Photo-Activated Sludge Denitrification EBPR (PASDEBPR) system

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Photo-Activated Sludge Denitrification EBPR (PASDEBPR) system

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Abstract

The complete removal of organic carbon, phosphorus and nitrogen was successfully achieved in a single bioreactor named Photo-Activated Sludge Denitrification Enhanced Biological Phosphorus Removal (PASDEBPR) system. This system was composed of algae, polyphosphate accumulating organisms (PAOs) and nitrifiers living in symbiosis. By this symbiotic relationship, the oxygen produced from algal photosynthesis was consumed by bacteria while contributing to the removal of phosphorus and nitrogen.

For the cultivation of PAOs and Nitrifiers, fresh activated sludge from Harnaschpolder wastewater treatment plant (WWTP) was utilized. While for the cultivation of algae, five algal species namely, Scenedesmus quadricauda, Anabaena variabilis, Chlorella sp., Chlorococcus sp., and Spirulina sp. were cultivated in 100 mL of diluted synthetic feed prior to start-up. The PASDEBPR was operated at a hydraulic retention time (HRT) of twelve hours and an overall solids retention time (SRT) of six days. Each sequential batch reactor (SBR) operational cycle lasted for six hours and followed the anaerobic/aerobic regime. The cycle had four phases: anaerobic phase (2 h), illuminated aerobic phase (3 h), settling phase (0.5 h), and decanting phase (0.5 h). For illumination, a provision of eight bulbs was made, each with a power rating of 70 W. The light intensity varied between 180 μmol/m².sec to 780 μmol/m².sec through the experimental phases.

In the first research phase, a steady-state model was developed to formulate the design basis for the experimental work. By fixing certain parameters (like influent phosphorus, etc.) in the model, a preliminary design of the SBR operation was established. Information such as duration of anaerobic and aerobic phases and range of solids concentration were predicted by the model. Using these information, the first experimental phase to establish the PAS-EBPR system was carried out successfully to achieve effluent phosphorus (PO₄³⁻) concentration of 2 mgP/L. Thereafter, the cultivation of nitrifiers was carried out to introduce nitrogen (NH₄⁺) removal in the system. The main mechanism of NH₄⁺ removal was nitrification by the algal-bacterial biomass, followed by algal uptake and nutrient requirements for the growth of bacteria. Furthermore, denitrification of nitrite (NO₃⁻) and nitrite (NO₂⁻) was found to be carried out possibly by denitrifying polyphosphate accumulating organisms (DPAOs), and through the phototrophic assimilatory mechanism by algae. In the end, a steady-state PASDEBPR was developed reaching effluent concentrations for nitrogen and phosphorus below 2 mg/L. The denitrifying activity resulted in the accumulation of nitrous oxide (N₂O), which is a usually observed in denitrifying EBPR bioreactors and demands further attention to suppress the emissions of this gas. Out of the five algal species cultivated at the beginning of experimental phases, only Scenedesmus quadricauda, Chlorella sp, and Spirulina sp. was observed in the PASDEBPR. Moreover, the effluent of the PASDEBPR was free of any solids or chlorophyll-a indicating that a good enmeshment of algae and bacteria in the bioreactor was achieved.

As a result, the bio-treatment was completely achieved without the supply of external aeration. By eliminating the need for external air supply, the PASDEBPR system can be considered as an alternative to wastewater treatment in a sustainable, efficient and cost-effective manner.
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Abbreviations

AOB: Ammonium oxidizing bacteria
ATU: Allyl-thiourea
BNR: Biological nitrogen removal
BOD: Biological oxygen demand
C: Carbon
CAS: Conventional activated sludge
COD: Chemical oxygen demand
DO: Dissolved oxygen
EBPR: Enhanced biological phosphorus removal
FP-PBR: Flat panel photo-bio reactor
FSA: Free saline ammonia
GC: Gas chromatography
HAc: Acetate
HPr: Propionate
HRAP: High rate algal pond
HRT: Hydraulic retention time
IC: Inorganic carbon
MLE: Modified Ludzack-Ettinger
MLSS: Mixed liquor suspended solids
MLVSS: Mixed liquor volatile suspended solids
N: Nitrogen
NOO: Nitrite oxidizing organisms
OHO: Ordinary heterotrophic organism
P: Phosphorus
PAO: Polyphosphate accumulating organism
PAS: Photo-activated sludge
PBR: Photo-bio reactors
PHA: Poly-β-hydroxyalkanoates
PHB: Poly-β-hydroxybutyrate
PHV: Poly-β-hydroxyvalerate

x
PSBR: Photo-sequencing batch reactor
RBCOD: Readily biodegradable chemical oxygen demand
SBCOD: Slowly biodegradable chemical oxygen demand
SBR: Sequencing batch reactor
SRT: Solids retention time
T: Temperature
TKN: Total kjeldahl nitrogen
TN: Total nitrogen
TOC: Total organic carbon
TPBR: Tubular photo-bio reactor
TSS: Total suspended solids
VFA: Volatile fatty acids
WWTP: Wastewater treatment plant
Chapter 1  Introduction

This introductory chapter lays down the foundation for this research study beginning with a brief background about the past developments in the field of wastewater treatment. Finally, the problem statement for this research is defined.

1.1 Background

Wastewater treatment was the most visible activity in the 20th century due to growing awareness about the health and nuisance risk wastewater carries in urban agglomerations (Henze, et al., 2008). In the first half of the 20th century, flowing water bodies like river, water streams etc. were considered as a medium for treatment of wastewater. But with the increase in population, these water bodies could not cope with continuous disposal wastewater and as a response, several treatment options were developed. Conventional activated sludge (CAS) system was one of the options dealing with removal of organic pollutants prior to disposal of wastewater. Later on, in the second half of the 20th century, a new problem was discovered: eutrophication of water bodies where treated wastewater was discharged. Eutrophication is caused by disposal of wastewater containing nutrients, both nitrogen (N) and phosphorus (P), into receiving water bodies. The adverse impact of eutrophication on ecology can be broadly classified as: (i) increased water toxicity, (ii) reduction of biodiversity and replacement of dominant species, and (iii) increased turbidity and decreased lifespan of water bodies.

To counter this issue, modified CAS system was developed which included processes like biological nutrient removal (BNR) and enhanced biological phosphorus removal (EBPR) in addition to the original CAS system. In BNR, biological removal of N takes place through a combination of nitrification and denitrification occurring in aerated and unaerated zones of a single reactor, respectively. Apart from N removal, the other advantage is the reduction in oxygen demand as facultative heterotrophic organisms, under anoxic conditions, utilize nitrate as the electron acceptor instead of dissolved oxygen for degradation of organic pollutants. The EBPR process also referred to as biological phosphorus removal (BPR) in literature, pertains to P removal in order to limit the growth of aquatic plants and algae, and thus control eutrophication. However, the aeration component of these activated sludge systems required around seventy-five percent of total plant energy (Tandukar, et al., 2007, Young and Koopman, 1991). In developing countries, especially in places having a low outreach of energy services, this proved to be a stumbling block for sanitation. Furthermore, the lack of clarity in selecting appropriate technology lead to the failure of low-cost technology in the developing world (Grau, 1996).

Meanwhile, since the early 1950s, the bioremediation benefits of algae (including cyanobacteria, micro and macro algae) was studied by considering wastewater as feed for algal growth, thereby addressing the problem of eutrophication in water bodies.
The major benefits of using algae for treatment of wastewater are: (i) the energy demand in algae-based wastewater treatment is lower as oxygen demand is supplied by photosynthesis instead of energy-intensive electromechanical air supply system; (ii) the algae removed nutrients from wastewater by assimilation and it could further be used as a source for recycling these nutrients in a biomass to be reused for energy production, raw chemicals, fertilizers, and other by-products; and (iii) simplistic and low operational costs (Abdel-Raouf, et al., 2012). Alcantara, et al. (2015) has cited several algal-mechanisms like nutrient uptake and aerobic degradation of organic matter, which support the idea behind the use of microalgae for wastewater treatment. Using this technical know-how, natural systems such as algae-based wastewater stabilization ponds were developed. But, these systems still required a large land area to achieve high performance efficiency and removal rates than activated sludge systems. This was further refined for developing compact wastewater treatment systems that did not require much land such as, photo-bioreactors (PBRs) or high rate algal ponds (HRAPs). These compact systems were advantageous since they resulted in a better performance at shorter hydraulic retention times than waste stabilization ponds (Craggs, et al., 2003, El Hamouri, et al., 2003). Despite the reduction in area requirement with respect to stabilization ponds, a study by Arashiro (2015) indicated that it was still not enough to replace activated sludge systems.

Further improvements in algal-based wastewater treatment to increase removal rates while reducing the footprint area and low operation costs were needed to make it attractive against conventional technologies. The potential of algae-bacteria synergy for wastewater treatment was assessed through several studies for treating wastewater leading to the development of photo-activated sludge (PAS) systems were made (de Godos, et al., 2010, Su, et al., 2012, van der Steen, et al., 2015). Photosynthesis by algae generates oxygen which is used by bacteria, while the carbon dioxide released through the carbonaceous oxidation process can be used by the algae (Muñoz and Guieysse, 2006). Also, the presence of bacteria along with algae in the biomass allows better settling thereby allowing control of solids retention time (SRT) and permits to operate the system at the shortest optimum SRT. By operating the system at optimum SRT, it is possible to have higher active biomass in the system to achieve high removal of organic and nutrient loads at a minimum value of hydraulic retention time (HRT) (Medina and Neis, 2007, Valigore, et al., 2012, Van Den Hende, et al., 2011).

1.2 Problem statement

By 2050, several forecasts indicate that there will be an increase of over fifty percent in water requirement in order to cater to the demand of global population (Coyle and Simmons, 2015, Godfray, et al., 2010). With almost three-quarters of the total global wastewater generated being discharged into water bodies without any treatment, dealing with wastewater has become an indispensable challenge. Lack of treatment prior to discharge of wastewater causes an excess of nutrient loadings into the ecosystem resulting in algal nuisance, low dissolved oxygen and undesirable pH levels (Correl, 1998). Even though activated sludge systems provide complete wastewater treatment to meet the effluent discharge standards, the consumption of energy is high thus making them costly processes.

By considering wastewater as a feedstock for production and harvesting of algae, the potential of algal-bacterial symbiosis could be tapped to address the challenge of wastewater treatment.
Studies have pointed out the prospective phycoremediation benefits by using algal-bacterial consortia to treat wastewater without the use of external aeration (Gonzalez, et al., 2008, Hernández, et al., 2013, Mulbry, et al., 2005, Pizarro, et al., 2006). The integration of BNR in an algae-based photobioreactor has been assessed extensively with positive outcomes to remove organic and nutrient pollutants (Karya, et al., 2013, Manser, et al., 2016, Rada-Ariza, et al., 2017, van der Steen, et al., 2015), while separate studies on coupling of algae and polyphosphate accumulating organism (PAO) for organic carbon and phosphorus removal has also been conducted (Mohamed, 2017).

However, an assessment of combined organic and total nutrient removal by integrating the two aforementioned algae-bacteria based systems in a single photo-bioreactor is yet to be made. Thus, the present research will focus on investigating the potential of a photo-activated sludge (PAS) system to treat wastewater using consortium algae, nitrifying organism, and PAO. This study will focus on achieving maximum removal efficiencies by identifying parameters that shall affect the symbiotic relationship of algae and bacteria. Also, these parameters may play a vital role in defining the design and operational parameters of the PAS system.

1.3 Research question

What are the effects of ammonium (NH$_4^+$) concentration on the complete nutrient (N & P) removal efficiency in a Photo-Activated Sludge Denitrification EBPR (PASDEBPR) system?

1.4 Research objective

To determine the relation between the ammonium concentration and the complete nutrient removal efficiencies of the PASDEBPR system.

1.5 Hypothesis

The PASDEBPR system will be able to perform complete nutrient removal with excess ammonium supplied to stimulate nitrification followed by denitrification through uptake by either PAOs or algae.
Chapter 2  Literature review

In this chapter, the EBPR system for only phosphorus removal and complete organic and nutrient removal is summarised followed by nitrification. Thereafter, the development of PAS system for the removal of organic carbon along with nutrient removal is described with supporting information from previous studies. Ultimately, a brief outlook is made to provide an insight into the potential behind coupling of algae, nitrifying organism and PAOs in a single reactor for the removal of both organic pollutants and nutrients (nitrogen and phosphorus).

2.1 Photo-activated sludge (PAS) system

2.1.1 PAS system for C removal

The symbiotic relationship between microalgae and bacteria was first studied for the bioremediation potential to treat waste in stabilization ponds by Oswald, et al. (1957) through laboratory tests in California. However, the possibility of utilizing algal-photosynthesis to support aerobic bacteria (mainly OHOs) for stabilizing organic wastes was studied in the early 1950s (Gotaas, et al., 1954, Oswald and Gotaas, 1954, Oswald, et al., 1953). Oswald, et al. (1953) defined photosynthetic oxygenation as “the process of producing oxygen through photosynthesis by microscopic green plants in the presence of light”.

![Figure 1: Photosynthetic oxygenation process as defined by Oswald, et al. (1953) (Source: Muñoz and Guieysse (2006)).](image)

The OHOs perform the bacterial action by oxidizing organic matter and release carbon dioxide through respiration, while algae could consume carbon dioxide as a carbon source and produce oxygen by undergoing photosynthesis in presence of light. Oswald, et al. (1957) conducted field research using shallow stabilization ponds in Richmond, California and recorded eighty-five percent organic matter removal as BOD. The study defined several critical operational parameters like critical photosynthetic efficiency, oxygenation factor, BOD loading rate etc. which were vital for the further development of algae-based wastewater treatment systems.
Oswald (1962) presented the idea of combining harvesting algae with wastewater treatment through controlling photosynthesis in a waste stabilization pond and termed it as High Rate Algal Ponds (HRAP). The principle behind the operation of HRAP system was utilizing the nutrients from wastewater for algal growth and produce photosynthetic oxygen for bacterial oxidation of organic matter. The result of this quasi-symbiotic relation between algae and bacteria was a reliable treatment of wastewater with the production of biomass, which if harvested, could be a potential source of fertilizers, bioproducts, etc. Furthermore, on applying this quasi-symbiotic concept to large shallow lagoons, Ellis and Mara (1983) recorded a BOD removal of around ninety-eight percent. However, due to lack of operational control as compared to HRAPs (Shelef, et al., 1980), this system in shallow lagoons did not allow any agitation resulting in low algal growth. Despite the extensive research undertaken in the HRAP technology, Abeliovich (1986) expected considerable efforts to obtain consistent and reliable results to consider it as a way to treat wastewater.

### 2.1.2 PAS system for complete N removal

Since HRAP system had the potential to remove organic waste and give biomass yield with reuse potentials, several studies were carried out to treat different wastewaters by algal systems to produce biomass having the potential to be used as animal feed (Goldman and Ryther, 1975, 1976, McGarry and Tongkasame, 1971, Shelef, 1982, Shelef, et al., 1977). While the development of energy-intensive CAS system was growing, Oswald (1970) described the potential of the algae-bacteria system to treat wastewater and control the nuisance of eutrophication. This was further supported through observations on the benefits of the HRAP system in terms of energy-saving as compared to the activated sludge process by Picot, et al. (1992).
Craggs, et al. (2003) and El Hamouri, et al. (2003) reported the removal efficiencies of BOD, TSS and faecal coliform to be better or comparable to that of stabilization ponds. However, removal of N was still a limitation as reported by Park, et al. (2011) in an HRAP system having eight days of HRT the effluent contained 13.5 mgN/L of dissolved nitrogen while the influent total nitrogen was 50 mgN/L. The reason behind this limited N-removal could be the inhibitory effect of high pH on nitrification in HRAPs (Craggs, et al., 2003) or effect of temperature on the algal assimilation mechanism (Nurdogan and Oswald, 1995). Karya, et al. (2013) reported efficient nitrification (ammonium conversion of 7.7 mg/L h) by utilizing an algal-nitrifying bacteria consortium which indicates the improved process control in a photo-bioreactor (PBR) system over HRAPs. van der Steen, et al. (2015) further improved the system of treating wastewater through PBR by introducing biomass recycle to obtain a well-settling biomass containing algae-nitrifiers consortia, which resulted in eighty percent removal of applied ammonium load. Moreover, the design of PBR has undergone rapid developments with promising configurations like flat-panel PBR (FP-PBR) and tubular PBR (TPBR) to be used for cultivation of microalgae (Wang, et al., 2012). Rada-Ariza, et al. (2017) reported an ammonium removal of around 100 mg/L d from artificial wastewater treated in a flat-panel PBR having a consortium of microalgae-bacteria.

To complete the process of N-removal, developments to include denitrification have been made by several research groups across the globe. A novel approach was made by Alcantara, et al. (2015) in their study in which anoxic-aerobic conditions were adopted to treat synthetic wastewater in an algal-bacterial photobioreactor. An HRT of two days and SRT of twenty
days resulted in removal efficiencies of around eighty percent for total organic carbon (TOC), inorganic carbon (IC) and total nitrogen (TN) (Alcantara, et al., 2015). Manser, et al. (2016) introduced another innovative process termed as ALGAMMOX (algal anaerobic ammonium oxidation). The potential of combining microalgae, ammonium oxidizing bacteria (AOB), and Anammox in a photosequencing batch reactor (PSBR) to treat high-ammonia strength wastewater (COD/TN around 3) was demonstrated (Manser, et al., 2016).

Figure 5: Conceptual diagram for ALGAMMOX process (Source: Manser, et al. (2016)).

2.1.3 PAS system for complete P removal

Phosphorous is an essential nutrient for algae to perform nucleic-acid synthesis and undertake cellular processes to produce high energy organic compounds (Martínez, et al., 1999). In fact, a study of nutrient concentrations in marine microalgae indicated that the C:N:P (molar ratio) in the biomass is about 106:16:1 (Redfield, 1958). However, Rhee (1978) reported that the nutrient content in the algal biomass varied depending upon the nutrient concentration present in the growth medium. Hence, the biological assimilation of phosphorus by algal species could be considered as one of the removal pathways. But, phosphorus removal from wastewater was still a limitation in stabilization ponds and HRAP systems (Picot, et al., 1992, Powell, et al., 2008). Powell, et al. (2008) discussed the lack of phosphorus uptake in stabilization ponds despite observing luxurious uptake in natural water bodies. For a HRAP system, the effluent with total phosphorus less than 2 mg/L could be obtained by maintaining the pH higher than nine (Picot, et al., 1992) through precipitation of phosphorus. But, this could inhibit nitrification, if simultaneous N-removal is to be achieved (Craggs, et al., 2003). However, microalgal assimilation of phosphorus of around ninety percent in a TPBR has been reported by Di Termini, et al. (2011).

Similar to N-removal, P-removal using microalgae-bacteria consortium has been investigated by several authors (de-Bashan, et al., 2002, de Godos, et al., 2009, 2010, Gonzalez, et al., 2008, González, et al., 2008, Gutzeit, et al., 2005, Perez-Garcia, et al., 2010). de Godos, et al. (2010) highlighted the complexity of P removal mechanisms in microalgae-based systems as the mechanism comprised of P precipitation (at high values of pH), microbial assimilation in the form of biomass, and phosphorylation. The study further outlined the sensitivity of process removal efficiencies due to variations to temperature, intensity of light and initial phosphate concentration (Nurdogan and Oswald, 1995, Powell, et al., 2008). Mohamed (2017) performed a study to understand the behaviour of microalgal-PAOs consortium in a PSBR under dark and light phase conditions to replicate anaerobic and aerobic conditions,
respectively. The study as compared to the aforementioned studies was important as it resulted in complete P-removal with a compact system (PSBR) without any external mechanical air supply (Mohamed, 2017).

2.2 Biological Nitrogen Removal (BNR)

2.2.1 Background

Since the nineteenth century, nitrogen was identified as a constituent of wastewater but it drew attention ever since the discovery of the first flow-through activated sludge system by Ardern and Lockett (1914). Sawyer and Bradney (1945) identified the problem of sludge rising in the final settling tanks which led to the first exhaustive studies into the need for nitrification and denitrification. This was followed by comprehensive research by Downing, et al. (1964a) for defining the SRT in excess of that required to prevent washout of slow-growing autotrophic bacteria. Further studies by Downing, et al. (1964b) and other researchers shed light on identifying limiting factors and compounds that prevented the application of nitrogen removal in activated sludge systems. In the 1950s, the process of denitrification gained importance as a process to attain complete nitrogen removal from wastewater. While Wuhrmann (1962) proposed a post-denitrification system using stored carbon, it was Ludzack and Ettinger (1962) who first proposed “Semi-aerobic Activated Sludge Process” to simulate single sludge nitrification-denitrification system. The results reported by Ludzack and Ettinger had variable rates for denitrification since the anoxic and aerobic zones were created in a single reactor resulting in limited control over the exchange of contents between the two zones. To overcome this limitation, Barnard (1973) modified the configuration by providing separate anoxic and aerobic reactors, recirculating both the mixed liquor from the aerobic reactor and sludge underflow from secondary settler to the anoxic reactor.

2.2.2 Principle of BNR

As stated above, it is clear that nitrification is the precursor for denitrification in order to have biological N removal. Nitrification is defined as the biological process whereby the oxidation of free and saline ammonia (FSA) to nitrite and nitrate takes place. Being a biological process, it arbitrated by certain autotrophic organisms which differ from the ordinary heterotrophic organisms (OHOs) (Ekama and Marais, 1984). The nitrifiers consume dissolved carbon-dioxide as carbon source while oxidizing ammonia to nitrite and nitrite to nitrate thereby generating energy for biomass synthesis. The autotrophic bacteria responsible for nitrification

![Figure 6: (left) Post-denitrification single sludge system for biological nitrogen removal suggested by Wuhrmann (1962). (right) Modified Ludzack-Ettinger (MLE) single sludge biological nitrogen removal system proposed by Barnard (1973) (Source: Ekama and Wentzel (2008)).]
are the ammonium oxidizing bacteria (AOOs) and the nitrite oxidizing bacteria (NOOs) apart from *Nitrosomonas* and *Nitrobacter* (Ekama and Marais, 1984, Ekama and Wentzel, 2008). The complete nitrification can be broken down into two sequential oxidation reactions: (i) conversion of FSA to nitrite by AOOs; and (ii) conversion of nitrite to nitrate by NOOs. The rate of conversion by AOOs is generally found to be much slower than NOOs which results in the direct conversion of any nitrite formed to convert immediately to nitrate. Hence, the limiting step in nitrification is the conversion of ammonia to nitrite by AOOs. This results in autotrophic nitrifiers having biomass growth coefficients much lower than the OHOs (Ekama and Wentzel, 2008). Also, the autotrophic nitrifiers require oxygen in order to carry out nitrification due to their obligate aerobic nature. Hence, the process of denitrification is added to nitrification systems by inclusion of anoxic zones to reactor systems but by increasing the sludge age so as to ensure complete nitrification (Barnard, 1973, Ludzack and Ettinger, 1962). Addition of unaerated zones should be done irrespective of nitrification requirements as they can be useful to achieve some biological P removal if BNR is not the desired outcome.

### 2.2.3 Biological N removal mechanism

In a BNR system, the nitrification converts the nitrogen from ammonia to nitrate with N being in liquid phase followed by denitrification where nitrate is converted to nitrogen with the transfer of N from liquid to gas phase and it escapes to the atmosphere. Hence, the at the end twenty percent of influent N is obtained in sludge mass while seventy-five percent of influent N is released as gas if complete denitrification occurs (Ekama and Marais, 1984). Under aerobic conditions for nitrification to occur, it is necessary to satisfy the oxygen demand by OHOs and AOOs as dissolved oxygen acts as the electron donor. On the contrary under unaerated conditions, it is necessary to ensure the presence of sufficient COD for denitrification of nitrates by NOOs. For denitrification to occur, the electron donors (COD) can be supplied either externally through chemical dosing or internally by using internal COD sources (van Haandel, *et al.*, 1981); the latter being the preferred choice due to low cost and absence of any chemical addition. The internal sources of COD as electron donors for denitrification are the two main forms of organics i.e. readily biodegradable organics (RBCOD) and slowly biodegradable organics (SBCOD) with an additional source of SBCOD through the endogenous respiration of biomass. Due to the difference in the degradation rates of RBCOD and SBCOD, there is variation in utilization of COD whereby the denitrification rate is affected. Studies have shown that the utilization rate of RBCOD is considerably faster than the rate of SBCOD hydrolysis which indicates that influent RBCOD is the favourable choice of organics for denitrification (Stern and Marais, 1974, van Haandel, *et al.*, 1981).

### 2.2.4 System configuration for BNR

Depending upon the different rates of degradations and utilization of RBCOD and SBCOD, it can be deduced that the provision of anoxic conditions is vital for denitrification to occur. Based on this, several configurations of nitrification-denitrification (ND) systems were developed based on the source of organics to be supplied as an electron donor. The initial developments in BNR process made by Wuhrmann (1962) and Ludzack and Ettinger (1962) were further improved by McCarty, *et al.* (1969), Schwin and Tozer (1974), Parker (1975), Balakrishnan and Eckenfelder (1970) and Barnard (1973). In fact, by assimilation of outcomes
from previous studies, Barnard modified the system given by Ludzack-Ettinger to overcome the shortcoming of nitrate removal by introducing a secondary anoxic reactor and called it the 4-stage Bardenpho system. In the MLE system, low content of nitrate was obtained in the effluent from the aerobic reactor which was now to be denitrified in the secondary anoxic reactor to generate a relatively nitrate-free effluent. Thereafter, a reaeration reactor was provided to strip off the nitrogen gas and nitrify any ammonia produced by denitrification.

Figure 7: 4-stage Bardenpho system for BNR developed by Barnard (1973) including pre and post denitrification system. (Source: Ekama and Wentzel (2008)).

Despite the theoretical validation of the system to provide complete nitrate removal by Barnard, practical results would indicate otherwise due to different influent TKN/COD concentration ratios. If this ratio is found to be low, it would lead to a low denitrification rate and ammonia release ultimately resulting in an inefficient performance in the secondary anoxic reactor. Hence, to avoid any competition between aerated and unaerated mass fractions, it was later decided to provide larger primary anoxic reactor by eliminating the secondary anoxic reactor and reaeration reactor.

2.3 Enhanced Biological Phosphorus Removal (EBPR)

2.3.1 Background

The key element behind eutrophication in aquatic environments is P as it supports the growth of algae and other photosynthetic microorganisms such as toxic cyanobacteria (blue-green algae). The control of P levels is significantly important to limit the risk of adverse effects on plant and animal communities in water bodies (Mainstone and Parr, 2002). With the formulation of stricter regulations to control P emissions, the removal of P in activated sludge systems has also increased. EBPR is one such method wherein the activated sludge is recirculated through anaerobic and aerobic conditions to achieve P removal without any chemical additives.

2.3.2 Principle of EBPR

The process of EBPR involves biological assimilation and removal of P by activated sludge systems in excess of the amount that is removed by aerobic activated sludge systems. In an aerobic activated sludge system, a sludge mass can uptake about 0.015 mgP/mgTSS which is only around twenty percent of P removal in a domestic wastewater treatment system. While
in an EBPR activated sludge system, the P uptake by the sludge mass increases to 0.05-0.10 mgP/mgTSS achieved through certain design and operational modifications to stimulate the growth of PAOs in addition to ordinary heterotrophic organisms (OHOs). The PAOs are able to store phosphate as intracellular polyphosphate, resulting in P removal from bulk liquid and obtained as biomass in waste activated sludge (Wentzel, et al., 2008).

Unlike the majority of other microorganisms, PAOs can take up sources of carbon under anaerobic conditions, and store them in the form of intracellular carbon polymers, namely poly-β-hydroxyalkanoates (PHAs). By the degradation of polyphosphate & glycogen and release of P, the energy is generated for the biotransformation to occur in the anaerobic zone of the system. While in the aerobic zone, PAOs utilize the stored PHA as a source of energy for biomass growth, P uptake, replenishment of glycogen and storage of polyphosphate. The overall P removal from such an EBPR activated sludge system is obtained by the discharge of waste activated sludge having a high content of polyphosphate.

![Figure 8: Schematic representation of activities by PAOs in an EBPR system (Source: adapted from Metcalf and Eddy, 2003).](image)

### 2.3.3 Biological P removal mechanism

In order to establish an EBPR system, the essential conditions required to achieve growth of PAOs are:

(i) A sequence of anaerobic followed by aerobic zones/reactors, and
(ii) Presence of VFAs in anaerobic zone/reactor, either through external supply or formation by fermenting bacteria.

There are several organisms present in the EBPR system in addition to PAOs which do not accumulate polyphosphate and they are termed as ordinary heterotrophic organisms (OHOs). In the anaerobic conditions, the lack of external electron acceptor, oxygen or nitrate limits the utilization of VFAs by OHOs. On the other hand, the PAOs can take up VFAs from the bulk liquid as intracellular storage to form long-chain carbon molecules of PHAs mainly as poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV). To form the PHAs from VFA uptake, PAOs require energy which is supplied through degradation of polyphosphate and glycogen. The degradation of polyphosphate leads to release of orthophosphate along with certain counter-ions of polyphosphate, such as potassium and magnesium. These compounds are accumulated within the cell and released by PAOs either for uptake of acetate or generation.

Figure 9: Simplified diagram of biochemical model for PAOs under anaerobic conditions (Source: Wentzel, et al. (2008)).

In the following aerobic zone, oxygen acts as an external electron acceptor for PAOs and they consume the stored PHAs as a carbon and energy source for further growth of new cells. Furthermore, P is consumed from the bulk solution to replenish the polyphosphate used in the anaerobic reactor and to supply polyphosphate to the new cells (Maurer, et al., 1997). This results in excess P consumption than it is released in the anaerobic zone, giving a net removal of P from the bulk liquid in the activated sludge system. In addition to P consumption, counterions such as potassium and magnesium are taken up to counter the negative charge of the polyphosphate polymer (Comeau, et al., 1986, Mino, et al., 1998, Oehmen, et al., 2007). The activated sludge now consists of biomass having PAOs filled with polyphosphate and it is removed from the system as waste activated sludge. A steady-state is achieved in the activated sludge system by maintaining a balance between the mass of PAOs (with stored polyphosphate) generated per day and mass of PAOs (with stored polyphosphate) wasted per day (Wentzel, et al., 2008).

Figure 10: Simplified diagram of biochemical model for PAOs under aerobic conditions (Source: Wentzel, et al. (2008)).

### 2.3.4 System configurations for EBPR

The initial observations made in P removal by activated sludge systems in excess of the metabolism requirements of microorganisms lead to the research and development of the EBPR technology. This has resulted in sever process configurations depending on the desired results for the removal of pollutants from wastewater. The first ever development of P uptake phenomenon was done by Levin and Shapiro (1965) through extensive batch studies to assess the effect of alternating anaerobic and aerobic conditions to demonstrate the biological nature
of EBPR. Levin, et al. (1975) further developed this system as PhoStrip process which entailed aeration of mixed liquor to cause uptake of dissolved phosphorus by activated sludge microorganisms in excessive of the amount required for growth. On switching the air supply off, the same microorganisms consumed the available dissolved oxygen and released the consumed phosphorus into the liquid phase. The PhoStrip utilized the chemical and biological P removal and was applied to non-nitrifying systems as a side-stream process.

![PhoStrip system configuration](image1)

Figure 11: PhoStrip system configuration developed by Levin (1965) and commercially marketed by M/s. Biospherics, USA (Source: Wentzel, et al. (2008)).

Even though with the development of EBPR technology and PhoStrip process, the potential P removal achievement if applied as a wastewater treatment process was yet to be proved or tested. Despite understanding the need for a sequence of anaerobic and aerobic conditions, the design and operational parameters of anaerobic conditions was not defined. To prove the microbiological aspects of EBPR, Fuhs and Chen (1975) discovered that the phenomenon was carried out by a consortium of several groups of organisms related to the *Acinetobacter* genus. They concluded that “anaerobic conditions preceding the aerobic conditions allowed the release of compounds like ethanol, acetate, and succinate, which served as a carbon source for *Acinetobacter* spp.”. Furthermore, the practical application of EBPR was first developed by Barnard (1975, 1974, 1976) during an investigation of a system designed for the nitrification/denitrification process. Barnard (1974) hypothesized that it is essential to have the mixed liquor passed through an anaerobic stage prior to the aerobic stage for phosphorus removal in an activated sludge system. Further studies on this hypothesis led to the development of the “Phoredox” method by Barnard (1976) which was a non-nitrifying EBPR activated sludge system. The configuration of this system was designed and controlled with short sludge age and high flow rate to prevent nitrification. The Phoredox system comprises of a sequence of an anaerobic and aerobic reactor (A/O) through which the influent and sludge underflow is recycled.

![Phoredox (A/O) system configuration](image2)

Figure 12: Phoredox (A/O) system configuration for EBPR (Source: Wentzel, et al. (2008)).
On the other hand with the increasing regulatory needs for nitrification in South Africa, Barnard developed activated sludge systems pre-dominantly for nitrification but incorporated Phoredox to develop the Bardenpho system. In one of these configurations, an anaerobic reactor was introduced to have EBPR in addition to N-removal. The sequence of reactors in this system was anaerobic-anoxic-aerobic reactors and it was called the 3-stage Bardenpho system (A2O).

![Diagram of 3-stage Modified Bardenpho (A2O) system configuration for EBPR](Source: Wentzel, et al. (2008)).

2.4 Factors affecting microalgal-nitrifiers-PAOs consortium in a PAS system

The wastewater fed to a PAS system acts as the growth medium for microalgal-bacterial consortium which makes the process efficiency of the system dependent on the constituents of wastewater. In addition to this, the growth of microalgae alongside bacteria depends on the biological, chemical, physical and operational parameters of the PAS system (Borowitska, 1998). Mata, et al. (2010) summarized several factors affecting the growth of microalgae which also govern the efficiency of the microalgae and bacteria to treat wastewater (Rawat, et al., 2011).

Table 1: Factors influencing algal growth (Source: Mata, et al. (2010)).

<table>
<thead>
<tr>
<th>Abiotic factors</th>
<th>Light intensity</th>
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<tbody>
<tr>
<td></td>
<td>Temperature</td>
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<td></td>
<td>Nutrient concentration</td>
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<td></td>
<td>pH</td>
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<td>Dissolved carbon dioxide</td>
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<td>Toxic chemicals</td>
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<tr>
<td>Biotic factors</td>
<td>Pathogenic microorganisms</td>
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<td>Competing algal species</td>
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<td>Operational factors</td>
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<td>Biomass retention</td>
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<td>Addition of bicarbonate</td>
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To identify the critical factors that affect the PAS system to have simultaneous N & P removal, the key parameters affecting growth of algae and microbial (nitrifiers and PAOs) activity are described below.

2.4.1 Factors affecting growth of algae

a) Intensity of light
The use of algae for wastewater treatment stems from the energy benefit obtained by the production of oxygen by the process of photosynthesis and it has been realised through treatment of domestic wastewater in waste stabilization ponds and facultative ponds (Abeliovich, 1986, Aziz and Ng, 1993, Mara and Pearson, 1998, Oswald, 1988, 1995). The algae consist of chlorophyll \(a\) as the principle photosynthetic pigment which is easily measurable and reproducible as indicated by (Mara and Pearson, 1998, Pearson, 1987). Mara (2003) explained the effect of light energy (photons) in algal photosynthesis which leads to fixing of carbon dioxide and production of oxygen as a by-product. Janssen and Lamers (2013) expressed the algal photosynthesis through a redox reaction equation given as:

\[
2\text{H}_2\text{O} + 2\text{NADP}^+ + 3\text{ADP} + 3\text{P}_i + 8\text{photons} \\
\rightarrow \text{O}_2 + 2\text{NADPH} + 2\text{H}^+ + 3\text{ATP} \quad (\text{oxidation})
\]

\[
\text{CO}_2 + 3\text{ATP} + 2\text{NADPH} + 2\text{H}^+ \\
\rightarrow \text{CH}_2\text{O} + 3\text{ADP} + 3\text{P}_i + 2\text{NADP}^+ + \text{H}_2\text{O} \quad (\text{reduction})
\]

From the above equations, it can be seen that theoretical equivalent of eight photons is a mole of oxygen through algal photosynthesis. The light absorbed by algae in the form of photons is within the 400-700 nm wavelength range and it is known as photosynthetically active range (PAR) (Mara, 2003). However, there is a direct relation between algal photosynthesis and light intensity. A light intensity between 50 and 300 W/m\(^2\) considered ideal to prevent photoinhibition (Barber and Andersson, 1992, Baroli and Melis, 1998, Mara, 2003, Melis, \textit{et al.}, 1998).

b) Retention time for algal biomass
The assessment of retention time related to algal growth is vital in order to understand the development of an algal population in a reactor system. The effect of solids retention time (SRT) is found to yield stable biomass with good settling properties at longer SRTs (Liao, \textit{et al.}, 2001, Medina and Neis, 2007, Valigore, \textit{et al.}, 2012). However, the increase in SRTs also leads to an increase in the solids concentration that could lead to the attenuation of light. In the case of the hydraulic retention time (HRT), the operation of HRAP was traditionally carried out at 2-6 d HRT (Mara and Pearson, 1986) and similar values were observed for enclosed PBRs by Essam, \textit{et al.} (2006) and Muñoz, \textit{et al.} (2005). Muñoz, \textit{et al.} (2004) reported a total removal of salicylate and high dissolved oxygen carbon (DOC) at 2.7 d HRT. But, this dropped when the HRT was reduced to 1.7 d indicating the
limitation of oxygen supply and therefore algal activity (Muñoz, et al., 2004). Medina and Neis (2007) outlined that short HRTs and high loadings favoured algal growth as these conditions stimulate their logarithmic growth phase. But, studies by Gutzeit (2006) and Valigore, et al. (2012) point out the risk of having too short HRTs which could lead to washout of biomass.

c) pH
The pH is one of the most important factors affecting the growth of algae as it ascertains the solubility and availability of essential nutrients and carbon dioxide, thereby having a major impact on the metabolism of algae (Chen and Durbin, 1994, Goldman and Shapiro, 1973). The algae consumes inorganic carbon causing the pH to rise in the growth medium with a maximum algal growth found to occur around neutral pH (Hansen, 2002). Altering the pH above the neutral value can have effects in terms of carbon uptake as reported by Chen and Durbin (1994). The increase in pH affects the algal mechanism to consume carbon dioxide and result in suppressed algal growth (Azov, 1982, Chen and Durbin, 1994). At higher pH, the affinity towards free CO$_2$ lowers as carbon is available in the form of carbonates (Azov, 1982, Nielsen, 1975, Rotatore and Colman, 1991). Under alkaline conditions, the algal cell must compensate the internal pH by depending on the active transport of HCO$_3^-$ instead of passive flux of CO$_2^-$ for storage of inorganic carbon (Azov, 1982, Moazami-Goudarzi and Colman, 2012). On the other hand, even an acidic pH can lead to negative outcomes such as alteration in nutrient uptake (Gensemer, et al., 2008) or create metal toxicity (Anderson and Morel, 1978, Sunda, 1975) and thereby affecting algal growth. Similar to alkaline conditions, the affinity of algae to consume CO$_2$ drops under acidic conditions (Nielsen, 1975, Rotatore and Colman, 1991). Similar outcomes were observed in algae-based wastewater treatment systems such as HRAPs where uptake of carbon dioxide by algae caused the pH to rise to high levels (Oswald, 1962). Even though this rise in pH was considered to be beneficial for pathogen inactivation, it was observed to affect the pollutant removal efficiency (Oswald, 1988, Schumacher, et al., 2003). Several studies, such as Mara and Pearson (1986), confirmed the negative impact of a high pH as it leads to bacterial inhibition at pH values around ten.

d) Temperature
Apart from pH, temperature is the second critical factor that affects the overall algal growth and metabolism. It has been observed that at non-optimal temperatures lead to inefficient carbon and nitrogen utilization rate by algae (Darley, 1982). An optimal temperature allows an ideal algal growth with minimal cell sizes (Harris, 1986, Rhee, 1982). A sub-optimal temperature results in changes in the cytoplasmic viscosity leading to a drop in carbon and nitrogen uptake by algae (Hope and Walker, 1975, Raven and Geider, 1988). Vonshak and Torzillo (2004) reported the alterations of algal mechanisms caused due to temperature that induced photoinhibition. The most commonly observed change due to drop in temperature is the increase in unsaturated fatty acids in their lipid membranes (Guschina and Harwood, 2009, Harwood, 1998, Thompson, 1996). Due to increase in unsaturated fatty acids, the algal cell membranes become vulnerable to
damage by free radicals (Nishida and Murata, 1996, Raven and Geider, 1988). However, the advantage of increasing the unsaturated fatty acids is that the photosynthetic machinery is protected against photoinhibition at low temperature (Nishida and Murata, 1996). Morris, et al. (1974) reported that the metabolism of *Phaeodactylum tricornutum* (marine algae) had a considerable rise in protein synthesis at night at lower temperatures, presumably because protein synthesis is the main component during night time in the metabolism of algae (Cuhel, et al., 1984). A similar observation was made by Rhee and Gotham (1981) in *Scenedesmus* sp. with a drop in temperature. On the other hand, the increase in temperature beyond the optimum value caused a drop in the protein synthesis efficiency (Rhee and Gotham, 1981). An increase in temperature is reported to degrade the starch produced (Mitsui, 1977, Nakamura and Miyachi, 1982b). The effect of temperature on the starch degradation in *Chlorella vulgaris* was observed by Nakamura and Miyachi (1982b). Algal photosynthesis involves carotenoids which are used to absorb light energy and rely on temperature for their formation (Armstrong and Hearst, 1996). Furthermore, they are involved in the photosynthetic reaction either in the energy-transfer process or protecting the chlorophyll from photodamage (Armstrong and Hearst, 1996). Several studies, such as Liu and Lee (2000), Tjahjono, et al. (1994) and Tripathi, et al. (2002), have indicated the temperature range of 20 – 35 °C as the optimal range for carotenoid accumulation to protect against the photodamaging and oxidative effects at high temperatures.

c) Nutrient concentration
The ecology of algae is strongly dependent on the size of algae as the algal biochemical composition, algal metabolism, processes that support growth and decay of algae are defined by the size of algae (Agustí, 1991, Agustí, et al., 1994, Banse, 1976, Harris, 1986, Runge and Ohman, 1982, Shuter, 1978, Watson and McCauley, 1988). The key element for algal metabolism is nitrogen due to its role in the formation of algal proteins and enzyme catalysts (Fang, et al., 1993, Hecky and Kilham, 1988, Lapointe, 1987). The rate of photosynthesis in small sized algae or micro algae is higher due to higher specific growth rates and faster transport of nutrients per unit of biomass (Hein, et al., 1995). The Michaelis-Menten equation and enzyme kinetics is used to describe the relationship between the uptake of nitrogen and nitrogen concentration (Dugdale, 1967). Redfield (1958) put forward the idea that the nitrogen uptake required by phytoplankton could not be limited by the lack of nitrogen availability as biological fixation of nitrogen could satisfy the nitrogen requirements of algae. However, this notion was opposed by Ryther and Dunstan (1971) as they proved that the inorganic nitrogen, not phosphorus, is the limiting component for algal growth. Oswald, et al. (1953) explained the process of photosynthesis in the presence of sunlight by using an equation given as:

\[
106 \text{CO}_2 + 236 \text{H}_2\text{O} + 16 \text{NH}_4^+ + \text{HPO}_4^{2-} \\
\rightarrow C_{106}H_{181}O_{45}N_{16}P + 118\text{O}_2 + 171\text{H}_2\text{O} + 14\text{H}^+
\]
This equation indicates that the production of algal biomass requires nitrogen in the growth medium. Oh-Hama and Miyachi (1992) concurred this outcome by reporting the growth of *C. vulgaris* on mainly ammonium and nitrate salts despite having the potential to grow by using different organic and inorganic nitrogen compounds. Despite feeding ammonium and nitrate together to *Chlorella* spp., algae preferred to consume ammonium-nitrogen and incorporate into the organic compounds produced by the microalgae (Oh-Hama and Miyachi, 1992).

On the other hand, Xin, *et al.* (2010) outlined the importance of both N & P in the growth medium on the basis of elementary composition of microalgal cells. The microalgae from Stumm empirical formula indicates a N:P ratio of 7.2:1. The importance of the N/P ratio on nutrient assimilation by microalgae was explained by Kapdan and Aslan (2008) in their study of *C. vulgaris*. The N/P ratio for *C. vulgaris* was 8:1 (Kapdan and Aslan, 2008) and for *Scenedesmus* sp. was in the range of 5:1 to 8:1 (Xin, *et al*., 2010). A similar study by Rhee (1978) on *Scenedesmus* sp. indicated that this ratio could range from 5:1 to 80:1 depending upon the limitation of either nitrogen or phosphorus. The minimum and maximum ratio for cellular N/P ratio in *Scenedesmus* sp. was 4 under N-limited conditions and 142 under P-limited conditions, respectively. The better indication of nutrient limitation is from wide range of N:P ratios in the algal cells instead of nutrient levels in the surrounding medium where the nutrient ratios are known (Rhee, 1978).

### 2.4.2 Factors affecting biological phosphorus removal

#### a) Wastewater composition

Influent wastewater composition should have minimal fluctuations and should be as stable as possible to prevent disturbances to the process (Brdjanovic, *et al*., 1998). If the influent organic composition is changed from VFAs to sugar, such as glucose, the growth of glycogen accumulating organisms (GAOs) might supersede the growth of PAOs leading to breakdown of P-removal process (Satoh, *et al*., 1994). Additionally, the COD loading rate is crucial to avoid deterioration the biological phosphorus removal process (Morgenroth and Wilderer, 1998). Chuang, *et al.* (1998) reported that high-P uptake is possible with low COD-SS loading rates. A high COD-SS loading rates causes the biomass to convert influent organic matter to 3-hydroxyvalerate, which cannot be used as a storage product by PAOs (Liu, *et al*., 1996).

#### b) Carbon sources

The carbon sources for PAOs include several organic compounds like volatile fatty acids (VFAs), carboxylic acids, sugars, amino acids, glucose, peptone, etc. But, VFAs are considered to play a major role in having an optimum biological phosphorus removal (Comeau, *et al*., 1996, Pitman, 1999, Ruel, *et al*., 2002). Barnard (1993) estimated that 7-9 mg of VFA are needed to have 1 mg of P-removal. Alternately, organic compounds other than VFAs like carboxylic acids, sugars, amino acids, glucose and peptone are found to support bio-P removal (Carucci, *et al*., 1999, Satoh, *et al*., 1996). However,
studies by Rustrian, et al. (1996) have shown that while acetate and butyrate are good carbon sources for P-removal, other sources of carbon like glucose, amino acids, propionate or starch supplementation have detrimental effects on P-removal. Randall and Khouri (1998) reported that acetic acid and isovaleric acid as carbon sources enhanced the removal efficiencies during short-term experiments.

c) pH
The process of biological P-removal is found to be efficient at high pH as shown by several studies. Under anaerobic conditions, the increase in pH under anaerobic conditions leads to an increase in rate of P-release (Smolders, et al., 1994). Bond, et al. (1998) reported that by operating an SBR without pH control in the anaerobic phase allowed improved P-removal as compared to an SBR having pH control. An acidic pH had a negative impact on the uptake of acetate and P-release in the anaerobic phase, while a highly alkaline pH prevented the uptake of acetate and stimulated a high P-release than under acidic conditions (Liu, et al., 1996). However, Converit, et al. (1995) reported that maintaining a stable, neutral pH was required to have stable biological phosphorus removal mechanisms in batch systems.

d) Temperature
Due to the effect of temperature on biological reaction rate constants, the assessment of temperature variations is vital to understand the biological treatment process (Mulkerrins, et al., 2004). The microbial growth rate is observed to double with every 10°C increase in temperature until the optimum temperature is achieved (Metcalf and Eddy, 1991). However, Brdjanovic, et al. (1997) reported multiple conflicting outcomes of temperature on bio-P removal. Studies, such as Converit, et al. (1995) and McClintock, et al. (1993), indicated the improved removal efficiency of phosphorus at higher temperatures of 20-37°C. Contrastingly, Florentz, et al. (1987) and Viconneau, et al. (1985), observed better P-removal efficiency at lower temperatures of 5-15°C. However, Brdjanovic, et al. (1997) provided a detailed analysis of the short-term (hours) and long-term (weeks) effects of temperature on the physiology of biological P-removal. Partial P-uptake was observed between 5 to 10°C while complete P-uptake was observed between 20 and 30°C (Brdjanovic, et al., 1997). However, good P-removal was observed at low temperatures with long anaerobic SRT.

e) Dissolved Oxygen (DO) concentration
The dissolved oxygen concentration mainly depends on the design criteria of the activated sludge systems due to different demands from the microbial community present in the system (Mulkerrins, et al., 2004). For a biological phosphorus removal process, the anaerobic zone should have no oxygen to prevent interference with the activity of PAOs (Shehab, et al., 1996). Shehab, et al. (1996) has recommended to observe a DO concentration of 3.0 and 4.0 mg/L. Brdjanovic, et al. (1998) reported the impact of excessive aeration leading to the termination of P-uptake due to the exhaustion poly-ß-hydroxybutyrate (PHB).
f) Solids Retention Time (SRT)

Biological phosphorus removal operation at different SRTs has been well documented. Efficient biological phosphorus removal at SRT higher than 3 days was observed by (Mamais and Jenkins, 1992). Chuang, et al. (1998) observed incomplete P-removal due to cessation of anoxic P-uptake while operating the system at a 5-day SRT but operating the similar system at a SRT of 10 days lead to anoxic and aerobic P-uptake. Also, studies of P-removal systems at various SRTs (5 to 20 days) resulted in the best P-removal efficiencies in systems operated at a SRT of 10 days (Chang, et al., 1996, Choi, et al., 1996). However, in case of complete nutrient removal systems, Furumai, et al. (1999) observed that SRTs longer than 21 days cause nitrate levels to increase in the anoxic phase thereby competing with PAOs for organic matter and preventing P-removal to proceed. In other studies, a sludge age of 10 days was found to contribute to reach maximum nutrient removal efficiencies (Chang and Hao, 1996, Kargi and Uygur, 2002).

g) Phosphorus load

Studies conducted with limited phosphate loadings have indicated the suppressed growth of PAO and causing the growth of GAO dominated microbial populations (Sudiana, et al., 1999). The importance of ratio of phosphorus to total organic carbon (P/TOC) in the feed of a system for selection of PAOs to perform optimally was observed by Liu, et al. (1997). Furthermore, the comparison between different P/TOC feeding ratio over growth of PAOs and GAOs suggested higher feeding ratios encouraged growth of PAOs over GAOs (Liu, et al., 1998). Converti, et al. (1993) in their study used a phosphate feed load of 70 mg/L and recorded a P-removal rate of around 90%. Similarly, Comeau, et al. (1996) operated an SBR system with feed concentration of 60 mgP/L to achieve an average removal of 88% for TP. Studies, such as Shin and Jun (1992) and Randall, et al. (1997), have indicated towards the redundancy in potential to cater to high influent phosphate load (>20 mgP/L).

2.4.3 Factors affecting nitrification

a) pH and alkalinity

The concentration of both hydrogen and hydroxyl ions play a major role in affecting the pH value of the bulk liquid and the unduly increase in their respective concentrations can have inhibitory effect on nitrification (Ekama and Marais, 1984). Ekama and Marais (1984) suggest that the optimal nitrification rates are expected within a range of 7.0 to 8.5 for pH as the growth of nitrifiers is extremely sensitive to pH of the growth medium. The stoichiometric equation given below for nitrification indicates that there is a release of hydrogen ions causing drop in alkalinity of the system (Ekama and Marais, 1984).

\[ \text{NH}_4^+ + 1.5\text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + 14\text{H}^+ \]

Lowenthal and Marais (1976) suggested that, by using the equilibrium chemistry of carbonate system the pH and alkalinity could be linked to produce the plot given in figure
14. Alkalinity below 40 mg/L as CaCO$_3$ causes pH to drop to low values causing poor nitrification process.

While Engel and Alexander (1958) reported a pH range of 7.0 to 9.0 as the optimal for the growth of nitrifiers, Meyerhof (1917) observed a peak of nitrification at a pH of 8.6. However, if the growth medium is free of any pH fluctuations then the nitrification rate would be optimal over the pH range 7.0-8.0; in all other cases the ideal pH range narrows down to 7.6-8.0 (Loveless and Painter, 1968).

b) Dissolved oxygen (DO) concentration

The effect of dissolved oxygen concentration on the nitrification rate has been defined by Stenstrom and Poduska (1980) using the following formula.

$$\mu_{AO} = \mu_{AM20} \frac{O_2}{K_O + O_2}$$

where, $O_2$ oxygen concentration in liquid (mgO$_2$/L)
$K_O$ half saturation constant (mgO$_2$/L)
$\mu_{AM20}$ maximum specific growth rate of nitrifiers (/d)
$\mu_{AO}$ specific growth rate of nitrifiers (mg/L.d)

The ideal range for $K_O$ is from 0.3 to 2.0 mgO$_2$/L and DO values below this would reduce the growth rate of nitrifiers to half the rate as compared to the rate when adequate oxygen concentration is available (Stenstrom and Poduska, 1980). Studies have also pointed out the significance of the range of $K_O$ with ceasing of nitrification below 0.3 mgO$_2$/L (Bragstad and Bradney, 1937, Downing, et al., 1964a). The effect of high oxygen concentration were initially observed to affect the nitrification rate as there was drop in growth of nitrifiers (Gundersen, et al., 1966, Schön, 1965). However, Charley, et al. (1980) reported that the drop in nitrification was observed at elevated DO levels for an
initial period. The nitrification process was restored once at high DO concentrations (38 mgO$_2$/L) once the nitrifiers had been acclimatized (Charley, et al., 1980).

c) Temperature
Downing, et al. (1964a) conducted experiments to show that the nitrification rate could be formulated in terms of the Monod equation. The Monod constants for maximum specific growth rate and half saturation coefficient for nitrifiers were reported to be sensitive to temperature (Ekama and Marais, 1984). The study by Ekama and Marais (1984) pointed out that with every 6°C drop in temperature the growth rate halved and caused the sludge age for nitrification to double. Similarly, the half-saturation coefficient (also termed as the affinity coefficient) doubled with every 6°C increase in temperature affecting the effluent ammonia concentration (Ekama and Marais, 1984). However, the temperature increase also allowed increase of growth rate to compensate for the impact caused by increase in affinity coefficient thereby causing the effluent ammonia concentration to decrease with an increase in temperature (Ekama and Marais, 1984).

d) Solids Retention Time (SRT)
The SRT (also known as sludge age) is a critical parameter in an activated sludge process. The time required for the growth of microorganisms performing biological activities in a reactor system is controlled by adjusting the SRT (Ekama and Wentzel, 2008). For nitrification, SRT plays a critical role due to the low biomass growth coefficients of nitrifiers (Ekama and Marais, 1984). The SRT for nitrification systems should be higher than the minimum value required to allow a sustained growth of nitrifiers in the system and avoid their wash out. Ekama and Marais (1984) described the effect of having sludge age higher than the minimum SRT on the effluent ammonia concentration. They reported that as soon as the system is operated at a SRT slightly more than the required sludge age there was a drastic drop in effluent ammonia concentration. The reason behind this is the low value of Monod half saturation concentration for nitrifiers (Ekama and Marais, 1984). The SRT is also vital to design the denitrification potential in a system as it affects the estimation of unaerated/anoxic requirements (Ekama and Marais, 1984).

2.4.4 Overview for assessing the feasibility of the photo-activated sludge denitrification EBPR (PASDEBPR) system

The potential of coupling algae with bacterial population has been proved successful through numerous studies. Studies specifically looking into establishing algal-bacterial consortia to achieve nutrient removal have also been done, but a PAS system for simultaneous nutrient removal is yet to be tested. The research by Mohamed (2017) provides the basis for developing a PAS system to have phosphorus removal without artificial aeration, while studies by Rada-Ariza, et al. (2017) and van der Steen, et al. (2015) reported the nitrification potential of a PAS system having algal-bacterial composition. However, in a PAS system to establish complete nutrient removal some critical factors shall be identified and studied in this research.

From the equation of algal photosynthesis given by Oswald, et al. (1953), it can be seen that the production of algal biomass requires nutrients (as NH$_4^+$ and PO$_4^{3-}$) in the growth medium.
to carry out the nutrient assimilation process. The nutrient requirement varies depending upon the algal species and it is represented by the C:N:P ratio. In this research study, the viability of PAS-EBPR system to perform denitrification shall be assessed through the variation in nitrogen concentration of the system. While in the study performed by Mohamed (2017) nitrification was prevented by the addition of allyl-thiourea (ATU), the ATU addition shall be stopped once a stable PAS-EBPR system is reached to proceed with the nitrogen removal process through nitrification.

In order to achieve nitrification, there should be an excess of ammonium in the growth medium for oxidation by nitrifiers apart from the ammonium requirements of algae. While the algal requirements of ammonium can be determined based on the stoichiometric analysis of photosynthesis process, the surplus ammonium required for nitrification is unknown and needs to be identified. The nitrification of this extra ammonium will form nitrite or nitrate and this can be determined through the stoichiometric analysis of nitrification process. The denitrification of the resultant products from nitrification shall have to be carried out to complete the biological nitrogen removal process. For the denitrification process, there are three probable pathways in the PASDEBPR system:

a) Denitrification by OHOs;
b) Denitrification by PAOs during the EBPR process; and
c) Algal uptake through phototrophic assimilatory mechanism.

The denitrification by OHOs is favoured by the presence of RBCOD as an organic source as reported by Ekama and Wentzel (2008). But, the RBCOD is consumed by PAOs under anaerobic conditions during the EBPR process and will not be available for OHOs to carry out the denitrification process. Hence, the probable pathways for completing nitrogen removal were denitrification by PAOs and uptake by algae as source of nitrogen.
This chapter explains the methodology adopted for understanding the development of the PASDEBPR system. This is followed by a brief description of the laboratory study performed to derive the findings of this research. Finally, an outline of the deductive approach is provided which justifies the research strategy.

3.1 Research approach

The research methodology was divided into four phases:

- **Phase 1** – In the first phase, a preliminary design of the system was established using a steady-state model. This model was useful to understand the behaviour of the PASDEBPR system based on several factors as well as foresee the possible outcomes.
- **Phase 2** – From the outcome of the steady-state model, a consortium of algae and PAOs were cultivated in a SBR setup. The reactor was operated under EBPR conditions to establish PAS-EBPR system. Initially, the dissolved oxygen concentration was maintained with external air supply during the aerobic phase. In addition to the artificial air supply, the growth of algae was stimulated by external illumination. Later on, the external aeration was discontinued to check the sustainability of the EBPR process based on photo-oxygenation by algae. Simultaneously, an identical SBR was operated to cultivate PAOs and was used as a positive control for the PAS-EBPR system.
- **Phase 3** – After achieving steady-state operation in PAS-EBPR system with no external oxygen supply, fresh activated sludge was added to introduce BNR in the same system.
- **Phase 4** – The factors affecting the performance of PASDEBPR system was identified in this phase. Additional experiments were carried out to identify the limiting factors affecting the nutrient removal efficiency in this system.

3.2 Apparatus and materials for experimental analysis

3.2.1 Laboratory setup

The experimental setup consisted of two reactor systems with features similar to the setup used by Saad, *et al.* (2016). The biomass was enriched in a double-jacketed cylindrical glass vessel with a working volume of 2.5 L. Each reactor was fed with synthetic wastewater having characteristics similar to those used by Mohamed (2017) and Saad, *et al.* (2016). The SBR was operated with four cycles per day with a duration of 6 hour per cycle. The reactor setup was operated automatically using an Applikon ADI controller along with BioXpert software for storing of online operating data (e.g., O\(_2\) and pH). During the initial stages for experimental operation, the dissolved oxygen was maintained with external air supply. For the SBR with algal-PAOs consortium, provision for illumination using eight lamps was made. The lamps
used in this experiment were Philips E27 series rated 75 W. The pH was maintained at 7.5 ± 0.1 by dosing 0.4 M HCl and 0.4 M NaOH. Temperature was controlled at 20 ± 1°C. The mixed liquor was mixed at 500 rpm during the anaerobic and aerobic phases. Six peristaltic pumps were utilised in the reactor setup for different purposes such as feeding of artificial wastewater, dosing of acid/base, etc. The HRT and SRT of the SBRs were maintained at 12 h and 8 d, respectively. For controlling the SRT at 8 d, 75 mL of mixed liquor was withdrawn every cycle and it was stored after each SRT as a backup during all the experimental phases.

![Diagram of laboratory setup](Source: The author)

### 3.2.2 Synthetic medium

The concentrated solution of synthetic feed was prepared in two separate bottles (10 L each) with de-mineralized water and autoclaved at 120 °C for 45 min. The first concentrated solution contained acetate and propionate as carbon sources, while the other contained the required nutrients, minerals and trace elements. Due to unavailability of magnesium sulphate during the research period, it was replaced by magnesium chloride and ammonium sulphate. The trace element solution was prepared as described by Smolders, et al. (1994). Initially, 2 mg/L of allyl-thiourea (ATU) was added to inhibit nitrification but in the later experimental phase it was discontinued for the PASDEBPR system. After dilution with de-mineralized water, the influent of the reactor was characterized as shown in the table given below.

<table>
<thead>
<tr>
<th>Mode of operation</th>
<th>SBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cycles</td>
<td>4</td>
</tr>
<tr>
<td>Time period for each cycle</td>
<td>6 hours</td>
</tr>
<tr>
<td>Anaerobic stage</td>
<td>2 hours</td>
</tr>
<tr>
<td>Aerobic stage</td>
<td>3 hours</td>
</tr>
<tr>
<td>Settling stage</td>
<td>0.5 hours</td>
</tr>
<tr>
<td>Decanting stage</td>
<td>0.5 hours</td>
</tr>
<tr>
<td>Hydraulic Retention Time (HRT)</td>
<td>12 hours</td>
</tr>
<tr>
<td>Solid Retention Time (SRT)</td>
<td>8 days</td>
</tr>
</tbody>
</table>

![Diagram of laboratory setup](Source: The author)
Table 2: Characteristics of synthetic feed wastewater

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chemical compound</th>
<th>Mass in 10 L g</th>
<th>Concentration mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Oxygen Demand (COD)</td>
<td>CH₃COONa.3H₂O</td>
<td>32.6</td>
<td>326</td>
</tr>
<tr>
<td></td>
<td>CH₃CH₂CO₂H</td>
<td>3.3 mL</td>
<td>0.033 mL</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>MgCl₂.6H₂O</td>
<td>63.0</td>
<td>75</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>CaCl₂.2H₂O</td>
<td>13.3</td>
<td>36</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>(NH₄)₂SO₄</td>
<td>4.5</td>
<td>25</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>NH₄Cl</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>NaH₂PO₄·H₂O</td>
<td>6.8</td>
<td>15</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>NaHCO₃</td>
<td>55.0</td>
<td>550</td>
</tr>
<tr>
<td>K⁺</td>
<td>KCl</td>
<td>3.6</td>
<td>19</td>
</tr>
<tr>
<td>EDTA</td>
<td>EDTA.Na₂</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>ATU</td>
<td>Allylthiourea</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>Yeast</td>
<td>Yeast</td>
<td>0.1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3: List of trace elements present in the synthetic feed (Smolders, et al., 1994)

<table>
<thead>
<tr>
<th>Chemical compound</th>
<th>Concentration in g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl₃.6H₂O</td>
<td>1.5</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.15</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.03</td>
</tr>
<tr>
<td>KI</td>
<td>0.18</td>
</tr>
<tr>
<td>MnCl₂.6H₂O</td>
<td>0.12</td>
</tr>
<tr>
<td>Na₂MoO₄.2H₂O</td>
<td>0.06</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>0.12</td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
<td>0.15</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
</tr>
</tbody>
</table>

3.2.3 Performance evaluation of biological activity

The biological activity of the two SBRs was regularly monitored by measuring different analytical parameters by collecting samples for influent and effluent as well as during the anaerobic and aerobic phases. Plastic syringes of 30 mL were used for collecting samples from the reactor. By using 0.45 μm pore size filters, the samples were filtered immediately after the collection for the analysis of dissolved substances like NH₄⁺-N, PO₄³⁻-P, NO₂⁻-N, NO₃⁻-N, and VFA. On the other hand, samples for measuring MLVSS, MLTSS and chlorophyll-a were collected and filtered as per the standard analytical procedures. The filtered samples were
preserved by refrigeration at 4°C as the analysis was not performed as soon as they were collected.

Cycle tests were conducted at the end of each experimental phase to understand the biomass activity when the SBR reached pseudo steady state conditions. For a cycle test, a total of 20 samples were collected (9 samples during the anaerobic phase and 11 samples during the aerobic phase). For the measurement of solids concentration, an additional sample was collected at the end of the aerobic phase. The frequency of sample collection was shorter at the beginning of each phase and it was increased at the end of the phase. Wasting of sludge was not carried out during the cycle test to compensate for the volume abstracted during the test to maintain the SRT.

Table. List of analytical parameters for performance evaluation of biological activity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Standard /Analytical method</th>
<th>Phase for sample collection/measurement</th>
<th>Frequency of sampling/measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO, pH and T</td>
<td>Online measurement</td>
<td>All (throughout SBR operation)</td>
<td>-</td>
</tr>
<tr>
<td>NH$_4^+$-N</td>
<td>Spectrophotometric analysis based on NEN 6472</td>
<td>All phases</td>
<td>2 times per week</td>
</tr>
<tr>
<td>PO$_4^{3-}$-P</td>
<td>Ascorbic acid method using spectrophotometer based on APHA (1992c)</td>
<td>All phases</td>
<td>2 times per week</td>
</tr>
<tr>
<td>HAc and HPr</td>
<td>Gas chromatography method using Varian GC-430</td>
<td>Anaerobic phase; sometimes aerobic phase</td>
<td>2 times per week</td>
</tr>
<tr>
<td>MLSS and MLVSS</td>
<td>Gravimetric methods based on EPA (1983)</td>
<td>Sampling phase</td>
<td>1 time per week</td>
</tr>
<tr>
<td>Chlorophyll-a</td>
<td>Sampling phase</td>
<td></td>
<td>1 time per week</td>
</tr>
<tr>
<td>Intensity of light</td>
<td>Manually using handheld photometer</td>
<td>Prior to start-up and end of effluent phase</td>
<td>Once per experimental phase</td>
</tr>
<tr>
<td>NO$_3^-$-N</td>
<td>APHA (1992a)</td>
<td>Aerobic phase</td>
<td>As per requirement</td>
</tr>
<tr>
<td>NO$_2^-$-N</td>
<td>APHA (1992b)</td>
<td>Aerobic phase</td>
<td>As per requirement</td>
</tr>
<tr>
<td>Identification of algal species</td>
<td>Microscopic examination</td>
<td>Sampling phase</td>
<td>As per requirement</td>
</tr>
<tr>
<td>Off-gas emission test</td>
<td>Salting-out method</td>
<td>All phases</td>
<td>As per requirement</td>
</tr>
</tbody>
</table>
Chapter 4  
Results and discussion

4.1 Research phase 1: Steady-state model

4.1.1 Introduction

The steady-state model is a crucial aspect of the research strategy behind the development of PASDEBPR. A preliminary design of the experimental analysis for establishing the basic SBR operation was formulated using the model. By varying the input values to simulate different scenarios, the desired outcome of simultaneous N & P-removal from a single photobioreactor system was predicted.

4.1.2 Design rationale for steady-state model

The fundamental principle behind the development of steady-state model in this research was to divide the photo-activated sludge into two categories of microbial communities:

1) PAOs, the phosphorus accumulating organisms and
2) Algae

Firstly, the growth of PAOs was prioritized to establish P-removal in the SBR. In an EBPR system, the operational conditions are very important as the biological activity could be dominated by either GAOs or PAOs. From the biochemical model of Wentzel, et al. (1986), the conditions required to produce an enhanced PAO culture was identified. These conditions provided information regarding the necessary anaerobic/aerobic sequence with sufficient anaerobic mass fraction, influent feed with acetate (HAc) and propionate (HPr) as substrate and with adequate macro- and micronutrients, and pH and temperature control during the aerobic phase. Furthermore, with the supply of acetate and propionate as organic substrate, it would be completely consumed during anaerobic phase. Hence, the chances for growth of OHOs during aerobic phase are negligible. Therefore, the expected outcome of the model with the above conditions was an enhanced culture of PAOs in the mixed liquor.

Since algae are fast growing organisms as compared to PAOs, their growth was initially not taken into consideration in the model. By limiting the model to favor growth of PAOs, the essential components of synthetic feed were obtained. The influent phosphorus was supplied in excess to allow optimal growth of PAOs and algae. Influent phosphorus concentration was first designed considering the maximum storage of poly-P in PAOs biomass i.e. 0.38 P/VSS. The remaining amount was supplied by considering the requirement for growth of algal biomass and in response to nitrogen (as NH₄⁺) consumption during algal photosynthesis. Also, the amount of effluent decanted from the SBR in each cycle was half the reactor volume. As a result, the concentration of dissolved compounds like PO₄³⁻, NH₄⁺, etc. in the reactor was equally contributed by the influent feed concentration and effluent concentration of the dissolved compounds.
The growth of algae was considered to occur during the aerobic phase with artificial illumination using lamps surrounding the SBR setup. The distribution of light intensity over the reactor was assumed to be uniformly distributed. Due to the cylindrical shape and double jacketed structure of reactor, it was difficult to achieve uniform distribution of light intensity at all points within the reactor. However, attempts to overcome this hurdle was made by surrounding the reactor using a box-type setup with provision to illuminate the reactor with eight lamps. Though the light intensity requirement was predicted in the model, the actual light intensity was higher than this value in order to avoid any limitations on algal growth.

The primary aim of the research was to establish a stable consortium of algae and PAOs to achieve EBPR without external air supply. Therefore, the synthetic media was supplied with allyl-thiourea (ATU) to suppress the growth of nitrifiers. As a result, the influent ammonium was available for uptake during algal photosynthesis.

The model developed here was based on the principles of the steady-state model for enhanced culture developed by Wentzel, et al. (1990). Table 4 gives the main input parameters considered for the model. The kinetic and stoichiometric parameters considered for the steady-state model alongwith some important output are provided in Appendix A.

<table>
<thead>
<tr>
<th>Reactor configuration</th>
<th>Diameter of reactor</th>
<th>12.5 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height of reactor</td>
<td>20.5 cm</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Characteristics of synthetic wastewater</th>
<th>COD (75% HAc and 25% HPr)</th>
<th>150 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH₄-N</td>
<td>25 mg/L</td>
</tr>
<tr>
<td></td>
<td>PO₄-P</td>
<td>15 mg/L</td>
</tr>
<tr>
<td></td>
<td>Alkalinity (HCO₃⁻)</td>
<td>550 mg/L</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Operational data for SBR operation</th>
<th>Length of anaerobic phase</th>
<th>2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length of aerobic phase</td>
<td>3 h</td>
</tr>
<tr>
<td></td>
<td>Length of settling phase</td>
<td>0.5 h</td>
</tr>
<tr>
<td></td>
<td>Length of decanting phase</td>
<td>0.5 h</td>
</tr>
<tr>
<td></td>
<td>Total number of cycles per day</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Aerobic SRT</td>
<td>4 d</td>
</tr>
<tr>
<td></td>
<td>Total SRT</td>
<td>8 d</td>
</tr>
<tr>
<td></td>
<td>Volume of influent feed per cycle</td>
<td>1250 mL</td>
</tr>
<tr>
<td></td>
<td>Volume of waste activated sludge discharged per cycle</td>
<td>75 mL</td>
</tr>
<tr>
<td></td>
<td>Volume of effluent discharged per cycle</td>
<td>1175 mL</td>
</tr>
</tbody>
</table>

Table 4: Main input parameters for the steady-state model
4.2 Research phase 2: Development of the PAS-EBPR and EBPR systems

4.2.1 Enrichment of PAOs and algae

Two identical SBRs were setup for cultivation of two different microbial communities. The first reactor (R1) was cultivated with activated sludge and five algal species (*Scenedesmus quadricauda*, *Anabaena variabilis*, *Chlorella sp.*, *Chlorococcus sp.*, and *Spirulina sp.*) to develop the PAS-EBPR. The second reactor (R2) was cultivated with only activated sludge to develop EBPR. The purpose of R2 was to act as a source of inoculum of PAOs for R1, in case of any difficulty faced while establishing phosphorus removal in R1. Therefore, R1 was inoculated with 1.24 L of activated sludge and 100 mL of algal species. On the other hand, R2 was inoculated with 1.25 L of activated sludge from an EBPR-based WWTP at Haranschpolder. The synthetic feed medium and operational conditions were identical for both reactors. The light needed for algal photosynthesis was provided by activating two lamps (75 W each) on the same side of the reactor. The only difference in SBR operation between R1 and R2 was the set-point for DO concentration. It was maintained at 20% in R2 while the same was set at 10% in R1. Any extra demand for oxygen in R1 was deemed to be supplied by algae. The development of biological activity was monitored from the beginning of reactor operation by analyzing samples collected over different phases during a cycle. The pseudo steady-state conditions were confirmed for both reactors, before proceeding to the next experimental phase, by the stable profile of pH and DO recorded alongwith the measured data for orthophosphate (PO$_4^{3-}$-P), VFA and MLSS.
4.2.2 Initial operation and cultivation of PAOs and algae

The development of a culture of PAOs in R1 took eighteen days from the start of the SBR operation. There was anaerobic sequestration of VFA together with release of $\text{PO}_4^{3-}$ during anaerobic phase with net $P$-released/VFA ratio at 0.38 P-mol/C-mol VFA. But due to incomplete uptake of VFA in the anaerobic phase, there was a leakage of organic substrate leaked into the aerobic phase during this time period. However, this overflow of organics gradually stopped as the enrichment of PAOs progressed. On the other hand, the aerobic uptake of $\text{PO}_4^{3-}$ was minimal at the beginning which indicated a slow development of PAOs. During the aerobic phase, the oxygen requirements of PAOs was aided by external oxygen supply. By coupling PAOs with algae, the oxygen requirements of PAOs was to be satisfied by algal photo-oxygenation process. Despite the growth of algae in the system, the online DO profile indicated that the external air was supplied continuously throughout the aerobic phase. To check the extent of coupling between algae and PAOs, a microscopic examination of biomass from R1 was conducted on day 12. The results of microscopy indicated a very sparse growth of algae (mainly *Scenedesmus quadricauda*, and *Chlorella sp.* was observed) and a low degree of coupling between algae and PAOs. In order to enhance the process of coupling, it was necessary to ensure that the algal biomass were retained in the reactor. Under the assumption that the algal biomass would remain in a suspended state, they would be susceptible to flow out of the reactor in the effluent. Hence, the effluent was recirculated in the system during the influent phase of each cycle from the beginning of the SBR operation.

![Microscopic image of the biomass from the PAS-EBPR on day 12. Green coloured biomass indicates the scattered algal biomass while the brown coloured biomass indicates the activated sludge. The image was captured using a semi-motorised fluorescence microscope with 20 times magnification.](image-url)
Figure 18: Concentration of VFA (HAc and HPr) observed in PAS-EBPR for different phases during the initial 18-day period.
Figure 19: Concentration of orthophosphate (PO$_4^{3-}$) observed in PAS-EBPR for different phases during the initial 18-day period.
Figure 20: Concentration of ammonium (NH$_4^+$) observed in PAS-EBPR for different phases during the initial 18-day period.
The concentrations of PO$_4^{3-}$ and NH$_4^+$ measured during the eighteen days reflect the progress towards stable conditions in R1. The recirculation of effluent caused an increase in the initial concentration of the compounds which were not fully consumed along the cycle. As a result, the profile of the dissolved substances were found to be unreliable to predict the status PAOs development. Only after discontinuing the recirculation of effluent, the improvement in the profile of the two parameters was observed. However, the system operation required ten days to stabilize and provide concrete evidence of PAO enrichment. On day 18, the PO$_4^{3-}$ concentration was 2 mgP/L and the NH$_4^+$ concentration was 15 mgN/L.

The development of PAOs in R2 was similar to that in R1. A regular monitoring of PO$_4^{3-}$ and VFA was carried out to understand the enrichment of PAOs in R2. On day 18, the PO$_4^{3-}$ concentration was measured at 13 mgP/L in the influent, 37 mgP/L at the end of anaerobic phase and 3 mgP/L at the end of aerobic phase. Furthermore, the average net-P release/VFA uptake ratio at the end of anaerobic phase was 0.51 ± 0.17 which indicated a typical PAO metabolism.

4.2.3 Problem identification - slow growth of algae and coupling with PAOs

The results show the gradual development of the PAOs culture in R1 and R2, respectively. The PO$_4^{3-}$ concentration in the end of the aerobic phase was 2 mgP/L after the first eighteen days of reactor operation. But, the P-removal activity was aided by external air supply instead of algal photo-oxygenation. For R1, the steady-state model predicted the required light intensity as 71 μmol/m$^2$.sec, considering illumination through the side-walls. Despite this, the actual light intensity was 180 μmol/m$^2$.sec (using two lamps on one side). This excess of light intensity was adopted to support algal photosynthesis without any limitation of light. Since the model was developed using several parameters obtained from different research studies, the projected light intensity was found to be insufficient. This can be corroborated by a similar study conducted by Mohamed (2017) when developing a steady-state PAS-EBPR. In that study, the reactor was illuminated using four lamps with a light intensity of 350 μmol/m$^2$.sec. Hence, on day 32 two additional lamps were added during the aerobic phase for R1. By using a total of four lamps during the aerobic (light) phase, the average light intensity was 580 μmol/m$^2$.sec.

4.2.4 Steady-state operation of the PAS-EBPR

With the increase in light intensity to increase algal growth, the online DO profile for R1 was monitored daily. By day 36, the positive impact of the increased light intensity was observed with a DO concentration exceeding the set-point of 10% during the aerobic phase. At the same time, analytical measurements were carried out for continuous monitoring of P-removal by PAOs in R1. From day 37, the external air supply was stopped to check the reliability of algae-PAOs symbiosis to perform EBPR. Steady-state conditions were confirmed after twenty-five days by the stable profile of pH and DO and measurement of PO$_4^{3-}$. Before proceeding to the next experimental phase, a cycle test on R1 was conducted to understand the parameters of biological activity performed by the algal-PAOs consortium.

- Cycle test on 28/01/2019 on PAS-EBPR (R1)

The cycle test on R1 was conducted on Monday 28/01/2019 (day 57). The measurements for VFA, PO$_4^{3-}$, NH$_4^+$ and solids concentration was carried out on the same day. From the profile of VFA, there was a complete uptake of organic substrate during the anaerobic phase. The propionate was consumed within an hour while the uptake of acetate took another thirty
minutes. The profile for $\text{PO}_4^{3-}$ during the cycle test exhibited a decent EBPR activity. The $\text{PO}_4^{3-}$ concentration at the end of anaerobic phase was 44 mgP/L and at the end of aerobic phase was 2 mg P/L. The P-release rate during the anaerobic phase was found to be 1.43 mmolP/gVSS.hr. Additionally, the $\text{PO}_4^{3-}$ uptake rate was 0.24 mgP/L/min in the initial thirty minutes of the aerobic phase and 0.22 mgP/L/min for the rest of the aerobic phase.

Eventhough the aim of ATU addition in the synthetic feed was to prevent nitrification, the profile exhibiting ammonium concentration is crucial to indicate growth of algae in R1. From the profile for ammonium concentration during the cycle test, it is evident the ammonium consumption during the anaerobic phase was minimal (0.9 mgN/L). The ammonium uptake rates during the aerobic phase was 0.061 mgN/L/min and 0.017 mgN/L/min. Since there was no nitrification occurring at this stage, the consumption of ammonium was attributed to the combination of two processes: algal photosynthesis and the orthophosphate uptake by PAOs as part of EBPR activity.
Figure 21: Concentration of VFA (HAc and HPr) observed in PAS-EBPR during the cycle test on 28/01/2019
Figure 22: Concentrations of orthophosphate ($PO_4^{3-}$) observed in PAS-EBPR during the cycle test on 28/01/2019. The first rate of release and uptake corresponds to the measurement carried out at a short time interval (5 min) in the initial 30 min of each phase. While the second rate of release and uptake corresponds to the measurement carried out at a longer time interval (>5 min) after the 30 minutes of each phase.
Figure 23: Concentrations of ammonium (NH₄⁺) observed in PAS-EBPR during the cycle test on 28/01/2019. The uptake rate 1 corresponds to the NH₄⁺ measurements at 5 min time interval in the first 30 min. The uptake rate 2 corresponds to the NH₄⁺ measurements at longer time interval (>5 min) after 30 min of aerobic phase.
The average MLTSS and MLVSS concentrations at the end of aerobic phase were 2060 mg/L and 1265 mg/L, respectively. The resulting average ratio of MLVSS/MLSS was 0.61, exhibiting a high ash content which is often associated with high poly-P content in enriched PAO cultures (Smolders, et al., 1995).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume</th>
<th>MLTSS</th>
<th>Average MLTSS</th>
<th>Average MLVSS</th>
<th>Average MLVSS</th>
<th>MLVSS/MLTSS</th>
<th>Average MLVSS/MLTSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1-1</td>
<td>10</td>
<td>2130</td>
<td>2060</td>
<td>1260</td>
<td>1265</td>
<td>0.59</td>
<td>0.61</td>
</tr>
<tr>
<td>R1-2</td>
<td>10</td>
<td>1980</td>
<td>1245</td>
<td>1265</td>
<td></td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>R1-3</td>
<td>10</td>
<td>2080</td>
<td>1290</td>
<td></td>
<td></td>
<td>0.62</td>
<td></td>
</tr>
</tbody>
</table>

Finally, the chlorophyll-a measurements for PAS-EBPR at the end of aerobic phase was performed when different number of lamps were used for artificial illumination. The chlorophyll-a measured on day 18 was 1.07 mg/L with two lamps used during aerobic (light) phase. While another two lamps were added on day 32, the online profile of dissolved oxygen concentration showed excess oxygen presence during the aerobic phase after four days. Therefore, the external air supply was cut-off on day 37 and the chlorophyll-a measurement at the end of aerobic phase was 3.09 mg/L.

**4.2.5 Overview of research phase 2 - successful development of PAS-EBPR**

A stable consortium of algae-PAOs was successfully cultivated after sixty days of SBR operation to establish the PAS-EBPR (R1) without external air support. The initial measurement of influent orthophosphate (PO$_4^{3-}$) was higher than the concentration in the synthetic feed because of the effluent recirculation in the system. The recirculation of effluent was carried out to mitigate the problem of algae overflowing from the system with the effluent. After eight days of SBR operation, the recirculation of effluent was discontinued in order to establish stable conditions in the system. On day 18, the PO$_4^{3-}$ concentration in the effluent was 2 mgP/L. However, the growth of algae was insufficient to supply the oxygen for PAOs through algal photosynthesis. By concluding that the projected light intensity was limiting the growth of algae, a total of four lamps were used during the aerobic phase from day 32. Increasing the light intensity to 580 $\mu$mol/m$^2$.sec resulted in an increase in chlorophyll-a thereby a rise in algal biomass. This increase in algal biomass was vital to allow the activity of PAOs to sustain biological activity, especially during the aerobic phase without external air support. An overview of the first experimental phase is represented by the profile of PO$_4^{3-}$ concentration, shown in figure 25. The EBPR (R2) was continuously operated in steady-state conditions during this experimental phase and the enriched culture was stored by collecting the waste every week.
Figure 24: Long-term measurements of orthophosphate ($\text{PO}_4^{3-}$) at different phases of SBR cycle representing steady-state conditions in PAS-EBPR. The first milestone (M1) on day 8 marks the end of effluent recirculation and the second milestone (M2) on day 32 indicates addition of two lamps to increase light intensity from 180 $\mu$mol/m$^2$.sec to 580 $\mu$mol/m$^2$.sec.
Figure 25: Images from microscopic examination of biomass from PAS-EBPR on day 47. The first image is a representation of coupling between algae (Scenedesmus quadricauda, and Chlorella sp.) and PAOs and the second image is a phase contrast view depicting the enmeshment of algae and PAOs. The images were captured using a semi-motorised fluorescence microscope at 10 times magnification.
4.3 Research phase 3: Development of PASDEBPR

4.3.1 Introduction

For the third phase of this research, further experimental work was carried out to introduce N-removal in the PAS-EBPR (R1). By combing the two processes, the system would permit simultaneous N and P-removal thereby yielding the PASDEBPR system. In this experimental phase, the performance evaluation of biomass activity was modified by including analysis of nitrite (NO$_2^-$) and nitrate (NO$_3^-$). The reactor (R2) containing an enriched culture of PAOs was modified to an algal-PAOs system by adding 120 mL of mixed liquor from PAS-EBPR. Again, the idea behind converting EBPR to PAS-EBPR was to have a control system for the PASDEBPR.

4.3.2 Cultivation of nitrifiers in the PASDEBPR system

Fresh activated sludge was used as inoculum for cultivation of nitrifiers in the PASDEBPR. On day 60, 120 mL of fresh activated sludge was added to PASDEBPR. As a new bacterial community was to be cultivated in the PASDEBPR, the total number of lamps illuminating the reactor during the aerobic phase was increased to five by adding another lamp (four on one side and one on opposite side). Additionally, the SRT of the system was reduced from eight to six days in order to speed up the development of the bacterial community. During the next three days, the excess oxygen production by algae was found to decline during the aerobic phase. However, on day 64 there was no excess oxygen production recorded during the aerobic phase. The average DO concentration during the aerobic phase was 1%. To determine the impact of this sudden drop in DO concentration, a sample was collected at the end of aerobic phase and analyzed for concentrations of dissolved substances. The concentration of PO$_4^{3-}$, NH$_4^+$, NO$_2^-$ and NO$_3^-$ were 48 mgP/L, 12 mgN/L, 0.7 mgN/L and 0.5 mgN/L, respectively. To prevent any negative impact on the PAOs, the external air supply was immediately re-started to recover the P-uptake activity during the aerobic phase. Since the light intensity was found to be a limiting factor during the earlier development of PAS-EBPR, the light intensity was increased to 775 μmol/m$^2$.sec by the addition of sixth lamp. During the measurement of light intensity using a photometer, the average light intensity was found to be negligible (~0 μmol/m$^2$.sec) around the center of the reactor at a radial distance of 1.8 cm. This finding indicated the existence of a dark zone within the reactor during the aerobic (light) phase. Visual observations indicated that the high solids concentration in the reactor could be the reason behind the occurrence of this dark zone. The measurement of solids was 3170 mgVSS/L and 4295 mgTSS/L at the end of aerobic phase.

Table 6: Measurement of solids concentration on 04/02/2019 for PASDEBPR

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume</th>
<th>MLTSS</th>
<th>Average MLTSS</th>
<th>Average MLVSS</th>
<th>Average MLVSS</th>
<th>MLVSS/MLTSS</th>
<th>Average MLVSS/MLTSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1-1</td>
<td>10 mL</td>
<td>4130</td>
<td>4295</td>
<td>3090</td>
<td>3170</td>
<td>0.75</td>
<td>0.74</td>
</tr>
<tr>
<td>R1-2</td>
<td>10 mL</td>
<td>4580</td>
<td></td>
<td>3260</td>
<td>3170</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>R1-3</td>
<td>10 mL</td>
<td>4180</td>
<td></td>
<td>3170</td>
<td></td>
<td>0.76</td>
<td></td>
</tr>
</tbody>
</table>
After three days of external air supply and increase in light intensity, the biological activity of PASDEBPR was recovered. The concentration of $\text{PO}_4^{3-}$ and $\text{NH}_4^+$ at the end of aerobic phase were 2 mgP/L and 3 mgN/L, respectively. With the $\text{NH}_4^+$ concentration being so low after the light phase, it was assumed that both nitrification and algal photosynthesis were responsible for its uptake. Additionally, the concentrations of $\text{NO}_2^-$ and $\text{NO}_3^-$ for the same sample were 1.1 mgN/L and 0.9 mgN/L, respectively. With the biological activity of PASDEBPR retained, the external supply of air was cut off on day 67.

The online DO profile was continuously monitored in the following days. Within eight days, the profile of the DO concentration showed signs of deterioration. By day 78, the average DO concentration during the aerobic phase was recorded as 1%. Once again, the concentration of $\text{PO}_4^{3-}$ and $\text{NH}_4^+$ at the end of aerobic phase were measured at around 49 mgP/L and 12 mgN/L, respectively. Interestingly, the time period for the breakdown of the biological activity was eleven days (day 67 to day 78), while the time period for improvement in biological activity was only three days (day 60 to day 63). During the short recovery period, the light intensity was increased and external oxygen was supplied. Since the light intensity was already higher than the values generally applied for a photo-activated sludge system, increasing light intensity by addition of lamps was ruled out. On the other hand, the gradual decline and immediate recovery of biological activity hinted towards the accumulation an unknown compound which proved to be toxic for the algae-bacteria symbiosis. Furthermore, the short recovery period under the presence of external air supply hinted that this effect was reversible and could be occurring by the accumulation of an intermediate compound (either a dissolved compound that needs to be oxidized or a gas that needs to be stripped out). Therefore, as a mitigation strategy, the aerobic phase of the PASDEBPR was modified on day 78. The modification included a change in the operational conditions of the aerobic phase. Thus, nitrogen was sparged in between the aerobic phase for five minutes one hour after the aerobic started.

Once again in a span of three days (day 81), the N and P-removal efficiencies of the system had improved. The effluent $\text{PO}_4^{3-}$ and $\text{NH}_4^+$ concentration were 1 mgP/L and 1 mgN/L, respectively. Also, the $\text{NO}_2^-$ and $\text{NO}_3^-$ concentrations at the end of aerobic phase were 1 mgN/L and 1.1 mgN/L, respectively. In order to check any denitrification activity in the system, an aerobic cycle test (with a length of 180 min) was carried out to analyze $\text{NH}_4^+$, $\text{NO}_2^-$ and $\text{NO}_3^-$. In this test, eleven samples were collected during the aerobic phase. The $\text{NH}_4^+$ declined as the phase progressed and was measured as 2 mgN/L in the end of the aerobic phase. On the other hand, the concentrations of $\text{NO}_2^-$ and $\text{NO}_3^-$ were observed to be low throughout the aerobic phase. To conclude this phase, measurements of the chlorophyll-a and solids concentrations were carried out for the effluent on day 89. This measurement was key to understand the extent of coupling between algae and bacteria. The average concentration of chlorophyll-a was 24 μg/L while the average concentration of MLSS was 0.13 g/L.
Figure 26: Concentrations of ammonium ($\text{NH}_4^+$), nitrate ($\text{NO}_3^-$) and nitrite ($\text{NO}_2^-$) observed in PASDEBPR during the aerobic cycle test on 22/02/2019
Figure 27: Images from microscopic examination of biomass from PASDEBPR on day 92. The first and third images are showing the coupling of algae with bacteria while the second and fourth images are the phase contrast views depicting the enmeshment of algae and bacteria. The images were captured using a semi-motorised fluorescence microscope at 10 and 20 times magnification.
4.3.3 Overview of research phase 3 – successful development of PASDEBPR

Eventhough the development of PASDEBPR was started on day 60, a reliable performance by the system in terms of N and P-removal was observed after day 78. From day 78 to day 93, the PASDEBPR was performing under steady-state conditions without any external oxygen supply. However, during the development of the PASDEBPR a limiting factor in the form of an unknown compound was found to impede the algal-bacterial symbiosis. This unknown compound was assumed to gradually accumulate in the system. By gradually accumulation, it was found to cause an inhibitory effect to either the oxygen production mechanism of algae or the intra-granular oxygen transfer between algae and bacteria. This toxic effect took a longer time period (eleven days) to appear and shorter time period (three days) to disappear in the presence of external air supply. Hence, it was presumed that this unknown compound could be present in the gas phase. The presumption of gaseous nature of this compound was based on the stripping effect caused by the sparging of oxygen and nitrogen during the two mitigation steps performed to retain the biological activity. To conclude, the PASDEBPR was successful in performing N & P-removal without artificial air supply; albeit with nitrogen supply during the aerobic (light) phase. Hence, after of continuous operation of the PASDEBPR for two weeks, steady-state conditions were confirmed by ensuring monitoring the $\text{PO}_4^{3-}$, $\text{NH}_4^+$, $\text{NO}_2^-$ and $\text{NO}_3^-$, respectively.

4.4 Research phase 4 – Identification of limiting factor

4.4.1 Introduction

In the final research phase, attempts were made to identify the factor limiting the successful development of PASDEBPR. As stated in the previous experimental phase, it was assumed that this limiting factor was an unknown compound present in the liquid phase – either a dissolved compound or a gas. Hence, in this experimental phase, a detailed investigation was carried out by studying the individual biological processes. The performance evaluation of PASDEBPR during this phase was similar to the previous research phase. However, based on the assumption that the unknown compound was a gas that stripped out by sparging of N$_2$, the measurement of dissolved gases in the mixed liquor was carried out. The emission of dissolved gases in the reactor was measured by the salting-out method described by Gal'chenko, et al. (2004).

4.4.2 Rationale behind the nature of the limiting factor

In the PASDEBPR, the algal-bacterial symbiosis was developed to achieve simultaneous N and P-removal through a combination of several processes. Initially, a PAS-EBPR system was developed wherein cultivation of an enriched culture of PAOs was carried out from scratch and combined with algae. Thereafter, the cultivation of nitrifiers was carried out and the addition of ATU in the synthetic feed was discontinued. As a result, the removal of NH$_4^+$ was observed in the steady-state PASDEBPR system. However, the pathway causing the removal of NO$_2^-$ and NO$_3^-$ was unclear.

During the development of a steady-state PAS-EBPR, no toxic compound limiting the EBPR activity was encountered. Hence, the occurrence of this limiting compound could only be possible with the introduction of nitrogen removal in the system. Furthermore, the presumption of the limiting compound being a gas was justified by understanding the pathways causing nitrogen removal.
As shown in the figure, the possible metabolic pathways for NH$_4^+$ removal can result in formation of several nitrogen compounds as intermediate products. Out of these several intermediates, some of them were measured during this research. In fact, nitrogen compounds found in gaseous state i.e. nitrogen (N$_2$), nitric oxide (NO) and nitrous oxide (N$_2$O) were to be measured during this phase. However, the measurement of nitrogen gas was not possible due to limitations of the analytical method.

The measurement of nitrate and nitrite in the earlier research phase indicated that these dissolved substances were formed in very low concentrations. For measurement of NO and N$_2$O in the PASDEBPR, the nitrogen supply during aerobic phase was stopped. This led to the accumulation of the gases allowing measurement and identifying the extent of their accumulation. Hence, the nitrogen supply was stopped on day 93. For the next eleven days, the off-gas emissions and concentration of dissolved substances was monitored daily. By day 96, the DO profile began to decline which was considered as a pre-cursor to the deterioration in biological activity. Correspondingly, the N$_2$O values at the end of aerobic phase was 70 ppm. This was significantly higher than 17 ppm recorded on day 92 at the end of aerobic phase. On day 96, the external nitrogen sparging was resumed to ascertain the effect of stripping on N$_2$O. As soon as the nitrogen sparging was restored, the N$_2$O measured at the end of aerobic phase dropped from 94 ppm to 16 ppm. Additionally, in these eleven days, the changes in biological activity were recorded by measuring the concentration of dissolved substances. After stopping of nitrogen supply on day 93, the PO$_4^{3-}$ concentration at the end of aerobic phase was found to increase slowly. On day 96, the effluent PO$_4^{3-}$ was measured as 14 mgP/L. With nitrogen supply restored on the same day, the biological activity was restored after seven days. On day 103, the concentrations of orthophosphate (PO$_4^{3-}$), ammonium (NH$_4^+$), nitrite (NO$_2^-$), nitrate (NO$_3^-$) and nitrous oxide (N$_2$O) was 1 mgP/L, 1 mgN/L, 1.1 mgN/L, 1 mgN/L and 16 ppm, respectively. Moreover, during this research phase, the tests for measuring off-gas emissions did not measure any nitric oxide (NO) at any stage of the reactor operation.
Figure 29: Concentrations of $\text{N}_2\text{O}$ emission, orthophosphate ($\text{PO}_4^{3-}$) and ammonium ($\text{NH}_4^+$) concentration observed during the experiment in research phase 4. On 05/03, the nitrogen supply during aerobic phase was stopped to check accumulation of $\text{N}_2\text{O}$ (indicated by milestone M1). With the orthophosphate and ammonium concentration increasing, the nitrogen supply was resumed on 08/03 (indicated by milestone M2). As the laboratory was inaccessible on 10/03, no measurements were made on that day.
Table 7: Nitrogen mass-balance calculation for PASDEBPR on day 93 and 103.

<table>
<thead>
<tr>
<th>Day</th>
<th>Concentration at the start of aerobic phase</th>
<th>Concentrations at the end of aerobic phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{NH}_4^+$</td>
<td>$\text{NH}_4^+$</td>
</tr>
<tr>
<td>93</td>
<td>12.00 mgN/L</td>
<td>3.00 mgN/L</td>
</tr>
<tr>
<td>103</td>
<td>9.00 mgN/L</td>
<td>1.00 mgN/L</td>
</tr>
</tbody>
</table>

From the table for nitrogen mass-balance, an important observation was drawn regarding the estimation of $\text{N}_2\text{O}$. From the measurements of $\text{N}$ compounds, the measurement of nitrous oxide is too high considering only 12 mgN/L as $\text{NH}_4^+$ was available at the beginning of aerobic phase on day 93. On the other hand, on day 103, the mass balance results in 0.81 mgN/L as $\text{N}_2$ due to denitrification. Eventhough, this balance works out mathematically, it raises questions in terms of the exchange of electrons during the nitrification and denitrification processes. Hence, the analytical measurement of $\text{N}_2\text{O}$ was appearing to have interference resulting in an inconclusive nitrogen mass-balance.

From the four different probable processes by which $\text{NH}_4^+$ was consumed, the pathways leading to generation of $\text{N}_2\text{O}$ were identified. From the aerobic cycle test conducted in research phase 3, the concentrations of $\text{NO}_2^-$ and $\text{NO}_3^-$ were low throughout the phase. Also, with the concentration of solids around 5 gTSS/L, the central portion of the reactor was observed to receive no artificial light during aerobic (light) phase. Hence, this dark zone could possibly act as an anoxic or anaerobic zone which causes denitrification of any $\text{NO}_2^-$ and $\text{NO}_3^-$. In a study by Fagerstone, et al. (2011), elevated $\text{N}_2\text{O}$ emissions during dark periods and reduced $\text{N}_2\text{O}$ emissions during light periods were observed in a system having microalgae-bacteria in symbiosis. The results pointed out $\text{N}_2\text{O}$ production was induced by anoxic conditions when nitrate was present suggesting $\text{N}_2\text{O}$ production due to denitrification (Fagerstone, et al., 2011). At the same time, $\text{N}_2\text{O}$ production have also been observed during nitrification and denitrification process caused by nitrifiers (Alcántara, et al., 2015, Guieysse, et al., 2013, Hatzenpichler, 2012). However, in the case of PASDEBPR, the probability of truncated denitrification pathways due to PAOs is highly likely. The DPAOs, a type of PAOs, are said to perform P uptake under anoxic (denitrifying) conditions. Studies by Kerrn-Jespersen and Henze (1993) and Meinhold, et al. (1999) have pointed out that the enrichment of DPAOs is reflected by the anoxic/aerobic P-uptake rate ratios. The first step to develop the denitrification potential in DPAOs require an exposure to $\text{NO}_3^-$ for stimulating the growth of nitrate reductase enzyme (Kuba and van Loosdrecht, 1996). However, the high levels of $\text{N}_2\text{O}$ production observed in many DPAO enrichment cultures indicate the lack of a complete denitrification process (Lemaire, et al., 2006, Meyer, et al., 2005). Gao, et al. (2019) conducted a genome-centric study using metagenomics for an enriched Candidatus Accumulibacter by supplying $\text{NO}_2^-$, instead of $\text{NO}_3^-$. In that study, it was observed that only one gene of Accumulibacter strains affiliated to clade IA had the potential to carry out complete denitrification.
4.4.3 Overview of research phase 4: Identification of the inhibitory compound

This last research phase was an attempt towards justifying the assumptions made at the end of research phase 3. Continuous monitoring of PASDEBPR under two scenarios of nitrogen supply was carried out in order to identify the inhibitory compound. From the analysis for off-gas emissions and the pathways for NH₄⁺ uptake, nitrous oxide (N₂O) was found to accumulate in the reactor. Also, the biological removal efficiency of PO₄³⁻ and NH₄⁺ was observed to decline after the N₂O was measured as 100 ppm. However, when nitrogen supply was resumed during the aerobic phase, the N₂O values dropped below 20 ppm. This clearly indicates that stripping of N₂O due to N₂ supply. Moreover, the N and P-removal capabilities of the PASDEBPR was regained with the concentration of N₂O around 20 ppm.
Chapter 5 Conclusions and recommendations

5.1 Conclusions

- The coupling of algae and bacteria for simultaneous nitrogen and phosphorus removal was successfully carried out to develop the Photo-Activated Sludge Denitrifying Enhanced Biological Phosphorus Removal (PASDEBPR) system.
- Complete removal of organic carbon, nitrogen and phosphorus was successfully achieved in the PASDEBPR system without external aeration.
- Development of the steady-state model was successful in establishing the basis for the initial operation and cultivation of PAS-EBPR system. Moreover, it indicated the correlation between the solids concentration and light intensity allowing the adjustment of light intensity as per the desired outcome for each research phase.
- The gradual increase in light intensity with each research phase was useful to have the required development of algal biomass to satisfy oxygen requirements of the nutrient removal processes.
- *Chlorella sp.* and *Scenedesmus sp.* were the dominant algal species in the reactor. *Spirulina sp.* was also found to be enmeshed with larger flocs of biomass.
- The increase in biomass hindered the penetration of light thereby creating zones of denitrification at the centre of the reactor.
- The effluent analysed for the steady-state PASDEBPR resulted in low concentrations of chlorophyll-a and solids. This indicates a successful coupling of algae and bacteria as well as a biomass with good settling properties.

5.2 Recommendations

- The supply of alkalinity and ammonium in the synthetic feed was in excess to ensure a successful development of the algal-bacterial consortia. However, the levels of alkalinity should be corrected for establishing the optimal conditions for operating a steady-state PASDEBPR. On the other hand, the ammonium concentration should be optimized based on the requirements of the individual microbial species as it would be consumed mainly by algae, PAOs and nitrifiers.
- Since the aim of this research did not involve any efforts towards introducing light optimization, it is an important aspect to be considered for further development of the PASDEBPR system. By the optimizing the artificial light supply, the ideal light intensity can be determined and utilized for carrying out pilot level tests under sunlight. At the same time, it would be necessary to understand the relation between effect of change in light intensities on the nitrogen and phosphorus removal activities.
While the steady-state model predicted an ideal light intensity for algal photosynthesis, the adopted light intensities in this research was higher. Hence, additional tests to determine the development of algal biomass based on a gradient of light intensity along the reactor configuration should be performed.

Documenting the actual kinetic and stoichiometric rates of biological activity in the PASDEBPR should be carried out by performing batch activity tests. These tests shall be used to measure the rates of activity by a target microbial specie while other species are inactivated. For example, by limiting light supply for algal growth and supplying external air, the aerobic P-uptake rate of PAOs can be determined. Similarly, the nitrification potential of the system can be determined by supplying a defined quantity of nitrate and nitrite at the beginning of aerobic (light) phase. Alternately, the parameters indicating algal activity can be checked by limiting compounds like organic substrate, etc. leading to inactivation of bacterial species.

Specific batch activity tests should be carried out to evaluate the denitrification capacity exhibited by PAO in the PASDEBPR system. These batch activity tests should be performed in a SBR reactor fed with organic substrate and operated under anaerobic-anoxic-aerobic regime. By determining the anoxic/aerobic P-uptake rate ratio, the level of denitrification exhibited by the biomass can be calculated.

Attempts to create a model based on the biological activities performed in the PASDEBPR system should be made. This should be done in a step-wise manner with simulating individual biological processes first. After establishing the individual microbial activities, different combinations should be tested by careful considerations of the mitigation measures adopted in this research. Once the model is established, the results from simulation should be compared with the results from laboratory experiments. By comparison of the outcomes, the model can be fine-tuned to increase its reliability and applicability. Furthermore, this model can be later used to simulate the behaviour of the system under different scenarios representing different types of wastewater.

Due to the property of auto-fluorescence exhibited by algal species, it is difficult to identify the bacterial populations by fluorescence in situ hybridization (FISH) technique. Though techniques like polymerase chain reaction (PCR) analysis have shown some success, further research should be carried out into establishing a universally accepted protocol for examining biomass having an algal-bacterial consortia.


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Appendix A

- Kinetic and stoichiometric rates considered for the steady-state model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO specific endogenous mass loss rate at temperature 20 °C</td>
<td>b_{PAO,20}</td>
<td>0.04</td>
<td>d⁻¹</td>
</tr>
<tr>
<td>Fraction of endogenous residue of the PAOs</td>
<td>f_{XE,PAO}</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Biomass yield of PAOs</td>
<td>Y_{PAO}</td>
<td>0.45</td>
<td>gVSS/gCOD</td>
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<tr>
<td>Maximum polyphosphate content in active PAO mass</td>
<td>f_{P,PAO}</td>
<td>0.38</td>
<td>gP/gVSS</td>
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<tr>
<td>Fraction of inorganic suspended solids of PAOs</td>
<td>f_{I,PAO}</td>
<td>0.15</td>
<td>gISS/gVSS</td>
</tr>
<tr>
<td>Nitrogen content of active PAO mass</td>
<td>f_{N,PAO}</td>
<td>0.1</td>
<td>gN/gVSS</td>
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<tr>
<td>Phosphorous content of in inert mass</td>
<td>f_{P,XE}</td>
<td>0.03</td>
<td>gP/gISS</td>
</tr>
</tbody>
</table>

- Main outputs from the steady-state model

1. Area of the reactor = 122.67 cm²
2. Volume of the reactor = 2500 cm³
3. Total duration of one SBR cycle = 2 + 3 + 0.5 + 0.5 = 6 h
4. Influent flow to reactor = \( \frac{\text{Volume}}{\text{Aerobic HRT}} = \frac{2500}{3} = 0.02 \text{ m}^3/\text{d} \)
5. Mass of PAOs in the reactor = \( Q_i S_{bi,PAO} Y_{PAO} SRT \) \( 1 + b_{PAO,20} SRT \)
   \[ MX_{PAO} = 2.73 \text{ g} \]
6. Endogenous mass (PAOs) in the reactor = \( f_{XE,PAO} b_{PAO,20} MX_{PAO} SRT \)
   \[ MX_{E,PAO} = 0.22 \text{ g} \]
7. Total mass of active PAOs = \( MX_{PAO} + MX_{E,PAO} \)
   \[ MX_V = 2.95 \text{ g} \]
8. Mixed liquor volatile suspended solids (MLVSS) = \( MX_V/\text{Volume} \)
   \[ MX_V = 1.18 \text{ gVSS/L} \]
9. Nitrogen requirement by PAOs (as NH$_4^+$) = $f_{N_{\text{PAO}}} \frac{MX}{Q_i \times SRT}$

N$_s$ = 1.84 mgNH$_4$-N/L

10. Phosphorus requirement by PAOs (as PO$_4^{3-}$) = $f_{P_{\text{PAO}}} \frac{(MX - MX_{\text{PAO}})}{Q_i \times SRT}$

P$_s$ = 0.04 mgPO$_4$-P/L

11. Phosphorous requirement in active PAOs = $f_{P_{\text{PAO}}} \frac{MX_{\text{PAO}}}{Q_i \times SRT}$

= 6.48 mgPO$_4$-P/L

12. Alkalinity generated by PAOs = 0.59 x $S_{bi_{\text{PAO}}} \times (\text{M.W. of CO}_2/\text{M.W. of HAc})$

= 32.45 mgCO$_2$/L

= 45 mgHCO$_3$/L

*The value 0.59 is the moles of carbon dioxide obtained at the end of EBPR according to the metabolic model of the EBPR process developed by Smolders, et al. (1995).*

13. Ammonium concentration available for algal uptake and nitrification

= N$_{ti}$ - N$_s$

= 10.67 mgNH$_4$-N/L