to 0.63 Kg BOD m^{-3} d^{-1} (4x) which might be due to the increased abundance of GAO in the biofilm (will be discussed in Section 6.3.5).

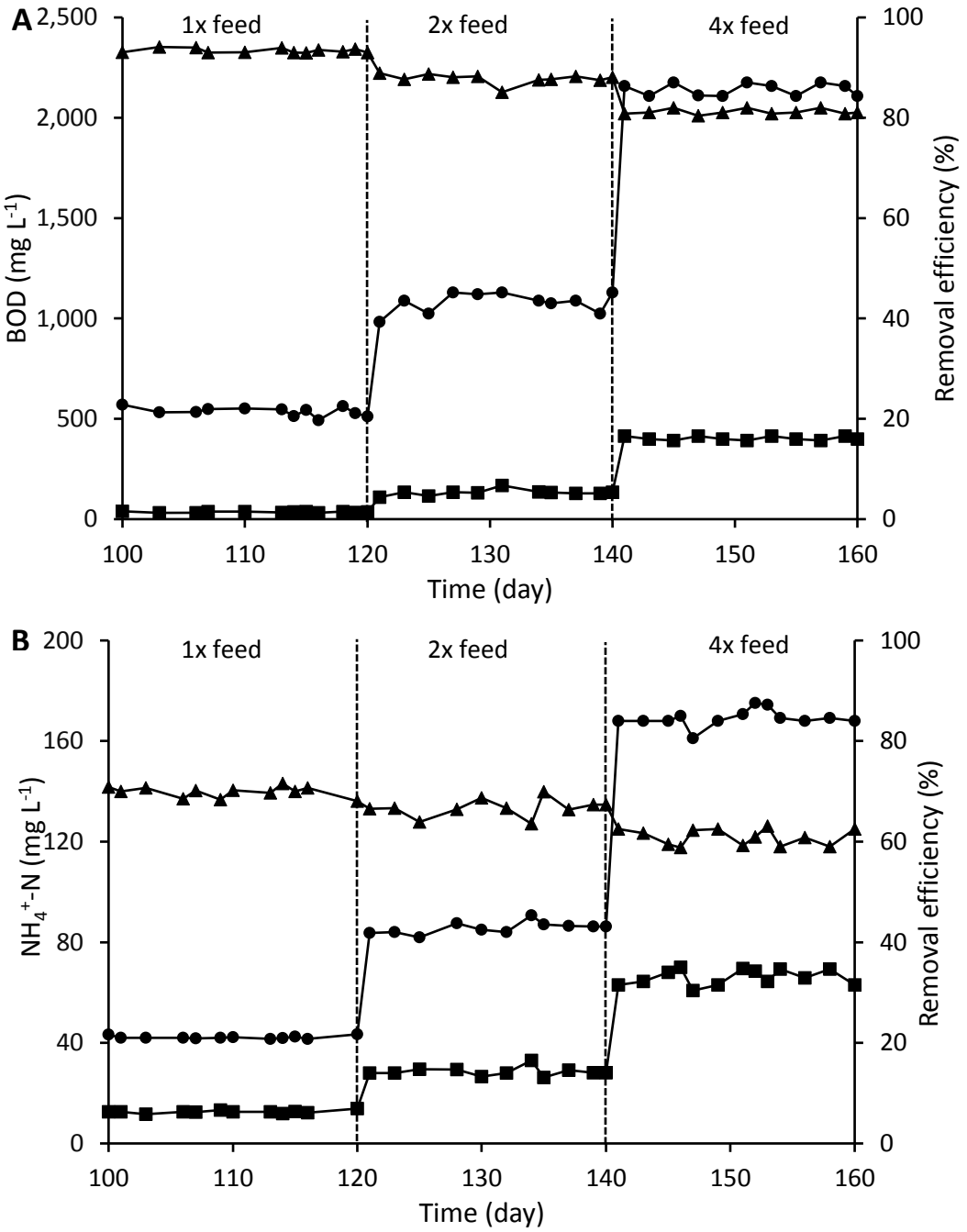

Figure 6.6: Performance of (A) organic carbon (BOD) and (B) nitrogen removal in the biofilm system under different feed concentrations: (●) influent, (■) effluent and (▲) removal efficiency.

In terms of nitrogen removal, the zeolite amended GAO biofilm showed a stable (70%) NH_4^+-N removal efficiency with standard-strength (1x) wastewater (Figure 6.6B). The removal efficiency slightly decreased to 67 % and 61% for double and quadruple strength wastewater, respectively. Nevertheless, the nitrogen removal rate (NRR) of the biofilm system (37.1 g m^{-3} d^{-1} at 4x feed) increased more than 2 times (17.04 g m^{-3} d^{-1} at 1x feed) over the period of the experiment demonstrating the stability of the described system to treat high-strength wastewater. It is worth to note that, nitrogen removal rate was not found to be negatively affected by the increase in nitrogen concentration, which may be due to the presence of zeolite in the biofilm as described in previous studies. He et al. (2007) reported that addition of zeolite powder could enhance the ability of the activated sludge in resisting the toxic shock load arising from organic compounds and ammonium.

6.3.5 Dynamic change of microbial communities with increased wastewater concentrations

The biofilm samples (day 120, 140 and 159) were analyzed with high-throughput methods to reveal the shifts in overall microbial community composition with the increase in influent BOD and NH_4^+-N concentrations. The initial bioinformatics analysis yielded a total of 219989 sequences which were assigned to different taxonomic levels. Overall, the richness of the bacterial community in the biofilm reactor declined over the experimental period, as evidenced by the decrease of both Chao 1 index and numbers of observed OTUs (Table 6.2). In addition, the microbial diversity also slightly decreased which is marked by the drop in Shannon index from 4.22 (day 120) to 4.02 (day 159).

Table 6.2: Community diversity indexes in different biofilm samples.

Samples	No of sequences	Chao 1	Observed OTUs	Shannon index
Day 120	82231	1213.14	693	4.22
Day 140	48041	1175.94	688	4.05
Day 159	89717	1004.16	637	4.02

The relative abundances of the top 11 genera in the biofilm samples from the different operational periods are shown in Figure 6.7. The genus of *Candidatus competibacter, Nitrospira,* and *Ignavibacterium* was distinguished by the difference of magnitude of their relative abundances over the experimental period. The most notable phenomenon in the community composition was the fraction of *Candidatus competibacter*, which increased from 20% (day 120) to 27% (day 159) as the influent wastewater concentrations increased 4 times within 40 days of operation. Members of the bacteria *Candidatus competibacter,* a known glycogen accumulating organisms (GAO), have been reported to uptake organic carbon anaerobically and store as PHA, but the cells accumulate glycogen instead of poly-phosphate under aerobic conditions (Zeng et al., 2002). The increased removal of BOD and accumulation of PHA by the biomass with concentrated wastewater (Figure 6.6) is associated with the increase in the proportions of GAO in the biofilm samples which is in accordance with previous reports (Hossain et al., 2017). In addition, the GAOs were also responsible for the denitrification reaction during the aerobic period since these bacteria are known to use their intracellular storage polymers (i.e., PHA, glycogen) as an electron source to reduce nitrite and/or nitrate (Bassin et al., 2012a; Coats et al., 2011).

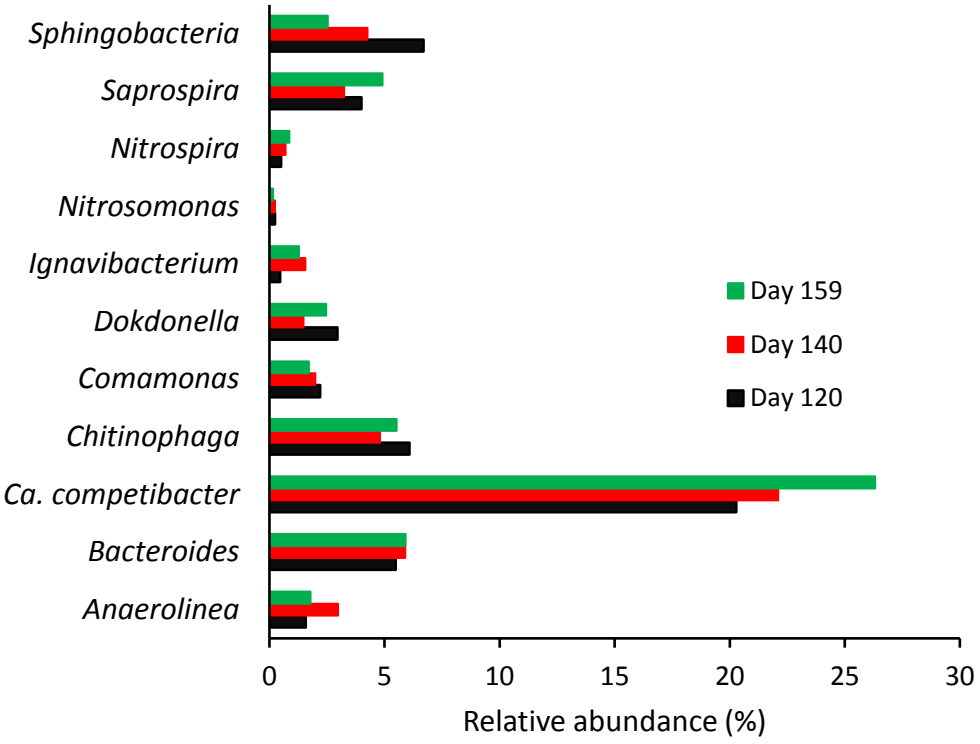

Figure 6.7: Relative abundances of the top 11 genera in the biofilm samples. The relative abundance for each genus was defined as the sum of OTUs assigned to a genus divided by the total OTUs of a biofilm sample.

Other microbial genera which showed a significant increase in their relative abundance includes *Ignavibacterium* and *Nitrospira*. The fraction of the microbial community associated with *Nitrospira*, a chemolithoautotrophic nitrite-oxidizing bacteria (NOB), increased about 40% when the influent NH_4^+-N concentration increased 4-times over the period of the present study. This observation is in accordance with the previous study (Tian et al., 2017). On the other hand, the abundance of *Nitrosomonas*, an ammonia-oxidizing bacteria (AOB) decreased from 0.24% to 0.16% during the experimental period.

6.3.6 Improvement of N removal efficiency by repeat treatment

The present study demonstrated that the zeolite amended GAO biofilm system could be an effective treatment option for high-strength wastewater. However, using a single treatment, the proposed technology was unable to remove nitrogen from wastewater

adequately (<72%) due to the limited capacity of the zeolite adsorbent and therefore required further treatment. Such proposed subsequent treatment trials have been assessed for the standard-strength wastewater treatment in Chapter 5 (Section 5.3.6.1). As explained previously, this subsequent treatment should not be done by a separate reactor as the lack of stored BOD present would not enable SND to occur (lack of electron donor for denitrification). To solve this problem, the effluent was returned to the same reactor in which residual stored PHA was still available.

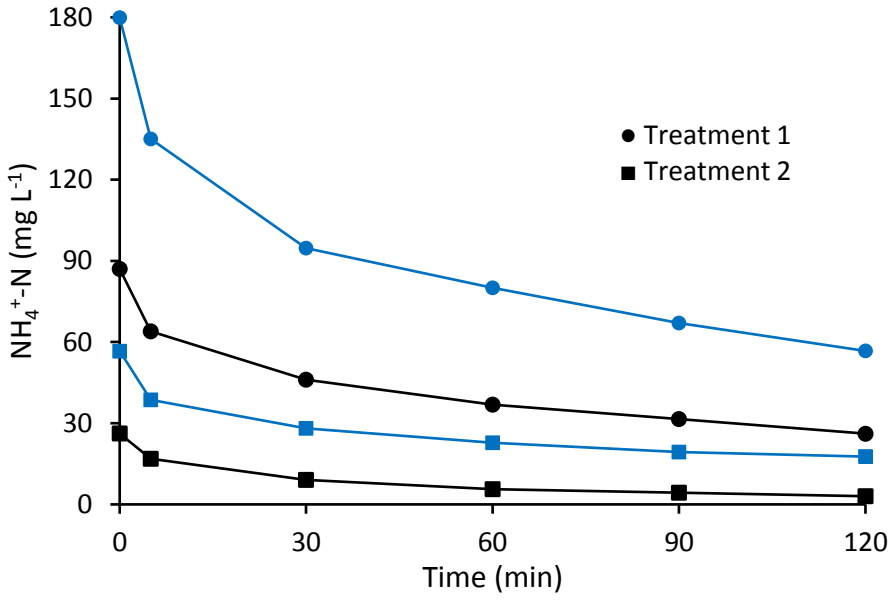

Figure 6.8: Treatment of double (black) and quadruple (blue) strength wastewater in the zeolite amended GAO biofilm in two successive treatment cycles (each treatment cycle consisted of anaerobic and aerobic phase).

With two successive treatments using the same reactor, the total nitrogen removal efficiency for both double and quadruple strength wastewater could be increased to more than 90% (Figure 6.8). This observation suggests that high-strength wastewater requires several repeated treatments before discharging to the environment. A key drawback of the repeat treatment of high-strength wastewater is that it will increase the total treatment time. Moreover, the repeated filling and draining of the wastewater will incur additional

energy costs. Since wastewater treatment in the described biofilm system avoids the costly and energy-intensive transfer of oxygen into the bulk wastewater, the total energy consumption might remain remarkably lower than that of the conventional activated sludge process.

6.4 Conclusions

Overall, the present study demonstrated that zeolite amended GAO biofilm could be an effective treatment option for high-strength wastewater. Based on the findings, the following conclusions could be drawn:

- Synthetic wastewater having 4-times higher concentration compared with the municipal wastewater could be successfully treated using the zeolite amended GAO biofilm.
- With high organic loading rate, the BOD uptake rate by the GAO biofilm increased accordingly resulting in higher accumulation of intracellular storage polymer (i.e., PHA).
- The influent NH_4^+-N was adsorbed onto zeolite during the anaerobic phase and removed via PHA-based simultaneous nitrification and denitrification in the subsequent aerobic period. The removal of high concentration of NH_4^+-N was benefited from the increased PHA storage.

Chapter 7

Optimization of Nitrogen Removal by Shortening and Repeating Cycles

Abstract

The limited nitrogen removal performance of the zeolite amended GAO biofilm (described in Chapter 5), requires a repeat treatment of wastewater resulting in doubling of total treatment time. This chapter describes and tests a concept that can make use of the effect of repeat treatment without extending the total treatment time. The approach involves treating a batch of wastewater in multiple anaerobic and aerobic phases (2, 4, and 8 times) in the zeolite amended GAO biofilm while maintaining the same total treatment time (8 h). Results showed that an increase in the treatment cycles from 2 to 8, results in increased nitrogen removal efficiency from 79% to about 100%. A short aerobic phase was found particularly suitable for preserving the reducing power (PHA) which could enhance the bioregeneration of zeolite via simultaneous nitrification and denitrification during the aerobic stage. Although a better nitrogen removal performance was found for 8-times treatment cycle, such multiple treatment options would incur an additional operational cost due to repeated filling and draining of wastewater from the reactor. A modification of rotating biological contactor such as low rotation speed to provide a biofilm alternating anaerobic and aerobic conditions, seems a promising alternative which requires extensive research.

7.1 Introduction

Conventional wastewater treatment process is extremely energy-consuming due to the need for active aeration of bulk wastewater. In order to reduce the cost associated with aeration, a zeolite amended glycogen accumulating organism (GAO) dominated biofilm system was established and operated under sequential anaerobic and aerobic conditions as described before (Chapter 5). This biofilm reactor demonstrated its stability for sustained removal of organic carbon (BOD) and nitrogen (NH_4^+-N) from wastewater. Although the biofilm system was found to remove >93% BOD from synthetic wastewater, the removal of NH_4^+-N was limited to about 70%. The remaining high concentration of NH_4^+-N (around 12 mg L^{-1}) doesn't meet typical standard limits (<3 mg N L^{-1}) (Foley et al., 2010) for the discharge of the effluent into the environment.

In order to improve the nitrogen removal efficiency and to meet the discharge limit set by the regulatory agencies, the effluent required further treatment. However, as the effluent from the first treatment contains limited residual BOD, the subsequent treatment in a second reactor is not feasible as BOD is necessary to help drive the denitrification process during simultaneous nitrification and denitrification (SND). To solve this problem, the effluent containing mainly ammonium was returned to the same reactor in which residual stored reducing substrate (i.e., PHA) is still available. The repeated treatment was found to increase the nitrogen removal efficiency up to 96%. However, treatment of wastewater twice resulted in doubling of the total treatment time (10 h).

In order to make use of the effect of repeat treatment without extending the total treatment time, a new approach is introduced in this chapter. Several anaerobic and aerobic phases were used while keeping the total treatment time the same. Furthermore, a numerical model is established to predict the treatment efficiency.

7.2 Materials and Methods

7.2.1 Zeolite

The natural zeolite used in this study was obtained from a local supplier (Zeolite Australia Pty. Ltd.) and composed mainly of clinoptilolite and quartz. The zeolites were repeatedly washed with deionized water to remove adhering dirt and soluble impurities, dried at 105°C for 24 h. The dried zeolite was ground in a milling machine to a fine powder and passed through British Standard Sieves (BSS). Zeolite particles with size of 75 μm was used in the current study.

7.2.2 Experimental setup and operation

The development of zeolite amended GAO biofilm reactor was described in Chapter 5 (Section 5.2.2). The zeolite amended GAO biofilm reactor was operated under sequential anaerobic and aerobic two-stage operation mode. During the stage one (anaerobic/ feast), the reactor was fully loaded with synthetic wastewater (full void volume) and remained at the anaerobic condition to remove organic carbon and ammonium. At aerobic (famine) stage, the anaerobically treated wastewater was drained out of the reactor by the gravity. The top of the reactor was open to enable oxygen passively entering into the reactor, whereby the synthesized biofilm was exposed to atmospheric oxygen. The drained and partially treated liquid was collected in a container to treat it multiple times in the PASND reactor. In this experiment, a total treatment time of 8 h was selected during which the wastewater was treated multiple times ranging from 2 to 8.

7.2.3 Synthetic wastewater

In this study, synthetic wastewater (Third et al., 2003b) was used to maintain tight control of influent constituents and enable full reproducibility for the establishment of a proof-of-concept study of low energy, passive aeration biological organic carbon and nitrogen

removal. The concentrated synthetic wastewater was prepared in two separate solutions using deionized (DI) water. The first solution contained acetate as carbon source, while the other contained the required nutrients, minerals and trace elements. After dilution with DI water, the influent wastewater contained (mg L^{-1}): CH_3COONa 660, NH_4Cl 160, KH_2PO_4 44, $NaHCO_3$ 125, $MgSO_4$. $7H_2O$ 25, $CaCl_2$. $2H_2O$ 300, $FeSO_4$. $7H_2O$ 6.25, yeast extract 50, and 1.25 ml L^{-1} of trace element solution, which contained (g L^{-1}): EDTA 15, $ZnSO_4$. $5H_2O$ 0.43, $CoCl_2$. $6H_2O$ 0.24, $MnCl_2$. $4H_2O$ 0.99, $CuSO_4$. $5H_2O$ 0.25, $NaMoO_4$. $2H_2O$ 0.22, $NiCl_2$. $6H_2O$ 0.19, $NaSeO_4$. $10H_2O$ 0.21, H_3BO_4 0.014 and $NaWO_4$. $2H_2O$ 0.050.

7.2.4 Analytical parameters of interest

The concentrations of chemical oxygen demand (COD), ammonium (NH_4^+-N), nitrite (NO_2^--N) nitrate (NO_3^--N), and orthophosphate (PO_4^{3-}-P) in the influent and effluent of the biofilm reactor were regularly measured to monitor system performance. Ammonium, nitrite, nitrate, orthophosphate, and COD concentrations were determined in accordance with Standard Methods (APHA, 2012). The acetate was measured by Gas Chromatography (Agilent, USA) according to the method used in Hossain et al. (2017). Freeze dried biomass was used to measure poly-hydroxyalkanoates (PHAs) and glycogen. PHAs were determined by the sum of poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV), which were analyzed as previously reported (Smolders et al., 1994a). Glycogen was analyzed according to the method used by Hossain et al. (2017). Total suspended solids (TSS), and volatile suspended solids (VSS) were measured according to Standard Methods (APHA, 2012). pH and DO were measured using a pH meter and a DO meter (Mettler-Toledo, USA), respectively.

7.2.5 Statistical analysis

Statistical analysis of the experimental data was conducted using the SPSS software package (IBM-SPSS v22). A one-way analysis of variance (ANOVA) was used to test whether a certain factor impacted an observed variable. Pearson's correlation coefficient was applied to quantify the relationship between two parameters. An $\alpha = 0.05$ and $p < 0.05$ indicated statistically significance.

7.3 Results and Discussion

7.3.1 Multiple treatment of wastewater

Having proven that repeated periodic treatment could achieve enhanced nitrogen removal, different frequencies of treatment (different number of the cycle of anaerobic/aerobic phase with one batch of wastewater treatment) was tested. In this experiment, a total treatment time of 8 h was selected during which the wastewater was treated multiple times ranging from 2 to 8 (Table 7.1).

Table 7.1: Experimental design and parameter changes.

Run	Number of cycles	Reactor operation		Total treatment time (h)
		Anaerobic (min)	Aerobic (min)	
I	2	120	120	
II	4	60	60	8
III	8	30	30	

7.3.1.1 Run I (2 treatment cycles)

In the case of 2 cycles (each cycle consisted of 2 h anaerobic and 2 h aerobic phase), the described biofilm system was found to be able to remove about 61% NH_4^+-N at a rate of 12.6 mg L^{-1} h^{-1} and 90% BOD during the anaerobic phase of the first cycle (Figure 7.1). This removal efficiency is low compared to the ammonium removal as described previously (Chapter 5). The reason for lower removal efficiencies obtained in this part of the thesis is the lower biomass and zeolite concentrations (about 5% lower), due to re-packaging of the reactor to overcome mixing limitations (Section 7.2.2). After the aerobic regeneration phase of the first cycle, the biofilm was found to be able to remove all the remained BOD and further 7.1 mg NH_4^+-N L^{-1} during the anaerobic phase of the 2nd cycle, resulting in a total of about 79% nitrogen removal from the wastewater. However, the

remained high concentration of ammonium (8.8 mg L^{-1}) suggests that the effluent is still not suitable for release into the environment and requires additional treatment.

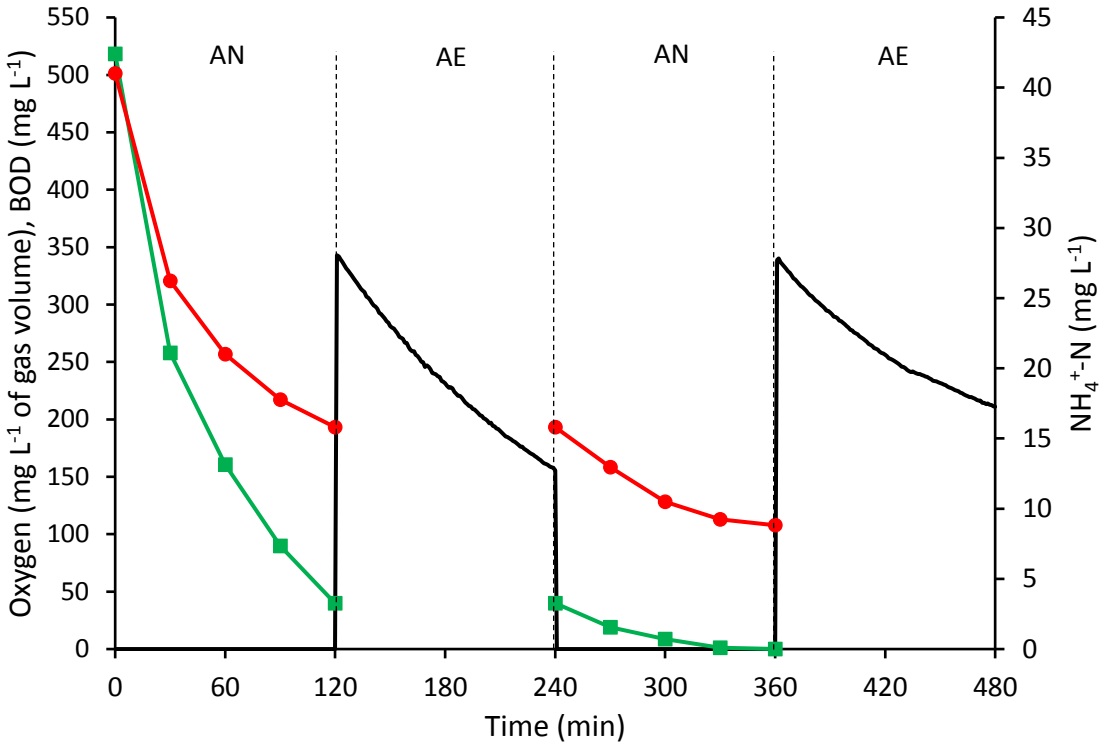

Figure 7.1: Profiles of BOD (■), NH_4^+-N (●) and oxygen (—) during the 4 h (2 h anaerobic and 2 h aerobic) treatment cycles: AN = anaerobic phase, AE = aerobic phase.

7.3.1.2 Run II (4 treatment cycles)

By increasing the number of treatment cycles to four, each cycle consisted of 1 h anaerobic and 1 h aerobic phase. During the first cycle anaerobic phase (0-60 min), about 18.1 mg NH_4^+-N L^{-1} (51.8%) was removed by adsorption onto the zeolite particles (Figure 7.2). The lower nitrogen removal efficiency compared to the 2 h anaerobic phase (Figure 7.1) is expected because of the shorter contact time between zeolite particles and ammonium ions as described previously (Section 4.3.1.2). However, continuous NH_4^+-N removal was found in the following anaerobic phases (2^{nd} to 4^{th} cycles) confirming that zeolite was regenerated and the ammonium adsorption capacity was recovered during the

relatively short 1 h aerobic phase. Overall, the 4 cycles of treatment within the same total operational period (8 h) resulted in about 93% nitrogen removal from wastewater.

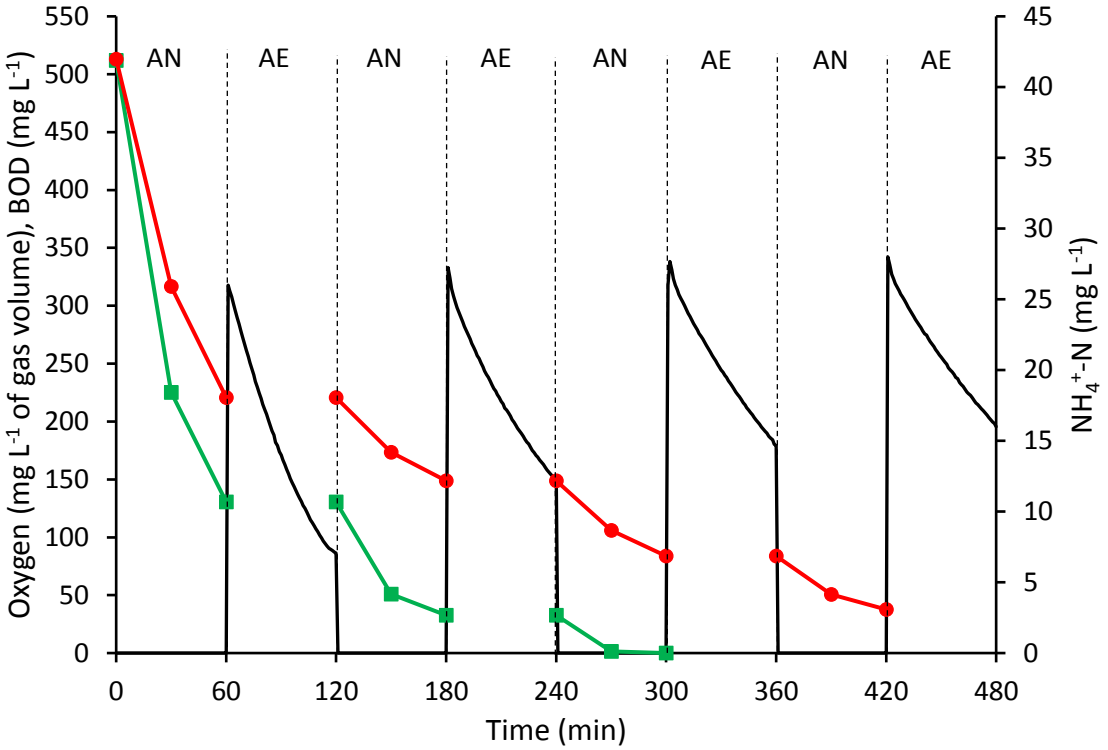

Figure 7.2: Profiles of BOD (■), NH_4^+-N (●) and oxygen (—) during the 2 h (1 h anaerobic and 1 h aerobic) treatment cycles: AN = anaerobic phase, AE = aerobic phase.

In the first anaerobic phase (cycle 1), the GAO biofilm removed about 381 mg BOD L^{-1} (74.5%), which again can be explained by the short time duration. It is worth to note that all the BOD was completely removed within the 3rd treatment cycle. Therefore, the denitrification reactions in the 4th aerobic phase (420-480 min) was principally driven by the residual PHA that was stored during the anaerobic phase of previous cycles (cycle 1 to 3). This observation suggests that short aerobic phases are particularly useful to preserve reducing power (i.e., PHA) which would result in efficient nitrogen removal via SND.

7.3.1.3 Run III (8 treatment cycles)

The zeolite amended GAO biofilms ability was further evaluated by increasing the number of treatment cycles to 8-times which involved 0.5 h anaerobic and 0.5 h aerobic phase for each cycle (Figure 7.3).

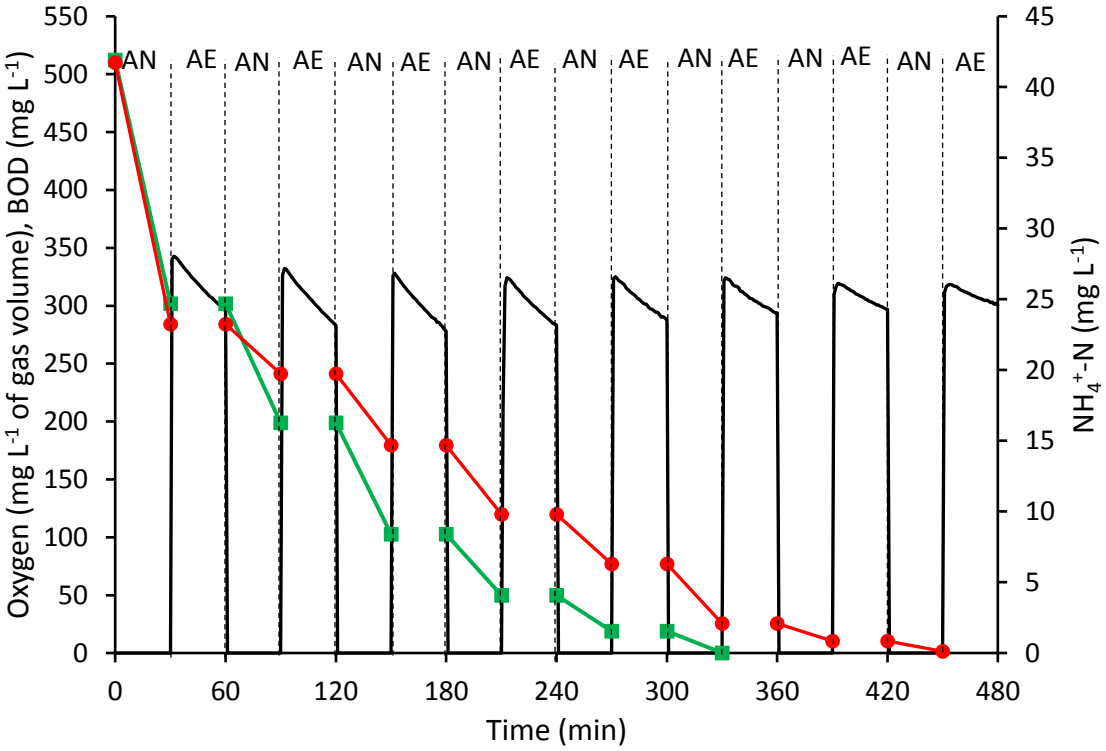

Figure 7.3: Profiles of BOD (■), NH_4^+-N (●) and oxygen (—) during the 1 h (0.5 h anaerobic and 0.5 h aerobic) treatment cycles: AN = anaerobic phase, AE = aerobic phase.

For nitrogen removal, the zeolite amended GAO biofilm used in this study removed only 44.3% NH_4^+-N in the first anaerobic phase (0-30 min). This reduced removal efficiency is due to the time limitation for the zeolite to reach the equilibrium. The adsorption of ammonium in the second anaerobic phase (60-90 min) was only 15.1%. This can be explained by the fact that the short aerobic time (30 min) applied was insufficient to regenerate the zeolite completely. However, over the subsequent anaerobic phases, continuous

removal of ammonium was achieved and the zeolite almost completely adsorbed all the ammonium (>99%) present in the wastewater within 8 h.

In terms of organic carbon removal, the biofilm system showed similar performance. About 41% BOD (210 mg L^{-1}) was removed during the first anaerobic phase, and >99% of the acetate (GC analysis), as well as COD (COD essay, Appendix 2) from wastewater, was removed within 5.5 h (6 cycles). Since no carbon was left in the last two cycles, denitrification was attributed to the use of PHA stored in the previous anaerobic phases as the electron donor.

7.3.2 Comparison of the treatment schemes

The treatment of one batch wastewater in repeated anaerobic and aerobic cycles resulted in increased nitrogen removal efficiency. When the wastewater was treated 2-times in 8 h period, the NH_4^+-N removal efficiency was about 79 % (Figure 7.4). On the other hand, the zeolite in the biofilm almost completely removed NH_4^+-N in 8 successive treatment cycles, suggesting that repeated treatment could potentially be an ideal option for efficient removal of organic carbon and nitrogen from wastewater.

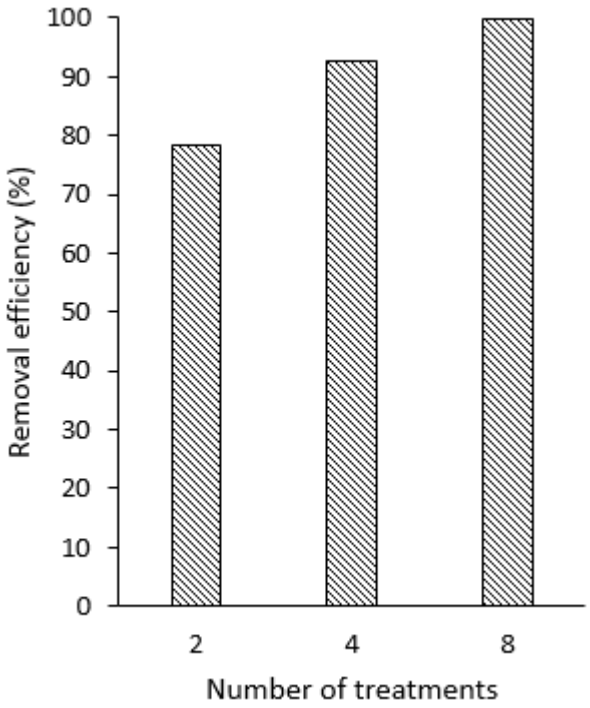

Figure 7.4: NH_4^+-N removal efficiency in different numbers of repeated treatment.

7.3.3 Modelling

A mathematical tool based on Langmuir ion-exchange model was developed in the current study to predict how rapidly and how effectively ammonium can be removed by using single or multiple cycles. The kinetic derivation of the model can be broken down into separate adsorption and desorption rate determination (Masel, 1996).

The adsorption of ammonium onto zeolite is driven by the ammonium concentration in aqueous solution, adsorption capacity of zeolite and the adsorption coefficient and can be expressed as follows:

$$r_{ad} = K_{ads}\,[S]\,(A_{cap} - A_{ads}) \qquad (7.1)$$

Where, r_{ad} is the rate of ammonium adsorption onto zeolite, K_{ads} is the adsorption coefficient (min^{-1}), [S] is the ammonium concentration in solution (mg L^{-1}), A_{cap} is the adsorption capacity of zeolite (mg NH_4^+-N g^{-1} of zeolite), A_{ads} is the ammonium adsorbed onto zeolite (mg NH_4^+-N g^{-1} of zeolite).

Similarly, the rate of desorption (r_{des}) can be expressed by the following equation:

$$r_{des} = K_{des} A_{ads} \quad (7.2)$$

Where, K_{des} is the desorption coefficient (min^{-1}) and A_{ads} is the ammonium adsorbed onto zeolite (mg NH_4^+-N g^{-1} of zeolite).

Both adsorption and desorption reactions occur at the same time and an equilibrium is reached when they become equal. After estimating K_{ads} and K_{des} from experimental results in which the biofilm was established (Table 7.2) simple numeric model predictions of ammonium uptake by zeolite can be made.

Table 7.2: Modelling parameters for ammonium adsorption and desorption from zeolite obtained from the experiment.

Parameters	**Value**	**Unit**
S	42	mg L^{-1}
K_{ads}	0.0025	min^{-1}
A_{cap}	1.474	mg NH_4^+-N g^{-1} of zeolite
K_{des}	0.1	min^{-1}

By using the above equations for numerical predictions, the net anaerobic ammonium adsorption onto zeolite could be adequately predicted leading to ammonium removal efficiencies and time courses that resemble experimental results (Figure 7.5). The observation that theoretical model predictions only allow up to 64% ammonium removal in a single treatment is because the zeolite reached its maximum adsorption capacity for that particular ammonium concentration used (See Section 4.3.1.4). Therefore, the zeolite needs to be regenerated before it adsorbs additional ammonium from wastewater

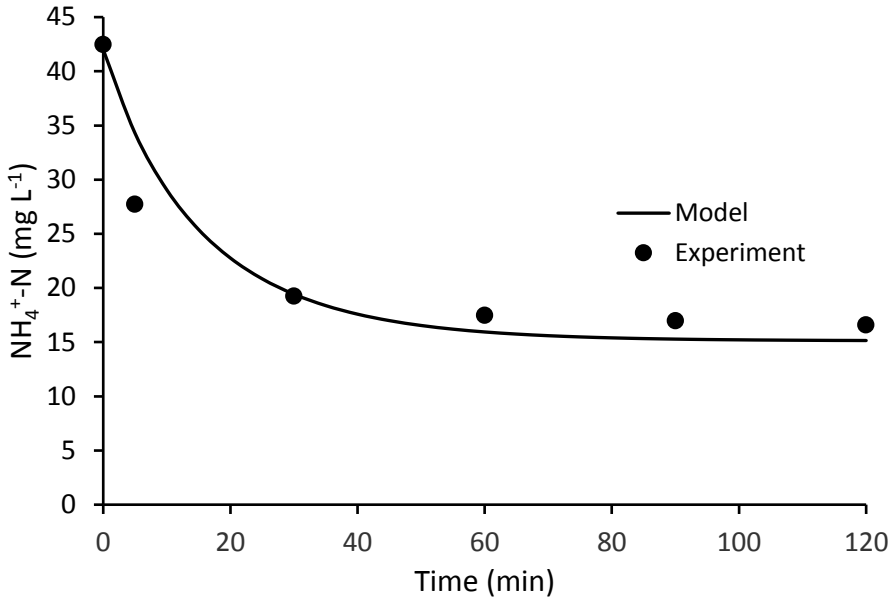

Figure 7.5: Kinetics of ammonium adsorption onto zeolite amended GAO biofilm and model prediction.

From the limited theoretical nitrogen removal efficiency of about 64.0%, one can conclude that repeat treatments will be essential to achieve a better nitrogen removal performance. From model predictions, a repeat treatment (assuming that during the aerobic stage between two anaerobic adsorption phases will completely regenerate the zeolite) should result in a similar percentage ammonium adsorption onto zeolite leading to a total maximum predicted nitrogen removal of 87%. However, the experimental repeat treatment of wastewater in the studied biofilm resulted in about 79% nitrogen removal (Figure 7.6).

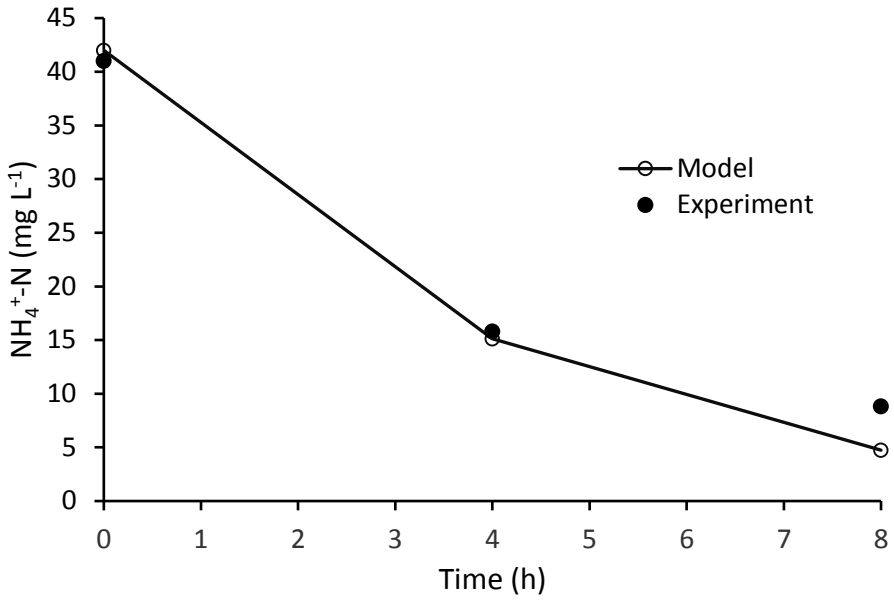

Figure 7.6: Optimized N removal performance during 8 h of total treatment time by using 2 cycles (2 h anaerobic and 2 h aerobic phase each) compared with model predictions. Model predictions for ammonium adsorption assume complete regeneration of zeolite during aerobic periods. Model predictions for percentage removal for each cycle were 64.0, and 68.7 %, respectively.

The model prediction of wastewater treatment in 4 repeated cycles is shown in Figure 7.7. In 4 repeat treatment cycle, the model predicts 98% ammonium removal, while about 93% removal performance was achieved in zeolite amended GAO biofilm. The reason behind this is that the developed model considered complete zeolite regeneration during the aerobic phase. In the case where ammonium is not completely removed during the aerobic phase, the amount of ammonium adsorbed in the subsequent cycles would be lower and depends on the residual ammonium concentration on the zeolite. Therefore, the low ammonium removal in the 2nd to 4th cycle (Figure 7.7) was due to incomplete zeolite regeneration. This observation suggests that the short aerobic phase time of 30 min was not enough for complete regeneration of zeolite.

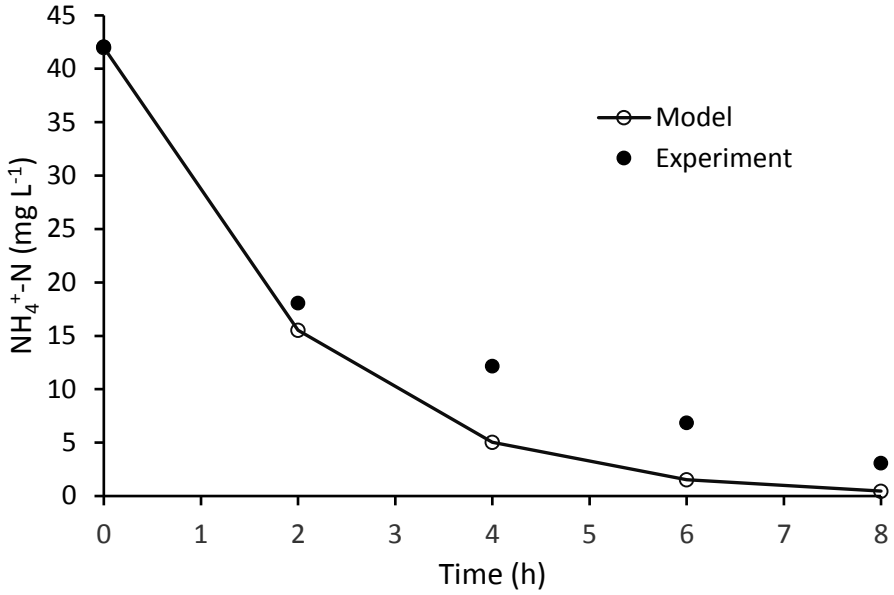

Figure 7.7: Optimized N removal performance during 8 h of total treatment time by using 4 cycles (1 h anaerobic and 1 h aerobic phase each) compared with model predictions. Model predictions for ammonium adsorption assume complete regeneration of zeolite during aerobic periods. Model predictions for percentage removal for each cycle were 63.0, 67.6, 69.3, and 69.9 %, respectively.

7.3.4 Practical implications

The repeated treatment of wastewater as described above resulted in the efficient removal of nitrogen from wastewater. However, the process involves repeated filling and draining of the reactor with the effluent. While the energy cost associated with the aeration of the bulk wastewater solution was avoided by providing passive aeration, the repeated filling and draining of wastewater will incur additional energy cost. This energy cost will increase proportionally for high-strength wastewater where more treatment cycles would be needed.

As an alternative option, the carrier material containing zeolite amended GAO biofilm could be lifted out of the water repeatedly rather than the solution filling and draining. This approach of the repeated lifting of biomass carrier out of solution is implemented by the well-known technology of rotating biological contactor (RBC) (Hassard et al., 2015).

By initiating half a turn of the RBC disks every two hours, the biofilm would experience a sequence of anaerobic and air exposure similar to experimental runs (Run I, Section 7.3.1.1) whereby the biofilm reactor was filled and drained every 2 hours (Figure 7.8).

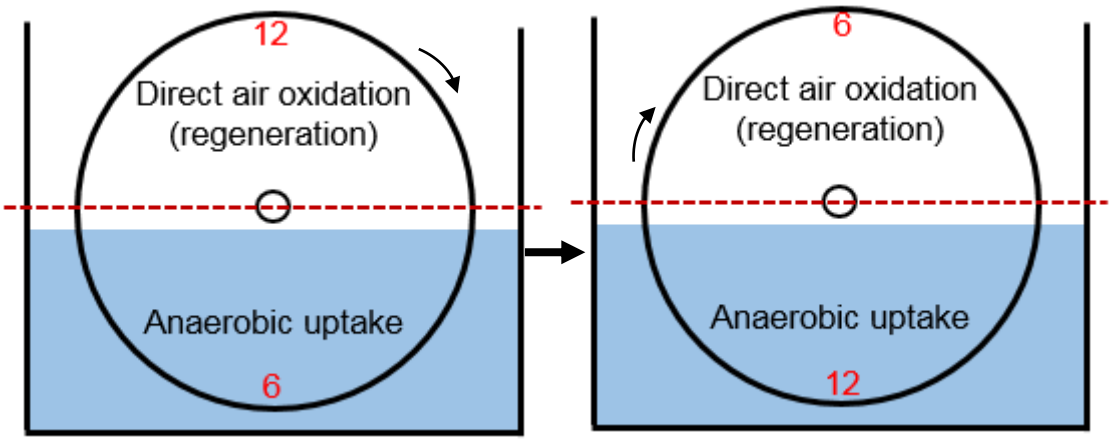

Figure 7.8: A schematic of the proposed modification of RBC operation that would simulate aerobic and anaerobic phases by a sudden 180º turn every two hours.

An alternative way of accomplishing the same result, namely exposure of each part of biofilm for the same period to the wastewater and air is to turn the RBC at low speed continuously. 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 after that to atmospheric air conditions for 30 min (Figure 7.9). By simply slowing down the rotation speed, similar results as the single zeolite amended biofilm reactor with periodic treatment could be achieved using the modified RBC.

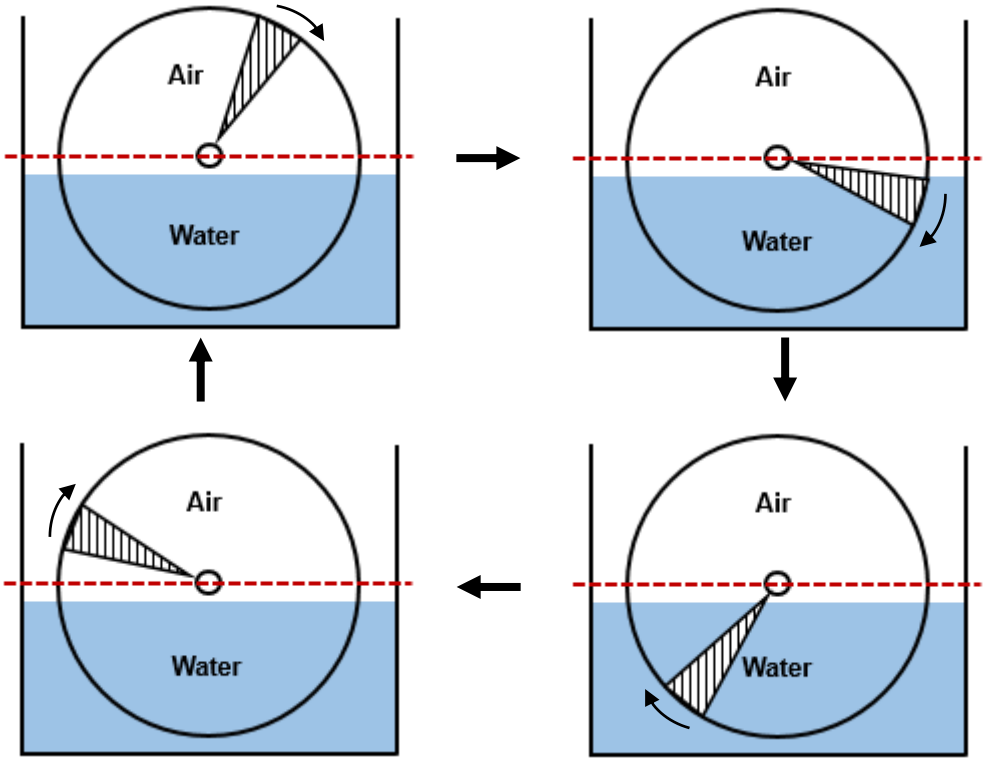

Figure 7.9: A schematic of the proposed modification of RBC operation that would simulate aerobic and anaerobic phases by rotating the disk at a speed of 1 rotation per hour: shaded area = biofilm.

7.4 Conclusions

The present study demonstrated that the developed treatment technology offers a better and efficient organic carbon and nitrogen removal performance if the wastewater is treated several times in the biofilm system. The biofilm reactor also provides a high degree of flexibility in the operation of the process where treatment cycle can be easily configured to achieve efficient performance. The duration of the anaerobic and aerobic phase can be extended depending on the strength of the wastewater to ensure optimization of the treatment process.

Chapter 8
Conclusions and Outlook

8.1 Potentials of the GAO Biofilm System

8.1.1 Use of nutrient containing wastewater for Irrigation

In order to reduce the energy input for wastewater treatment, McCarty et al. (2011) proposed a low-energy mainline (LEM) process, which involves treatment of wastewater through the anaerobic process (such as anaerobic membrane bioreactor) to remove organic compounds only. The remaining nutrients could be used for irrigation, and hence biological nutrient removal is avoided. Since the proposed GAO biofilm system effectively removes organic carbon, it could be an ideal option for wastewater treatment where effluent containing nitrogen and phosphorus could potentially be used for irrigation purposes.

8.1.2 Resource recovery

The PHA-rich GAO biofilm could be a suitable alternative for resource recovery in the form of bioplastics (Guest et al., 2009). This is particularly feasible for nutrient deficient wastewaters (such as pulp and paper mill effluent, dairy effluent, etc.) rich in soluble organic compounds. In addition, the PHA enriched biofilm could be subjected to anaerobic digestion as a source of methane gas because PHA (0.65 L g^{-1}) has higher methane potential than for example carbohydrates (0.45 L g^{-1}) and is readily biodegradable (Wang et al., 2016). Moreover, increased PHA content is known to enhance methane production by promoting cell disruption and soluble protein conversions (Wang et al., 2015a). Thus, the GAO dominated biofilm rich in PHA obtained in this work can guide engineers to develop improved WWTP operational strategies, which provide strong support to the ongoing paradigm shift in wastewater management from pollutant removal to resource recovery.

8.1.3 Removal of phosphorus

The passive aeration simultaneous nitrification and denitrification (PASND) biofilm process presented in this research demonstrated its ability to remove organic carbon and nitrogen (e.g., NH_4^+-N) efficiently from wastewater. However, the developed process could not remove phosphorus. To meet the strict discharge limits, set by the regulatory authorities, phosphorus need to be removed as well.

A simple phosphorus removal mechanism is chemical precipitation. Phosphorus can be easily precipitated using iron ($FeCl_3$), aluminium ($AlCl_3$) or lime $Ca(OH)_2$. $FeCl_3$ and $AlCl_3$ have a similar removal efficiency with a difference of <10%. However, phosphate precipitation with Fe^{3+} and Al^{3+} requires an acidic pH (e.g., 3.6-6.2), while calcium precipitates phosphate over a pH range of 8.0 to 9.0 (Tchobanoglous et al., 2014). Chemical precipitation (after removal of carbon and nitrogen) would be an ideal process for the effluent of the proposed reactor because the absence of organic compounds in the wastewater improves phosphate precipitation. Alternatively, phosphorus can be removed after the treatment of wastewater in PASND biofilm by adsorption (Sengupta & Pandit, 2011) which would lead to recovery of phosphorus as a usable fertilizer.

8.2 Limitations to Current Research

This research has demonstrated that GAO biofilm development simply from activated sludge is feasible in order to remove organic carbon and nitrogen from wastewater with low energy expenses. However, there are still many limitations and questions to be answered before the technology can be applied in large-scale. For example, synthetic wastewater used in this study contained mainly acetate which microorganisms can easily convert to PHA. However, real wastewater contains complex organics which are not readily removed by bacteria via PHA accumulation. When the GAO biofilm's performance

was evaluated with real wastewater (Appendix 3), COD removal efficiency (about 66%) was found poor than that with synthetic wastewater. Compared to the conventional treatment technologies, such COD removal efficiency is relatively low which can be explained by the high amount of non-biodegradable organics present in the real wastewater. A long-term adaptation of the GAO biofilm system to real wastewater is expected to alleviate the problem. Real wastewater also contains a large amount of suspended organic material which reduces the performance of the system by accumulating into the biofilm. Although such effect was not observed in the short-term study, it may pose a risk in long-term operation of the biofilm with real wastewater. The lack of such study may delay the application of the developed technology in large-scale.

The biofilm system described in the current study has only been shown at the laboratory-scale, and there was no indication of how such a biofilm concept can be scaled up such that it can handle a considerable amount of wastewater. For example, zeolite was integrated into the current study by trickling a suspension of zeolite particles over the biofilm. However, in a large-scale application, it is not feasible to trickle a large amount of zeolite into the biomass, and it is unsure in which way zeolite will be able to be integrated into the biofilm. Moreover, zeolite might fall off from the biomass which will increase the operational costs of wastewater treatment plants due to the continuous purchase of zeolite.

In the current research, potential greenhouse gas such as N_2O emission was not investigated. N_2O emission can occur at different steps of biological nitrogen removal process such as during denitrification (Kampschreur et al., 2009; Ni et al., 2011). Previous studies have reported that PHA based denitrification carried out by denitrifying polyphosphate accumulating organism (dPAO) and denitrifying glycogen accumulating organism

(dGAO) results in N_2O emission (Domingo-Félez et al., 2017; Zeng et al., 2003a). Compared with dPAO, N_2O accumulation rate is considerably higher in dGAO culture due to its low nitrogen oxides reduction rates. Moreover, nitrite inhibits the nitrous oxide reduction in dGAO (Ribera-Guardia et al., 2016), which also contributes to N_2O accumulation. Since the studied biofilm was predominated by GAO and denitrification was principally driven by PHA, it is possible that this biofilm system emits N_2O but to what level was not investigated in the current study due to time constraint. A detailed study, hence, is needed to evaluate the N_2O emission from this biofilm system.

8.3 Future Work

The limitations of this research have provided direction for future research needed in this area. In short, future work should focus on investigating the performance of the proposed biofilm technology with real wastewater, emission of greenhouse gas such as N_2O, and developing a field-scale biofilm unit to evaluate the feasibility of the proposed biofilm technology on a larger scale. Such a study would be an important aspect of the continued development and commercialization of the proposed wastewater treatment technology.

8.4 Concluding Remarks

The passive aeration simultaneous nitrification and denitrification (PASND) biofilm process can be easily developed using activated sludge as the sole biological starting material. This observation suggests that wastewater treatment plant operators can convert activated sludge systems readily into a “passive aeration” biofilm that avoids costly oxygen transfer to bulk wastewater solution.

The GAO dominated biofilm system has been shown to generate a very little amount of excess sludge (10-times less than that of activated sludge process) in long-term operation. The low sludge yield may lead to reduced sludge management and disposal cost of

wastewater treatment plants. Therefore, this treatment technology could be particularly suitable in densely populated areas where the land area for sludge disposal is scarce.

The proposed biofilm technology has shown its feasibility to treat wastewater with up to 4-times of municipal strength. This means that more concentrated wastewater that cannot be treated with conventional aerobic and anaerobic treatment technologies due to prohibitive aeration cost and inadequate nutrient removal could potentially be treated effectively using the described biofilm process.

The organic carbon and nitrogen removal efficiency of the PASND process can be optimized by using several shorter repeat treatments which could reach (>90%) nitrogen removal from synthetic wastewater. However, it is unsure what will be the total nitrogen removal efficiency of the biofilm system with real wastewater which contains organic nitrogen species as well. Since zeolite only adsorbs NH_4^+-N from wastewater, the nitrogen removal performance is expected to be low compared with synthetic wastewater. Therefore, a long-term study of the PASND process using real wastewater is needed.

Overall, the novel biofilm technology developed in this research has shown promises to effectively remove organic carbon and nitrogen from wastewater with low aeration cost but needs to be verified in field-scale before it is proven to worth in real-world applications.

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Appendix 1

Low-Energy Wastewater Treatment via Passive Aeration SND Using a Novel Zeolite Amended Biofilm Reactor

Abstract

The current paper describes a novel passive aeration simultaneous nitrification and denitrification (PASND) zeolite amended biofilm reactor that removes carbon and nitrogen from wastewater with extremely low-energy consumption. Next to ammonium oxidizing bacteria (AOB) this reactor contained naturally enriched Glycogen Accumulating Organisms (GAOs) and zeolite powder to initially adsorb BOD (acetate) and ammonia from synthetic wastewater under anaerobic conditions. Draining of the treated wastewater exposed the biofilm directly to air enabling low-energy oxygen supply by passive aeration. This allowed the adsorbed ammonium to be oxidized by the AOB and the produced nitrite and nitrate to be reduced simultaneously by the GAOs using the adsorbed BOD (stored as PHAs) as carbon source. Overall, with operation mode of 1 hour anaerobic and 4 hours aerobic phase, the nutrients removal efficiency after single treatment was about 94.3 ± 4.3% for BOD and 72.2 ± 3.1% for total nitrogen (TN) (NH_4^+-N, NO_2^--N and NO_3^--N). As high-energy aeration of the bulk solution for oxygen supply is completely avoided, the energy requirement of the proposed PASND biofilm reactor can be theoretically cut down to more than 50 % compared to traditional activated sludge process.

Introduction

Conventional wastewater treatment using activated sludge has been reliably and extensively used to remove organic matter and nutrients from wastewater since its introduction about 100 years ago. However, this treatment technology is energy intense and could be considered unsustainable because of high costs for oxygen supply and its dependency on a continuous energy supply. Approximately 60-65% of the total energy consumption is used for aeration for direct oxidation of soluble organic carbon (Foley et al., 2010). The reasons for such a high energy requirement lie in the poor solubility of oxygen (around 8 mg L^{-1}), the high volumes of airflow needed because of relatively low standard oxygen transfer efficiency (SOTE) ranging from 4.8% to 34.1% (Groves et al., 1992), and the need to supply compressed air against a water head of about 5 m (e.g., the depth of the reactor). A recently described biofilm process has demonstrated in laboratory experiments that instead of oxygen transfer into the bulk solution, direct uptake of oxygen from air by the biofilm by what is termed 'passive aeration' is possible. By avoiding the use of compressed air and oxygen transfer to the bulk solution more than 70% energy savings were achieved (Flavigny & Cord-Ruwisch, 2015; Hossain et al., 2017).

The direct oxygen uptake by the biofilm from air via passive aeration to a fixed bed bioreactor involves the presence of high concentrations of glycogen accumulating organisms (GAO) enriched in the biofilm (Flavigny & Cord-Ruwisch, 2015; Hossain et al., 2017). During an initial anaerobic phase, the submersed biofilm took up biological oxygen demand (BOD) from solution in the form of acetate and stored it as poly-hydroxyalkanoates (PHAs), a well-documented process in wastewater research (Ciggin et al., 2013; Third et al., 2003a; Van Loosdrecht et al., 1997a). Subsequently after draining the fixed bed reactor, biofilm was exposed to air enabling the direct uptake of oxygen, without the need of compressed air supply or indeed, in some cases any air supply other than that present in

the void spaces left by draining the wastewater (Flavigny & Cord-Ruwisch, 2015; Hossain et al., 2017). During the passive aeration phase the GAOs oxidized PHAs as long as electron acceptors are present (i.e., oxygen) to regenerate new BOD storage capacity in the following anaerobic phase (Flavigny & Cord-Ruwisch, 2015; Hughes et al., 2006; Liu et al., 1996; Van Loosdrecht et al., 1997a).

Although the previously described passive aeration biofilm reactor was able to remove BOD efficiently by relying on the sequence of anaerobic uptake followed by aerobic regeneration via passive aeration, it did not remove significant amounts of nitrogen (Flavigny & Cord-Ruwisch, 2015; Hossain et al., 2017). Nitrogen, which is removed from wastewater by nitrification and denitrification, cannot be readily stored by bacteria to enable anaerobic storage followed by aerobic regeneration using the passive aeration. However, it is well known that zeolite, an ion-exchange material can adsorb ammonium, which has also been shown to allow microbial regeneration of the ion-exchange capacity by nitrifying bacteria (Jung et al., 2004; Jung et al., 1999; Park et al., 2002). Therefore, to address the aforementioned disadvantages of high energy consumption of conventional wastewater treatment process using the previously established passive aeration fixed bed GAOs biofilm technology, we explore an option of introducing zeolite into the GAO biofilm to create a real energy-efficient wastewater treatment technology for both BODs and ammonium (NH_4^+-N) removal.

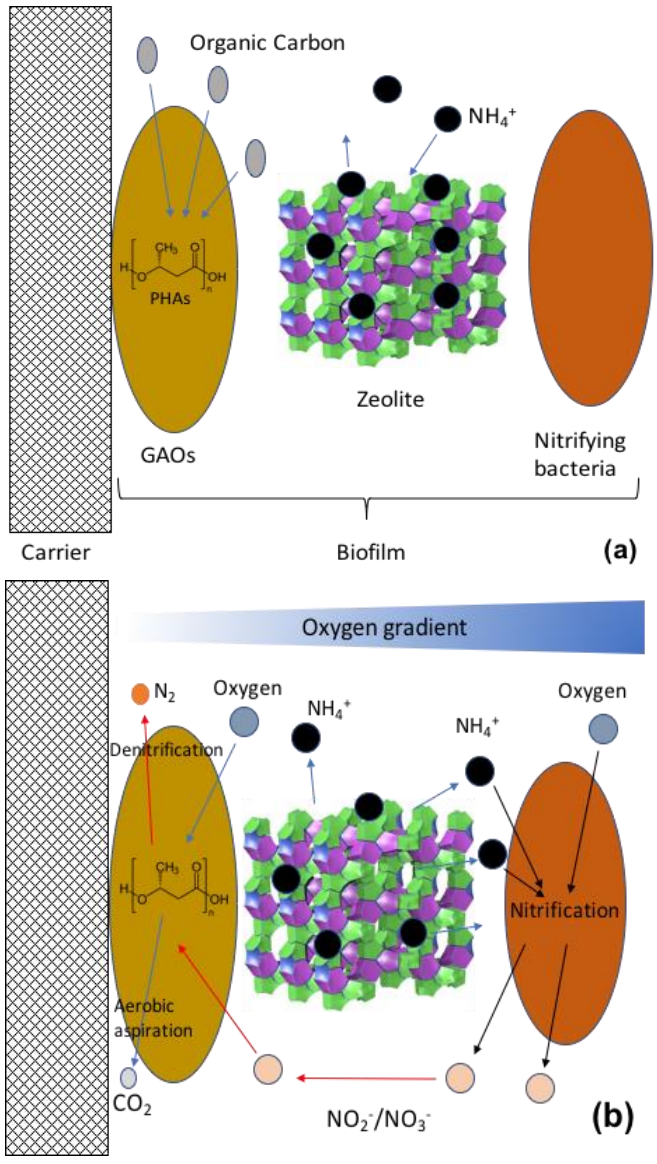

Figure A1.1: Illustration of wastewater treatment using sequential (a) anaerobic adsorption of organic carbon and ammonia and (b) oxygen dependent regeneration of storage capacity of GAOs and zeolite by including aerobic carbon and ammonium oxidation as well as denitrification by GAOs.

In the current study, a novel biofilm reactor consisting of GAOs, nitrifying bacteria, and zeolite was invented, named as passive aeration simultaneous nitrification and denitrification (PASND). Proposed mechanisms for BODs and ammonium removal of the invented PASND zeolite amended biofilm reactor is illustrated in Figure A1.1. In this system, the reactor is filled with wastewater, the dissolved organic carbon and ammonium is

removed simultaneously under anaerobic conditions via anaerobic carbon storage by GAOs and adsorption by zeolite, respectively (Figure A1.1a). Then, the treated wastewater is discharged from the reactor allowing the synthesized biofilm to be exposed to air and regenerated under passive aeration for the next batch of wastewater treatment. The two-stage bio-regeneration is achieved by nitrifying biofilm oxidation of zeolite adsorbed ammonium and subsequently GAOs biofilm denitrification of nitrifying products (nitrite and nitrate) via storage carbon (i.e., PHA) under anoxic conditions (Figure A1.1b). The mechanisms and capacity of C and N removal of the invented PASND biofilm reactor by a sequence of anaerobic storage followed by aerobic regeneration using passive aeration were thoroughly investigated in this study.

Materials and Methods

Synthetic wastewater

In this study, synthetic wastewater was used to ensure a well-controlled operation condition to be consistent throughout the whole study. A widely used synthetic wastewater was prepared (Third et al., 2003a), which consisted of (mg L^{-1}): CH_3COONa 672, NH_4Cl 160, $NaHCO_3$ 125, KH_2PO_4 44, $MgSO_4$ $7H_2O$ 25, $CaCl_2$ $2H_2O$ 300, $FeSO_4$ $7H_2O$ 6.25, yeast extract 50, and 1.25 mL L^{-1} of trace element solution, which contained (g L^{-1}): ethylenediamine-tetra-acetic acid (EDTA) 15, $ZnSO_4$ $7H_2O$ 0.43, $CoCl_2$ $6H_2O$ 0.24, $MnCl_2$ $4H_2O$ 0.99, $CuSO_4$ $5H_2O$ 0.25, $NaMoO_4$ $2H_2O$ 0.22, $NiCl_2$ $6H_2O$ 0.19, $NaSeO_4$ $10H_2O$ 0.21, H_3BO_4 0.014 and $NaWO_4$ $2H_2O$ 0.050.

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 dried zeolite was ground in a milling machine to gain fine powder with grain size ranging from 50 - 80 μm.

Set-up of PASND zeolite amended biofilm reactor

Plastic biofilm carrier material coated with enriched, active GAO biofilm (approximately 5.1 g dry weight), obtained from the previously established GAO biofilm reactor (Flavigny & Cord-Ruwisch, 2015), was packed in a tubular reactor with an inner diameter of about 68 mm and a height of 140 mm. The total volume of the reactor was about 400 mL. Then, 15 g of the zeolite powder (50 - 80 μm) was suspended in a synthetic wastewater solution and recirculated over the GAO biofilm for 24 h until most of the suspended zeolite powder (>99%) was attached onto the biofilm (indicated by the optical density). The ammonium adsorption capacity of the zeolite powder used was 1.68 mg-N g^{-1} zeolite when the NH_4^+-N concentration was 42 mg L^{-1}.

This zeolite coated biofilm was then coated by a layer of concentrated nitrifying bacterial enrichment to produce the sandwiched layer of GAO/zeolite/nitrifying bacteria. 2 L of the concentrated nitrifying bacterial enrichment culture was continuously trickled over the zeolite amended GAO biofilm for 24 hours at a rate of 120 mL min^{-1} until the OD_{600} value of the effluent decreased to about 0.1, indicating more than 95% of nitrifying bacteria were attached onto the carriers. The enrichment culture had been operating with ammonium enrich synthetic wastewater (approximately 140 mg NH_4^+-N L^{-1}) as the feed medium for more than 12 months under sequencing batch operation resulting in a suspension of granular sludge highly enriched for nitrifying bacteria. The volatile suspended solids level was 5.4 g L^{-1} with a maximum ammonium oxidation rate of 16.8 mg NH_4^+-N L^{-1} h^{-1}. This zeolite amended biofilm reactor is hereafter referred to as the passive aeration SND (PASND) biofilm reactor. The temperature of the reactor was kept at around 25^{o}C at all times and the pH value of the reactor was monitored.

General experimental set-up and operation

The wastewater treatment process was carried out in the PASND biofilm reactor using synthetic wastewater via sequencing anaerobic and aerobic/anoxic two-stage operation mode (Figure A1.2). In anaerobic phase (stage one), one full void volume of synthetic wastewater was up-flushed into the reactor and remained there for an anaerobic phase of 24 hours to remove organic carbons and ammonium. In aerobic phase (stage two), the anaerobically treated wastewater was drained from the reactor by gravity. The top of the reactor was left open to enable oxygen passively entering the reactor, whereby the synthesized biofilm was exposed to air for 24 hours. For the purpose of regular sampling and online monitoring, a minimum amount of residual liquid (less than 50 mL) was retained and slowly circulated through the column from the top to the bottom. Repeated cycles of the anaerobic and aerobic phases were carried out for the evaluation of system stability.

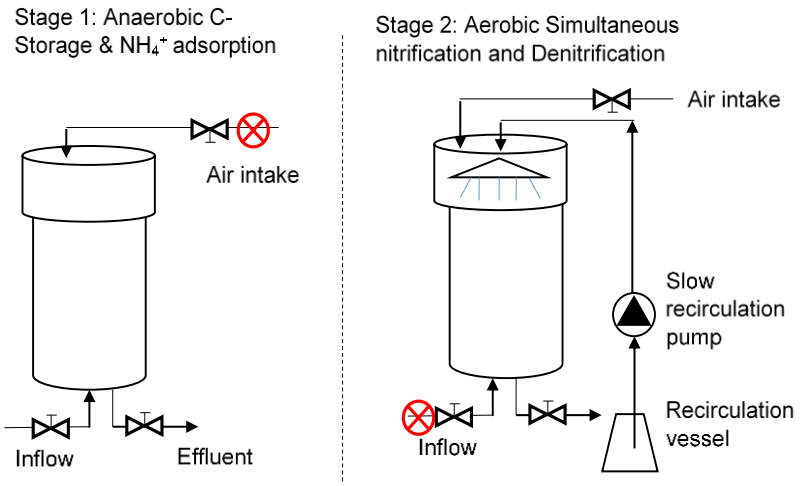

Figure A1.2: Schematic diagram of the reactor operation including anaerobic phase of stage one and aerobic phase of stage two.

Nitrogen gas production

Because of background N_2 in air, the demonstration of N_2 produced during denitrification required a N_2 free atmosphere, which was generated after transferring the biofilm on its carrier material to a 150 mL Schott bottle sealed with a rubber stopper. After placing a known weight of the synthesised biofilm carriers (containing approximately 3.12 g dry weight biofilm) in the air-tight Schott bottle, the biofilm was exposed to 3 batches of wastewater under anaerobic conditions (24 hours for each batch) to maximize organic carbon and ammonium storage for the subsequent nitrification and denitrification test of the biofilm under direct exposure to 20% oxygen. The measurement of N_2 production at the aerobic phase was conducted by removing all the liquid from the reactor and allowing the biofilm to be exposed to a gas mix consisting of 80% Helium (99% purity) and 20% oxygen. Gas samples (100 µL) were taken using a 500 µL gas-tight GC syringe through the rubber stopper. To verify that the whole procedure including sampling method did not introduce N_2 from atmosphere into the system, a control trial was conducted in the same manner without biomass. All gas samples were analysed in duplicate and an average of the value was used for determining the N_2, O_2 and CO_2 content.

Analysis and Monitoring

The concentration of acetate, ammonium (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N) in the inflow and outflow of the zeolite amended biofilm reactor was regularly measured to monitor the system performance. In this study, total nitrogen (TN) refers to sum of NH_4^+-N, NO_2^--N, and NO_3^--N. Ammonium, nitrite, and nitrate concentrations were determined in accordance with standard methods (APHA, 2012). The acetate was measured by Gas Chromatography (GC) (Agilent 7820A, USA) according to the method used in Flavigny and Cord-Ruwisch (2015). The N_2, O_2 and CO_2 content determination was also done through a GC.

Results and Discussion

Simultaneous anaerobic ammonium and organic carbon removal

The PASND biofilm reactor was operated and monitored under sequential anaerobic and aerobic conditions immediately after the synthetic biofilm was prepared, and the capacity of anaerobic adsorption of C and N was monitored (Figure A1.3a). Almost 100% BOD removal (acetate) and 80% ammonium removal was observed (Figure A1.3a). As the synthesized zeolite amended biofilm was rich in GAOs, the BOD removal under anaerobic conditions was mainly driven by the storage of the organic carbon (i.e., acetate) as polyhydroxyalkanoate (PHA) as previously described (Flavigny & Cord-Ruwisch, 2015). GAOs have demonstrated to uptake VFAs anaerobically with the required reducing power and energy both be derived from the glycolysis of glycogen (Zeng et al., 2002).

The rapid decrease in NH_4^+-N concentration in the first hour can be explained by the ammonium adsorption onto the zeolite particles. This is in line with the previous published work, which also showed a rapid adsorption of ammonium by zeolite within the first hour (Cooney et al., 1999; Halim et al., 2010). The results indicated that the PASND biofilm reactor was able to store both organic carbon and ammonium, through biological and chemical mechanisms respectively. However, to enable repeated adsorption and regeneration of both zeolite and GAO biomass, aerobic oxidation of the stored compounds is needed.

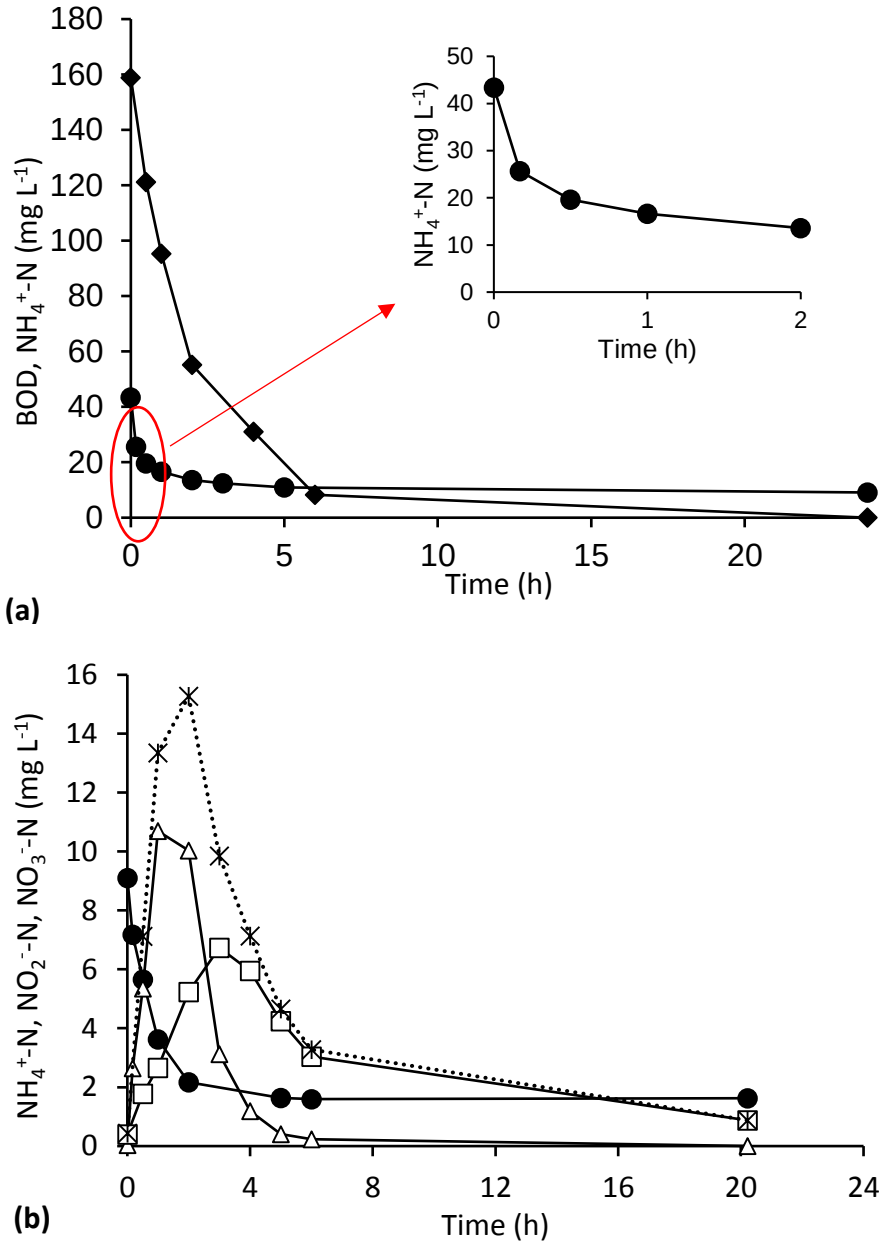

Figure A1.3: (a) Anaerobic BOD (♦) and ammonium (●) removal from wastewater by the zeolite amended GAO rich biofilm; (b) Time course of ammonium oxidation during direct air exposure of the zeolite amended biofilm in the first cycle operated after synthesis of the biofilm. Ammonium (●), nitrite (△), nitrate (□), and sum of nitrite and nitrate (∗) at aerobic phase.

Simultaneous nitrification and denitrification under passive aeration

In the subsequent aerobic regeneration stage, the draining of treated wastewater enabled oxygen uptake by the biofilm directly from air by what can be termed "passive aeration", as the provision of compressed air is not necessary. As expected, the entry of oxygen into the voids between the biofilm coated carrier material via passive aeration allowed nitrification of ammonium to NO_2^--N and NO_3^--N (Figure A1.3b), driven by the ammonium oxidizing biomass incorporated into the biofilm. Note that the total concentration of N produced as NO_2^--N and NO_3^--N during the first 2 hours seemed to be higher than the total concentration of NH_4^+-N available in the retained solution at the beginning of the aerobic phase. This can be explained by the fact that soluble NH_4^+-N monitored only represents a faction of total NH_4^+-N available, with the major part of all NH_4^+-N is always adsorbed onto the ion-exchanger zeolite according to Langmuir isotherm predictions. The relatively high intermediate accumulation of NO_2^--N and NO_3^--N also shows that indeed the NH_4^+-N adsorbed from the zeolite was being nitrified (Figure A1.3b).

Within the aerobic phase, NO_2^--N and NO_3^--N decreased to reach values less than 1 mg L^{-1} after about 20 hours. Since there was no organic carbon in the remaining liquid, the results indicated that denitrifying glycogen-accumulating organisms (DGAO) present in the synthesized biofilm enabled denitrification under the passive aeration conditions. It has been demonstrated that under anoxic conditions such intracellular stored organic carbon (i.e., PHA) could be used by DGAOs as an endogenous electron donor for NO_2^--N and NO_3^--N reduction, termed "storage carbon driven denitrification" (Beun et al., 2000; Hughes et al., 2006; Third et al., 2003a; Zeng et al., 2003c). Jones et al. (1990) reported that the GAOs are capable of carrying out denitrification without acclimatization time, which would be beneficial for enabling a rapid start-up of the current system in real applications.

The occurrence of denitrification under full atmospheric oxygen conditions is not likely to be "aerobic denitrification" but can be explained by a steep oxygen gradient in the biofilm. Poor oxygen penetration into biofilms is a well-known phenomenon. For example, oxygen was depleted completely at a depth of approximately 175 microns into a 220-micron-thick biofilm (Stewart & Franklin, 2008). Such oxygen depletion along the depth of biofilm was likely due to the active respiration by the bacterial cells in the upper layer of the biofilm. After detaching a portion of the synthetic biofilm from the carriers and suspending it in a fully aerated solution, complete inhibition of denitrification was observed (data not shown). This observation suggests that in the PASND biofilm reactor, the denitrification could occur because of the lack of oxygen penetration to the biofilm, rather than denitrifying biomass being tolerant to oxygen (Oh & Silverstein, 1999). While trace amounts of NH_4^+-N (< 2 mg L^{-1}) were observed in the retained circulated solution during the first cycle, after months of operation of the biofilm reactor, no residual ammonium was detected at the end of aerobic phase.

N_2 and CO_2 production during the aerobic phase

To verify whether denitrification was truly active during full exposure of the biofilm directly to the atmospheric oxygen partial pressure, the production of N_2 gas during aerobic phase was recorded (Figure A1.4).

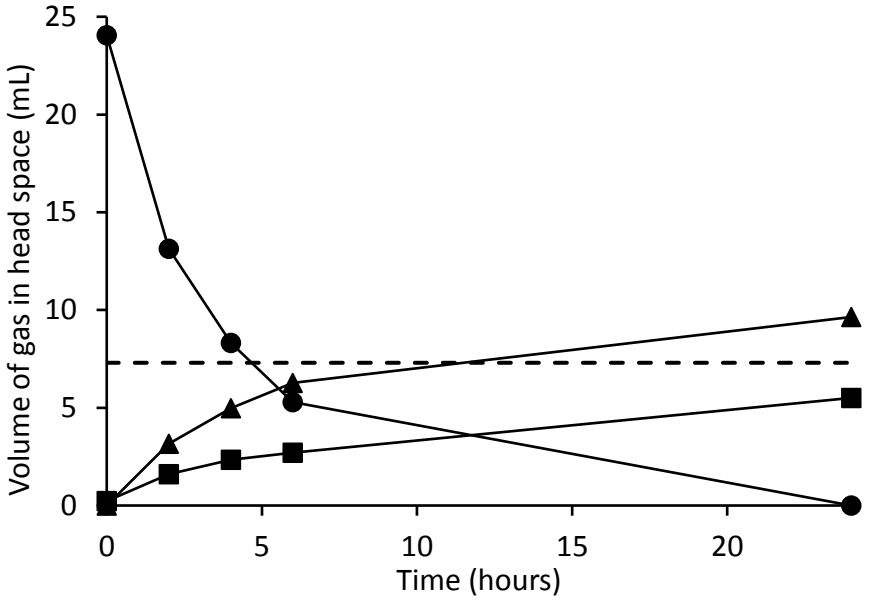

Figure A1.4: Batch production of nitrogen (■) and CO_2 (▲) and consumption of oxygen (●) by the drained zeolite amended biofilm during aerobic phase in N_2 free (80% helium, 20% oxygen) atmosphere. The expected nitrogen production (- -) was calculated from the ammonium adsorbed by the zeolite in the anaerobic phase.

The off-gas resulting from denitrification during the aerobic phase was measured after placing the biofilm that had been operated in the PASND reactor for about 2 months (22 cycles) in a 150-mL sealed bottle with helium (80%) and oxygen (20%) atmosphere. Over a period of 24 hours, 5.5 mL of N_2 was produced, which represented a recovery as N_2 of about 75.3% of the total adsorbed ammonium onto the zeolite (about 0.596 mmol of NH_4^+-N absorbed equivalent to about 7.3 mL of N_2 production if completely nitrified and denitrified) (Figure A1.4).

CO_2 was also produced as a result of oxidation of the stored intracellular PHAs (no other organic carbon was available) with both oxygen and NO_3^--N as electron acceptors. This test demonstrated CO_2 and N_2 as the key end products of the process and explain how the aerobic phase reactions regenerated the capacity of both ammonium adsorption by zeolite and organic carbon storage by GAO in the next anaerobic phase.

Sustained nutrient removal

Considering the described biofilm was synthesized by adding separately produced ammonium oxidizing biomass rather than selectively enriched, the longer-term nitrogen removal needed to be evaluated and the reactor was operated as described above for 22 cycles. During continuous operation for 6 weeks, the nitrogen removal performance of cycle 1, 14, and 22 was compared (Figure A1.5). About 82 % of NH_4^+-N in the inflow was removed consistently during the anaerobic phase and only 1.4 mg L^{-1} NO_3^--N remained in the aqueous phase at the end of aerobic phase (i.e., the 22^{nd} cycle) (Figure A1.5a and A1.5b). BOD (i.e., acetate) removal efficiency of about 98% during the anaerobic phase stayed constant throughout the test period (Figure A1.5a). This showed that the synthesized biofilm could sustain constant organic carbon and nitrogen removal from synthetic wastewater by a simple operation in which energy expensive use of compressed air for oxygen transfer to the bulk solution was avoided.

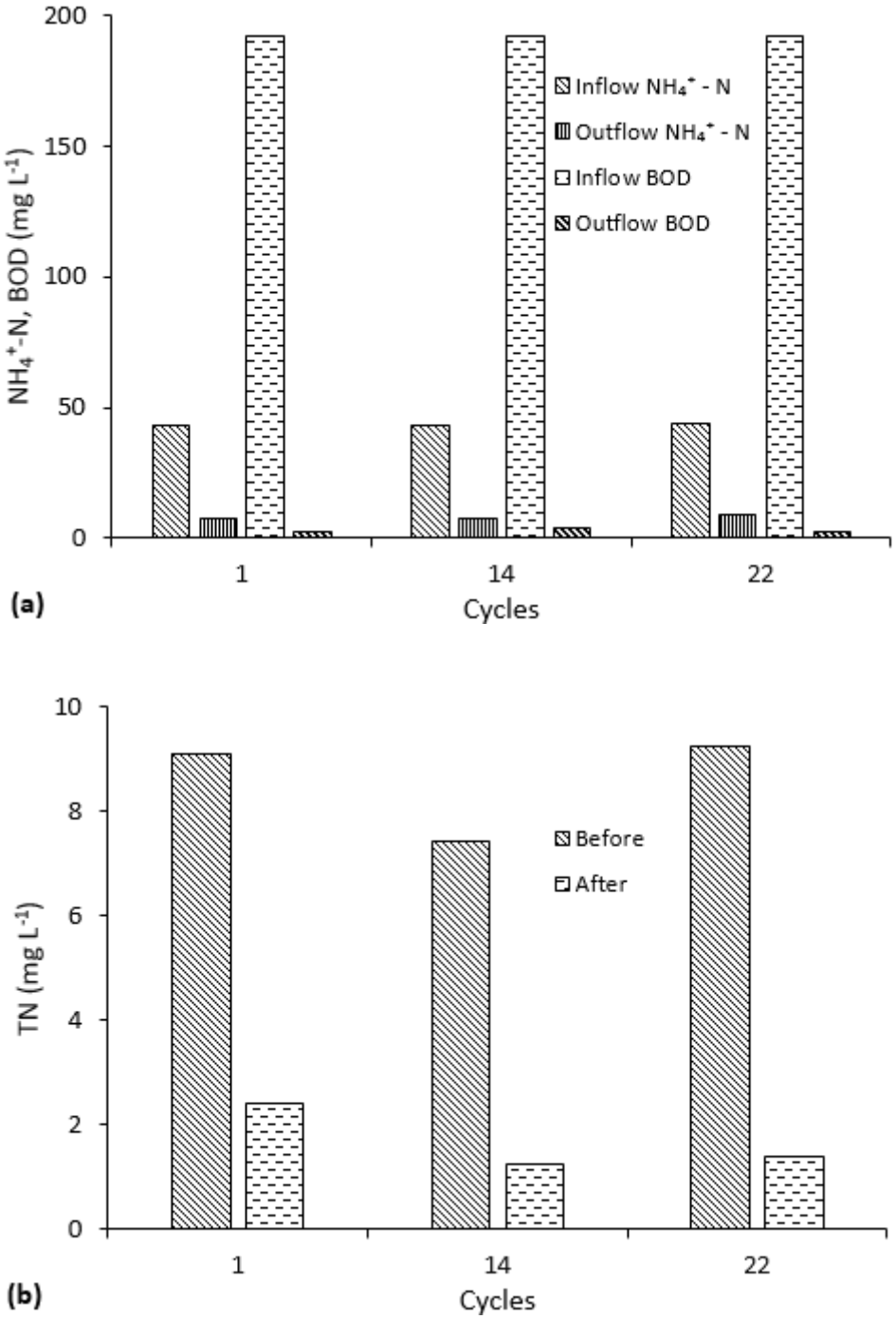

Figure A1.5: Concentrations of nutrients during a full cycle (24 h anaerobic phase and 24 h aerobic phase) of treatment over a long period of operation. a), concentration of ammonium and total organic carbon (BOD) in the inflow and the outflow after the anaerobic treatment (anaerobic phase-stage 1); b) total nitrogen (TN) (sum of NH_4^+-N, NO_2^-- N and NO_3^--N) in the aqueous phase before and after the aerobic treatment (aerobic phase-stage two). Before the treatment, TN refers to NH_4^+-N (left from the stage one). After the treatment, TN included only 1.4 mg NO_3^--N L^{-1} (i.e., cycle 22) while the concentration of NO_2^-- N was negligible.

Effect of shortening of total treatment time

The longer-term operation of the reactor under the original long total treatment time of 48 h (24 hours of anaerobic phase and 24 hours aerobic phase) showed an average BOD and TN removal efficiency of 97.4% and 81.3%, respectively (Figure A1.6, Cycles 1-22, Table A1.1). Given that the majority of the ammonium removal occurred within the first hour of the anaerobic phase (Figure A1.3a), a much shorter total treatment time (TTT) of 5 h (1 h anaerobic phase and 4 h aerobic phase) was tested. Initially this shortening to TTT decreased BOD and TN removal efficiency to 87.8% and 76.7%, respectively (Figure A1.6, Cycle 23). This was probably due to the insufficient contact time during the anaerobic phase for biological organic carbon uptake as well as chemical ammonium adsorption.

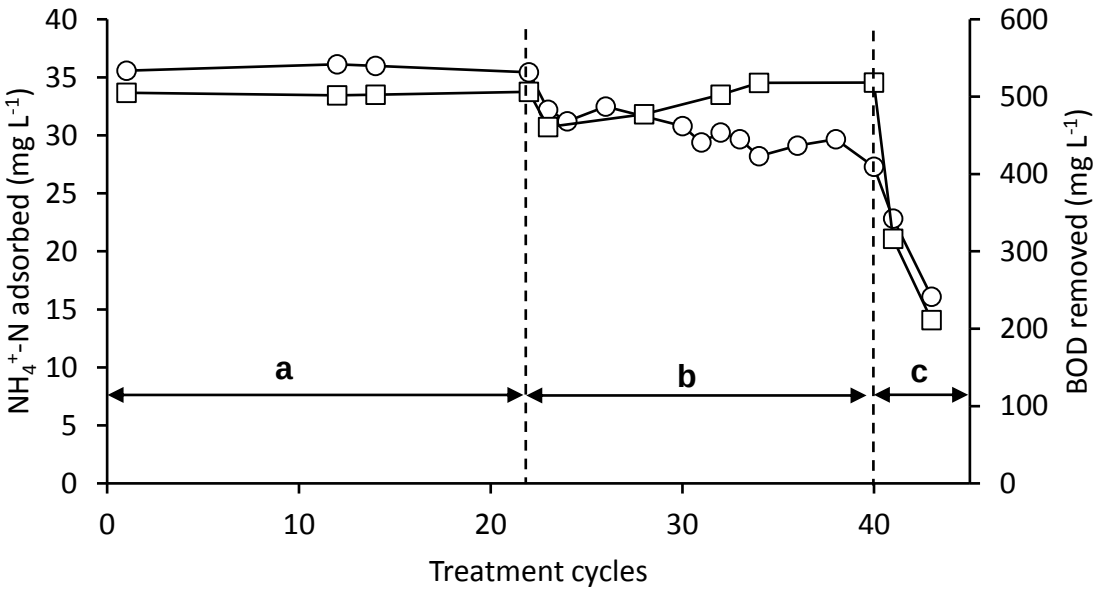

Figure A1.6. Summary of long-term performance of BOD (□) and NH_4^+-N (○) removal from synthetic wastewater under different operational conditions: (a) general operation: 24 h anaerobic + 24 h aerobic phase; (b) short TTT: 1 h anaerobic and 4 h aerobic phase; (c) no aerobic phase: 1 h anaerobic and no aerobic phase.

After prolonged operation under this short TTT of 5 hours (Figure A1.6, Cycles 23 - 40), the BOD removal efficiency recovered to about 98%, while the TN removal efficiency stayed between 65% -70%. At this short TTT of 5 h the TN removal efficiency did not recover and remained at this lower level. It is explained by the combination of shorter anaerobic adsorption time of 1 h and the limited aerobic regeneration time of 4 h. For real-world plants improved N removal would need to be established, possibly utilizing a repeat treatment, which according to Langmuir isotherm modelling (not shown) would predict about 90% N removal. A simple repeat treatment would bring the TTT to 10 h which is comparable with the hydraulic retention times used in activated sludge plants.

During extended operation at 5 h TTT, the BOD removal rate (i.e., carbon storage) increased from 460 (23rd cycle) to 518 mg L^{-1} h^{-1} (Cycle 40), which suggests that the biological carbon storage capacity improved during long-term operation, likely due to a continued growth of GAOs (Beun et al., 2000). This rapid BOD removal was about 40-100 times faster than that obtained for traditional trickle reactors (i.e., 4 to 11.7 mg L^{-1} h^{-1}) (Doan et al., 2008; Forster, 2003; Gray, 2004) that also save energy by using direct exposure of biofilms to air. It should be noted that if considering the length of aerobic phase, the overall BOD removal rate (approximately 103 mg L^{-1} h^{-1}) is still about 10 times faster than the traditional trickle reactors, and similar to that described for sequencing batch reactor (i.e., 85.2 mg L^{-1} h^{-1}) (Zhao et al., 2016).

Table A1.1: Summary of average performance of wastewater treatment under different operational modes (average values and standard deviation are presented).

Test	Aerobic phase	Anaerobic phase	Effluent[1] (mg L^{-1})		BOD removal (%)	TN removal (%)
			BOD	TN		
General operation (Cycle 1-22)	24 h	24 h	12.8 ± 9.4	7.8 ± 0.8	97.4 ± 1.8	81.3 ± 2.0
Short TTT (Cycle 23 – 40)	1 h	4 h	29.5 ± 2.27	11.7 ± 1.3	94.3 ± 4.3	72.2 ± 3.1
No aerobic phase[2] (Cycle 41 → 43)	1 h	0 h	201.8→306.8	19.2→25.9	60.3→40.4	54.1→38.2

[1] Effluent means the discharged anaerobically treated wastewater after the aerobic phase

[2] Performance not stable but steadily deteriorating. Drift in data indicated by arrows

In a separate experiment where the aerobic phase was intentionally omitted (Figure A1.6, Cycle 41-43), the efficiency of BOD and TN removal immediately decreased to 60% and 54% and further down to 40% and 38%, respectively, indicating a continued deteriorating trend and proving that the aerobic phase was essential for regeneration of both BOD and TN adsorption capacity (Table A1.1). When including the aerobic phase back into operation the normal adsorption behavior resumed within 2 cycles of operation (data not shown).

Although in the current study the TN removal efficiency by a single PASND reactor was only about 70% (short TTT), the remained TN can be further removed by a 2nd PASND reactor or other downstream treatment process, whereby the single PASND could be considered as an upfront treatment to enable energy efficient removal of the majority of pollutants

Practical implications

Passive aeration enables low-energy wastewater treatment

Studies have been shown that the energy consumption of aeration system can account for 40-60% of total energy usage in wastewater treatment plants (Foley et al., 2010). This high energy requirement is caused by (i) the need for compressed air: each air bubble has to be pressurized against the pressure of the whole water column (e.g., 5 m) by its own height; (ii) the poor solubility of oxygen (only 8 mg L^{-1} at 25^{o} C and 1 atm); (iii) low standard oxygen transfer efficiency (SOTE) ranging from 4.8% to 34.1% (Groves et al., 1992).

In the wastewater treatment process presented in the current study, aeration of bulk solution as usually required in traditional activated sludge system was avoided leading to a potential quantum leap for saving in energy consumption. Therefore, the energy requirement of the proposed PASND biofilm reactor can be theoretically cut down by 60-65% compared to traditional AS process. This suggests also potential higher energy efficiency in comparison with other widely used wastewater treatment technologies, such as rotating biological contactor (RBC), trickling filter (TF), and membrane bioreactor (MBR), which energy consumptions are about 50%, 70% and 150-300% of that used in AS process, respectively (EPRI, 2002; Krzeminski et al., 2012).

SND enables sustained zeolite regeneration

The use of zeolite has been suggested in the literature to aid in microbial N removal from wastewater. It is well known that the regeneration of zeolite can be accomplished by biological ammonium oxidation via nitrifying bacteria (Jung et al., 2004; Jung et al., 1999; Park et al., 2002). However, this process is not sustainable due to the acid build up

(Lahav & Green, 1998). Regeneration of zeolite by SND as shown can avoid proton accumulation and its well-known inhibitory effect on ammonium oxidation hence is a potentially promising way to enable a sustainable nitrogen (i.e., ammonium) removal by zeolite.

Practical operational considerations

In the current study, the experimental tests were conducted on synthetic wastewater instead of real municipal wastewater in order to obtain reproducible results. However, municipal wastewater has various types of dissolved organic carbon sources, apart from the simple organic carbon (i.e., acetate) used in this study. These more complex organic carbon compounds may affect the types of biological stored carbon and the GAOs' activity and growth (Bengtsson, 2009; Krasnits et al., 2013; Smolders et al., 1995). For example, wastewater organic carbon does not always breakdown to acetate but to alternative products. Some of the predominant compounds such as propionate and butyrate can also be stored in the cells as PHA (for example poly-β-hydroxyvalerate (PHV) (Dircks et al., 2001) or poly-β-hydroxy-2-methylvalerate (PH2MV) (Oehmen et al., 2005), while the storage capacity of other compounds (e.g., lactic acid, ethanol, amino acids and other fermentation end-products, next to aromatic compounds) remains to be tested. Nevertheless, the storage of the bulk BOD of real wastewater by bacterial storage as PHA has been demonstrated (Hughes et al., 2006; Kargi et al., 2005; Puig et al., 2007; Rustrian et al., 1996) and hence the energy efficient removal of the majority of BOD in real wastewater is expected but needs to be quantified.

Another component that may affect the process efficiency in practical operation is suspended solids (SS) of real municipal wastewater. SS contains mostly colloids that are difficult to settle and must be removed prior to entering the biofilm reactor. It has been

reported that in Biological Aerated Filters (BAFs) the SS could be adsorbed on the biofilm reducing the nitrification efficiency by up to 50 % (Boller et al., 1994), which might also be encountered when running the PASND biofilm reactor. Therefore, in order to maintain steady performance, a comprehensive primary treatment is necessary to eliminate SS.

Future study

In current study, GAO and ammonium oxidizing bacteria were enriched separately before combining to form the synthesized biofilm. However, this design generates a biofilm artificially enriched in ammonium oxidizing bacteria (AOB) which cannot be reproduced in large scale operation. A further study of using activated sludge as seeding biomass to develop the same system is needed. Microbial community analysis is also recommended to reveal the dynamic changes of the microbial community structure during the operation to provide further solid evidence on the change in nutrient removal capacity and nutrients degradation pathways. Therefore, further study to gather more comprehensive operational data is needed to obtain detailed analysis of capital and operational cost.

Conclusions

This study has described a novel concept of removing organic carbon and nitrogen from wastewater without the need for conventional extensive aeration, suggesting that the concept could be further explored to accomplish low-cost wastewater treatment. The concept uses: (i) GAO bacteria in combination with zeolite to adsorb carbon and N from wastewater; (ii) the principle of SND to regenerate the capacity of carbon and N anaerobic adsorption; (iii) avoidance of costly oxygen transfer to the bulk solution. For future application, it is necessary to use naturally enriched biofilms from activated sludge to verify real-world reproducibility of the concept.

Appendix 2 (Supplementary information to Chapter 7, Section 7.3.1.3)

In Chapter 7 (Figure 7.3), the zeolite amended GAO biofilm almost completely removed organic carbon from wastewater. In order to confirm that COD was also efficiently removed, COD analysis was carried out in parallel to acetate analysis (using Gas Chromatography) of the effluent (Figure A2.1). Results showed that wastewater contained no organic species other than acetate which was completely removed by the GAO biofilm within 6 treatment cycles (each cycle consisted of 30 min anaerobic and 30 min aerobic phase).

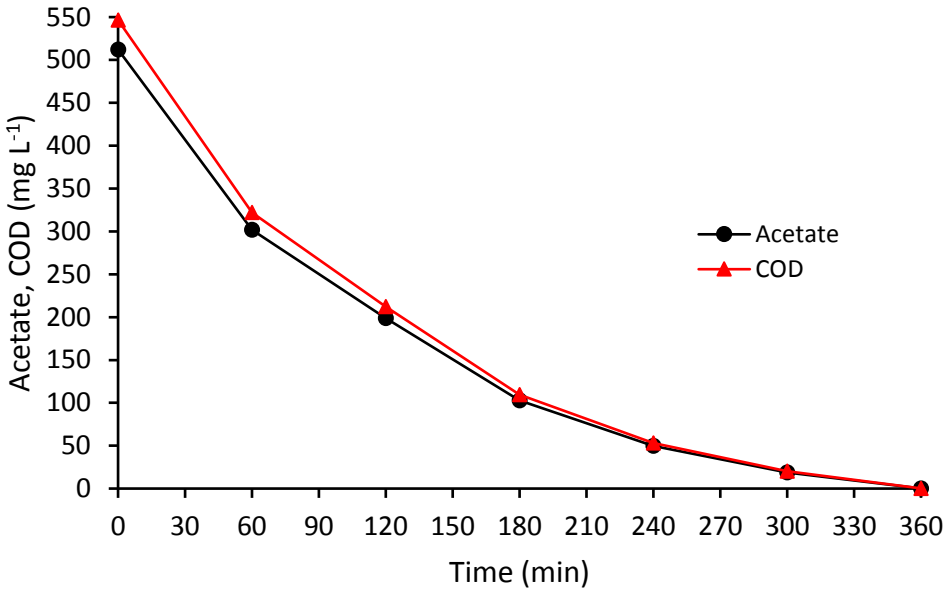

Figure A2.1: Comparison of anaerobic organic carbon uptake via acetate and COD by the zeolite amended GAO biofilm.

Appendix 3 (Supplementary information to Chapter 8, Section 8.2)

Treatment of Real Wastewater in a Zeolite Amended GAO Biofilm

In the present study, the organic carbon and nitrogen removal capability of the zeolite amended GAO biofilm was evaluated using synthetic wastewater which contains acetate and ammonium as the main sources of organic substrate and nitrogen, respectively. However, in real wastewater acetate is not the principle dissolved organic carbon substrate and nitrogen may be present in other forms. Therefore, the performance of the GAO biofilm system is expected to be different.

In order to investigate the applicability of the proposed technology, municipal wastewater from a local wastewater treatment plant (Woodman Point Wastewater Treatment Plant, Perth, Western Australia) after primary treatment was collected and fed to the biofilm reactor. Because of time constraints, the present study could only investigate an example of real waste treatment for about 50 cycles. The COD and NH_4^+-N concentrations of the wastewater were 236-316 mg L^{-1} and 47-66 mg L^{-1}, respectively and the C: N ratio ranged between 4.2 to 5.4. The treatment of wastewater with such low C: N ratio by the conventional process is uneconomical, due to the requirement for costly addition of carbon supplements for efficient nitrogen removal. The duration of anaerobic and aerobic phase was 2 and 3 h, respectively giving an overall effective HRT of 5 h as described in Chapter 5.

The GAO biofilm showed a low COD removal efficiency of only about 66% from real wastewater (Figure A3.1). Such COD removal performance is substantially lower than observed with synthetic wastewater which may be due to the complex organic substrates present in real wastewater.

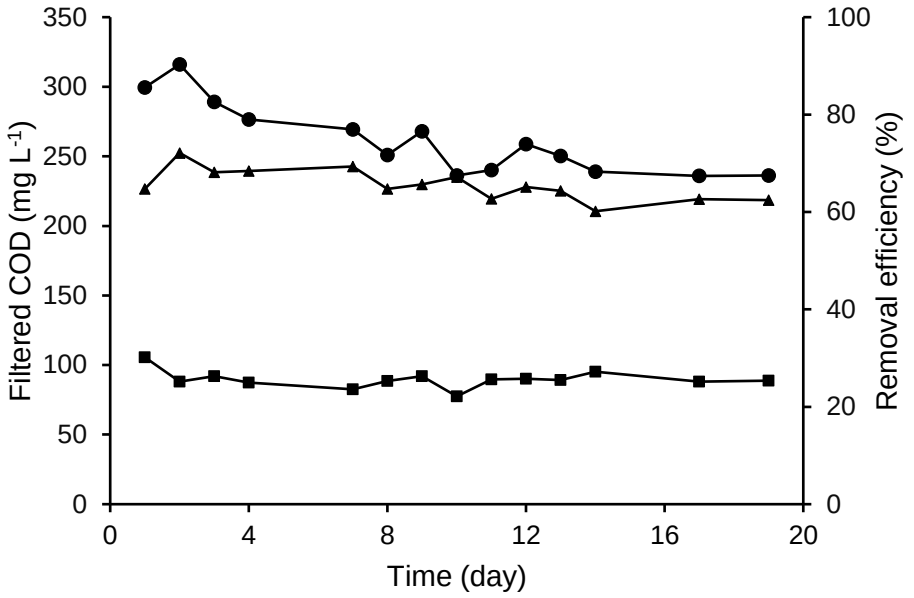

Figure A3.1: COD removal performance of the zeolite amended GAO biofilm from municipal wastewater during 50 cycles of operation: (●) Influent; (■) Effluent and (▲) Removal efficiency.

Despite the low COD removal performance, the zeolite amended GAO biofilm demonstrated a steady nitrogen removal. Although the ammonium concentration was about 1.5 times higher in real wastewater compared to synthetic wastewater, the zeolite presents in the biofilm showed a similar ammonium removal performance of about 66% (Figure A3.2). While the study period of 20 days (50 cycles) was too short to convincingly conclude about treatment efficiencies of real wastewater, the fact that the N removal was comparable to that in synthetic wastewater suggests that the integration of zeolite powder in biofilms treating real wastewater has promised to enable energy efficient N removal.

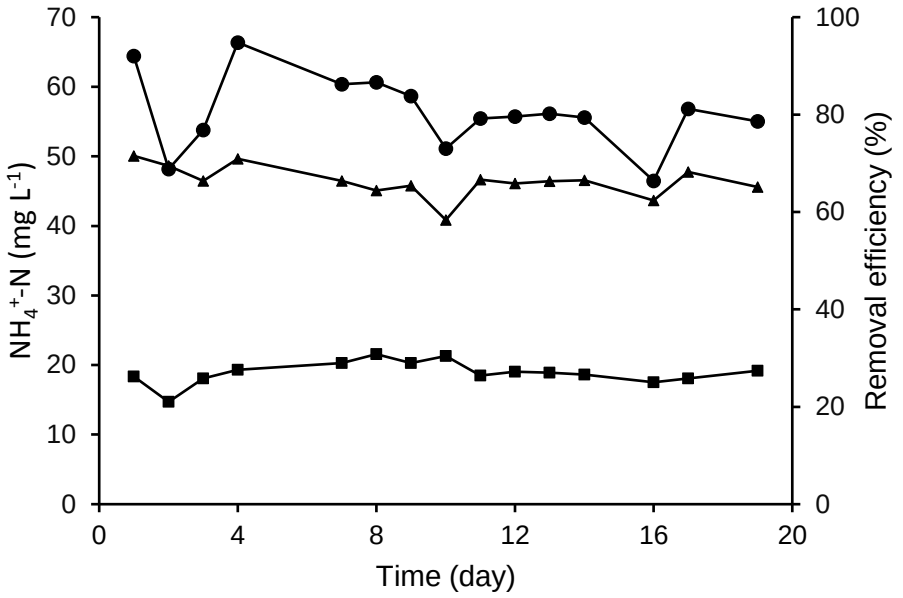

Figure A3.2: Nitrogen removal performance of the zeolite amended GAO biofilm from municipal wastewater during 50 cycles of operation: (●) Influent; (■) Effluent and (▲) Removal efficiency.